1 Science without Borders
T. R. Ceci, Howard Hughes Medical Institute, Chevy Chase, MD
Borders between disciplines. Borders between science and medicine. Borders that wall-off women and minority scientists from full participation. Those are some of the borders that we need to erase - or, failing that, at least make it easier to cross. With respect to interdisciplinarity, I’ll mention my own research experiences, talk about the challenges to cross-discipline research in the universities, and then discuss HHMI’s efforts. For example, HHMI’s Janelia Farm Research Campus now has physicists and computer scientists working shoulder-to-shoulder with neuroscientists. With respect to Science/Medicine, I’ll discuss our programs in research and in medical student and graduate student training. Finally, I’ll share some thoughts about women in science, and I’ll review HHMI’s Exceptional Research Opportunities Program.

2 In Vivo Interactions between Migrating Cells and the Micronvironment
D. J. Montell, J. McDonald, X. Wang, A. Jung, M. Starz-Gaiano, J. Kim, H. Yin, T. Bridges, M. Melani, Y. Chang, W. Yoon, M. Prasad; 1Department of Biological Chemistry, Johns Hopkins Medical School, Baltimore, MD, 2Department of Cell Biology, Scripps Research Institute, La Jolla, CA
My laboratory studies the molecular mechanisms that control cell migration in development and disease. We have developed the border cells in the Drosophila ovary as a model system to study the developmental regulation of cell migration and its coordination with other developmental events. We have described multiple extracellular signals that are required for the correct cells to move in the proper direction, at the appropriate stage. These signals include a steroid hormone (ecdysone), growth factors that activate receptor tyrosine kinases, and a cytokine that stimulates the JAK/STAT pathway. Each signal emanates from a distinct anatomical source, insuring proper coordination of border cell migration with other cell types and developmental events. We have recently shown that spatial localization of Notch activity can be achieved by localized Kuzbanian gene expression, a previously unrecognized mechanism by which Notch activity can be localized during development. Ongoing screens continue to identify new genes, including Par-1, that control additional aspects of border cell migration. We have also shown that a number of these genes contribute to ovarian cancer. Recently we have defined conditions that allow us to observe border cell migration directly, in living organ culture, using time-lapse movies. This allows us to combine powerful genetic manipulations with live-imaging to decipher and observe the mechanisms by which cell migration is regulated in vivo.

3 Integrating the Dynamics of Actin, Myosin, and Adhesions in Cell Migration
C. M. Waterman-Storer; Department of Cell Biology, Scripps Research Institute, La Jolla, CA
Cell migration is driven by forces generated by myosinII-based tension in an actin filament (F-actin) network linked via molecularly complex focal adhesions (FAs) to the extracellular matrix (ECM). The relationship between myosinII activity, F-actin and FA dynamics, and the nature of the molecular linkages between F-actin and the ECM via FAs are unclear. It is established that cells exhibit a biphasic migration velocity response to increasing adhesion strength, with fast migration occurring at intermediate adhesion strength and slow migration occurring at low and high adhesion strength. A simple mechanical model has been proposed to explain this, in which too little adhesion does not provide sufficient traction whereas too much adhesion renders cells immobile. We sought to determine if distinct organizational states of F-actin, myosin II, and FAs accompany adhesion-dependent changes in cell velocity. We characterize a unique phenotype for optimal migration, entailing rapid F-actin flow convergence and local depolymerization, local myosin II activation, rapid renewal of FA components, and intermediate FA lifetime and turnover rates. We recapturate this phenotype and fast migration at non-optimal adhesion strength by manipulating myosinII activity. Thus, in contrast to simple models, we find that a complex spatiotemporal feedback between the F-actin, myosin II, and FAs mediates the biphasic migration velocity response to increasing adhesion strength, so that a specific balance between adhesion and contraction induces maximal migration velocity. We also developed Correlational Fluorescence Speckle Microscopy to measure the coupling of FA proteins to the actin cytoskeleton during cell migration. Different classes of FA structural and regulatory molecules exhibit varying degrees of correlated motions with actin filaments, indicating differential transmission of actomyosin motion through FA. Our results suggest that transient interactions between FA proteins and actin filaments constitute a friction clutch between the cytoskeleton and the extracellular environment that is regulated during morphodynamic transitions of cell migration.

4 Integrin and 3D Matrix Dynamics in Cell Migration and Morphogenesis
K. M. Yamada; CDBBB, NIH/NIDCR, Bethesda, MD
Cell adhesion, migration, and tissue remodeling depend critically on coordinated interactions of integrins and the cytoskeleton with the extracellular matrix. These interactions are highly dynamic, with integrins, cytoskeletal proteins, and matrix proteins all undergoing distinct translocalizations. Particularly central roles are played by integrin α5β1, actin, and myosin IIA in coordinated integrin-matrix and myosin-microtubule functions that regulate cell adhesion and migration. Conversely, whether cell interactions with the extracellular matrix occur in two-dimensional (2D) versus 3D matrix microenvironments has substantial effects on cell adhesion, migration, proliferation, and signaling. For example, human fibroblasts in a 3D matrix show altered total Rac activity, which activates a general cellular mechanism that switches cell migration from random and exploratory to directionally persistent using a mechanism distinct from chemotaxis. At the tissue level, developmentally regulated, local production and movements of a 3D matrix were found to regulate organ formation. Highly directional, local translocation of a cytokine that stimulates the JAK/STAT pathway. Each signal emanates from a distinct anatomical source, insuring proper coordination of border cell migration with other cell types and developmental events. We have recently shown that spatial localization of Notch activity can be achieved by localized Kuzbanian gene expression, a previously unrecognized mechanism by which Notch activity can be localized during development. Ongoing screens continue to identify new genes, including Par-1, that control additional aspects of border cell migration. We have also shown that a number of these genes contribute to ovarian cancer. Recently we have defined conditions that allow us to observe border cell migration directly, in living organ culture, using time-lapse movies. This allows us to combine powerful genetic manipulations with live-imaging to decipher and observe the mechanisms by which cell migration is regulated in vivo.

5 Working Together to Improve Life Science Education
A. Campbell; Department of Biology, Davidson College, Davidson, NC
Life science education, like basic research, is a collaborative venture. I have benefited from many collaborations, but those associated with the Genome Consortium for Active Teaching (GCAT; www.bio.davidson.edu/GCAT) have been among the most enjoyable and productive. GCAT began in 2000 and during its first six years, GCAT has provided DNA microarrays to over 5,000 undergraduates on campuses serving diverse populations. These students show significant learning gains in knowledge, and improved attitudes towards research. Faculty enjoy the network of colleagues and the excitement of genomics in their teaching. Now GCAT is branching out to new areas. Collaborating with Dr. Sarah Elgin at Washington University, undergraduates will sequence genomes as part of original research projects. Working with Dr. Drew Endy at MIT, GCAT students participated in the 2006 International Genetically Engineered Machines Jamboree (http://parts2.mit.edu/wiki/index.php/Main_Page) along with students from 36 schools around the world. GCAT is working with high school teachers to develop genomics curriculum for high school students. It is important to assess these educational innovations, both to guide the development process and to demonstrate the strength of this lab-intensive, research-oriented approach to teaching in life sciences. By establishing a publishing standard that requires assessment, CBE Life Sciences Education has grown to be an important journal for sharing our educational resources and outcomes. (Supported by NSF, HHMI, Waksman Foundation for Microbiology, and Davidson College).

6 Bringing Research into the Undergraduate Curriculum
S. C. R. Elgin, C. Shaffer, W. Leung; 1Department of Biology, Washington University, St. Louis, MO; 2Department of Computer Science & Engineering, Washington University, St. Louis, MO; 3Department of Genetics, Washington University, St. Louis, MO; 4Department of Psychology, Grinnell College, Grinnell, IA
Undergraduate research experiences are a critical part of our biology curriculum, enabling students to understand how new knowledge is generated in their field. Undergraduate students often enter research through an individual apprenticeship during the summer. This has been found to generate a spectrum of intellectual and social gains, including greater understanding of the research process and the strategies and tools that scientists use to work on real problems (SURE survey data; D. Lopatto, 2004, Cell Biol. Educ. 3: 270). An alternative to summer research is a one semester, upper-level laboratory course built around a research problem. Bio 4342, Research Explorations in Genomics, is taught by collaborating faculty at Washington University from Biology, Computer Science, and the WU Genome Sequencing Center (see http://www.nslc.wustl.edu/elgin/genomics). Students in Bio 4342 join a research team working on sequencing and annotating the distal portion of a dot chromosome from a Drosophila species. The region is of interest because chromatin packaging in this region varies among the different species (resulting
publication: Slawson et al, 2006, Genome Biology 7: R15). Students report similar intellectual gains, particularly in developing problem-solving skills, but gain greater confidence in working as a group rather than individually. They are enthusiastic about the course, and particularly like the practice in reasoning from evidence, defending their conclusions in written and oral reports. Similar research-oriented courses elsewhere have focused on using other genomics tools, for example microarrays to sample gene expression (see A.M. Campbell, 2003, Cell Biol. Edu 2: 98).

An effort is underway to expand Bio 4342 through participation of students and faculty at other colleges and universities. As sequencing becomes cheaper, and the need for finishing and annotation increases (activities requiring human input that students can provide), the time is right for a student-scientist partnership in genomics. Funded by grants from HHMI.

8 Endless Flies Most Beautiful: The Role of Cis-Regulatory Sequences in the Evolution of Morphological Diversity

S. Carroll, HHMI/Laboratory of Molecular Biology, University of Wisconsin, Madison, WI

One of the general issues concerning the evolution of form is the relative contribution of gene duplication, protein sequence evolution, and regulatory sequence evolution to the generation of morphological variation and diversity. We have analyzed the gain, loss, and modification of pigmentacion patterns in Drosophila as models of morphological evolution. Six cases have involved regulatory changes at the pleiotropic pigmentation gene yellow. These include the surprising independent gains of similar wing patterns via the co-option of distinct cis-regulatory elements and the gain and loss of abdominal patterns via the gain and loss of Hex binding sites. Our results demonstrate how the functional diversification of the modular cis-regulatory elements of pleiotropic genes contributes to evolutionary novelty and the independent evolution of morphological similarities.

9 Fishing for the Secrets of Vertebrate Evolution

D. Kingsley, Department of Developmental Biology, HHMI/Stanford University, Stanford, CA

The molecular mechanisms that underlie the diversity of living organisms are still unknown. We have been developing stickleback fish as a model system for studying how new traits evolve in natural populations of vertebrates. These fish have undergone a dramatic radiation in postglacial lakes and streams throughout the Northern Hemisphere. Ancestral marine sticklebacks can still be crossed with newly established freshwater populations, making it possible to carry out a detailed study of the genetic basis of many dramatic morphological, physiological, and behavioral differences that have evolved in the last 10,000 years. We have developed a large set of genetic and genomic resources for mapping and isolating the genetic changes that underlie evolutionary change in sticklebacks. Whole genome linkage mapping shows that both gain and loss traits can be traced to particular chromosome regions, with a distribution of phenotypic effects much larger than predicted by "infiniteisles" models of evolutionary change. Detailed cloning and sequencing studies have revealed the actual genes that control major changes in pelvic development and arm plate patterning. In both cases, major evolutionary change has occurred by making regulatory alterations in key developmental control genes. Comparative studies suggest that many of the conclusions from initial study populations can be generalized to mechanisms that underlie evolution of similar traits over wide geographic and evolutionary distances. Further application of forward genetic approaches should thus help elucidate general rules about the number, location, and type of genes and mutations that control evolutionary change in nature.

10 The Molecular Evolution of Neural Circuits for Vocal Learning

E. D. Jarvis; Department of Neurobiology, Duke University Medical Center, Durham, NC

Vocal learning, the behavioral substrate for human language, is a rare trait found to date in only three distantly related groups of birds (parrots, hummingbirds, and songbirds) and four distantly related groups of mammals (humans, bats, cetaceans, and elephants). We have been studying the molecular biology and evolution of vocal learning brain systems. Here I will present our findings on the dynamic cascades of behaviorally regulated genes in vocal learning neural circuits and the implications these findings have for understanding the evolution of these circuits. We found that production of learned vocalizations is accompanied by activation of cascades of genes involved in both plastic and homeostatic cellular functions. These cascades consist of overlapping, but unique sets of genes in each vocal nucleus in different brain subdivisions. We believe that these genes are used to shape and maintain cellular homeostasis and circuits specific to each brain subdivision so that the animal is ready to produce learned vocalizations or other behaviors in other circuits at a moments notice. The differences between vocal learning and non-vocal learning circuits are not in the presence or absence of specific genes, but in the specialized expression of ancient genes in new ways in the vocal learning circuits. These gene activation patterns revealed remarkably similar vocal learning neural pathways across distantly related vocal learning birds, and have helped gain insight in the neural pathways for vocal learning in humans. The resultant hypothesis suggest that it may take just a few genetic mutations to convert a pre-existing non-vocal brain circuit into a vocal learning brain circuit that then uses an ancient set of genes in new ways to regulate the learning and production of vocalizations.

12 The Nuclear Membrane Protein Nesprin-2 Giant Plays a Key Role in Nuclear Positioning during Centrosome Reorientation and Cell Migration

E. R. Gomes, E. C. Vintinner, I. Karakesisoglou, G. G. Gundersen, Anatomy and Cell Biology, Columbia University, New York, NY; Center for Biochemistry, Medical Faculty, University of Cologne, Cologne, Germany

Migrating fibroblasts reorient their centrosome towards the leading edge by moving the nucleus away from the leading edge while the centrosome remains at the cell center. The rearward movement of the nucleus is mediated by Cdc42, myotonic dystrophy kinase-related Cdc42-binding kinase and myosin II-dependent actin retrograde flow, while the maintenance of the centrosome at the cell center is mediated by Cdc42, Par3, aPKC and dynein (Gomes et al., Cell 2005). Most nuclear movements are mediated by microtubules raising the question of how actin retrograde flow moves the nucleus away from the leading edge in migrating cells. We found that the outer nuclear membrane protein nesprin-2 giant is required for rearward nuclear movement during centrosome reorientation in 3T3 fibroblasts. Nesprin-2 giant is the largest nesprin-2 isoform and contains a transmembrane KASH domain at its C-terminus, which targets it to the outer nuclear membrane. We have performed the first live imaging of subcellular events to examine the two-stroke model in brain slices using cerebellar granule cells or tangentially migrating neurons as models of morphological evolution. Six cases have involved regulatory changes at the pleiotropic pigmentation gene yellow. These include the surprising independent gains of similar wing patterns via the co-option of distinct cis-regulatory elements and the gain and loss of abdominal patterns via the gain and loss of Hex binding sites. Our results demonstrate how the functional diversification of the modular cis-regulatory elements of pleiotropic genes contributes to evolutionary novelty and the independent evolution of morphological similarities.

13 Triple Labeling of Migrating Neural Progenitor Cells in Live Brain Tissue Reveals Novel Roles for Cytoplasmic Dynine and LIS1 in Centrosomal, Nuclear, and Microtubule Behavior

J. Tsai, R. B. Valle; Pathology and Cell Biology, Columbia University, New York, NY

During neocortical development, neurons arising at the ventricular zone migrate along radial glial fibers to the cortex. In vitro studies using cerebellar granule cells or tangentially migrating neurons have shown that the movement is saltatory and comprises two "strokes": forward centrosomal movement into a swelling in the leading process, followed by the soma (Nat Neurosci, 7:1195; J Neurosci, 25:103652). We previously reported that LIS1 RNAi inhibits somal movement but not process growth of neural progenitors in vivo (JCB, 176/935), suggesting the importance of cytoplasmic dynein in somal movement; however, its specific role remained underdetermined. By electrophoretically raising the stroke model in brain slices and its underlying motor machinery. Nuclei exhibited both rapid jumps and slower continuous movements. However, centrosomes moved at relatively constant rates. GFP-EB3 imaging revealed microtubules to concentrate from and move in association with the centrosome. Nuclei showed microtubule-dependent distortion prior to advancing into the migratory process, indicative of strong, discontinuous pulling forces. In cells expressing LIS1 or dynine siRNAs, centrosomal and nuclear movements were both disrupted. In partially-inhibited cells, remarkable wandering excursions of the centrosome toward the swelling were seen, whereas nuclear movement was independently inhibited. Blebbistatin blocked somal translocation as previously reported, but centrosomal movement was unaffected. Immunocytochemistry showed dynine specifically concentrated in the swelling and the leading process of freely migrating neurons. These data have led to a comprehensive model for the unique migration mechanism used by neural progenitors: cytoplasmic dynine pulls from the leading process on the centrosome-anchored microtubules; the nucleus is then transported cooperatively by dynein as cargo along the trailing microtubules and by myosin II acting from the rear. Supported by HD40182.
The EGF receptor (EGFR) is essential for the growth and differentiation of many types of epithelial cells. To understand how signaling through this receptor is regulated, we are developing models of the ERK pathway have previously indicated the possibility of oscillations because of the negative feedback loop between ERK and

Intracellular pH-dependent Polarity and Actin Assembly in Migrating Cells


Computational Biology, Pacific Northwest National Laboratory, Richland, WA

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Analysis and Modeling of the Rapid ERK Oscillations That Occur in Response to EGF Receptor Activation

D. L. Ippolito, H. Shankaran, H. Resat, G. J. Newton, W. B. Chreiter, L. K. Opreško, H. Wiley. Biological Sciences, Pacific Northwest National Laboratory, Richland, WA,

C. Frantz, L. Dominguez, D. Zaghi, M. P. Jacobson, D. L. Barber. Department of Cell and Tissue Biology, University of California, San Francisco, San Francisco, CA,

2Computational Biology, Pacific Northwest National Laboratory, Richland, WA

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Formation of Functional Invadopodia Requires the Actin-binding Repeats and Tyr-Phosphorylation of Cortactin

B. A. Webb, L. Jia, R. Eves, A. Mak. Biochemistry, Queen's University, Kingston, ON, Canada

The Biology of FAK: Implications in the Maintenance of Cell Tension and Polarity

R. W. Tilghman, Z. Rajfur, M. Iwanicki, C. R. Cowan, K. Jacobson, J. T. Parsons. Microbiology, University of Virginia, Charlottesville, VA, 2University of North Carolina, Chapel Hill,

D. Zaghi, M. P. Jacobson, D. L. Barber. Department of Cell and Tissue Biology, University of California, San Francisco, San Francisco, CA,

Regulation of chemotaxis requires the proper control of cell adhesion and cell movement to the substrate. We have investigated the role of Rap1 in Dictyostelium and demonstrate that Rap1 is required for proper regulation of cell adhesion and chemotaxis through the control of Myosin II (MyoII). Rap1 is activated rapidly and transiently in response to chemotactant stimulation and activated Rap1 is preferentially found at the leading edge of chemotaxing cells. The spatial distribution of Rap1-GTP extends more laterally than that of Rac-GTP and the kinetics of activation are slower. Cells expressing constitutively active Rap1 (Rap1CA) are highly adhesive and flattened. These cells chemotax very slowly and produce a large number of lateral pseudopodia. Like wild-type cells, Rap1CA cells rapidly disassemble myosin in response to chemotactant stimulation but reassemble MyoII very slowly. Consistent with this, we observe a very delayed reassociation of GPP-Mysillin response to chemotactant stimulation in Rap1CA cells. These results suggest that Rap1 negatively controls MyoII assembly. In Dictyostelium, MyoII assembly/disassembly is regulated by phosphorylation of 3 Thr residues in the MyoII tail. We show that the kinase Phg2, a putative Rap1 effector, colocalizes with Rap1-GTP at the leading edge of chemotaxing cells and is required for MyoII phosphorylation in vitro assay. Phg2 null cells exhibit defects in MyoII assembly in vivo and show severe chemotaxis defects. We suggest that Phg2 is a new component of the rapidly-activated, leading edge regulatory network that, in this case, controls MyoII phosphorylation and its assembly/disassembly. In conclusion, our findings suggest that Rap1 is a key upstream spatial regulator of MyoII assembly, and the function of Rap1, in part, is mediated by Phg2. Our results provide new insights into the function of Rap1 in controlling the leading edge and posterior of chemotaxing cells.

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The Biology of FAK: Implications in the Maintenance of Cell Tension and Polarity

R. W. Tilghman, Z. Rajfur, M. Iwanicki, C. R. Cowan, K. Jacobson, J. T. Parsons. Microbiology, University of Virginia, Charlottesville, VA, 2University of North Carolina, Chapel Hill,

Focal adhesion kinase (FAK) plays an important role in cell migration during key physiological processes such as cancer metastasis, wound healing, and embryonic development, yet the mechanism by which FAK regulates cell migration is unclear. Fibroblasts derived from FAK -/- mice are highly contractile and show an increased amount of large Rho-dependent focal adhesions, suggesting that FAK is a negative regulator of the Rho pathway, and FAK is required to release adhesions to promote focal adhesion turnover. However, in wild-type fibroblasts and endothelial cells, FAK is highly active when cells are under tension, suggesting that FAK may also play a role in focal adhesion growth or maintenance. To resolve this apparent paradox in FAK signaling, we employed two ways of activating FAK in wild-type cells: siRNA of FAK to rapidly inhibit its expression, and the inhibition of FAK activity using a novel specific small compound kinase inhibitor (PF-228). Treatment of fibroblasts with FAK siRNA or PF-228 did not produce the morphology that is characteristic of the FAK -/- cells; rather, they formed a proper leading edge, and instead they formed multiple thin protrusions and became elongated. Both treatments resulted in cells that lost their ability to spontaneously polarize on fibronectin. Surprisingly, treatment of fibroblasts with PF-228 resulted in a decrease in myosin light chain phosphorylation, suggesting that FAK is important in the generation or maintenance of contractility. To measure changes in cellular tension, fibroblasts were grown on flexible polyacrylamide substrates embedded with beads to measure forces exerted by the cells. Treatment with PF-228 resulted in a rapid decrease in cellular tension, suggesting that FAK activity is required to maintain tension across the cell. These results point to FAK’s role in mechanotransduction to establish cell polarity during cell migration by inducing resistance at critical points in the cell.

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2006 ASCB Annual Meeting Abstracts
Quantitative Models of Embryonic Development
S. Y. Shvartsman, Lewis Sigler Institute and Department of Chemical Engineering, Princeton University, Princeton, NJ
The development of tissues and organs is regulated at a variety of levels, from gene sequence to anatomy. At this level of complexity, modeling becomes essential for integrating data and guiding future studies. Any attempt to establish quantitative models of embryogenesis is confronted by the high level of structural and parametric uncertainty, a large number of components, and the multiscale nature of development. At the same time, an expanding arsenal of experimental tools can constrain models and directly test their predictions, making the modeling efforts not only necessary, but feasible. I will illustrate this paradigm using two examples from our work on the quantitative analysis of fruit fly development. Both examples are related to the concept of a morphogen, a chemical that induces multiple cell fates in a concentration-dependent manner. In the first case, we will use a parameter estimation approach to infer the experimentally inaccessible concentration profile of the Gurken morphogen in a developing egg. In the second case, a combination of imaging, genetic, and modeling approaches will be used to probe the information processing capabilities of the Torsos signaling cascade in the early embryo.

Multicellular patterns and structures emerge as cells execute instructions received from multiple cues in their microenvironment. How cells integrate these cues to achieve an organized, functional structure is a fundamental question in development biology with important biomedical implications. Developing strategies to guide multicellular structure formation is a central challenge in tissue engineering and regenerative medicine. Thus, our lab seeks to understand how cellular and molecular networks qualitatively interpret pattern-guiding environmental cues. In this talk, I will present our study of cell patterning during adult-phase development in C. elegans, a process that involves molecular signals with close homology to those underpinning human development and disease. A feature of this system is that patterning depends not only on a spatial gradient of a soluble factor (a morphogen gradient), but also on direct cell-cell contact. Our computational analysis shows that the signaling network coupling neighboring cells amplifies cellular perception of the morphogen gradient, thereby enhancing fate segregation beyond that achieved in an uncoupled system. In addition to elucidating how the topology of the signaling network confers qualitative advantages in signal processing, our mathematical model accurately predicts wild-type and mutant phenotypes. This ability to predict phenotypes has allowed us to conduct in silico genetics experiments, offering answers to intriguing questions such as what new phenotypes are possible, and what perturbations render such phenotypes.

Automatic Parameter Identification via the Adjoint Method, with Application to Understanding Planar Cell Polarity
R. Raffard, K. Amontlirdviman, I. Axelrod, C. Tomlin; Stanford University, Stanford, CA
A key focus of systems biology has been the development of models, at the appropriate level of abstraction, to help understand different biological processes. This development usually proceeds in iterative fashion, in which the structure of the model is chosen to represent certain hypotheses about how the system operates and parameters for this structured model are chosen. Often, the first experiment is to ask if a robust set of parameters exists so that the model reproduces all or most of the observed biological data. The model is tested against this actual data and for its predictive capabilities. As new data and/or new understanding arises, the structure of the model may be altered, and new parameters selected. In protein regulatory networks, the number of states to model is typically large and depends on the number of proteins of interest, the parameter spaces are large, and the most appropriate models are nonlinear functions of the states. Thus it is becoming increasingly important to develop fast, efficient, scalable methods for large scale parameter identification. This paper presents an adjoint-based algorithm for performing automatic parameter identification on differential equation based models of biological systems. The algorithm solves an optimization problem, in which the cost reflects the deviation between the observed data and the output of the parameterized mathematical model, and the constraints reflect the governing parameterized equations themselves. Results of the application of this algorithm to a previously presented mathematical model of planar cell polarity signaling in the wings of Drosophila melanogaster are presented.

Functional Grouping of Kinetochore Proteins Based on Their Regulation of Kinetochore Microtubule Dynamics
K. Jaqaman,1 J. F. Dorn,2 E. Marco,2 G. S. Jelson,3 P. K. Sorger,2 G. Danuser1; 1Department of Cell Biology, The Scripps Research Institute, La Jolla, CA, 2Department of Biology, Massachusetts Institute of Technology, Cambridge, MA
To test the hypothesis that kinetochore proteins regulate kinetochore microtubule (kMT) dynamics, we measured single kMT dynamics in the budding yeast S. cerevisiae and compared them between wild type (WT) and strains carrying kinetochore protein mutations. We established autoregressive moving average (ARMA) model parameters as a unique and complete set of descriptors of kMT dynamics that allowed us to distinguish between subtle phenotypes associated with gene deletions and temperature-sensitive mutations. ARMA models extracted the dependence of kMT length on its history and on a related white noise series which embodied the stochastic nature of kMT dynamics. Multiple kMT length series from each condition were fitted together, taking into account observational error and missing observations, to achieve robust parameter estimation. We also estimated the variance-covariance matrices of ARMA descriptors and used them to compare descriptors between different conditions within a statistical hypothesis testing framework. The p-values from the statistical tests revealed which conditions had different dynamics, and also provided us with a proximity measure that we used for clustering kMT dynamics. This allowed us to classify kinetochore proteins within functional groups. We found that kinetochore proteins do indeed regulate kMT dynamics. For instance, kMT dynamics in the mutants okp1-5 and kip3- delete are different from those in WT. Furthermore, we found that the proteins Ipl1p, Dam1p and Kip3p form one functional group, where the dynamics resulting from their mutation are equivalent and significantly different from dynamics in WT. In addition to their classification power, ARMA descriptors are ideal intermediate statistics for matching experimental and simulated kMT dynamics for calibrating stochastic mechanistic models of kMT regulation by kinetochore proteins.

System Level Analysis Identifies Timing of Force Integration during Mitosis
R. Wollman, G. Civelekoglu-Scholey, J. M. Scholey, A. Mogilner; Center for Genetics and Development, University of California, Davis, Davis, CA
Mitotic spindle development is characterized by consecutive transitions between distinct morphological states that can be characterized by increasing spindle length and distances between sister chromatids. For example, during Drosophila embryonic development, the spindle poles and chromosomes separate in a well-defined and quantitatively reproducible fashion. While a number of molecular perturbations have revealed the basic mechanisms of multiple motor and microtubule actions underlying spindle elongation, a complete picture of how motor and microtubule forces are integrated is still lacking. We performed an In Silico model screen to identify all potential mechanisms of force integration that will reproduce Drosophila's pole and chromosome separation phenotype. We 'trained' the computer to assemble a set of models based on different combinations of forces and molecular on/off switches regulating motor and microtubule dynamics. We screened the models in a 39-dimensional parameter space (11 timing parameters and 28 kinetic and mechanical parameters). To identify models that fit experimental data we used stochastic optimization and genetic algorithms. We found that only 1000 different models that can quantitatively describe pole and chromosome separation in Drosophila. Cluster analysis of these different models shows that there are potentially 11 qualitatively different ways to integrate spindle forces and reproduce experimental results of wild-type embryos. We show that in all 11 strategies, the timing of force activity must be fine tuned, in contrast to the kinetic and mechanical parameters that show robustness to change. Using additional data from mutant embryos and inhibition experiments we show that changes in sliding forces, structure of the microtubule mid-zone overlap region, and microtubule pole depolymerization must be integrated in a particular way to reproduce both wild type and mutant data. System modeling results in quantitative understanding of force integration in the spindle and is the crucial step toward elucidating the regulatory biochemical network governing transitions during mitosis.

The Epigenetics of Cancer Etiology
A. Feinberg; Department of Medical Genetics, Johns Hopkins University School of Medicine, Baltimore, MD
In over 20 years since the discovery of altered methylation in cancer, many epigenetic alterations have been found in human cancer, including global and specific gene hypomethylation, hypermethylation, altered chromatin marks, and loss of genomic imprinting. Cancer epigenetics has been limited by questions of cause and effect, since epigenetic changes can arise secondary to the cancer process and associated changes in gene expression. Furthermore, mutations in the DNA methylation machinery have not been observed in tumors, while they have for chromatin modification. To address the issue of human cancer etiology, we have more recently focused on identifying epigenetic changes in normal cells that predispose to cancer. One line of investigation has been to investigate loss of imprinting (LOI) of the insulin-like growth factor-II gene (IGF2) associated with a personal history of colorectal neoplasia, as well as a family history of colorectal cancer (CRC). We developed an animal model showing that LOI of IGF2 increases the frequency of neoplasms caused by mutations in the Apc gene, suggesting that a major component of cancer risk involves epigenetic changes in normal cells that increase the probability of cancer after genetic mutation. Furthermore, we found that LOI increases and alters the progenitor cell compartment, likely accounting for this increased risk. These data are consistent with an “epigenetic progenitor” model of human cancer, and they suggest a model of cancer prevention that involves the epigenetic analysis of normal cells for risk stratification and cancer prevention strategies, similar to the widely used approach of modifying risk of cardiovascular disease by treating apparently healthy individuals with detectable biochemical indicators of enhanced risk.

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Suz12 Silences Large Regions of the Genome in a Cell Type-specific Manner
P. J. Farnham,1 S. Squazzo,1 H. O'Geen,2 V. Komashko,3 V. Jin, R. Green, Genome & Biomedical Sciences Facility, University of California, Davis, Davis, CA, 2NimblerGen Systems Inc, Madison, WI
Suz12 is a component of the Polycomb Group Complexes 2, 3, and 4 (PRC 2/3/4). These complexes are critical for proper embryonic development, but very few target genes have been identified in either mouse or human cells. Using a variety of ChIP-chip approaches, we have identified a large set of Suz12 target genes in several different human and mouse cell lines. Interestingly, we found that Suz12 target promoters are cell type-specific, with transcription factors and homeobox proteins predominating in embryonal cells and glycoproteins and immunoglobulin-related proteins predominating in adult cells. We have also characterized the localization of other components of the PRC complex with Suz12 and investigated the overall relationship between Suz12 binding and markers of active vs. inactive chromatin, using both promoter arrays and custom tile arrays. Surprisingly, we find that the PRC complexes can be localized to discrete binding sites or spread through large regions of the mouse and human genomes. Current studies are focused on determining the site-specific factors that are involved in recruiting Suz12 to discrete sites vs. large chromosomal domains. Towards this goal, we have shown that some Suz12 target genes are bound by OCT4 in embryonal cells and suggest that OCT4 maintains stem cell self-renewal, in part, by interacting with PRC complexes.

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DNA Methylation Regulates Insulin Gene Expression
T. Yoshioka, S. Shinya, O. Jun, S. Kunio; Faculty of Agriculture, The University of Tokyo, Tokyo, Japan
There are two insulin genes in mouse; Ins1 on chromosome 19 and Ins2 on chromosome 7. Ins2 has the same exon-intron-structure as human INS while Ins1 is different. Ins2 expression is specific to beta cells of the islet of Langerhans in pancreas. Ins2 has a promoter (~400 bps) with a TATA box and several transcription factor binding sites such as Pdx1(-209), HNF4 (-231, -106) and Maf(-122). DNA methylation profile of the tissue-dependent differentially methylated regions (T-DMRs) is unique in each tissue or cell type including embryonic stem cells (ESCs), somatic cells and germ cells. Therefore, DNA methylation profiles of T-DMRs are useful to evaluate and characterize the cells. In present study, the DNA methylation status of the upstream region (~440 to ~65) of Ins2 which includes the promoter region was examined in insulin-producing MIN6 cells and non-producing ESCs using bisulfite sequencing. All the three CpGs (-408, -176, -171) within the region were unmethylated in MIN6 but were hypermethylated in ESCs. In addition an ectopic expression of Ins2 was induced in ESCS after a two-day culture with a DNA methylation inhibitor, 5-aza-2'-deoxycytidine. Thus, we found that Ins2 has a T-DMR. We analyzed DNA methylation status of the Ins2 promoter in the islet of Langerhans by laser microdissection and confirmed that the Ins2 T-DMR was totally unmethylated in the islet, in contrast it was hypermethylated in whole pancreas. The methylation status of the pancreas reflected insulin-non-expressing exocrine cells, since they account for more than 95% of the pancreatic cells. Besides that, the T-DMR was also hypermethylated in other non-expressing tissues including kidney, liver and heart. Promoter assays using lac-reporter system in MIN6 cells showed that the promoter activity was severely decreased when the promoter construct that was methylated in vitro was used. In conclusion, the Ins2 expression is regulated by DNA methylation.

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Tumor Suppressor P16INK4A Regulates Polycomb-mediated DNA Hypermethylation
T. D. Tlsty,1,2 P. A. Reynolds,1,2 M. Sigaroudinia,1,2 G. Zardo,1,2 M. B. Wilson,1,2 G. M. Benton,1,2 C. J. Miller,1,2 C. Hong,1,2 J. Fridlyand,1 J. F. Costello; 1Comprehensive Cancer Center, University of California, San Francisco, San Francisco, CA, 2Department of Pathology, University of California, San Francisco, San Francisco, CA, 3The Brain Tumor Research Center and Department of Neurological Surgery, University of California, San Francisco, San Francisco, CA
To understand dynamic changes in gene expression mediated by steroid receptors, our laboratory has taken a direct visualization approach to analyze steroid hormone action. Using GFP-tagged glucocorticoid receptor and RNA polymerase II supports formation of chromatin loops in the interphase nucleus of living mammalian cells. As the ligand-bound receptor functions to regulate steroid hormone-responsive gene transcription, we have analyzed the ability of the glucocorticoid receptor to recruit steroid receptor co-activators, basal transcription factors, and RNA polymerase II to an amplified array of a specific target gene. While different fluorescently labeled factors are recruited at varying efficiencies following hormone treatment, two different types of localization are observed—diffuse or highly localized. For a basal transcription factor, hormone treatment results in its recruitment to the amplified array. Interestingly, we found that Suz12 target promoters are cell type-specific, with transcription factors and homeobox proteins predominating in embryonal cells and glycoproteins and immunoglobulin-related proteins predominating in adult cells. We have also characterized the localization of other components of the PRC complex with Suz12 and investigated the overall relationship between Suz12 binding and markers of active vs. inactive chromatin, using both promoter arrays and custom tile arrays. Surprisingly, we find that the PRC complexes can be localized to discrete binding sites or spread through large regions of the mouse and human genomes. Current studies are focused on determining the site-specific factors that are involved in recruiting Suz12 to discrete sites vs. large chromosomal domains. Towards this goal, we have shown that some Suz12 target genes are bound by OCT4 in embryonal cells and suggest that OCT4 maintains stem cell self-renewal, in part, by interacting with PRC complexes.

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Direct Visualization of Glucocorticoid Receptor and RNA Polymerase II Supports Formation of Chromatin Loops in the Interphase Nucleus of Living Mammalian Cells
H. Hsu, R. S. Singh, W. J. Dixon, T. Lee, C. Langer, Y. K. Lee; Ob-Gyn and Molecular & Medical Pharmacology, David Geffen School of Medicine at UCLA, Los Angeles, CA
To understand dynamic changes in gene expression mediated by steroid receptors, our laboratory has taken a direct visualization approach to analyze steroid hormone action. Using GFP-labeled steroid receptors, we previously showed that both estrogen and glucocorticoid receptors undergo subcellular changes in their organization in response to activation by steroid hormones. As the ligand-bound receptor functions to regulate steroid hormone-responsive gene transcription, we have analyzed the ability of the glucocorticoid receptor to recruit steroid receptor co-activators, basal transcription factors, and RNA polymerase II to an amplified array of a specific target gene. While different fluorescently labeled factors are recruited at varying efficiencies following hormone treatment, two different types of localization are observed—diffuse or highly localized. For a basal transcription factor, hormone treatment results in its recruitment to the amplified array, giving rise to a diffuse fluorescent signal that surrounds and encases a highly localized RNA polymerase II signal. Such strikingly different localization patterns most likely reflect intrinsic differences in the mechanism of factor concentration at the amplified array. The glucocorticoid receptor also shows a highly localized pattern, which varies in its overlap with that of RNA polymerase II. A “candy cane”-like structure is observed in cases of limited overlap, whereas, a single RNA polymerase II track, approximately 2-micron in length, is sandwiched between two glucocorticoid receptor tracks. The proximal end of the polymerase track contacts the proximal end of one receptor track, while the distal end contacts the distal end of the other receptor track. The central segment of the polymerase track shows no overlap with the receptor site. The segregation of fluorescent signals suggests a model of receptor and polymerase binding at different segments of a repeated chromatin loop, providing direct visual evidence for the presence of chromatin loops in the interphase nucleus of living mammalian cells.

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Targeting Heterochromatin Formation in Drosophila melanogaster
S. C. R. Elgin,1 K. A. Haynes, S. D. Findley, B. Brower-Toland, A. A. Caudy, H. Lin; 1Biology, Washington University, St. Louis, MO, 2Cell Biology, Duke University Medical School, Durham, NC, 3Lewis-Sigler Institute for Integrative Genomics, Princeton University, Princeton, NJ
Drosophila melanogaster is an excellent system for studying the potential chromatin packaging that leads to heterochromatin formation and gene silencing. Using a P element reporter containing an hsp70-driven copy of the white gene, we observe a variegating phenotype when the reporter has inserted into the pericentric heterochromatin, the small fourth chromosome, or the telomeres. Detailed analysis of the fourth has indicated that the repetitious element 1360 can serve as a target for heterochromatin formation, insertion of a reporter within 10 kb of a 1360 element correlates with variegation. 1360 is derived from a DNA transposon, and incomplete copies are widely distributed in D. melanogaster heterochromatin. A Northern
blot shows short RNAs (21-23 nt) corresponding to 1360, indicating processing by the RNAi machinery. Using a P element construct carrying a single copy of 1360 upstream of the hsp70-white reporter, we find that this 1360 element contributes to HP1- and RNAi-dependent variegation at a pericentric insertion site, as demonstrated by a decrease in silencing after HLP-mediated removal of the 1360 element. These results suggest that heterochromatin formation at transposable element remnants is targeted by the RNAi system. We have found that PIWI, a protein broadly implicated in the RNAi system, interacts specifically with HP1 in a yeast two-hybrid screen, via interaction of a PIWI PeVXL motif with the HP1a chromo shadow domain dimer. PIWI is detected by immunofluorescent staining of polytene chromosomes both at sites in the euchromatic arms, and in a punctate pattern in the pericentric heterochromatin. The latter staining, not the former, is sensitive to RNase H, suggesting association through an RNA-DNA hybrid. The direct interaction between PIWI and HP1a provides a link between the RNAi machinery and epigenetic regulation. Supported by NIH grants GM073190 and GM068388 to SCRE and HD33760 to HL.

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Asymmetric Cell Divisions in the Epidermis
T. Lechler, E. Fuchs; 1Cell Biology, Duke University Medical Center, Durham, NC, 2Laboratory of Mammalian Cell Biology and Development, Rockefeller University, New York, NY

The epidermis is a stratified squamous epithelium which forms the barrier that excludes harmful microbes and retains body fluids. To perform these functions, proliferative basal cells in the innermost layer periodically detach from an underlying basement membrane of extracellular matrix, move outward and eventually die. Once suprabasal, cells stop dividing and enter a differentiation program to form a barrier. The mechanism of stratification is poorly understood. Although in vitro studies have led to the view that stratification occurs through delamination and subsequent movement of epidermal cells, most culture conditions favor keratinocytes that lack the polarity and cuboidal morphology of basal keratinocytes in tissue. Such features could be important in considering an alternative mechanism, that stratification occurs through asymmetric cell divisions in which the mitotic spindle orients perpendicular to the basement membrane. We have found that basal epidermal cells use their polarity to divide asymmetrically, generating a committed suprabasal cell and a proliferative basal cell. We further demonstrate that integrins and cadherins are essential for apical localization of PKC, the Par3/LGN/Inscuteable complex and NuMA/dynactin to align the spindle.

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The Agrin/Perlecan-related Protein Eyes Shut Is Essential for Epithelial Lumen Formation in the Drosophila Retina
N. Husain, 1 M. Pelikika, 1 H. Hong, 1 T. Klimentova, 1 K. Choc, 1 T. R. Clandinin, 1 U. Tepass 1; 1Department of Cell and Systems Biology, University of Toronto, Toronto, ON, Canada, 2Department of Neurobiology, Stanford University, Stanford, CA

The formation of epithelial lumina is a fundamental process in animal development. Each ommatidium of the Drosophila retina forms an epithelial lumen, the interrhabdomeral space, which has a critical function in vision as it optically isolates individual photoreceptor cells. Ommatidia containing an interrhabdomeral space have evolved from ancestral insect eyes that lack this lumen as seen, for example, in bees. In a genetic screen we identified eyes shut (eys) as a gene that is essential for the formation of interrhabdomeral space. Eys is a predicted proteoglycan related to agrin and perlecan and a component of the apical extracellular matrix secreted by photoreceptor cells. The honeybee orthologue of eys is not expressed in photoreceptors, suggesting that recruitment of eys expression may have made an important contribution to insect eye evolution. Our findings indicate the deposition of an apical extracellular matrix is critical for the formation of a luminal cavity.

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The Role of Merlin and the ERM Proteins in Membrane Organization
M. Curot, 1 B. Cole, 1 C. Liu, 1 L. Saotome, 1 A. W. Chan, 1 A. I. McClatchey 1, 2; 1Massachusetts General Hospital Center for Cancer Research, Charlestown, MA, 2Department of Pathology, Harvard Medical School, Boston, MA

The neurofibromatosis type 2 tumor suppressor, Merlin and related ERM (Ezrin, Radixin, Moesin) proteins are thought to provide regulated linkage between the membrane and actin cytoskeleton, thereby organizing cortical domains that interface with the extracellular environment. Through mouse modeling, we have found that both Merlin and Ezrin play an important role in remodeling cell-cell contacts during tissue morphogenesis and tumorigenesis. We found that Ezrin is critically required for proper assembly of apical membranes and dynamic regulation of intercellular contacts during villus morphogenesis in the developing mouse intestinal epithelium. Both Ezrin overexpression and Merlin loss-of-expression have been linked to metastatic progression. At the membrane:cytoskeleton interface, Merlin is poised to modulate the transmission of mitogenic signals from the extracellular environment, in contrast to other tumor suppressors that function intracellularly to control the cell division cycle. We previously demonstrated that Merlin mediates contact-dependent inhibition of proliferation by organizing or stabilizing cell-cell junctions. In subsequent work we found that upon cell-cell contact Merlin coordinates the process of adherens junction stabilization with concomitant negative regulation the Epidermal Growth Factor Receptor (EGFR) by restraining the EGFR into a membrane compartment from which it can neither signal nor be internalized. This activity requires precise compartmentalization of Merlin itself into a defined membrane compartment. As a consequence, physiologic EGFR activation persists in confluent N2-/- cells, driving their continued proliferation. Our results suggest that excess EGFR signaling is critical for the hyperproliferation of N2-/- cells and tumors and reveal a novel mechanism of tumor suppressor action. Indeed, specific EGFR inhibitors such as Gefitinib (Iressa) block the proliferation of N2-/- cells, suggesting a therapeutic strategy for N2-mutant tumors. Our studies indicate that this model of Merlin function applies to several different cell types, including Schwann cells.

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Tissue Architecture Sculpts Inhibitory Gradient(s) to Determine Sites of Mammary Branching Morphogenesis
C. M. Nelson, 1 M. van Duijn, 1 J. L. Inman, 1 D. A. Fletcher, 1 M. J. Bissell 1; 1Life Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA, 2Department of Bioengineering, University of California, Berkeley, CA

The tree-like structures of many organs, including the mammary gland, are generated by branching morphogenesis, a reiterative process of branch initiation and invasion from a pre-existing epithelium. Using a micropatterning approach to control the initial three-dimensional architecture of mammary epithelial tubules, combined with an algorithm to quantify the extent of branching, we find that the geometry of tubules dictates the position of branch site initiation. We predict numerically and confirm experimentally that branch initiation occurs at sites with a characteristic geometry of tubules. Time-lapse confocal analysis verifies that branch site initiation is determined rather than stochastic, and shows that cells within the engineered tubules behave in a dynamic, coordinated manner. These results reveal that tissue architecture defines the local cellular microenvironment to control organ morphogenesis.

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The C. elegans Tropomodulin Homolog, TMD-1, Regulates Adherens Junctions during Epidermal Morphogenesis
E. A. Cox, J. Hardin; Zoology, University of Wisconsin, Madison, WI

Association between the cadherin-catenin complex and the actin cytoskeleton is essential for epithelial development, but how this is accomplished and regulated is poorly understood. C. elegans is an excellent model system for exploring this issue, since its adherens junctions contain a highly conserved cadherin-catenin complex that is essential for epithelial morphogenesis. To identify proteins that regulate the cadherin-catenin complex during embryonic development we have performed a screen to look for enhancers of α-catenin loss-of-function allele, hmp-1(fe4). In this screen, we identified a tropomodulin homolog, tmd-1. Tropomodulins cap the slow growing, pointed ends of actin filaments. In vertebrates, tropomodulins regulate various actin networks, including those present in muscle sarcomeres, lamellipodia, and the cortical actin-spectrin cytoskeleton (reviewed in Fisher and Fowler, 2003, Trends. Cell Biol., 13, 593-601). Our work on TMD-1 indicates a new role for tropomodulins as regulators of adherens junctions during morphogenesis. RNA interference (RNAi) directed against tmd-1 does not yield lethality on its own, however, it enhances the embryonic lethality of hmp-1(fe4) homozygotes. We find that hmp-1(fe4);tmd-1(RNAi) embryos fail to properly undergo epidermal elongation, a process by which contraction of the exterior epidermis helps to squeeze the embryo into a worm-like shape. This involves the junctional anchorage and contraction of an ordered array of actin bundles. Interestingly, the adherens junctions of hmp-1(fe4);tmd-1(RNAi) embryos do not maintain their strict apical-lateral position, and develop a frayed appearance as the embryos attempt to elongate. These areas of perturbed cadherin-catenin complex localization correlate with areas of perturbed actin morphology, which likely accounts for the failed elongation of these embryos. Together, these results suggest that TMD-1/tropomodulin acts with HMP-1/tropomodulin to promote junctional integrity during epidermal morphogenesis.
A Requirement for Crumbs-dependent Cell Polarity during Epithelial Morphogenesis

K. A. Campbell, H. Skaer; Department of Zoology, University of Cambridge, Cambridge, United Kingdom

Many epithelial tissues undergo dramatic morphogenetic changes, yet maintain their apicobasal polarity. This occurs during the development of the epithelial renal tubules in Drosophila melanogaster. Organogenesis of these tubes involves both cellular rearrangement by convergent extension movements and integration of an additional cell population into the epithelium from the surrounding mesoderm. Thus the tubules face two challenges: 1) They have to maintain their polarity during these potentially destabilising events 2) The integrating cells must polarise with respect to their new position within the tube. To understand how they do this we have analysed the molecular basis for cell polarity in both cell types throughout tubule development. We find two stages in the maintenance of polarity in the tubules, the first is independent of the apical protein complex, Crumbs, whereas the second phase requires its activity. In contrast, the other major protein complexes, Bazooka and Scribble are required for polarity during tubule development. In the absence of Crumbs, the tubules localise polarity markers during early development and form Zonula Adherens (ZA) junctions. However, at the onset of cell integration and rearrangement, the cells lose polarity and the ZA junctions disperse. In the epidermis Crumbs is required at the onset of germband extension. We suggest that the temporal difference in the requirement for Crumbs between tissues underlines its role in maintaining polarity during the remodelling of cell-cell contacts. Maintaining cell polarity during cell rearrangement is also critical for the correct integration of the second cell population. When the apical protein Crumbs or the non-ECRGA PRC are overexpressed in tubule cells, integrated with their new neighbours they invade the lumen. We are examining this further using real time imaging in both mutant embryos and in embryos where polarity is selectively disrupted in either tubule or integrating cells.

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The Function and Regulation of Podosomes in Dendritic Cells

M. A. West, H. Svensson, R. Zarra, A. R. Prescott, C. Watts; Division of Cell Biology and Immunology, University of Dundee, Dundee, United Kingdom

Podosomes are dynamic adhesive structures expressed in monocyte derived cells (macrophages, dendritic cells & osteoclasts) and some transformed cells. They are thought to play a role in cell migration and tissue invasiveness. We have been investigating the possible functions and control of podosome formation in murine dendritic cells. Our earlier studies showed that TLR signalling in dendritic cells (DC) triggers a reversible disassembly of podosomes that is mirrored by a reversible supramolecular complex formed by talin and RIAM. Finally, thrombin receptor stimulation of human platelets resulted in endogenous talin co-immunoprecipitating with RIAM. Thus, we have ordered a pathway from Rap1. In addition, shRNA-mediated knockdown of RIAM blocked complex assembly and integrin activation. By subcellular fractionation and immunofluorescence imaging, Rap1A(G12V) bypassed the requirement for PKC, establishing that Rap1 is downstream of PKC. Talin binding to integrins mediates Rap1-induced activation because Rap1A(G12V) failed to activate αIIbβ3 in cells expressing the integrin binding-defective talin(W59A). Over-expression of the Rap effector, RIAM, activated αIIBβ3 and bypassed the requirement for PKCα and Rap1. In addition, shRNA-mediated knockdown of RIM blocked complex assembly and integrin activation. By subcellular fractionation and immunofluorescence imaging, Rap1A(G12V) expression led to redistribution of talin to the plasma membrane and increased association with αIIbβ3. Furthermore, activated Rap1A increased the abundance of an ‘activation complex’ containing Talin and RIAM. Finally, thrombin receptor stimulation of human platelets resulted in endogenous talin co-immunoprecipitating with RIAM. Thus, we have ordered a pathway from agonist stimulation to integrin activation and established the Rap1-induced formation of an ‘integrin activation complex’, containing RIAM and talin, that binds to and activates the integrin.

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Distinct Mechanism of Lytic Granule Release by Gα12/13-Cytotoxic T Lymphocytes

A. M. Beauvais, N. M. Simc, R. Varzi, V. P. Thomas, M. L. Dustin, Y. Sykulev; 1Thomas Jefferson University, Philadelphia, PA, 2Skirball Institute of Biomolecular Medicine, New York, NY, 3Blood System Research Institute, San Francisco, CA

Cytotoxic T lymphocytes (CTL) are capable of killing virus-infected and transformed cells with extreme selectivity and sensitivity by releasing cytolysins that deliver a lethal hit to target cells. Growing evidence suggests that mechanisms of granule release may vary for different cytotoxic lymphocytes and may influence the sensitivity of specific lysis. We have previously shown that sensitivity of lysis by CD8+ CTL is dependent on the peripheral adhesion ring (pSMAC) of the immunological synapse (IS) which functions as a gasket to focus the granules to the target cell membrane. We have also observed that the granules are directed to the central supramolecular cluster (cSMAC) and that disruption of pSMAC formation abrogates effective lysis. We now show that the CD4+ CTL are less efficient lytic effectors than CD8+ CTL despite similar expression levels of lytic granules. Nevertheless, granules isolated from CD4+ CTL can lyse target cells comparable to granules isolated from CD8+ CTL suggesting that CD4+ CTL have a distinct mechanism for targeting of cytolysins. To compare granule release by CD8+ and CD4+ CTL, we visualised IS formation and granule redistribution by CD4+ and CD8+ CTL using glass-supported lipid bilayers to mimic target cells. Analysis of lytic granule redistribution revealed that CD4+ CTL polarize granules towards the interface similar to CD8+ CTL. However, we show that the IS of CD8+ CTL is unlike that of CD4+ CTL in cSMAC granule localization. Further, using TIRFM we have found that CD4+ CTL demonstrate accumulation of secretory lysosomal membrane proteins mostly in the pSMAC while the CD8+ CTL have central accumulation in the cSMAC surrounded by the pSMAC ring. Our data show that CD4+ CTL utilize a distinct pathway of granule release to the periphery of the IS resulting in less effective granule delivery to the target cell.

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Vav Proteins Control Integrin- and Adhesion-dependent ROI Production in Phagocytes

D. B. Graham, A. V. Milelic, V. Montgrain, K. Fujikawa, J. S. Brugg, R. Xavier, W. Swa; 1Department of Pathology & Immunology, Washington University School of Medicine and Siteman Cancer Center, St. Louis, MO, 2Department of Biochemistry, Hokkaido University School of Medicine, Sapporo, Japan, 3Department of Cell Biology, Harvard Medical School, Boston, MA, 4Department of Medicine, Harvard Medical School, Boston, MA

Reactive oxygen intermediates (ROI) generated by phagocytes possess potent antimicrobial properties, and patients bearing mutations in NADPH oxidase subunits develop chronic granulomatous disease (CGD) associated with recurrent, life-threatening infections. On the other hand, excessive ROI generation can contribute to tissue pathology in diverse settings, including sepsis and rheumatoid arthritis. More recently, ROI have also been implicated in intracellular signal transduction as second messengers which potentiate tyrosine kinase cascades by reversible oxidation of active site cysteine residues in protein tyrosine phosphatases. In phagocytes, the primary source of ROI produced during inflammatory responses is the NOX2 (gp91phox) NADPH oxidase complex. Unlike NOX1, NOX3 and NOX4 (which are constitutively active and regulated primarily by expression levels), or NOX5 and DUOX (which are activated by Ca2+), NOX2 is inactive in unstimulated phagocytes and becomes activated after exposure to inflammatory mediators as a result of assembly of cytosolic subunits with transmembrane gp91phox. Although the inductible assembly and function of the NOX2 is relatively well understood and involves translocation of regulatory subunits (p40, p47, p67, and Rac2) from the cytosol to plasma membrane-associated catalytic subunits (p91 and p22), the signaling pathways regulating NOX2 activation remain to be elucidated. A particular issue is the identification of GEF(s) responsible for the activation of Rac2 downstream of integrin or Toll-like receptors (TLRs). Herein, we identify the Vav family of Raf-related GEFs as critical regulators of integrin- and TLR-dependent ROI induction. We present data showing that Vav proteins exert global control over oxidant dependent ROI generation by regulating integrin-mediated “priming” of cytoxic NOX2 components and promoting GTP exchange on Rac2 in response to adhesion- or TLR-dependent ROI-generation in phagocytes. Strikingly, we demonstrate that Vav is specifically required for integrin-, but not TNFα- or GPCR-induced priming of NOX2, and that Vav-dependent ROI production is essential for neutrophil firm adhesion and spreading, and activation of several signaling effectors including the G protein-coupled receptors (GPR37A) that are important for the production of ROI.

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A Roadmap to Integrin Activation

C. J. Lim, J. Han, N. Watana, A. Soriani, B. Ramikov, D. A. Calderwood, W. Puron-McLaughlin, E. M. Lafuente, V. A. Boussiotis, J. S. Shattil, M. H. Ginsberg; 1University of California, San Diego, La Jolla, CA, 2Pharmacology, Yale University School of Medicine, New Haven, CT, 3Division of Hematology and Oncology and Transplantation Biology, Massachusetts General Hospital, Boston, MA

Agonist stimulation of integrin receptors, composed of transmembrane α and β subunits, leads cells to regulate integrins’ affinity (“activation”) thus controlling cell adhesion and migration, extracellular matrix assembly, and contributing to angiogenesis, tumor cell metastasis, inflammation, the immune response, and hemostasis. A final step in integrin activation is the binding of the cytoskeleton to integrins to form integrin-activated cytoskeleton complexes. Various lines of evidence that regulate integrin affinity have been described, yet a pathway connecting agonist stimulation to talin binding and activation has not been mapped. We used forward, reverse, and synthetic genes to engineer and order an integrin activation pathway in a model CHO cell system expressing a prototype activating integrin, platelet αIIbβ3. Phorbol myristate acetate (PMA) activated αIIbβ3 only after the increased expression of both recombiant protein kinase Cz (PKCz) and talin to levels approximating those in platelets. Inhibition of Rap1 GTPase by Rap1GAP overexpression reduced αIIbβ3 activation while expression of the constitutively active Rap1A(G12V) bypassed the requirement for PKCz, establishing that Rap1 is downstream of PKCz. Talin binding to integrins mediates Rap1-induced activation because Rap1A(G12V) failed to activate αIIbβ3 in cells expressing the integrin binding-defective talin(W59A). Over-expression of the Rap effector, RIAM, activated αIIBβ3 and bypassed the requirement for PKCz and Rap1. In addition, shRNA-mediated knockdown of RIM blocked complex assembly and integrin activation. By subcellular fractionation and immunofluorescence imaging, Rap1A(G12V) expression led to redistribution of talin to the plasma membrane and increased association with αIIbβ3. Furthermore, activated Rap1A increased the abundance of an ‘activation complex’ containing Talin and RIAM. Finally, thrombin receptor stimulation of human platelets resulted in endogenous talin co-immunoprecipitating with RIAM. Thus, we have ordered a pathway from agonist stimulation to integrin activation and established the Rap1-induced formation of an “integrin activation complex”, containing RIAM and talin, that binds to and activates the integrin.
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CD2-CD58 Interaction Activates Microdomain Formation and Signaling in the Absence of T-Cell Receptor Activation in Jurkat T Cells

Y. Kaizu, A. D. Douglass, R. D. Vale; Cellular and Molecular Pharmacology, University of California, San Francisco, San Francisco, CA

Activation of T-cell by antigen presenting cells (APC) is thought to involve the ligation of the T-cell receptor (TCR) and a subsequent tyrosine phosphorylation cascade, which includes activation of a critical scaffold protein called CD2. Another T cell surface protein called CD2, which interacts with human CD58 on the APC, also has been implicated in signaling but its role and requirement has been controversial and poorly understood. Through real time imaging in a reconstituted system consisting of Jurkat T-cells and a planar lipid bilayer (as a mimic of an APC), we found that activated TCR and CD2 separate into distinct microdomains and LAT tends to colocalize with the CD2 domains. In addition, CD58 alone, in the absence of TCR activation, caused co-clustering of CD2 and LAT into microdomains. The ACD8/D2 microdomains displayed robust phosphotyrosine staining, indicating a signaling function. These results suggest an important role of CD2 in T-cell activation, through the organization and activation of signaling molecules in the T-cell membrane.

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E-cadherin/β-catenin Signaling Defines a Distinct Pathway of Dendritic Cell Maturation

A. Jiang, S. Ono, J. Untermaier, S. Jiang, J. A. Whitney, J. Connolly, J. Banchereau, M. Mellman; 1Department of Cell Biology and Section of Immunobiology, Ludwig Institute for Cancer Research, Yale University School of Medicine, New Haven, CT, 3Genzyme, Framingham, MA, 4Baylor Institute for Immunological Research, Dallas, TX

The maturation of dendritic cells (DCs) following exposure to microbial products, immune modulators, or inflammatory cytokines plays a critical role in initiating the immune response. We now find that maturation can also occur under non-inflammatory conditions, triggered by alterations in E-cadherin-mediated cell adhesion. Disruption of E-cadherin-mediated interactions results in all of the morphological correlates of DC maturation, including upregulation of MHC class II, costimulatory molecules, and CCR7, a chemokine receptor important for migration of DCs from sites of residence in peripheral tissue to draining lymph nodes. These events apparently occur at least in part through activation of the β-catenin signaling pathway, based on activation of TCP-1/EFT-LEF-dependent transcription and maturation induced by transfection of β-catenin. Inhibition of GSK3β, which normally suppresses β-catenin signaling by phosphorylation, also induces DC maturation. Remarkably, however, E-cadherin/β-catenin-stimulated DCs exhibit an overall transcriptional profile highly distinct from DCs matured by microbial products (Toll-like receptor [TLR] ligands). Notably, they fail to produce any inflammatory cytokines (e.g. IL-6, IL-12 and TNF-α), suggesting that these DCs are unable to initiate immune responses. Although E-cadherin-matured DCs are as effective as TLR-stimulated cells in presenting antigen to T cells in vitro, in vivo, immunization with E-cadherin-stimulated vs TLR-stimulated DCs produced entirely distinct T cell responses. E-cadherin-stimulated DCs generated IL-10-producing CD4+CD25+ T cells (possibly regulatory T cells that suppress immune responses), while LPS-matured DCs generated INF-γ-producing effector T cells that mediate immune responses. Thus, DCs matured in an E-cadherin-like manner may represent the elusive "tolerogenic DCs" present in vivo under steady state conditions. We propose that the steady state migration of DCs from peripheral tissues to lymph nodes may generate the E-cadherin-stimulated mature phenotype as peripheral DCs break E-cadherin contacts with their neighbors upon emigration from resident epithelia.

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Regulation of Axonal Dynein Activity by a Calmodulin and Radial Spoke Associated Complex (CRC)

E. E. Dynes, F. E. Smith; Department of Biological Sciences, Dartmouth College, Hanover, NH

Calcium-induced modulation of ciliary motility involves regulation of dynein-driven microtubule sliding. Numerous studies indicate that calmodulin localizes to the axoneme and is a key calcium sensor. To understand the role of calmodulin in modulating dynein activity, we first used an immunoprecipitation approach to identify calmodulin interactors. In addition to the radial spokes and calmodulin, nine polypeptides were specifically precipitated using anti-calmodulin antibodies. These nine polypeptides form two distinct complexes, one of which localizes to the C1a projection of the central apparatus (Wargo et al., 2005). Three polypeptides (CAMP-IP2, -3, and -4) form a second complex that is precipitated from both central pairless and radial spokeless mutants. Using mass spectrometry we determined that IP2 is the Chlamydomonas homologue of AAT-1, a protein first identified in testis that forms a complex with an A-kinase anchor protein (AKAP) (Yukitake et al. 2002). Using several biochemical approaches we determined that CAMP-IP2 interacts with the radial spoke protein RSP3, an AKAP that links the spoke to the doublet microtubule. These results indicate that the CalmIP2, -3, -4 complex most likely localizes to the base of the radial spokes near the dynein regulatory complex. We then tested whether this complex is involved in regulating dynein activity using isolated axonemes in a microtubule sliding assay. Addition of CaM-IP2 antibodies to mutant axonemes with reduced dynein activity which lack either the radial spokes (pf14) or central apparatus (pf18) restores dynein activity to wild-type levels. Importantly, addition of CaM-IP2 antibodies also reduces dynein activity in axonemes lacking both the central apparatus and the inner arm dynein heavy chains (pf16a/b) but not inner arm dynein (pf16a/b). Based on these combined results, the calmodulin and spoke associated complex (CRC) most likely mediates regulatory signals between the central apparatus/ radial spokes and inner arm dynein.

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Chlamydomonas Hydin Is a Central Pair Protein Required for Flagellar Motility

K. F. Lechtreck, G. B. Witman; University of Massachusetts Medical School, Worcester, MA

Mutations in HYDIN cause lethal congenital hydrocephalus in mice, and HYDIN is a strong candidate for causing hydrocephalus in humans. In mice, expression is high in spermatozoa and in ciliated epithelia of ovary, lung and brain, suggesting a role for hydin in the development or function of motile cilia. The gene is well conserved in many ciliated and flagellated species, including the green alga Chlamydomonas. An antibody raised against a portion of Chlamydomonas hydin was specific for a protein of ~540 kD in western blots. In immunofluorescence and electron microscopy, the antibody specifically decorated the central pair apparatus of Chlamydomonas. Hydin was strongly reduced in axonemes of pf15 and pf18, which lack the central pair apparatus. An 80% knock-down of HYDIN resulted in short and paralyzed flagella. Residual motility was often limited to one of the two flagella, and the two flagella of a given cell were frequently arrested asymmetrically, with one flagellum in the 'hands up' and one in the 'hands down' position. Electron microscopy of HYDIN knock-down strains revealed defects in the central pair apparatus, ranging from missing projections to the loss of microtubules. The central pair Kinesin-like protein 1 was strongly reduced in axonemes from HYDIN knock-down cells, suggesting that its localization to the central pair requires hydin. A combination of biochemical and immunocytochemical analyses showed that hydin is anchored on both central microtubules and partially encompasses the central pair. In conclusion, Chlamydomonas hydin is a central pair protein required for flagellar motility. Hydrocephalus caused by mutations in HYDIN likely involve malfunctioning of brain cilia due to a defect in the central pair. Currently, we are analyzing the effect of a HYDIN mutation on central pair structure and flagellar motility in the mouse.

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IFT46 Transports Outer Arm Dynein into Flagella

Y. Hou, H. Qin, J. Follit, G. Pazour, J. Rosenbaum, G. Witman; 1Cell Biology, UMASS Medical School, Worcester, MA, 2Biological Sciences, Texas A&M University, College Station, TX, 3Program in Molecular Medicine, UMASS Medical School, Worcester, MA, 4Department of Molecular, Cellular, and Developmental Biology, Yale University, New Haven, CT

Intraflagellar transport (IFT) is responsible for transporting outer arm dynein into flagella. We isolated flagellae from three different strains of Chlamydomonas, with deletions of RRF46, MRC161, and MRC163, respectively. The IFT46 mutant showed defects in outer arm dynein localization. Immunofluorescence microscopy revealed that complex A and complex B proteins occurred in distinct but overlapping compartments in the peribasal body region; in the absence of IFT46, complex B protein IFT172 no longer overlaps with complex A, indicating that it does not interact directly with complex A in the cell body. Biochemical and MS analysis showed that IFT46 is phosphorylated at three sites. However, when the three sites were mutated to alanines or aspartic acids, the mutant constructs rescued IFT46 to wild-type swimming. Deflagellation experiments on the rescued cells showed that these phosphorylations are not critical for IFT46's function in flagellar assembly.
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A Core Complex of BBS Proteins Promotes Ciliary Membrane Biogenesis

M. V. Nachury,1 A. V. Loktev,2 F. Bazan,3 V. C. Sheffield,4 P. K. Jackson1,2; 1Department of Tumor Biology and Angiogenesis, Genentech, South San Francisco, CA, 2Department of Pathology, Stanford University School of Medicine, Stanford, CA, 3Department of Protein Engineering, Genentech, South San Francisco, CA, 4Department of Pediatrics and Howard Hughes Medical Institute, University of Iowa, Iowa City, IA

The Primary Cilia Play an Essential Role in Cell Migration through PDGFR-α Signaling

L. Schneider,1 A. Schwab,2 C. Stock,2 G. Pazour,3 E. Hoffmann,1 P. Satir,4 S. Christensen1; 1Department of Biochemistry, The August Krogh Institute, University of Copenhagen, Copenhagen, entrance are inhibited. In 1977 Albrecht-Buhler observed that primary cilia predominantly oriented parallel to the direction of movement in migrating 3T3 fibroblasts and hypothesized that Specific (gas) protein PDGFR-alpha localizes to the primary cilium and its signaling is regulated through the cilium in quiescent cells. Wt MEFs re-enter cell cycle upon incubation with Primary cilia are essential in PDGFR-alpha signaling during growth arrest in mouse embryonic fibroblasts (MEFs) (Schneider et. al. 2005 Curr Biol. 25;15(20):1861-6). The Growth Arrest network. Finally, the dense network seals to form a double lipid bilayer. Our data suggest that NE formation does not involve vesicle fusion but rather tethering, branching and fusion of tubules interact with DNA and eventually tubules align with the chromatin surface. In a second step, immobilized membrane tubules expand by branching and fuse to form a dense tubular plane above a flat chromatin by 4D-confocal microscopy. High-resolution time lapse imaging of NE reorganization events suggest that the NE forms by at least three steps. First, the tips of ER method for imaging nuclear envelope formation in real time. We immobilized dsDNA on a glass surface and transformed the spotted DNA into chromatin by the addition of Xenopus egg experiments will test for BBSome-dependent transport of known and novel ciliary signaling factors. As predicted, αRNA-mediated depletion of PCM1 disintegrates centriolar satellites and prevents the assembly of primary cilia. Conversely, loss of the BBSome subunit BBS1 keeps centriolar satellites functionally intact while still preventing ciliogenesis. These results place PCM1 upstream of the BBSome and imply that the BBSome is a critical ciliogenic cargo of centriolar satellites. To further understand the ciliogenic function of the BBSome, we performed structural modeling on the most conserved subunit and uncovered two Pleckstrin Homology (PH) folds. PH domains usually recognize phosphatidylinositol phosphate and protein-lipid interaction assays confirmed that this subunit recognize specific phospholipids. In vivo, BBSome-lipid interactions appear to take place at the ciliary membrane since detergents selectively extract the ciliary BBSome pool. In conclusion, we propose that centriolar satellites deliver the BBSome to the basal body where it is transferred onto the ciliary membrane and may coordinate the intrflagellar transport of signaling factors involved in weight regulation, phototransduction and kidney homeostasis. Future experiments will test for BBSome-dependent transport of known and novel ciliary signaling factors.

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The Roles of Cilia in Mouse Developmental Signaling Pathways

K. Anderson, D. Huangfu, T. Caspary, J. Oshima; Developmental Biology, Memorial Sloan-Kettering Cancer Center, New York, NY

Although the core of the Hedgehog signal transduction pathway is conserved between Drosophila and vertebrates, recent studies have identified at least a dozen proteins that are required for Hedgehog signaling in the mouse, but not in Drosophila. Among these vertebrate-specific components of the pathway are the Intraflagellar Transport (IFT) proteins, which are known to be required for the assembly and maintenance of cilia. Mouse mutants that lack any one of several different IFT proteins fail to specify ventral neural cell types, which depend on Sonic hedgehog signaling. Genetic analysis shows that the IFT proteins are required for cells to respond to Hedgehog and that they control the activity of the Gli transcription factors that mediate Hedgehog responses. The data indicate that IFT proteins are required for Hedgehog signaling because Hedgehog signal transduction components are localized to non-motile primary cilia. Evidence from our lab and other labs has suggested that cilia are required for other signaling pathways as well. We are testing whether cilia are required for canonical and noncanonical Wnt signaling, as well as other developmental signaling pathways.

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The Primary Cilia Play an Essential Role in Cell Migration through PDGFR-α Signaling

L. Schneider,1 A. Schwab,2 C. Stock,2 G. Pazour,3 E. Hoffmann,1 P. Satir,4 S. Christensen1; 1Department of Biochemistry, The August Krogh Institute, University of Copenhagen, Copenhagen, Denmark, 2Institut für Physiologie, Muenster, Germany, 3Molecular Medicine, University of Massachusetts, Worcester, MA, 4Department Anatomy and Structural Biology, Albert Einstein College of Medicine Yeshiva University, Bronx, NY

Primary cilia are essential in PDGFR-alpha signaling during growth arrest in mouse embryonic fibroblasts (MEFs) (Schneider et. al. 2005 Curr Biol. 25;15(20):1861-6). The Growth Arrest Specific (gas) protein PDGFR-alpha localizes to the primary cilium and its signaling is regulated through the cilium in quiescent cells. Wt MEFs re-enter cell cycle upon incubation with PDGFR-AA, which is a specific ligand for PDGFR-alpha. In growth arrested Tg3774E mutant MEFs, which have defects in cilary assembly PDGFR-alpha cascades leading to cell cycle entrance are inhibited. In 1977 Albrecht-Buhler observed that primary cilium predominantly oriented parallel to the direction of movement in migrating 3T3 fibroblasts and hypothesized that they are involved in directional control of these cells. Benzing and Walz (2006) hypothesized that the primary cilium in acts as a cellular GPS. Here we investigated a possible role of the primary cilium in PDGFR-alpha-mediated regulation of cell migration in fibroblasts. Initially we used wound healing assays to show that the primary cilium in growth arrested wt MEFs orient parallel to one another and perpendicular to a wound within 30-60 minutes after wounding. Migration speed is lowered during quiescence compared to cells in interphase growth. PDGFR-AA incubation increases the migration speed and translocation directionality of wt cells during cell growth arrest. Growth arrested Tg3774E MEFs have significantly higher migration speed compared to wt MEFs, and migration speed is unaffected by PDGFR-AA incubation. Further Tg3774E MEFs have decreased translocation directionality. Using micro-pipettes to inject small amounts of PDGFR-AA creating a gradient, we show that growth arrested wt MEFs respond immediately to PDGFR-AA and migrate towards the pipette. Tg3774Epcp cells do not react to gradients of PDGFR- AA. These results strongly indicated that the primary cilium is necessary for PDGFR-alpha-influenced cell migration and control of directional movement, which is essential in developmental processes and wound-healing.

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Role of Mmr1p and Cortical ER in Retention of Mitochondria at the Cell Poles during Inheritance in Budding Yeast

T. Swayne,1 I. Boldogh, 1 S. Thoms, 2 R. Erdmann, 2 L. A. Pon 1; 1Department of Anatomy/Cell Biology, Columbia University College of Physicians & Surgeons, New York, NY, 2Institute of Physiological Chemistry, University of Bochum, Bochum, Germany

During cell division, yeast mitochondria move to and are anchored at opposite cellular poles, the bud tip and mother cell tip. These processes contribute to equal segregation of the organelle during inheritance. We find that two proteins implicated in mitochondrial inheritance, a type V myosin (Myo2p) and a mitochondrial protein that binds to Myo2p (Mmr1p), are not required for poleward mitochondrial movement leading to inheritance or for normal mitochondrial membrane organization detected by EM. In contrast, mutation of these proteins results in reduced retention of mitochondria in the bud tip, and enhanced retention of mitochondria in the mother tip. Mislocalization of Myo2p from the bud tip does not result in release of accumulated mitochondria from that site. Thus, Myo2p does not interact directly with mitochondria in the bud tip. Rather, ultrastructural analysis indicates that mitochondria interact with cortical ER (cER), flattened sacs of ER that underlie the yeast cell cortex, during retention of mitochondria at the poles. Mitochondria-associated microsomes (MAM), ER-derived membranes that co-fractionate with yeast mitochondria and are released from the organelle by low pH, have been implicated in phospholipid delivery from ER to mitochondria. We find that 1) Mmr1p is recovered with mitochondria and MAM during subcellular fractionation, 2) Mmr1p and MAM are released from the mitochondrial fraction by low pH, and 3) deletion of MMR1 results in a decrease in the amount of MAM recovered with mitochondria during subcellular fractionation. Deletion of MMR1 has no obvious effect on cER localization or morphology. Rather, our findings support a role for ER-mitochondrial interactions in anchorage of mitochondria at the poles during cell division and inheritance, and for Mmr1p in this process. Finally, Myo2p may contribute to Mmr1p function as a motor to drive movement of Mmr1p from the mother cell tip to the bud tip.

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Real Time Imaging of Nuclear Envelope Formation

M. W. Hetzer, D. J. Anderson; Molecular and Cell Biology Laboratory, Salk Institute for Biological Studies, La Jolla, CA

Nuclear envelope (NE) reformation on the surface of chromatin is a massive membrane-restructuring event that occurs at the end of mitosis in metazoans. Two models have been proposed to explain this poorly understood process. Electron and fluorescence microscopy studies on fixed nuclear assembly intermediates supported the idea that NE formation involves the fusion of chromatin-bound nuclear vesicles. In contrast, in vivo data suggest that the NE re-emerges from the tubular ER network. To discriminate between these possibilities we have developed a novel method for imaging nuclear envelope formation in real time. We immobilized dsDNA on a glass surface and transformed the spotted DNA into chromatin by the addition of Xenopus egg extracts. In the presence of fluorescently-labeled membranes a NE containing nuclear pore complexes formed specifically on the chromatin spot. NE formation was monitored in a single x-y plane above a flat chromatin by 4D-confocal microscopy. High-resolution time lapse imaging of NE reorganization events suggest that the NE forms by at least three steps. First, the tips of ER tubules interact with DNA and eventually tubules align with the chromatin surface. In a second step, immobilized membrane tubules expand by branching and fuse to form a dense tubular network. Finally, the dense network seals to form a double lipid bilayer. Our data suggest that NE formation does not involve vesicle fusion but rather tethering, branching and fusion of membrane tubules.
A Novel Mitotic Function for Rab5 in Structural Reorganization of the Endoplasmic Reticulum and Nuclear Envelope Disassembly

A. Audhya, A. Desai, K. Oegema; Cellular and Molecular Medicine, Ludwig Institute for Cancer Research, La Jolla, CA

Membranes of the endoplasmic reticulum (ER) typically exist in two morphologically unique forms, either a tubular network that extends toward the cell periphery or as sheets that encircle the nucleus. This fundamental property relies on a number of factors that are enriched on either the peripheral or nuclear ER. However, when the nuclear envelope disassembles during mitosis, the ER must transiently reorganize and lose this distinction. Inner nuclear membrane proteins are redistributed to the peripheral ER, where they reside until nuclear envelope reformation. How this transition is regulated remains unclear. Here we identify two pathways required for ER remodeling that involve a class of integral membrane ER proteins, DJ1/NogoA and Reticulon, and the Rab-type GTPase Rab5. We show that disruption of either pathway perturbs ER morphology in a phenotypically similar fashion, which is most pronounced during mitosis when the ER is normally restructured and compacted. Importantly, in the absence of ER remodeling, nuclear envelope disassembly fails. Our results argue that ER reorganization is a necessary step essential for complete nuclear envelope disassembly, and indicate a novel role for Rab5 in ER reorganization that is independent of its previously characterized functions during endocytosis.

The Mother Centriole Plays an Instructive Role in Defining Cell Geometry

J. L. Feldman, S. Geimer, W. F. Marshall; 1Biochemistry, University of California, San Francisco, San Francisco, CA, 2Biologie/Elektronenmikroskopie, Universität Bayreuth, Bayreuth, Germany

A fundamental question in cell biology is how cell geometry is established and maintained. Precise organelle positioning is critical for signaling, growth, division, and in establishing cell polarity. The centriole provides an ideal case for investigating the problem of organelle positioning. Unlike more complex organelles, centriole position can be easily measured and described. Moreover, centriole position is known to respond to cell polarity and developmental cues, and is thought to link such signals to the arrangement of the cytoskeleton. Currently, little is known about the mechanism by which centrioles are properly positioned within the cell. Using a computer-aided screen in the unicellular green alga Chlamydomonas reinhardtii, we identified thirteen mutants, which we term askev (ask), with defective centriole positioning. Combining genetic analysis, three-dimensional imaging, and a novel algorithm for quantifying cellular geometry, we demonstrate that the mother centriole guides the daughter centriole to the proper subcellular location. Specifically, in mutants where mother and daughter centrioles are separated, only mother centrioles localize properly. We further show that in mutants in which the centrioles are detached from the nucleus, the nucleus becomes randomly positioned while the mother centrioles retain correct positioning, indicating that the nucleus depends on the mother centriole for proper positioning within the cell and not vice-versa. Classic experiments in ciliates have demonstrated that local cell geometry is controlled by a stable structure with defined orientation that can propagate positional information over many generations. Our data indicate that the mother centriole serves this purpose, acting as a node to coordinate the positioning of many subcellular structures. We have identified flanking sequence to the insertion site in ask1, ask2, and ask7 and will present our progress towards identifying the affected loci in these mutants.

The P21-activated Kinases Cla4 and Ste20 Regulate Vacuole Inheritance in the Budding Yeast S. cerevisiae

C. R. Bartholomew, C. F. J. Hardy; Cell and Developmental Biology, Vanderbilt University School of Medicine, Nashville, TN

The vacuole (lysosome) in budding yeast is partitioned along actin cables, using the type V myosin, Myo2, via a tubular segregation structure that enters the bud and fuses to the daughter vacuole. The vacuole-specific Myo2 receptor, Vac17 is degraded in the bud during mitosis, preventing vacuole movement to the bud neck later in the cell cycle. The P21-activated kinases (PAKs) Cla4 and Ste20 accumulate specifically in the bud site of polarized bud growth. Here we show that Cla4 also accumulates in the bud at a perivacuolar site associated with the segregation structure. Cla4 does not interact with vacuoles in the mother or with vacuoles in mutants defective for segregation structure formation and vacuole inheritance. In support of a role for PAKs in regulating vacuole inheritance, cells over expressing CLA4, and to a lesser extent STE20, fail to form segregation structures. Interestingly, over expression of CLA4 results in Vac17 degradation an event which prevents segregation structure formation and vacuole inheritance. Strikingly, these defects are suppressed in cells expressing a non-degradable form of Vac17 (Vac17APEST). In addition, we find that PAK activity is required for the degradation of Vac17 during mitosis. These results suggest that segregation of negative regulators of vacuole movement to the bud positions the cell to inactivate the organelle partitioning machinery after, but not before, the vacuole has been partitioned to the bud.

Pex11β Functions as a Peroxisomal Scaffold Protein Involved in Membrane Elongation of Pre-Existing Peroxisomes Prior to Organellar Fission

B. Agricola, H. K. Dellel, G. H. Luers, M. Schrader; 1Department of Cytobiology, University of Marburg, Marburg, Germany, 2Department of Anatomy and Cell Biology, University of Marburg, Marburg, Germany

Members of the Pex1 family of peroxisomal membrane proteins have been implicated in the regulation of peroxisome abundance, division and proliferation, but their biochemical properties are still a matter of debate. We have shown recently that Pex1p, one of the three mammalian isoforms, is primarily involved in membrane elongation of peroxosomes instead of directly mediating peroxisome division. To further elucidate its function, and to manipulate peroxisomal division at different steps, we have examined N- and C-terminally modified versions of Pex1p. Removal of the C-terminal transmembrane domain or the cytoplasmic tail did not interfere with targeting but inhibited elongation of peroxisomes. Addition of a fluorescent tag to the C-terminal (but not to the N-terminus) led to the formation of tubular peroxisomal aggregates (TPAs), which had lost their ability to constrict or divide. The number of peroxisomes was drastically reduced, often leaving the cell with only one large peroxisomal structure. Ultrastructural and fluorescence studies revealed that the TPAs were composed out of highly ordered elongated peroxisomal membranes which each originated from a pre-existing peroxisome. Pex11β localized predominantly to the tail-like membrane compartment, whereas matrix proteins and PMP70 were exclusively targeted to the connected mature peroxisome. ER marker proteins were absent from these membrane structures. Time course experiments revealed that Pex1p early on localized to the membranes of pre-existing, spherical peroxisomes, where it was observed to concentrate at the side of tubule formation. These observations imply that Pex11β is a peroxisomal scaffold protein required for the deformation and elongation of the peroxisomal membrane. Our data further demonstrate that peroxisomal growth and division is a complex multistep process where the consecutive steps are linked to each other, and may be triggered by an assembly of distinct machineries at the peroxisomal membrane.

Kinetics of Morphogen Gradient Formation

M. González-Gaitán1,2; 1Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany, 2Department of Biochemistry, University of California, San Francisco, San Francisco, CA, 2Biochemistry, CNRS UMR 6061, Rennes, France, 3Biologie, CNRS UMR 8542, Ecole Normale Supérieure, Paris, France

During the larval development of Drosophila the morphogen Decapentaplegic (Dpp), a TGF-beta homolog, provides cells with information about their position along the anterior-posterior axis of the developing wing. Dpp is expressed in a restricted source of cells from where it spreads to form a gradient of concentration in the target tissue. How the morphogen molecules move and form graded profiles of concentration is not fully understood. It has been proposed that two different modes of transport through the wing epithelium play a role in Dpp gradient formation - extracellular diffusion and intracellular trafficking (transcytosis). To determine the relative contribution of each of these modes of transport to the establishment of a stable and robust gradient, high-resolution quantitative data about the kinetics of morphogen spreading are necessary. We have approached the kinetic analysis of Dpp gradient formation at the tissue level by employing FRAP (Fluorescence Recovery After Photobleaching). Using this method, we have been able to determine the effective diffusion coefficient, degradation rate, production rate and mobile fraction of Dpp and other morphogens. These crucial kinetic parameters provide a full quantitative description of the morphogen gradient formation. In addition, by applying FRAP in different conditions, we have gained insight on the cell biological processes that are involved in Dpp transport, as well as in maintaining the stability and robustness of the gradient.

Activation of Notch Signalling by Transcytosis

R. Le Borgne, F. Schweiguth; 1Genétique et Développement, CNRS UMR 6061, Rennes, France, 2Biologie, CNRS UMR 8542, Ecole Normale Supérieure, Paris, France

Cell-cell signalling is an essential process in the formation of multicellular organisms. Notch is the receptor of an evolutionarily conserved signalling pathway regulating numerous developmental decisions. Notch and its transmembrane ligands are distributed widely throughout development, yet Notch activity is tightly controlled and restricted in time and space. In Drosophila, Notch signalling regulates binary fate decisions at each asymmetric cell division in sensory organ lineages. Following division of the sensory precursor cell (pI), Notch is activated in one daughter cell (pI1a) and inhibited in the other (pIb). The E3 ubiquitin ligase Neuralized segregates unequally in the pIb cell. Neuralized upregulates endocytosis of the Notch ligand Delta in the pIb cell and acts in the pIb cell to promote the activation of Notch in the pIa cell by a yet unknown mechanism. We observed that in the pIb cell, Delta mostly resides in endosomal structures at steady state. By contrast, in the pIa cell, Delta localizes predominantly at the basal-lateral cortex and is physically separated from Notch that resides at the apical cortex. In neuralized mutant cells, Delta accumulates at the basal-lateral cortex in both pl daughters preventing its interaction with apically localized Notch. We propose that in order to become
active. Delta has to be transported to the basal membrane towards the apical cortex by a transcytotic route, and that Neuralized promotes the first step in this intracellular transport event. Regulating the sorting of the receptor and its ligands along the apico-basal axis could be an efficient mechanism to control the activation Notch signalling in a spatio-temporal manner.

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FGF Mediates Asymmetric Heart Specification in the Primitive Chordate Ciona intestinalis
B. Davidson, M. Levine; UC Berkeley, Berkeley, CA
Asymmetric cell divisions are often crucial for specification of embryonic cell lineages. However, no such role has been demonstrated during vertebrate cardiac specification. Here we employ the simple chordate, Ciona intestinalis, to investigate the role of asymmetric cell divisions in chordate heart development. Midway through embryogenesis the four cells of the Ciona heart lineage undergo an asymmetric division, generating four small rostral heart precursor cells and four large caudal tail muscle precursors. We demonstrate that this division is associated with an FGF driven specification event. Targeted expression of a dominant negative FGF receptor blocks the asymmetric division and heart specification. Specification is also blocked by inhibition of MAPK signaling, or targeted expression of a constitutive repressor form of the downstream transcriptional effector Ets1/2 (Ets-WRPW). Conversely, application of bFGF to isolated heart lineage cells or targeted expression of a constitutively active form of Ets1/2, Ets-VP16, doubles the number of heart progenitor cells. Intriguingly, blocking the MAPK pathway or Ets activation does not appear to inhibit asymmetric cell division. We are currently investigating this apparent uncoupling of specification and asymmetry downstream of FGF. We are also exploring the functional importance of asymmetric cell division in Ciona cardiogenesis and whether this role is conserved in the vertebrates.

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Retinoic Acid Signaling Limits Atrial and Ventricular Cells through Different Cellular Mechanisms
J. S. Waxman, B. R. Keegan, D. Yelon; Developmental Genetics, Skirball Institute of Biomolecular Medicine/NYU School of Medicine, New York, NY
All vertebrate hearts begin as a simple, linear tube composed of a single atrium and ventricle. The proper proportion of atrial and ventricular cells needs to be determined in order to establish a normal, functional heart. Work from chicks and mice has suggested that retinoic acid (RA) signaling determines the relative proportions of the atrial and ventricular chambers through instilling atrial identity. Here, we use loss and gain of function experiments to test the hypothesis that RA signaling is necessary and sufficient for determining atrial cell identity in the zebrafish heart. To our surprise, loss of function experiments indicate that RA signaling is not required to create atrial cells in zebrafish. Instead, loss of RA signaling results in increased numbers of both atrial and ventricular cells. Using fate mapping to examine the cellular mechanisms underlying these increases, we find that the increase in atrial and ventricular cells occur through different mechanisms. Loss of RA signaling causes increased numbers of progeny from atrial progenitors and also causes increased specification of ventricular progenitors. In complementary gain of function experiments, we find that high RA signaling can eliminate all atrial and ventricular cells. However, modest increases in RA signaling can increase the number of atrial cells without affecting the number of ventricular cells, also suggesting there may not be a tradeoff between atrial and ventricular cells. Therefore, our results indicate that the relative proportions of cardiac chambers in the zebrafish heart is determined through independent effects of RA signaling upon atrial and ventricular cells.

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Functional Roles of Wnt and Slug Target Genes during Avian Neural Crest Development
L. A. Tanyhill, M. Bronner-Fraser; Biology, California Institute of Technology, Pasadena, CA
The vertebrate neural crest is a population of migratory cells that arises, in part, via an inductive interaction between neural plate and ectoderm. Previous work in our lab has demonstrated that Wnt signals are necessary and sufficient for induction. However, the molecular nature of downstream targets of Wnt signaling are unknown, and few quantitative studies have examined the expression of candidate genes involved in this process. To this end, we screened the Affymetrix microarray to identify novel targets of Wnt signaling during neural crest development, employing intermediate neural plate tissue cultured in collagen in the presence of either Wingless or control conditioned medium as the starting material. Secondary in situ hybridization analysis of those genes that are induced by Wnt signaling > 2-fold reveals many novel markers of the neural crest. One Wnt target gene is the transcriptional repressor, Slug, known to regulate various genes in the pre-migratory neural crest and be involved in epithelial-to-mesenchymal transitions. We find that depletion of Slug using morpholino antisense oligonucleotides results in the up-regulation of many dorsal neural tube genes, as assessed by QPCR. Moreover, the cell adhesion molecule cadherin6B is de-repressed by 30 minutes after blocking Slug translation. Examination of the chick cadherin6B genomic sequence revealed the presence of three pairs of clustered E boxes, representing putative Slug binding sites. Furthermore, in vivo and in vitro biochemical analyses demonstrate that Slug directly binds to these sites and regulates cadherin6B transcription. These results are the first to describe a direct target of Slug repression in vivo and in the context of the epithelial-to-mesenchymal transition that characterizes neural crest development. Collectively, these experiments have enabled us to generate a molecular description of genes activated by Wnt signaling that facilitate the transformation of a non-motile precursor cell into a migratory neural crest cell.

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Lipoproteins in Hedgehog Signaling
S. Eaton, D. Panavoko, C. Eugster; Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany
Mechanisms controlling the spread and signaling activity of morphogens are critical for growth and pattern formation. Morphogens of the Wnt and Hedgehog families are covalently linked to lipids, which are essential for normal trafficking and signaling of these proteins. While the lipid anchors confer a strong affinity for membranes, we have shown that Wingless and Hedgehog can be released from the cell membrane on the Drosophila lipoprotein Lipophorin. This association is necessary for normal long-range signaling. Our recent data show that Lipophorin does not just affect Hedgehog transport, but has an integral role in the Hedgehog signaling pathway. Lipophorin particles themselves are required for the degradation of Cubitus interruptus that occurs in the absence of Hedgehog. Lipophorin may stabilize CI, at least in part, by binding to Lipophorin and altering its signaling activity. Furthermore, binding of the glypicanc Dally to Lipophorin increases Hedgehog signaling efficiency without affecting its spread. Thus, Lipophorin is a multicomponent signaling particle - our data raise the possibility that lipoprotein association may just affect Hedgehog transport, but has an integral role in the Hedghog signaling pathway. Lipophorin particles themselves are required for the degradation of Cubitus interruptus that occurs in the absence of Hedgehog. Lipophorin may stabilize CI, at least in part, by binding to Lipophorin and altering its signaling activity. Furthermore, binding of the glypicanc Dally to Lipophorin increases Hedgehog signaling efficiency without affecting its spread. Thus, Lipophorin is a multicomponent signaling particle - our data raise the possibility that lipoprotein association may increase the complexity of signals that can be generated by morphogens.

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Intraflagellar Transport (IFT) and Flagellar Assembly: New Insights into the Role of Cilia/Flagella in Health and Disease
J. L. Rosenbaum; Department of Molecular Cellular/Developmental Biology, Yale University, New Haven, CT
Much of the new research interest in cilia and flagella has been derived from the discovery of the Intraflagellar Transport (IFT) process in 1993. The molecular basis of IFT, required for the assembly of almost all eukaryotic cilia and flagella, has led to the discovery of genes involved in a variety of human diseases in which the role of cilia was unsuspected. Among these is the most common life threatening inherited disease of humans, PKD (polycystic kidney disease) which has been shown to involve the absence or abnormal function of the primary cilium of kidney tubules. In the retina, the primary cilium gives rise to the rod outer segments, and it is now known that a defective IFT process within the connecting cilium is related to rod outer segment degeneration and blindness (RP, retinitis pigmentosum). In addition, all developmental condition, Situs Inversus, (organs misplaced on wrong side of body), has been related to the absence or abnormal function of cilia on the embryonic node. Recent genomic and proteomic analyses have led to the discovery that cilia are also involved in pathologies such as Bardet-Biedl Syndrome (BBBS) and Congenital Hydrocephalus. New work centers on the role of cilia in the control of the cell cycle. Almost all cells in the Go stage of the cell cycle have a primary cilium that is resorbed prior to cell division, when the ciliary basal bodies duplicate and become the poles of the mitotic apparatus. This resorption may be involved in the cell cycle, as recent results indicate that there are ciliary IFT proteins required for the completion of cell division. Finally, IFT may be directly involved in the sensory processes of primary cilia as IFT is required for the movement of channels and receptors in the plane of the ciliary membrane. (Support, NIH-GM-38135).

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Preformed Homo- and Heterodimers of EGF/ErbB2 Receptors as Major Forms on the Cell Surface
T. Sudhaharan, P. Liu, R. Koh, L. C. Hwang, T. Wohland, I. Maruyama; Centre for Molecular Medicine, Singapore, 2Chemistry, National University of Singapore, Singapore, 3Institute of Molecular and Cell Biology, Singapore, 4Molecular Neuroscience Unit, Okinawa Institute of Science and Technology, Okinawa, Japan
The epidermal growth factor (EGF) /ErbB receptor family plays pivotal roles in the development of organisms ranging from worms to humans. Dysregulation of the receptor’s activity is frequently implicated in a variety of cancers. It is widely thought that ligand binds to the monomeric form of the receptor and induces its dimeric form for activation. Prior to ligand binding, however, it remains controversial whether the receptor has a monomeric or dimeric structure. We have recently found that the majority of the EGF receptor (EGFR) and ErbB2, another
member of the family also called HER2 or Neu, have preformed homo- and heterodimeric structures at physiological expression levels (<10^12 molecules per cell) on the cell surface. When EGF and ErbB2 fused with a fluorescent protein (FP) were expressed on the cell surface at physiological levels, fluorescence resonance energy transfer (FRET) was clearly observed between the donor and acceptor FPs. Furthermore, cross-correlation between FPs separately fused to EGF or ErbB2 was also observed by fluorescence cross-correlation spectroscopy (FCCS), indicating EGF and ErbB2 molecules diffuse together as homo- or heterodimers in the cell membrane. Our results demonstrate that the unactivated cell-surface receptors can form spontaneously homo- and heterodimers as major forms.

62 Interaction of EGF-R and COX-2 Signaling Pathways in Human Fetal Intestine
R. M. A. Kajumte, P. Lindkainen, S. Leppä, A. Ristimäki, P. Miettinen; Molecular and Cancer Research Program, University of Helsinki, Helsinki, Finland, 2Hospital for Children and Adolescents, Helsinki University Central Hospital, Helsinki, Finland, 3Department of Oncology, Helsinki University Central Hospital, Helsinki, Finland, 4Program for Developmental and Reproductive Biology, University of Helsinki, Helsinki, Finland

Hydrocortisone (HC) has been used in clinical trials to improve the outcome of preterm infants and indomethacin, an inhibitor of both cyclooxygenase-1 (COX-1) and -2 (COX-2), is used to treat patent ductus arteriosus (PDA). Side-effects related to NSAID treatment include gastric lesions in the mouse, necrotizing enterocolitis (NEC) and spontaneous intestinal perforation (SIP). The identification of the cellular pathways in the development of NEC and gastric lesions is critical. Data pertaining to the role of indomethacin and glucocorticidal drug treatment in the development of gastric lesions is scarce. The aim of this study was to elucidate the crosstalk between EGF-R, glucocorticoids and COX-2 expression during human intestinal development and the interplay between them in the intestine using 1) normal small intestine cell line 2) paraffine sections of human fetal intestine, wt and EGF-R null mice. We showed that HC, indomethacin or PGE_2 alone had no significant effect on cell proliferation. However, when HC was combined with EGF, they synergistically increased cell proliferation and migration but HC had no effect on MAPK (pERK) or PI3-K (pAK) pathway when combined to EGF. High doses of indomethacin also induced apoptosis. EGF upregulated COX-2 mRNA and this could be inhibited by EGF-R, MAPK and PI3-K inhibitors. EGF-induced COX-2 mRNA upregulation was suppressed by HC, but HC alone had no significant effect. Correlating with these results, also PGE_2 production was increased by EGF and blocked by EGF-R inhibitor. Furthermore, HC reduced EGF-induced PGE_2 production and indomethacin suppressed it to baseline. Also, COX-2 immunoreactivity was greatly reduced in the EGF-R (-/-) jejunal suggesting that EGF-R signalling is needed for COX-2 expression. These results suggest that EGF-R regulates COX-2 expression and activity in the intestine.

63 Spatial Localization of EGFRs Dictates Activation of Growth Versus Apoptotic Pathways
B. P. Ceresa, D. Hyatt; Cell Biology, University of Oklahoma Health Sciences Center, Oklahoma City, OK

The epidermal growth factor receptor (EGFR) plays physiological roles in regulating cell growth, proliferation, DNA and protein synthesis, and differentiation. Ligand binding to the EGFR induces the dimerization of two monomers, activation of the intrinsic kinase, and transphosphorylation of carboxyl terminal tyrosines on its receptor pair. These phosphoryrosines are docking sites for downstream signaling proteins that, when activated, integrate to produce receptor specific physiologies. In addition to signaling, ligand binding to the EGFR initiates the internalization of the receptor through clathrin-coated pits and into the endocytic pathway. Here the receptor is sorted for its proper fate, such as recycling to the plasma membrane or lysosomal degradation. Endocytic trafficking is known to be an important regulator of EGFR-mediated effector activation, but there is little data to indicate these changes have a biological consequence. Both the non-specific effects of the inhibitors of EGFR internalization, as well as the limited cell responses have restricted the interpretation of how endocytosis affects EGFR-mediated cell physiology. We have re-examined this important question using an approach that specifically inhibits EGFR internalization without perturbing other endocytic processes and used a cell line that robustly responds to EGFR stimulation. In order to determine the physiological consequence of limiting EGFR signaling to a single cellular location, we have used EGF covalently conjugated to 900 nm polystyrene beads (EGF-beads). EGF-beads can bind and stimulate the EGFR, but do not allow entry via clathrin-coated pits (~50-100 nm). EGF induced apoptosis has been well established in MDA-MB-468 cells. When these cells are treated with EGF-beads, despite comparable levels of EGFR phosphorylation, cell growth is enhanced as measured by 3H-thymidine incorporation. Further, intracellular EGFR can stimulate caspase-3 activity whereas cell surface EGFRs inhibit caspase-3 activity. These data demonstrate that EGFR internalization can convert signaling from an anti-apoptotic to a pro-apoptotic phenotype.

64 Tenascin-C EGF-like Repeats - Novel Matrix Kinesin Ligands for EGFR
A. Iyer, K. Tran, L. Richardson, C. Borysenko, C. Camacho, H. Blair, I. Bahar, A. Wells; 1Molecular Pathology, University of Pittsburgh, Pittsburgh, PA, 2Chemical Engineering, Massachusetts Institute of Technology, Cambridge, MA, 3Molecular Genetics & Biochemistry, University of Pittsburgh, Pittsburgh, PA, 4Computational Biology, University of Pittsburgh, Pittsburgh, PA

Recently, EGF-like (EGFL) repeats of matrix proteins have been shown to signal via the EGFR receptor (EGFR), though only at concentrations higher than those for classical soluble ligands such as EGF. The molecular nature of these interactions between EGFR repeats and EGFR has not yet been elucidated. We modeled the 14th repeat of tenascin-C (Ten14) using structure prediction servers to produce a tight molecule with truncated loops, and generated two bound conformations with EGFR. Analysis of the generated bound structures using the Gaussian Network Model and FastContact suggested that Ten14 is much more labile in the ligand-binding pocket of EGFR compared to prototypical ligands EGF/TGFα, implying both low affinity and flexibility enabling ligandation even when embedded within the ECM. Such low-affinity binding should result in distinct biochemical/biophysical and cellular responses as compared to EGF/TGFα. Radio-ligand binding assays showed that recombinant Ten14 did not bind stably to EGFR, with no significant internalization of Ten14 over either the short or long term, both in contrast to EGF. Immunofluorescence and flow cytometry analysis confirmed Ten14-mediated restriction of activated EGFR at the cell surface. Surface plasmon resonance analysis yielded a K_d of 74 µM for Ten14, three logs higher than EGF. Surface-compartmentalization of EGF due to matrix-constrained presentation of EGFR ligands should result in predominant activation of the membrane-restricted PLCγ1, resulting in enhanced cell migration. Indeed, sustained migration over 2D surfaces was observed at levels of Ten14 that failed to stimulate proliferation, concomitant with potent peri-plasma membrane PLCγ1 and m-caspin signaling, but weak activation of intracellular ERK/MAPK, p90Rsk and Elk1 signaling. Thus, with this study, we present a comprehensive analysis of a novel ligand for EGFR in a matrix-constrained environment, and how its signaling impacts overall cellular behavior. This has implications for cell responses during normal tissue regeneration, a situation in which tenascin-C is upregulated.

65 The Effect of PTHrP on EGFR Activation in Co-cultures of Mouse Mammary Epithelial and Stromal Cells
T. T. Nguyen, W. J. Shive, M. E. Dunbar; Biology, Penn State University-Berks Campus, Reading, PA

Parathyroid hormone related protein (PTHrP) is a normal product of mammary epithelial cells where it plays a role during both embryonic and post natal mammary development. We have previously shown that PTHrP overexpression during puberty results in defects in estrogen induced epithelial cell growth during puberty. It is our hypothesis that PTHrP regulates estrogen induced growth by modulating the effects of estrogen in the mammary stroma. To explore the effects of PTHrP on estrogen induced growth of mammary epithelial cells, we developed a cell culture system to recapitulate the effects of PTHrP on estrogen stimulation of cell proliferation in vitro. Co-cultures of mouse mammary epithelial and stromal cells respond to 17β-estradiol with an increase in cell proliferation. This increase in cellular proliferation is blocked by pre-treatment with PTHrP. Therefore, this cell culture system mimics the situation in vivo. Much prior work has implicated stromal epidermal growth factor receptor (EGFR) in estrogen induced ductal elongation during puberty. Therefore, we examined the effects of PTHrP on estrogen induced expression/activation of EGFR in our co-culture system. Co-cultures were treated with either 17β-estradiol or epidermal growth factor (EGF) in the presence or absence of PTHrP. Western blot analysis was performed to determine the levels of expression and/or activation of EGFR. Treatment of co-cultures with either estrogen or EGF did not result in an increase in expression of EGFR. However, there was a marked increase in the level of activated EGF in co-cultures treated with either 17β-estradiol or EGF. Pre-treatment with PTHrP blocked EGFR activation in co-cultures treated with estrogen. Together, these results suggest that PTHrP inhibits estrogen induced proliferation of mammary epithelial cells by inhibiting the activation of EGFR in the mammary gland.

66 TGFβ Role on the Gastric Epithelium: From Signaling to Cell Cycle Control
E. R. De Andrade Sá, B. Bitencourt, E. P. Alves, P. Gama; Cell and Developmental Biology, University of Sao Paulo, Sao Paulo, Brazil

The growth of the gastric mucosa is coordinated by a complex interaction of hormones, growth factors, milk- borne molecules and genetic program. We have shown that epithelial cell proliferation can be stimulated by fasting specifically during suckling phase, suggesting that milk potentially regulates the process. In addition, when milk-borne hormones are administered
concomitant with fasting, the stimulatory response is reversed. The inhibitory role of transforming growth factor beta (TGFβ) on gastrointestinal cell proliferation has been mostly studied in vitro. Our aim was to identify the mechanisms of TGFβ function in the gastric epithelium in vivo. We evaluated the activation of signaling cascade, DNA synthesis and cell cycle control in stomach of suckling rats. TGFβ1 was administered by gavage (1 ng/g body weight) to 14-ldl old rats during fasting. The stomach was collected in time-course experiments to evaluate signaling by immunohistochemistry, as well as cyclin E, Cdk2 and p27 levels by western blotting and immunoprecipitation. To estimate DNA synthesis index, pups were injected with BrDU. Active TGFβ1 increased in the gastric mucosa concomitant with constant levels of TJRII. The labeling index (LI) for Smad2/3 increased more rapidly at 30 min (P<0.05). Smad7 was restricted to surface epithelial cell. Next, we observed that TGFβ1 inhibited DNA synthesis by 20% (P<0.05). Although Cdk2 level increased after 2 h, cyclin E was unchanged and the complex cyclin E-Cdk2 was not altered. The concentration of p27 also increased after 2 h and exceeded cyclin E-Cdk2. TGFβ1 triggered Smad pathway and inhibited DNA synthesis, possibly through the modification of p27/cyclin E-Cdk2 levels. We suggest that the potential of TGFβ1 to regulate a hyperproliferative process should be more explored in the progression of gastric diseases during the stages when cells are still responsive.

67 Anti-inflammatory Effect of TGF-beta Is Mediated by Smad6 in Primary Peritoneal Macrophages

Y. Lee, S. Kim, S. Park; In Ha University, Incheon, Republic of Korea, NIH, Bethesda, MD

The transforming growth factor-beta (TGF-beta) is a potent anti-inflammatory cytokine. We here demonstrated that the increased expression of Smad6, upon treatment of TGF-beta, inhibited the expression of pro-inflammatory genes in primary peritoneal macrophages. The reduced expressions of pro-inflammatory genes were due to the disruption of Myd88-IRAK-Pellino-1-TRAF6 signaling complexes via direct binding of Smad6 to Pellino-1. The blockade of IRAK-mediated signaling complexes by Smad6 decreased the DNA binding activity of NF-kB transcription factor, resulting in the decreased expressions of pro-inflammatory genes in primary macrophages. These results strongly indicate that the anti-inflammatory effect of TGF-beta is mediated by Smad6.

68 Phosphorylation of p66Shc by TGF-β Type I Receptors Attenuates Its Subsequent Phosphorylation by EGF Receptors

M. K. Lee, S. M. Smith; Center for Craniofacial Molecular Biology, University of Southern California, Los Angeles, CA

Shc adapter proteins participate in multiple signaling pathways. Canonically, Shc isoforms are phosphorylated by receptor tyrosine kinases at two sites in the CH1 domain to initiate the Erk MAP kinase signaling cascade. Shc proteins are also directly phosphorylated by activated TGF-β receptors. TGF-β stimulation induces strong Shc serine phosphorylation and weak tyrosine phosphorylation, resulting in low-level Erk MAP kinase activation. Because they are substrates of two different receptors with different specificities, Shc proteins may represent a point at which EGF and TGF-β pathways regulate one another. We hypothesize that TGF-β-mediated Shc phosphorylation attenuates its subsequent phosphorylation by EGF receptors. To evaluate this postulate, bacterially-produced p66Shc was phosphorylated in vitro by either wild-type or T 204D constitutively active TGF-β Type I Receptors. We speculate that the activation of Erk MAP kinases by Shc signaling is dependent on the order of receptor activation.

69 Pro- and Anti-Angiogenic Cytokines Modulate FGFR-1 Isoform Splicing in an Opposing Manner

S. S. Kelpke, J. A. Thompson; Surgery, University of Alabama at Birmingham, Birmingham, AL

Inflammation and angiogenesis are co-dependent processes, wherein several cell types, cytokines, and growth factors interact to promote repair and maintenance of vascular integrity. During inflammation, activated T helper (Th) cells control angiogenesis by polarizing to different phenotypes (Th1/Th2) and producing specific Th1- or Th2-like cytokines that promote or antagonize angiogenesis in a manner analogous to their classification as pro- or anti-inflammatory. The coordination of angiogenesis in vivo includes the responsibility of the fibroblast growth factor (FGF) family and interactions with their high affinity cell surface receptors. Structural variants of FGF receptors (FGFR-1) are generated by alternative splicing, resulting in major FGFR-1 isoforms containing either three (FGFR-1α) or two (FGFR-1β) Ig-like extracellular domains. In the adult, vascular structures primarily express FGFR-1α, whereas during angiogenesis and wound healing, isoforms switching to FGFR-1β is observed. FGF ligand activation of these FGFR-1 isoforms induces distinct signal transduction cascades that differentially modulate vascular cell morphology, growth, migration, and responses to oxidative stress. To examine the ability of inflammatory-angiogenic cytokines to modulate alternative splicing of FGFR-1, primary rat carotid adventitial fibroblasts (AFs) were treated (24hrs) with 1.0 ng/ml of either IFN-γ (Th1; antiangiogenic) or IL-6 (Th2; proangiogenic). IFN-γ treatment resulted in a 15-fold increase in FGFR-1α mRNA (qRT-PCR) and protein (Western) that was accompanied by a 22-fold increase in Srp55 mRNA and protein. In contrast, IL-6 treatment resulted in a 10-fold increase in FGFR-1β mRNA and protein that was accompanied by a 50% decrease in Srp55 mRNA and protein. Transfection of siRNA recognizing Srp55 confirmed that normal expression of FGFR-1α required this splicing factor, which appears to be differentially modulated by Th1 and Th2 cytokines. Following individual cytokine treatments, AFs demonstrated FGF-dependent responses consistent with distinct signal transduction cascades. These results provide insights into new mechanisms potentially regulating the resolution of inflammation and angiogenesis.

70 RhoA-Mediated Cell Death Promoted by Fibroblast Growth Factor-2 (FGF2) in Mouse Tumor Cells Transformed by ras Oncogenes

F. L. Forti, H. A. Armelin; Biochemistry, Institute of Chemistry, University of Sao Paulo, Sao Paulo, Brazil

The Rho-GTPases family (Rho, Rac and Cdc42) regulates architecture and activity of the actin cytoskeleton to control important cellular functions like adhesion, migration, cell cycle progression and proliferation. Several growth factors were reported to activate RhoA in many cell types, promoting cell migration and proliferation. Here we show for the first time that recombinant FGF2 (18kDa) causes a RhoA-dependent drastic morphological modification, leading to death of two mouse tumor cell lines (Y1 adrenal cortical cells carrying overexpressed K-Ras-GTP and Balb3T3 fibroblasts, B61, transformed by the H-ras-V12 oncogene), but not of normal Balb3T3 fibroblasts. In both tumor cell lines, RhoA-GTP is undetectable in G0/G1-arrested cells maintained in SFM (serum-free medium); serum addition elicits RhoA activation, cell migration, cell cycle progression and proliferation. FGF2 also causes RhoA activation and cell migration, but leads to cell death, irrespective of serum presence. In addition, Y1 tumor cells treated with FGF2 display activation of caspase-3, -7 and PARP. Overexpression of the dominant negative mutant RhoA-N19, but not of the constitutively activated RhoA-V14, renders both tumor cell lines resistant to cell death induced by FGF2. Stable transfectant clonal sub-lines Y1 and B61-RhoA-N19 are unable to migrate but grow well in serum supplemented medium and respond to FGF2 with growth stimulation. Altogether, these novel results demonstrate that FGF2 can trigger a RhoA-dependent death in tumor cells in sharp opposition to the largely accepted notion that FGF2 is a bona fide oncogenic factor.

71 Identification of Novel Gab1-Interacting Proteins Downstream from the Met Receptor Tyrosine Kinase

G. N. Palouras, M. Park; Experimental Medicine, McGill University, Montreal, PQ, Canada

The Met receptor tyrosine kinases (RTK) is frequently dysregulated in human cancer and plays a significant role in its development and progression. Growth factor-stimulated cell growth is dependent on the activation of multiple structural and enzymatic proteins. Stimulation of the Met receptor by its ligand HGF (hepatocyte growth factor) results in a remodeling of the actin cytoskeleton of epithelial cells that is essential for cell morphogenesis, cell migration and invasion. This process downstream from the Met receptor requires the scaffold protein Gab1, which we have shown to be a critical modulator of the morphogenic response induced by Met and is important for tumorigenesis. Gab1 is a member of a family of Gab family of adaptor/scaffold proteins linking a broad range of growth factor, cytokine, and antigen signals to intracellular signaling pathways. Gab1 has been found to recruit and activate proteins, including Shp2, the p85 subunit of PI3K, Phospholipase Cγ and Crk which have been found to be involved in cellular migration, invasion, adhesion, apoptosis and proliferation. To identify novel Gab1-protein complexes following Met receptor activation, we have generated a Gab1-TAP-tag fusion protein and have used this in a proteomic screen to identify novel Gab1 protein-protein interactions. Preliminary proteins identified by mass spectrometry of Gab1-associated proteins ranged from cytoskeletal assembly proteins, to proteins that regulate cell migration. Understanding the molecular events that regulate Gab1 signaling will identify the Gab-dependent signaling pathways required for epithelial cell tumorigenesis and invasion and new Gab protein interactions will be presented.
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**VEGF Is Critical in Resolution of Inflammation during Wound Healing**

M. L. Petreaca, M. Yao, M. Martins-Green; Cell Biology and Neuroscience, University of California, Riverside, Riverside, CA

Wound healing processes show a sequential series of events, including inflammation, granulation tissue formation, and remodeling. Appropriate resolution of inflammation is essential for normal healing, as alterations in the extent and/or duration of inflammation are responsible for many pathological healing conditions. However, little is known about resolution of inflammation and the factors important in regulating this process. Here, we present evidence that Vascular Endothelial Growth Factor (VEGF), a key angiogenic molecule known to function in the development of the granulation tissue, may play an important role in resolution of inflammation during wound healing. Inhibition of VEGF or its receptors during the healing of rabbit wounds markedly increased the number of macrophages at the wound site. As macrophages normally disappear from the wound site via apoptosis, we investigated a possible role for VEGF in stimulating macrophage apoptosis. We found that VEGF can induce apoptosis in cultured macrophages, as shown by a decreased number of cells remaining in culture after treatment and by an increased macrophage annexin V staining. This apoptosis is accompanied by increases in the activation of caspases 3 and 9, as shown by immunostaining and immunoblot analysis, and does not occur immediately after treatment. Therefore, we hypothesized that VEGF-mediated macrophage apoptosis occurs via the stimulation of pro-apoptotic molecules rather than proceeding from VEGF stimulation directly. Microarray and RT-PCR data reveal that VEGF strongly induces the expression of at least one pro-apoptotic gene that belongs to the Tumor Necrosis Factor ligand superfamily; we are currently investigating the possibility that this molecule is important in VEGF-mediated macrophage apoptosis. Taken together, our results identify a novel role for VEGF in the induction of macrophage apoptosis during wound healing, and thus in the resolution of inflammation.

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**VEGF-related Protein Isolated from Vipera Palestinae Venom, Promotes Angiogenesis**

M. Higgins,1 I. Staniszewska,1 I. Oliva,1 J. Calveci,1 P. Lazarovici,1 C. Marcinkiewicz;1 1Department of Neuroscience, Temple University, Philadelphia, PA, 2C.S.I.C., Instituto de Biomedicina, Valencia, Spain, 3Department of Pharmacology, Hebrew University, Jerusalem, Israel

Vascular endothelial growth factor (VEGF) plays a crucial role in promotion of angiogenesis. The mechanism of this promotion includes induction of endothelial cell migration and proliferation. We isolated a new protein, vpVEGF, from *Vipera palestinae* venom that structurally shows high homology with human VEGF but is analogous to human VEGF, however, vpVEGF is missing the heparin binding domain. The effect of vpVEGF on angiogenesis-related activity of endothelial cells was assessed using dermal vascular endothelial growth factor (VEGF). boyden assay. The effect of vpVEGF on DmVEC migration was performed in two assays, in Boyden chamber and radial migration in collagen gel. In both assays we observed vpVEGF-induced migration of endothelial cells. The analysis of signal transduction induced by vpVEGF showed phosphorylation of MAPK Erk1/2, which is characteristic for VEGF interaction with endothelial cells. This signaling process has been blocked by a specific inhibitor of VEGFR2 (Flk-1), k525a, suggesting that vpVEGF is a ligand for this cell surface receptor. This finding was confirmed using siRNA technique by silencing a Flk-1 gene in DmVEC. The “knockout” cells lost the ability to induce proliferation and cell signaling following treatment with vpVEGF. The pro-angiogenic activity of vpVEGF was confirmed in vivo using CAM assay of the embryonic quail system. In this assay, vpVEGF increased the ratio of vascularization to the same level as human microvascular endothelial cells (dHMVEC), which are important in several pathologies of skin, including melanoma. vpVEGF significantly induced proliferation of dHMVEC in the BrdU assay. The effect of vpVEGF on DmVEC migration was performed in two assays, in Boyden chamber and radial migration in collagen gel. In both assays we observed vpVEGF-induced migration of endothelial cells. The analysis of signal transduction induced by vpVEGF showed phosphorylation of MAPK Erk1/2, which is characteristic for VEGF interaction with endothelial cells. This signaling process has been blocked by a specific inhibitor of VEGFR2 (Flk-1), k525a, suggesting that vpVEGF is a ligand for this cell surface receptor. This finding was confirmed using siRNA technique by silencing a Flk-1 gene in DmVEC. The “knockout” cells lost the ability to induce proliferation and cell signaling following treatment with vpVEGF. The pro-angiogenic activity of vpVEGF was confirmed in vivo using CAM assay of the embryonic quail system. In this assay, vpVEGF increased the ratio of vascularization to the same level as human recombinant VEGF. However, addition of k525a completely abolished vpVEGF-induced angiogenesis, confirming that Flk-1 is a receptor for vpVEGF.

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**Insulin and Cell Medicine: Leading to Acceleration of Wound Healing**

Y. Liu, M. Yao, M. Martins-Green; Cell Biology & Neuroscience, University of California Riverside, Riverside, CA

Wound healing is a complex process that involves an inflammatory response, re-epithelialization and angiogenesis and, is governed by the local production of cytokines and growth factors. Our recent studies have shown that the topical application of insulin significantly enhances the wound healing process in vivo. To determine the cell and molecular basis of insulin acceleration of wound healing, we studied the effects of insulin on keratinocytes (important in re-epithelialization) and endothelial cells (important in angiogenesis). Insulin stimulates keratinocyte proliferation and migration in a dose- and time-dependent manner and stimulation is dependent on activation of Akt, PI3-K and steroid regulatory element binding proteins (SREBPs) but not on EGFR and its receptor. Our studies on the effects of insulin on microvascular endothelial cell proliferation and migration, show that this protein stimulates endothelial cell migration but not proliferation and that this is independent of VEGF. In microvascular endothelial cells, insulin stimulates Src-phosphorylation very rapidly and this stimulation is followed by phosphorylation/activation of Akt and ERK1/2 which is inhibited by the Src inhibitor SU6656. Furthermore, SREBPs are also involved in the effects of insulin on endothelial cell migration, in particular the precursor and mature form of SREBP1. Our results show that insulin stimulates keratinocytes and endothelial cells to migrate and that in both cases Src-Pi3K-Akt-SREBP are critical molecules. Taken together these results show that insulin is another factor that accelerates wound healing and hence it should be considered as potential treatment for impaired wounds.

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**Prokineticin Receptors Localize to Caveolae Membranes**

E. L. Bradshaw,1 E. J. Smart2 1Physiology, University of Kentucky, Lexington, KY, 2Pediatrics, University of Kentucky, Lexington, KY

Hyposia-induced angiogenesis is a compensatory mechanism to supply blood to ischemic tissues. In the peripheral vasculature, ischemia-initiated angiogenesis is controlled by a variety of growth factors, which allow for endothelial permeability, proliferation, and migration. LeCouter et al. recently described a new angiogenic factor, endocrine gland-derived vascular endothelial growth factor (EG-VEGF). EG-VEGF causes proliferation, migration, and fenestration of the endothelium causing massive vascularization. To date, prokineticin receptor (EG-VEGF receptor) localization within the plasma membrane and protein-protein interactions have not been elucidated. Caveolae are cholesterol and sphingolipid rich invaginations of the plasma membrane, which are characterized by caveolin-1. Caveolae are recognized as centers for signal transduction, and caveolin modulates cellular signaling cascades. Immunofluorescence data shows caveolin-1 and prokineticin receptors colocalize, suggesting that prokineticin receptors localize in caveolae. As a complementary method, subcellular fractionation studies show that prokineticin receptor-1 and -2 are enriched in the caveolae membrane fraction along with caveolin-1. Furthermore, immunoprecipitation data from CHO cells as well as murine tissues shows that prokineticin receptors-1 and -2 immunoprecipitate caveolin-1. This potentially has interesting implications for prokineticin receptor function because caveolin-1 has been shown to negatively regulate and inhibit signaling of G-protein coupled receptors as well as tyrosine kinase receptors. Overall, caveolae-localization of prokineticin receptors allows for close proximity to many signaling molecules, which may facilitate its robust angiogenic response.

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**Ephedra sinica Friction Inhibits Hepatocyte Growth Factor (HGF)/c-Met Signaling and HGF-induced Tumor Cell Migration and Invasion**

C. Hyun-Young1, L. Ming Hong2, K. Inhae,1 K. Myeongdeok,1 R. Jae-Ha,1 L. Jae-Ho; 1Department of Biochemistry and Molecular Biology, Ajou University, Suwon, Republic of Korea, 2College of Pharmacy, SooMyung Women’s University, Seoul, Republic of Korea

Aberrant hepatocyte growth factor (HGF) c-Met signaling has been known to be involved in many human cancer development and progression. During the search for an effective inhibitor of HGF/c-Met signaling with the herbal extracts, we have found that Ephedra sinica fraction (ESM) showed inhibition of HGF-induced MDCK-2 cell scattering. Studies were performed to address whether ESM inhibits HGF/c-Met signaling, to find the underlying mechanisms, and to evaluate its therapeutical potential. As mentioned, ESM effectively blocked HGF-induced scattering and uPA activation in MDCK-2 cells, with 50% inhibition at 2.5 μg/ml concentration and complete inhibition at around 12.5 μg/ml. Inhibition by ESM was accompanied by the decrease of HGF-induced tyrosine phosphorylation of Met as well as HGF-mediated activation of p44/42 mitogen-activated protein kinase, but not epithelial growth factor (EGF)-induced tyrosine phosphorylation of EGFR, suggesting the specificity of ESM to HGF/c-Met signaling. In addition, at high doses, ESM seemed to reduce the expression of c-Met protein. This inhibition leads to decrease in HGF-induced migration of B16F10 murine melanoma cells. Also, the decrease in HGF-induced invasion of SK-LMS-1 human leiomyosarcoma cells was also observed, showing the possible application of ESM for preventing HGF-induced cancer cell migration or invasion. In conclusion, ESM specifically inhibits c-Met phosphorylation induced by paracrine HGF stimulation which leads to decrease in cell migration and invasion in vitro. These data suggest the feasibility of selectively targeting c-Met with ESM to inhibit aberrant HGF/c-Met signaling in human cancer.
Activation of the C-jun NH2-terminal Kinase Determines Mitotic Entry and Paclitaxel Sensitivity in Human Hepatoma Cells

S. Chae,1,2 W. Lim,1 H. Cho1,2,1 Biochemistry and Molecular Biology, Ajou University School of Medicine, Suwon, Republic of Korea, 2Chronic Inflammatory Disease Research Center, Ajou University, Suwon, Republic of Korea, 3Department of Molecular Science and Technology, Ajou University, Suwon, Republic of Korea

Paclitaxel is a class of anti-microtubule agent and currently used in the chemotherapeutic treatment of patients with ovarian, breast and lung carcinomas. However, paclitaxel resistance in liver cancer often renders the drug ineffective. Here, we investigated the molecular mechanism underlying paclitaxel resistance in HCC cell lines. Human hepatoma cell lines, SNU series were used to investigate the mechanism of paclitaxel resistances. Two out of six HCC cell lines (type I) underwent active cell death upon paclitaxel treatment whereas four cell lines showed paclitaxel resistance (type II). We observed that type I cells tended to arrest at G2 phase upon paclitaxel treatment, whereas type II cells entered mitosis and died, which was accompanied with Bcl-2 phosphorylation. In type I cell paclitaxel treatment induced activation of the c-Jun NH2-terminal kinase (JNK) and paclitaxel-induced cell death in these cells was significantly inhibited by SP600125, a specific inhibitor of JNK, but not with inhibitors of Erk1/2 (U0126) or mTOR (rapamycin). In addition, treatment with SP600125 suppressed cells entering mitosis. On the contrast, in type II cells, caffeine, an ATM/ATR partially rescued paclitaxel resistance evidenced by increase of mitotic entry and cell death. These results suggest that activation of the JNK mainly determines mitotic entry and paclitaxel sensitivity in human hepatoma cells.

Activin Regulates Myofibroblast Proliferation and Differentiation Following Liver Injury

F. Kalantar2, J. Lebrun,1 E. Chevet1,2,3 H. Vail,4 P. Auguste1,2, Anatomy & Cell Biology, McGill University, Montreal, PQ, Canada, 2Surgery, McGill University, Montreal, PQ, Canada, 3Medicine, McGill University, Montreal, PQ, Canada, 4Inserm e362, Universite Bordeaux 2, Bordeaux, France

Transplantation remains the only viable treatment for end-stage liver diseases. Liver transplantation is often associated with numerous ischemia-reperfusion related complications including calcification. We have previously demonstrated that one major mechanism involved in this process is linked to the proliferation and differentiation of myofibroblasts. Here, we show that the expression of activin receptors is up-regulated in myofibroblasts near calcified regions of the liver. We also present that, activin-associated smad signal transducers are activated and co-localized with activin receptors. Since proliferating and differentiating myofibroblasts show activated activin signaling, we hypothesize that activin may stimulate myofibroblasts differentiation and/or proliferation leading to calcification of liver tissues. To assess the role of activin on myofibroblasts, we studied the impact of activin on the proliferation and differentiation of C3H/10T1/2 cells and compared these results with those obtained from TGFI experiments. Activin signaling was analyzed by measuring the activation of the Smad pathway after C3H/10T1/2 stimulation. The results of this experiment revealed an increase in p-Smads which was specifically correlated with an activin-mediated cell proliferation. These data were validated using dominant negative Smad2 and Smad3 stable cell lines where activin signaling is blocked. The same approach was used to evaluate cell proliferation and differentiation. In conclusion, our work demonstrates the involvement of activin as a critical regulator of myofibroblasts proliferation and differentiation and could represent a potential therapeutic target to reduce fibrosis in the liver. In addition, as both activin and TGFI play a role in bone formation, their possible involvement in liver calcification should be explored.

Novel Modification of the Ran Exchange Factor, RCC1, and Its Function in Mitosis

T. Chen1, T. Muratore,1 C. Schaner-Tooley,1 D. Hunt,1 F. Macara1, Microbiology, University of Virginia, Charlottesville, VA, 2Chemistry, University of Virginia, Charlottesville, VA

RCC1 is the only known guanine nucleotide exchange factor for the Ran GTPase, and plays pivotal roles in nucleocytoplasmic transport, mitosis, and nuclear envelope assembly. RCC1 can bind to chromatin through histones H2A and H2B, in a Ran-regulated manner. Mitotic phosphorylation of RCC1 can also modulate chromatin binding. We now report a unique modification on RCC1. An enzyme activity specific for this modification of RCC1 is present in soluble nuclear extracts from HeLa cells. Modification-defective mutants of RCC1 are unable to function in certain aspects as effectively as the wild type protein, which results in mitotic defects. These defects are additive to those caused by a mutation that disrupts exchange activity.

The Expression of Dvl2 Increases Frizzled-7 Dimers and Prevents Frizzled-7 C-terminus Cleavage at the Plasma Membrane

C. D. Mao, I. T. Strewing; GCNS, University of Kentucky, Lexington, KY

The frizzled (Fz) proteins are seven-transmembrane G-protein coupled receptors involved in the control of cell polarity and Wnt-signaling. Though there is increasing evidence that Wnt endocytosis with its receptors, Frizzled, and co-receptors, LDL-receptor like protein-5/6, is required for full activation of the downstream Wnt-signaling pathways, the role and mechanisms of Frizzled endocytosis and turnover at the plasma membrane have not been identified. We have recently shown that Fz7 receptors are subjected to a specific C-terminus cleavage at the plasma membrane that can be increased by PMA and calcium in endothelial cells and attenuated by both clathrin-dependent and caveolin-dependent endocytosis inhibitors. However, Wnt13A and Wnt5A, two putative endogenous ligands for Fz7 in endothelial cells, had no effect on the appearance of Fz7 C-terminus cleavage, suggesting that Fz7 is subjected to constitutive endocytosis and down-regulation. On the other hand, co-expression of Fz7 with the Wnt-downstream effector, dvl2, resulted in a decrease of Fz7 C-terminus cleavage and an increase of the formation and/or stabilization of Fz7 dimers. These data are also consistent with a constitutive Fz7 endocytosis and down-regulation modulated by the levels of dvl2, though it remains to determine whether dvl2 prevents Fz7 C-terminus cleavage and/or Fz7 endocytosis. For this purpose, we have identified the cleavage site within the 3rd intracytoplasmic loop and designed various mutations of the putative cleavage site. However all the Fz7 mutants identified so far that displayed a reduced C-terminus cleavage were also defective for inducing dvl2 translocation to the plasma membrane, which included the Fz7 receptor mutated within the conserved KTLXXW sequence in the cytoplasmic tail. Therefore, our results altogether emphasize the intricate link between Fz7 C-terminus cleavage and Fz7 signaling.

Differential Transmission of Actin Motion within Focal Adhesions

K. Hu, L. Ji, K. Thompson, G. Danuser, C. Waterman-Storer; Dept. of Cell Biology, Scripps Research Institute, La Jolla, CA

Cell motility requires transmission of forces generated in the actin filament cytoskeleton through molecularly complex integrin-based Focal Adhesions (FAs) to the extracellular environment. Although it is well-accepted that spatiotemporal coordination between FA and actin is required for the regulation of cell migration, the dynamics of molecules within these two arrays have not been analyzed simultaneously in living cells. In this study, we have successfully visualized the dynamics of molecules within individual FA in living cells, using TIR-FSM, an imaging technique that combines Total Internal Reflection fluorescence microscopy with Fluorescent Speckle Microscopy. To determine if FA molecules dynamically interact with actin filaments within living cells, we developed a novel technology, Correlative Fluorescent Speckle Microscopy. This new method allows quantitative analysis of the degree of correlation between the motions of two molecule population labeled with spectrally distinct fluorophores. Monte Carlo simulations have confirmed that a high degree of correlated speckle motion indicates an interaction between the molecules. We screened a cross-section of known FA proteins (ECM binding proteins: α1/β1 integrin; F-actin binding FA proteins: α-actinin, vinculin and talin; and FA “core” proteins, that do not bind F-actin or ECM directly, but include structural and signaling molecules: paxillin, zyxin, focal adhesion kinase (FAK)), using this technology to analyze their correlated motions with actin. Our results illustrate that different classes of FA proteins display different dynamic behaviors. We find that motion of the actin cytoskeleton is differentially transmitted through focal adhesions, with the efficiency of transmission gradually decreasing from actin-binding proteins to integrins, thus defining the FA as a hierarchical molecular friction clutch. Interestingly, the degree of this transmission is spatially and temporally regulated, and modulation of transmission correlates with specific events in the cell migration cycle. Therefore, FA internal molecular dynamics represents a key element in the mechanics of cell morphogenesis during migration.

Human Insulin Receptor (hIR) and Glucose Transporter (GLUT4) Trafficking in Transfected CHO Cells: Effects of the Inhibitors of PI-3 and MAP Kinases and That of the Actin-Microtubules

A. A. Sattar, P. Berhau; Endocrinology/Internal Medicine, Wayne State University School of Medicine, Detroit, MI

The binding of insulin to insulin receptor (IR) triggers intracellular insulin signaling and GLUT4 redistribution from the intracellular compartment to the plasma membrane, where it facilitates entry of glucose into the cell. We sought to determine insulin-induced IR and GLUT4 trafficking in transfected CHO cells as a function of time and with respect to the inhibitory effects of the inhibitors of PI3 and MAP kinases and that of the actin-cytoskeleton to better understand the dynamics of their trafficking by analyzing immunostained cells using confocal microscopy. Insulin (30 nM) -stimulated IR trafficking from cell periphery to perinuclear sites (up to 25% of the maximal) within 20 min, reached a maximum at 30 min and then declined to 42% of the maximal at 60 min. On the other hand, GLUT4 trafficking from perinuclear to peripheral sites (up to 73% of the maximal) within 10 min, gradually to a maximum at 30 min, and then declined to 85% at 60 min. Pretreatment of the cells either with 100 nM wortmannin or 100 µM PD98059 reduced insulin-mediated intracellular IR trafficking by ~60% and ~55%, respectively; whereas contrast, in type II cells, caffeine, an ATM/ATR partially rescued paclitaxel resistance evidenced by increase of mitotic entry and cell death. These results suggest that activation of the JNK mainly determines mitotic entry and paclitaxel sensitivity in human hepatoma cells.

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GLUT4 trafficking decreased by ~95 and ~25%, respectively. Pretreatment of the cells with 1 μM latrunculin A reduced insulin-induced IR prenuclear localization by ~50%, and 13 μM nocodazole apparently had no consequence on IR trafficking. On the other hand, both latrunculin A and nocodazole disrupted insulin-mediated GLUT4 trafficking by ~95 and 55%, respectively. These results demonstrate: 1) a lack of time-dependent correlation between insulin induced IR and GLUT4 trafficking, 2) although PI-3 and MAP kinases regulate intracellular IR trafficking, GLUT4 trafficking is mostly regulated via PI-3 kinase, and 3) intact microtubule may not be essential for insulin-induced IR trafficking to the perinuclear region but microtubule and F-actin networks are required for GLUT4 trafficking to the cell periphery.

83 Interleukin 6 Up-regulates Angiogenin Secretion from Human Prostate Cancer Cells, LNCaP
M. Arakawa, M. Kawada, D. Ikeda; Drug Development Unit, Microbial Chemistry Research Center Numazu Bio-Medical Research Institute, Numazu, Japan
Objective: Androgen-dependent human prostate cancer LNCaP cells rarely form tumors without prostate stromal cells in SCID mice, and even if the tumor is formed, the tumor growth is very slow. In recent studies, we have established the cytokine-resistant LNCaP cells (LNCaP-CR) that acquired high tumorigenicity by the selection of survived cells on the co-culture with the normal fibroblast in the presence of IL-1 β (Kawada et al, 2006). LNCaP-CR exhibited not only higher tumorigenicity but also higher secretion of angiogenin (ANG), one of the angiogenesis factors, than the parental LNCaP. Methods: To examine effects of several cytokines (IL-1β, TNF-α, IL-6, IFN-γ) and signal transduction inhibitors (MEK inhibitors, JAK2 inhibitor, IP3K inhibitor) on ANG secretion in culture media of LNCaP-CR or LNCaP, we assessed ANG secretion using human ANG ELISA. Results: IL-6 (50 ng/ml) significantly increased ANG secretion from LNCaP-CR (345 ±47 pg/ml, 24h) compared with that of untreated-LNCaP (140 ±37 pg/ml, 24h), while it had a slight effect on LNCaP-CR. Moreover, IFN-γ (200 ng/ml) decreased the IL-6 (50 ng/ml)-induced ANG secretion from LNCaP-CR, but it had little effect on that of LNCaP-CR. In addition, JAK2 inhibitor, AG490 (50 μ M), significantly decreased ANG secretion from both LNCaP and LNCaP-CR. Conclusions: These results suggest that the production of ANG from IL-6-treated LNCaP may be regulated through the activation of JAK/STATs (Janus kinase/signal transducers and activators of transcription) pathways. Furthermore, the disruption of the JAKs/Stats pathways may be involved in the high secretion of ANG from the cytokine-resistant LNCaP-CR.

84 Anillin-mediated Targeting of Peanut to Pseudocleavage Furrows Is Regulated by the Ran Pathway
R. V. Silverman-Gavrilova, A. Wilde; Medical Genetics and Microbiology, University of Toronto, Toronto, ON, Canada
Pseudocleavage furrows are required to maintain the separation between microtubule spindles during nuclear divisions in the syncytial Drosophila embryo. The pseudocleavage furrow is analogous to the cytokinetic furrow in somatic cells and invagination of the membrane is similarly driven by the actin cytokinetic ring. Failure to form pseudocleavage furrows allows neighboring microtubule spindles to make contact and fuse, thereby disrupting chromosome segregation. We observed this phenotype during a screen to identify mitotic processes regulated by the GTPase, Ran. Further investigation found that whilst the septin Peanut failed to be targeted to the nascent cleavage furrow, other furrow components were correctly targeted, including Actin, Anillin and Sep2, another septin family member. Septins are thought to be targeted to furrows by interacting with the PH domain of Anillin. Indeed we found that both Peanut and Sep2 bind to the PH domain of Anillin. However, in the presence of nuclear transport receptors Peanut failed to bind to the PH domain of Anillin whereas Sep2 did. These data suggest a molecular mechanism by which the Ran pathway can regulate furrow ingress. Furthermore, these data suggest that the PH domain of Anillin contains at least 2 distinct septin binding sites that bind distinct classes of septins.

85 Physiological Role of the Oxidative Stress-sensitive TRPM2 Ca2+ Channel in Immune Cells
S. Yamamoto, S. Shimizu, T. Wajima, Y. Mori; Kyoto University, Kyoto, Japan, 3Showa University, Tokyo, Japan
TRPM2 is a Ca2+ permeable channel activated by redox status changes such as oxidative stress. Although it has been suggested that Ca2+ influx via TRPM2 mediate cell death, its physiological significance is still elusive. TRPM2 is expressed in immune cells such as monocytes and lymphocytes. In monocyteic cell line U937, although it is known that hydrogen peroxide (H2O2) induces chemotactic cytokine interleukin-8 (IL-8) production via Erk / NF-κB pathway and that IL-8 is produced via intracellular Ca2+ increases, the detail mechanism is not known. Here, we investigate if oxidative stress-sensitive TRPM2 Ca2+ channels participate in H2O2-induced IL-8 production in U937. Addition of H2O2 to U937 triggered Ca2+ influx, and both Ca2+ influx and TRPM2 expression were attenuated by the treatment of TRPM2 siRNA. H2O2-activated Erk was amplified by Ca2+ influx. The amplified Erk translocated NF-κB to the nucleus, which mediated IL-8 production. The amplification of Erk, nuclear translocation of NF-κB, and IL-8 production induced by H2O2 were inhibited by the treatment with TRPM2 siRNA. Thus, Ca2+ influx via TRPM2 is involved in IL-8 production via Erk / NF-κB pathway. Additionally, we investigated the physiological significance of the above role played by TRPM2 in vivo by utilizing TRPM2 knockout mice.

86 The Armadillo-protein P0071 Regulates Rho-signalling during Cytokinesis
A. Wolf, R. Keil, M. Hatzfeld; Division of Pathobiochemistry, Institute for Pathophysiology, Halle/Saale, Germany
Cytokinesis requires the spatio-temporal coordination of cell cycle control and cytokinetic reorganization. Members of the Rho-family of GTPases are crucial regulators of this process and assembly of the contractile ring depends on local activation of Rho-signalling. We show that p0071, a member of the armadillo-family of proteins previously implicated in regulation of cell cell adhesion, in addition plays an essential role in Rho-signalling during cytokinesis. In contrast to the closely related p120ctn, p0071 was localized at the centrosome throughout the cell cycle and at the midbody during cytokinesis. Both, knockdown and overexpression of p0071 interfered with normal cell growth and survival due to cytokinesis defects with formation of multinucleated cells and induction of apoptosis. This failure of cytokinesis apparently correlated with the deregulation of RhoA-activity in response to altered p0071 expression, as shown by FRET-measurements based on biosensors and by GST-chtoekin pull-down assays. To determine where in the molecular signalling cascade p0071 modulates Rho-activity we analyzed its interaction with RhoA itself as well as upstream regulatory proteins. We show a direct association of p0071 with RhoA with a preference for activated RhoA which occurs at the region of furrow ingression during mitosis. Moreover, we found a physical and functional interaction of p0071 with Ect2, the one Rho-guanine nucleotide exchange factor (GEF) essential for cytokinesis. Testing RhoA activation in an GDP-GTP-exchange assay revealed that p0071 stimulates RhoA exchange activity in conjunction with Ect2. These results suggest that both, p0071 and Ect2 are necessary for full activation of RhoA during cytokinesis. Our findings indicate that p0071 serves an essential role in regulating spatially restricted Rho-signalling during cytokinesis.

87 Phosphorylation Motif Clustering as a Signature for Protein Kinase Substrates
H. Aldaz, M. Joachimiak, P. Houshmand, G. Barnes, D. G. Drubin; Molecular and Cell Biology, UC Berkeley, Berkeley, CA
Protein phosphorylation is a widely used form of cellular regulation. The identification of new kinase substrates, however, has proven challenging. Though many protein kinases exhibit a preference to phosphorylate certain consensus motifs, use of these motifs in a guide to identifying new substrates is often impractical due to the common occurrence of such motifs. Here is reported the observation that phosphorylation consensus motifs are organized within the primary sequences of many protein kinase substrates in a manner best described as clusters. Substrates of the budding yeast protein kinase lpllp were determined to be quantitatively enriched with lpllp consensus motif clusters, relative to the rest of the yeast proteome. Quantitation of consensus motif clustering showed little correlation with the number of motifs in a given protein, supporting this as a novel metric with which to judge potential kinase substrates. Experimental validation of this approach is underway, with early studies supporting this as a method to predict the identities of novel lpllp substrates. A set of proteins previously assayed for their ability to be substrates of another kinase with a known consensus motif, Cdc28p, was subjected to a similar analysis. The results revealed a strong correlation between identified substrates and proteins enriched with Cdc28p minimal consensus motif clusters, suggesting that consensus motif clustering may be a signature for the substrates of many protein kinases. This approach should aid in the discovery of new kinase substrates when only a general consensus motif is known. These results also suggest the possible existence of a general strategy of phosphoregulation.

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88 Motific Phosphorylation of Mis13 Is Required for Kinetochore Assembly and Proper Chromosome Segregation

Y. Yang,1 T. Ward,2 Z. Dou,3,4 F. Wang,5 Y. Ke,6 K. Jiang,3 N. Li,3 J. Yao,7 X. Yao;8 1Physiology, Morehouse School of Medicine, Atlanta, GA, 2Cellular Dynamics, Hefei National Laboratory, Hefei, China

Chromosome segregation in mitosis is orchestrated by dynamic interaction between spindle microtubules and the kinetochore, a multi-protein complex assembled onto centromeric DNA of the chromosome. Our previous studies show that kinetochore is composed of several interactive protein subcomplexes such as Zw10, Hecl and CENP-E. However, the mitotic regulation of these protein-protein interaction networks remains elusive. To identify the proteins that regulate Mis13 in mitosis, we carried out proteomic search for Mis13-interacting protein in mitotic cells. To this end, mitotic extracts from HeLa cells stably expressing FLA-Mis13 were purified using a FLAG antibody-affinity chromatography. Among various known Mis3-binding proteins identified, Aurora B was found to be retained on Mis13 affinity beads. The Aurora B-Mis13 interaction was then confirmed with recombinant Mis13 and Aurora B proteins in test tubes. Significantly, Aurora B co-localizes with and phosphorylates Mis13 in vivo. This Aurora B-mediated phosphorylation of Mis13 was confirmed in vitro using recombinant proteins. Interestingly, suppression of Aurora B kinase by siRNA or kinase activity inhibitors reduced the level of Mis13 associated with kinetochore. Expression of non-phosphorylatable Mis13 resulted in a delay in prometa-phase-meta-phase transition. Immunofluorescence analyses revealed that localization of the outer plate proteins such as CENP-E, CENP-F and Hecl was severely reduced in the kinetochore expressing non-phosphorylatable Mis13. Moreover, our studies show that the absence of Mis13 or inhibition of Mis13 phosphorylation reduces tension at kinetochores of bi-oriented chromosomes. These results indicate that Aurora B-mediated phosphorylation of Mis13 links mitotic kinetochore assembly to faithful kinetochore-microtubule attachment, which is essential for chromosome segregation.

89 An Allosteric Model of Circadian KaiC Phosphorylation

J. S. van Zon,1 P. Altena,1 D. K. Lubensky,6 P. ten Wolde1 1Centre for Integrative Systems Biology, Imperial College London, London, United Kingdom, 2FOM Institute for Atomic and Molecular Physics [AMOLF], Amsterdam, The Netherlands, 3Department of Physics, Vrije Universiteit Amsterdam, Amsterdam, The Netherlands

Cyanobacteria show 24 hour rhythms in gene expression. In the cyanobacterium Synechococcus elongatus three genes, kaiA, kaiB and kaiC are essential for this circadian rhythm. In higher organisms, it is known that these circadian oscillations are the result of transcriptional feedback loops. However, it was recently shown in vitro KaiC undergoes a stable circadian oscillation in its degree of phosphorylation when incubated with KaIA, KaiB and ATP. Here, we present a model of how the experimentally observed interactions between the Kai proteins together with allosteric transitions within KaiC proteins can give rise to such stable oscillations.

90 Bdm-1, a Phosphducin-like Protein Required for G-protein Signaling in the Filamentous Fungus Cynodon cymosa, Is a Phosphoprotein Targeted by Casein Kinase II

J. A. Salamon-Kozubowska, A. L. Dawe; Biology, New Mexico State University, Las Cruces, NM

In this study we have explored the nature of a post-translational modification to a protein that is required for functional G-protein signaling. G-protein signaling modulates many responses in fungi, including virulence of plant and animal pathogens. Previous studies with the filamentous fungal plant pathogen Cryptocapsa parasitica, the chestnut blight fungus, have identified components that include a phosphducin-like protein (BDM-1). Perturbation of the G-protein signaling pathways has been shown to affect virulence, pigmentation and sporulation. It has also been shown that deletion of the beta subunit CGBP-1 in BDM-1 affects these characteristics in an identical manner and caused similar alterations in transcriptional profile. Furthermore, G-proteins have also been shown to be reduced in accumulation by infection of C. parasitica with virulence-attenuating dsRNA viruses (hypoviruses). We have approached the study of BDM-1 by first showing that CGBP-1 protein levels are undetectable in the absence of BDM-1, thus explaining the identical phenotypes of the two null-mutants. Tagging BDM-1 with the FLAG epitope and transfection of the Bdm-1 strain demonstrated that the FLAG-tag does not affect BDM-1 function. Using this tool we show by treatment with phosphatase that BDM-1 exists in a phosphorylated form. Re-phosphorylation of BDM-1 can be accomplished in the presence of total protein lysates, but this activity is inhibited by the casein kinase II specific inhibitor DMAT, indicating that BDM-1 is substrate for this kinase. These tools are now possible to explore the effects of phosphorylation on BDM-1 function and determine if this modification is altered by the presence of hypoviruses, thereby providing a model for exploring the molecular events associated with viral-mediated changes of eukaryotic cell signaling pathways.

91 Autophosphorylation-deficient Mutant of Protein Kinase C delta Mutant Has Higher Kinase Activity and Apoptosis Activity Than Protein Kinase C delta Wild Type

J. Jang,1 H. Kwon,1 D. Park,1 J. U. Kazi,1 Y. Lee,1 Y. Lee,1 J. Soh;1 1Department of Chemistry, Inha University, Incheon, Republic of Korea, 2Korea Institute of Radiological and Medical Sciences, Seoul, Republic of Korea

PKC-delta is a protein kinase which is reported to mediate apoptosis. Upon activation, PKC-delta undergoes autophosphorylation and translocates to membrane and induces apoptosis. HSP25 (Heat Shock Protein 25) is known to bind to PKC-delta and inhibit phosphorylation of PKC-delta, which leads to inhibition of apoptosis. Recent studies identified V5 region of PKC-delta as a binding site of HSP25. And there are several autophosphorylation sites in V5 region of PKC-delta. Therefore, we designed an experiment with PKC-delta-WT and PKC-delta-S662A mutant, an autophosphorylation-deficient mutant of PKC-delta. And we compared apoptosis activities and kinase activities between PKC-delta-WT and PKC-delta-S662A mutant. We found that the kinase activity and apoptosis activity of PKC-delta-S662A mutant was higher than PKC-delta-WT. We observed that PKC-delta-S662A mutant has more phosphorylation on tyrosine 311 than PKC-delta-WT. We also found that PKC-delta-S662A mutant translocates to membrane faster than PKC-delta-WT after PMA treatment. Detailed biochemical mechanisms by which autophosphorylation regulate kinase activity of PKC-delta is under investigation.

92 Mechanisms Underlying Rho GTPass Signaling Specificity in Mammalian Cells

A. B. Jaffe,1 A. Schmidt,1 A. J. Self,1 A. Hall1;1 Cell Biology, Memorial Sloan-Kettering Cancer Center, New York, NY, 2MRC Laboratory for Molecular Cell Biology, University College London, London, United Kingdom

The precise temporal and spatial regulation of signaling pathways is essential for a wide range of cellular processes, such as migration, proliferation, and morphogenesis. The Rho family of small GTPases are key players in these processes, through their ability to control the rearrangement of the actin and microtubule cytoskeletons, the activation of gene expression, and the various phases of the cell cycle. In response to extracellular cues, Rho GTPases switch from the inactive, GDP-bound form, to the active, GTP-bound form via guanine nucleotide exchange factors (GEFs). One active, Rho GTPases transduce their signals by interacting with target proteins, termed ‘effectors’. Two crucial questions in Rho GTPase signaling are how specificity downstream of the GTPasses is controlled, and how the Rho GTPass-dependent signaling pathways are coordinated during various cellular processes. We have found that the scaffold protein hCnK1 can associate with a subset of Rho-specific GEFs, as well as components of the JNK MAPK pathway, and that hCnK1 is specifically required for agonist-induced JNK activation downstream of Rho. We are currently investigating how the hCnK1 complex is spatially and temporally regulated in response to extracellular signals, and how its regulation affects Rho GTPase-dependent signaling pathways.

93 Identification of Activation Mechanism and Signaling Pathway for Orphan Tyrosine Kinase Receptor 2 (Ror2)

Y. Liu,1 J. F. Ross,1 P. Bodine,1 J. Billiard1 1Women’s Health & Musculoskeletal Biology, Wyeth R&D, Collegeville, PA, 2Biological Technologies, Wyeth R&D, Cambridge, MA

Ror2 is an orphan receptor tyrosine kinase important for developmental morphogenesis, particularly of the skeleton. To investigate the Ror2 activation mechanism, we tested if the Ror2 receptor can form homo-dimers and whether this can lead to receptor activation similar to other receptor tyrosine kinases. Using an interaction assay between flag-tagged and his-tagged Ror2 over-expressed in U2OS cells, we demonstrated that Ror2 does form homo-dimers and that the extent of dimerization can be greatly enhanced by treatment with a bivalent Ror2 antibody. The antibody-induced dimerization leads to activation of the Ror2 tyrosine kinase, as measured by Ror2 autophosphorylation. We next investigated potential Ror2 binding partners. Ror2 was immunoprecipitated from Ror2-over-expressing U2OS cells lysates and Ror2 binding proteins were analyzed by mass spectrometry (MS). This analysis identified a scaffold protein 14-3-3β as a potential target. The interaction between Ror2 and 14-3-3β was confirmed by co-immunoprecipitation from cell lysates and by in-vitro binding assay using purified GST-Ror2 and 14-3-3β. We also found that Ror2 phosphorylates tyrosine residue(s) on 14-3-3β in both the cell and in vitro and that treatment with Ror2 antibody potentiates this effect. Furthermore, we performed MS analysis of 14-3-3β interactors in absence or presence of Ror2. We found several proteins that interacted with 14-3-3β in Ror2-dependent manner suggesting that interaction with Ror2 may have functional consequences on 14-3-3β signaling. Previously, we demonstrated that Ror2 stimulates osteoblast commitment and differentiation. Here, we found that treatment with a bivalent Ror2 antibody dose-dependently enhanced osteogenesis of human mesenchymal stem cells. Furthermore, treatment of neonatal mouse calvarial bones with the Ror2 antibody ex-vivo increased...
the rate of bone formation by 50% compared to IgG control. In conclusion, this work shows that antibody-induced dimerization of Rock2 leads to receptor activation and identifies 14-3-3β scaffold protein as the first substrate for Rock2 tyrosine kinase.

94  Strech-induced Tyrosine Phosphorylation of PECAM-1 in a Detergent-extracted Cell Model

Y. Chiu,1,2 E. McBeath,1 K. Fujimura1; Cardiovascular Research Institute, University of Rochester, Rochester, NY, 1Department of Biochemistry and Biophysics, University of Rochester, Rochester, NY

PECAM-1 (Platelet, Endothelial Cell Adhesion Molecule-1) is concentrated at interendothelial cell adhesions and interacts homophilically between neighboring cells. When a monolayer of endothelial cells is exposed to fluid shear stress, hypertonic shock, or cyclic stretch, PECAM-1 is rapidly tyrosine phosphorylated. Furthermore, we have shown that directly pulling on cell surface PECAM-1 will tyrosine phosphorylate the molecule and proposed that PECAM-1 is a mechanoresponsive protein of endothelial cells. To further investigate the mechanism of mechanical force-induced PECAM-1 phosphorylation, we made an extracted cytoskeletal model that could be mechanically activated. A confluent monolayer of bovine arterial endothelial cells cultured on an elastic silicon membrane was briefly treated with a Triton X-100 containing buffer to permeabilize the plasma membrane and release soluble cellular components. Immunofluorescence imaging showed that the resulting PECAM-1-actin complexes were able to undergo 2-fold increases in filamentous actin (F-actin) content, and we also identified three other mutant Actins whose ectopic expression favored F-Actin formation, nuclear accumulation of MAL and activation of SRF in the absence of external signals. The activation had similar or even shorter kinetics compared to control cells, whereas nuclear Elk-1 activation was decreased. Thus, the spatial organization of endosomes contributes to the cell cycle progression of human melanoma cells. We have demonstrated that the endosomal compartment provides spatial resolution of signal transduction which has implications for the understanding of cell cycle regulation.

95  TULA: A Novel Protein That Affects Protein Tyrosine Kinases’ Activation and Degradation

R. Agrawal, A. Tsyanov; Microbiology and Immunology, Temple University, Philadelphia, PA

TULA (T-cell Ubiquitin Ligand) is a novel protein of a family of 2 proteins. The proteins consist of UBA and SH3 domains; and the C-terminal half contains homologies to PhosphoGlycerate Mutase catalytic domain and also nuclear localization signal. TULA is the first protein described to contain both the UBA and SH3 domains. TULA is a primarily lymphoid protein, although the second protein of the family is ubiquitously present. TULA was shown to bind c-Cbl, ubiquitin and ubiquitylated proteins. As c-Cbl is an E3 ubiquitin ligase and is known to downregulate activated protein tyrosine kinases (PTKs) by ubiquitination, involvement of TULA in this process was studied. It was found that TULA protects receptor PTKs from c-Cbl mediated ubiquitilation. Double knockout mice for the two proteins of this family were viable and normal. The double knockout T cells had enhanced proliferative ability. Further analysis showed that in the double knockout T cells, p70, a S6 kinase kinase, was activated. Therefore, TULA protects an effect on PTKs but its molecular mechanism is not known. Hence, to understand these cellular mechanisms we chose to study the effect of TULA on Syk, a non-receptor PTK. Syk is found in both B and T cells, binds to c-Cbl and is ubiquitylated by c-Cbl. Our studies have shown that TULA activates Syk. However, the effect of the second protein is opposite to TULA’s effect on Syk. Therefore, in contrast to knockout data, which suggest both the members of this family have similar/overlapping effects, our studies show that these proteins may have different effects and act through different functional mechanisms. The details of the differential effects and mechanisms of the TULA proteins will be discussed.

96  Regulation of SRF-mediated Transcription by Monomeric Actin and MAL/MRTF

G. Posern, M. Bode, S. Fiehn; Dept. of Molecular Biology, Max-Planck-Institute of Biochemistry, Munich, Germany

Signal-induced activation of the transcription factor Serum Response Factor (SRF) requires Rho-mediated alterations in Actin dynamics. Direct evidence demonstrates that monomeric G-Actin itself participates specifically in SRF regulation. The molecular link between G-Actin and SRF was identified as the MAL protein, which serves as a Rho-regulated transcriptional coactivator for SRF. MAL was first described in the leukemic fusion protein OTT-MAL, which is a potentially deregulated oncogene. MAL translocates from the cytoplasm to the nucleus upon stimulation. The translocation is blocked by β-ACTin or non-polymiserable Actin mutants. MAL directly associates with G-Actin via N-terminal RPEL motifs with high affinity. Stimuli which activate SRF resulted in dissociation of the MAL-Actin complex. This suggests a titration model in which monomeric Actin negatively regulates SRF via its interaction with MAL. However, we also identified three other mutant Actins whose ectopic expression favoured F-Actin formation, nuclear accumulation of MAL and activation of SRF in the absence of external signals. The three mutants share several biochemical properties, e.g. increased binding to profilin, reduced binding to Gelsolin (Segment 4-6), and stabilised F-Actin formation. Two of the mutants interact with MAL, but activate SRF in a Rho- and Actin-treadmilling independent manner. Thus, the spatial organization of endosomes contributes to the cell cycle progression of human melanoma cells. We have demonstrated that the endosomal compartment provides spatial resolution of signal transduction which has implications for the understanding of cell cycle regulation.

97  Phosphotyrosine-3 Kinase Regulatory Subunit P55γ Interacts with Rb and Regulates Cell Cycle

W. Wang, T. Deh, M. Johnson, R. Dickson; Oncology, Georgetown University Medical Center, Washington, DC

Phosphotyrosine-3 Kinase Regulatory Subunit P55γ (P55γ) plays an important role in regulating the proliferation of the cells of the mammary gland. P55γ is a regulatory subunit of PI3K. In contrast to the widely studied 85 kDa regulatory elementsubunits p85α and p85β, p55γ is encoded by a distinct gene, p55γ3, and is expressed mostly in the brain, testis, kidney, and fatty tissues. The role of p55γ in breast tissue has not been studied in detail. Western blot analysis confirmed that p55γ is expressed at various levels in cells of mammary origin, normal or malignant mammary epithelial cells. An earlier report demonstrated that the NH2-terminal of p55γ contains retinooblastoma protein (Rb) binding ability. In MCF7 cells, overexpression of the NH2-terminal amino acids leads to cell cycle arrest. We hypothesize that p55γ might regulate cell cycle progression through its interaction with Rb. The aim of this study was to test this hypothesis and to determine the role of p55γ in cell cycle progression in human cells. We generated wild-type and epitope-tagged expression constructs for p55γ to study its role in epithelial cells. The interaction between full-length p55γ and Rb was studied by co-immunoprecipitation and western blot analysis. Mammalian cells overexpressing p55γ as a result of stable or transient transfection were subjected to cell cycle analysis by FACS. Using the constructs we generated have confirmed the binding between Rb and full-length p55γ protein. Preliminary results also suggested that overexpression of p55γ alters cell proliferation primarily by shortening the time of S phase entry in cell cycle. This is the first report indicating cellular function of a full-length p55γ protein in cultured mammalian cells, based on its interaction with Rb, and we continue to examine the underlying mechanisms. Supported by individual DOD predoctoral traineeship award #W81XWH-04-1-0408 (YW).

98  The Late Endosomal Compartment Provides Spatial Information for MAPK Signaling Pathways

N. Taub, D. Ten, L. A. Huber; Division Cell Biology, Biocenter Innsbruck, Innsbruck, Austria

Signal transduction through the MAPK cascade is regulated by the assembly of different scaffold complexes at distinct subcellular locations like plasma membrane or endosomes. Thus it appears likely that spatial resolution of signal transduction is a critical parameter to define signaling specificity. Therefore, we investigated if the endosomal compartment provides spatial information for MAPK signaling. To study the crucial signaling parameter space we used the dynemin subunit p50 to change the intracellular position of endosomes. Overexpression of p50 mislocalized late endosomes, LAMP-1 and LIPA positive compartments, to the cell periphery, whereas the distribution of early endosomes, EEA1 positive structures, was unaffected. This dearrangement of the late endosomal compartment also dramatically affected the transport of the EGFR and resulted in a prolonged EGFR activation on peripherally mislocalised endosomes. Moreover, the endosomal compartment also alters the kinetics of EGFR phosphorylation on peripherally mislocalised endosomes. This indicates that the endosomal compartment provides spatial resolution of signal transduction which has implications for the understanding of cell cycle regulation.

99  Stretch-induced Tyrosine Phosphorylation of PECAM-1 in a Detergent-extracted Cell Model

Y. Chiu,1,2 E. McBeath,1 K. Fujimura1; Cardiovascular Research Institute, University of Rochester, Rochester, NY, 1Department of Biochemistry and Biophysics, University of Rochester, Rochester, NY

PECAM-1 (Platelet, Endothelial Cell Adhesion Molecule-1) is concentrated at interendothelial cell adhesions and interacts homophilically between neighboring cells. When a monolayer of endothelial cells is exposed to fluid shear stress, hypertonic shock, or cyclic stretch, PECAM-1 is rapidly tyrosine phosphorylated. Furthermore, we have shown that directly pulling on cell surface PECAM-1 will tyrosine phosphorylate the molecule and proposed that PECAM-1 is a mechanoresponsive protein of endothelial cells. To further investigate the mechanism of mechanical force-induced PECAM-1 phosphorylation, we made an extracted cytoskeletal model that could be mechanically activated. A confluent monolayer of bovine arterial endothelial cells cultured on an elastic silicon membrane was briefly treated with a Triton X-100 containing buffer to permeabilize the plasma membrane and release soluble cellular components. Immunofluorescence imaging showed that the resulting PECAM-1-actin complexes were able to undergo 2-fold increases in filamentous actin (F-actin) content, and we also identified three other mutant Actins whose ectopic expression favored F-Actin formation, nuclear accumulation of MAL and activation of SRF in the absence of external signals. The activation had similar or even shorter kinetics compared to control cells, whereas nuclear Elk-1 activation was decreased. Thus, the spatial organization of endosomes contributes to the cell cycle progression of human melanoma cells. We have demonstrated that the endosomal compartment provides spatial resolution of signal transduction which has implications for the understanding of cell cycle regulation.
99  Profiling of Signal Pathway Activation during In Vitro Lymphangiogenesis  
L. V. Leak,1 V. S. Calver,2 J. D. Walkshulte,3 E. F. Petricoin, III,1 L. A. Liotta1; Anatomy, Howard University, Washington, DC; 1Molecular and Microbiology, Center for Applied Proteomics and Molecular Medicine, George Mason University, Manassas, VA; 1George Mason University, Manassas, VA; 1Molecular and Microbiology, George Mason University, Manassas, VA

Results from our laboratory and others have shown that VEGF-C is a potent mitogen for the lymphatic endothelium and a key lymphangiogenic factor. However, the biochemical signaling pathways activated via VEGFR-3 upon stimulation by VEGF-C in the lymphatic endothelium (LEC) are not well characterized. Likewise, the molecular mechanisms that regulate the discrete phenotypic modulations of proliferation leading to the formation of new lymphatic vessels from existing vessels during the process of lymphangiogenesis have not been defined. To profile and map multiplexed protein phosphorylation and determine the linkage of signal transduction events during these various stages, we have employed an in vitro cell culture model of lymphangiogenesis exhibiting all four stages, coupled with reverse-phase protein microarrays and phosphor-specific antibodies. The goal is to examine the activation status of key molecular proteins and determine the roles played by others. By employing this high throughput technology for the analysis of signal pathway profiling in LEC during lymphangiogenesis it will be possible to gain further insight into the molecular mechanism of signals in the lymphatic endothelium during various physiological and pathological conditions.

100  Retinoylation: Formation of Retinoylated Protein from Retinyl-CoA  
Y. Kubo, T. Obha, M. Naito, N. Takahashi; Laboratory of Physiological Chemistry, Institute of Medicinal Chemistry, Hoshi University, Tokyo, Japan

Retinoylation (acylation of protein by retinoic acid) is considered as one mechanism of retinoic acid (RA) action occurring in cells in vitro and in vivo. Previously our studies have shown that in rat tissues the formation of retinoyl-CoA from RA, the first step of retinoylation required ATP, CoA and MgCl2. In the current study, we examined whether the transfer of retinoyl-CoA into protein, the second step of retinoylation, occurs in rat tissues. [3H]-Labelled-retinoyl-CoA bound covalently to proteins in rat liver, kidney, testis, and brain. The levels of incorporation of retinoyl-CoA into proteins were higher in vitrino than in vivo. A distinct peak of RA was detected in rat liver, and the formation of retinoylated protein depended on the incubation time and the concentrations of retinoyl-CoA and homogenate. The reaction was suppressed by fatty acyl-CoAs and palmitic acid, but not by arachidonic acid. Vmax and Km values for retinol-CoA in the formation of retinoylated protein using crude liver extract were estimated to be 2597.3 pmol/min/mg protein and 9.5 x 10^-5 M respectively. Retinoylated proteins formed from retinyl-CoA, including a 17 kDa protein with high radioactivity, disappeared in the presence of 2-mercaptoethanol, indicating that RA was linked to protein by thioester. These results demonstrate that retinoylation in rat tissues occurs via retinyl-CoA formed from RA. This process may play a significant physiological role in cells.

101  Essential Amino Acids Determine Lifespan in Yeast  
P. Gomes, B. Sampaio-Marques, P. Ludovico, F. Rodrigues, C. Leão; Life and Health Sciences Research Institute (ICVS), Braga, Portugal

In virtually every organism, nutrient sensing and caloric intake regulate aging and longevity. In yeast, interventions resembling caloric restriction, either by reduction of glucose or non-essential amino acids content in the medium, prolong lifespan and retard aging, suggesting that the energetic status of the cell is the main responsible for the lifespan extension. In addition, it has been shown that subtle, often unrecognized amino acid limitations lead to much lower final biomass concentration in cultures of different commonly used auxotrophic Saccharomyces cerevisiae strains. In this study, we have examined the role that essential amino acid supplementation of auxotrophic S. cerevisiae mutants plays in determining yeast lifespan and stress resistance. Our results performed in yeast cells cultured in regular amino acid content showed that this is insufficient amino acid supplementation, being accompanied by a reduced final biomass and phenotypes of premature aging. These included shorter lifespan and a decreased resistance to oxidative stress, without significant changes in heat or acid stress resistance. These results indicate that starvation for essential amino acids results in cell stress, which may finally trigger apoptosis. In conclusion, our findings point to the fact that subtle amino acid limitation has severe consequences on cell physiology, lifespan and stress resistance, therefore presenting a note of caution for studies using auxotrophic yeast strains.

102  Gene Expression of hrp2+ Gene Related to SNF2 Family in Yeast  
I. Choi; Life Science, Silla University, Pusan, Republic of Korea

The SNF2/SW12 family comprises proteins from a variety of species with in vivo functions, such as transcriptional regulation, maintenance of chromosome stability during mitosis, and various types of DNA repair. This study was aimed at the characterization of hrp2+ gene which was isolated by PCR amplification using the conserved domain of SNF2 motifs. Sequence analysis of hrp2+ gene showed striking evolutionary conservation among the SNF2 family of proteins. The transcript of hrp2+ gene was found to be a 4.7 kb as identified by Northern hybridization. In addition, to determine the transcription initiation site of hrp2+ gene, primer extension analysis was performed. This result showed the band of 64 bp. The transcriptional start point was mapped to a position of 47 base pair from the first ATG codon of translational initiation codon. In order to investigate the inducibility of hrp2+ gene, transcript levels were examined after treating the cells to various DNA damaging agents. The transcripts of hrp2+ were induced by UV-irradiation. But the transcripts were not induced by treatment of 0.25% Methylmethane sulfonate (MMS). These results implied that the effects of damaging agents are complex and different regulatory pathways exist for the induction of this gene.

103  Response of the HgI MAPK Pathway in S. cerevisiae Shows an Ability to Learn from Previous Stimulation  
T. Ursell, K. Yasutis, B. B. Kaufmann, A. van Oudenaarden; Physiology Course 2006, Marine Biological Laboratory, Woods Hole, MA

In order to survive a wide range of osmotic conditions the budding yeast S. cerevisiae has developed a combined signaling and genetic circuit that, under hypertonic shock, temporarily up-regulates glycerol synthesis. In response to hyperosmotic stress, the MAPK HgI translocates to the nucleus upon phosphorylation by its upstream kinase Hence, nuclear localization of HgI is a direct measurement of activation of this protein cascade. Using YFP labeled HgI and GFP labeled nuclear protein Nrd1, the nuclear amount of HgI was tracked as a function of time. Cells were subjected to 250 mM pulses of NaCl for various periods from 4 minutes to 80 minutes. Simultaneous tracking of cell size gave a readout of the end product of the circuit as glycerol production increased and osmotic pressure equalized. Upon osmotic shock, initial Hog1 nuclear localization is transient, but subsequent identical shocks yielded an amplified response, and the ability of such protein circuits to learn from previous stimuli may be an important evolutionary tool for survival in diverse environmental conditions.

104  Dissecting the Glucose Signaling Network in Saccharomyces cerevisiae  
S. Zaman1, L. Schnerer,1 X. Zhang,2 K. M. Shokat,2 J. R. Bresch3; 1Department of Molecular Biology, Princeton University, Princeton, NJ; 3Department of Cellular and Molecular Pharmacology, University of California, San Francisco, CA

Glucose is an important nutrient that is used as the primary source of energy in most organisms. Misregulation in glucose utilization can cause diseases in humans such as diabetes mellitus. Glucose signals transcriptional changes through a number of different pathways. Identification of the transcriptional targets of the pathways will contribute towards the understanding of how mutations in specific pathways perturb the system to cause diseases. Interestingly, the proteins that make up the glucose signaling pathways are highly conserved from humans to yeast. The budding yeast, Saccharomyces cerevisiae, is a genetically tractable organism and we have used it to dissect out how the glucose signal feeds through the different signaling pathways and
investigate how the pathways interconnect with each other. *Saccharomyces cerevisiae* has at least four pathways, Ras/PKA, Snf1, Rgt1 and Sch9 that have been implicated in signaling changes to glucose availability. By using inducible and repressible mutants in the Ras/PKA, Snf1, Rgt1 and Sch9 pathways and following the changes in the transcriptional profile of the cells with microarrays after activation/repression of these pathways, we have made several new discoveries. Our studies show that selective inhibition of Snf1, the yeast homolog of mammalian AMPK, changes expression of only a few genes involved in carbohydrate acid metabolism. On the other hand, the C-terminally truncated pathway and Sch9 protein kinase, homologue to mammalian Akt, redundantly mediates most of the transcriptional changes that occur in response to glucose addition. However, the Ras/PKA pathway plays a bigger role than the Sch9 pathway because selective inhibition of PKA reduces but does not eliminate the response of cells to glucose. Inhibition of Sch9 pathway does not affect the response of cells to glucose. This study contributes towards the understanding of how the cell processes the glucose signal and opens up novel routes for intervention for disease treatment.

105 Cell Cycle Regulation of MAP Kinase Cascade Signaling in a Yeast Differentiation Pathway

P. Pyciak, S. Strickfaden, M. Winters, R. Lamson; University of Massachusetts Medical School, Worcester, MA

Control of cell fate by external signals often occurs in the context of cell division. Thus, it may be generally important to coordinate the response to differentiation signals with cell cycle status. In budding yeast, mating pheromones arrest cells in the G1 phase of the cell cycle by preventing passage through Start. Conversely, when cells pass Start and commit to a new cell cycle, they become resistant to pheromone arrest. In particular, signal transduction through the pheromone-responsive MAP kinase pathway is actively inhibited by G1 CDKs. But the target, mechanism, and physiological role of this inhibition has remained enigmatic. Here, we show that the target of this inhibition is Ste5, the MAPK cascade scaffold protein. G1 CDK phosphorylation of Ste5 occurs at a cluster of sites surrounding a small, basic membrane-binding motif. The added phosphates disrupt the electrostatic interaction of this Ste5 domain with the plasma membrane, which is required for pheromone resistance. Effective inhibition of Ste5 signaling requires phosphorylation at multiple (8) sites and a substantial accumulation of negative charge, suggesting that Ste5 acts as a sensor for high G1 CDK activity. Thus, Ste5 is an integration point for signals from both external (pheromone) and internal (cell cycle) sources. When Ste5 cannot be phosphorylated, its activity is no longer tied to the cell cycle, and pheronome now triggers an aberrant arrest at inappropriate stages of the cell cycle. This ectopic arrest occurs either in the presence or absence of the CKD inhibitor protein Far1. Overall, these findings define a mechanism and physiological benefit of restricting antiproliferative signaling to G1. Furthermore, by requiring phosphorylation at multiple CDK sites in Ste5, this regulatory circuit demands high G1 CDK activity, providing the cell with a mechanism for decisively shutting off pheromone signaling only when conditions are appropriate for cell cycle entry.

106 ATG1 Regulates Filamentous Growth in *Saccharomyces cerevisiae* Independent of Autophagy

M. D. Seya, M. Snyder, S. P. Dinesh Kumar; MCDB, Yale University, New Haven, CT

In response to nitrogen limitation, diploid *Saccharomyces cerevisiae* utilizes distinct signaling mechanisms for survival including pseudohyphal growth and autophagy. Moderately low nitrogen concentrations trigger diploid cells to undergo pseudohyphal differentiation while extremely low nitrogen concentrations trigger cells to undergo bulk protein degradation via autophagy for cell survival. Although these distinct processes operate within a relatively narrow physiological range of nitrogen availability, they must be stringently controlled to prevent cross activation yet flexible enough to facilitate a rapid and fluid response during nitrogen consumption. Here we present evidence that ATG1, a serine/threonine kinase required for autophagy, is also needed for pseudohyphal formation. 

107 Identification of Short Membrane-Targeting Domains in Yeast Cdc42 Effectors

S. Takahashi, P. M. Pyciak; University of Massachusetts Medical School, Worcester, MA

Signaling proteins often contain multiple interaction modules, which in principle can collaborate to control protein function. The *S. cerevisiae* p21-activated kinase (PAK) Ste20 functions in multiple MAPK signaling pathways as well as in actin organization, cell polarity and cell cycle transitions. Ste20 kinase activity and localization are controlled by the GTPase Cdc42, which binds an autoinhibitory region in Ste20 called the CRIB motif. We have identified a new domain, adjacent to the Ste20 CRIB motif, that interacts directly with plasma membrane lipids and is critical for Ste20 function. This region, termed the basic-rich (BR) domain, is a short (~27 residue) motif that can target GTP to the plasma membrane in vivo and bindsPIP2-containing liposomes in vitro. Mutations in the BR domain severely disrupt the localization and function of Ste20. In addition, the Ste20 BR domain can be functionally replaced with foreign lipid-binding domains, such as the PH (pleckstrin homology) domain from mammalian PLCγ. Thus, both membrane and protein interactions act in concert to regulate membrane-localized activation of Ste20 by Cdc42. Consistent with this model, the BR domain becomes dispensable when Cdc42 binding is bypassed by disrupting the autoinhibitory conformation of Ste20 with separate mutations. This situation is analogous to a mammalian Cdc42 effector, N-WASP, whose activation requires both Cdc42 and a polybasic PIP2-binding motif. Furthermore, we find that two other Cdc42 effectors in yeast, Gic1 and Gic2, also have BR-like motifs that are critical for their localization and function. These observations suggest a common theme for Cdc42 effectors in which a membrane-binding domain is required to help target the protein to its activator. Interestingly, some BR-like domains have the additional capacity to confer nuclear localization, raising the possibility that BR-like motifs may be generally useful for targeting proteins to both nucleus and the plasma membrane.

108 Triad3A Interacts with RIP1 through a TIR Homologous Domain

C. Fears, Q. Pan, J. C. Mathisson, T. Chuang; Immunology, The Scripps Research Institute, La Jolla, CA

Triad3A is a RING finger E3 ubiquitin-protein ligase identified in a yeast two hybrid screening using the *TIR* domain of TLR9 as a bait. This E3 ligase promotes ubiquitination and proteolytic degradation of TLR4 and TLR9, and negatively regulates their activation by LPS and CpG-DNA, respectively. Given that Triad3A is capable of binding to the TIR domains of TLR4 and TLR9, we reasoned that Triad3A may also be able to regulate proteolytic degradation of TIR domain-containing adapter molecules. In the present study, we investigated its activity on adapter molecules downstream of TLRs and TNF-α receptor 1. Triad3A promoted down-regulation of two TIR domain-containing adapter proteins, TRAP and TRIF, as well as a RIP1, but had no effect on other adapter molecules in either the TLRs or TNF-α signaling pathways. RIP1 is essential for TNF-α induced NF-κB activation. This adapter protein contains an N-terminal kinase domain, a RHIM motif, and a C-terminal death domain, but does not contain a TIR domain. However, the interaction between Triad3A and RIP1 suggested to us that RIP1 may have a domain with some conserved motif homologous to a TIR domain that allows for interaction with Triad3A. We performed multiple sequences alignment analysis using amino acid sequences from the TIR domain of each of the MyD88 family members and RIP1. The results indicated that the C-terminal domain of RIP1 contains a TIR homologous domain, and mutation of amino acid residues in this domain identified three residues critical for its interaction with Triad3A. Moreover, reduction of Triad3A expression by siRNAs rendered cells hyperresponsive to TNF-α stimulation. Conversely, overexpression of Triad3A in cells blocked TNF-α induced cell activation. These results suggest that Triad3A interacts with RIP1 through a TIR homologous domain, and negatively regulates TNF-α activation.

109 Suppressing Plus-end Microtubule Dynamics Leads to Loss of MTOC Cohesion at the Onset of Mitosis

J. Hornick, E. H. Hinchcliffe; Biological Sciences, University of Notre Dame, Notre Dame, IN

Cells treated with Taxol are observed to assemble multiple mitotic asters, resulting in abnormal spindle assembly and defects in cell division. Taxol preferentially stabilizes the plus-ends of microtubules ([Rogov et al. 1999 MTCB 18:947]), and inhibits minus-end directed transport, which requires the recruitment of dynein/dynactin to the dynamic plus-ends of the microtubules. However, it remains unclear how stabilization of the plus end leads to the observed mitotic defects. We examined Taxol spindle assembly in vivo using BSC1-otubGFP cells. Live-cell video microscopy of Taxol-treated cells entering mitosis reveals that the majority of microtubules dissociate from the centrosome just before NEB, and intact microtubules rapidly migrate to the cell periphery, where they become embedded in the cortex. The cortical arrays of microtubules are then bundled into large sheets, which bud off the cortex and form large, 20
hollow asters. These asters migrate toward the mass of chromosomes, where they fuse together to form a multipolar spindle. While some small asters form on the nuclear envelope, asters are not observed appearing de novo in the cytoplasm, contrary to the results of in vitro experiments. To examine whether microtubule dissociation from the centrosome is a result of microtubule stabilization or inhibition of transport, we treated cells with Taxol for 5 min and then observed cells at NEB. This 5 min treatment is sufficient to suppress plus-end dynamics and block “tip-tracking”, but does not cause microtubule dissociation from the centrosome at the onset of mitosis. Our results suggest that suppression of plus-end microtubule dynamics during interphase results in the inhibition of microtubule-based transport, leading to the depletion of “key factors” necessary to maintain the cohesion of the microtubule network at the centrosome as the cell transitions into mitosis.

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Proteasome Inhibition Leads to Centrosome Fragmentation and Loss of Chromosome Motility
B. K. Carney, B. Becker, L. Cassimere; Biological Sciences, Lehigh University, Bethlehem, PA
We are interested in how the mitotic spindle assemblies and maintains a bipolar shape. We find that treatment of Hela or LLCPK cells with the proteasome inhibitor MG132 disrupts centrosome integrity, leading to centrosome fragmentation within 2 - 3 hours. MG132-treated cells also assemble multipolar spindles, consistent with a previous report (Ehrhardt and Sluder, 2005. J. Cell Physiol. 204: 808). In MG132-treated Hela cells ubiquitin is localized to an aggregate at the centrosome, suggesting that proteasomes are localized there. Live cell imaging of Hela cells expressing GFP-CENPA demonstrated loss of kinetochore directional instability 2 - 3 hours after MG132 addition and relaxation of tension between sister kinetochores. These results demonstrate that MG132 treatment disrupts both normal spindle structure and chromosome motility and suggest that the proteasome may function in these processes.

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Laser Ablation of Mitotic Spindle in Anaphase Can Prevent Progression to Cytokinesis
N. M. Baker,1 G. Wu,1 L. Shi,1 E. Botvinick,2 M. Berns;2 1University of California, San Diego, La Jolla, CA; 2Beckman Laser Institute, University of California, Irvine, Irvine, CA
In previous studies we have shown that the second harmonic (532 nm) from a picosecond frequency doubled Nd:YAG laser can be used to cleanly and selectively ablate and sever fiber microtubules in live cells (Botvinick et al 2004, biophys. J. 87:4303-4212). It was shown that ablating only one half spindle (severing microtubule connections between pole and chromosome at the metaphase plate) after the immediate onset of anaphase did not affect chromosome movement to the poles and subsequent cytokinesis. In this current study on PTK2 cells, we have observed that immediate complete ablation across both half spindles immediately after anaphase onset results in the prevention of cytokinesis even though the chromosomes still undergo normal anaphase movements from the metaphase plate to the poles. Cells of the rat kangaroo line (PTK2) were stably transfected with YFP-tubulin and Histone-CFP and imaged and ablated using the automated RoboLase microscope (Botvinick and Berns, 2005, Micros. Res. Tech 69/65-74). Fluorescent (in metaphase-anaphase transition were irradiated with 0.2447 nJ/micropulse, corresponding to an irradiance of 1.4496*10^7 W/m^2. The irradiated cells were followed for up 24 hours with no observed cytokinesis. Other cells were fixed and stained immediately after laser ablation in order to determine whether or not the prevention of cytokinesis was due to disruption of the microtubules or due to effects on key proteins that require microtubules as a ‘rail’ for transport. The laser microablating system and experimental observations could contribute significantly to elucidating the cellular dynamics of anaphase mitotic spindles.

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Cell Division Defects Associated with Exposure to Bisphenol-A
O. L. George,1 C. Corona,2 J. Arterburn,2 C. B. Shuster;1 1Molecular Biology and Biology, New Mexico State University, Las Cruces, NM, 2Chemistry, New Mexico State University, Las Cruces, NM
There is an increasing concern that mammalian reproduction may be adversely affected by exposure to artificial compounds resembling endogenous hormones. In particular, the ubiquitous component of polycarbonate plastics, Bisphenol-A (BPA), has shown to have weakly estrogenic properties. Additionally, rodent colonies accidentally exposed to BPA resulted in increased meiotic aneuploidies (Current Biol 13:546-553), suggesting that BPA may also have profound effects on cell division. In an effort to characterize its effects on cell division at the cellular and molecular level, we examined BPA effects on sea urchin embryo development, whose rapid cell cycles and lowered checkpoint responsiveness make them more sensitive to environmental pollutants. Examination of Lytechinus pictus and Strongylocentrotus purpuratus eggs and embryos exposed to BPA displayed dose-dependent effects on microtubule organization and cytokinesis. Live cell analyses suggest that while a bipolar spindle formed in the presence of 500nM BPA, the spindle fails to correctly direct contractile ring formation, resulting in ectopic furrows in the polar and equatorial cell surfaces. However microtubule polymerization in Xenopus oocyte extracts was not affected by BPA at any dose, suggesting that BPA is not directly affecting microtubule polymerization. Morphological examination of BPA-treated embryos revealed several general classes of phenotypes, including ectopic cortical microtubules, multipolar spindles, cleavage failure, and delayed mitosis. Pulse treatment of blastula-stage embryos resulted in an increase of mitotic figures, suggesting that BPA was delaying mitotic progression. Lastly, fractionation of mitotic extracts with biotinylated BPA analogs has identified several candidate BPA-binding proteins, including the cohesin subunit, SMC1. Together, these results provide additional evidence that in addition to its properties as an estrogenic molecule, BPA may adversely affect normal development by interfering with embryonic cell divisions.

Using Single-Fluorophore Speckle Microscopy to Examine Microtubule Dynamics in Xenopus Extract Spindles
G. Yang,2 B. Houghteling,3 J. Gaetz,2 J. Liu,2 G. Damaser,1 T. Kapoor;1 1Laboratory for Computational Cell Biology, The Scripps Research Institute, La Jolla, CA, 2Laboratory of Chemistry and Cell Biology, Rockefeller University, New York, NY
Fluorescence speckle microscopy (FSM) is a powerful technique for quantitatively tracking polymerization and transport dynamics of cytoskeletal proteins in a range of different cellular structures. In metaphase spindles, FSM has provided insightful into the spatial and temporal organization of microtubule flux and revealed the co-existence of different populations of microtubules. Under commonly used labeled tubulin concentration (~50nM for Xenopus extract spindles), each speckle represents a cluster of 2-8 fluorophores. When the fraction of labeled tubulin is reduced by about two orders of magnitude, approximately 80% of the trackable speckles (i.e. those with trajectories lasting 4 frames) correspond to single fluorophores as confirmed by photobleaching and intensity analyses. We report a comprehensive comparison of microtubule flux measurement under conventional, ultra-low labeling ratios and preliminary results on using single-fluorophore imaging to probe the structure of microtubule network. Although the ultra-low labeling is accompanied by much lower speckle density and thus reduced spatial resolution, we found microtubule dynamics that are not detectable using standard FSM conditions. Analysis of single-fluorophore speckles revealed a population of fast moving (~4-6 micron/min) microtubules, up to 3-fold faster that the rate of microtubule poleward flux. We are combining single-fluorophore FSM with perturbations of dynin and mitotic kinesins to examine the molecular basis for this fast transport in the spindle. In general, these results show that FSM at ultra-low labeling ratio provides an important complement to existing methods in reporting microtubule flux dynamics and further insight into the spatial and temporal heterogeneity of microtubule dynamics in metaphase spindles.
DNA Repair Pathways Involved in Anaphase Bridge Formation

C. Acilan,1 D. M. Potter,2 W. S. Saunders1; 1Biological Sciences, University of Pittsburgh, Pittsburgh, PA, 2Biostatistics, University of Pittsburgh, Pittsburgh, PA
Cancer cells frequently exhibit gross chromosomal alterations such as translocations, deletions, or gene amplifications, an important source of chromosomal instability in malignant cells. One of the better-documented examples is the formation of anaphase bridges - chromosomes pulled in opposite directions by the spindle apparatus. Anaphase bridges are associated with DNA double strand breaks (DSBs). While the majority of DSBs are repaired correctly, to restore the original chromosome structure, incorrect fusion events also occur leading to bridging. To identify the cellular repair pathways used to form these aberrant structures, we tested a requirement for either of the two major DSB repair pathways in mammalian cells: homologous recombination (HR) and non-homologous end joining (NHEJ). Our observations show that neither pathway is essential, but NHEJ helps prevent bridges. When NHEJ is compromised, the cell appears to use HR to repair the break, resulting in increased anaphase bridge formation. Cancer cells with high NHEJ activity are less likely to form bridges. In addition, the level of the NHEJ component XRCC4 can be used to estimate the tendency of different cancer cell lines to form bridges from chromatid breaks.

The Contribution of Chk1-dependent Centrosome Amplification to Mitotic Catastrophe and DNA Damage-induced Cell Death

E. Bourke,1 H. Dodson,1 S. P. Wheatley,2 A. Merdes,1 L. Currie,1 G. Zachos,1 M. Walker,1 D. Gillespie,1 C. Morrison1; 1National University of Ireland, Galway, Galway, Ireland, 2Genome Damage and Stability Centre, University of Sussex, Brighton, United Kingdom, 3CNRS-Pierre Fabre, Toulouse, France, 4Beatoson Institute for Cancer Research, Glasgow, United Kingdom
Centrosomal abnormalities are frequently observed in cancers. We used light and electron microscopy to show that DNA damage induces centrosome amplification in human cells. Caffeine abrogated this amplification in both ATM- and ATR-defective cells, suggesting a complementary role for these DNA damage-responsive kinases in promoting centrosome amplification. Inhibition of CHK1 by RNAi or drug treatment suppressed DNA damage-induced centrosome amplification. Radiation-induced centrosome amplification was abrogated in Chk1−/− DT40 cells, but occurred at normal levels in Chk1+ cells transgenically expressing Chk1. Using hypothermia, we observed that G2 arrest without DNA damage was insufficient to potentiate centrosome amplification. Interestingly, overexpression of Cyclin E in Chk1−/− cells restored DNA-damaged centrosome amplification, suggesting that Chk1 control of Cdk2-Cyclin E activity contributes to DNA damage-induced centrosome aberrations. To determine the contribution of centrosome aberrations to mitotic catastrophe and to analyse the extent to which mitotic catastrophe is involved in cell death after irradiation, we performed timelapse microscopy on human cells that expressed GFP-centrin1 and histone H2B-GFP following irradiation. We found a radiation dose-dependent increase in the frequency of cells failing in M, with > 50% of cells failing to complete mitosis successfully after 10 Gy (N = 107). The cellular outcome of having multiple centrosomes also depended on the levels of DNA damage. After 5 Gy, < 20% of cells with multiple centrosomes entered M and only 20% of these cells failed in their first mitosis (N = 32). Following 10 Gy, < 20% of cells with multiple centrosomes entered M and some 60% of these cells failed in M phase (N = 44). These findings demonstrate that mitotic catastrophe is a significant contributor to radiation-induced lethality and that centrosome amplification is a potential source of mitotic failure.

Tumor Formation via Loss of a Molecular Motor Involved in Maintenance of Chromosome Structure

M. Mazumdar, J. Lee, K. Sengupta, T. Ried, S. Rane, T. Misteli; National Cancer Institute, NIH, Bethesda, MD
Microtubule-associated kinesins are a diverse group of molecular motors that have been implicated in transport of membranes, chromosomes and viruses. The chromosome-associated chromokinesin KIF4 plays multiple roles in mitosis and its loss leads to mitotic defects including aneuploidy and genomic instability characteristic of many human cancers (Mazumdar et al., ICB, 2004). We have now taken advantage of the direct formation of aneuploidy in the absence of KIF4 to ask whether loss of a molecular motor and generation of aneuploidy can trigger tumorigenesis. We find that embryonic stem cells genetically depleted of KIF4 support anchorage-independent growth and form tumors in nude mice. Down-regulation or loss of KIF4 is physiologically relevant since reduced KIF4 levels are present in many human cancers from several tissues. In cells lacking KIF4 mitotic spindle checkpoints and DNA damage response pathways are activated in similar patterns as observed in early cancer cells (Mazumdar et al., Curr. Biol., 2006). Although the detrimental role of KIF4 loss becomes most evident in mitosis, we find that KIF4 also plays a critical role in interphase chromatin structure. Our results suggest that KIF4 is unique amongst known molecular motors in that it maintains both interphase and mitotic chromosome structure. Its functional loss can trigger tumor formation, possibly via alteration of chromatin organization, and they support the notion that aneuploidy can act as a primary trigger of tumorigenesis.

Aurora B Is Enriched at Merotelic Attachment Sites Where It Regulates MCAK

A. L. Knowlton, W. Lan, T. Stukenberg; Biochemistry, UVA, Charlottesville, VA
Often kinetochores form merotelic attachments, in which a single kinetochore is attached to microtubules from both spindle poles. These attachments can result in improper chromosome segregation, and are a significant source of aneuploidy, a hallmark of cancer. Aurora B kinase and the kinesin-13 microtubule depolymerase Mitotic Centromere Associated Kinesin (MCAK) are required to release improper microtubule attachments. Aurora B regulates MCAK's activity and localization. We set out to understand why MCAK and Aurora B are more abundant at some metaphase aligned chromosomes, but are present at low amounts on most others. We find that members of the Aurora B complex are specifically enriched at merotelic attachment sites. We also find that Aurora B does not appear to become enriched at these sites, however, inhibition of Aurora B activity causes a significant increase in the number of merotelic attachments per cell. Aurora B activity enriches MCAK at merotelic attachments and phosphorylates MCAK on residues that regulate its microtubule depolymerase activity. These data demonstrate that proteins which resolve the defect are specifically localized to merotelic attachments, where their enzymatic activities are regulated.

Human Tumor Cells with Chromosome Instability and Aneuploidy Display Elevated Rates of Merotely during Mitosis

S. L. Thompson, D. A. Compton; Biochemistry, Dartmouth Medical School, Hanover, NH
Chromosomal instability (CIN) is a distinct phenotype of some tumor cells that is characterized by frequent loss and/or gain of whole chromosomes. CIN contributes to aneuploidy in tumor cells, but the underlying mechanism of chromosome mis-segregation in CIN is unknown. Here, we use fixed and live cell imaging to examine chromosome segregation in human cancer cell lines with CIN. Using fixed cell assays, we found a significant increase in the number of anaphase cells displaying lagging chromatids in tumor cell lines with CIN (HT29, Caco2, and MCF-7) compared to tumor cells without CIN (HCT116). These lagging chromatids frequently displayed microtubule attachments to both spindle poles consistent with merotic attachment. Using live cell assays, we directly monitored chromosome segregation with GFP-histone H2B. As expected, the cell line without CIN (HCT116) aligned and segregated chromosomes faithfully greater than 90% of the time. The tumor cell lines with CIN (HT29 and Caco2) also aligned all chromosomes prior to anaphase onset and no cells entered anaphase with mis-aligned chromosomes. This demonstrates that these tumor cells with CIN have an intact spindle checkpoint, and rule out chromosome mis-segregation due to failure in spindle attachment. However, 23% and 86% of HT29 and Caco2 cells, respectively, displayed lagging chromatids at anaphase consistent with our fixed cell analyses. These data demonstrate that human cancer cell lines with CIN have elevated rates of merotely that are likely to contribute to aneuploidy. Interestingly, most Caco2 cells underwent transient monopolar spindle formation prior to bipolarization raising the possibility that syntely may precede merotely. Consistent with this idea, significant increases in lagging chromatids at anaphase were observed in near-diploid cells following recovering from monopolar spindles (via monastathal washout). We are currently testing if persistent merotely is sufficient to induce CIN in an otherwise normal diploid cell.

Functional Genomic Analysis of Genes Required for Clustering Supernumerary Centrosomes: A Novel Strategy for the Development of Selective Anticancer Drugs

M. Kwon, S. A. Godinho, N. S. Chandhok, D. Pellman; Pediatric Oncology, Dana Farber Cancer Institute, Boston, MA
Effective cancer therapeutics must exploit biological differences between tumor cells and the normal cells from which they arose. One striking feature of many cancers is the presence of extra centrosomes but efficiently form bipolar spindles (~90%). Using automated microscopy and visual inspection for multipolar spindle phenotypes upon RNAi, our primary screen identified 696 genes corresponding to ~3% of genome that are required for centrosome clustering. Among those, 44% have mammalian homologues. Potentially interesting candidates include genes involved in the regulation of actin and microtubules, cell polarity and cell adhesion. To determine if the actin cytoskeleton plays a role in centrosome clustering in mammalians, we used drugs that depolymerize actin or disrupt myosin II-dependent contractility in three different human breast cancer cell lines: one with a relatively normal centrosome
content (MCF-7) and two with extra centrosomes (MDA-231 and T47D). The MDA-231 cells, but not T47D cells, showed efficient clustering of extra centrosomes (40% of the bipolar spindles). Actin disruption selectively induced an increase of multipolar mitosis in MDA-231 cells but not in other cells. Currently we are performing automated image analysis and quantitative morphometrics measuring centrosome number, spindle length, pole width, and chromosome area for ~350 genes of our special interest. This will potentially enable the identification of different functional groups defining different mechanisms that promote centrosome clustering.

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Identification of Novel Proteins Involved in Mitotic Spindle Assembly

J. Torres, 1, 2 D. Anderson, 3 P. Jackson 1, 2, 3
Tumor Biology and Angiogenesis, Genentech, Inc., South San Francisco, CA, 3Pathology, Stanford University School of Medicine, Stanford, CA, 3Institute of Molecular Biology, University of Oregon, Portland, OR

Although recent identification of factors involved in mitotic spindle assembly has enriched our understanding of this enigmatic structure, much remains to be understood about the formation, stabilization and checkpoints that ensure fidelity of mitotic spindle assembly. To elucidate novel proteins involved in proper mitotic spindle formation, we undertook a mass spectrometry analysis of mitotic microtubule-associated proteins. Mitotic HeLa cell extracts were used for in vitro microtubule polymerization reactions and proteins co-pelleting with microtubules were identified through 2D LC/MS/MS. We identified a set of 556 microtubule-associated proteins. About 1/3 of these were previously characterized proteins involved in various aspects of spindle dynamics. The remaining 2/3 are unknown or have been described to have functions outside of mitotic spindle assembly. We have systematically knocked down the expression of this set of genes by SirNA in human HeLa cells and analyzed mitotic spindle defects through fluorescence microscopy. We have identified a set of 25 novel gene knockdowns displaying mitotic spindle defects. These defects range in severity, penetrance and mitotic stage. Currently we are analyzing the mechanisms that lead to these defects and we hypothesize that these novel genes have a role in mitotic spindle assembly and will lend insight into mitotic fidelity.

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Identification of Proteins That Contribute to Spindle Bipolarity in C. elegans Oocytes

S. M. Wigmill, A. M. Villeneuve; Developmental Biology, Stanford University, Stanford, CA

During cell division in most cell types, duplicated centrosomes nucleate microtubules, move apart, and ultimately form the poles of the spindle. However, oocytes of many species lack centrosomes and therefore spindles form through a different mechanism, where microtubules are nucleated around the chromosomes and then sorted into a bipolar array. One motor that is essential for both types of spindle assembly is Eg5, which is required for spindle bipolarity. Surprisingly, in C. elegans it has been previously reported that a deletion strain of the Eg5 homolog bmk-1 is homozygous viable, and spindle morphology appears normal in both oocytes (lacking centrosomes) and embryos (with centrosomes). Therefore, bipolarity must be established by a different mechanism. We performed an RNAi-based screen designed to identify genes required for spindle formation in C. elegans oocytes. Intriguingly, we found two genes whose depletion resulted in a phenotype reminiscent of Eg5 inhibition in other organisms: microtubules formed a single aster with the chromosomes towards the outside. In order to determine the arrangement of microtubules within the asters, we obtained an antibody to the worm homolog of Asp, a Drosophila MAP that localizes to spindle poles. As predicted, C. elegans Asp concentrates at spindle poles in both oocytes and embryos of wild type worms. Interestingly, Asp also localizes at the center of the asters observed in our screen, suggesting that they are monopolar spindles. The two genes with this phenotype are klp-18 (a Klp2 family kinesin), and a novel gene that we named mesp-1 (meiotic spindle 1). In contrast to Eg5, neither of these proteins is required for spindle bipolarity when centrosomes are present: spindle morphology in embryos is normal. Therefore, we have identified new factors that promote acentriolar spindle bipolarity; ongoing studies of these proteins should shed light on the mechanisms by which bipolarity is established.

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Monastrol Causes Inhibition of Shortening or Lengthening of K-fibers during Anaphase in Crane-fly Spermatocytes

J. R. LaFontaine, R. Oldenbourg; 1Biological Sciences, University at Buffalo, Buffalo, NY, 2Marine Biological Laboratory, Woods Hole, MA

Anaphase A in crane-fly spermatocytes is MT flux-based: kinetochore (k) MTs add subunits to their plus ends and lose subunits from minus ends, yet kinetochore (K-) fibers shorten and attached chromosomes move poleward because the off-rate at poleward ends of kMTs is greater than the on-rate at kinetochore. We employed monastrol, a kinesin-5 inhibitor, to investigate what drives anaphase in spermatocyte spindles. Monastrol inhibited anaphase: after 3 hrs in 100µM monastrol, anaphase segregation velocities were 0.5µm/min vs. 1.0µm/min in controls; attached chromosomes move poleward because the off-rate at poleward ends of kMTs is perturbed by monastrol. Thus, because of the partial or complete inhibitory effect of monastrol on subunit loss from minus ends and the stabilization and checkpoints that ensure fidelity of mitotic spindle assembly. To elucidate novel proteins involved in proper mitotic spindle formation, we undertook a mass spectrometry analysis of mitotic microtubule-associated proteins. Mitotic HeLa cell extracts were used for in vitro microtubule polymerization reactions and proteins co-pelleting with microtubules were identified through 2D LC/MS/MS. We identified a set of 556 microtubule-associated proteins. About 1/3 of these were previously characterized proteins involved in various aspects of spindle dynamics. The remaining 2/3 are unknown or have been described to have functions outside of mitotic spindle assembly. We have systematically knocked down the expression of this set of genes by SirNA in human HeLa cells and analyzed mitotic spindle defects through fluorescence microscopy. We have identified a set of 25 novel gene knockdowns displaying mitotic spindle defects. These defects range in severity, penetrance and mitotic stage. Currently we are analyzing the mechanisms that lead to these defects and we hypothesize that these novel genes have a role in mitotic spindle assembly and will lend insight into mitotic fidelity.

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Microtubule Pulling at the CellTip Cortex Drives Meiotic Nuclear Oscillation in Schizosaccharomyces pombe

S. K. Vogel, I. Tolic-Norrelykke; MPI-CBG, Dresden, Germany

During meiotic prophase, the fission yeast Schizosaccharomyces pombe shows a striking phenotype where the nucleus continuously oscillates between the two ends of the zygote for a few hours prior to the meiotic divisions. We set out to determine which forces drive the nuclear oscillation and how the force generation is spatially and temporally regulated. The nuclear oscillation is dependent on astral microtubules (MTs) radiating from the spindle pole body (SPB) and on cytoplasmic dynein, a minus end directed MT motor. Based on the observation that dynein is localized at the SPB, along MTs, and at certain cortical sites, two main models of force generation can be proposed: In the “pulling model” dynein resides immobilized at the cell cortex and generates a pulling force by walking along the astral microtubules towards their minus-ends, which are at the SPB. In the “pushing model” dynein is localized at the SPB and produces a pushing force on the SPB by moving along rearward extending MTs. By cutting single MTs using laser nanosurgery, we can distinguish between these two models and identify a subset of MTs that generates nuclear oscillation. Our data provide direct evidence that the main forces contributing to the nuclear oscillation are pulling forces, which are typically generated at the zygote ends, and that the event of force generation is driven by the interaction of forward extending MTs with the cell cortex.

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Chromosome-directed Cortical Actomyosin Contractile Ring Assembly During Polar Body Extrusion in Mouse Oocytes

M. Deng, P. Suraneni, R. Li; Stowers Institute for Medical Research, Kansas City, MO

It is well known that the microtubule-based spindle is responsible for the equatorial assembly of an anactinomyosin contractile ring after initiation of anaphase in mitotic cells. Astral microtubules or spindle midzone are well recognized cues for inducing a cleavage furrow during cytokinesis. However, mammalian oocytes lack astral microtubules and centrosomes. Our data show that close-range extrusion to completion. We find that microtubule-based actomyosin ring assembly during metaphase of meiosis, long before anaphase onset. Injection of DNA beads to egg cortex mimics the meiotic chromosomes in inducing the actomyosin ring formation even in the absence of microtubule assembly, suggesting that the signals responsible for actomyosin ring assembly come from chromatin but not any microtubule-based structures. The chromatin-induced actomyosin ring contracts and creates an initial cleavage furrow during the early phase of polar body extrusion. The initiation of this early furrow does not require the presence of a bipolar spindle, suggesting that both actomyosin contractile ring assembly and the initial furrow formation are independent of spindle microtubules. However, further construction and closure of the contractile ring requires chromosome segregation and the anaphase spindle positioned perpendicular to the cortex, with the midzone encircled by the contractile ring. When microtubules are disrupted with nocodazole, even though the chromatin-induced actomyosin ring contracts and generates the initial furrow, polar body extrusion never completes and the initial furrow gradually regresses. In the case when the anaphase-spindle is positioned in parallel to the cortex, an extra furrow is formed at the cap, resulting in abortive polar body extrusion, suggesting that the neither the chromatin-defined initial furrow nor the midzone-induced ectopic furrow alone is able to drive the polar body extrusion. We propose that corticospinal signals derived from chromatin and midzone are spatiotemporally coupled to accomplish the highly asymmetric cytokinesis during meiosis in mammalian oocytes.
Two Mutants with Novel Meiotic Phenotypes in \textit{C. elegans}

S. Sover-Gri, A. Dernburg, Department of Molecular and Cell Biology, University of California at Berkeley and Lawrence Berkeley National Laboratory, Berkeley, CA

Diploid cells undergo meiosis to produce haploid gametes. Before the first meiotic division occurs, homologous chromosomes must pair, synapse, and recombine. Homologous chromosomes separate in the first meiotic division, followed by the sister chromatids in the second meiotic division. The failure of chromosomes to properly segregate leads to aneuploidies and genomic instability. In \textit{C. elegans}, the missegregation of \textit{X} chromosomes during meiosis in hermaphrodites (XX) results in an elevated number of male progeny, relative to the 0.2% of males self-progeny produced by wild type animals. We are studying two genes that give rise to this male bias: \textit{Cib1} (germline-enhanced At-Hook protein-1) and \textit{plk-2}, which is one of three polo-like kinases in \textit{C. elegans}. Neither of these mutants dramatically perturbs pairing, synapsis, or recombination, and these genes therefore illuminate other mechanisms that are required for accurate meiotic chromosome segregation. We noted that both \textit{Cib1} and \textit{plk-2} males produce 20-25% male progeny, and 40% of the hermaphrodites are completely sterile. These mutants are proficient in crossover formation and usually display 6 bivalents. However, the normal deconstruction of the synaptonemal complex elements seems to be perturbed in these mutants. \textit{plk-2} (tm1359) hermaphrodites display a weaker \textit{His} phenotype, producing 5-7% of males. They show some chromosome asynapsis, an abnormal number of bivalents in diakinesis and mislocalization of synaptonemal complex elements along the chromosomes. We are currently working to understand how these defects arise and why they give rise to the high levels of nondisjunction. One possibility is that the \textit{gak-1} and \textit{plk-2} genes may be regulating the disassembly of the synaptonemal complex. Further characterization of these mutants will be presented.

CIB1 Is Essential for Mouse Spermatogenesis

W. Yuan, T. Leisner, A. McFadden, S. Clark, S. Huller, N. Maeda, D. A. O'Brien, L. V. Parise, Pharmacology, UNC at Chapel Hill, Chapel Hill, NC, 3Pathology and Laboratory Medicine, UNC at Chapel Hill, Chapel Hill, NC, 3Cell and Developmental Biology, UNC at Chapel Hill, Chapel Hill, NC

\textit{Cib1} is a 22 kDa calcium-binding, regulatory protein with ~50% homology to calmodulin and calcineurin B. \textit{CIB1} is widely expressed and binds to a number of effectors such as integrin α3β1, p21 activating kinase 1, DNA-dependent protein kinase and polo-like kinases in different tissues. However, the in vivo functions of \textit{CIB1} are not well understood. To elucidate the function of \textit{CIB1} in whole animals, we used homologous recombination in embryonic stem cells to generate \textit{Cib1−/−} mice. We found that although \textit{Cib1−/−} mice grow normally, the males are sterile due to a disruption of differentiation during the haploid phase of spermatogenesis. This is associated with reduced testis size and numbers of germ cells in seminiferous tubules, increased germ cell apoptosis, appearance of multinucleated cells and the loss of elongated spermatids and sperm in \textit{Cib1−/−} male seminiferous tubules. In contrast, male \textit{Cib1+++} and female \textit{Cib1−/−} mice exhibited no obvious impairment of growth, development or reproductive performance. We confirmed that \textit{CIB1} is expressed in isolated pachytene spermatocytes, round spermatocytes, sperm and cultured Sertoli cells.

Furthermore, we found that \textit{Cib1−/−} females display a significantly increased mRNA and protein expression of the cell cycle regulator Cdc2/CDk1 (cyclin dependent kinase 1) relative to \textit{Cib1+++} females. However, \textit{Cib1−/−} and \textit{Cib1+++} tests show comparable mRNA expression levels of other spermatogenesis stage-specific markers such as \textit{Sperm-1} (Pou-homeodomain domain), \textit{Cre} (CAMP-responsive element modulator), \textit{Trf2} (TRBP related factor 2), \textit{Pmn-2} (protease 2), \textit{Trp-1} (transition protein 1 and 2). In addition, mouse embryonic fibroblasts (MEFs) derived from \textit{Cib1−/−} mice exhibit a higher sensitivity to apoptosis when compared to \textit{Cib1+++} MEFs. Taken together, these results indicate that \textit{CIB1} is essential for the normal process of spermatogenesis and point to a role for \textit{CIB1} in regulating the cell cycle or differentiation of spermatogenic stem cells and/or the supporting Sertoli cells.

Translational Unmasking of Emi2 Directs Cytostatic Factor Arrest in Meiosis II

P. K. Jackson, J. S. Tong, K. Padmanabhan, J. D. Richter, Chemical Biology and Pathology, Stanford University School of Medicine, Palo Alto, CA, 3Genentech Inc., South San Francisco, CA, 3University of Massachusetts Medical School, Worcester, MA

Cytostatic Factor (CSF) arrests unfertilized vertebrate eggs in metaphase of meiosis II to prevent parthenogenesis. By inhibiting the Anaphase Promoting Complex/Cyclosome (APC/C) from targeting cyclin B for ubiquitin-mediated destruction, CSF activity sustains Maturation Promoting Factor (MPF), the biochemical engine of meiosis composed of cyclin B/Cdc2. The APC/C inhibitor Emi2/XErlp satisfies a number of historic criteria for the molecular identification of CSF: Emi2 is sufficient and necessary for CSF arrest, and is rapidly degraded by a calcium-dependent mechanism upon egg activation. How CSF is activated selectively in meiosis II is the remaining unresolved criterion. Here we show that Emi2 is expressed specifically in meiosis II through translational de-repression or “unmasking” of its mRNA, thus explaining how CSF activity appears during oocyte maturation and peaks in meiosis II. We find that Emi2 protein is absent in immature G2 oocytes and accumulates ~90 minutes after germinal vesicle breakdown (GVBD), in contrast to earlier work reporting that Emi2 is present at relatively constant levels throughout oocyte maturation. Furthermore, premature expression of ectopic Emi2 blocks the meiosis I to meiosis II transition (MI-MII), very likely through APC/C inhibition. This suggests that the APC/C is required for MI-MII in Xenopus oocytes contrary to previous findings and demonstrates the need to delay Emi2 accumulation until meiosis II. We show that the 3′ untranslated region (3′UTR) of Emi2 mRNA contains cytoplasmic polyadenylation elements (CPEs) that directly bind the CPE binding protein (CPEB) and confer temporal regulation of Emi2 polyadenylation and translational unmasking of a meiosis II-specific APC/C inhibitor directs CSF arrest.

The Paromycin Sensitive Aminopeptidase, PAM-I, Is Required for Meiotic Exit and Anterior-Posterior Polarization in the \textit{C. elegans} Embryo

R. Lycra, J. Zweier, C. Snyder, L. Kalovits, A. Beauty, B. Bowerman, Biology Department, Ursinus College, Collegeville, PA, 3Institute of Molecular Biology, University of Oregon, Eugene, OR

In the nematode \textit{Caenorhabditis elegans}, sperm entry into the oocyte triggers the completion of meiosis and the establishment of the embryonic anterior-posterior (A-P) axis. Axis establishment occurs in tight succession with completion of meiosis and the sperm donated centrosome coordinates this process through destabilization of the cortical actomyosin network. How the early embryo makes the transition from a meiotic cyclin to a mitotic zygote and coordinates cell cycle changes with axis formation remains unclear. We have discovered roles for the \textit{C. elegans} paromycin-sensitive aminopeptidase, PAM-I, in both cell cycle progression and A-P axis formation, further implicating proteolytic regulation in these processes. \textit{C. elegans} PAM-I is highly related to human PSA. \textit{pam-1} mutant embryos exhibit a delay in exit from meiosis; thus, this peptidease is required for progression to mitotic interphase. In addition, the centrosomes associated with the sperm pronucleus fail to closely associate with the posterior cortex in \textit{pam-1} mutants, and the A-P axis is not specified. The meiotic exit and polarity defects are separable, as inactivation of the B-type cyclin, CYB-3, in \textit{pam-1} mutants rescues the meiotic exit delay but not the polarity defects. Thus \textit{PAM-I} may target CYB-3 to regulate meiotic exit but presumably targets other protein(s) to regulate polarity most likely by ensuring cortical/centrosome association. The degradation of proteins through ubiquitin-mediated proteolysis has been previously shown to regulate the cell cycle and A-P axis formation in the \textit{C. elegans} zygote. Our analysis of \textit{PAM-I} requirements show that a paromycin sensitive peptidease also is required for proteolytic regulation of the oocyte to embryo transition.

Live Imaging Reveals That Chiasmata Ensure Timely Coorientation during \textit{Drosophila} Meiosis

J. L. Cottia, S. Hughes, W. Gilliland, R. S. Hawley; Stowers Institute for Medical Research, Kansas City, MO
Anillin and the Septins Break Symmetry within the Actomyosin Cytoskeleton to Promote Unilateral Furrowing during Cytokinesis

A. S. Maddox, L. K. Lewellyn, A. Desai, K. F. Oegema; Cell and Molecular Medicine, LICR / UCSD, La Jolla, CA

Anillin and the septins are widely conserved structural components of the cortical cytoskeleton. Septins heteroligomers are thought to polymerize to form a membrane-associated filament system. Anillins are cytoskeletal crosslinkers that can bundle actin filaments and bind active myosin II and septin filaments. Inhibition of anillin or septin function in C. elegans causes stochastic but strikingly similar defects in growth, maturation and tissue morphogenesis, suggesting that they function together during development. We have examined the cortical localization of anillin and septins during the first cytokinesis of the C. elegans early embryo, where anillin is required to position the septins in the contractile ring. The first mitotic cleavage furrow is highly asymmetric: initially, a smooth indentation deforms the entire cell equator, then the furrow dips in at one location and sweeps across the division plane unilaterally. Unilateral furrowing is prevalent among species and cell types, including marine invertebrate eggs, mammalian cultured cells, and C. elegans somatic cells. In other systems, the eccentric position of the mitotic spindle, the presence of cell-cell and cell-substrate adhesions, or other existing axes likely contribute to unilateral furrowing. Importantly, in the C. elegans one-cell embryo, none of these possible confounders appear to exist, suggesting the existence of an intrinsic mechanism for unilateral furrowing. A novel four-dimensional imaging regime yielding an end-on view revealed that during unilateral ingestion, cytoskeletal components of the contractile ring are asymmetrically distributed around the ring, with greatest accumulation at the fastest-increasing side. Depletion of anillin or the septins did not alter the kinetics of contractile ring closure, but caused both furrow ingestion and contractile ring structure to become symmetric. We conclude that in the contractile ring, anillin and the septins act together to break the symmetry of the actomyosin cytoskeleton, an activity that may be important for neuronal path finding or other developmental events.

Microtubules Negatively Regulate Cortical Actin during Cytokinesis of Adherent Cells

K. T. Murphy, C. J. Fagerstrom, P. Wadsworth; Biology Department, University of Massachusetts, Amherst, Amherst, MA

During anaphase, an F-actin and myosin II containing contractile ring is assembled at the equatorial cortex and functions in cytokinesis. Microtubules are responsible for signaling the cortex for contractile ring assembly, but the mechanism remains poorly understood. LLC-PK1 epithelial cells expressing GFP-actin were treated with nocodazole during anaphase to disassemble microtubules, and the organization and dynamics of actin were examined. Addition of nocodazole within 1 minute of anaphase onset results in complete microtubule disassembly and blocks cytokinesis. Addition of nocodazole >1 minute after anaphase onset eliminates astral, but not interzonal microtubules, and cytokinesis proceeds to completion, demonstrating that astral microtubules are dispensable once incoordination is initiated. In these cells, the contractile ring appeared wider than in control cells, and actin from distal regions of the cortex contributed to ring formation. In nocodazole treated cells, loss of microtubules resulted in novel wave-like behavior of cortical actin. Waves could be initiated at various sites and moved rapidly across the cortex (~25-60 μm/min). FRAP experiments on nocodazole treated cells showed that the half-time for recovery was decreased 2-fold. To determine if the changes in actin behavior following microtubule disassembly are mediated by Rho, we examined the distribution of RhoA in nocodazole treated cells. In nocodazole treated cells that divided, RhoA was localized to the ring as in controls, but was also mislocalized to other regions of the cortex. Similar results were obtained by imaging C. elegans GFP-RhoA in living cells. Our results demonstrate that loss of microtubules results in increased accumulation of actin in the contractile ring, abnormal cortical behavior, increased actin turnover, wave-like behavior of cortical actin and mislocalization of RhoA. We conclude that microtubules negatively regulate actin behavior in the cortex of cultured epithelial cells.

Imaging Recruitment of Myosin and Actin to the Cytokinetic Furrow by Total Internal Reflection Fluorescence Microscopy (TIRF-M)

M. Zhou, Y. Wang; Department of Physiology, University of Massachusetts Medical School, Worcester, MA

Cytokinesis involves the recruitment and organization of actin and myosin filaments along the equatorial cortex, likely as part of the contractile process to drive the separation of daughter cells. Although many studies have attempted to address the mechanism of equatorial recruitment of actin and myosin, there has been little direct information on the dynamic process, partially due to the limitation of imaging techniques and the high background from the abundance of myosin and actin in the cytoplasm. In this study, we used total internal reflection fluorescence microscope (TIRF-M) to image the dynamics of GFP-actin II and GFP-actin during the assembly of equatorial cortex in dividing NRK cells. TIRF-M effectively reduced the cytoplasmic fluorescence background and highlighted the cortical structure. We found that myosin and actin followed clearly different patterns of recruitment into the equatorial region. While there was a striking flux of actin filaments toward the equator, myosin structures showed no directed movement but appeared to assemble de novo along the equator, initiating slightly ahead of the onset of actin flux. Actin flux was abolished by blebbistatin, a myosin ATPase inhibitor, although this did not inhibit the concentration of equatorial actin, suggesting that there is a flux-independent process for actin recruitment. In contrast, the recruitment of equatorial myosin during early cytokinesis did not appear to require F-actin, as the process was unpainted in cells treated with latrunculin. Our results suggest distinct mechanisms for the equatorial organization of actin and myosin. In addition, the recruitment of actin may involve both a myosin-dependent flux mechanism, and a flux-independent, de novo mechanism.

Cytokinesis Requires the Regulation of Actin Cytoskeleton by α-actinin in Mammalian Cells

S. A. Mukhina, Y. Wang, M. Murata-Hori; 1Temasek Life Sciences Laboratory, Singapore, 2University of Massachusetts Medical School, Worcester, MA

Recent advances in the regulation of cytokinesis have implicated the modulation of actin cortex during cytokinesis, which likely involves a number of actin modulating proteins. Concentration of an actin cross-linking protein α-actinin to the cleavage furrow has been reported over the past decades. However, its function during cytokinesis remains poorly understood. We have analyzed the function of α-actinin during cytokinesis by a combination of time-lapse imaging and molecular manipulations. α-actinin accumulated along the equator underwent gradual dissipation from the cleavage furrow as cytokinesis progressed. FRAP analysis demonstrated that α-actinin along the cleavage furrow was highly dynamic (τ1c = 8.4 ± 0.4 s), while being relatively stable at the subequatorial region (τ1s = 23.1 ± 4.3 s). Overexpression of α-actinin caused decelerated cytokinesis and cytokinesis failure due to excessive bundling of actin filaments and precocious formation of focal adhesions. In addition, we observed abnormal rope-like structures of myosin II along the cleavage furrow in cells overexpressing α-actinin. Conversely, global depletion of α-actinin by siRNA caused a decrease in the density of actin filaments throughout the cell cortex, surprisingly inducing not only accelerated cytokinesis but also ectopic furrowing. Furthermore, local disruption of α-actinin by chromophore-assisted laser inactivation resulted in the formation of wider and slightly deeper furrow at the site of irradiation. These observations suggest that furrowing requires remodeling and solution of the actin filament network and that equatorial-specific modulation of actin filaments mediated by α-actinin ensures proper cytokinesis in mammalian cells.

The Cell End Factor Pomp1p Inhibits Mid1p in Specification of the Cell Division Plane

N. N. Padte, M. S. Martin, M. Howard, F. Chang; Microbiology Department, Columbia University College of Physicians and Surgeons, New York, NY, 3Mathematics Department, Imperial College London, London, United Kingdom

Intrinsically spatial cues ensure the proper placement of the cell division plane. In the fission yeast Schizosaccharomyces pombe, the position of the nucleus helps to direct the medial positioning of contractile ring assembly and subsequent cell division. An important factor in this process is mid1p (anillin-like protein), which is a peripheral membrane protein that forms a broad cortical band of dots overlaying the nucleus in interphase and recruits myosin in early mitosis. How mid1p localizes to this cortical band and tracks the nucleus is not clear, especially as its localization is independent, de novo mechanism. We provide evidence that pom1p, a Dyrk family protein kinase, forms an inhibitory concentration gradient emanating from the non-growing cell end. In pomp1 mutants, mid1p is distributed over the entire cell plane over the non-growing cell end. Pomp1 is a well characterized cell polarity factor. However, this phenotype is not due simply to altered cell polarity, since other mutants with similar polarity defects positioned mid1p normally at the medial cortex. The abnormal mid1p distribution is established in a dynamic manner in interphase and leads to misplaced or multiple contractile rings. In

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addition, we provide evidence that pom1p works in conjunction with a second inhibitor at the growing cell tip. Our computational and experimental results support a new model in which both positive cues from the medial nucleus and negative cues from the cell tips specify the position of the division mechanism. Further understanding of this interplay promises to provide new insights into the global spatial regulation of cell division.

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Characterization of Cytokinesis Mutants in Saccharomyces cerevisiae
E. A. Vallen,1 L. Tha,2 C. Palmer,2 M. Lippincott,1 H. Han,1 H. Bhattachar,1 J. Luo,2 C. Dravis,1 E. Bi,2 1Biology, Swarthmore College, Swarthmore, PA, 2Cell and Developmental Biology, University of Pennsylvania School of Medicine, Philadelphia, PA

Cytokinesis in Saccharomyces cerevisiae occurs via the coordinated action of the actomyosin contractile ring and the septum-formation process. The actomyosin contractile ring is not essential in some strain backgrounds. However, it is required for cytokinesis and viability in our strain in the presence of a deletion of HOFL, a gene important for coordinating the ring function and septum formation (Vallen, Caviston and Bi, 2000 Mol. Biol. Cell, 11:593-611). A screen for mutants synthetically lethal with ho1p was utilized to identify and characterize proteins required for the function of the actomyosin ring pathway. Approximately forty HOFL-independent mutants were identified by screening 33,000 mutagenized colonies using a sectoring assay and other genetic tests. Complementation testing, linkage analysis and plasmid-linked suppression indicate that mutations were isolated in RHO1, RHO2, CDC12, CYK3, ELM1, GNA1, MCL1, MYO1, and PSE1. Some mutants have yet to be characterized. A subset of the myo1p alleles has been characterized in more detail. A number of the isolated mutations are premature stop codons occurring in the C-terminal quarter of the protein. Preliminary data analyzing GFP-myo1p+ suggests these proteins localize to the bud neck and that at least some of the mutant strains have actin rings as visualized by staining with rhodamine-labeled phallolidin. This suggests that these mutations are not complete loss-of-function alleles.

Identification of Cytokinesis Proteins by Dosage-Suppressor Screens in S. cerevisiae
N. Ko,1 R. Nishihama,2 J. R. Pringle1 1Department of Genetics, Stanford University School of Medicine, Stanford, CA, 2Department of Biology, University of North Carolina, Chapel Hill, NC

In the yeast Saccharomyces cerevisiae, a ring of myosin II (Myo1p) forms in a septin-dependent manner at the presumptive budding site in late G1. At the onset of cytokinesis, actin is recruited to the Myo1p ring; the resulting actomyosin ring then contracts, and a septum of cell wall concurrently completes cytokinesis. Three other proteins involved in cytokinesis are Iqg1p, Cyk3p, and Ho1p. Iqg1p is the only S. cerevisiae IQGAP; it promotes actomyosin-ring formation and is required for primary-septum formation and hence for normal cytokinesis. Because the actomyosin ring itself is not essential for primary-septum formation, Iqg1p must have at least one other cytokinetic function. Cyk3p was identified previously on the basis of its ability to suppress iog1Δ growth defects without restoring the actomyosin ring; thus, Cyk3p may function downstream of Iqg1p, or an actomyosin-ring-independent pathway for cytokinesis. Previous studies suggest that Ho1p may function as an adapter linking the actomyosin ring to the machinery that synthesizes the primary septum. To identify additional proteins involved in the septin-dependent, actomyosin-ring-independent pathway of cytokinesis, we have conducted screens for dosage suppressors of the myo1Δ cyk3Δ and myo1Δ ho1Δ synthetic-lethal phenotypes.

We have isolated several genes including EG12 (which encodes a GTP-anchored cell-wall endoglucanase thought to be involved in cell separation), ECM3 (which encodes a GPI-anchored protein that may function in cell-wall organization), and a truncated CDC24 (which encodes the GEF for the Rho-type GTPase Cdc42p) that lacks the C-terminal PDB domain. Overexpression of Ecm3p or the truncated Cdc24p can also suppress iog1Δ growth defects and restore the formation of primary septa. Because overexpression of Cdc42p does not rescue the iog1Δ growth defects, Cdc24p may have a cytokinetic function that is independent of Cdc42p. Further studies of these and other genes identified in the screens will be presented.

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The Cdc14/Cdc1p Phosphatase Regulates Phosphorylation and Dynamics of Fission Yeast Contractile Ring Components through a Midl-dependent Mechanism
D. M. Clifford Hart,2 B. A. Wolfe,1 R. H. Roberts,1 W. H. McDonald,2 J. R. Yates III,2 K. L. Gould1 1Cell and Developmental Biology, HHMI and Vanderbilt University Medical School, Nashville, TN, 2Department of Genetics, Stanford University School of Medicine, Stanford, CA

In eukaryotes, progression through the cell cycle is directed by the activity of cyclin-dependent kinases (cdks). Reversal of cdk-dependent phosphorylation events involves the highly conserved Cdc14-family of phosphatases. The fission yeast Cdc14 homolog, Cdc1p/Fpl1p, localizes to many subcellular compartments, such as kinetochores, the mitotic spindle and the division site where it is involved in several facets of cell cycle control. However, the mechanism by which Cdc1p/Fpl1p functions at these distinct sites is unclear. To investigate the role of Cdc1p at the division site, we used a proteomics approach to identify the mechanism of Clp1 recruitment to the CAR and describe the functional consequence of its recruitment by examining the phosphorylation status and dynamic properties of key CAR components. We present evidence that Clp1/Fpl1 association with Mid1, an anillin-related protein required for correct cytokinetic actin ring (CAR) positioning, is necessary for Cdc1p/Fpl1 localization to the ring. Once localized at the CAR, Cdc1p activity regulates the complete dephosphorylation of the essential CAR component Cdc15 and influences the dynamic properties of both Cdc15 and myo1p to provide stability to this structure. Our findings underscore the significance of Cdc14 catalytic activity in the regulation of CAR dynamics and offer an explanation for the requirement of Cdc1p to ensure the fidelity of S. pombe cytokinesis.

Adaptive Evolution of Cytokinesis Pathways in Budding Yeast
N. Pavelka, G. Rancati, R. Li, Stowers Institute for Medical Research, Kansas City, MO

As pathogens evolve elegant immune escape mechanisms and eventually develop antibiotic resistance, cancer cells evade immune responses and eventually develop resistance to chemotherapy. In fact, adaptability to perturbations is common to all biological systems. As an example, Saccharomyces cerevisiae cells lacking a functional copy of the MTO1 gene, whose product is essential for forming the contractile ring during cytokinesis, are able to evolve alternative mechanisms of cell division. We have used a combination of adaptive evolution experiments and computational methods to investigate how yeast cells rapidly evolve novel, myosin-independent strategies for cell division. 45 myo1Δ yeast strains were allowed to independently evolve over hundreds of generations until they either died out or stabilized their improved growth ability. Growth curves, cytokinesis proficiency, DNA content, cellular size and viability of 29 stable evolved strains have been analyzed by a combination of methods, including optical density, microscopy and flow cytometry. Principal Component Analysis of these viability data revealed the emergence of recurring phenotypic classes, suggesting convergent evolution to a limited number of solutions. Surprisingly, among the strains that best recovered the capacity to undergo cytokinesis, all showed a change in their ploidy. In fact, we found that six of the 29 evolved strains had become diploid, four were tetraploid, ten were aneuploid, and the nine most cytokinesis-deficient strains were still haploid. This is reminiscent of the genomic aberrations that are typically observed in cancer cells and may suggest a selective advantage, e.g. in terms of increased adaptability, for cells that increase their ploidy. In order to gain further molecular insights into the adaptation strategies that these strains have evolved, we are in the process of applying a combination of microarray, proteomics and computational analyses to identify the genes whose differential expression correlates with genomic rearrangement and phenotypic improvement.

Promotion of Cytokinesis by the Iqg1p (IQGAP)-Cyk3p Pathway in Yeast
R. Nishihama, N. Ko, J. T. D. Perdue, J. R. Pringle 1Department of Genetics, Stanford University School of Medicine, Stanford, CA, 2Department of Biology, University of North Carolina, Chapel Hill, NC

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. Cytokinesis in Saccharomyces cerevisiae involves such a contractile ring, which functions in conjunction with the formation of a chitinous primary septum (PS) by the chitin synthase Chs2p to complete cytokinesis. Null mutations of MTO1 (the only myosin II gene) block actomyosin-ring formation but are nonetheless nonlethal. Electron microscopy revealed that myo1Δ cells form PS-like, chitin-containing septa, but not the membrane invagination that occurs in wild-type cells. In contrast, deletion of CHS2 causes complete loss of the PS. Thus, Chs2p-mediated PS formation may drive the membrane invagination. In a screen for dosage suppressors of myo1Δ phenotypes, we recovered IQG1 (encoding an IQGAP essential for actomyosin-ring formation) and CYK3 (overexpression of which can suppress iog1Δ growth defects). Both gene products form rings at the division site, and increased expression of either gene caused a proliferation of PS-like structures. Electron microscopy showed that cyk3Δ cells have simultaneous formation of PS and secondary septa; although a ring of Chs2p was assembled properly at the division site, its constriction, as well as that of the actomyosin ring, proceeded much more slowly than in wild-type cells. Iqg1p was also dispensable for Chs2p localization, but overexpression of Cyk3p could restore PS formation in such cells. These results suggest that Iqg1p and Cyk3p are components of a pathway that promotes membrane deformation, PS formation, or both.

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Phosphorylated on Ser-19 Residue of Vps74p Is Involved in the Apical Growth

F. S. Lee, J. Jung, L. Chang, Y. Liu. Institute of Molecular Medicine, National Taiwan University, Taipei, Taiwan

In Saccharomyces cerevisiae, apical bud growth occurs for a brief period in G1 when the deposition of membrane and cell wall is restricted to the tip of the growing bud. Vps74p (product of YDR372C) was recently reported to alter the elongated bud morphology of cdc34-2 cells arrested in the apical growth phase. Here, we show that Vps74p is phosphorylated on serine-19 in a growth-phase-dependent manner. Cdc28 kinase is required for Vps74p phosphorylation. Both alanine and aspartate substitutions in serine-19 directly affect elongated bud morphology of cdc34-2 cells. The alanine substitution abolished Vps74p functional activity on apical growth. In contrast, the aspartate substitution stimulates cellular elongated buds formation. Localization of Vps74p at the Golgi is not dependent on the N-terminal phosphorylation, but on its C-terminal domain. Vps74p is involved in the transportation of GPI-anchored protein Gas1. In addition, deletion of C-terminus, but not N-terminus, of Vps74p affects cell wall integrity. Together, we infer that the distinct functional outcome from Ser-19 phosphorylation modulates Vps74p activity in apical growth, but not in cell wall integrity.

The SIN Signaling Pathway Acts to Inhibit Interphase Polarity Machinery until Cytokinesis Is Completed

S. Ray, J. Nordberg, D. Hirata, D. McCollum. Molecular Genetics and Microbiology, University of Massachusetts Medical School, Worcester, MA, 2Graduate School of Advanced Sciences of Matter, Hiroshima University, Higashihiroshima, Japan

Maintenance of genomic stability requires precise coordination of cell cycle events. We have found that cytokinesis is coordinated with cell cycle progression through a cytokinesis checkpoint in the fission yeast S. pombe. If cells have delays in completion of cytokinesis, the cytokinesis checkpoint arrests nuclear division, keeping cells in a binucleate state. In addition, the checkpoint acts to maintain the actomyosin ring and inhibit return to interphase polarity until cytokinesis is complete. The SIN signaling pathway and the Cdc14-like phosphatase Clp1p are essential for this checkpoint. We have investigated the relationship between the SIN and Clp1p further, in particular whether Clp1p is required for checkpoint functions if the SIN is constitutively activated in the absence of Clp1p. We show that the SIN can carry out checkpoint functions independently from Clp1p, and activation of the SIN in interphase can block cells in G2 phase with disrupted interphase polarity and inhibited growth. G2 arrest is only observed if the SIN is activated early in the cell cycle, when Clk1 activity is low, suggesting an antagonistic relationship between the SIN and Cdk1. Consistent with this, we observe Cdk1 phosphorylation of the SIN kinase Sid2p. The G2 arrest induced by SIN activation is not due to a cell size checkpoint. Inactivation of Wee1 overrides the G2 arrest, but cells still have disrupted interphase polarity. We also show that interphase SIN activation disrupts localization of several polarity markers located at cell tips, including the Cdc42 protein, which is part of the Orb1 kinase pathway. Together these results suggest that the SIN not only promotes cytokinesis, but also inhibits cell cycle progression and the interphase polarity machinery until cytokinesis is complete.

Chemical Inhibitor Studies of Polo Kinase Function during Anaphase and Cytokinesis in Mammalian Cells

I. M. Brennan, U. Peters, T. M. Kapoor, A. F. Straight. Department of Biochemistry, Stanford University, Stanford, CA, 2Laboratory of Chemistry and Cell Biology, Rockefeller University, New York, NY

Polo-like kinase 1 (Plk1) is a key regulator of mitosis and cytokinesis. Genetic and RNA interference studies have determined that Plk1 is required for multiple processes during cell division including mitotic entry, spindle pole maturation, bipolar spindle assembly, and the mitotic checkpoint. These studies have also demonstrated that deficiency in Plk1 activity induces failure in cytokinesis yielding binucleate cells. However, the specific function of Plk1 in cytokinesis has remained difficult to deduce with genetic experiments due to extensive defects early in mitosis. We are investigating the role of Plk1 during cytokinesis using a specific small molecule inhibitor of polo kinase function. The high degree of temporal control afforded by the use of a small molecule allows for rapid inhibition of Plk1 during cytokinesis without disrupting prior mitotic events. Here we show that suppression of Plk1 activity in mammalian cells by inhibitor treatment at anaphase onset causes defects in cytokinesis. Interestingly, Plk1 inhibition also disrupts anaphase-B spindles elongation, affecting chromosome movements to the spindle poles during anaphase-A. The activities of polo kinase during furrow ingression and anaphase-B are separable; when the small molecule is washed out after inducing a complete block of anaphase-B and furrow ingression, the cells complete cytokinesis without spindle pole separation. These data imply separate and critical roles for Plk1 in anaphase spindle elongation and cytokinesis in mammalian cells.

A Critical Role for Novel PKC Epsilon Phosphorylations during Cytokinesis


To identify proximal PKCε targets we undertook a yeast 2-hybrid screen of a cDNA library using the PKCε regulatory domain (V1-V3) as bait. Among the positive clones identified was the scaffold protein 14.3.3β. We have confirmed in mammalian cells that 14.3.3β binds to PKCε in a phosphorylation dependent manner. Specifically, we have shown that binding is mediated through two previously unidentified phosphorylation sites within the V3 hinge region of PKCε, Ser346 and Ser368. Moreover, we have characterised Ser368 as a PKCε autophosphorylation site in vitro and Ser346 as a GSK3β site that requires an initial priming phosphorylation by p38-MAPK kinase at Ser350. The acutely induced phosphorylation of these sites has been monitored in distinct cell types and under different growth conditions, revealing that occupation of these sites may be involved in multiple processes. Notably however, immunofluorescence studies have revealed that phosphorylation of these sites also occurs in cells undergoing mitosis. These phosphorylations and the subsequent association of 14-3-3 is critical for cytokinesis as evidenced by an increased degree of binucleation and an extended time to mitotic cell separation in PKCε knockout MEFs expressing GFP-PKCε mutants incapable of binding 14-3-3 when compared to wild type GFP-PKCε. A catalytic requirement for PKCε in this process is demonstrated by the inhibition of cytokinesis following expression of a kinase dead PKCε or by specific inhibition of PKCε. Furthermore, this inhibition of cytokinesis via inhibition of PKCε correlates with the accumulation of PKCε at the furrow during the final stages of telophase, suggesting that PKCε activity is required for furrow progression and exit from this compartment. These data show that a series of regulated PKCε phosphorylations and subsequent 14-3-3 association is critical for furrow progression and separation of the daughter cells at the end of cytokinesis.

Regulation of the Small G-proteins Rho1 and Cdc42 during Cytokinesis and Polarized Growth

S. Yoshida, K. Kono, D. Lowery, S. Bartolini, D. Pellman. Pediatric Oncology, Dana-Farber Cancer Institute, Boston, MA, 2University of Tokyo, Kashiwa, Japan, 3Laboratory of Chemistry and Cell Biology, Rockefeller University, New York, NY

Budding yeast is an important model for asymmetric cell division where polarized morphogenesis must be coordinated with cell division. In the early part of the cell cycle Rho-type GTPases and downstream targets such as formins promote the growth of the daughter cell (the bud). During mitosis, these proteins are redirected to the cell division site to enable cytokinesis. Although mitotic Cdk1 activity is implicated in activation of Rho-family GTPases, the molecular mechanisms remain poorly understood. Here we present evidence that Polo-like kinase Cdc5 controls cell polarity and cytokinesis by affecting the activity and/or localization of Rho1 and Cdc42. We found that Cdc5 phosphatases and targets Rho1 Guanine nucleotide exchange factors (GEFs) to the division site, which is a critical step for cytokinetic actin ring assembly. In addition, we find that Cdc5 phosphorylates a Cdc42 GTPase activating protein (GAP) Bem3. As Cdc42-GTP accumulates to the distal bud cortex and cells hyper-elongates in the cdc5 mutant, regulation of Cdc42 by Cdc5 may be important for cell morphogenesis. Our results suggest a coordinated regulation of Rho1, Cdc42 and mitotic exit by Polo-like kinase, revealing a novel mechanism for regulating the cortical cytoskeleton during cell division.

The Requirement for Rho Kinase in Recruitment of Myosin II to the Cleavage Furrow Is to Phosphorylate the Myosin Regulatory Light Chain

S. O. Dean, J. A. Spudich. Biochemistry, Stanford University, Stanford, CA

Myosin II is recruited to the cytokinetic furrow of Drosophila S2 cells independently of F-actin, but the small GTPase Rho1 and Rho Kinase (Rok) are essential for this recruitment. Rok has been shown to phosphorylate multiple proteins involved in cytokinesis including the myosin II Regulatory Light Chain (RLC), Pten, and ERM proteins. Using phosphorylation state mimic constructs expressed in Drosophila S2 cells we show that phosphorylation of the RLC is indeed required for myosin II recruitment to the furrow and, furthermore, the only essential role of Rok...
in cytokinesis is to phosphorylate the RLC. Phospho-mimic RLC is fully functional in these cells demonstrating that while the presence of phosphorylated RLC is essential for cytokinesis, the ability to regulate the phosphorylation state spatio-temporally is not. In addition, we demonstrate that the essential role of Citron kinase is not phosphorylation of the RLC despite its ability to do so in vitro.

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The Evi5 Oncogene Binder Bab1 in a GTP-Dependent Manner, and Cooperates with Bab1 in Regulation of Cytokinesis

C. J. Westlake,1 J. R. Junutula,2 R. Prekeris,2 M. Pilli,1 R. H. Scheller,1 P. K. Jackson,2 A. G. Eldridge1; 1Genentech, South San Francisco, CA, 2Cellular and Developmental Biology, University of Colorado Health Sciences Center, Denver, CO

The Evi5 oncogene has recently been shown to serve as a stabilizing factor for Emi1, an inhibitor of the Anaphase-Promoting Complex/Cyclosome (APC/C) that controls cell cycle progression in mitosis and G1. Evi5 stabilizes Emi1 by binding Emi1 and preventing its phosphorylation by the Polo-like kinase Plk and its association with the SCFβTrCP ubiquitin ligase. Sequence analysis of Evi5 revealed a TBC domain, which has been shown to act as a GTPase-activating protein (GAP) domain for the Rab family of small Ras-like GTPases. Here we describe the identification of Bab1 as a candidate target of Evi5. Like other Rab GTPases, Bab1 has been shown to regulate intracellular transport and vesicle trafficking. In addition to mediating endosome recycling, Bab1 has also been shown to be required for the completion of cytokinesis. By immunoprecipitation and Bisacore analysis, we demonstrate that Evi5 binds to Bab1 in a GTP-dependent manner. Evi5 co-localizes with both Bab1a and Bab1b, but not Bab1, in vivo. Furthermore the Bab1b effector protein FIP3 was found to modulate Evi5 localization at the centrosome.

Interestingly, in vitro binding studies suggest that Bab1 effector proteins compete with Evi5 for binding to Bab1. Consequently we have investigated the potential for Evi5 to act as a Bab1 GAP. Finally, ablation of Evi5 through RNA interference causes a multimuculate phenotype, indicative of cytokinesis failure, similar to that previously seen following perturbation of Bab1 and FIP3 function. These data argue that Evi5 cooperates with Bab1 in the regulation of critical aspects of cytokinesis.

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A Novel RhGAP Functions in the Regulation of Cytokinesis

A. V. Poth, A. Gromley, X. Pan, D. Lambright, S. Dessey; Molecular Medicine, University of Massachusetts Medical School, Worcester, MA

Cytokinesis is the final step in the cell cycle when two daughter cells are physically separated in a process known as abscission. We have previously identified centrinol as a protein necessary for the completion of cytokinesis. Centrinol localizes to the maternal centriole and to the midbody ring of telophase cells. Depletion of centrinol results in cytokinesis defects, most notably persistent intracellular bridges that remain between daughter cells. In an effort to determine how centrinol functions in cytokinesis, we performed a yeast two-hybrid screen using a centrinol domain homologous the S. cerevisiae protein, nud1. We identified, cloned, and sequenced a previously uncharacterized cDNA encoding an ~150kDa protein with homology to RhoGTPase activating proteins (GAPs). In an effort to identify the GTPase target of the GAP, we performed GAP assays which show specific activity of the GAP toward the RhoGTPase family (RhoA, cdc42 and Rac1) with the highest activity toward Rac1. This activity can be inhibited by expression of an arginine finger mutation in the GAP domain. To verify the interaction of centrinol and the GAP, we have performed coimmunoprecipitation assays demonstrating an interaction between endogenous centrinol and the GAP. Centrosomal localization of the GAP throughout the cell cycle suggests a role in cytokinesis both by antagonistic protein and transient overexpression of epitope-tagged protein. Functionally, we found that overexpression of the GAP results in an increased population of telophase cells. We are currently working to identify the mechanism of action of the GAP during cytokinesis.

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GTPase Flux Maintains a Tightly Focused Zone of Rho Activity During Cytokinesis

A. L. Miller, W. M. Beam; Zoology, University of Wisconsin-Madison, Madison, WI

Microtubules of the mitotic spindle provide a signal that specifies the position where the actomyosin-based contractile ring should form during cytokinesis. The nature of this signal from microtubules remains mysterious, although emerging evidence implicates the small GTPase RhoA. We recently showed that a precisely-bounded, microtubule-dependent zone of high RhoA activity forms during cytokinesis in echinoderm and Xenopus embryos. Moreover, work from other labs has shown that Ect2 (a RacA GEF also known as LET-21 or Pbl), MgcRacGAP (a RacA GAP also known as CYK-4, RacGAP50c, or Tumbleweed), and MKLP (a kinesin also known as ZEN-4, Pavarotti, or CHO1) are localized at the furrow and are important regulators of cytokinesis. Taken together, these results suggest that microtubules may specify the position of the contractile ring by precisely localizing regulators of RhoA activity, which then direct formation of a localized RhoA activity zone. Therefore, we are currently examining the roles of the RhoA regulators Ect2 and MgcRacGAP in modulating the RhoA activity zone during cytokinesis in Xenopus embryos. Importantly, both Ect2 and MgcRacGAP are necessary for cytokinetic RhoA activity zone formation and maintenance. Specifically, disruption of MgcRacGAP’s GAP activity leads to RhoA activity zones with increased intensity and breadth compared to controls. Additionally, disruption of MgcRacGAP’s GAP activity promotes the accumulation of a downstream target of RhoA, myosin-2, at the furrow. We propose that Ect2 locally activates RhoA, while MgcRacGAP locally inactivates RhoA, resulting in the constant flux of RhoA through the GTPase cycle. In this way, cells can maintain a tightly focused zone of RhoA activity to drive furrow formation and contraction.

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Intermolecular Interactions of B. subtilis MreB

J. A. Mayer,1 G. J. Bean,2 K. J. Amann1; 1Cell and Molecular Biology, UW-Madison, Madison, WI, 2Molecular Biology, UW-Madison, Madison, WI

The bacterial actin homolog MreB has been implicated in the regulation of cell shape changes including the establishment of cell polarity and the condensation and segregation of chromosomes. We hypothesize that MreB carries out these roles both through its own direct action and through interactions with other bacterial proteins; however few such molecular interactions have been confirmed biochemically. We have overexpressed in E. coli and purified Bacillus subtilis MreB. We have studied polymerization dynamics using light scattering and sedimentation assays showing significant pH-,ionic-,cationic-, and temperature-dependences. At present, we are using this purified MreB to identify interacting molecules that may mediate the proposed cellular roles for MreB. We have purified putative binding partners and are testing them for direct interactions with MreB polymers and monomers using light scattering, sedimentation and fluorometric assays. We will quantitatively determine the nature of interaction of each ligand with MreB, particularly any influence on the polymerization dynamics, nucleotide use or polymer structure. As the details of these interactions are revealed, they will form a more complete picture of the cellular functions of the bacterial actin homolog MreB and its similarities and differences to eukaryotic actin.

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Interaction of Actin with Translation Factors and the Role of Actin in Translation Fidelity

P. L. Cathcart, K. A. Kandl; Biology, St. Olaf College, Northfield, MN

Previous work demonstrated that actin plays a role in translation fidelity, as yeast strains with a subset of actin mutations (for example, act1-122) show read through of nonsense codons and sensitivity to paromomycin, an inhibitor of translation elongation (Kandl et al., 2002. Mol Genet Genomics 268: 10-18). We are testing the hypothesis that the fidelity defects seen in yeast with actin mutations are caused by altered physical or functional interactions between actin and translation factors known to affect translation fidelity. A likely candidate is the elongation factor eEF1A. eEF1A delivers aminoacyl-tRNAs to the ribosome, ensuring the incorporation of the correct amino acid into the elongating polypeptide, and eEF1A has been shown to bind and bundle actin in vitro. A cosedimentation assay, in which actin bound by eEF1A pellets after low speed centrifugation, showed that the mutant actin purified from yeast expressing actin alleles with act1-122 severe in the assay with eEF1A and eEF1B, the mutant actin remained banded by eEF1A, suggesting that the act1-122 mutant actin has a higher affinity for eEF1A than the wild type actin does. This result could explain the translation fidelity defects seen in act1-122 yeast, and experiments will be done to study this. This result also suggests that the strongest interaction between actin and eEF1A is not necessarily optimal, and this may have implications for understanding actin cytoskeleton dynamics.
Nucleotide-mediated Conformational Changes of Monomeric Actin

X. Zheng, D. Sept; Department of Biomedical Engineering and Center for Computational Biology, Washington University in St. Louis, St. Louis, MO

The nucleotide state of actin is an important regulator within the cell since it not only affects the polymerization of actin, but also the interaction of actin monomers and filaments with other proteins. Recent crystal structures of actin indeed show some structural differences between the two forms, but in order to gain some idea of the dynamical differences between the two nucleotide states, we performed long molecular dynamics (MD) simulations. Our MD simulations reveal many significant secondary structure changes in the DNase binding loop in subdomain 2. Comparisons between the two simulations as well as implications for potential differences in actin filaments will be presented. The effect of the cation (Mg versus Ca) in the nucleotide binding cleft will also be discussed.

Active Micro rheology of Actin with Laser Tweezers


Nucleotide-mediated Conformational Changes of Monomeric Actin

P. Dalhaimer, B. Nolen, T. Pollard; Yale University, New Haven, CT

The conformation of monomeric actin depends on the bound nucleotide: ATP, ADP-P, or ADP. Most crystal structures of actin with bound ATP have the central cleft closed around the bound nucleotide and a disordered DNase-binding (DB) loop. A crystal structure of rhodamine actin with bound ADP also has a closed cleft but an n-helical DB loop. We used molecular dynamics simulations to study the conformations of monomeric actin with bound ATP or ADP without nucleotide (apo-actin) in solution with Ca2+. Assuming equilibrium formalisms, the fraction of time each complex is in an open versus closed conformation for ATP, ADP, and apo is 10^{-6}, 0.1, and 29Δapo ~ kBT. Assuming equilibrium formalisms, the fraction of time each complex is in an open versus closed conformation for ATP, ADP, and apo is 10^{-6}, 0.1, and 29Δapo ~ kBT.

Active Micro rheology of Actin with Laser Tweezers

A. Pomerance, E. Rericha, W. Losert; Institute for Research in Electronics and Applied Physics, University of Maryland, College Park, MD

A living cell’s actin network is capable of exerting and responding to forces of several pN. In order to understand and model cell processes which that exert forces, such as in a crawling cell, it is necessary to characterize the mechanical response of the cytoskeleton under load. We use laser tweezers to locally apply forces of tens of pN to beads embedded in a purified, in vitro actin network. We show that for imposed strains below 4 microns, the beads recoil to their equilibrium position, suggesting that the actin network is undamaged by the strain. When beads are pulled beyond 4 microns, however, the beads do not recoil to their initial positions. We find the strain threshold is independent of pulling speed. The characteristic recoil time of strained beads, however, is a function of the strain rate and the total strain and obeys a power law in the total strain time with an exponent ~ 2/5. Finally, pulling a bead less than the threshold and holding it at beyond 4 microns, however, the beads do not recoil to their initial positions. We find the strain threshold is independent of pulling speed. The characteristic recoil time of strained beads, however, is a function of the strain rate and the total strain and obeys a power law in the total strain time with an exponent ~ 2/5. Finally, pulling a bead less than the threshold and holding it at its destination changes the recoil dynamics. During the holding time, the network relaxes and the force required to hold the bead in place lessens. For waiting times greater than 50s, the network fully relaxes and the bead no longer recoils. The strain relaxation in the long wait time experiments is explained by diffusion of filaments, however this is not sufficient to explain the observed strain threshold for larger strains and another mechanism of remodeling the actin network may be at work.

Residue Size at Position 204 of the Pointed-End in Actin Controls Polymerization

S. Yates, J. Dawson; Molecular and Cellular Biology, University of Guelph, Guelph, ON, Canada

Previous work has shown that the double mutant, A204C/C374A (AC), in yeast actin lacks the ability to polymerize in vitro. To understand the importance of the 204 position, a series of actins with a single mutation at this position, with Cys-374 retained, were created. The attempted substitutions were Gly, Ser, Cys, Asp, Ile, Lys, Phe and Trp. Plasmid shuffling was used to introduce the mutant actin containing plasmids into yeast. No viable yeast were obtained containing the Asp, Ile, Lys, Phe and Trp substitutions, suggesting that these mutations are lethal. The viable strains were those expressing A204G, A204S or A204C-actin. The A204G and A204S strains were slightly sensitive to cold-temperature and hypersensitivity, while the A204C strain, like AC-actin, shows much larger effects on its growth under these conditions. Fluorescence microscopy of yeast stained with DAPI, Calcofluor or rhodamine-phalloloid revealed only slight differences between wild-type and the A204G- and A204S-AC1 expressing cells. Cells expressing A204C-AC1 showed the anomalies as were previously observed for AC-actin, including abnormal actin structures. The similarity of the A204C and AC-actin strains illustrates that the mutation at position 204 alone is responsible for polymerization-deficient actin. Light scattering was used to monitor polymerization of these actins in vitro. A204G and A204S have increased critical concentrations of 12 and 13-fold, respectively, relative to wild-type. An investigation into the lag phase showed that A204G and A204S, like wild-type, nucleates through a trimmer. All the mutations, viable or lethal, were modeled into the Holmes model of F-actin and energy
minimization was performed. A correlation between size of the residue and stability of the modeled filament was observed. Non-viable strains contained actin with larger, bulkier 204 residues that would severely impair filament formation, whereas smaller substitutions at 204 could still be accommodated within the actin polymer interface.

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Actin Isoform Specific Conformational Differences Observed with H/D Exchange and Mass Spectrometry
E. Stokasimov, P. A. Rubenstein; Biochemistry, University of Iowa, Iowa City, IA

Yeast actin appears to be biochemically more conformationally dynamic than muscle actin. The difference could arise from a predisposition of muscle actin for a more closed state than yeast actin. We used amide proton hydrogen/deuterium exchange detected by mass spectrometry to analyze conformational differences between yeast and muscle actins. After short labeling times, G-actin subdomain 3 and 4 peptides have 20-40% of their amides protons exchanged for yeast and 0-20% for muscle actin. After 3 hours of labeling, the peptides with the most extensive exchange in both yeast and muscle G-actin (40-60%) are in subdomain 4, the area around the DNase I loop in subdomain 2, the linker region between subdomains 1 and 3, and near the C-terminus. However, muscle actin shows less uptake (0-20% vs. 20-40% for yeast) in an outer helix between subdomains 1 and 2, and two helices in subdomain 4, one of which is in the nucleotide binding cleft. These results indicate that yeast G-actin is conformationally more flexible than muscle. Yeast F-actin incorporates about 20 deuteriums less per monomer than G-actin, observed throughout the molecule for short labeling times. After 3 hours of labeling, peptides in subdomain 3, around the DNase I loop, and towards the C-terminus show 11-27% less exchange compared to G-actin. Similar results are observed for muscle actin. For F-actins after 6 minutes, regions in subdomain 4 and between subdomains 1 and 2 exchange more slowly in yeast than muscle actin. At 3 hours labeling there are no differences in exchange between yeast and muscle F-actins except for a helix and a peptide in subdomain 1 (residues 94-125). In this helix-loop region the helix exchanges more in yeast and the loop more in muscle actin. This technique allows regional assessment of actin behavior in response to imposed forces on the protein.

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Polycation Induced Actin Polymerization
A. Muhlrad, D. Pavlović, E. Reisser; Institute of Dental Sciences, Hebrew University, Jerusalem, Israel, Dept. of Chemistry and Biochemistry and the Molecular Biology Institute, University of California, Los Angeles, CA

Polycations, including basic proteins as myelin basic protein, polymerize very efficiently actin. This polymerization was proposed to be initiated by formation of antiparallel actin dimers that nucleate filament assembly (Bubb et al., 2002. J. Biol. Chem. 277:20999). We studied the factors affecting polycation induced actin polymerization by using pyrene labeled G-actin and polycation as a polycation model. As shown by excimer formation, antiparallel dimers are formed at the onset of polycation polymerization, but not upon polymerization of actin by Mg. At low ionic strength the polymerization of actin by polycation is very fast, but becomes slower as the ionic strength increases. 100 mM KCl completely abolishes the acceleration of polymerization by polycation. Inorganic phosphate, independently of the ionic strength, significantly inhibits actin polymerization in the presence of polycation. Divalent cations, Mg or Ca, are necessary for efficient polycation induced polymerization, which is optimal in the presence of 0.2 mM Mg or 2 mM Ca. The speed of polymerization depends also on the actin-bound cation and nucleotide and it decreases in the order MgATP > CaATP > NaATP and NaADP. This reduces the critical concentration for actin polymerization by increasing the rate of association and decreasing the rate of dissociation of monomers from the actin filament. The polymerization by polycation is accompanied by bundling of actin filaments even at very low actin concentrations. These results indicate that polycations may significantly affect the dynamics of actin in the cell.

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Biological Thermal Phase Separation in Living Cells
C. Cheng, P. Leduc; Carnegie Mellon University, Pittsburgh, PA

The cytoskeleton in living cells is critical as it strongly influences many cellular functions such as motility, organelle transport, mechanotransduction and mitosis. This structural network is also known to respond to a variety of factors including chemical, mechanical, and scaffolding environments. In our studies, we are interested in the effects of localized cytoskeleton organization, with a particular focus on the actin filaments and their response to the thermal environment as heat shock responses have been shown to be critical in cell behaviors such as the apoptosis. We found that this mode of stimulation produces distinct reversible phases as the cytoskeleton will reorganize its network under defined reproducible thermal conditions. We first globally controlled the thermal environment of NIH 3T3 fibroblasts and after applying an increase in temperature of 5 °C, we observed a change in the actin filament network as these single molecular polymers depolymerized. When we reversed the process and lowered the temperature back to 37 °C, the actin repolymerized indicating a reversible phase that is controlled by the thermal environment. However, when examining this stimulation with respect to microtubules and intermediate filaments, little or no alterations in these structures were found. We also characterized the presence of F-actin and G-actin for these phases through analyzing the associated intensities in immunofluorescence studies. We also probed localized thermally-induced response within living cells through creating a thermal gradient. These studies suggest that cells not only have distinct phases or patterns, but also maintain a reversible equilibrium with respect to the thermal environment. These results have implications in a variety of fields such as cellular organization, biopolymer dynamics, and biothermal environments.

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In Vitro Reconstitution of DNA Segregation: The Modulation of ParM Dynamic Instability Drives Plasmid Segregation
E. Garner, C. Campbell, D. Mullins; Biochemistry, UCSC, San Francisco, CA

The R1 par operon constructs a minimal DNA-segregating spindle from three components, the centromeric DNA sequence parC, the DNA binding protein ParR, and the actin homolog ParM. In vitro, ParM filaments exhibit rapid spontaneous nucleation, symmetrical elongation, and dynamic instability. In vivo, the ParC complex is thought to couple ParM polymerization to plasmid segregation. To determine how these three components combine to form a bipolar spindle we reconstituted this system in vitro from purified components. We combined parC conjugated beads with ParR and fluorescently labeled ParM. Isolated parC-coated beads short, dynamic, and radial symmetrical asters of ParM. The size of these asters is determined the difference in kinetic polarity between the stabilized, ParR/C bound filaments and the unbound dynamically unstable filaments in solution provides the monomer differential that drives bead segregation. The direct attachment of ParR/C to the ParM filament occurs between pairs of parC-coated beads, suggesting that both filament ends are bound and stabilized by the ParR/C complex. We directly observe this bipolar attachment by electron microscopy.

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Cytosplasmic γ-Actin Null Mice Are Viable but Deaf
K. J. Sonnemann, K. W. Prins, S. Ikeda, A. Ikeda, J. M. Ervasti; 1Department of Physiology, University of Wisconsin, Madison, WI, 2Department of Medical Genetics, University of Wisconsin, Madison, WI

The six mammalian actin isoforms comprise a family of highly conserved cytoskeletal proteins that play fundamental roles in all aspects of cell biology. The "non-muscle" β,- and γ-cyto-actin isoforms are abundantly expressed and sorted to distinct locations within a variety of polarized cell types despite differing at only 4 out of 375 amino acids. A ubiquitous expression pattern, the conservation of actin sequences across species, and the inherent importance of the actin cytoskeleton have led to the theory that both β-cyt- and γ-cyt-actin are essential for cell viability. In contrast to mouse models which identified β-cyt-actin as an essential gene (Shawlot et al., Transgenic Res., 1998; Schmerling et al., Genesis, 2005), we show here that a substantial fraction of γ-cyt-actin null mice (Actγ1-/-) were viable, fertile, and survived beyond 1 year of age. Consistent with skeletal muscle-specific ablation of β-cyt-actin (Sonnemann et al., Dev Cell, In Press), Actγ1-/- mice exhibited an overt skeletal myopathy characterized by hind limb contractures and progressive cell necrosis and regression. Actγ1-/- mice also presented with age- and frequency-dependent hearing loss, closely mimicking a form of human deafness caused by mutations in γ-cyt-actin. The progressive phenotype observed both in muscle and the inner ear implies that γ-cyt-actin is essential for the long-term maintenance, but not establishment, of polarized cell types in vivo. Surprisingly, we found a compensatory upregulation of muscle actins in several Actγ1-/- tissues, which novelty suggests that muscle and non-muscle actins may be partially redundant in vivo. These results challenge the idea that both β-cyt- and γ-cyt-actin are essential for the development of polarized cell types and suggest more functional overlap between muscle and non-muscle actins than previously thought. Actγ1-/- mice thus provide a valuable tool to investigate how actin isoforms regulate the cytoskeleton and how actin mutations lead to human disease.
Abil Gene Silencing by Short Hairpin RNA Impairs Bcr-Abl-induced Cell Adhesion In Vitro and Leukemogenesis In Vivo

W. Yu, N. Clough, X. Sun, Z. Dai; Department of Medicine, University of Colorado Health Sciences Center, Aurora, CO

Actin polymerization, a core process in cytoskeletal mechanics, is closely associated with cell shape, adhesion, and migration. This dynamic cellular process is spatially and temporally regulated in normal cells. Its dysregulation, however, is often associated with cellular transformation and tumorigenesis. The mechanism by which tumor cell overrides normal control of actin polymerization is not fully understood. In this study, we explored the role of Abi1 in actin polymerization, in Bcr-Abl-induced abnormal actin polymerization, cell adhesion, and leukemogenesis. We introduced Abil short hairpin RNA (shRNA) into Bcr-Abl-transformed Ba/F3 cells and monitored leukemia development in NOD/SCID mice which received these cells. The expression of Abi1 shRNA resulted in a stable reduction of Abi1 protein to undetectable level, as analyzed by Western blot. Abil gene silencing by shRNA does not affect Bcr-Abl-stimulated cell proliferation and IL-3 independent growth. However, it attenuates the Bcr-Abl-induced abnormal actin remodeling and cell adhesion to fibronectin. The mice injected with Bcr-Abl-transformed cells developed leukemia with an average of 15.7 +/-2.1 day survival time, whereas the mice injected with Bcr-Abl-transformed cells expressing Abil shRNA survived longer with an average of 25.9+/2.3 day survival time. In contrast to Bcr-Abl-transformed cells, which induced splenomegaly when injected into NOD/SCID mice, Bcr-Abl-transformed cells expressing Abil shRNA failed to induce splenomegaly. Thus, these data provide direct evidence that Abil pathway contributes to the pathogenesis of Bcr-Abl-induced leukemia.

166 Fibroblasts in Wound Healing: Transformation, Motility, and Proliferation Differences in Syndecan-1-deficient Mice

R. A. Jurjus, S. Pal-Ghosh, Y. Liu, G. Tadvalkar, A. Sekaran, M. Stepp; Anatomy and Cell Biology, George Washington University, Washington, DC

Activated fibroblasts have several important functions in wound healing and express α smooth muscle actin (αSMA). Syndecans are transmembrane proteoglycans that play a major role in cell adhesion. Syndecan-1 (sdc-1) is upregulated in epithelial cells in response to injury and sdc-1 deficient mice show delayed corneal and skin wound healing. In this study we determine whether loss of sdc-1 on primary dermal fibroblasts altered the proliferation, motility, and proliferation of fibroblasts to myofibroblasts. Primary sdc-1 null (-/-) and wild type (+/+) fibroblast cultures were monitored to day 8 for nSMA expression, motility, migration, and proliferation by calculating the mitotic index (MI). Results show that +/- fibroblasts begin to convert to the myofibroblasts (αSMA+/-ve) at day 1 while +/- fibroblasts take longer. In time lapse migration studies, the velocity of +/- fibroblasts was significantly higher than that of the +/-/+ (p<0.0001) between 2 days and 3 identical between day 3 and 4. The MI of +/-/+ fibroblasts was higher at day 3 and decreased by day 5 when compared to +/-/- and +/-/+ fibroblasts were also treated with TGF-β at 10 ng/ml and monitored up to day 8 by analysis of nSMA expression, cell motility, and MI index. TGF-β accelerated fibroblast to myofibroblast conversion at earlier timepoints but the effect was more pronounced in +/- cells. The velocity of +/-fibroblasts after treatment with TGF-β at day 3-4 remained similar to +/-/. The MI decreased for both +/-/+ and +/- but with the increase remaining for +/-/+ cells. In conclusion, the sdc-1/-/- fibroblasts migrated faster than +/-/+ and their MI was transiently increased compared to +/-/. When sdc-1/-/- fibroblasts were treated with TGFβ, both their migration rate and MI decreased and were similar to TGFβ treated +/-/+ cells.

A Functional Role for the Smooth Muscle Specific Isoform of Actin

N. D. Weymouth, D. C. Rockey; Internal Medicine, University of Texas Southwestern Medical Center, Dallas, TX

Smooth muscle α-actin is one of six highly conserved mammalian actin isoforms that appear to exhibit functional redundancy. Nonetheless, we have postulated a specific functional role for the smooth muscle α-actin specific isoform. Given smooth muscle α-actin’s implied role in myofibroblast contraction and the predominance of this isoform in myoepithelial cells, we hypothesized that smooth muscle α-actin is essential for myoepithelial cell function and tested this postulate by using a smooth muscle α-actin deficient mouse. Here, we show smooth muscle α-actin deficient mice have a novel mammary gland phenotype such that dams lacking smooth muscle α-actin are unable to nurse their offspring effectively and nursing pups die shortly after birth due to malnutrition. The phenotype was rescued in cross-fostering experiments with wild type mice, excluding a developmental defect in smooth muscle α-actin null pups. Null mammary glands exhibited ductal distension during lactation as well as reduced milk stasis, continued ductal dilation, and premature involution during lactation. Premature involution occurred in null mammary glands shortly after parturition within 4-6 days and was marked by a massive increase in the number of apoptotic cells as revealed by TUNEL analysis (at 6.5 days postpartum null (-/-) glands exhibited 12.3% versus wild type (+/+) glands which exhibited 0.95% TUNEL positive cells, p<0.001. The mechanism for the underlying lactation phenotype is due to an apparent developmental arrest in and an impaired contractility of myoepithelial cells in smooth muscle α-actin null mammary glands, despite normal expression of other actin species. Contraction in isolated myoepithelial cells from null mammary glands was severely compromised (53% reduction compared to +/-/+ cells, p<0.001, n=6) when measured on collagen lattices. These findings reveal an absolute requirement for smooth muscle α-actin in myoepithelial cell contraction, which appears to be necessary for lactation.

Three-dimensional Culture induces F-actin Dependent Cytoplasmic Extensions

M. L. Marquette, L. Vergara, D. Byerly, M. Sognier; 1Neuroscience and Cell Biology, University of Texas Medical Branch, Galveston, TX, 2NASA-Johnson Space Center, Houston, TX, 3Universities Space Research Association, Houston, TX

Identifying cellular and morphological changes of myoepithelial cells during differentiation in three-dimensional (3D) culture may provide novel insight into skeletal muscle morphogenesis. Our previous observations indicate that numerous cytoplasmic extensions (podula) are present on single cells in 3D culture conditions. Similar structures were described on cells during microgravity/modeled microgravity exposure. References to cytoplasmic extensions have also been reported in other culture conditions; i.e., when substrate adhesion is prevented in anchorage-dependent cells. The purpose of these studies was to determine if 3D culture increased podula formation in single cells. C2C12 cells, a murine myoblast cell line, were grown in 3D conditions using Synthecon’s Rotary Cell Culture System (RCCS) for 3, 6, 9 hours, fixed, and stained. Cells cultured in Petri dishes, precoated to prevent cell adhesion, served as controls. Immunofluorescent staining and confocal microscopy analysis revealed that the podula of cells cultured in the RCCS were significantly more numerous than those in suspension control (p<0.05, n=15). In rat myoepithelial cells, RCCS culture resulted in an absolute increase in podula formation compared to 2D control (p<0.05, n=15). In conclusion, this study reveals an absolute requirement for smooth muscle α-actin in myoepithelial cell contraction, which appears to be necessary for lactation.

S100B Protein Regulates the Rho/ROCK Axis and Akt via P33-K in Giblollastoma Cells: Implications for Astrocyte Activation and Tumor Growth

F. Brozzi, C. Arcuri, R. Donato; Department of Experimental Medicine & Biochemical Science, University of Perugia, Perugia, Italy

Contrary to the abundant information about the regulatory effects of the astrocyte, Ca2+-modulated protein of the EF-hand type, S100B, in neuroprotection and neurodegeneration once released by astrocytes, little is known about the functional role(s) of S100B within astrocytes in relation to their proliferation and participation in inflammation and/or tumor growth. In the normal brain tissue, S100B is an abundant protein that is expressed in astrocytes. In addition, S100B is expressed in a variety of normal and abnormal cells including neurons, glia, inflammatory cells, and tumors. In the current study, we examined the changes in S100B expression, motility, migration, and proliferation by calculating the mitotic index (MI). Results show that S100B deficient mice have a novel mammary gland phenotype such that dams lacking smooth muscle α-actin are unable to nurse their offspring effectively and nursing pups die shortly after birth due to malnutrition. The phenotype was rescued in cross-fostering experiments with wild type mice, excluding a developmental defect in smooth muscle α-actin null pups. Null mammary glands exhibited ductal distension during lactation as well as reduced milk stasis, continued ductal dilation, and premature involution during lactation. Premature involution occurred in null mammary glands shortly after parturition within 4-6 days and was marked by a massive increase in the number of apoptotic cells as revealed by TUNEL analysis (at 6.5 days postpartum null (-/-) glands exhibited 12.3% versus wild type (+/+) glands which exhibited 0.95% TUNEL positive cells, p<0.001). The mechanism for the underlying lactation phenotype is due to an apparent developmental arrest in and an impaired contractility of myoepithelial cells in smooth muscle α-actin null mammary glands, despite normal expression of other actin species. Contraction in isolated myoepithelial cells from null mammary glands was severely compromised (53% reduction compared to +/-/+ cells, p<0.001, n=6) when measured on collagen lattices. These findings reveal an absolute requirement for smooth muscle α-actin in myoepithelial cell contraction, which appears to be necessary for lactation.
Myosin-Ic Binding to Hair-Cell Stereotipic Tips Is Mediated by a Calmodulin-Free Second IQ Domain and Does Not Occur in Cadherin 23 Mutant Mice

K. R. Phillips, R. Goodyear, S. Tong, G. P. Richardson, J. L. Cry; Sensory Neuroscience Research Center, West Virginia University School of Medicine, Morgantown, WV, School of Life Sciences, University of Warwick, Falmer, Brighton, United Kingdom, Sensory Neuroscience Research Center and Departments of Otolaryngology and Biochem. and Mol. Pharmacology, West Virginia University School of Medicine, Morgantown, WV.

In hair cells, the sensory receptor cells of the inner ear, deflections of the hair bundle due to sound or head movements opens cation-selective transduction channels located at the tips of stereocilia, actin-filled processes that comprise the mechanosensitive hair bundle. In the prevailing model of transduction, hair-bundle deflection tenses fine interstereociliary extracellular linkages called tip links, which in turn opens transduction channels. During prolonged stimuli, transduction-current magnitude decreases within tens of microseconds in a process called adaptation. Adaptation is powered by myosin-Ic (MyoIC), which is located in stereocilia and is part of the transduction complex. We have developed an in situ binding assay to visualize the binding of recombiant MyoIC fragments to the tips of stereocilia, the site of hair-cell transduction. Using this assay in previous work, we have ascertained that the calmodulin-binding neck region of MyoIC is sufficient for binding to stereotipic tips. In our current work we have determined that the second IQ domain of MyoIC mediates this interaction and also mediates interactions with phosphatidylinositol 4,5-bisphosphate (PIP 2) in a nitrocellulose-based assay. Furthermore, we find that MyoIC binding to stereotipic tips is sensitive to calcium chelators and lanthamide ions, conditions that are known to break tip links, and does not occur in hair cells of mice lacking the protein cadherin 23, a proposed component of the tip link, although these cells do contain PIP 2.

Myosin-Ia Powers the Sliding of Apical Membrane along Microvillar Actin Bundles

R. E. McConnell, M. J. Tsuda; Cell and Developmental Biology, Vanderbilt University Medical Center, Nashville, TN

Myosin-Ia (MyoIA) is a plus-end directed, actin-based motor found in brush border (BB) microvilli. Within the microvillus, MyoIA forms a highly-ordered array of bridges that link the plasma membrane to the underlying actin core. MyoIA molecules in this array are uniformly oriented due to the polarized nature of the actin filaments in the supporting bundle. Based on this unique arrangement, we expect the microvillar population of MyoIA to exert substantial plus-end directed forces on the apical membrane. To test for the presence of these forces, we attempted to reactivate the mechanical activity of MyoIA in isolated BBs. When millimolar ATP alone was added to fluorescently labeled BBs, time-lapse spinning disk confocal microscopy revealed a rapid and robust loss of apical membrane from microvilli. Kymograph analysis of time-lapse data showed that membrane loss is the result of tip-directed (plus-end directed) translation; upon reaching microvillar tips, membrane is released or "shed" into solution in the form of small vesicles. Ultrastructural analysis demonstrated that ATP addition increases the length of exposed actin bundle at the base of microvilli and confirmed the striking vesiculation of membrane at microvillar tips. Membrane shedding is insensitive to blebbistatin, suggesting that this activity is independent of myosin-2, the most abundant myosin in the BB. The rate of membrane shedding does, however, scale with ATP concentration and is significantly inhibited by low levels of ADP, a predicted feature of MyoIA motor activity. Finally, BBs isolated from MyoIA KO mice exhibit significantly lower levels of membrane shedding compared to WT preparations. Taken together, these data indicate that: (1) ATP activates the plus-end directed translation of apical membrane along microvillar actin bundles, and (2) MyoIA powers this movement. We anticipate that the membrane translation and "shedding" described here may hold significant implications for MyoIA function in vivo.

Acanthamoeba Myosin IC Has Equal Affinity for Phosphatidyserine and Phosphatidylinositol Bisphosphate

K. Hwang, F. Mahmoudian, J. Gruschus, E. D. Korn, H. Brezsa; Laboratory of Biophysical Chemistry, NHLBI, NIH, Bethesda, MD, Laboratory of Cell Biology, NHLBI, NIH, Bethesda, MD

Acanthamoeba myosin IC (AMIC) binds to acidic lipids through the basic region of its motor (Bubb et al. Cell Motil. Cytoskelet. 39, 134-46, 1998). Recent work shows that mammalian myosin IC tail binds PIP 2 with high affinity and specificity. By NMR and homology modeling, we have investigated the affinity of AMIC for unilamellar phosphatidylcholine vesicles containing charge-equivalent amounts of PIP 2 or phosphatidylserine (PS). AMIC has a high affinity for PIP 2, K1/2=2.3 μM based on surface-accessible PIP 2, similar to the affinity of mammalian MIC for PIP 2. However, in contrast to mammalian MIC, the affinity of AMIC is equally high for PS, when corrected for its 5-fold difference in negative charge, i.e. K1/2=28 μM for vesicles containing 25% PS. Similarly, AMIC binds with equal affinity, K1/2=2-3 μM, to vesicles containing 5% PIP2-25% PS and vesicles containing 50% PS. The binding affinities are the same for constructs that contain only the basic region of the tail, confirming that the acidic lipid-binding site is in the basic region, and demonstrating that folding of the full-length tail, as shown by cryo-electron microscopy (Ishikawa et al. PNAS 101, 12189-94, 2004) and NMR, does not affect the affinity of AMIC for acidic lipids. The fact that AMIC has 10-fold higher affinity than phospholipid vesicles containing 5%PIP2+25%PS than for vesicles containing only 25%PS suggests that under physiological conditions AMIC might be targeted to regions of membranes enriched in PIP 2, which concentrates at the leading edge of migrating Acanthamoeba (Bubb et al. Cell Motil. Cytoskelet. 39, 134-46, 1998).

Myosin I Substrate Specificity of the Dictyostelium Pak Family

S. W. Crowley, Y. Yang, A. Bigg, G. P. Côté; Biochemistry, Queen's University, Kingston, ON, Canada

In vitro and in vivo studies indicate that activation of the motor activity of myosin requires a negative charge at the TEDS site in the motor domain. While all the myosin I isoforms from Dictyostelium (D. discoideum) contain a serine or threonine at this site and are therefore subject to regulation by phosphorylation. We have previously purified a p21-activated kinase, PakB, which is capable of phosphorylating and activating the Dictyostelium class I myosin MyoD. MyoD is also capable of being phosphorylated and activated by other members of the Pak family including the budding yeast Paks Ste20p and Cla4p and mammalian Pak3. Interestingly, however, the closely related class I myosin MyoB is not a substrate for either Dictyostelium PakB or mammalian Pak3. Dictyostelium possess two other characterized Pak isoforms, PakA and PakC. In order to define the substrate specificity of the Pak family we have expressed in Dictyostelium and purified, using actin and FLAG-tag affinity steps, five class I myosin head constructs (MyoA, B, C, D, and E) and tested them with the catalytic domains of Dictyostelium PakA, B and C expressed and purified from E. coli. In conjunction with this, we have performed studies to investigate which myosin I isoformes directly interact with PakB. In pull down assays, a GST-fusion of the SH3 domain of MyoB, but not MyoC or MyoD, was shown to bind to an N-terminal fragment of PakB containing residues 1-120. This may indicate that, although MyoB is not a substrate for PakB, it may function to localize the kinase to the leading edge of chemotaxing cells.

Myosin Binds PIP2 through a PH Domain

D. E. Hokanson, J. M. Laakso, T. Lin, E. M. Ostap; Physiology, University of Pennsylvania School of Medicine, Philadelphia, PA

MyoIC is a member of the myosin superfamily that binds PIP2, links the dynamic actin cytoskeleton to the membrane, and plays roles in mechanosignal transduction and membrane trafficking. We located and characterized two distinct membrane binding sites within the regulatory and tail domains of this myosin. We found that the tail domain binds tightly and specifically to PIP2, in a non-cooperative manner. It binds with slightly higher affinity to Ins(1,4,5)P3 as well as other inositol phosphates which may act as inhibitors to membrane binding in the cell. By sequence and secondary structure analysis, we identified this phosphoinositide binding site in the tail to be a putative pleckstrin homology (PH) domain. Point mutations of residues known to sequence and secondary structure analysis, we identified this phosphoinositide binding site in the tail to be a putative pleckstrin homology (PH) domain. Point mutations of residues known to be essential for phosphoinositide binding in previously characterized PH domains inhibit myoIC binding to PIP2 in vitro and eradicate correct localization and membrane binding in vivo. The extended sequence of this binding site is conserved within many other myosin-Is across species, suggesting they too contain a putative PH domain. We also characterized a previously identified membrane binding site within the IQ motifs in the regulatory domain. This region is not phosphoinositide specific, but binds anionic phospholipids in a calcium dependent manner; nevertheless, this site is not essential for in vivo membrane binding. As a result, we have determined that myoIC contains two lipid binding sites, a polybasic region that binds to high levels of PS in a calcium dependent manner, and a putative PH domain that binds tightly and specifically to phosphoinositols.

Calmodulin Binding to the MyoIC Regulatory Domain

S. D. Mancheva, T. Lin, H. Pham, Y. E. Goldman, E. M. Ostap; Physiology, University of Pennsylvania School of Medicine, Philadelphia, PA
Myo1c is an unconventional short-tailed myosin-I involved in cell signaling and membrane dynamics. Calcium binding to calmodulin (CaM) bound to the myo1c regulatory domain affects myo1c motor properties. However, the effect of calcium on myo1c kinetics, mechanics, and function are not fully understood. In order to characterize the effect of calcium and CaM on the regulation of myo1c, we must first understand the biochemical parameters that define the CaM-regulatory-domain interaction. We have performed fluorescence spectroscopy and stopped-flow kinetics to measure the binding and stoichiometry of CaM to the three IQ motifs in the myo1c regulatory domain. We measured the binding of a fluorescently labeled CaM mutant (N111C) to recombinant myo1c and peptides containing the three IQ motifs. In the absence of calcium, the CaM bound to the IQ motif adjacent to the motor domain (IQ1) has the slowest dissociation rate, and the IQ motif adjacent to the tail domain (IQ3) has the fastest dissociation rate. In the presence of saturating concentrations of calcium, IQ1 has the fastest rate of dissociation. Calcium accelerates the actomyosin ATPase rate, but this increase is suppressed by the addition of > 1 μM calmodulin. As shown previously, calcium inhibits actin gliding in the in vitro motility assay, but is rescued by addition of 1 μM calmodulin. Our data suggest that the CaM bound to the IQ motif adjacent to the motor domain is rapidly exchangeable in the presence of calcium and is responsible for regulation of myo1c ATPase and motility activity.

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Biochemical and Structural Characterization of Calcium Regulation of Myo1b
Myo1b is an unconventional myosin which has a proposed role of mediating membrane tension. Being located close to the cell membrane subjects myo1b to rapid and drastic changes in calcium concentration. Since the lever arm of myo1b binds the light chain calmodulin, calcium could be a potent regulator of myo1b activity. This supposition was borne out when the steady-state ATPase rate was found to be calcium dependent, increasing with increasing calcium concentration. Moreover, it was found that calcium abrogates actin motility in gliding assays using myo1b. In order to determine a mechanism for calcium regulation, the calcium dependence of key steps in the ATPase cycle of a myo1b with a truncated lever arm was explored at 37°C using a stopped-flow apparatus. In this study, it was found that while calcium had no effect on ADP release and only a minimal effect on ATP binding, the rate of phosphate release was increased nearly four-fold. For multiple myo1b motors acting in concert, introduction of calcium could create a considerable increase in the effective duty ratio. To determine if the calcium effect occurs on a physiologically relevant time scale, the rate of calcium binding to myo1b during phosphate release was determined. The basis of calcium regulation is likely a conformational change in each of the IQ motifs that is triggered by calcium binding.

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Roles for Drosophila melanogaster Myo1B in Maintenance of Enterocyte Brush Border Structure and Resistance to Bacterial Pathogens
P. S. Hegaz1, V. Merrell, M. S. Mooseker2; MCD Biology, Yale University, New Haven, CT, 2MCD Biology, Cell Biology, Pathology, Yale University, New Haven, CT
Drosophila myosin IB (Myo1B) is one of two class I myosins in the Drosophila genome. In the larval and adult midgut enterocyte, Myo1B is present within the microvilli (MV) of the apical brush border (BB) where it forms lateral tethers between the MV membrane and underlying actin core. Expression of GFP-Myo1B tail in the larval gut showed that the tail domain is sufficient for localization of Myo1b to the BB. A deletion mutation was created in the Myo1B gene in order to assess the role of Myo1b in the larval midgut. Basic gut physiology (food uptake and clearance, gut pH) appears normal. However, there is a 3 fold increase in the number of apoptotic cells in the Myo1B mutant midgut epithelium based on TUNEL staining of enterocyte nuclei. Ultrastructural analysis of mutant gut revealed many perturbations in the BB, including irregular MV length, MV vesiculation, and membrane tethering defects. Biochemical purification of BBs showed that the loss of Myo1b causes the BB to become destabilized as compared to heterozygous control guts. Ultrastructural analysis of purified mutant BBs shows the loss of cross bridges between the actin core and plasma membrane, which appears to cause the filaments of the central actin core of the MV to be less tightly bundled than in control BBs. Although Myo1B is not essential for the absorptive functions of the gut, it may play a critical role in gut barrier functions. Myo1B mutant larvae exhibit enhanced sensitivity to infection by the bacterial pathogen Pseudomonas entomophila. Survival of orally infected Myo1b mutant larvae is decreased by up to 50%, and severe cytoskeletal defects are observed in the BB of epithelial cells soon after infection. Resistance to P. entomophila infection is restored in Myo1b mutant larvae expressing a Myo1B transgene.

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Myosin Surface Loop 4 Modulates Association of Myosin IB with Actin-Tropomyosin
A. M. Lieto-Trivedi, S. Dash, L. M. Coluccio; Boston Biomedical Research Institute, Watertown, MA
Structural studies of the class I myosin, Myo1, led to the predictions that loop 4, a surface loop near the actin-binding region that is longer in class I myosins than in other myosin subclasses, might limit binding of myosins I to actin when actin-binding proteins, like tropomyosin, are present; and might account for the exclusion of myosin I from stress fibers. To test these hypotheses, mutant myosins with the related mammalian class I myosin, Myo1b, in which loop 4 was truncated (from amino acid sequence RNMGLDEE to NGGLD) or replaced with the shorter and distinct loop 4 found in Dictyostelium myosin II (GAGEGA), were expressed in vitro and their interaction with actin and actin-tropomyosin was tested. Increasing amounts of expressed fibroblast tropomyosin-2 or tropomyosin-5a resulted in a decrease in the actin-activated Mg 2+-ATPase activity of wild-type Myo1b, but had little or no effect on the actin-activated Mg 2+-ATPase activity of the two mutants. In motility assays, few actin filaments bound tightly to Myo1b-coated cover slips when tropomyosin-2 was present; however, actin filaments bound and were translocated by Myo1b-NGLD or Myo1b-GAGEGA in both the presence and absence of tropomyosin-2. When expressed in mammalian cells, like wild type, the mutant myosins were largely excluded from tropomyosin-containing actin filaments indicating that in the cell additional factors besides loop 4 determine targeting of myosins I to specific subpopulations of actin filaments.

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Analysis of Intracellular Functions and Binding Partners of Myosin IE
M. Krendel, M. S. Mooseker; Molecular, Cellular, and Developmental Biology, Yale University, New Haven, CT
Class I myosins are actin-dependent motor molecules expressed in various organisms from yeasts to humans. All class I myosins contain a head (motor) domain, a light chain-binding neck domain, and a tail, which can be either short, consisting only of a membrane-binding TH1 domain, or long, including an SH3 domain in addition to TH1. In mice and humans, there are eight myosin I (Myo1) heavy chain genes, which encode both short- and long-tailed myosins. Little is known about the intracellular functions of long-tailed myosins I in vertebrates and no binding partners for their SH3 domains have been identified to date. We characterized intracellular functions, localization, and binding partners of a human long-tailed myosin, myosin IE (Myo1E). Using dominant-negative constructs and RNAi, we observed that inhibition of Myo1E led to disruption of receptor-mediated endocytosis of transferrin. We also found that Myo1E interacted with two endocytic proteins, synaptojanin-1 and dynamin, via its SH3 domain. Using TIRF microscopy, we observed colocalization of myosin IE with clathrin-coated vesicles and dynamin, which required presence of both TH1 and SH3 domains in Myo1E tail. These findings suggest that Myo1E is involved in clathrin-dependent endocytosis and interacts with components of endocytic machinery via its tail domain.

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Interaction of Chara Myosin with Actin
T. Sokkenari, Y. Ichikawa, K. Ito, K. Yamamoto; Biology, Chiba University, Chiba, Japan
Chara myosin is the fastest motor protein known so far. It can move actin filaments at the maximum velocity of 50 μm/sec in the in vitro motility assay. This velocity is about 10 times higher than that of the skeletal muscle myosin. Although such high velocity can mostly be explained by the kinetic parameters such as ADP release rate from AM-ADP complex or the rate of ATP induced AM dissociation, there are reports suggesting that the surface interaction between Chara myosin and actin is quite different from that between skeletal muscle myosin and actin. For example, muscle tropomyosin completely inhibited the interaction between Chara myosin and actin whereas it did not affect that between skeletal muscle myosin and actin. Such studies were done using crude Chara myosin. Since we recently succeeded in expressing fully functional recombinant Chara myosin2, we studied its interaction with actin by chemical cross-linking with water-soluble carbodiimide and also studied the effect of tropomyosin on its interaction with actin. It was found that (1) major cross-linked site of Chara myosin to actin was not loop 2 and (2) tropomyosin inhibited the motility of Chara myosin but it affected the actin-activated ATPase activity only partially. 1 Sugie Higashin-Fujime et al. Proc. Jpn. Acad. 76, 118–122 (2000). 2 Kohji Ito et al. Biochem. Biophys. Res. Commun. 312, 958–964 (2003).
Isomorphic Specific Functions of Nonmuscle Myosin II in MDA-MB 231 Breast Cancer Cell Adhesion

V. Betapudi, T. T. Egloff; Biology and Physiobiology, Case Western Reserve University, Cleveland, OH

Cell migration in Limulus is critical for the formation of lamellipodia, formation of focal complexes, translocation of cell body and posterior end detachment. Most of these steps are force and tension dependent and are widely believed to be driven by actin polymerization; however, the exact mechanical roles of nonmuscle myosin II during these events remain remarkably poorly understood. Earlier, we reported a significant enhancement and reduction of lamellipodial extension by myosin II and IIB isoforms-depleted MDA-MB 231 breast cancer cells, respectively by unknown mechanism. Here, we present our further studies to understand isomorphic specific functions of myosin II in the formation of focal complexes and focal adhesions during spreading. Our preliminary results show reduced attachment to fibronectin coated surface when cells are treated with myosin II inhibitors ML-7, Blebbistatin and Y-27632 or transfected with myosin IIA and HB sRNAs. Cells treated with ML-7 or transfected with myosin IIB sRNA form focal complexes/focal adhesions in cortical regions but not in the central region of the cells. However, there was a significant reduction in the formation of focal complexes/focal adhesions by cells experienced in the presence of Y-27632 or transfected with myosin IIA sRNA. Western blot analysis of cells depleted with myosin II isoforms reveals reduced phosphorylation of vinculin but no change in the expression of vinculin. However, paraxin expression decreases when myosin IIB is depleted in the cells. These results suggest that both the isoforms are critical for the mechanism of cell migration, with myosin IIA appearing to have roles in both the assembly process of focal complexes and focal adhesions whereas myosin IIIB may be contributing to the maturation of focal complexes into focal adhesions.

Kinetics of the Kinase and Motor Domains of Human Myosin IIIA

J. E. Moore, 1 S. Ananthanarayanan, 1 B. Burns2, A. C. Dose, 1 C. M. Yengo1; 1Biology, University of North Carolina at Charlotte, Charlotte, NC, 1Molecular and Cell Biology, University of California at Berkeley, Berkeley, CA

Myosin IIIA is specifically expressed in photoreceptors and coelchea and is important for the phototransduction and hearing processes. In addition, myosin IIIA contains a unique N-terminal kinase domain and C-terminal actin binding motif. We examined the kinetic properties of baculovirus expressed human myosin IIIA containing the kinase, motor, and two IQ domains. The rate of autophosphorylation by the kinase domain was found to be linearly dependent on ATP concentrations in the range of 0.1 to 1 mM, while manATP binding to the kinase domain was relatively fast. Thus, autophosphorylation activity may be limited by an ATP-dependent conformational change. The maximum actin-activated ATPase rate is relatively slow (VMAX = 0.77 ± 0.08 s⁻¹), and high actin concentrations are required to fully activate the ATPase rate (KATPase = 34 ± 11 µM). However, actin co-sedimentation assays suggest that myosin IIIA has a relatively high steady-state affinity for actin (Kact = 7 µM). The rate of ATP binding to the motor domain is quite slow both in the presence and absence of actin (KATPase = 0.020 and 0.001 µM⁻¹ s⁻¹, respectively). The rate of actin-activated phosphate release is more than 100-fold faster (85 s⁻¹) than the VMAX, while ADP release in the presence of actin follows a two-step mechanism (7.0 s⁻¹ and 0.6 s⁻¹). Thus, our data suggests a transition between two actomyosin ADP states is the rate limiting step in the actomyosin ATPase cycle. Our kinetic model indicates that both the actin attached hydrolysis and the Pi release steps determine the over-all cycle rate. While the stable steady state intermediates of acto-myosin IIIA ATPase reaction are not typical strong actin binding intermediates, the affinity of the stable intermediates for actin is much higher than conventional weak actin binding forms. The present results suggest that myosin IIIA can spend a major of its ATP hydrolysis cycling time on actin.

Myosin III Loop 2 Phosphorylation In Vivo: A Mass Spectrometric Study

H. L. Cardasis, K. E. Kempler, S. M. Stevens, B. A. Battelle; 1Proteomics Core, ICBR, University of Florida, Gainesville, FL, 2Chemistry, University of Florida, Gainesville, FL, 3The Whitney Laboratory of Marine Bioscience, University of Florida, St. Augustine, FL

Myosin III is a photoreceptor specific protein in Limulus. Phosphorylation of this protein by cyclic AMP dependent kinase (PKA) correlates with changes in photoreceptor structure and function. Our objective in this work was to verify the in vivo phosphorylation sites in Limulus Myosin III and to determine the effect of the circadian clock on levels of phosphorylation at each of these sites in vivo. Both lateral eyes from the each animal were used. The lateral optic nerve to one eye on each animal was cut to eliminate clock input, while the optic nerve to the control eye was kept intact. Eyes were extracted one week later at midnight (when circadian input is at a maximum) and under infrared illumination (to avoid the influence of light on the biochemistry of the eye). Eyes were homogenized in protease inhibitors, phosphatase inhibitors, and reducing agents, and Myosin III was purified by SDS-PAGE and digested in-gel with trypsin. Myosin III digests were analyzed by liquid chromatography - tandem mass spectrometry (LC-MS/MS) in conjunction with both differential labeling and label-free methods for relative quantitation. Three phosphorylation sites were verified in endogenous Limulus Myosin III preparations (S-796, S-841, S-846). Two of these sites are located within loop 2, the actin binding region of the protein (S-841 and S-846), while the third site (S-796) is located just upstream of loop 2. In response to circadian clock input, S-796 and S-846 demonstrate a significant 2-fold increase in their levels of phosphorylation. Both of these sites are putative substrates for PKA. S-841, a predicted substrate for protein kinase C (PKC), showed no change in levels of phosphorylation in response to clock input. Our findings support our hypothesis for an actomyosin interaction that is regulated at night by the circadian clock via loop 2 phosphorylation.

Myosin III Changes Distribution in Limulus Photoreceptors with Circadian Clock Input and Light

B. Battelle, N. Robinson; Whitney Laboratory, University of Florida, St. Augustine, FL

Limulus myosin III (LpMyoIII) is phosphorylated in photoreceptors in response to signals from a central circadian clock. Clock signals reach the eyes via a octopaminergic effector projection that is activated nightly by the central clock. OA activates a AMP cascade causing the PKA-phosphorylation of LpMyoIII at sites within and near loop 2, reducing the net charge in this region. In other myosins, actin affinity increases with the net positive charge of loop 2. Therefore clock input and other treatments that decrease the net positive charge of loop 2 via PKA phosphorylation of LpMyoIII may reduce its affinity for actin. Light appears to reverse the effect of PKA. Our findings support our hypothesis for an actomyosin interaction that is regulated at night by the circadian clock via loop 2 phosphorylation.

Functional Analysis of MYO7A in Human Fetal Primary RPE Cells: An Ex Vivo Model for Usher Syndrome 1B

D. Gibbs, T. Dieck; J. Khamodsre, C. Lillo, A. Klomp, J. Hsu, D. Bok, D. S. Williams; 1Pharmacology and Neurosciences, UCSD School of Medicine, La Jolla, CA, Jules Stein Eye Institute, UCLA, Los Angeles, CA

Mutations in the gene encoding MYO7A underlie Usher syndrome 1B. In the mouse retina, the majority of MYO7A is expressed in the apical region of RPE cells, associated with melanosomes. In homologous Zombie 46265B mutant mice, MYO7A protein levels are undetectable. In the absence of MYO7A, melanosomes in the RPE are mislocalized in vivo, and have been reported to fuse with melanin particles.
aberrant transport dynamics in primary culture. Abnormal localization and digestion of ingested phagosomes is also evident. To test if MYO7A functions similarly in the human retina we have determined the distribution of MYO7A in human retinas, and analyzed the expression and function of MYO7A in human primary fetal RPE cells (HRFPE). The distribution of MYO7A in human retina was comparable to mouse retina. HRFPE cells were also found to express MYO7A at a level comparable to mouse primary RPE cells, and with a similar pattern of distribution. Analysis of phagocytic ability identified HRFPE cells as active phagocytes of rod outer segments. Live cell imaging demonstrated that the melanosomes in HRFPE cells were moving, and exhibited the same constrained, rapid movements seen in primary mouse RPE. Using siRNA we have successfully knocked down MYO7A in a human RPE cell line (ARPE19). These siRNAs are being used to knockdown MYO7A in HRFPE primary cells. These results confirm that MYO7A expression is similar between human and mouse retinas. Cellular processes dependent on the function of MYO7A are also preserved in cultures of primary HRFPE cells. Primary cultures of HRFPE cells can represent a physiologically relevant model system of human origin for the study of RPE function and disease, and will be useful for testing therapies for Usher 1B. The comparable expression, distribution and function of MYO7A in HRFPE cells and primary mouse RPE cells validate the Shaker1 4626SB mouse as a model for Usher 1B.

**Functional Analysis of Dynactin Mutations Associated with Amyotrophic Lateral Sclerosis**

R. Dixit, M. Tokio, E. Holzbaur; Physiology, University of Pennsylvania, Philadelphia, PA

The molecular motor cytoplasmic dynein and its associated activator dynactin are critical components of normal axonal transport, and their functional disruption is linked to neurodegenerative disease. Dynactin facilitates dynein processivity and cargo recruitment, and may also regulate motor activity. The p150<sup>Glued</sup> subunit of dynactin binds to both microtubules and dynein. A G59S mutation in p150<sup>Glued</sup> results in decreased microtubule affinity and enhanced p150<sup>Glued</sup> aggregation and is associated with motor neuron disease in humans. Recently, additional p150<sup>Glued</sup> missense mutations (M571T, R785W, R1101K, and T1249I) were identified in amyotrophic lateral sclerosis patients; however, the functional consequences of these mutations are not understood. To test whether these p150<sup>Glued</sup> mutations lead to functional disruption of dynein-dynactin activity, we have used myc-tagged versions of mutant proteins to assay their in vivo and in vitro activities. Our results indicate that these mutations do not perturb microtubule binding of p150<sup>Glued</sup> when expressed in COS7 cells and in vitro microtubule binding assays. Likewise, when overexpressed in COS7 cells, both the wild-type and mutant p150<sup>Glued</sup> proteins disrupt normal Golgi distribution; indicating that the mutant forms of p150<sup>Glued</sup> retain the ability to poison the endogenous dynein-dynactin complex. Sucrose density gradient fractionation of COS7 cell lysates showed no difference in the level of incorporation of wild-type and mutant human p150<sup>Glued</sup> in COS7 cell dynactin-dynein complex. Additionally, none of these mutations results in aberrant p150<sup>Glued</sup> aggregation when expressed in COS7 or PC12 cells. The M571T and R785W mutations reside in the region of p150<sup>Glued</sup> known to interact with the dynein intermediate chain (DIC). However, DIC pulldown assays showed that none of the four mutations significantly decreased p150<sup>Glued</sup>-DIC binding affinity compared to wild-type controls. Our results suggest that these p150<sup>Glued</sup> mutations result in relatively subtle defects in dynactin function or that these mutations represent polymorphisms in the p150<sup>Glued</sup> sequence.

**Mechanism of Dynactin - Microtubule Interactions**

A. D. Stephens, T. L. Culver, S. A. Lex, S. J. King; School of Biological Sciences, University of Missouri-Kansas City, Kansas City, MO

Dynactin, a multi-subunit protein complex, links cytoplasmic dynein and other motors to cargo, and is involved in organizing radial microtubule arrays (Schroer, TA, 2004; Quintyne, NJ, 1999). The largest subunit of dynactin, p150<sup>Glued</sup>, binds the intermediate chain of cytoplasmic dynein and has an N-terminal microtubule-binding domain. To examine the role of microtubule binding by p150<sup>Glued</sup> in vivo, we replaced the wild-type p150<sup>Glued</sup> in cultured Drusenella S2 cells with mutant ΔN-p150, lacking amino acid residues 1-200. ΔN-p150 is unable to bind microtubules. Strikingly, while movement of both membranous organelles and mRNP complexes by cytoplasmic dynein and conventional kinesin is absolutely dependent on dynactin, the role in the dynamics of dynactin-dependent organelle transport. To further analyze the regulation of MT-binding and release of p150<sup>Glued</sup> dynactin function. Removal of the acidic tail of tubulin via subtilisin treatment lowers the relative number of binding events but does not prevent the CAP-Gly domain or the basic sequence.

**Microtubule Binding by p150<sup>Glued</sup> Is Not Required for Cargo Transport but Essential for Spindle Assembly**

H. Kim, 1 C. K. C. Rogers, 2 C. R. Berg, 1 P. R. Selvin, 1 S. L. Rogers, 1 V. I. Gelfand 1; 1Cell and Molecular Biology, Northwestern University, Chicago, IL, 2Biology, University of North Carolina, Chapel Hill, NC, 3Biophysics, University of Illinois, Urbana, IL

Microtubule (MT) tip-tracking is a controversial behavior thought to reflect the dynamic binding of proteins to MT plus-ends. Phosphorylation has been implicated as one mechanism of release and aggregation when expressed in COS7 or PC12 cells. The M571T and R785W mutations reside in the region of p150<sup>Glued</sup> known to interact with the dynein intermediate chain (DIC). However, DIC pulldown assays showed that none of the four mutations significantly decreased p150<sup>Glued</sup>-DIC binding affinity compared to wild-type controls. Our results suggest that these p150<sup>Glued</sup> mutations result in relatively subtle defects in dynactin function or that these mutations represent polymorphisms in the p150<sup>Glued</sup> sequence.

**Dynamic Binding of p150<sup>Glued</sup> to Microtubules Dictates the Function of Tip-Tracking**

M. Raycroft, P. S. Vaughan, K. T. Vaughan; Biological Sciences, University of Notre Dame, Notre Dame, IN

The N-terminus of dynactin p150 contains two microtubule-binding domains that exhibit different properties. The p150 CAP-Gly domain binds stably to microtubules and can inhibit dynein motility along microtubules. The p150 basic domain binds to microtubules but can exhibit one-dimensional diffusion along the microtubule. Additionally, the basic domain increases the processivity of the cytoplasmic dynein motor. Therefore, the p150 basic domain, but not the CAP-Gly domain, helps dynein to act as a second molecule processivity factor for cytoplasmic dynein. Here, we are investigating the mechanism by which these microtubule-binding domains interact with microtubules. In particular, we are examining the role of the acidic C-terminus of tubulin upon dynactin function. Removal of the acidic tail of tubulin via subtilisin treatment lowers the relative number of binding events but does not prevent the CAP-Gly domain or the basic domain from binding microtubules.

**mTOR Kinase Regulates Tip-Tracking Proteins and Cytoplasmic Dynein**

L. Casaletti, K. T. Vaughan; Biological Sciences, University of Notre Dame, Notre Dame, IN

The mammalian target of rapamycin (mTOR) modulates a large number of major cellular processes and has been identified recently as another potential kinase that regulates microtubule (MT) dynamics. mTOR modulates both microtubule plus-end specificity and dynein IC phosphorylation complexity, and the coordination of other tip-tracking proteins during tip-tracking. Our lab has proposed that lagging recruitment of MAP4 with CLIP-170, EB1, and p150<sup>Glued</sup> is the basis of MT plus-end specificity in overexpression experiments. To test the “lagging recruitment” model we co-transfected MAP4 with CLIP-170, EB1, and p150<sup>Glued</sup>. MAP4 decorated the MT lattice but displayed differential binding at MT plus-ends; compared to p150<sup>Glued</sup> MAP4 lagged during recruitment and was slower to label plus-ends during elongation. This supports the possibility that delayed recruitment of dynactin kinases plays an important role in the dynamics of dynactin-dependent organelle transport. To further analyze the regulation of MT-binding and release of p150<sup>Glued</sup>, we engineered a G59S mutation linked to late-onset motor neuron disease into p150<sup>Glued</sup> and assessed the impact on dynein-driven transport. As an initial assessment, the G59S mutant failed to bind MTs in tip-tracking assays consistent with previous work. The G59R mutation also failed to rescue normal transport after siRNA-driven depletion of normal p150<sup>Glued</sup>, suggesting that MT binding is critical to dynactin function. Imaging of p150<sup>Glued</sup>, EB1 and CLIP-170 in fixed cells has suggested that all three colocalize on MT plus-ends. Because only a subset of these proteins is preserved after fixation, we co-expressed each combination for live imaging. EB1 labeled the very plus-end of the MTs, whereas p150<sup>Glued</sup> and CLIP-170 lagged behind slightly. p150<sup>Glued</sup> overlapped with some of the EB1, however CLIP-170 was most clearly separated. This suggests a hierarchy of plus-end binding and coordinate regulation.

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rapamycin-treated COS-7 cell extracts. Rapamycin-treated extracts decreased the affinity of p150Glued for MTs (via p150Glued phosphorylation) consistent with our imaging results. Together these results suggest that multiple pathways regulate dynein and dynactin and that mTOR kinase regulates phosphorylation complexity through a new mechanism.

192 Neuronal Targeting of Cytoplasmic Dynein through Isoform-specific Phosphorylation of Intermediate Chains S. Tautaha, 1 W. S. Lane, 1 K. K. Pfister, 1 P. S. Vaughan, 1 K. T. Vaughan, 1 1Biological Sciences, University of Notre Dame, Notre Dame, IN, 2Biological Sciences, Harvard University, Cambridge, MA, 3Cell Biology, University of Virginia Health Sciences Center, Charlottesville, VA

Neuronal cytoplasmic dynein is a motor protein implicated in axonal transport. Although a single dynein heavy chain is expressed in neurons, a total of six dynein intermediate chain (IC) isoforms are expressed through the expression of two gene products and alternative splicing of each. The function of this IC isoform expansion is unclear, although the IC may function in the assembly or dimerization of the IC. We observed formation of heterodimers in the absence of dynein LCs, but the formation of IC homodimers when LCs were present. The potential significance of this assembly emerged during analysis of motile dynein where we determined that the phosphorylated form of the ICs is responsible for dynein targeting. We performed MS/MS analysis on each neuronal IC isoform and determined that each isoform displays phosphorylation in the p150Glued-binding domain of the ICs. Interestingly, the specific sites of phosphorylation were unique to each isoform and appear to be recognized by different kinases. To test if these phosphorylated peptides bound different binding partners, we used squid axolotl to phosphorylate each protein and measured binding using overlay assays. Squid axolotl recapitulated the phosphorylation specificity we mapped by MS/MS analysis and provided biochemical chemical analysis of the phospho-protein. Binding assays demonstrated that the phospho-version of each protein reduced binding to p150Glued and revealed interactions with new binding partners. This suggests that neuronal dynein modulates cargo specificity through differential phosphorylation of IC isoforms.

193 In Vitro Interactions of the N-terminal Region of BicD2 with Dynactin and Dynactin E. W. Schmitt, 1 D. Splinter, 1 A. Akhmanova, 2 S. J. King 1 1University of Missouri-Kansas City, Kansas City, MO, 2Department of Cell Biology and Genetics, Erasmus Medical Center, Rotterdam, The Netherlands

BicD2 is a mammalian homologue of Bicaudal D (BicD), a coiled coil, evolutionarily conserved protein that acts in the same pathway as the microtubule based motor protein dynein and its activator dynactin. Recent work has shown that targeting the N-terminal region of BicD2 (BicD2-NT) to mitochondria and peroxisomes induced their relocalization via dynein mediated transport. Recent work suggests that BicD2 helps dynein and dynactin to form a higher order complex with each other. However, it is unknown whether BicD2 accomplishes this by binding directly to each megadalton complex or by working through other cellular proteins. We have used purified proteins in in vitro biochemical assays to test BicD2 binding to purified dynein and dynactin. We now report the direct interaction of purified BicD2-NT to purified dynein and dynactin when all three proteins are present. We are now investigating how BicD2-NT affects dynein motor functions.

194 Defective Cytoplasmic Dynein-driven Transport as the Basis of Niemann Pick Type C Disease K. Huelg, K. T. Vaughan; Biological Sciences, University of Notre Dame, Notre Dame, IN

Niemann Pick Type C (NPC) disease is characterized by the formation of abnormal lipid storage organelles in neurons and hepatocytes. Mortality arises from neuronal defects, although the etiology of these defects remains controversial. The identification of two genes (NPC1 & 2) linked to NPC disease has focused most work on defects in cholesterol metabolism. However, because the defective membranes in NPC disease resemble membranes induced by expression of dynein phosphorylation site mutants, we assessed the impact of NPC1 mutations on the morphology and motility of membranes by cell live imaging. Using a GFP-NPC1 expression construct, we observed that NPC1 membranes were highly motile and produced tubulo-vesicular extensions which probed several membrane compartments. Membrane motility and extension were ablated by nokedazole treatment, indicating that NPC1 membranes require an intact microtubule array. Expression of dominant-negative dynactin (p50 dynamitin) and cytoplasmic dynein intermediate chain (S84A) mutants blocked normal bidirectional membrane transport and lead to the accumulation of engorged NPC1 membranes at the plasma membrane. To test if NPC1 mutations disrupt normal NPC1 function by blocking motility, we generated GFP-NPC1 harboring the I1061T mutation and compared wild-type and mutant NPC1 in living cells. In contrast to the behavior of the wild-type protein, I1061T mutant NPC1 membranes were immobile, engorged in cholesterol and lacked the formation of tubulo-vesicular extensions. These phenotypes could not be rescued by cholesterol depletion. Analysis of additional NPC1 mutants (C98S, P692S, and Y635C) confirm the loss of motility and tubule formation as a common theme among mutant NPC1 membranes. Together these studies suggest that NPC1 mutations affect lipid metabolism by perturbing motor-driven transport of NPC1 membranes.

195 Molecular Analysis Reveals a Role for the Cargo-binding Domain of Cytoplasmic Dynein Heavy Chain in Mitochondrial Transport D. Varma, R. B. Vallee; Cell Biology and Pathology, Columbia University, New York, NY

Cytoplasmic dynein is a complex motor protein (1.2 MDa) involved in a wide range of cellular functions. The dynein heavy chain consists of an ~120 kDa N-terminal cargo-binding and self-association domain, and a 380 kDa motor domain. We have analyzed the cargo binding domain both for its potential value as a motor-dead (“headless”) form of dynein and for an understanding of cargo binding control. In previous work we evaluated a series of dynein heavy chain N-terminal truncation mutants (MBC 14:313a). We found that the longer constructs decorated kinetochores without displacing other dynein subunits, dynactin, or L151, and potently inhibited mitosis. We now find that live imaging of mitotic cells transfected with a minimal headless fragment arrested in prometaphase with severe defects in chromosome alignment. In monopolar spindles, both monotectically and symmetrically attached kinetochores were observed, all of which were positive for the headless dynein construct. These results support a role for dynein in its own removal from kinetochores. We also tested the effect of headless dynein on mitotic or microtubule motility, for which a role for dynein has been relatively difficult to establish. Cos7 cells overexpressing this fragment showed ~ 40% increase in the number of cells with dispersed mitochondria in comparison to control GFP overexpressing cells where they were focused towards the cell center. Low to medium overexpression of the GFP-tagged fragment in polarized neuronal CAD cells specifically interfered with the minus-end directed motility of mitochondria which was reduced ~ 4 fold in comparison to control cells. The effect was stronger in high overexpressers, which induced mitochondrial clumping within axonal processes and producing a complete inhibition of all mitochondrial movements. Together these results indicate an important role for cytoplasmic dynein in mitochondrial transport and suggest that headless dynein should be valuable tool for studying dynein function in mitosis. Supported by GM47434

196 Biochemical Analysis of a Dynactin-independent Interaction between Cytoplasmic Dynein and Huntingtin J. P. Caviston, J. L. Ross, M. S. Antony, M. K. Tokito, E. L. F. Holzhaar; Physiology, University of Pennsylvania School of Medicine, Philadelphia, PA

To further our understanding of how the minus-end directed microtubule motor dynein targets vesicular cargo, we conducted a two-hybrid screen to identify dynein-interacting proteins. We isolated a 162aa fragment of Huntingtin (Htt) that interacts with dynein intermediate chain (DIC). Htt is a vesicle-associated protein thought to play a role in vesicular transport. Huntington Associated Protein 1 (HAP-1) has previously been shown to interact with dynactin, suggesting that Htt and dynactin could interact in a complex with HAP-1 and dynein to facilitate dynein-based vesicular transport. We loaded mouse brain cytosol onto an affinity column generated using a recombinant fragment of Htt (536-698) and demonstrated that both dynein and dynactin bind specifically. We also observed that FPLC-purified dynein, which lacks dynactin, bound to the affinity matrix, indicating that dynactin is not required to mediate the interaction with Htt. To identify the Htt binding site of DIC, we analyzed a series of DIC truncation constructs and demonstrated by yeast two-hybrid that 1-283aa of DIC are required for binding to Htt. To map the DIC binding site of Htt, four constructs spanning the full length of Htt were in vitro translated and incubated with beads conjugated to DIC. Htt fragment 1-1483 bound specifically to the DIC beads, thus confirming that the region of Htt encompassed by 600-698aa is both necessary and sufficient for binding to DIC. Htt and DIC are both present on vesicles purified from mouse brain and in vitro vesicle motility assays suggest that Htt acts to mediate binding of vesicles to microtubules. Cellular studies also support a functional role for Htt in vesicular transport. Together, these findings indicate that Htt binds to dynactin independently of dynactin and may act in a complex to recruit dynactin to vesicles and facilitate vesicular transport. Supported by NIH GM48661.
Cytoplasmic Dynein Motility In Vivo: Transport of and Regulation by Growth Factor Receptors

J. Ha,1 M. K. Humm,1 K. R. Myers,1 T. M. Carr,1 K. W. Lo,1 R. A. Segel,1 K. K. Pfister;1 Cell Biology, University of Virginia, Charlottesville, VA, 2Neurobiology, Harvard Medical School, Boston, MA

Cytoplasmic dynein is the motor for retrograde microtubule-based transport in cultured cells, as well as neurons. To directly analyze dynein behavior and regulation in vivo, dynein intermediate chain (IC)-green fluorescent protein (eGFP) fusion proteins were constructed and stable PC12 cell lines expressing low levels of the fusion proteins were made. Biochemical characterization of the fusion proteins demonstrated that GFP-ICs were incorporated into functional dynein complexes. IC-2C is the ubiquitous intermediate chain isoform. In PC12 cell neurites, punctate dynein with GFP-IC-2C co-localized with vesicle markers, including early endosomes. When living cells were imaged, movement of these GFP-dynein puncta in both the anterograde (0.4 +/- 0.05 μm/sec) and retrograde (0.6 +/- 0.05 μm/sec) directions was observed (~10% switch direction). The partial co-localization of dynein puncta with kinesin and secretory vesicle markers, as well as the significant difference between the anterograde and retrograde dynein velocities (p< 0.001), suggest that the anterogradely moving dynein is transported as cargo by kinesin. Internalization and retrograde transport of growth factor receptors and their transporters is essential for axonal survival. Addition of nerve growth factor, which activates the TrkA tyrosine kinase receptor, resulted in a two-fold increase in the relative number of dynein puncta moving in the retrograde direction, and there was partial overlap of IC-2C and IC-2B dynein with the TrkA receptor. Hippocampal neurons were co-transfected with GFP-TRK growth factor receptor and either IC-2C or IC-1A monomeric red fluorescent fusion proteins. When their axons were observed in real time with two color imaging, the co-incidental movement of TRK and dynein was observed. This is the first direct visualization of the transport of a physiological cargo by vebtrate dynein in vivo. Importantly, neuronal TrkB is more likely to be transported by dynein containing a neuron specific intermediate chain, IC-1A, than dynein containing the ubiquitous 2C intermediate chain.

Regulation of Dynein Motor Function by Num1 and PP1/Glc7

J. K. Moore, J. A. Cooper, Cell Biology, Washington University, St Louis, MO

In many eukaryotes the microtubule motor dynein directs the placement of organelles relative to the microtubule cytoskeleton. For the budding yeast Saccharomyces cerevisiae, dynein provides the force to draw the mitotic spindle toward the nascent daughter cell, and across the plane of cytokinesis. This requires that dynein motor activity be tethered to specific sub-domains of the cell cortex in order to translocate the spindle along the axis of division. To determine how dynein motor activity is coordinated with the asymmetry of this cell division, we sought to characterize the role of Num1 in this process. Genetic analyses suggest a role for Num1 in linking dynein to the cell cortex. Here we show that Num1 directs dynein to the tip of the bud late in the cell cycle. Our results suggest that while the cortical localization of Num1 is dependent on a carboxy-terminal PH domain, PIP2-binding does not account for the localization of Num1 to the bud tip. In addition, we provide evidence that the association of dynein with cortical Num1 is regulated by the type 1 protein phosphatase, Glc7.

Kinetochores Coordinate Two Populations of Cytoplasmic Dynein to Control the Spindle Assembly Checkpoint

J. Bader,1 J. Whyte,1 S. Taudaha,1 M. Raycroft,1 J. Hornick,1 E. Hinchcliff1, W. S. Lane,1 G. Chan,1 K. Pfister,1 P. Vaughan,1 K. T. Vaughan1,1 Biological Sciences, University of Notre Dame, Notre Dame, IN, 2Biological Sciences, Harvard University, Cambridge, MA, 3Oncology, University of Alberta, Edmonton, AB, Canada, 4Cell Biology, University of Virginia Health Sciences Center, Charlottesville, VA

Kinetochores play several crucial roles during mitosis, including microtubule capture, chromosome movement and generation of potent anaphase inhibitors. Defects in kinetochore function result in aneuploidy and chromosomal instability. It remains unclear how kinetochores coordinate these different tasks, however cytoplasmic dynein is implicated in each. In this study we identified multiple populations of dynein at kinetochores and defined their functions during mitosis. Proteomic mapping of the cytoplasmic dynein intermediate chains revealed a novel phosphorylation site which regulates binding to the dynactin complex. Despite reduced affinity for dynactin, phosphorylated dynein is recruited to kinetochores during prometaphase and in nocodazole-treated cells. Kinetochores retain phosphorylated dynein until metaphase where dynein undergoes dephosphorylation that is linked to chromosome alignment. Dephosphorylation was blocked using taxol to relieve tension or calicin to A inhibit phosphorylation. To determine the impact of changes in phosphorylation, we identified binding partners for both forms of dynein. Consistent with previous work, dephosphorylated dynein bound to p150<sub>glued</sub>. In contrast, phosphorylated dynein bound directly to the checkpoint protein zw10. To elucidate the specific roles for each dynein population, we inhibited phosphorylation or dephosphorylation dynein to kinetochores selectively and assessed mitotic defects. Kinetochores which could not recruit phosphorylated dynein displayed diminished recruitment of BubR1 and initiated premature anaphase onset. Kinetochores that could recruit phosphorylated dynein but not retain dephosphorylated dynein completed congress but failed to shed BubR1 after chromosome alignment resulting in metaphase arrest. These findings suggest that phosphorylation defines two populations of kinetochore dynein and that each plays an important role in the spindle assembly checkpoint.

Defining Elements of Yeast Cytoplasmic Dynein Required for Motility

A. P. Carter, C. Cho, S. L. Reck-Petersen, R. D. Vale; Cell and Molecular Pharmacology, UCSF, San Francisco, CA

Cytoplasmic dynein is a microtubule motor protein with many important functions in eukaryotic cells. The dynein heavy chain consists of an N-terminal tail domain responsible for cargo binding and dimerization and a C terminal motor core. The motor core is made up of a linker element and a ring of six AAA+ domains and binds microtubules via a ~18nm coiled coil stalk. In order to understand how dynein generates movement it would be helpful to address which elements of the motor core are required for motility. To address this we have used S.cerevisiae as an expression system, which has the advantage that homologous recombination can be used to readily manipulate the genomic copy of cytoplasmic dynein. Starting with a 331KD fragment of yeast dynein (equivalent to the 380KD construct defined by labs working with dictyostelium and rat dynein) we made a series of truncations along the linker region towards the first AAA+ domain in order to determine changes in phosphorylation, we identified binding partners for both forms of dynein. Consistent with previous work, dephosphorylated dynein bound to p150<sub>glued</sub>. In contrast, phosphorylated dynein bound directly to the checkpoint protein zw10. To elucidate the specific roles for each dynein population, we inhibited phosphorylation or dephosphorylation dynein to kinetochores selectively and assessed mitotic defects. Kinetochores which could not recruit phosphorylated dynein displayed diminished recruitment of BubR1 and initiated premature anaphase onset. Kinetochores that could recruit phosphorylated dynein but not retain dephosphorylated dynein completed congress but failed to shed BubR1 after chromosome alignment resulting in metaphase arrest. These findings suggest that phosphorylation defines two populations of kinetochore dynein and that each plays an important role in the spindle assembly checkpoint.

The Role of ATP Hydrolysis at Distinct ATP Binding Sites in Cytoplasmic Dynein

S. L. Reck-Petersen,1 C. Cho,1 D. Applewhite,1 E. Riehaca,2 R. Vale1; Cellular and Molecular Pharmacology, UCSF, San Francisco, CA, 2Cell and Molecular Biology, Northwestern Medical School, Chicago, IL, 3Physics, University of Maryland, College Park, MD

Dynein is a member of the AAA+ superfamily of ATPases with a characteristic ring structure composed of four functional ATP binding modules. Dynein differs from the other cytoskeletal motor proteins, kinesin and myosin, which both contain a single ATP binding site per motor domain. To elucidate the role of dynein’s multiple nucleotide binding sites, we expressed a panel of recombinant dynein dimers in S. cerevisiae predicted to block ATP hydrolysis in each of dynein’s 4 conserved AAA+ domains. Minimal functional dimers were created by fusing the motor domain to glutathione-S-transferase (GST), which forms a stable homodimer. For each mutant, we assayed the motor function by ATPase activity, speed in a multiple motor microtubule gliding assay, and processivity and speed of single dynein dimers in a total internal reflection- based single molecule motility assay. As expected, mutation of AAA1 blocks dynein motor activity. Surprisingly, we find that mutation of AAA4 dramatically enhances dynein processivity, but has no effect on the motor’s single molecule velocity. Our results suggest that the AAA4 mutant has a higher affinity for microtubules, indicating that nucleotide hydrolysis at this site functions to coordinate dynein’s microtubule association with its mechanochemical cycle.

Reversion Analysis of the Cytoplasmic Dynein Motor: A Trip Around the Ring

M. Plamann, D. Madole, R. Schnitker, E. Wulf; School of Biological Sciences, University of Missouri-Kansas City, Kansas City, MO

Cytoplasmic dynein is a large, microtubule-associated motor complex that facilitates minus-end-directed transport of various cargoes. Dynein heavy chain (DHC) is ~4000 residues in length, with the last two-thirds of the heavy chain forming the motor head. Six domains within the dynein motor exhibit varying degrees of homology to the AAA+ superfamily of ATPases. These domains are followed by a distinct C-terminal domain and together form a ring-like structure from which a microtubule-binding domain (MTBD) protrudes. Using a genetic assay, we have isolated over 50 DHC mutants of Neurospora that produce full-length proteins that are defective in function. We have identified DHC point mutations in nearly all domains within the dynein motor. Consistent with previous work, dephosphorylated dynein bound to p150<sub>glued</sub>. In contrast, phosphorylated dynein bound directly to the checkpoint protein zw10. To elucidate the specific roles for each dynein population, we inhibited phosphorylation or dephosphorylation dynein to kinetochores selectively and assessed mitotic defects. Kinetochores which could not recruit phosphorylated dynein displayed diminished recruitment of BubR1 and initiated premature anaphase onset. Kinetochores that could recruit phosphorylated dynein but not retain dephosphorylated dynein completed congress but failed to shed BubR1 after chromosome alignment resulting in metaphase arrest. These findings suggest that phosphorylation defines two populations of kinetochore dynein and that each plays an important role in the spindle assembly checkpoint.

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motor head. We have now isolated revertants for a subset of these DHC mutants and have identified the respective intragenic suppressor mutations in >100 revertants. Interestingly, we have found that two mutations within the MTBD are suppressed by mutations in various domains around the ring. These results suggest that the conformational states of the MTBD and motor head are tightly coupled. Most DHC mutations examined to date revert exclusively by intragenic suppression. However, 30 to 90% of the AAA6 and C-terminal domain DHC revertants result from extragenic suppression. These results suggest that AAA6 and C-terminal domain mutations are readily bypassed by mutations in other genes.

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Characterization of the Dynein Microtubule Binding Domain

D. S. Razansky, D. Madole, R. Schnittker, C. T. Cuppock, M. C. Martinez, M. Plamann, S. J. King; School of Biological Sciences, University of Missouri, Kansas City, MO

Dynein is a minus-end directed microtubule motor protein which functions to carry a variety of cargoes, including nuclei, throughout a cell. Dynein is composed of multiple polypeptides including heavy (DHCl), intermediate, light intermediate and light chains. The DHCl is ~4500 amino acids in length and contains a single ~125 amino acid microtubule binding domain (MTBD). The MTBD is located on the end of a long anti-parallel coiled-coil with the microtubule contact region at the tip. We have characterized the wild-type MTBD using biochemical and biophysical techniques such as gel filtration, co-sedimentation, Raman spectroscopy, analytical ultracentrifugation, and single molecule assays. We identified a number of mutations within the MTBD of Neurospora crassa that cause a ropy hyphal growth phenotype, indicative of a non-functional dynein motor. In addition to the ropy phenotype, we observed altered nuclear migration in all of these MTBD mutants. We also identified intragenic suppressors that rescue these mutant phenotypes to varying degrees. Using these approaches we hope to determine how the MTBD functions as a unit of the DHCl.

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The ATPase-dependent ON/OFF Switching of MT Binding of Cytoplasmic Dynein and Its Coordination with the Tail Swing

R. Ohkura, K. Sutoh, K. Inamura, T. Kon, K. Sutoh; Department of Life Sciences, University of Tokyo, Tokyo, Japan

Cytoplasmic dynein is a minus-end directed microtubule (MT) motor protein. It moves along MT filament with repetitive dissociation/re-association cycle that is tightly coupled with the ATP hydrolysis cycle. Here, we measured the nucleotide-dependent MT affinity of a single-headed cytoplasmic dynein, which clearly demonstrated ON/OFF switching of the MT binding that takes place in the course of ATP hydrolysis. By means of mutational analysis, we determined that AAA1 ATPase site is responsible for the MT binding among multiple ATPase sites of dynein. It has been indicated previously that this ATPase site is also responsible for the tail swing, and thus the ON/OFF switching and the tail swing are regulated by the identical ATPase site, enabling the tight coordination of the two processes as walking along MT filament. Indeed, recovery stroke of dynein is ensured by staggering timing of the ON-to-OFF transition and the tail swing. Furthermore, we provide here direct evidences that stalk head is a solo MT-binding site of dynein that activates dynein ATPase upon MT binding and senses nucleotide state of the ATPase site, suggesting that communication between the ATPase site and the MT-binding site is actually carried out via stalk coiled-coil as has been generally expected.

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Distinct Response of Kinesin and Dynein to Microtubule Cross-Over Versus Illuminates Their Differential Cellular Functions

J. L. Ross, H. Shuman, E. L. F. Holzbaur, Y. E. Goldman; Pennsylvania Muscle Institute, University of Pennsylvania, Philadelphia, PA

Kinesin and cytoplasmic dynein are microtubule-based motor proteins that actively transport material throughout the cell. The microtubules form a polarized network that is primarily radial, but microtubules intersect at a variety of angles both near the nucleus and at the cell periphery. The behavior of kinesin and dynein at these intersections has implications for transport efficiency and accuracy. Intersecting microtubules serve as tracks for direction alteration but also as obstacles to motion. In order to test motor function at microtubule intersections, cross-overs of microtubule tracks were arranged in vitro using flow to orient successive layers of filaments. Kinesin and cytoplasmic dynein-dynactin were fused with green-fluorescent protein and observed one at a time during translocation using total internal reflection fluorescence microscopy. Kinesin is most likely to pass or dissociate at an intersection, while dynein-dynactin has a variant response that includes reversing direction and switching between the intersecting tracks. When many kinesin molecules are attached to artificial head cargos (0.8-1 µm diameter), the transporters were forced to pass or switch at an intersection, independent of motor density on the head. Interestingly, dynein-dynactin cargos are more likely to pass the intersection or switch microtubules at low surface density, but becomes more likely to pause or stop at higher density. This result implies that a simple mechanism of adding motors could change a motile cargo into an anchored one at intersections, consistent with dynein’s proposed tethering functions in the cell. The different actions of dynein and kinesin when encountering an intersection most likely reflect their inherent structural differences, and give new insight into the functions of these motors in the complex cellular environment. Supported by NIH grant AR051174.

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The Interaction of Cytoplasmic Dynein with Bub3 Is Mediated by the DYNLT3 Light Chain: A Link for Kinetochore to Pole Transport of Spindle Checkpoint Proteins

K. W. Lo, J. M. Kogoy, K. K. Pfister; Cell Biology, University of Virginia, Charlottesville, VA

Cytoplasmic dynein is composed of six subunits: heavy chain, intermediate, light chain, protraction, and two additional regulatory proteins, as a binding partner of DYNLT3. The spindle assembly checkpoint delays mitosis until all kinetochores are attached to spindle microtubules. Several proteins including Mad1, Mad2, Bub1, Cdc20, and Bub3 assemble into the checkpoint complex. This complex undergoes cytoplasmic dynein-dependent depletion from kinetochores in response to the formation of kinetochore microtubules (Howell et al., 2001, JCB, 155:59). However, the molecular link between the checkpoint protein complex and dynein was unknown. GST-bub3 pull-down assays probing lysates from tissue culture cells expressing either myc-DYNLT3 or mCherry-DYNLT3 demonstrated the specific binding of Bub3 with DYNLT3. GST-Bub3 binds endogenous dynein from mitotic cell extracts. Bub3 co-immunoprecipitates with the dynein heavy chain from mitotic cell extracts, confirms that Bub3 interacts with the cytoplasmic dynein complex, not just DYNLT3. DYNLT3 co-localized with both the CREST antigen and Bub3 on kinetochores at prometaphase, but not at later mitotic stages. DYNLT3 was also depleted from the kinetochore as mitosis proceeded. Overexpression of DYNLT3-GFP in LLCPK cells disrupted mitosis, causing an increase in the number of cells in a prometaphase-like state. Preliminary results suggest that siRNA knockdown of DYNLT3 also cause a mitotic defect in NRK cells. These data support a model for the binding of checkpoint proteins to the cytoplasmic dynein complex via DYNLT3, in order to transport checkpoint proteins from the kinetochore to the pole. These results also support the hypothesis that subunit isoforms contribute specificity to dynein cargo interaction.

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NudE and NudEL Are Required for Mitotic Progression and Play a Novel Role in Dynein Recruitment to Kinetochores

S. A. Stehman, Y. Chen, R. B. Valle´; Pathology, Columbia University, New York, NY

NudE and NudEL are related proteins that interact with cytoplasmic dynein and LIS1, a dynein regulator responsible for the brain developmental disease, lissencephaly. LIS1 and dynein play known roles in mitotic progression (NCB 2:784, JCB 170:935) and recent work has implicated a similar role for NudE in this process (Neuron 44:279). Little is known, however, about the mitotic functions of NudE and NudEL at the cellular level. We previously observed that NudE and NudEL each localize to mitotic kinetochores prior to dynein and LIS1 and remain at these sites until early anaphase, well after dynein, dynactin, and LIS1 have departed (MBC 15:394a). To determine the significance of this early localization, we injected an anti-NudE/EL antibody into mitotic cells and followed them by phase-contrast microscopy. This antibody specifically blocks the binding of NudE/EL with dynactin but not LIS1 and potentially interferes with mitosis. 83.3% of injected cells were defective in mitotic progression with the most prominent phenotype being metaphase arrest followed by cell death. We saw no defects in congression, unlike with LIS1 inhibition, and no defects in chromosome segregation, in contrast to ZW10 mutant cells. Localization analysis showed that NudE and NudEL, as well as ZW10, remained at the kinetochores of injected cells but, surprisingly, dynein and dynactin were lost. Additionally, many injected cells exhibited misoriented sister chromatids and defective microtubule attachment. Live analysis of cells expressing the kinetochore targeting region of NudE exhibited similar mitotic defects. To determine the mechanism for NudE-mediated kinetochore anchoring, we coexpressed it with dynein subunits and found a striking interaction with dynein intermediate chain. Although NudE and NudEL exhibit very different brain developmental phenotypes, we show a common localization site for both proteins at mitotic kinetochores and identify a novel role for NudE and NudEL in recruiting dynein to kinetochores and regulating mitotic progression.

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Hexon-mediated Dynein Recruitment to Adenovirus Reveals Mechanistic Differences between Normal and Pathogenic Cargo

K. H. Bremer, R. B. Valle´; Pathology, Columbia University, New York, NY

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Cytoplasmic dynein is the predominant minus end-directed microtubule motor in interphase cells and transports a range of subcellular cargoes. It has also been implicated in the translocation of a number of viruses to the nucleus, including adenovirus. The aim of this study was to define the molecular basis of the adenovirus-dynein interaction in order to further our understanding of dynein cargo binding mechanisms in general, and determine the extent to which pathogenic forms of cargo utilise physiological mechanisms. We have previously observed colocalization of both dynein and adenovirus on the surface of ciliates (75%) of incoming adenovirus particles (MBC 8:60a). We have since found colocalization with adenovirus particles of polypeptides N1α, N1β, N2α, N2β, Nuc, but not with N10 or L11. In studies of other processive motors, a mechanism has been proposed in which two identical motor domains alternately swing forward at each step while one motor domain remains attached on intermediate zone upon knockdown of LIC2 alone. Collectively, these results strongly argue that the LICs have both co-related and distinct functions, but are uniquely extraneous in dynein function at the Golgi, suggesting their involvement in a specific subset of dynein functions, thereby allowing for more refined modulation of dynein activity.

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Conformational Changes of Inner-Arm Dynein-f from Chlamydomonas Coupled with Phosphorylation of Its Intermediated Chain IC138

S. H. Sakakibara,1 S. Burgess,2 Y. Sakai,1 K. Oiwa1; 1Kobe Advanced ICT Research Center, National Institute of Information and Communications Technology, Kobe, Japan, 2Institute of Molecular and Cellular Biology, University of Leeds, Kobe, United Kingdom

Inner-arm dynein subcomplexes f (dynein-f or the 1 complex) of Chlamydomonas is a double-headed dynein composed of two different heavy chains, three intermediate chains and a number of light chains. It has been shown that an axonemal casein kinase 1 (CK1) phosphorylates the 138K intermediate chain (IC138) and that this phosphorylation is involved in regulation of flagellar activity in phototaxis. To elucidate the mechanism of regulation of IC138 phosphorylation, we compared molecular conformation of dynein-f molecules with and without IC138-phosphorylation using negative-staining electron microscopy and single-particle image processing. When images of dynein-f were aligned with respect to the tail domain and averaged the two heads of IC138-phosphorylated dynein-f appeared more smeared than those without IC138-phosphorylation. Although no systematic conformational changes coupled to IC138-phosphorylation were observed, the differences found in averaged images suggest that IC138-phosphorylation increased flexibility of the molecule. We have confirmed these observations using dynein-f molecules purified from the mutant strain 212, a photo-taxis mutant that has high level of the phosphorylated form of IC138. The change in flexibility of the molecule could modify the interactions between the two heads and/or between dynein-f and the adjacent B-tubule in the axoneme.

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Motility of the 1α and 1β Heavy Chains of the Inner Arm Dynein 11 of Chlamydomonas Flagella

S. Tosa,1 H. Sakakibara,1 M. E. Porter,2 W. S. Saku,2 K. Oiwa,1 Kove Advanced ICT Research Center, National Institute of Information and Communications Technology, Kobe, Japan, 1Institute of Molecular and Cellular Biology, University of Leeds, Kobe, United Kingdom

Inner arm dynein 11 of Chlamydomonas flagella, also known as dynein 1, is a protein complex composed of two distinct heavy chains, three intermediate chains and a number of light chains. We performed conventional in vitro motility assays showing that dynein 11 translocated microtubules at the comparatively low velocity of about 1.5 μm/sec. The velocity increased slightly as surface density of dynein 11 increased. From this dependence, we estimated the duty ratio of dynein 11 to be 0.6-0.7. Moving microtubules observed at low dynein densities rotated erratically about a vertical axis through a fixed point on the surface, at which a single dynein 11 complex is presumably located, while still progressing. Thus, we concluded that dynein 11 is a processive motor. In studies of other processive motors, a mechanism has been proposed in which two identical motor domains alternately swing forward at each step while one motor domain remains attached on the axonemal dynein. In the dynein 11, however, its heterogeneous motor domains do not need to behave the same to perform the role of each motor domain in processive movement, we took advantage of mutants lacking the 1α or 1β motor domain (Myster et al., 1999, Perrone et al., 2000). Negative-staining electron microscopy and area-gel electrophoresis confirmed that the mutant molecule was intact except lacking one head domain. The 1β and the 1α translocate microtubules smoothly on glass surface at 0.8 μm/sec and 1.0 μm/sec at 0.5 mM MgATP, respectively. Thus, we concluded that the motor domain of the 1α and the 1β are each sufficient for microtubule translocation. The velocities of these mutants are, however, significantly lower than those of intact dynein 11. A certain coordination mechanism may exist between each motor domain of an intact dynein 11 complex, such that one head assists power stroke of the other.

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Image-based Quantitative Scattering Analysis Reveals Differential Effects of Drugs on Adhesion and Motility

D. Loerke,1 J. De Rooij,1 A. Kerstens,2 C. Waterman-Storer,3 G. Danuser2; 1The Scripps Research Institute, La Jolla, CA, 2Department of Cell Biology, Netherlands Cancer Institute, Amsterdam, The Netherlands, 3College of Engineering, The University of Texas at El Paso, El Paso, TX

In response to HGF growth factor stimulation, cultured MDCK cells lose cell-cell junctions and acquire a migratory phenotype, causing cell clusters to scatter in space. This process is a commonly used in vitro model system for cancer metastasis, and the antimitastatic potential of a drug can be tested by evaluating its efficiency in inhibiting MDCK cell scattering. The time course of MDCK cell scattering can be evaluated over a range of hours with phase-contrast imaging, which has the advantage that it is non-invasive and avoids the phototoxic effects or bleaching associated with fluorescence probes. However, phase-contrast image analysis is non-trivial, and quantitative analysis of scattering (as well as the evaluation of potential scattering inhibitors)
has previously been limited to relatively small sample sizes, or to those select parameters that lend themselves to automated analysis, notably cell centroid velocity. We developed a software package that enhances velocity information by automated motion correlation and spatial clustering analysis. It performs time-resolved quantitative analysis of the spatial dynamics of the scattering process on large sample sizes, allowing us to perform a screen of selected inhibitors of signaling intermediates downstream of HGF. Focusing on MAPKinase signaling inhibitors, we find that different drugs differentially affect motility-related and adhesion-related aspects of the scattering process. This highlights the importance of analyzing these contributions separately, particularly since using an automated velocity-only-based approach may incorrectly predict a high inhibitory potential for drugs with low efficiency in preventing the disruptions of cell-cell adhesions. Thus, our live-cell microscopy-based assay permits the differential evaluation of the contributions of migration regulation and cell-cell adhesion disruption in HGF-treated MDCK cells, and can be used as a useful tool for screening new potential pharmacological agents against tumor metastasis.

214 Role of Slit2 in Neural Crest Cell Migration
M. E. de Bellard, 1 M. Brommer-Fraser, 1 L. Correa, 1 A. Ochou; 1Biology, CSUN, Northridge, CA; 2Biology, Caltech, Pasadena, CA

The neural crest are cells that emerge by delamination from the dorsal neural tube shortly after neural tube closure and give rise to the wide variety of neuronal, glial and non-neuronal derivatives that make a large number of head structures and the peripheral nervous system. These cells go from a non-motile, epithelial-adherent cell type, to a highly motile one that allows them to migrate rapidly throughout the embryo and reach distant areas where they differentiate. Little is known about the underlying mechanism directing these processes. The following experiments tried to further examine the role of Slits and their Robo receptors may have in selective neural crest delamination and migration and the dual function of Slit in both inhibiting and stimulating motility of neural crest cells. In order to determine the function of Slit expression by non-migrating neural crest cells, I constitutively expressed Slit2 in or Slit2 in pre-migratory neural crest by electroporating plasmids in chicken embryos. I found that ectopic expression of both Slit molecules in trunk neural tube caused defects in their epithelial to mesenchymal transition, as shown by the reduced numbers of neural crest cells in the sympathetic ganglia by the aorta and the reduced migration in cultured neural tubes after Slit electroporation. However, control transfectied cells were observed to migrate in larger numbers and farther away than those from Slit electroporated neural tubes. In order to determine what was happening to the cells after Slit electroporation, neural crest cells were visualized under confocal microscopy live imaging. Movies showed that control GFP electroporated cells were migrating normally, while Slit expressing cells were behaving differently, moving less smoothly, were slower and were rounding-up and extending at intermittent times. Thus Slit molecules may play a role in keeping neural crest cells from delaminating from the neural tube ahead of time.

215 Enhanced Ordering of Interacting Filaments by Molecular Motors
P. Kralikvski, 1 R. Lipowsky, 1 J. Kierfeld; 1Theory, Max Planck Institute of Colloids and Interfaces, Potsdam, Germany; 2University of Connecticut Health Center, Farmington, CT

We theoretically study the cooperative behavior of cytoskeletal filaments in motility assays in which immobilized motor proteins bind the filaments to substrate surfaces and actively pull them along these surfaces. Because of the repulsive interaction of filaments, the motor-driven dynamics of filaments leads to a nonequilibrium phase transition which generalizes the isotropic-nematic phase transition of the corresponding equilibrium system, the hard-rod Liquid. Long-time dynamics simulations and analytical theory show that the motor activity enhances the tendency of filaments to arrange in a nematic order, which is responsible for the formation of focal adhesions. We develop a theory for the location of the phase boundary as a function of motor density. At high detachment forces of motors, we observe the formation of filament clusters because of blocking effects; at low detachment forces, cluster formation can be controlled by the density of inactive motors.

216 Kinking, Rather Than Corkscrewing, Generates Thrust in Spiroplasma Motility
J. Y. Lee, J. W. Shaevertz, D. A. Fletcher; UC Berkeley, Berkeley, CA

Prokaryotes use a variety of mechanisms to maneuver through their environments. One family of bacteria, called Spiroplasma, is capable of swimming in the absence of flagella by changing their cell shape. In a previous study, we showed that Spiroplasma can swim by rotating to unwind their helical bodies, resulting in a progressive change in helicity that forms kinks in the cell axis. Because Spiroplasma lack cell walls, an internal cytoskeleton must be responsible for the dynamic cell shape they use for motility. However, the hydrodynamic details of this propulsive mechanism remained unclear since motility involves both rotation of the helical cell body and kinking of the cell axis. Rotation of a helix may generate thrust by causing a cell to “corkscrew” through liquid medium at a velocity related to helical pitch. Processive kinking of the cell axis from front to back, on the other hand, propels the cell forward at a velocity weakly dependent upon pitch. To determine the relative importance of pitch, kink angle and kink propagation speed, we measured cell kinematics of four morphologically varied species of Spiroplasma during free-swimming. Our results indicate that cell velocity varies between species and that this cell velocity correlates with kink propagation speed. However, changes in cell velocity are not correlated with morphological parameters such as helical pitch and kink angle. Because cell velocity appears to be independent of morphological changes, thrust is most likely dominated by the kinking of the cell axis rather than the rotation of the helical body.

217 Modifying Cellular Microenvironments in Real Time with 3D Lithographic Microfabrication
B. Kaehr, R. Nelson, J. Shear; Chemistry and Biochemistry, UT Austin, Austin, TX

The ability to control physical and chemical properties of cellular microenvironments is important in addressing a range of compelling biological questions. Various lithographic techniques, for example, have been developed for patterning adherent cells through modification of surface properties, providing a level of control over the spatial relationships between adherent cells. However, current approaches cannot readily confine and direct active, motile or developing cells. To overcome these limitations, we have developed microfabrication strategies to confine cells in well-defined three dimensional microstructures under conditions that remain suitable for monitoring and perturbing cell growth and development. Moreover, our techniques typically allow microfabrication in the presence of living cells. Using two-photon photochemistry, we are able to fabricate high resolution matrices (with feature dimensions of less than 1 µm) composed of crosslinked proteins that commonly retain catalytic and physical functionalities. Using this approach, we are thus able to define local topographic and chemical microenvironments of cultured cells in real time. Here, we demonstrate an ability to corral and redirect neurite outgrowth in situ - an important step towards engineering inter-cellular networks, and show that microstructures can be fabricated on tissue scales fast enough to confine actively motile cells such as E. coli and fish keratocytes. These results demonstrate new capabilities for directing, trapping and incubating individual cells of interest - an advance that will enable key questions regarding cell motility and development to be addressed.

218 Enhanced EGFP-CALI Using Deficient Cells Rescued with Functional EGFP-fusion Proteins: Application to Capturing Protein and Mena
E. A. Vitriol, 1 A. Uetrecht, 1 F. Shen, 2 K. Jacobson, 1 J. E. Bear; 1Department of Cell and Developmental Biology, University of North Carolina at Chapel Hill, Chapel Hill, NC; 2Department of Pharmacology, University of North Carolina at Chapel Hill, Chapel Hill, NC

Chromophore Assisted Laser Inactivation (CALI) is a loss of function approach that offers precise spatiotemporal control of protein inactivation. EGFP has been used effectively for CALI and of Pharmacology, University of North Carolina at Chapel Hill, Chapel Hill, NC of the RhoA free for normal regulation by GDI (Sequence in chain = RhoA-CFP-Citrine-RBD, where RBD is Rhotekin binding domain that binds only to activated RhoA). We have recently developed a single chain FRET biosensor of RhoA activation, used to examine the spatio-temporal dynamics of RhoA activity during cell morphological changes and growth factor stimulation. Unlike other single chain biosensor designs, the two GFP mutants undergoing FRET (CFP and Venus) were placed on the internal portion of the chain, leaving the C-term of RhoA free for normal regulation by GDI (Sequence in chain = RhoA-CFP-Citrine-RBD, where RBD is Rhotekin binding domain that binds only to activated RhoA). We have
extended this approach by substituting other target proteins and corresponding binding domains to produce biosensors for RhoC and for Rhog. FRET response was optimized through use of circularly permuted GFP mutants and optimization of linker lengths. The spectral characteristics and biological applications of these two new biosensors will be presented. Extension of this design to study Rho, Rac and Cdc42 interaction with GDI will also be discussed.

220 Monitoring Global Protein Conformational Changes within Cells under Mechanical Force
C. Johnson, D. Discher, A. Engler, D. Speicher; Chemical and Biomolecular Engineering, University of Pennsylvania, Philadelphia, PA, Wistar Institute, Philadelphia, PA
Changes in protein structure in response to mechanical forces are believed to mediate a variety of cellular responses, and may take the form of either rearrangement in the fold of the protein, or dissociation of protein-protein complexes. Recent studies have attempted to identify force sensitive proteins and the induced conformational states associated with mechanotransduction. However, there is currently no facile methodology to scan for mechanical force induced conformational changes on a global level within the cell, and no direct in vivo evidence exists. To address this shortcoming, we have developed an in-cell technique that allows for the probing of force induced conformational changes in a high throughput fashion. As an initial test of our method, erythrocyte ghosts were monitored for signs of shear induced spectrin unfolding. Subsequently, the methodology has also been applied to nucleate cells in order to identify mechanosensitive proteins involved in cardiomyocyte contraction and stem cell differentiation.

222 Direct Observation of Microtubule Dynamics in Live Brain Tissue
J. Tsai, R. B. Vallee; Pathology and Cell Biology, Columbia University, New York, NY
Recent studies from our laboratory have used in vitro electrophoresis of neural progenitor cells to determine the cellular roles of the lissencephaly protein LIS1. We monitored the behavior of GFP-tagged neural progenitors in 400μm thick brain slices under control and LIS1 RNAi conditions, and identified multiple roles of LIS1 during neurogenesis (JCB, 176:935). We are now using a variety of subcellular markers to understand neural progenitor subcellular behavior in vivo (Tsai and Vallee, MBC abst., in press). The behavior of cytoskeletal networks and their associated motor proteins in these cells have been largely beyond the reach of conventional microscopic methods. Here we describe methods we have developed to image microtubule dynamics in neural progenitors migrating through live brain tissue. Using in vitro electrophoresis, we have labeled neural progenitors with a GFP-tagged version of the microtubule end-binding protein EB3 and RFP-centrosome. To solve the problem of scattering in thick slices and from conventional culture methods, we embedded the brain slices in Matrigel directly on a coverglass to enable direct fluorescent imaging with high N.A. objectives. EB3 "surfing" movements were readily observed throughout the cell. In bipolar cells undergoing glia-guided migration, microtubules exhibited a relatively constant rate of surfing revealed microtubules to emanate from an astrom from and move in association with the highly motile centrosome during migration. Microtubules in the leading process were arranged unidirectionally and in parallel, whereas the trailing microtubules were clamped and formed a cage-like network around the nucleus, suggesting different forces acting on the microtubule network in the two cases. We are currently investigating the dynamics of the microtubule network at different steps during neural progenitor migration and its changes in cells subjected to LIS1 and dynein RNAi treatments. Supported by HD40182.

222 Intrinsic Dynamic Behavior of Fascin in Filopodia
Y. S. Aratyn, D. Vignjevic, O. Dancu, J. Pelsyin, S. Koijima, E. Taylor, G. Borisy; Cell and Molecular Biology, Northwestern University, Chicago, IL, Equipe de Morphogenese et Signalisation cellulaires, CNRS/Institut Curie, Paris, France, Cell and Molecular Biology, University of Wisconsin, Madison, WI
Fascin is required for the formation of tightly cross-linked actin bundles in filopodia. We performed fluorescence microscopy techniques to investigate the incorporation and dynamics of fascin cross-links in order to model the disassembly of filopodia formation. First, fluorescence photobleaching revealed no fluorescence recovery of GFP-fascin in B16/F1 mouse melanoma cells along with rapid fluorescence recovery of GFP-fascin, with t1/2 = 9 ± 6 seconds (n = 41) and 96 ± 37% fluorescence recovery, indicating that fascin undergoes cycles of dissociation and association in filopodia. Photobleaching experiments were repeated in S2A mouse neuroblastoma cells and revealed rapid exchange of GFP-fascin in filopodia, t1/2 = 7 ± 3 seconds (n = 18), confirming that dynamic cross-links are a general phenomenon in filopodia. Second, in vitro fluorescence photobleaching experiments of Alexa488-fascin in reconstituted filopodia-like bundles yielded rapid fluorescence recovery, with t1/2 = 19 ± 18 seconds (n = 18) and 96 ± 25% fluorescence recovery, suggesting that fascin exchange is an intrinsic property of the fascin molecule. Lastly, photobleaching recovery data indicate the existence of mobile and immobile fractions of cross-links prompting us to investigate the proportion of bound fascin molecules in filopodia. On average, we found that filopodia contain one fascin cross-link per 20 actin monomers and that about 90% of those cross-links are bound. These results indicate that fascin exchange in filopodia is primarily governed by mobile, highly dynamic cross-links.

222 Reconstitution In Vitro of MSP-based Filodipodium Extension in Nematode Ascaris suum Sperm
L. Miao, K. Yi, J. M. Mackey, N. Rowley, T. M. Roberts; Department of Biological Science, Florida State University, Tallahassee, FL
The MSP motility system in nematode sperm is best known for propelling the movement of mature sperm, where it has taken over the role usually played by actin in amoeboid cell motility. However, MSP filaments also drive the extension of filopodia, which are transient organelles, each with a core bundle of MSP filaments, that form in the late in sperm development. We have reconstituted this process in vitro whereby thin bundles of MSP filaments, then enveloped by a membrane sheath at the growing end, elongate at rates up to 18 μm/min. These slender protrusions often exceed 500 μm in length but are comprised of filaments that are only 1 μm long. The reconstituted filopodia assemble in the same sperm extract that produce MSP fibers, columnar meshesworks that assemble behind a membrane vesicle and reconstitute the dynamics of the lamellipodial filaments system that propels sperm movement. Indeed, filopodia and fibers both have a membranous structure at their growing end, share five MSP accessory proteins, and respond identically to agents that modulate protein phosphorylation. However, filopodia are much thinner than fibers and elongate 3-4 fold faster. The reconstitution of filopodial extension shows that, like actin, MSP filaments can adopt two architectures, bundles and meshesworks, each capable of pushing membranes, In the bundle condition the filaments exert force against a concave membrane surface whereas MSP filament meshesworks push against a flat membrane sheet. Supported by NIH Grant R37 GM29994

224 Time-lapse Videography of Photomovement in Filamentous Blue-Green Alga Oscillatoria sp
S. J. Lee, G. H. Kim; Biology, Konji National University, Konji, Republic of Korea
Filament motility in Oscillatoria was recorded more than a century ago, but the basic underlying mechanisms are still obscure and pose a major biological research problem. The effects of monochromatic light and cytoskeletal inhibitors on the photomovement of Oscillatoria sp. were studied using time-lapse videography and image analysis program (Image pro 5.1). Under blue light (peak at 470 nm), the filaments showed faster gliding movement than blue light. The glding movement was much faster under UV light, showing negative photokinetics. The pattern of photomovement was similar under two types of UV light (254 and 365 nm). Moreover, filaments took determined orientation and quickly moved away from the original position. A reversible inhibition of the movement was observed when the cyanobacteria were treated with myosin inhibitor, IDM (50~100 μM), or Ca²⁺ inhibitor, EDTA (10 μM), indicating that the movement was based on acto-myosin system. The filament motility in Oscillatoria was observed for the first time with FITC-phalloidin. Actin has never been reported as present in prokaryote cells, but some similar kind of proteins are present in prokaryotes instead of actins. This is first report of acto-myosin system working in filamentous blue green algae.

225 Seeing More by Seeing Less: New Actin Cytoskeletal Dynamics Revealed by Photoactivatable Thresholding
C. G. Galbraith, A. Galbraith; NIDCR, NIH, Bethesda, MD, NINDS, NIH, Bethesda, MD
During cell migration, the cytoskeleton undergoes rapid movement, assembly, and disassembly. Visualizing and analyzing cytoskeletal dynamics has always been challenging - dense labeling of the cytoskeleton obscures the visualization of movement, while sparse labeling requires extensive computational analysis and information about the protein trajectory within the cytoskeletal network. To investigate actin dynamics at the leading edge of migrating cells, we exploited the properties of photoactivatable GFP (PaGFP) to adjust the amount of protein that is visible in an individual cell. By regulating the intensity of the activating light, we can titrate the level of photoactivation to continuously adjust the "threshold" of visible cytoskeleton during cell migration.

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226 Collectible Contractile Dynamics in Confluent Epithelial Cells Are Highly Coherent
T. E. Angelini; Department of Engineering and Applied Science, Harvard University, Cambridge, MA

We have studied the collective contractile dynamics of confluent Cos-7 epithelial cells during migration in several contexts. We patterned cells in single file lines on confluent PDMS rubber bands, and quantified substrate deformation by tracking embedded fluorescent particles over the course of approximately 10 hours. Deformations confined to one dimension, well over ten microns in magnitude, correlated over distances exceeding the millimeter scale were observed. On unpatterned PDMS, collective substrate deformations in two dimensions were over ten times smaller, and exhibited acoustic-like spectral properties. Three dimensional matrix deformation was studied by embedding cells at high density in 1mg/ml collagen. Since collective network deformations are difficult to quantify in the microscope, a dynamic small angle light scattering technique was adapted. With this technique, we have spectrally characterized the three dimensional mechanical network deformations during cell migration, and observed collective behavior similar to the measurements on compliant surfaces.

227 Cytoplasmic Pressure Differential in Migrating Adherent Cells
T. Iwashaki, Y. Wang; University of Massachusetts Medical School, Worcester, MA

Amoeboid movement is known to involve a pressure differential along the cell length, with contractions in the posterior region driving cytoplasmic streaming toward the front. While this process is believed to play a critical role in amoeboid cells, a parallel transport process has yet to be demonstrated in cultured adherent cells during migration. To probe the distribution of intracellular pressure, we microinjected high molecular weight, linear polyacrylamide (PAA) as a passive force gauge into migrating NIH3T3 fibroblasts. Injected PAA appeared as amorphous aggregates that underwent shape changes and directional movements in response to differential forces exerted by the surrounding environment. PAA injected into the posterior region immediately moved toward the front, while PAA in the anterior region remained stationary relative to the cell center. This forward movement was observed only in moving cells. In addition, disruption of myosin II activity by blebbistatin inhibited the forward translocation of PAA, while cell migration persisted in a disorganized fashion. These results suggest that myosin II in the posterior region generates a gradient of cytoplasmic pressure, which may be responsible for the transport of some essential cytoplasmic components to the anterior region. This myosin II-dependent cytoplasmic flow may in turn play an important role in maintaining the directional stability of cell movement.

228 Actin Meshwork Pore Size in the Lamellipodium Varies with Local Actin Barbed End Density
K. Keren,1 P. T. Yam,1 G. Marriott,1 J. A. Theriot1; 1Dept. of Biochemistry, Stanford University, Stanford, CA, 2Dept. of Physiology, University of Wisconsin, Madison, WI

The actin meshwork in the lamellipodium of moving cells has a central role in propelling the cell forward and is also the major determinant of the cytoarchitecture in that region. While the basic biochemical mechanisms involved in its assembly are largely known, the large-scale organization of this dynamic structure and the relationship between the structure and its function as the motility apparatus are still not understood. Using various probes, we have characterized the actin meshwork structure and its influence on the distribution of soluble cytosolic components in the lamellipodium of epithelial keratocytes. By examining both stationary and moving cells we were able to study and compare the lamellipodium in two different cell states using the same cell type. We measured the size dependent distribution and diffusion of inert soluble components (different sized dextrans, PEG-coated quantum dots) in the lamellipodium of live cells and correlated that with actin barbed end distribution in live cells labeled with TRITC-kabramide1c and with F-actin distribution in fixed cells labeled with phalloidin. In stationary cells we found a dramatic enhancement in the concentration of large soluble probes compared to smaller ones at the lamellipodial periphery, suggesting that the effective pore size was larger there. This peripheral enhancement correlated with more organized and slightly lower density of F-actin and a higher concentration of barbed ends We also observed an enhancement of large soluble probes at the leading edge of some moving cells. This correlated with a higher concentration of barbed ends but not with changes in the overall F-actin distribution, again demonstrating the influence of local actin meshwork structure on the distribution of inert soluble components. Our results reveal a substantial degree of heterogeneity within the lamellipodium and indicate that in both stationary and moving keratocytes there are at least two distinct structures in the lamellipodium.

229 Ena/VASP Anticapping Activity Modulates Cell Morphology and Migration
C. I. Lacayo,1 Z. Finchus,1 M. van Duin2,1 C. Wilson,1 D. Fletcher,1 F. Gertler,1 A. Mogilner,1 J. A. Theriot1; 1Biochemistry, Stanford University, Stanford, CA, 2Bioengineering, University of California at Berkeley, Berkeley, CA, 3Biological, Massachusetts Institute of Technology, Cambridge, MA, 4Mathematics and Center for Genetics and Development, University of California at Davis, Davis, CA

Variations in cell migration and morphology are consequences of changes in underlying cytoskeletal organization and dynamics. Since Ena/VASP proteins play critical roles in cell migration and actin dynamics, we examined their role in the migration and morphology of fish epithelial keratocytes, which are among the fastest locomoting eukaryotic cells. Keratocytes have broad, flat lamellipodia and generally migrate persistently in a gliding fashion while exhibiting a characteristic smooth-edged “canoe” shape, but they may also exhibit less regular morphologies with rougher edges and less persistent movement. We found that VASP strongly localizes as a thin line at the “smooth” but not “rough” leading edges in lamellipodia. Ena/VASP mislocalization and overexpression altered the proportion of “smooth” and “rough” keratocytes within a population as well as trajectory curvature. When different keracteyte morphological and behavioral features were examined quantitatively, we found that they correlated with each other and described a spectrum of coordinated phenotypes. One extreme of this spectrum contained canoe-shaped, smooth-edged, fast, straight moving keratocytes with high VASP and peaked F-actin distributions along the leading edge. The opposite extreme encompassed more circular-shaped, rough-edged, slow, turning keratocytes with low VASP at the leading edge and flat F-actin distributions. We termed these extremes of the spectrum “coherent” and “decohere,” respectively, and found that individual cells could be converted from “coherent” to “decohere” behavior by increasing actin barbed end capping activity. After mechanically displacing VASP from the leading edge by stalling protrusion, we observed that the leading edge of nascent lamellipodia accumulated VASP in irregular protruding microregions that subsequently matured into “smooth” morphology. We have developed a mathematical model that fully accounts for the observed spectrum of coordinated cell morphologies and movement behaviors as a function of leading edge by stalling protrusion, we observed that the leading edge of nascent lamellipodia accumulated VASP in irregular protruding microregions that subsequently matured into a smooth-edged, slow, turning keratocytes with low VASP at the leading edge and flat F-actin distributions. We termed these extremes of the spectrum “coherent” and “decoherent,” respectively, and found that individual cells could be converted from “coherent” to “decoherent” behavior by increasing actin barbed end capping activity. After mechanically displacing VASP from the leading edge by stalling protrusion, we observed that the leading edge of nascent lamellipodia accumulated VASP in irregular protruding microregions that subsequently matured into “smooth” morphology. We have developed a mathematical model that fully accounts for the observed spectrum of coordinated cell morphologies and movement behaviors as a function of leading edge.

230 A Computational Model for the Cell Adhesion Clutch: Theoretical Prediction and Direct Observation of Substrate Oscillations
C. E. Chan, D. J. Odde; Biomedical Engineering, University of Minnesota, Minneapolis, MN

F-actin retrograde flow and localized traction forces are prominent features of the exploratory, leading edge structures of many cells undergoing substrate-dependent motility. The motor-clutch hypothesis of cell motility explains these features by assuming that F-actin networks are pulled rearward by myosin motors, molecular clutches engage the network and transmit traction forces to the substrate to drive the cell forward. To investigate the relationship between retrograde clutch flow and traction forces, we constructed a computational model for the motor-clutch system by assuming: 1) a force-dependent motor sliding velocity, and 2) spring-like molecular clutches that fail in a force-dependent manner. We found that the model successfully replicates the F-actin retrograde flow velocities that we observed experimentally in filopodia of GFP-actin-transfected embryonic chick forebrain neurons (ECNFs) growing on polyethyleneimine-coated glass substrates. Although the vast majority of cell migration studies have been conducted on such stiff glass substrates, cells crawl over substrates of far lower stiffness in vivo. Interestingly, under these in vivo conditions, the model predicts that the system develops traction forces that are periodically lost as clutches abruptly lose engagement. This causes the underlying substrate to repeatedly load and relax, a behavior we refer to as substrate oscillation. To test whether oscillations occur on soft substrates, we plated ECNFs on soft polyacrylamide gel substrates embedded with fluorescent beads and found that beads near filopodia oscillated (period of ~10-100 seconds) as predicted by the computational model. The model also predicts that the retrograde flow rate on soft substrates will be reduced, compared to stiff substrates, which was confirmed experimentally. Further computational studies revealed that maximal force transmission, minimal...
Coordinating Actin Meshwork Flow during Persistent Cell Movement and Turning
C. A. Wilson,1 P. T. Yam,2 L. J.3 G. Danuser,4 J. A. Theriot1; 1Biochemistry, Stanford University, Stanford, CA, 2Cell Biology, The Scripps Research Institute, La Jolla, CA

The persistent movement of crawling cells depends on the proper spatial coordination of actin polymerization, actin depolymerization and myosin contraction, with actin polymerization biased toward the cell’s leading edge and myosin contraction strongest at the rear. We characterize the coordination of F-actin flow dynamics across regions of cells undergoing different movement behaviors such as transient and sustained turns, and in the presence of drugs such as the myosin-II inhibitor blebbistatin. Notably, ciliated keratocytes moving at steady state do not lose polarity or stop moving when treated with blebbistatin alone. However, when jasplakinolide, which slows actin depolymerization, is combined with blebbistatin, cell motion and actin movement cease. This arrest cannot be achieved in these cells by treatment with jasplakinolide alone, but is a synergistic effect of the two drugs. This suggests that myosin activity contributes to rear retraction and forward movement not by directly pulling the cell rear forward but by promoting actin meshwork disassembly in the rear. Myosin activity is also implicated in directional persistence, which is altered in the presence of blebbistatin. We propose that imbalances in myosin-driven contractility and/or meshwork disassembly at the cell rear may contribute to changes in cell orientation and movement direction, and may be involved in coupling orientation with direction such that the cell maintains its shape even as it turns. Overall these results demonstrate that events at the rear of the cell are critical to the overall coordination of cytoskeletal organization and dynamics in cell motility, contributing to persistent forward motion and choice of direction.

The Initiation of Cell Polarization Involves Asymmetry of Actin Retractosome Flow, Which Depends on Myosin Activity, and Is Stabilized by Microtubules
S. Bohet1, S. Schub,2 J. Meister,1 A. B. Verkhovsky1; 1Lab of Cell Biophysics, Swiss Federal Institute of Technology, Lausanne, Switzerland, 2UMR 144, Institut Curie, Paris, France

Cell polarization and directional locomotion are believed to involve asymmetry in actin assembly, contractility, and substrate adhesion. We investigated how this asymmetric behavior first develops during random polarization of fish epidermal keratocytes, which were rendered isotropic and then induced to polarize by first depleting and subsequently restoring bivalent cations in culture media. We analyzed the distribution and dynamics of actin and myosin II using fluorescence speckling, conventional fluorescence microscopy and computer tracking, and studied the dynamics of substrate adhesions with interference reflection microscopy. In the isotropic state, the cells exhibited lamellipodia with the uniform rate of actin retractosome flow all around the cell perimeter. Upon initiation of directional movement, retractosome flow accelerated at the prospective rear of the cell, but the lamellipodia initially persisted all around the perimeter. The assembly of actin network also continued all around the cell. Surprisingly, the rate of network extension (calculated as the sum of the rate of advance of the cell edge and the rate of actin retractosome flow) was even higher in the rear lamellipodia than in the front lamellipodia. In contrast to the smooth lamellipodia at the cell front, the lamellipodia at the rear developed filopodia-like ridges, which transformed into the retraction fibers when the rear lamellipodia eventually retracted. Interference reflection microscopy indicated local areas of detachment from the substrate at the base of rear lamellipodia. Treatment with myosin inhibitor blebbistatin blocked the polarization nearly completely, while depolymerization of microtubules with nocodazole resulted in attenuated polarization, which was usually preceded by a stage when protrusion/retraction wave traveled around the cell periphery without net cell movement. The results suggest that initial polarization results from asymmetry in actin retractosome flow, which is induced by myosin-dependent contraction and stabilized by microtubules. Supported by Swiss National Science Foundation.

Modeling Yeast Cell Polarization Induced by Pheromone Gradients
T. Yi, Developmental and Cell Biology, UCI, Irvine, CA

Yeast cells respond to spatial gradients of mating pheromones by polarizing and projecting up the gradient toward the source. It is thought that they employ a spatial sensing mechanism in which the cell compares the concentration of pheromone at different points on the cell surface and determines the maximum point, where the projection forms. Here we constructed a spatial mathematical model of the yeast pheromone response that describes the dynamics of the heterotrimeric and Cdc2p G-protein cycles, which are linked in a cascade. Two key performance objectives of this system are (1) amplification -- converting a shallow external gradient of ligand to a steep internal gradient of protein components and (2) tracking -- following changes in gradient direction. We used simulations to investigate amplification mechanisms that allow tracking. We identified specific strategies for regulating the spatial dynamics of the protein components (i.e. their changing location in the cell) that would enable the cell to achieve both objectives. We have experimentally examined some of the modeling predictions.

Polarization of Yeast Cells in Microfluidically-Generated Spatial Alpha-Factor Gradients
T. Yi1, T. I. Moore,1 N. Jeon2; 1Developmental and Cell Biology, UCI, Irvine, CA, 2Biomedical Engineering, UCI, Irvine, CA

Yeast cells were exposed to spatial gradients of alpha-factor generated in microfluidics chambers. Segall was the first to report this type of response in yeast using a micropipette to administer the alpha-factor. Microfluidics offer the advantage of greater control over the properties of the gradient. We reproduced many of Segall’s findings, verifying that wild-type cells have the capability of detecting shallow gradients over a broad range. We compared the response of cells containing mutations in the pheromone response pathway versus wild-type cells. In addition, we found that yeast will employ alternative strategies to improve the accuracy of the gradient-sensing. Finally, we imaged specific proteins tagged with GFP to determine if localization of mating response components are similar to yeast cells exposed to uniform alpha-factor.

The Cell Polarity Protein Par-1 Regulates Cell Migration and Adhesion in Drosophila
J. A. McDonald,1 C. Brooks1, D. J. Montell1; 1Dept. of Molecular Genetics, Cleveland Clinic Foundation, Cleveland, OH, 2Dept. of Biological Chemistry, Johns Hopkins University School of Medicine, Baltimore, MD

The serine/threonine kinase Par-1 (also known as MARK/EMK/KIN1) plays a well-conserved role in regulating the polarity of many cell types in species as diverse as C. elegans, Drosophila, Xenopus, mice, and humans. In addition, Par-1 homologs are known to regulate microtubule stability and Wnt signaling. Here, we present the finding that Drosophila Par-1 regulates the migration of border cells, a group of cells that undergoes a process analogous to tumor invasion and metastasis. In a genetic screen, we identified three alleles of par-1 that disrupted border cell migration. Further analysis revealed that about half of border cells mutant for par-1 failed to detach properly from the epithelium. In addition, clonal analysis indicated that par-1 functions in both border cells and adjacent epithelial cells. While Par-1 protein localized to the basolateral membrane of all epithelial and border cells, a phosphorylated form of Par-1 was specifically enriched in migrating border cells. This phosphorylation site is important for Par-1 function, because expression in the ovary of the non-phosphorylatable mutant form of Par-1 resulted in both border cells and adjacent epithelial cells. While Par-1 protein localized to the basolateral membrane of all epithelial and border cells, a phosphorylated form of Par-1 was specifically enriched in migrating border cells. This phosphorylation site is important for Par-1 function, because expression in the ovary of the non-phosphorylatable mutant form of Par-1 resulted in both border cells and adjacent epithelial cells. This arrest cannot be achieved in these cells by treatment with jasplakinolide alone, but is a synergistic effect of the two drugs. This suggests that myosin activity contributes to rear retraction and forward movement not by directly pulling the cell rear forward but by promoting actin meshwork disassembly in the rear. Myosin activity is also implicated in directional persistence, which is altered in the presence of blebbistatin. We propose that imbalances in myosin-driven contractility and/or meshwork disassembly at the cell rear may contribute to changes in cell orientation and movement direction, and may be involved in coupling orientation with direction such that the cell maintains its shape even as it turns. Overall these results demonstrate that events at the rear of the cell are critical to the overall coordination of cytoskeletal organization and dynamics in cell motility, contributing to persistent forward motion and choice of direction.
polarized. Our previous data showed that Rho/ROCK inhibited cultures migrate farther and faster as observed with Wnt inhibition data, but outgrowth cells are not polarized. Currently we are investigating the source and type of Wnt responsible for F9 parietal endoderm migration and plan to perturb other modulators of the PCP pathway.

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S-adenosylhomocysteine Hydrolyase Concentration with F-actin at the Front of Chemotaxing Dictyostelium and Neutrophils Suggests a Role for Transmethylation during Chemotaxis S. Shu, X. Liu, D. Mahadeo, C. A. Parent, W. Liu, E. D. Korn; Laboratory of Cell Biology, NHLBI, NIH, Bethesda, MD, Laboratory of Cellular and Molecular Biology, NCI, NIH, Bethesda, MD

Bacterial chemotaxis requires regulated methylation of chemoreceptors. However, despite considerable effort in the 1980s, transmethylation has never been established as a component of eukaryotic cell chemotaxis. S-adenosylhomocysteine (SAH), the product formed when the methyl group is transferred from the universal methyl donor, S-adenosylmethionine, to an acceptor molecule, is a potent inhibitor of all transmethylation reactions. In eukaryotic cells, this inhibition is relieved by hydrolysis of SAH to adenosine and homocysteine, a reaction catalyzed by S-adenosylhomocysteine hydrolase (SAHH). Therefore, SAHH is an important component of the methylation machinery. Recent studies showed that SAHH is sequestered into actin rods in Dictyostelium spores but diffusely distributed in the cytoplasm of vegetative amoebae. We have confirmed, by immunofluorescence microscopy and live cell imaging of GFP-fused SAHH, the diffuse distribution of SAHH in the cytoplasm of non-motile cells, but find that SAHH concentrates with F-actin at the front of motile, chemotaxing Dictyostelium amoebae and human neutrophils. Interestingly, SAHH concentrates in pseudopods but not in filopodia or at the very leading edge, in contrast to coronin, an F-actin binding protein that localizes with F-actin in all these regions. Tuberculid, an inhibitor of SAHH, substantially inhibits chemotaxis of Dictyostelium and neutrophils at concentrations that have little effect on cell viability. This inhibition is specific as tuberculid does not inhibit phagocytosis or capping of concanavalin A receptors in Dictyostelium, nor does SAHH localize with F-actin in either of these activities. Tuberculid treatment does not affect the starvation-induced expression of the cAMP receptor, cAR1, nor does it affect the ability of cAMP to activate adenyl cyclase activity and polymerize actin in Dictyostelium. These results add SAHH to the list of proteins that redistrubute in response to chemotactic signals, and strongly suggest a role for transmethylation in chemotaxis of eukaryotic cells, although the methyl acceptor(s) has yet to be identified.

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Embryonic morphogenesis requires the coordinated polarization of cells and tissues. We are using the morphogenic movements of the C. elegans embryonic epidermis as a model system to understand the initiation of polarity in migrating tissues. These movements include cell shape changes and migrations of the epidermal tissue as it encloses the football-shaped C. elegans embryo. We have identified mutations that affect these initial polarization of the cells that allows directed tissue movement, but they do not interfere with the cell identity of the tissue. This allows us to test a fundamental question in developmental biology: how are tissues that have assumed the correct cell fate polarized to move during morphogenesis? Some of the genes we have identified encode homologs of the WAVE/SCAR complex that regulates actin dynamics in multicellular organisms from plants to humans. Others genes we have identified are not yet molecularly identified but likely represent newly discovered regulators of the complex. Our phenotypic studies suggest all of the WAVE/SCAR components contribute to complex function. In vivo imaging suggests that epithelial cells can form protrusions but they fail to migrate, suggesting a more specific roles for these regulators of the Arp2/3 complex in actin regulation during morphogenesis. Our biochemical studies suggest that all components of the WAVE/SCAR complex contribute to complex stability while some complex components appear to be modified but only when they are in the complex. These studies suggest that some of the regulation of the WAVE/SCAR complex components may be due to in vivo modifications that regulate the activity of individual components of the WAVE/SCAR complex. Supported by grants from the American Heart Association (00415424T).

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The Regulation and Function of Lamellipodin in Directed Cell Migration M. Michael, M. Krause; Randall Division of Cell and Molecular Biophysics, King's College London, United Kingdom

Lamellipodin (Lpd), a member of the MRL (MIG-10, RIPM, Lamellipodin) family of proteins is localised at the leading edge of migrating cells. It contains a Ras GTPase and a phospholipid binding domain that is specific for PI(3,4)P2. Furthermore Lpd interacts with Efa/VASP proteins, important regulators of the actin cytoskeleton. There is good evidence that both Ras GTPases and 3' phosphorylated inositol lipid products are required for the establishment of cell polarity downstream of chemotactic receptors. Therefore, Lpd may link membrane signals from chemotactoic receptors to direct effectors of the actin cytoskeleton. The aim of this study is to investigate the role of Lpd in directed cell migration and the mechanisms by which upstream signals regulate its function. We are using biochemistry and functional studies to unravel the mechanism by which Lpd is regulated either through its interaction with Ras GTPases or by phosphorylation. Lpd has been implicated in the regulation of lamellipodial protrusion, an important step in cell migration. We generated Lpd overexpression and knockdown Rat2 fibroblasts cell lines to test the role of Lpd in directed cell migration using video microscopy imaging techniques in the scratch/wound healing assay. Our results suggest that Lpd plays an important role in directional cell migration.

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A Phosphorylation-independent Mechanism of Cofilin Activation Involving PIP; during EGF-stimulated Carcinoma Cell Motility J. van Rheezen, Anatomy & Structural Biology, Albert Einstein College of Medicine, Bronx, NY

Lamellipodial protrusion and directional migration of carcinoma cells towards chemoattractants depend upon the spatial and temporal control of actin dynamics. Cofilin, an actin severing protein, has been implicated to be important for the initial steps of actin remodeling in response to epidermal growth factor (EGF). However, the spatial and temporal control of cofilin activity during chemotaxis is not fully understood. In contrast to neutrophils and other motile cells, cofilin is initially activated following EGF stimulation of carcinoma cells by a mechanism that does not involve serine 3 dephosphorylation (Song et al. (2006) J. Cell. Sci. 119:2871). Alternatively, in vitro studies have suggested that the plasma membrane phospholipid PIP2; inhibits cofilin’s severing activity, however, this regulatory mechanism has not been demonstrated to be important in cells. Using several chemical and biophysical techniques, we have identified a membrane-bound fraction of cofilin in carcinoma cells. Upon EGF-stimulation, PIP2; is rapidly hydrolysed, resulting in the release of the membrane-bound fraction of cofilin, and subsequent increase in cofilin severing activity. In conclusion, we propose that the initial activation of cofilin after EGF involves the release of a pool of membrane-bound cofilin through PLC-dependent PIP2; hydrolysis thereby determining the direction of cell motility in metastatic cancer cells.

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Cytosplastic Polyadenylation Element Binding Protein Regulates Beta Catenin Synthesis and Localization to the Leading Edge of Migrating Astrocytes K. J. Jones, E. Korb, D. G. Wells; MCDL, Yale University, New Haven, CT

Directed cell migration involves the coordination of several cellular processes including cell polarization, the rearrangement of cytoskeletal elements, and the clustering of signaling components to the leading edge. One such protein that localizes to the leading edge of a migrating cell is beta catenin (Etienn-Manneville and Hall 2003). Since beta catenin has many diverse functions in the cell, we investigated whether this increase in beta catenin protein could be the result of local mRNA translation at the leading edge. Using in situ hybridization, we show that beta catenin mRNA is localized to the leading edge of migrating astrocytes, where it co-localizes with the RNA binding protein cytoplasmic polyadenylation element binding protein (CPEB). Further, we demonstrate that the increase in beta catenin protein at the leading edge is a result of new mRNA translation. CPEB binds to the 3'-untranslated region of specific mRNAs, and regulates translation by controlling polyadenylation. Therefore, we examined the poly(A)-tail length of beta catenin mRNA and show an increase in polyadenylation precedes beta catenin protein localization. Consistent with this, a polyadenylation inhibitor, cordycepin, blocks beta catenin protein localization to the leading edge. Finally, expression of a dominant negative CPEB mutant protein also inhibits localization of beta catenin protein to the leading edge. These results suggest a role for CPEB mediated local protein synthesis in astrocyte migration.

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Investigating the Role of the Arp2/3 Complex during C. elegans Gastrulation M. Rob, B. Goldstein; University of North Carolina, Chapel Hill, NC

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Dictyostelium discoidium signaling during chemotaxis.
Cellular actin architecture is remodeled based on signalling events and upstream actin regulators in diverse cell types. One such regulator is the Arp2/3 complex. This complex acts to nucleate new actin filaments off existing actin filaments and, thus, affects overall actin organization. In C. elegans, actin organization is regulated by the expression of two endosomal precursor cells, Ea and Ep. The apical region of the E cells construct, thereby pulling in the neighboring cells such that they cover the E cells as the E cells ingress into the interior (Lee and Goldstein, 2003). In addition, myosin is also activated in the apical region of the E cells, as indicated by an accumulation of phosphorylated myosin light chain (Lee et al., submitted). Depleting C. elegans Arp-related proteins, ARX-2 and ARX-3, result in gas- trulation defects. In these embryos, gastrulation is not initiated and the E cells divide on the surface of the embryo (Severson et al., 2002). Currently, the effects of Arp2/3 components on the actin cytoskeleton and/or polarity of the cells, particularly Ea and Ep, are unknown. However, our preliminary evidence suggests that active myosin still accumulates in the apical region of the E cells, but the E cells still fail to ingress (D. Marston, pers. comm.). Using a combination of live and fixed microscopy, we are determining whether the polarity is altered in Ea and Ep. We are also determining which cells require Arp2/3 function through blastomere isolation techniques, and we are analyzing the effects of depleting Arp2/3 components on actin architecture.

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FRAP Experiments Reveal Differences in Binding Rates of PTEN between Front and Back of Polarized Dictyostelium Cells

L. Yang, 1 C. Janetopoulos, 1 P. N. Devreotes, 2 P. A. Iglesias; 1Electrical Engineering, Johns Hopkins University, Baltimore, MD, 2Biological Sciences, Vanderbilt University, Nashville, TN, 3Cell Biology, Johns Hopkins Medical Institutions, Baltimore, MD

Dictyostelium cells are able to sense shallow chemotactic gradients and respond with highly polarized changes in cell morphology and motility during chemotaxis. The complementary regulation of PKB/Akt and PTEN binding sites through parallel Local Excitation and Global Inhibition (LEGI) mechanism is able to account for the spatially amplified PTEN(3,4,5)P3 response observed in Latrunculin-treated Dictyostelium cells. However, models based on this complementary regulation do not exhibit the same degree of polarized response measured in moving cells. We hypothesized that differences between polarized and unpolarized cells in the binding rates of these enzymes could account for the observed differences in the amplification. To test this hypothesis we measured the binding rates of PTEN using Fluorescence Recovery After Photobleaching (FRAP). In vegetative and unpolarized cells we found two distinct populations differentiated by the binding rates: approximately 75% of PTEN had binding off rates with a time constant of ~6 seconds; the remaining fraction of PTEN had sub-second binding off rates. In polarized cells, we again observed two populations. However, the relative fraction between fast and slow binding off rates differed significantly between the front and rear of the cell. These results suggest a model in which PTEN(3,4,5)P3, found in greater abundance at the anterior part of the cell, regulates the binding rate of PTEN, making PTEN less abundant at the front. Mathematical models reveal that this positive feedback scheme can increase the degree of PT(3,4,5)P3 amplification by up to 50%. It is reasonable to suppose that other feedback pathways also exist and would act to improve the amplification factor.

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Quantitative Analysis of Chemotactic Responses in C. Elegans

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

Chemotaxis is an important cellular response that mediates cell movements during development and the immune response and is a critical factor in many forms of cancer and disease. Experiments in the social amoeba, D. discoideum and neutrophils have shown that local accumulations of PI(3,4,5)P3 mediate the ability of cells to migrate directionally. During directional sensing, it is a discrete step in the signaling pathway, downstream of G protein activation but upstream of the accumulation of PI(4,5)P2, that controls the initial asymmetry. We have previously shown that the localization is dependent on the relative chemotactant gradient, and the final distribution of PI(3,4,5)P3, is amplified, even without the presence of an actin cytoskeleton. Various stimuli paradigms on latrunculin treated cells can lead to bi-modal responses, which can be extinguished and redistributed by shifting the concentration gradients. The enzymes PI3K and PTEN regulate local levels of PI(3,4,5)P3 and move to the anterior and posterior of the cell, respectively. This PI(3,4,5)P3 polarization circuit is essential for proper migration and disrupting the regulation of these enzymes has the ability to completely change cell morphology and reduce motility. We have previously characterized these PI(3,4,5)P3 responses by quantitatively measuring the dynamic distribution of GFP-tagged signaling markers in a rapid perfusion chamber and by the use of multi-stimulus inputs. Our results suggest that a local excitation, global inhibition mechanism is at work during gradient sensing.

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Physical Obstacles Redirect PIP3, Distribution and Migration of Chemotaxing Cells

M. M. vanDuijn, 1 J. A. Theriot, 1 D. A. Fletcher; 1Dept of Bioengineering, UC Berkeley, Berkeley, CA, 2Dept of Biochemistry, Stanford University, Stanford, CA

Migrating cells navigate through challenging environments in order to reach their destination. Chemical gradients can provide cues to the cell for directional control, but cells in vivo are often prohibited from taking a direct path to the source of the chemotactant. This can require navigating around physical structures the cell encounters. Not much is known about the way structural features affect the migration of chemotaxing cells, nor about how the signaling pathways that specify polarity adapt to changes in the physical environment. Here, we investigated HL-60 cells chemotaxing on a micropipette with a chemotactant on a substrate decorated with a maze of microfabricated obstacles. When the obstacles prevented movement along a direct path towards the source, we found that the cells could circumnavigate the obstacles by tracing their edge, which required migrating at an angle to the gradient. In freely chemotaxing cells, phosphorylatedinositol-3,4,5-trisphosphate (PIP3) is enriched at the leading edge of the cell, facing towards the gradient. Using a fluorescent probe, we investigated the distribution of PIP3 in cells tracing the edge of an obstacle. The formation of PIP3 by phosphatidylinositol-3-kinase, is directly downstream of the chemotactant receptor. Interestingly, we found that PIP3 was enriched at the leading edge of these cells, rather than remaining oriented towards the chemical gradient. This observation shows that the contact with a physical structure can affect the signal that generates PIP3, and specifies the direction of migration. We propose that these effects are due to the actin-dependent positive feedback loops that enhance PIP3 polarization. As actin is prevented from polymerizing by the presence of a barrier, the feedback loop is disrupted. Positive feedback can then reinforce polymerization at sites that do allow productive protrusion. At chemotactic edges, a combination of physical cues create a direct path towards the source when possible, and navigation around obstacles when necessary.

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Dynamics of PI3K- and PKC-dependent Motility Processes during Cell Spreading

M. Weiger, J. Haugh; North Carolina State University, Raleigh, NC

Cell spreading and migration involve surface attachment and cytoskeletal reorganization, leading to membrane extension and ultimately cell polarization, processes that are stimulated and coordinated through intracellular signal transduction pathways. In fibroblasts and many other cells, activation of the phosphoinositol-3-kinase (PI3K) pathway is required for motility. PI3Ks generate several lipid products, which act as downstream second messengers, and the spatial pattern of 3' PI density in the membrane is thought to control the directionality of membrane protrusion and cell migration. Using total internal reflection fluorescence microscopy in conjunction with a fluorescent 3' PI probe, we have observed spontaneous activation of PI3K during spreading of mouse fibroblasts that is independent of integrin-mediated adhesion (fibronectin vs. poly-lysine) and is frequently localized to areas of active, membrane protrusion. Inhibition of PI3K blocks active cell spreading, whereas inhibition of actin polymerization halts spreading but not PI3K signaling. Inhibition of phosphatidylcholine polymerization gives rise to a dynamic motility phenotype that is also sensitive to PI3K inhibition. Inhibiting protein kinase C (PKC), isoforms of which are known mediators of PI3K signaling, prevented cell spreading and, surprisingly, led to a significant decrease in PI3K activity that could not be rescued by stimulation with platelet-derived growth factor. The kinetics of these effects suggests roles of PKC both upstream and downstream of PI3K. Taken together, we conclude that PI3K and PKC are spontaneously and dynamically activated during cell spreading, in a manner that is independent from known receptor-mediated stimulation or feedback from F-actin/microtubules, and PI3K/PKC signaling is required for actin-based protrusion.

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PKA Regulates Phospholipid Dynamics during Growth Factor-induced Membrane Ruffling

P. B. Deming, S. L. Campbell, L. C. Baldor, A. K. Howe; Pharmacology, University of Vermont, Burlington, VT

Previously, our laboratory has shown that the activity of the CAM-dependent protein kinase (PKA) is required for chemotactic cell migration. PKA plays an important role in growth factor-induced cytoskeletal re-organization in part by sustaining the activity of Rac at the leading edge. Consistent with these findings, live cell imaging revealed that inhibition of PKA activity significantly altered the dynamics of PDGF-induced peripheral membrane ruffles. Given the established role for phospholipids in membrane and cytoskeletal dynamics during ruffling, the connection between PKA and phospholipid metabolism in growth factor-stimulated cells was investigated. Imaging of live cells expressing the PH domain of PLC-gamma suggested that inhibition of PKA activity decreased the dynamics of phosphatidylinositol-4,5 bisphosphate (PIP2) in PDGF-induced membrane ruffles. Immunofluorescence of fixed cells using a PIP2
antibody inhibition that showed that PKA attenuated PDGF-induced accumulation of PI(2) while arrest activation of PKA by treatment with forskolin enhanced basal PI(2) immunoreactivity in the absence of growth factor. Moreover, PI(2)K, an enzyme that regulates PI(2) synthesis, was markedly enriched and phosphorylated by PKA in pseudopodia purified from chemotactic cells. Assessment of phosphatidylinositol 3, 4, 5 triphosphate (PI(3)P) via immunofluorescence demonstrated that PKA can also modulate PI(3)P levels. Pretreatment of cells with forskolin dramatically increased PI(3)P localization to membrane ruffles following PDGF stimulation, while inhibition of PKA reduced the levels of PDGF-induced PI(3)P. Together, the data support a model in which PKA can govern phospholipid dynamics at the leading edge which may, in turn, regulate growth factor-induced membrane ruffling during cell migration.

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Light-induced Repression of Direction Change during Diatom Movement
S. A. Cohn, D. Hans, K. Marabar, J. E. Shurbo; Biology, DePaul University, Chicago, IL
Previous work has indicated that exposure to high irradiance light at the leading tips of moving diatoms can induce a reversal of cell direction (Diat. Res. 19:167). Initial characterization has shown that several different diatom species display this behavior, each with species-specific sensitivities to the wavelength of the irradiating light. Our current work has shown that generation of a light-induced direction change represses additional direction changes in the cell for about one minute and that high irradiance exposure to the trailing end of moving diatoms can similarly repress subsequent light-stimulated direction changes for about one minute. In addition to tracking the cell migration, the fluorescence intensity of nuclear H2B-GFP provided a DNA analog for tracking ploidy through the cell division cycle. We extend the algorithms to hESCs to track their migration and establish cell lineages during differentiation. In contrast to the 3T H2B-GFP model, differentiating stem cell colonies are usually composed of all cell layers and cell types. In particular, we have observed and immunohistochemically verified differentiation of hESCs into endothelial blood vessel-like structures as well as neurons. To investigate formation of these biological units, we acquired three-dimensional time-lapse (3-D) movies of H2B-GFP-transfected differentiating hESCs and generated "migration maps". We also incorporated deconvolution methods to assist in removing out-of-focus light per z-plane, which aids in subsequent image processing routines in Matlab for the generation of 3-D tracks and cell lineages.

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Migrational Behavior of Differentiating Human Embryonic Stem Cells in Culture
J. Russo, N. Pirozhina, A. Pekurovsky, J. Price; Barnham Institute for Medical Research, La Jolla, CA
Differentiation of human embryonic stem cells (hESC) in culture most likely follows a subset of developmental codes that the inner cell mass executes during embryogenesis. It is known that during normal development, the cells composing the inner cell mass undergo coordinated migration and differentiation program. While studying embryodiff culture is fairly difficult, analysis of hESCs behavior in culture provides a simplified model system to investigate the relationship between migration and differentiation. We previously (Shen et al, 2006) developed automated tracking algorithms and demonstrated them on multi-field two-dimensional time lapse movies of 3T3 cells expressing fluorescent marker histone 2B-GFP (H2B-GFP). In addition to automatically tracking the cell migration, the fluorescence intensity of nuclear H2B-GFP was used as an analog for tracking ploidy through the cell division cycle. Recent evidence indicates that chemotaxis requires spatial regulation of signals that drive morphological polarization characterized by the formation of a leading pseudopodium and a rear cell body compartment. Howev
by the breakdown of the paternal and maternal pronuclei. This data suggests that Aurora-A acts as, or is directly responsible for, a soluble signal at centrosomes that promotes timely NEBD.

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Centrosome Attachment to the Nucleus Requires the SUN-1 Mediated Localization of ZYG-12 to the Outer Membrane of the Nuclear Envelope

I. Minn, C. J. Malone; Biochemistry and Molecular Biology, The Pennsylvania State University, University Park, PA

Although the localization of proteins specifically to the outer membrane of the nuclear envelope is thought to be required for attachment of the centrosome to the nucleus, the mechanism of this localization is unknown. Work from our lab and others has recently identified a novel class of proteins with KASH domains that specifically localize to the outer membrane of the nuclear envelope via an interaction with SUN proteins. We have shown that two isoforms of the C. elegans protein ZYG-12, which contain KASH and transmembrane domains, localize to the outer membrane of the nuclear envelope in a SUN-1 (also called MTF-1) dependent manner. ZYG-12 is required for attachment of the centrosome to the nucleus in C. elegans. Both epistasis accessibility and fluorescent protease protection assays using in vivo nuclei in combination with protease protection assays using microsomes revealed that ZYG-12 and SUN-1 are type II membrane proteins of the outer and inner membrane of the nuclear envelope, respectively. In support of ZYG-12 being a type II membrane protein, ZYG-12 physically interacts with the cytoplasmic protein dynein via the N-terminus of ZYG-12 and is required for the localization of dynein to the nucleus. SUN-1 directly interacts with KASH domain of ZYG-12. The interaction occurs between the KASH domain and the internal domain of SUN-1 as mapped by a membrane based two-hybrid assay and a pull-down assay. The functional importance of this interaction was demonstrated in vivo by driving the localization of ZYG-12 to the ER of cells expressing chimeras of SUN-1 and the ER resident protein RAMP4. ZYG-12 at the outer nuclear membrane is necessary for centrosome attachment. Our results suggest that the inner nuclear membrane protein SUN-1 confines localization of ZYG-12 to the outer nuclear membrane via direct binding in the lumen of the nuclear envelope.

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DdCenB, an Unusual Centrin with Predominant Nuclear Localization

S. Mana-Capelli, D. A. Larochelle; Department of Biology, Clark University, Worcester, MA

Centrins are a subclass of calcium-modulated proteins with a typical array of 4 helix-loop-helix domains (EF-hands). These proteins typically localize to the centrosome and may function in centrosome duplication, among other things. In Dicystostelium discoideum, a divergent member of this family had previously been described (DdCcrp). More recently, a second centrin-related protein has been identified through the Dicystostelium genome sequencing project and has been named DdCenB (DdCrp has been renamed to DdCenA). In order to study DdCenB we have cloned and expressed the coding sequence for this protein behind the red fluorescent protein tag (RFP). Indirect immunofluorescence using anti-DdCP224 and anti-tubulin antibodies was used to visualize centrosomes. DAPI staining was carried out to visualize nuclei. Interestingly, RFP-DdCrp shows no centrosomal localization. Rather, it is found predominantly in the nucleus in interphase cells. Furthermore, this nuclear localization disappears as the cells enter mitosis (recall that Dicystostelium cells undergo a closed mitosis in which the nuclear envelope does not breakdown). DdCenA, on the other hand, does localize to the centrosome, although it also displays a cell cycle dependent re-localization in which it is lost from the centrosome during mitosis.

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Identification and Isolation of Maternal Procentrosomes from Oocytes of Spisula solidissima

J. Guo, D. E. Crane, D. Hull, X. Wu, T. Ohita, R. Kuriyama, R. E. Palazzo; 1 2 Biology and Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY, 1 Department of Molecular Biosciences, University of Kansas, Lawrence, KS, 2 Dept. of Genetics, Cell Biology, and Development, University of Minnesota, Minneapolis, MN, 3 Wadsworth Center New York State Dept. of Health, Albany, NY, 4 Marine Biological Laboratory, Woods Hole, MA

We have identified procentrosomes in oocytes of the surf clam Spisula solidissima. Oocytes are arrested at the G2/M border of meiosis I and can be activated to complete meiosis I and II by fertilization or treatment with KCl. No centrosomes have previously been identified in unactivated oocytes and de novo centrosome assembly and maturation were thought to be induced by oocyte activation. We isolated centrosomes from KCl activated oocytes (activated centrosomes) to generate anti-centrosome antibodies. Some of these recognize a 300-350 kDa centrosome protein (Spicen). Immunofluorescence with Spicen antibodies revealed the presence of two procentrosomes in oocytes. Comparison of isolated procentrosomes to activated centrosomes revealed a) procentrosomes are smaller and of lower density as determined by migration in sucrose density gradients, 2) unlike activated centrosomes, procentrosomes are unable to nucleate microtubules and 3) procentrosomes may not contain centrioles while activated centrosomes clearly do. Importantly, treatment of procentrosomes with oocyte lysate derived from activated eggs induces microtubule nucleation, while treatment with lysate from unactivated eggs has no affect. We conclude that Spisula solidissima oocytes contain maternal centrosome precursors, which we term procentrosomes, that are induced to undergo maturation upon oocyte activation. Centrosome maturation includes a) recruitment and assembly of centrosome components to increase centrosome mass, b) induction of microtubule nucleation potential, and c) the assembly and duplication of centrioles.

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Function and Regulation of γ-Tubulin and Its Associated Proteins

B. Raynaud-Messina, C. Vérollet, N. Colombi, T. Daunon, M. Wright, A. Merdes; CRPS, UMR2587 CNRS-Pierre Fabre, Toulouse, France

γ-tubulin is critical for initiation and regulation of microtubule assembly at the centrosome. In Drosophila, it acts within two main cytosolic complexes: the γ-TuSC (γ-Tubulin Small Complex, ~300kDa) and the γ-TuRC (γ-Tubulin Ring Complex, ~3000kDa). In metazoans, it is assumed that nucleation of microtubules at the centrosome depends on the fully assembled γ-TuRC. However, the mechanisms of γ-tubulin recruitment and regulation have not been fully investigated. To approach the functional specificities of γ-TuRC components and the roles of the cytosolic complexes, we took advantage of the efficiency of RNAi in cultured cells and the availability of mutant strains. γ-TuSC proteins are essential for viability and the assembly of a functional bipolar spindle. While these proteins do not appear redundant, their levels are closely co-regulated, indicative of the importance of the integrity of this complex. In contrast in cells lacking γ-TuRC, γ-tubulin is still recruited to the poles but mitotic processes are partly disrupted, leading to a transient mitotic accumulation and a poor density of microtubules. Therefore, we suggest that, in Drosophila, organization and partial functionality of the mitotic spindle could be achieved even in the absence of fully assembled γ-TuRC. In parallel to the role of γ-TuRC in microtubule assembly, γ-TuSC could be recruited to the poles and perform at least partially microtubule nucleation activity.

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HCα66, a Novel Centrosomal Protein Regulating γ-Tubulin Stability

X. Fant, A. Merdes; Wellcome Trust Centre for Cell Biology, University of Edinburgh, Edinburgh, United Kingdom

The centrosome is the main microtubule organising centre in metazoan cells. It contains a pair of centrioles surrounded by pericentriolar material (PCM), a fibrous mass containing proteins involved in microtubule nucleation. Several proteins of the PCM exhibit a dynamic association with the centrosome in a cell cycle dependent manner. For example, gamma-tubulin has been shown to accumulate at the centrosome before mitosis, resulting in an increase of microtubule nucleation during spindle formation. To investigate changes in the composition of the PCM during the cell cycle, centrosomes were isolated at different stages, during G1 and early S phase. Pericentriolar material was solubilised by salt-stripping of centrosomes, and analysed by gel electrophoresis followed by MALDI-TOF mass spectrometry. We identified HCα66 as a novel protein that localises temporarily to the centrosome from S to M-phase, and that also resides in the nucleus. The sequences responsible for centrosomal and nuclear localization were mapped using GFP-fusion constructs. Overexpression of the centrosome-targeting region leads to an increase in centrosome mass, b) induction of microtubule nucleation potential, and c) the assembly and duplication of centrioles.

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NEDD1 Targets γ-Tubulin to the Centrosome in Mammalian Cells

L. Haren, M. Remy, I. Bazin, M. Wright, A. Merdes; UMR2587, CNRS, Toulouse, France
260 A Kinesin-9 Family Member Ki66 Localizes to Centrosomes and Is Required for γ-tubulin-mediated Microtubule Nucleation K. Wang,1 C. E. Walczak;1 2Department of Biology, Indiana University, Bloomington, IN, 2Medical Sciences, Indiana University, Bloomington, IN
Ki66 is a Kinesin-9 family member that has been identified in various vertebrate organisms, yet the function of Ki66 is not understood. We identified and characterized both human and Xenopus laevis Ki66. Ki66 has its conserved kinesin motor domain at the N-terminus and a non-conserved stalk-tail (ST) domain at the C-terminus, which contains two predicted coiled-coil domains. Immunofluorescence staining showed that Ki66 localized on the mother centrosome during G1 through early G2 but was found on both centrosomes at late G2/phase. As the cells entered mitosis Ki66 staining decreased at centrosomes/poles, but accumulated at midzone/midbodies during late anaphase/early telophase. Overexpression of human Ki66 constructs in U2OS and HeLa cells showed that GFP-Ki66ST localized to centrosomes better than full-length GFP-Ki66, indicating that the centrosome-targeting domain of Ki66 is contained within the ST domain. Interestingly, Ki66 overexpression caused a decrease in gamma tubulin on centrosomes, and inhibited microtubule reorganization by interphase centrosomes after cold treatment. These results suggest that Ki66 is necessary for gamma-tubulin mediated centrosomal microtubule nucleation. The overexpression of Ki66ST caused a similar albeit stronger defect, supporting the idea that the ST domain of Ki66 is required for targeting and thus the function of Ki66. Consistent with our findings in U2OS and HeLa cells, depletion of Ki66 from meiotic Xenopus egg extracts inhibited the formation of asters induced by addition of purified centrosomes. Together our data support a model in which Ki66 helps tether gamma tubulin to centrosomes via its stalk-tail domain. This activity is then critical for proper microtubule nucleation both during interphase and in mitosis.

261 γ-Tubulin Is Essential for Noncentrosomal Microtubule Nucleation P. Binarova,1 J. Pochankova,1 J. Vole,1 V. Cenklka,1 G. Pihal;1 E. Bogre;1 2Institute of Microbiology ASCR, Prague, Czech Republic, 3Institute of Experimental Botany ASCR, Oломouc, Czech Republic, 4School of Biological Sciences, Royal Holloway, University of London, Egham, United Kingdom
It has recently emerged that microtubules (MTs) nucleated independent of defined microtubule organizing centres such as centrosomes or spindle pole bodies play an important role in designing cytoskeleton architecture. γ-Tubulin is required for MT nucleation at defined microtubule organizing centres but its role in nucleation of noncentrosomal MTs is much less understood. In higher plants, where all somatic organs are flanked by a centrosomal, there are several microtubule arrays and girders, which are organized during cell cycle progression from undefined dispersed sites. Well characterized γ-tubulin ring complexes that are essential for centrosomal MT nucleation in animal cells have not yet been identified in plants. Rather we found the presence of heterogeneous protein complexes of γ-tubulin in cytoplasm, in association with membranes and MTs. Large γ-tubulin complexes (>1MD) were active in microtubule nucleation, but nucleation activity was not observed for the smaller complexes. To further analyze the role of γ-tubulin, we conditionally downregulated γ-tubulin by inducible expression of RNAi constructs in Arabidopsis thaliana. After induction of RNAi γ-tubulin was gradually depleted from all known cellular locations including the microtubular and the microtubular fraction. We found that γ-tubulin acts as a component of cortical nucleation templates guides cortical MTs. The regrowth of MTs from perinuclear membrane rich region after drug depolymerization was delayed in cells with reduced γ-tubulin levels. Similarly, immunodepletion of γ-tubulin from A. thaliana extracts strongly compromised the in vitro polymerization of MTs. Almost complete RNAi depletion of γ-tubulin led to the absence of microtubules. In summary, we showed that γ-tubulin is essential for MTs nucleation from dispersed sites in acentrosomal plant cells. Further characterization of γ-tubulin forms and their protein interactions is under progress. Supported by grants PC L54-MSMT CR and A3020302 from GA CAS.

262 Characterization of NIP2/Centrinob, a Novel Substrate of Nek2, and Its Potential Role for Microtubule Stabilization Y. Jeong, J. Lee, S. Kim, K. Rhee; School of Biological Sciences, Seoul National University, Seoul, Republic of Korea
Nek2 is a mitotic kinase whose activity oscillates during the cell cycle. It is well known that Nek2 is involved in centrosome splitting. A number of experimental evidence indicated that Nek2 is also critical for maintaining the integrity of noncentrosomal microtubule structure and the microtubule nucleation activity. In the present study, we report that NIP2, previously identified as Centrinob, is a novel substrate of Nek2. NIP2 was daughter centrosome-specific, but was also found in association with stable microtubule network of cytoplasm. Ectopic NIP2 formed aggregates but was resolved by Nek2 into small pieces and eventually associated with microtubule. Knockdown of NIP2 showed significant reduction of microtubule organizing activity, cell shrinkage, defects in spindle assembly and abnormal nuclear morphology. Based on the results, we propose that NIP2 has a role in stabilizing the microtubule structure. Phosphorylation may be critical for mobilization of the protofibril to a fresh microtubule and stabilizing it.

263 Identification and Analysis of γ-Tubulin Complex Proteins of Aspergillus nidulans Y. Xiong, T. Nayak, E. Szewczyk, H. Edgerton, B. R. Oakley; Molecular Genetics, Ohio State University, Columbus, OH
Although γ-tubulin has a well-documented role in microtubule nucleation in both animal and plant cells, its role in mitotic and cell cycle regulation, the functions of proteins that form complexes with γ-tubulin (γ-tubulin complex proteins or GCPs) are not well understood. Indeed, the complexity of γ-tubulin complexes varies among organisms. Saccharomyces cerevisiae has only two γ-tubulin complex proteins, Spc97p and Spc98p. These proteins are ubiquitous in eukaryotes and their homologs in animal cells are called GCP2 and 3. Schizosaccharomyces pombe has two additional γ-tubulin complex proteins and animals have five GCPs, homologs of GCP2 and 3 plus three additional GCPs. A search of the Aspergillus nidulans genome revealed homologs of all of five animal GCPs. We have designated these proteins AnGCP2-6. Animal γ-tubulin complexes contain an additional protein that contains WD40 repeats, but identification of an A. nidulans homolog of this protein is problematic because this motif is found in many proteins. We have confirmed that AnGCP2-6 are, indeed, γ-tubulin complex proteins by GFP tagging their C-terminal and observing their localizations in living cells by single-time-point and time-lapse microscopy. Like γ-tubulin, they localize to the spindle pole body throughout the cell cycle. To investigate their functions, we have deleted each of the genes. AnGCP2 and 3 are essential, but AnGCP4-6 are not. In vivo observations with an mCherry γ-tubulin fusion reveal, not surprisingly, that AnGCP4-6 are not required for γ-tubulin localization at the spindle pole body. Although AnGCP2 and 3 are essential, heterokaryon rescue was used to determine the effects of AnGCP2 and 3 deletions on γ-tubulin localization. Each deletion eliminated γ-tubulin localization at the spindle pole body. These data indicate that in A. nidulans, GCP2 and 3 homologs are essential for γ-tubulin localization but the other GCPs have other, inessential, functions. Supported by the NIGMS.

264 The RSA Protein Complex Targets Phosphatase Protein 2A to Centrosomes and Regulates Mitotic Spindle Assembly in Caenorhabditis elegans Embryos A. Schlaits,1 M. Srayo,1 A. Dammermann;2 N. Wielsch;3 A. Shevchenko;1 K. Oegema;2 A. A. Hyman;2 2Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany, 3Department of Cellular and Molecular Medicine, Ludwig Institute for Cancer Research, University of California, San Diego, CA
The microtubule cytoskeleton undergoes an extensive reorganization during the cell cycle in order to assemble the mitotic spindle. Mitotic kinases are known to regulate many events leading to spindle formation, however, little is known about their counteracting phosphatases. Moreover, it is unclear how the cell coordinates and integrates the various processes that lead to spindle formation. We have identified a Protein Phosphatase 2A (PP2A) complex in C. elegans that is required for mitotic spindle assembly. This phosphatase complex consists of the new protein RSA-1 (Regulator of Spindle Assembly 1), which is a targeting regulatory subunit for the phosphatase catalytic subunit LET-92, and the previously uncharacterized RSA-2 protein, which binds RSA-1 and thereby recruits it to centrosomes. While the PP2A catalytic subunit LET-92 has multiple functions in the early C. elegans embryo, RSA-1 and RSA-2 are specifically required for microtubule outgrowth from centrosomes and spindle assembly. The correct targeting of PP2A catalytic activity by the RSA complex may regulate these functions by controlling the localization of two key mitotic effectors, TPXL-1 and KLP-7, the C. elegans homologs of TPX2 and MCAK. The RSA complex is recruited to the centrosome by a phospho-dependent interaction with the centrosomal motor protein KLP-7 at centrosomes and might thereby allow the appropriate number of microtubules to grow out. In addition, the RSA complex recruits the critical regulator of spindle assembly TPXL-1 to centrosomes, thus promoting spindle assembly. By affecting the centrosomal localization of two essential players of mitotic spindle assembly, the RSA-PP2A complex may constitute a tool for coordinating different events in spindle assembly. In summary, we show that the RSA-1/RSA-2 protein complex recruits the PP2A catalytic subunit LET-92
to centrosomes. We suggest that this complex thereby regulates a subset of PP2A functions that coordinate microtubule outgrowth from centrosomes and kinetochore microtubule stability in the forming mitotic spindle.

265 Using Monopolar Spindles in Cenarchochitlids elegans to Study Bipolar Spindle Function

The faithful segregation of genetic material is the ultimate goal of mitosis. Cells employ a microtubule-based bipolar spindle to ensure correct chromatin separation and the production of genetically identical daughter cells. This same structure is also used to introduce an asymmetry in the size of these daughter cells by displacing slightly toward the posterior during anaphase. Thus key events in the early embryo are a result of bipolar spindle orientation and positioning. While it is assumed that the separation of the poles is largely responsible for chromatid separation in anaphase, we wanted to investigate the role of forces that might push chromatids apart independently of pole-kinetochore forces. To investigate this problem, we study chromatid movement in monopolar spindles. A temperature-sensitive mutation in the kinase ZYG-1 abrogates its activity under non-permissive conditions. Up-shifted embryos are essentially normal in the first cell division due to the sperm contributed pair of centrioles, but the lack of ZYG-1 prevents subsequent centriole duplication. Therefore daughter cells inherit only one centriole from the mother and form monopolar mitosis. Using zyg-1 (dby-1 ts) worms we observe that sister chromatids congress, align on a metaphase plate and form a monopolar spindle which is half the size of a bipolar spindle. A reproducible separation of the sister chromatids occurs and we are investigating what forces are causing this movement. We have also analysed the positioning of the single centrosome and the monopolar spindle. Each monopolar spindle in zyg-1 ts embryos is orientated orthogonal to that of the wild type spindle. Furthermore we see increased oscillations of the single centrosome in the posterior cell of a two-cell stage embryo lacking functional ZYG-1, indicating that second centrosome is required to stabilize the position of the centrosome-nuclear complex.

266 The Microtubule Anchoring Activity Is Regulated by Centrosomal Kinases
G. Mao, J. Baxter, A. M. Fry; Department of Biochemistry, University of Leicester, Leicester, United Kingdom

Centrosomes are the centre of microtubule organization in animal cells. They are essential for cell division and cell structure. Microtubule nucleates from and anchors at centrosome. This involves numerous centrosomal proteins including ninein like protein, Nlp. Nlp recruits γ-tubulin and stimulates microtubule regrowth in vivo. Nlp interacts with and is co-ordinately regulated by two centrosomal kinases, Nek2 and Pkl1. Regulation of Nlp at the transition of G2/M is important for microtubule dynamics at mitotic entry of eukaryotic cell. The coiled-coil(CC) domain of Nlp interacts with γ-tubule and is phosphorylated by Nek2 and Pkl1. Overexpression of this CC-rich domain does not affect the localization of CNap-1 but γ-tubulin and like full length wild type Nlp, its overexpression can cause dramatic reorganization of the cellular microtubule network at microtubule regrowth, which is not associated with centrosome. Importantly, this domain alone can form aggregates and be displaced from centrosome by phosphorylation of Nek2. Truncation study shows Nlp may interact with other proteins in order to anchor at centrosome. It is concluded that Nlp stimulates microtubule regrowth by recruiting γ-tubulin. This interaction is regulated by phosphorylation of Nlp CC domain by Nek2 and Pkl1. This is important for microtubule structure changes at mitotic entry that lead to the formation of the mitotic spindle.

267 A Genome-wide Visual RNAi Screen to Identify New Centrosome Components
J. Dubbelaere, J. Raff; Gordon Institute, Cambridge, United Kingdom

The centrosome is the main microtubule organising center in animal cells, and it plays an important part in cell cycle progression, organisation of the mitotic spindle and cell division. The centrosome consists of a pair of centrioles surrounded by an amorphous pericentriolar material (PCM) that is the site of microtubule nucleation. During interphase, the centrosome organises a small amount of PCM and nucleates a small number of microtubules. As cells enter mitosis, however, centrosomes "mature" and there is a dramatic recruitment of PCM around the centrioles, and the centrosomes organise many more microtubules. Our goal is to understand the function of the centrosome during the cell cycle and how it is regulated during these different phases. To identify new components and regulators of the PCM, we designed a genome-wide RNA interference screen in Drosophila S2R+ cells. Cells treated with a specific dsRNA for 4 days are stained with a Centrosomin- (Cnn, a PCM Marker) and a phospho-histon3 antibody (H3P, marks mitotic cells). We developed automated protocols to plate the cells, to microscope different wells and to score defects (using Cell Profiler). In addition, manual scoring is performed to detect subtle defects. Initial screening of the genome revealed the PP2A phosphatase as a major regulator of Centrosome function. At this stage we are completing the genomic screen and analysing PP2A centrosome function in more detail.

268 Dissecting the Centriole Proteome
L. C. Keller,1 E. Romijn,2 I. Zamir,1 R. D. Sloboda,3 J. R. Yates,2 W. F. Marshall;1 Biochemistry, UCSF, San Francisco, CA, 1Cell Biology, The Scripps Research Institute, La Jolla, CA, 2Biological Sciences, Dartmouth College, Hanover, NH

We used mass-spectrometry-based MudPIT to identify the protein composition of basal bodies (centrioles) isolated from the green alga Chlamydomonas reinhardtii. This analysis detected the majority of known centriole proteins, including centrin, epsilon-tubulin, and the cartwheel protein BLD10p. Through a bioinformatics cross-validation approach, we identified two classes of proteins: Basal-body proteins with L preregulated Genes (BUGs) and Preregulated of The Centriole proteins (P ocs). We have confirmed centriolar localization for many of the human homologs of candidate proteins in both Hela and U2OS cells. We are in the process of using microscopy, RNA-interference, and biochemical methods to examine the centriole assembly pathway in both Chlamydomonas and in centriole-overduplicating mammalian cell lines.

269 Proteomic Characterization of Tetrahymena thermophila Basal Bodies
C. L. Kilburn,1 C. G. Pearson,1 E. Romijn,2 T. Giddings,1 J. R. Yates,2 M. Winery;1 Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, Boulder, CO, 1Department of Cell Biology, Scripps Research Institute, San Diego, CA

Centrosome duplication is an important event in cell cycle progression. Errors in this process are evident in the majority of tumor cells. However, centrosome duplication is not a well-understood process, partly because many of the molecular components of this organelle are not known. Centrioles form the core of the centrosome and are structurally identical to basal bodies for ciliary function. We used shotgun proteomics to define the basal body proteome in the ciliated protozoan Tetrahymena thermophila and novel components were then studied by immunofluorescence and immunoelectron microscopy. Mass spectrometry analysis of two separate basal body enrichment preparations was used to identify novel basal body proteins. First, cell cortices were isolated in wild-type cells and cells in which basal bodies had been genetically disrupted. In this approach, both samples were analyzed with Multidimensional Protein Identification Technology (MudPIT) to identify the proteins present in these complex mixtures. The identified proteins found only in the sample with basal bodies were retained for the study. Second, a basal body preparation was isolated from oral apparati, an organelle enriched for basal bodies, and subjected to MudPIT. Using the two methods a total of 357 proteins were identified. An organellar enriched fraction was used for MudPIT. The used methods a total of 357 proteins were identified. The organellar enriched fraction was used for MudPIT. The identified proteins were classified using GO Codes, homology and domain searches. We identified 97 proteins (27% of the total list) as known centriole components or potential candidate proteins in both Hela and U2OS cells. We are in the process of using microscopy, RNA-interference, and biochemical methods to examine the centriole assembly pathway in both Chlamydomonas and in centriole-overduplicating mammalian cell lines.

270 The Basal Bodies/Centrioles as a Giardia lamblia Cellular Control Center
T. Lauwaet,1 D. Reiner,1 E. Romijn,2 S. R. Birkeland,3 C. J. Michael,1 D. Palm,3 B. Davids,3 S. Pacocha,3 S. Svard,3 A. McArthur,3 J. Yates,2 F. D. Gillin1; 1Department of Pathology, Division of Infectious Diseases, UCSD, San Diego, CA, 2The Scripps Research Institute, La Jolla, CA, 3Marine Biological Laboratory, Woods Hole, MA, 5Woods Hole Oceanographic Institution, Woods Hole, MA

Eccystation and excystation are differentiations crucial to the pathogenesis of Giardia lamblia, a major protozoan cause of waterborne diarrheal disease. The signaling underlying the dramatic remodeling of the cytoskeleton throughout the giardial life cycle in response to rapidly changing signals from the host, is still poorly understood. Previous studies have shown that signaling
molecules involved in growth cessation during encystation and in the resumption of motility and cytokinesis during excystation, all localize constitutively to the basal bodies/centrioles, while their localization to other cytoskeletal structures varies. We propose that the basal bodies may be a cellular control center while other cytoskeletal elements may have specialized effector functions that are reflected in their distinct protein compositions. We isolated basal bodies from *Giardia* cytoskeletons with sucrose density fractionation. Fractions were analyzed by immunofluorescence analysis and ELISA for the presence of known giardial basal body proteins (calmodulin, PKA, PP2A and centrin). The protein composition of the basal body enriched fraction was analyzed by Multidimensional protein identification technology (MudPIT) and revealed a total of 400 proteins. Comparative genomic analysis showed that about 25% of these proteins are homologous to basal body, centrosomal, centriolar or spindle pole body proteins in other organisms. Selected proteins with no homology to any other protein were cross-validated by epitope tagging and all localize to the basal bodies. Moreover, transcriptome analysis demonstrated that 24 of the proteins found in our basal body fraction are highly upregulated in cysts and early excystation. The protein composition of the *Giardia* basal bodies will help in unraveling the complex signaling in the *Giardia* life cycle and increase global understanding of conserved basal body/centriole function.

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**Genes Required for Basal Body Assembly Show Hypersensitivity to Taxol**

J. M. Esparza, Genetics, Washington University School of Medicine, St. Louis, MO

Basal bodies are morphologically complex structures. We identified mutations in two genes, *TNS1* and *BLD11*, through a non-complementation screen and an inserational mutant screen, which result in morphological abnormalities of basal bodies in *Chlamydomonas*. In wild-type cells, a ring of amorphous material (<40 nm in depth) assembles at the proximal end of basal bodies and we hypothesize that it participates in basal body microtubule nucleation. In the *tns1-1* strain, the amorphous material extends 200 nm throughout the length of a staggered, aberrant full-length basal body. The defect appears to arise during assembly as basal bodies have a normal ring of amorphous material. The *bld11-1* mutant strain lacks basal body microtubules but contains amorphous material. Fibers associated with the basal bodies become thicker and longer. Additionally, these mutant strains show increased sensitivity to taxol compared to wild-type cells. The *tns1-1* and *bld11-1* strain arast on taxol-containing medium at a concentration of 0.75 µg/ml, whereas wild-type *Chlamydomonas* strains arrest at concentrations of 8 µg/ml. We are currently testing if the mutant strains contain hyperstabilized microtubules using an antibody to acetylated α-tubulin and if the mutant strains are sensitive to microtubule destabilizing drugs. The mutant strain, *tns1-1*, is a linked dominant enhancer of the *bld2* mutation, which encodes α-tubulin. Interestingly, three independent fragments of DNA rescue the mutant phenotypes of *tns1-1* cells. Included in these fragments is e-tubulin, which plays an essential role in basal body assembly (Dutcher et al., 2002). Dosage-dependent suppression of the *tns1-1* mutant phenotype by a few genes may compensate for the absence of functional *Tns1p* to allow for flagellar assembly. We are currently assaying the mutant and rescued strains by immunofluorescence using antibodies to basal body proteins to interrogate the assembly process in these strains.

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**Rotating of Centrioles and Detachment from the Centrosome in Drosophila centrosin (cnn) Mutants**

E. P. Lucas, J. W. Raff; Wellcome Trust/CR-UK Gurdon Institute, Cambridge, United Kingdom

Centrosomes are major organizers of animal cell division and their dysfunction has been implicated in several human diseases, such as cancer and primary microcephaly. The Drosophila Centrosin (Cnn), a putative ortholog of the human microtubule protein MCPH3/CDK5RAP2 and Myomegalin, has been implicated in centrosome organization and microtubule nucleation during mitosis, although its ability to directly recruit and anchor PCM components to the centrosome has not been demonstrated. In contrast to previous reports, here we show that PCM components can, in fact, be recruited to the centrosome in the absence of Cnn. High-resolution live imaging of several PCM markers shows that, in *cnn* mutant embryos, the PCM is initially recruited but then abnormally dispensed from the centrosome in a microtubule-dependent manner. Strikingly, dual imaging of several GFP-PCM components and GFP-centrosomes reveals that, in *cnn* mutant embryos, the centrosomes move abnormally and are not tightly linked to the PCM, in contrast to wild-type, but in most cases still remain attached to the dispersed PCM. Additionally, in live *cnn* mutant neuroblasts, centrosomes also move abnormally and are not properly positioned at the spindle poles and therefore, are unequally partitioned between dividing cells, leading to an asymmetric cell division failure. Furthermore, centriole missegregation and spindle defects occur in male meiosis. Thus, unlike what was shown previously, we demonstrate that Cnn is not required for the recruitment of PCM components to the centrosome, but instead, maintains the structural integrity of the centrosome by keeping an intact and strong connection between the centriole and the PCM.

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**Role of Mammalian Homologues of Drosophila centrosin in Centrosome Function and Asymmetric Cell Division**

J. A. Barrera, L. K. Kao, R. E. Hammer; 1Pharmacology, UT Southwestern Medical Center, Dallas, TX, 2Biochemistry, UT Southwestern Medical Center, Dallas, TX

Autosomal recessive primary microcephaly (MCPH) is a developmental disorder resulting in the reduction of cranial circumference due to reduced development of the central nervous system, and the greatest impact on the cerebral cortex. Six genes have been linked to MCPH. Four of these encode centrosomal proteins, implicating a centrosomal mechanism underlying early neuronal development. Spindle orientation plays a key role in the process of asymmetric cell division of neural precursors, and is, in part, centrosome dependent. Studies of mutations in two MCPH gene homologues in *Drosophila* and *Disco* (homologue of *CENP-D*), did not show reduced brain size, suggesting different requirements for centrosomal proteins in these two systems. A homologue search for *Drosophila* cnn revealed two mammalian genes: Cyclin Dependent Kinase 5 Regulatory Associated protein 2 (*CDK5RAP2*) and Myomegalin. An in vivo rescue assay is being used to determine functional homology among these genes with MCPH gene homologues in *Drosophila* and *Disco*.

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**MTOC Reorientation in Macrophages**

E. W. Y. Eng, A. Bettio, J. Ibrahim, R. E. Harrison; Department of Life Sciences, University of Toronto at Scarborough, Scarborough, ON, Canada

The microtubule organizing center (MTOC) plays many important roles in cells, including microtubule nucleation, organelle positioning, cell division, as well as in cell polarization during T cell activation and the migration of fibroblasts in wounded monolayers. Recently, we have observed that the MTOC in macrophages reorients during the process of phagocytosis. The MTOC movement is initiated shortly after particle binding and continues after the particle is ingested. Key cytoskeletal and signalling elements that have been previously implicated in MTOC reorientation in other cells were investigated in RAW 264.7 macrophages using transfection, immunofluorescence, live imaging, and frustation phagocytosis assays. It was found that the reorientation of the MTOC towards the phagocytic site of IgG-sheep red blood cells (sRBCs) in macrophages requires an intact microtubule and actin network. In addition, signalling elements that have been previously implicated in MTOC reorientation in other cells were investigated in RAW 264.7 macrophages using transfection, immunofluorescence, live imaging, and frustrated phagocytosis assays. It was found that the reorientation of the MTOC towards the phagocytic site of IgG-sheep red blood cells (sRBCs) in macrophages requires an intact microtubule and actin network. In addition, signalling molecules, such as cdc42 and phosphatidylinositol 3-kinase (PI3K), and the microtubule motor, dynein, all appear to contribute to MTOC reorientation in macrophages. More extensive studies are currently underway to determine the role of MTOC reorientation during phagocytosis in macrophages.

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**The a and β Subunits of G, Family Heterotrimeric G Proteins Are Both Required but Function Independently to Regulate Centrosome Reorientation**

E. S. Folker, G. G. Gundersen; Anatomy and Cell Biology, Columbia University, New York, NY

Centrosome reorientation to a position at the leading edge of motile cells is among the first steps in preparation for directed cell migration. In serum starved fibroblasts, lysophosphatidic acid (LPA) stimulates centrosome reorientation via two Cdc42 regulated processes: actin dependent nuclear movement away from the leading edge and microtubule dependent maintenance of the centrosome at the cell centroid (centrosome centration) (Gomes et al., Cell, 2005). We explored the possibility that heterotrimeric G proteins were involved in centrosome reorientation given their role in spindle positioning during asymmetric cell divisions in invertebrates. Pertussis toxin (PTX), an inhibitor of G i/o family heterotrimeric G proteins, blocked centrosome reorientation by inhibiting both nuclear movement and centrosome centration. PTX prevented activation of Cdc42 GTP by LPA, yet constitutively active Cdc42 did not overcome PTX inhibition, suggesting roles for G i/o proteins both upstream and downstream of Cdc42. Experiments to perturb G protein signaling using G ao, βARK, and LGN/mPins indicated that the G and Gβγ subunits of G, regulate different facets of centrosome reorganization. Gao and the C-terminus of βARK both inhibit reorientation by blocking nuclear movement. Both reagents bind and sequester Gβγ indicating that Gβγ activity regulates nuclear movement. Expression of LGN/mPins, a protein with C-terminal GoLoco domains that bind Gao and release Gβγ, and N-terminal TPR domains that interact with other proteins, was sufficient to induce both nuclear movement and centrosome centration in the absence of LPA resulting in centrosome reorientation. The C-terminal GoLoco domains alone induced only nuclear movement, suggesting that the TPR domains are essential for linking Gao to microtubules to regulate centrosome centration. These data support a model where LPA receptor activation releases Gβγ to initiate nuclear movement. LGN/mPins then binds Gao, preventing Gβγ reassociation, maintaining Gβγ activity. Additionally LGN/mPins serves as a bridge between Gao and the microtubules regulating centrosome centration.
Vinculin is a major component of cell adhesion sites anchoring actin filaments to the adhesion plaque. The protein consists of five alpha-helical bundle domains (Vd1-Vd5), which perform an uniform gradient. In this study we propose that ablation of the centrosome will result in random migration of the structurally polarized cells as opposed to a directed migration. HL60 cells were in a uniform concentration (non-gradient) of chemoattractants have the ability to polarize and move. Previous studies have show n that microtubules control the polarity of neutrophils in such a cDNA with sequence identity to the coding region of IK1. The IK blocker clotrimazole significantly inhibited swelling-activated K+ currents, and currents were inhibited by transfection with a opening of 50 pS K+-permeable channels, suggesting the involvement of intermediate conductance (IK) channels. Consistent with I K expression, we isolated from rat liver and HTC cells a hepatocytes, the molecular characteristics of the channels involved in volume regulation have not been determined. We found that hypotonic exposure of HTC rat hepatoma cells evoked the restoration of cell volume in the continued presence of osmotic stimuli is essential, particularly in liver, which swells in response to nutrient uptake. Responses to swelling involve the Ca2+-dependent activation of K+ channels, which promote electrolyte and fluid efflux to drive volume recovery. Although several classes of Ca2+-activated K+ channels have been detected in hepatocytes, the molecular characteristics of the channels involved in volume regulation have not been determined. We found that hypotonic exposure of HTC rat hepatoma cells evoked the opening of 50 pS K+-permeable channels, suggesting the involvement of intermediate conductance (IK) channels. Consistent with IK expression, we isolated from rat liver and HTC cells a cDNA with sequence identity to the coding region of IK1. The IK blocker clotrimazole significantly inhibited swelling-activated K+ currents, and currents were inhibited by transfection with a dominant interfering mutant of IK1. Clotrimazole also inhibited volume recovery. By contrast, neither the small conductance K+ channel blocker apamin nor the large conductance K+ channel blocker tetraethylammonium chloride affected swelling-activated K+ currents. Because IK1 can be regulated by products of phosphatidylinositol kinases, which are produced in lipid rafts, we sought to determine whether IK1 and lipid rafts undergo volume-sensitive association. In HTC cells transfected with a GFP fusion of IK1, there was a time dependent increase in colocalization between IK1 and a lipid raft marker, ganglioside GM1, on the plasma membrane, which subsequently decreased with volume recovery. Treatment of cells with filipin abolished plasma membrane GM1 localization and altered the plasma membrane signal of IK from discrete patches to continuous. Filipin also led to a loss of sustained volume recovery after hypotonic exposure. Collectively, these findings support the hypothesis that IK1 regulates compensatory responses to hepatocellular swelling, and that suggestion of cell volume involves coordination of lipid raft signaling and IK function.

Centrosome Ablation in Neutrophils Affects Cell Polarity and Migration
N. M. Wakida, J. Xu, L. Z. Shi, E. L. Botvinick, A. Dvornikov, M. W. Berns, H. R. Bourne; Beckman Laser Institute, University of California, Irvine, Irvine, CA, Departments of Cellular and Molecular Pharmacology and Medicine and Cardiovascular Res. Institute, University of California, San Francisco, San Francisco, CA, Department of Bioengineering, University of California, San Diego, San Diego, CA
Pathways for initiation and regulation of neutrophil polarization and chemotaxis in response to chemoattractants are of considerable interest. Numerous studies have been undertaken to elucidate the interactions between cytoskeletal elements (actin, myosin, microtubules), various Rho proteins, and other regulatory factors that control the migration of single cells. Neutrophils in a uniform concentration (non-gradient) of chemoattractants have the ability to polarize and move. Previous studies have shown that microtubules control the polarity of neutrophils in such a way that microtubules are required for cell polarization and chemotaxis. In this study we propose that ablation of the centrosome will result in random migration of the structurally polarized cells as opposed to a directed migration. HL60 cells were differentiated to a granulocytic morphology by treatment with DMSO allowing for studies on polarization and chemotaxis. Transient transfections by electroporation with GFP-Clip170 or β-arrestin were used to label the centrosome of the differentiated cells. (Xu et al., 2005) Cells were allowed to recover and attach to fibronectin plated coverslips for 1-2 hours before laser targeting. A uniform chemoattractant was added immediately after exposure of femtosecond laser pulses to specific regions of the cell. 10 of 10 cells in which laser pulses were directed at the centrosome region failed to polarize, 5 of 5 control cells where the laser was targeted to a random region in the cytoplasm exhibited neither a change in polarization nor directed movement. These results indicate that the centrosome plays a significant role in cell polarization and subsequent cell migration.

Association of the Ca2+-activated K+ Channel IK1 with Lipid Rafts Is Required for Hepatocellular Volume Regulation
E. Barfod, A. L. Moore, M. W. Roe, S. D. Lidoňsky; Pharmacology, University of Vermont, Burlington, VT, Medicine, University of Vermont, Burlington, VT
Restoration of cell volume in the continued presence of osmotic stimuli is essential, particularly in liver, which swells in response to nutrient uptake. Responses to swelling involve the Ca2+-dependent activation of K+ channels, which promote electrolyte and fluid efflux to drive volume recovery. Although several classes of Ca2+-activated K+ channels have been detected in hepatocytes, the molecular characteristics of the channels involved in volume regulation have not been determined. We found that hypotonic exposure of HTC rat hepatoma cells evoked the opening of 50 pS K+-permeable channels, suggesting the involvement of intermediate conductance (IK) channels. Consistent with IK expression, we isolated from rat liver and HTC cells a cDNA with sequence identity to the coding region of IK1. The IK blocker clotrimazole significantly inhibited swelling-activated K+ currents, and currents were inhibited by transfection with a dominant interfering mutant of IK1. Clotrimazole also inhibited volume recovery. By contrast, neither the small conductance K+ channel blocker apamin nor the large conductance K+ channel blocker tetraethylammonium chloride affected swelling-activated K+ currents. Because IK1 can be regulated by products of phosphatidylinositol kinases, which are produced in lipid rafts, we sought to determine whether IK1 and lipid rafts undergo volume-sensitive association. In HTC cells transfected with a GFP fusion of IK1, there was a time dependent increase in colocalization between IK1 and a lipid raft marker, ganglioside GM1, on the plasma membrane, which subsequently decreased with volume recovery. Treatment of cells with filipin abolished plasma membrane GM1 localization and altered the plasma membrane signal of IK from discrete patches to continuous. Filipin also led to a loss of sustained volume recovery after hypotonic exposure. Collectively, these findings support the hypothesis that IK1 regulates compensatory responses to hepatocellular swelling, and that suggestion of cell volume involves coordination of lipid raft signaling and IK function.
release intramembranous bonds and allow vinculin activation. Acidic phospholipids bind to Vδ4 and contribute to the regulation of vinculin-ligand interactions in competition with actin filaments. While conformational changes in the vinculin head upon binding of vinculin-binding-site (VBS) peptides are known, and a model for actin filament binding to Vδ4 has been suggested based on cryo-electron microscopy data, alterations in the Vδ4 structure induced by phospholipids remain largely uncharacterized. We studied the Vδ4 structure by electron paramagnetic resonance spectroscopy (EPR) employing single and double cysteine mutants which were site-specifically labeled with a commonly used paramagnetic spin label (Mnt). Peptide-derivatized proteins were tested for binding-induced activity. EPR analyses identified the interaction surface of the Vδ4 helical bundle with PtdSer-vesicles and the depth of penetration into the vesicle surface. In contrast to predictions based on the crystal structure of Vδ5, which suggested unfurling of the helical bundle, vesicle binding is accompanied by a movement separating helices H2 and H3 of Vδ5, with conservation of the remaining structure, to generate a new interaction surface with the lipid bilayer. The structural changes observed provide a rational for the strongly reduced binding of acidic phospholipids to the tail of metavinculin, a splice variant with an amino acid insert affecting this precise region.

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CD98hc Activates Integrin Signals at the Embryo-Maternal Interface
M. Kabir-Salmani,1,2 M. Fukuda,3 M. Kanai-Azuma,4 K. Sugihara,1 M. Iwashita1, Basic Science, National Institute for Genetic Engineering and Biotechnology, Tehran, Iran (Islamic Republic of), 1The Department of Obstetrics and Gynaecology, Kyorin University School of Medicine, Tokyo, Japan, 2Gycochemistry Program, Cancer Research Center, Burnham Institute, La Jolla, CA, 2Anatomy, Kyorin University School of Medicine, Tokyo, Japan

CD98 heavy chain is an actin-associated type II plasma membrane protein highly expressed in human tumors and placenta, implicated as functional contributor to cell motility. Since αβ3 integrin serves as a dominant integrin of the focal adhesions of invasive extravillous trophoblasts, we investigated the potential role of CD98 and mechanisms by which it may promote αβ3 integrin activation. Immunocytochemistry revealed that CD98hc is co-localized with integrin αβ3, phospho-FAK, vinculin and with phospho-FAK in focal adhesions. Co-immunoprecipitation demonstrated a strong association between CD98hc and its transmembrane domain with β integrin. When serum-starved CD98 negative cells were stably transfected for CD98hc, crosslinking of CD98 enhanced activation of αβ3 integrin inside-out and outside-in signals, cell adhesion, and migration. Similar events occurred when these cells were transfected with a mutant of irrelevant type II membrane protein in which the transmembrane domain had been replaced with that of CD98hc. The CD98-induced effects were inhibited in knock down trophoblastic cells and upon treatment with specific inhibitors of CD99, αβ3 integrin, phosphoinositide 3-OH kinase (PI3-K) and RhoA. Our results suggest a novel role of CD98hc at the embryo-maternal interface. The association of CD98hc transmembrane domain with β integrin provides a new target for cancer therapy and assisted reproductive technology.

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Rap1 Regulates a Feed Forward Signaling Pathway Required for β1-integrin-dependent Phagocytosis of Collagen
P. D. Arora,1 K. Burridge,2 P. A. Marignani,3 M. R. Philips,4 J. A. Cooper,5 C. A. McCulloch1, CHIR Group in Matrix Dynamics, University of Toronto, Toronto, ON, Canada, 1Department of Cell Biology, University of North Carolina, Chapel Hill, NC, 3Department of Biochemistry and Molecular Biology, Dalhousie University, Halifax, NS, Canada, 4Department of Medicine, New York University, New York, NY, 2Fred Hutchinson Cancer Research Center, Seattle, WA

Collagen phagocytosis is a crucial, αβ3 integrin-dependent process that mediates extracellular matrix remodeling by fibroblasts. We have shown previously that gelsolin is required for Rac1 activation in the initial binding step of collagen phagocytosis. Objective: To define the roles of the Ras related GTPase, Rap1, and the GTP exchange factor Vav2, in regulation of Rac1 during collagen phagocytosis. Methods: Cultured mouse fibroblasts were incubated with collagen beads to ligate alpha2 beta1 integrins. Cells were transfected with plasmids expressing small GTPase regulatory proteins and studied by confocal microscopy, immunoblotting and Rac1 activation assays. Results: Collagen bead binding required Rac activation but not cdc42 or RhoA. Rac activity was also increased following collagen bead binding. Accordingly, we focused on the upstream regulation of Rac in the collagen binding step of phagocytosis. Collagen bead binding promoted phosphorylation of Vav2 at Y172. Cells transfected with constitutively active Vav2 and Rap1 exhibited Rac activation and enhanced collagen bead binding while dominant-negative Vav2 and Rap1 blocked Rac activity and bead binding. Recruitment of active rac to bound collagen required active Vav2. Immunoprecipitation studies showed interactions between constitutively endogenous Vav2 and active Rap1. Transfection with dominant negative Rap1, with or without co-transfection with constitutively active Vav2, inhibited collagen bead binding. In contrast, cells co-transfected with constitutively active Rap1 and constitutively active Vav2, exhibited enhanced collagen bead binding. In cells transfected with constitutively active, but not dominant negative Rap1, incubation with collagen beads promoted translocation of constitutively active Vav2 to collagen bead binding sites. Conclusion: Rap1 interacts with constitutively active Vav2 to localize Vav2 activity to integrins and to enhance collagen binding by activating Rac.

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Restoration of Dystrobrevin to the mdx Sarcolemma Does Not Attenuate Muscular Dystrophy
M. E. Adams, Y. Tesch, J. M. Percival, D. E. Albrecht, K. Anderson, S. C. Froehner; Physiology and Biophysics, University of Washington, Seattle, WA

Restoration of dystrobrevin to the mdx sarcolemma does not significantly reduce the muscular dystrophy phenotype of these animals. We conclude that dystrobrevin, even when expressed at normal levels, cannot rescue the muscular dystrophy of dystrobrevin-null mice. Synaptic integrity is significantly reduced in dystrobrevin-null mdx mice, and expression of either dystrobrevin isoform prevented the muscular dystrophy normally observed in the dystrobrevin null mouse and restored centrally nucleated fiber counts to levels similar to controls. On the mdx background that lacks dystrophin, only the palmitoylated form of dystrobrevin was present on the sarcolemma. The non-palmitoylated form did not accumulate at the sarcolemma. The palmitoylated form of dystrobrevin was able to restore α-syntrophin to the sarcolemma, however, nNOS remained absent. This suggests that nNOS requires dystrophin as well as syntrophin for proper localization. Furthermore, counts of centrally nucleated fibers showed that the presence of the dystrobrevin and syntrophin on the sarcolemma of mdx mice did not significantly reduce the muscular dystrophy phenotype of these animals. We conclude that dystrobrevin, even when directed to the sarcolemma, is not sufficient to restore a functional protein complex capable of preventing muscular degeneration.

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A Novel Role of the Septins during the Spore Membrane Morphogenesis in Schizosaccharomyces pombe
M. Onishi,1 T. Koga,1 A. Hirata,2 H. Tachikawa,3 T. Nakamura,2 H. Asakawa,2 J. Bähler,2 J. Wu,3 C. Shimoda,1 J. Pringle,1 Y. Fukui1, Graduate School of Agriculture & Life Science, University of Tokyo, Tokyo, Japan, 1Department of Integrated Biosciences, Graduate School of Frontier Science, University of Tokyo, Chiba, Japan, 2Department of Biology, Graduate School of Science, Osaka City University, Osaka, Japan, 3Wellcome Trust Sanger Institute, Hinxton, Cambridge, United Kingdom, 4Department of Molecular, Cellular, and Developmental Biology, Yale University, New Haven, CT, 5Department of Genetics, Stanford University, Stanford, CA

Septins are a family of conserved, filament-forming proteins that has been shown to play roles in diverse cellular events in various organisms. We propose a novel role of the septins in the spore membrane morphogenesis of the fission yeast Schizosaccharomyces pombe. The S. pombe accomplishes gametogenesis by two tightly linked processes, meiosis and spore-envelope formation. At prometaphase of meiosis II, membrane vesicles begin to fuse at the cytoplasmic surfaces of the spindle-pole bodies to form the forespore membranes (FSMs), which then extend to form the spore membrane surrounding each haploid nucleus and associated cytoplasm. Formation of the FSMs is an interesting model for the de novo synthesis of biological membranes. In particular, understanding how the FSM extends and closes to form a sac might provide general insights into the mechanisms of membrane formation. The S. pombe genome encodes seven septins. As their possible roles in sporulation have not been examined in detail. We found that deletion of spm2, spm3, spm6, or spm7, but not of the others, dramatically affected sporulation formation. The orientation at the beginning of FSM extension was frequently abnormal, resulting in the formation of aberrant numbers of spores per ascus as some FSMs failed to enclose nuclei. The spore morphology is affected interdependently to a horseshoe-like assemblage along each FSM, which extended to form a ring in the end. We also found that Spm2p and Spm7p bound to the phosphoinositides PtdIns(4P) and PtdIns(5P) in vitro, and that PtdIns(4P) was enriched in the FSM. Introduction of a mutation in the phosphoinositide-binding region of Spm2p abolished the association of the septin assemblage with the FSM in vivo. We suggest that the septin assemblage contributes to the proper orientation of FSM extension by interacting with PtdIns(4P) in the FSM. Biochemical analyses of the sporulation-specific septin complex will be also presented.

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Characterization of TIMP, the Potential Regulatory Subunit of Protein Phosphatase 1 in Human Endothelial Cells
I. Czirka,1,2 G. Oláh,1 C. Csortos,1,2 A. D. Verin; 1Department of Medicine, University of Chicago, Chicago, IL, 2Department of Medical Chemistry, University of Debrecen, Debrecen, Hungary
Reversible phosphorylation of numerous cytoskeletal proteins has a crucial role in the maintenance of appropriate alignment of the cytoskeleton. We have shown earlier that protein phosphatase 1c (PP1c), the catalytic subunit of myosin phosphatase (MP), associates with microfilaments and directly is involved in endothelial cell (EC) contractility and barrier regulation through the dephosphorylation of myosin light chain. In the present work we studied TIMAP, a potential regulatory subunit of PP1. It is highly abundant in EC and it was described as a membrane associated protein. Indeed, using an anti-peptide polyclonal antibody by immunofluorescence (IF) staining we observed TIMAP being enriched in a membrane in human pulmonary arterial EC (HPAEC) but also in the cytosol, mostly around the nucleus. We showed protein-protein interaction in HPAEC between TIMAP and PP1c by immunoprecipitation. Moreover, in pull-down assay using bacterially expressed recombinant GST-TIMAP we found for the first time that PP1c delta isoform binds to TIMAP with higher affinity then the alpha isoform. Thrombin (20 nM) treatment evoked increased IF staining of TIMAP at the membrane. In parallel, we detected enrichment of moesin, a member of the ERM family, as well as phospho-moesin at the cell membrane. These results suggest that TIMAP may take PP1 activity to the cell-membrane where it can participate in ECM driven membrane dynamics. Supported by NIH grants HL067307, HL58064, and OTKA grant T043133

288 Chromium Picolinate Displays Membrane Phospholipid and Actin Cytoskeleton Protective Effects Against Hyperinsulinemia-Induced Insulin Resistance in 3T3-L1 Adipocytes E. M. Horvath, J. S. Elmedina; Cellular and Integrative Physiology, Indiana University School of Medicine, Indianapolis, IN Analyses reveal that membrane phosphatidylcholines 4,5-bisphosphate (PIP2) and cortical filamentous actin (F-actin) are diminished by chronic insulin exposure and this loss contributes to insulin-induced insulin resistance in fat and skeletal muscle. Here we tested whether chromium picolinate (CrPic) could improve insulin sensitivity in 3T3-L1 adipocytes rendered insulin resistant by hyperinsulinemia (12 h, 5 nM), as accumulating evidence suggests that CrPic may alleviate high glucose and lipid abnormalities in diabetes. Insulin’s acute (30 min, 100 nM) effect on GLUT4-regulated glucose transport was diminished in hyperinsulinemic adipocytes. Concomitant with the insulin-resistant state, plasma membrane PIP2 and F-actin were decreased in hyperinsulinemic cells. We describe if CrPic could correct the insulin resistant state, cells were cultured in the absence or presence of CrPic. Insulin-regulated glucose transport improved in CrPic-treated hyperinsulinemic adipocytes and this was paralleled by an increase in plasma membrane PIP2; immunofluorescent detection and cortical F-actin polymerization. As study has demonstrated that CrPic improves insulin responsiveness by decreasing plasma membrane cholesterol, we next sought to assess potential cholesterol/PIP2 interaction. Labeling of cholesterol/caveolin-1-enriched membrane microdomains revealed a clear PIP2 co-localization in these regions. Furthermore, this juxtaposed nature of caveolin-1 and PIP2 intensifies in hyperinsulinemic cells and it appears as though CrPic treatment may remove PIP2 from the cholesterol-enriched caveolae microdomains. Immunofluorescent labeling also showed a clear co-localization of actin and caveolin-1 in these domains, which was diminished in hyperinsulinemic cells. Interestingly, treatment of resistant cells with CrPic restored the presence of actin/caveolin-1 structures. Our results support a novel paradigm in which plasma membrane lipid abnormalities induced by hyperinsulinemia impair regulation of the glucose transport system by insulin. Concomitant glucose membrane remodeling that occurs in response to CrPic favors insulin-regulated glucose transport. We propose that CrPic corrects plasma membrane abnormalities associated with insulin resistance.

289 Identification and Characterization of a Cardiac-specific Isoform of Alpha-spectrin Y. Zhang, W. G. Resnek, C. Lee, D. H. Catin, W. R. Randall, R. J. Bloch, J. A. Ursitti; Physiology, University of Maryland, Baltimore, MD, Pharmacological and Experimental Therapeutics, University of Maryland, Baltimore, MD, Medical Biotechnology Center, University of Maryland, Baltimore, MD Spectrin is a large, flexible protein that functions to stabilize membranes and organize proteins and lipids into microdomains in intracellular organelles and at the plasma membrane. Alternative splicing occurs in spectrins, and yet it is not clear if the small variations in structure caused by alternative splicing alter the functions of spectrin. Three splice variants have been identified previously for α-spectrin. Here we describe a new alternative splice variant: a 21 amino acid sequence in motif 21. This new splice variant (cardi+) is only expressed in significant amounts in cardiac muscle and occurs within the high affinity nucleation site for binding of all-spectrins to β-spectrin. Long-range RT-PCR demonstrates that the insert is expressed randomly in heart tissue. This indicates that the expression of the cardi+ insert is not dependent on the presence or absence of the other alternatively spliced sequences. In vitro studies of recombinant all-spectrin polypeptide complexes reveal two alternative splice variants of α-spectrin and significant differences in affinity for the complementary site on β-spectrin with values of -1 nM for both, as measured by surface plasmon resonance (SPR) in a Biacore 3000. Remarkably, however, the αβ-cardi+ form shows significantly higher levels of binding than the αβ-cardi- form, both by SPR and blot overlay. This suggests that the 21-amino acid insert prevents some of the αβ-cardi- form from interacting with ββ-spectrin, even in assays requiring minutes to hours to complete. Small constructs expressing the αβ-cardi- sequence are insoluble in solution and aggregate in COS cells, consistent with the possibility that this insert removes a significant portion of the protein from the population that can bind β subunits. We are continuing our studies of this unique splice variant of cardiac α-spectrin to determine its specific function in heart.

290 Tropomodulin Is Required for Anterior Lens Fiber Cell Organization and Lens Focusing R. Nowak, R. S. Fischer, J. R. Knues, V. M. Fowler; Cell Biology, Scripps Research Institute, La Jolla, CA, Ophthalmology and Pathology, Rush Medical College, Chicago, IL Tropomodulin (Tmod) caps the pointed ends of actin filaments in the lens fiber cell plasma membrane skeleton and is proposed to regulate lens fiber cell elongation and morphogenesis. To understand the role of Tmod and actin filament capping in the lens, we have studied the optical properties and fiber cell membrane organization in lenses from normal mice and in lenses lacking Tmod1. Immunoblotting and immunofluorescence staining show that both Tmod1 and Tmod3 isoforms are expressed in cortical but not nuclear fiber cells of mouse lenses. Biochemical fractionation of lens cortical fiber cells by immunoblotting demonstrates that Tmod1 is predominantly associated with fiber cell membranes whereas Tmod3 is cytoplasmic. Immunofluorescence staining and confocal microscopy of cryosections of P14 lenses also demonstrates that Tmod1 is enriched on lateral membranes of fiber cells, whereas Tmod3 is cytoplasmic. In lenses lacking Tmod1, Tmod3 protein levels are unchanged, and Tmod3 remains in the cytoplasm and does not become enriched on fiber cell membranes, suggesting that membrane-associated actin-actin interactions may be stabilized in the absence of Tmod1. Scanning electron microscopy of lenses lacking Tmod1 shows a decrease in membrane protrusion complexity along the long axis of cortical fiber cells when compared to wild-type, indicating that cell-cell and/or membrane stability is decreased in the absence of Tmod1. H & E staining of semi-thin sections of lenses lacking Tmod1 demonstrates defects in anterior fiber cell layer organization and packing relative to wild-type lenses. Cataracts do not form in the absence of Tmod1, but laser scan analysis reveals decreased lens optical quality as indicated by increased back focal length and back focal length variability relative to wild-type lenses. We conclude that Tmod1 function is required for normal anterior fiber cell interactions and layer organization, which determines lens optical quality and normal lens focusing function.

291 F-Actin Componentalisates the Plasma Membrane to Support Polarized Membrane Growth during Drosophila Cellularization A. Sokol, E. Wieschaus; Molecular Biology, Princeton University, Princeton, NJ, Molecular Biology, HHMI/Princeton University, Princeton, NJ In Drosophila, apical-basal blastic cell boundaries are formed by a thin sheet of F-actin that is a modification of the landmarks of a polarized epithelium. Surface labeling of ingressing furrows during cellularization previously suggested that new membrane insert at discrete domains along the plasma membrane and so could contribute to the generation of apical/basal polarity (Lecuit and Wieschaus, 2000). Using confocal microscopy to follow multiple membrane probes in fixed and living fly embryos, we now show that the actin/myosin-2 furrow canal at the basal tip of the ingressing furrow indeed comprises a membrane domain that is distinct from the adjacent, growing, apical/lateral plasma membrane. Contrary to prior models that named cell-cell junctions at the interface between these domains as possible barriers to lateral diffusion, our analysis of maternal/vgycogenic β-catenin mutants demonstrates that the membrane domains are maintained even in the absence of junctions. Instead, we find that a myristoylated, natively-disordered protein Nullo, that binds PH(3,4,5)P2, in vitro and concentrates at the cell-cell junction region in vivo, defines and/or maintains these discrete plasma membrane domains. Nullo drives the local accumulation of cortical F-actin, and when F-actin is disrupted in either null mutants or with cytochalasin-D, components of the apical/lateral plasma membrane invade the furrow canal domain. Mixing between the domains precedes the regression of some furrows, and that persists, the cell-cell junctions disperse throughout the membrane. Thus, Nullo compartmentalizes the plasma membrane via cortical F-actin to support membrane growth and to cluster polarity cues such as cell-cell junctions. Intriguingly, the interaction of Nullo with PH(3,4,5)P2 suggests that specific phosphoinositides may also contribute to plasma membrane compartmentalization during the establishment of this embryonic epithelium.

292 Probing the Fluid Environment at and Near the Cell Surface by Rotating Laser Trapped Microspheres E. Botvinick, H. Rubinstein-Dunlop, M. Berns; Beckman Laser Institute (UCI), Irvine, CA, Department of Physics/Centre for Biophotonics and Laser Science, The University of Queensland, Brisbane, Australia
A microscope system has been developed to apply ultra-localized fluid shear to sub regions of a cell’s surface. Spherical vaterite crystals are rotated near the cell surface by applying optical torques with circularly polarized light. Measurement of crystal rotation rate and changes in circular polarity as light exits the crystal allows measurement of fluid apparent viscosity near the crystal surface. The fluid flow field is generally 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 mapped out as a crystal approaches the cell surface and begins to interact with the glycoalyx and with the plasma membrane. The effects of the glycoalyx on the fluid environment outside the cell were measured for a set of controls including serum starvation and heparinase III treatment. It was found that degradation of the glycoalyx suppressed (with respect to normally cultured cells) the increase in apparent viscosity observed as a crystal is brought towards the cell from a sufficiently distal location. The rotating crystals are also used to apply physiological-magnitude shear to local regions of the cell surface. Using apparent viscosity as an indicator of the flow-field’s proximity to the plasma membrane or glycoalyx, it is possible to selectively apply fluid shear to the glycoalyx. In this way fluid shear mechanotransduction of the glycoalyx can be measured independently from that of the plasma membrane.

293 Modulation of Free PI(4,5)P2 Levels In vivo by MARCKS
J. E. Sable, 1 G. Di Paolo, 1 M. P. Sheetz; 1 Biological Sciences, Columbia University, New York, NY, 1Department of Pathology, Columbia University Medical Center, New York, NY

Phosphatidylinositol-3',4',5'-trisphosphates (PI(4,5,5)P3) have important roles in many cellular processes but how cytoplasmic PI(4,5)P2 levels are regulated is still relatively unknown. Myristylated alanine-rich protein kinase C substrate (MARCKS) is an abundant cellular protein that strongly binds PI(4,5)P2 in vitro and may be responsible for regulation of PI(4,5)P2 levels in vivo by localized sequestration/release mechanisms. We find that over-expression of MARCKS decreases membrane-lysosome adhesion, but increased PI(2,3)P2 synthesis via PI(4,5)P2 Inhibits prevents this decrease. We have also attempted to test MARCS +/- MEFs using optical tweezers but failed to obtain tethers due to presumably high membrane tension. Phosphorylation of MARCKS by PKC activation dramatically decreases its affinity for PI(4,5)P2 and the plasma membrane, restores normal membrane tension and causes increased recruitment of PLC-PIP2-GFP to the plasma membrane as shown by TIRF microscopy. In primary MARCKS +/- cells, biochemical analyses show a significant increase in the rate of turnover of PI(2,3)P2 and PI(4,5)P2 versus wild-type. MARCKS +/- cells also show a significant increase in the rate of labeling of PI(2,3)P2 (but not PI(4,5)P2) in vivo in in vitro experiments with liposomes and γ-ATP. These cells also have altered actin morphology (radial arrays of filopodia-like structures) and exhibit uncoordinated protrusions and retractions during cell spreading. We have also shown that MARCKS protein migrates towards EGF gradients in cells, which could provide localized increases of PI(2,3)P2 to the leading edge of the lamellipodia. Therefore, we suggest that MARCKS is critical to regulation of free PI(4,5)P2 levels and intracellular actin cytoskeleton dynamics by functioning as a critical reservoir of phosphoinositides at the plasma membrane.

294 Phosphatidylinositol-3’-OH-Kinase Coordinates Cdc42 and Rac GTPases by a Signal Transition and Integrates FcγR Signals during Phagocytosis
P. J. BeenMiller, 1 A. Hoppel, 1 A. Swanson; 1 Cellular and Molecular Biology, University of Michigan, Ann Arbor, MI, 2Microbiology and Immunology, University of Michigan, Ann Arbor, MI

Fc receptor (FcγR)-mediated phagocytosis of large particles (>3 μm diameter) by macrophages requires the activity of phosphatidylinositol-3',4',5'-trisphosphate (PI(4,5,5)P3) in the phagosome membrane. Macrophages treated with inhibitors of PI(3)K inhibit actin polymerization and pseudopod extension but fail to close phagosomes. The relationship between PI(3)K activity and the regulation of the Rho-family GTPases Cdc42, Rac1 and Rac2, essential regulators of the actin cytoskeleton and phagocyte oxidase complex, during phagocytosis has not been examined. We hypothesized that PI(3)K activity initiates a signal transition at the phagosome that coordinates the deactivation of one set of GTPases (including Rac1 and Cdc42) with the activation of a second set of GTPases (including Rac2). To determine how PI(3)K coordinates the activity of these GTPases during phagocytosis, we examined the coordinated GTP-changes that occurred during the transition from Cdc42, Rac1 and Rac2 to Cdc42, Rac1 and Rac2. In Cdc42, Rac1 and Rac2, we measured the increase in Cdc42 and Rac2 GTPase activity and decrease of Rac1 GTPase activity. In Cdc42, Rac1 and Rac2, we measured the increase in Cdc42 and Rac2 GTPase activity and decrease of Rac1 GTPase activity. These results suggest that PI(3)K-dependent Cdc42 GAP activity is required to initiate the signal transition, and that PI(3)K activity integrates FcγR signals during phagocytosis.

295 Coordination of Microtubules and the Actin Cytoskeleton Is Important during Ruffle Formation in Macrophages
P. C. Patel, R. Harrison; Department of Life Sciences, University of Toronto at Scarborough Campus, Toronto, ON, Canada

The exposure of cells to a variety of external signals causes rapid changes in plasma membrane morphology. Treatment of macrophages phorbol myristate acetate (PMA) rapidly induces the formation of conspicuous membrane ruffles, which can be visualized by phase and DIC microscopy as waves arising at the leading edge of lamellipodia that move centripetally towards the main cell body. Although ruffles have been studied in the context of macroopinocytosis for decades, ruffle formation and the mechanisms leading to their formation remains largely unknown. It is known that membrane ruffle formation, is highly dependent on the actin cytoskeleton but it remains unclear to what degree microtubules contribute to this process. We studied the interaction between microtubules and the actin cytoskeleton in ruffle formation. The treatment of macrophages with cytochalasin D, an F-actin-depolymerizing reagent, inhibited the process of ruffle formation and macroinocytosis, which was visualized by immunofluorescence microscopy, live DIC analysis and SEM. The role of the microtubule cytoskeleton in ruffle formation was tested using 10μm of nocodazole to disrupt microtubule polymerization. We found that MT depolymerization impaired membrane ruffles, which was reversed following nocodazole washout. Interestingly, treatment of macrophages with the microtubule stabilizing drug taxol resulted in ruffle formation in the absence of phorbol ester activation of PKC. These results were confirmed by live imaging, immunofluorescence and SEM. These findings suggest that coordination of the actin cytoskeleton and microtubules is important for the ruffle formation. Thus it appears that ruffle formation is a microtubule-dependent process and is likely facilitated by the actin reorganization.

296 Intracellular Trafficking in Activated Macrophages
M. G. Binker, R. E. Harrison; UTSC, Toronto, ON, Canada

Classically, macrophage activation is a process developed in response to different inflammatory and or infectious conditions. Several stimuli including Interferon-gamma (IFN-γ) and Lipopolysaccharides (LPS) are known to activate macrophages. As a consequence of this novel process, resting macrophages undergo several changes including microtubule cytoskeleton reorganization. The specific function of this change is unclear. Using acetylated-α-tubulin as a marker, we found a significant increase in the stabilization of microtubules when resting RAW 264.7 cells were activated through both IFN-γ and LPS alone or in combination. By confocal microscopy, an intimate contact was observed between these acetylated-microtubules and vesicles of the endocytic pathway characterized by the presence of Rab5 and Rab7 on its membranes. Moreover, Rab7-Interacting Lysosomal Protein (RILP) was present in large vesicles colocalizing with “the core” formed by these stable microtubules. We also noticed a sizeable difference in phagocytosis in activated macrophages. Based on our results, we hypothesize a differential trafficking between the cellular cortex and the microtubule organizing center (MTOC) when macrophages are activated.

297 Microtubule Dynamics and Microtubule-based Motors Contribute to the Formation of a Specialized Endocytic Compartment in Cells Infected with Salmonella Typhimurium
T. Dam, T. A. Schroer; Biology, The Johns Hopkins University, Baltimore, MD

Upon entering the host cell, the intracellular pathogen Salmonella typhimurium traffics through the endocytic pathway until it reaches a late endocytic compartment known as the Salmonella-containing vacuole (SCV). Bacterial-effectors proteins are then delivered into and through the SCV membrane. Acting in ways that are not yet fully established, these effector proteins cause dramatic changes in the structure and dynamic behavior of this late endosome-derived-derived. Interaction with other organelles is altered and the structure extends elongated, interconnected membranous tubules known as Salmonella-induced filaments (SIFs). SIF formation is known to be microtubule dependent. We have used fluorescent protein (FP) technology to image SIF formation in living HeLa cells. We observe SIFs to be extremely dynamic structures that can extend, branch, and rapidly retract. Low doses of taxol or nocodazole, conditions that suggest microtubule dynamics do not cause loss or rearrangement of the microtubule cytoskeleton, interfere with SIF formation, suggesting that dynamic microtubules play a role in tubule extension. The plus end-directed, microtubule-based motor, kinesin-2, is also required for normal SIF formation. These findings suggest that SIF formation is driven by the combination of kinesin-2 dependent movement of membrane tubules along microtubules and the association of these membranes with the ends of growing microtubules.
Epithelial Cytoskeletal and Cell Surface Response to Pathogenic Fungi

M. Sabanero,1 G. Sandoval-Bernal,2 A. Perez,2 G. Barbosa-Sabanero,3 K. Barbosa-Sabanero,3 V. Tsutsui Fujiyoshi,3 1Instituto de Investigación en Biología Experimental, Universidad de Guanajuato, Guanajuato, Mexico, 2Facultad de Medicina, Universidad Nacional Autónoma de México, México, México, Mexico, 3Instituto de Investigaciones Médicas, Universidad de Guanajuato, Guanajuato, Mexico, 4Patología Experimental, Centro de Investigación y de Estudios Avanzados - IPN, México, Mexico

Changes in the organization of the cell cytoskeleton during the interaction with the microbial and other organisms are complex and varied, and much still remains to be elucidated, specially in terms of the molecules that signal and bring about the reorganizations that are often observed. In this work, we developed a cell culture model of infection of polarized epithelia cultured yeasts of Sporothrix schenckii (S.s) on fibroblasts monolayer and examining changes in cellular morphology and organization of cytoskeleton induced by S. s. infection. The results show that the yeasts of S.s are physically associates with epithelial surface, the adhesion of yeast are time depend i.e. 5h - 31%, 24h - 47%. We monitored changes induced by S.s infection in the organization of cytoskeleton by immunofluorescence with beta tubulin antibodies. We observed that after infection the microtubules (MT) distribution are dramatically altered. Tubulin is visible as small dots or a few MT. Both the dots and end of MT are associated with the cortex membrane. Long time (48h-72h) infection on epithelial cells results in displastic changes in cellular morphology with alterations of monolayer continuity and cell - cell junctions. SEM revealed budding and blebs on epithelial cell surface during the infection by S.s yeast. These results suggest that the interaction of the pathogen with host cell generate an uptake signals that are able to alter the host cytoskeletal and cell surface. It is possible that pathogens induce modulation of different host cell pathways in order to evade host defenses and to faster their own proliferation. Preliminary results indicate that four glycoproteins are involved in the adhesion of pathogens to epithelial cells. The work was supported by the Universidad de Guanajuato. (Grant No. 18 to MSL)

Characterization of Membrane-associated APC Pools in Motile versus Non-Motile Epithelial Cells

K. A. Siemers,1 H. Y. Caro-Gonzalez,2 W. J. Nelson,2,3 A. I. M. Barth; 1Molecular and Cellular Physiology, Stanford School of Medicine, Stanford, CA, 2Department of Biological Sciences, Stanford University, Stanford, CA

The adenomatous polyposis coli (APC) protein has an important role in directed cell migration and localizes to the tip of extending membranes in response to growth factor or integrin-mediated signals. Here we used a biochemical approach to analyze the association of APC and its binding partners with membranes in motile and non-motile epithelial cells. In non-polarized motile Madin Darby Canine kidney (MDCK) epithelial cells, homogenized under conditions that disrupt the microtubule cytoskeleton, the majority of APC and its binding partners Asef and β-catenin were recovered in the membrane fraction whereas tubulin was cytosolic. To further analyze the different cytosolic and membrane-associated APC pool we used a self-forming iodixanol density gradient. APC was enriched in two membrane fractions: a lower density membrane fraction that co-distributes with E-cadherin and a higher density membrane fraction that co-distributes with Asef, IQGAP and Par-3. In polarized non-motile MDCK epithelial cells the majority of APC was enriched in the lower density membrane fraction with E-cadherin. Disruption of cell-cell adhesion causes a shift of APC from this lower density “E-cadherin”-membrane fraction into the higher density “IQGAP/Asef”-membrane fraction. These results indicate that APC complexes that distribute in these higher density fractions may have a role in cell motility.

Deciphering the Relationship between Bik1p (CLIP-170) Localization and Function in Yeast

F. Caudron, A. Andrieux, D. Job, C. Boscheron; INSERM, Grenoble, France

We have previously demonstrated impairment of Bik1p localization at microtubule ends in yeast cells expressing Glu tubulin, with resulting defects in spindle motion. Here, in Glu tubulin cells Bik1 deletion further suppressed Bik’s localization at microtubule ends, where it became undetectable. However, Bik1 deleted Glu tubulin clones were fully viable, which was surprising since a double Bik1 deletion was lethal and since Bik1p function is thought to be intimately related to association with microtubule ends. We provide evidence for an unexpected conservation of dynactin localization at microtubule ends in Bik1 deleted Glu tubulin clones. Surprisingly, additional deletion of the kinesin Kip2p was still compatible with cell viability despite suppression of both dynactin association with microtubule ends and detectable interactions of astral microtubules with the cell cortex. In Bik1/Kip2p deleted Glu tubulin spindles, spindle positioning was achieved through a new mechanism in which the spindle elongated in the mother cell and was orientated through apparent geometrical constraints. Thus, Bik1p can mediate both dynactin positioning at microtubule ends and cell division without detectably accumulating at microtubule ends.

Characterization of a Novel Protein, Linking Membrane and Microtubules, “CLIP-76”

G. T. Ugrinova, A. J. Martin, H. Y. Goodson; Chemistry and Biochemistry, University of Notre Dame, Notre Dame, IN

The microtubule cytoskeleton plays a central role in cell organization and membrane traffic in higher eukaryotes. Cytoplasmatic linker proteins (CLIPs) have been implicated in both membrane-microtubule interactions and regulation of microtubule dynamics. We are studying two vertebrate-specific proteins related to CLIP-170. One of them, CLIP15-59 localizes to the Golgi apparatus and is involved in the control of early endosome/TGN transport. (JCB, 156(4), 631-642, 2002). We are presently focusing on the second protein, CLIP76. At least two alternatively spliced isoforms of CLIP76 exist. Both contain four ankyrin-like repeats at the N-terminus. One is widely expressed and has three CAP GLY motifs, while a second (CLIP76T) has two CAP GLY motifs and may be tissue-specific. Serial deletion mutants of CLIP76 have been placed into mammalian and bacterial expression vectors to allow characterization of its domains in vivo and in vitro. Full-length CLIP76 localizes to the endoplasmic reticulum (ER) and alters ER morphology at high expression levels. Detailed serial deletion analysis of CLIP76 showed that the information necessary for ER targeting resides within a domain containing the second CAP GLY motif and the serine-rich region. Overexpression of mutants containing only the CAP GLY motifs strongly perturbs ER and ER-Golgi intermediate compartment (ERGIC) morphology, causing strong colocalization with microtubules. Preliminary data show that VSVG transport between ER and Golgi is inhibited in cells expressing these mutants. These data suggest a role for CLIP76 in membrane transport, specifically ER-Golgi transport. Ongoing studies will further investigate the effects of CLIP76 mutations on their fragments on membrane transport in vivo and on microtubule assembly in vitro.

In Vitro Model Systems for Studying Tissue Impedance

J. P. Frampton,1 M. R. Hynd,2 A. Gupta,2 C. Bjornsson,2 G. Lin,2 B. Roysam,2 W. Turner;2 SUNY Albany, Albany, NY, 1Wadsworth Center, Albany, NY, 2Rensselaer Polytechnic Institute, Troy, NY

Mammalian cell culture typically is performed on planar substrates. While such strategies allow observations of individual cells that are difficult to make in vivo and have provided information on dynamic cellular processes, cells cultured in two-dimensions behave differently from cells in their native three-dimensional (3-D) environments. Functionalized alginate scaffolds were used to make culture systems for 3-D systems. Astroglia and neurons were used. Tissue impedance spectroscopy and biochemistry were used to describe organization of 3-D tissue constructs. Impedance spectroscopy provides a real time method for assessing organization in living cultures. Immunocytochemistry and 3-D imaging, and image analysis permit mapping of cells within scaffolds. We will correlate impedance spectroscopy and image analysis data to determine how cell density, cell type, and changes in cell morphology contribute to tissue impedance spectra. These data can be used to test computer-generated models for predicting changes in cell and tissue organization following tissue damage or responses to infection or disease. Alginate was functionalized by covalent attachment of a GRDGR peptide using aqueous carbodiimide chemistry. 3-D hydrogel constructs were created by suspending cells at various densities in alginate solutions. Alginate was polymerized by exposure of gel solutions to 200 mM CaCl2 through MilliPore tissue culture inserts. Hydrogels were polymerized on planar microelectrode arrays and the thickness of the 3-D construct was controlled by fixing the total volume of the cell-alginate mixture. Cell densities were varied from 1000 cells/μl to >20,000 cells/μl, thus approaching estimates of cell densities in the brain. Computer reconstructions of cell distribution in the in vitro 3-D constructs were created and compared to similar reconstructions of brain tissue where astrocytes, microglia, neurons, and vascular elements are mapped. Work was supported by the Nanobiotechnology Center (NBTC), an STC program of the NSF under Agreement Number ECS-9876771, CenSyS (NSF), NIH, NS044287, and CNCT (EB002030).
Tumor Cell Motility in 3D Matrices: Parsing the Roles of Proteolysis, Adhesion, and Mechanics
M. H. Zaman, D. Lauffenburger, P. Matsudaira; Biomedical Engineering, University of Texas at Austin, Austin, TX, Bioengineering, MIT, Cambridge, MA, Biology, Whitehead Institute, Cambridge, MA

Cell migration on 2D surfaces is governed by a balance between counter-acting tractive and adhesion forces. While receptor-ligand interactions and signaling through adhesion complexes have been studied in detail in a 2D context, the critical biochemical and biophysical parameters that affect cell migration in 3D matrices have not been quantitatively investigated. Using a combination of high-resolution and high-throughput imaging, rheological measurements and novel computational modeling, we demonstrate that in addition to adhesion and tractive forces, matrix stiffness is a key factor that influences cell movement in 3D. Our 3D migration assays with varying concentrations of the matrix, fibronectin and integrin activity shows that the migration speed of DU-145 human prostate carcinoma cells is a balance between tractive and adhesion forces. Unlike the 2D systems, where migration is a balance between adhesion ligand concentration and integrin activity, we note that in 3D systems, matrix stiffness and structure plays an equally important role, along with adhesion and integrin expression, in regulating motility. These novel observations have also been predicted by a recent force-based computational model of cell movement in a 3D matrix (Zaman et al., Biophys. J. [2005]). Additionally, 3D motility through an extra-cellular environment of pore size much smaller than cellular dimensions does depend on proteolytic activity as broad-spectrum MMP inhibitors limit the migration of DU-145 cells and also HT-1080 fibroswroma cells. Our hybrid approach, combining high-resolution experimental and computational techniques demonstrate an original discovery of a novel set of balances of cell and matrix properties that govern the ability of tumor cells to migrate in 3D environments. Reference: Zaman et al, Proc. Natl. Acad. Sci. USA. 2006, 103; 10889-10894. Zaman et al, Biophysical Journal, 2005. 89; 1389-1397.

Probing the Role of Extracellular Matrix Composition and Tensional Homeostasis in Single Mammary Epithelial Cells by Atomic Force Microscopy
J. Alcaraz, C. Nelson, V. Spencer, R. Xu, H. Mori, C. Bustamante, M. Bissell; Cell and Molecular Biology, Lawrence Berkeley National Laboratory, Berkeley, CA, Physics and Cell and Molecular Biology, Howard Hughes Medical Institute and University of California, Berkeley, CA

The extracellular matrix (ECM) has profound influence on cellular phenotype. Although the mechanism by which ECM integrates form and function are largely unknown, recent findings suggest that ECM may control cellular phenotype by modulating intracellular tension as part of a mechanical homeostasis mechanism. To dissect how ECM regulates endogenous tension in relation to function, we cultured single mammary epithelial cells on top of gels with different composition but similar stiffness comparable to what normal cells experience in vivo. Using atomic force microscopy we measured how single cells store and dissipate mechanical energy, processes that are related to intracellular tension and structural disorder, respectively. We found that ECM composition rather than gel stiffness or cell shape per se controls intracellular tension. Functional differentiation, assessed by β-casem protein expression, was modulated by tension in a biphasic manner. Moderate levels of tension, mediated by binding of β1-containing integrin to the basement membrane protein laminin, correlated with β-casem expression and enhancement of β-casem protein. In contrast, β-casem was down-regulated by conditions that elicited either low or high mechanical tension. Intriguingly the structural disorder remained essentially stable for all substrata except basement membrane ECM gels, even after blocking binding of β1 and of-and containing integrins to laminin, thereby suggesting that structural disorder is regulated by non-integrin laminin receptors. Our findings indicate that ECM integrates cell shape, intracellular tension, and structural disorder through different receptors, and suggest that these signals contribute to cell function in fundamentally different ways. Specifically our data suggest that a moderate level of tension, transduced by β1-containing integrins, is necessary for functional differentiation.

The Use of Surface Protein Micropatterning for Directing Neural Cell Growth on a Novel Biomimetic Surface
M. R. Hynd, J. F. Frampton, N. M. Dowell-Mesfin, J. N. Turner, W. Shan; Department of Health, Wadsworth Center, Albany, NY, Department of Biomedical Sciences, SUNY at Albany, Albany, NY

Critical to the development of patterned neural networks is the ability to control the spatial localization of molecules on biocompatible materials. Hydrogels are a class of synthetic, non-toxic, hydrophilic polymers well-suited for use in biomedical applications. Here we describe a method for patterning of hydrogel surfaces to control and direct neural cell growth and attachment. A novel approach was used to create streptavidin-functionalized hydrogel surfaces capable of binding biotin-labelled molecules. Hydrogels were formed by co-polymerization of an aqueous solution of acrylamide, poly(ethylene glycol) diacrylate and streptavidin-acrylamide. Microcontact printing (µCP) was used to pattern the biotinylated proteins, laminin and fibronectin, and the laminin epitope (biotin-IKVAV), onto hydrogel surfaces. Proteins were patterned using PDMS stamps with patterns corresponding to either 10 μm-wide relief structures and 90 μm gap spacings or orthogonal 2 μm-wide lines connecting 15 μm diameter nodes, with a repeat spacing of 150 μm. As a biological assay, LRM55 astroglia and primary rat hippocampal neurons were plated on patterned hydrogels. LRM55 cells were found to selectively adhere to regions patterned with biotin-conjugated proteins. After 24 hours, LRM55 cells were well spread along the patterned regions and exhibited process-bearing morphologies. Primary rat hippocampal neurons on patterned hydrogels were found to attach predominantly to the 15 μm nodal regions of the stamped grid pattern, with few cells attaching to the orthogonal 2 μm-wide lines. Neurons were found to self-organize into networks by 10 days and develop functionally active synapses as measured by uptake of the dye, FM 1-43FX. Neurons survived on peptide-modified hydrogel surfaces for at least 4 weeks. Results from this study suggest that hydrogel surfaces can be patterned with multiple proteins to direct cell growth and attachment. This work was supported in part by the Nanobiotechnology Center (NBTC), an STC Program of the National Science Foundation under Agreement No. ECS-9676771.

Adhesion and Proliferation of Primary Hepatocytes on Synthetic Peptides
N. Takahashi, Y. Kikkawa, M. Nomizu; Tokyo Univ. of Pharm. and Life Sci., Hachioji, Japan

Liver transplantation is a curative treatment for hepatic dysfunction such as cirrhotic liver and hepatocellular carcinoma. However, it is limited by severe shortage of donors. Therefore, a bioartificial liver is expected to provide the critical hepatic functions. Design of effective biomaterial for hepatic adhesion is important for development of a bioartificial liver. Previously, we have identified 20 cell adhesive sequences in the mouse laminin-111, a multifunctional glycoprotein in basement membranes, using 673 synthetic peptides. Here, we screened hepatocyte-adhesive peptides using the 20 cell adhesive peptides to apply as a biomaterial for development of bioartificial liver. Rat hepatocytes were isolated by two-steps collagenase perfusion and used for cell adhesion assay. When we tested the 20 synthetic peptides in a hepatocyte adhesion assay, only two peptides, A13 (RQVFGQAYIYIK, mouse laminin α1 chain residues 121-133) and C16 (KADFITYYRLK, mouse laminin γ1 chain residues 139-150) exhibited the activities in a dose-dependent manner. The morphology of hepatocytes on the A13- and C16-coated plates was similar. Further, we tested inhibitory effects of EDTA and heparin on cell adhesion activity of the peptides to characterize the cellular ligands for the peptides. Both EDTA and heparin inhibited the adhesion of the hepatocytes to the peptides, suggesting that the cellular interaction was dependent on a divalent cation and potentially interact with heparin/heparan sulfate proteoglycans. We also evaluated the effect of the A13 and C16 peptides on proliferation of hepatocytes. The C16 peptide promoted DNA synthesis in hepatocytes but A13 did not affect, suggesting that the cellular effect of the peptides was different. These results suggest that the A13 and C16 peptides could control the cell attachment and proliferation of hepatocytes. The peptides have a potential to maintain hepatic function in long-term culture and may be useful for development of bioartificial liver as a biomaterial.

Fibronectin Heparin III Binding Domain (III4-5 Repeats) Is Involved in Fibronectin Fibrillogenesis
A. Maqueda, J. V. Mooyan, D. M. Peters, A. Garcia-Pardo; Immunology, Centro de Investigaciones Biologicas, CSIC, Madrid, Spain, Department of Pathology and Laboratory Medicine, University of Wisconsin, Madison, WI

Fibronectin (Fn) is an important component of the extracellular matrix, from where it regulates cell adhesion and migration, growth, and differentiation. Fn matrix assembly involves interactions among various regions of the molecule, which contribute to elastogen and stabilization of the fibrils. We previously showed that the heparin II domain of Fn (repeats III12-14) is involved in Fn fibrillogenesis (Bultmann et al., J. Biol. Chem. 273: 2601, 1998). Because of the functional similarities between Fn heparin-binding domains II and III (repeats III4-5), we have now studied whether the heparin III region participates in Fn fibrillogenesis. The recombinant fragment FNI11-5 blocked Fn fibril formation and 121-Fn incorporation into fibroblast cell layers, as effectively as the amino-terminal domain of 20 kDa fragment, a well known competitor in these assays. Binding assays using a biosensor revealed that FNI11-5 bound to Fn and to the amino-terminal receptors of 70 kDa and 29 kDa. FNI11-5 also bound to itself, indicating that the fragment containing sequences in FNI11-5, KLDAPT(II)2 site and WTPPRA...TGYRL...TGLTR (HBP/III5 site) (Mooyan et al., J. Biol. Chem. 274: 135, 1999), were involved in these interactions and in the matrix inhibitory effect, we mutated specific amino acids in the II and II5 sites and studied their functional effect. While mutations on the II site had no effect, mutating the first two arginine residues in HBP/III5 abolished the ability of FNI11-5 to bind to itself, Fn or 70 kDa. In contrast, the last two arginine residues in HBP/III5 were required for the matrix inhibitory effect.
activity of FNIII4-5, and for blocking 125I-29 kDa incorporation into cell layers. Altogether these results established that IIIA-5 repeats constitute a novel Fn region involved in self-association and Fn fibrogenesis, and that both functions require different arginine residues in the HBP/III5 sequence.

308 The Homogeneous Mutant Collagen in Brit/Mov Compound Mice Is Associated with a Milder Osteogenesis Imperfecta Phenotype Than Occurs in Heterozygous Brit/+ Mice, Despite Increased Matrix Insufficiency Due to the Mov13 Null Allele

T. E. Uveges,1 J. A. Meganck,1 E. L. H. Daley,1 S. A. Goldstein,1 J. C. Marin1; 1BEMB, NIH/NICHD, Bethesda, MD, 2Ortho Res Lab, University of Michigan, Ann Arbor, MI

The Brit mouse (Brit/+), a model for dominant-negative type IV osteogenesis imperfecta (OI), has a glycine substitution (G349C) in one col1a1 allele and a heterogeneous population of collagen forms, with 0, 1 or 2 mutant α1(I) chains. Surprisingly, mice homozygous (Britl/Britl) for this dominant mutation have a milder phenotype, with growth, BMD, and femoral geometry and mechanical properties intermediate between wt and Britl/+.

Britl/Britl synthesize homogenous collagen with only mutant α1(I) chains (Forlino, 2005 EJHG 13(S1):61-65:C12), suggesting that heterogeneity of type I collagen may be an essential factor in OI severity. To examine collagen homogeneity by an alternative route; we crossed Britl/+ with Mov13/+ mice, which have a null col1a1 allele. Analyses of the offspring indicate that Britl/Mov mice also have a milder phenotype than Britl/+ mice. Britl/Mov, like Britl/Britl, has normal perinatal survival vs 30% lethality in Britl/+.

On skeletal staining, Britl/Mov and Mov13/+ do not have the in vitro rib fractures of Britl/+.

Growth curves of Britl/Mov and Mov13/+ are intermediate between wt and Britl/+.

At 2 months of age, Britl/Mov and Mov13/+ dermal collagen fibril diameters were significantly smaller than Britl/+ or wt fibrils. Britl/Mov fevms have normal BMD and the same geometric adaptations found in Mov13/+ (increased cross-sectional area and cortical thickness) rather than the improved material properties found in Britl/Britl. Britl/Mov fevms also have improved mechanical properties (energy to failure and brittleness) compared to Britl/+. Since Britl/Mov femoral geometry and mechanics resemble Mov13/+, we cannot rule out the possibility that the type I collagen insufficiency associated with the mov13 allele is disorganizing the matrix in a way that masks the weakening effect of the structurally abnormal collagen. However, the data on Britl/Mov and Britl/Britl are most consistent with the interpretation that a homogeneous population of mutant collagen is associated with a milder OI phenotype.

309 Annexin V / Collagen Interactions Regulate Mineralization of Growth Plate Chondrocytes

H. Kim, T. Kirsch; University of Maryland, Baltimore, MD

Mineralization of growth plate cartilage is crucial for normal bone formation. Therefore, an understanding of the mechanisms regulating mineralization is important. We have shown that an annexin (II, V and VI) form Ca2+ channels in growth plate chondrocytes enabling the initiation of mineralization. In addition, binding of annexin V to type I, II, and X collagen stimulates annexin V Ca2+ channel activities. In this study, we hypothesized that type II or type I collagen matrix stimulates mineralization of growth plate chondrocytes via binding and activating annexin V Ca2+ channels. To test this hypothesis, we treated growth plate chondrocytes with vitamin C to stimulate mineralization events in the absence or presence of dihydroxyproline, which inhibits collagen fibril formation, and measured intracellular Ca2+ concentration (Ca2+), alkaline phosphatase activity and the degree of mineralization. On the other hand, we cultured growth plate chondrocytes on type I or II collagen coated dishes. Vitamin C stimulated type II and X collagen synthesis, alkaline phosphatase activity and mineralization of growth plate chondrocytes, whereas dehydroxyproline inhibited the stimulation of these events in vitamin C-treated cells. Vitamin C also led to an increase of [Ca2+]i in growth plate chondrocytes, whereas dehydroxyproline inhibited this increase. Flow cytometric analysis and cell attachment assays revealed that ~ 40% of annexin V were located on the outer membrane surface and that cell attachment of growth plate chondrocytes to type II and type I collagen was inhibited with annexin V specific antibodies. On the other hand, culturing growth plate chondrocytes on type I or II collagen coated dishes stimulated their phosphatase activity and the degree of mineralization compared to cells cultured on culture dishes. In conclusion, our findings reveal that type I and II collagen via binding to annexin V stimulate annexin-mediated Ca2+ influx, alkaline phosphatase activity and mineralization of growth plate chondrocytes.

310 Localization of Pdlim2 at Cell-Matrix Adhesion Sites Requires an Intact Actin Cytoskeleton and Rho Activity

A. Gatesman-Ammer, B. M. Helfer, D. C. Flynn, S. M. Frisch; Mary Babb Randolph Cancer Center, West Virginia University, Morgantown, WV

The homogeneous mutant collagen in Britl/Mov compound mice is associated with a milder osteogenesis imperfecta phenotype than occurs in heterozygous Britl/+ mice, despite increased matrix insufficiency due to the Mov13 null allele.

Pdlim2 cDNA, encoding a protein containing an N-terminal PDZ motif and a C-terminal LIM domain, was previously identified in a rat eye iridocorneal angle cDNA library. It is highly expressed in the corneal epithelium and is associated with α-granules, filamin A, myosin VI, and MYH9.

In the present study, we further explore its potential involvement in cell adhesion, motility, migration, and actin cytoskeleton in Human Corneal Limbal and Epithelial cells (HCL) by Total Internal Reflection Fluorescence Microscopy (TIRFM).

The results showed that GFP-Pdlim2 was specifically localized to cell-matrix adhesion and actin attachment sites at the periphery of cells grown on fibronectin. Cell controls transfected with GFP only did not acquire any specific TIRF signals. Cytochalasin D treatment completely obliterated Pdlim2-associated structures at the cell periphery, while treatment with C3, a Rho family inhibitor, changed the distribution of Pdlim2 from cell adhesion sites to lamellipodia. Immunofluorescence showed co-localization of Pdlim2 with actin cortical filaments and phospho-S19 myosin light chain regulatory chain (MLC II) at the cell periphery. We also identified active Rho, Pdlim2, phospho-S19 MLC II, and β1 integrin in protein complexes isolated by Rhoptenkin RBD affinity chromatography.

Consistent with these findings, immunofluorescence of corneal whole mounts detected Pdlim2 protein only in the corneal epithelium in association with actin cytoskeletal structures. These findings demonstrate that localization of Pdlim2 at cell adhesion sites requires an intact actin cytoskeleton and Rho activity, and suggest that Pdlim2 plays a role in corneal epithelial cell adhesion and migration.

311 Caspase-2 Regulates Cell-Matrix Adhesion

B. M. Helfer, A. Gatesman-Ammer, D. C. Flynn, S. M. Frisch; Mary Babb Randolph Cancer Center, West Virginia University, Morgantown, WV

Caspase-2 plays important roles in DNA damage-induced, detachment-induced, and heat shock-induced apoptosis. Additional biologic functions are likely; while the developmental phenotype of caspase-2 knockout mice is normal, it is often the case that the same gene knockout examined in different mouse strains has varying effects. Here, we demonstrate that caspase-2 has additional roles in the cell beyond apoptosis. Caspase-2 promotes cell-matrix adhesion and protects the fidelity of cytoskeleton as well as affecting cell motility. Mechanisms for the regulation of cytoskeleton by caspase-2 are being explored and will have important ramifications for normal and tumor cell behavior.

312 Laminin 411 Expression and Localization in Human Erythromegakaryocytic Cell Lines and Blood Platelets

S. Ingerup,1 T. Geberthow,2 E. Junoren,1 M. Pook,1 M. Meiesar,1 T. Mainets,1 M. Patartoy,2 1Institute of Molecular and Cell Biology, University of Tartu, Tartu, Estonia, 2Department of Odontology, Karolinska Institute, Stockholm, Sweden, 3Institute of General and Molecular Pathology, University of Tartu, Tartu, Estonia

Laminins are a family of heterotrimeric proteins with cell adhesive and signaling properties. Laminin 411 (α4β1γ1) is a major laminin isoform of blood vessels and also found in platelets, monocytes, lymphocytes and neutrophils, and mediates their adhesion via α6β1 and αMβ2 integrins. We have explored the synthesis and presence of laminin 411 in erythromegakaryocytic HEL and DAMI cells. In HEL cells, transcripts for α4, β1 and γ1 laminin chains were detected by RT-PCR. Metabolic labeling of HEL cells and immunoprecipitation of lysate with anti-β1 and γ1 chain mAbs revealed corresponding laminin subunits in precipitate. Immunofluorescence flow cytometry demonstrated reactivity of mAbs to laminin β1 and γ1 chains with permeablized cells. Confocal microscopy studies on HEL and DAMI cells have demonstrated laminin localization in cytoplasm being assembled mainly in membrane skeleton area. Abovementioned facts suggest that blood platelet laminin originates from bone marrow megakaryocytes. We have shown also that human platelets contain and following activation secrete laminin 411. The mAbs to laminin α4 chain give positive staining in resting and glass-activated human platelets as well as in bone marrow megakaryocytes predominantly to granules but also the overall cytoplasm reactivity is noticeable. However, fibrinogen matrix-activated platelets expose positive staining of laminin in the center of the cell. Rearrangements upon activation are typical to other platelet α-granule adhesive proteins which accumulate in the cell center when α-granules coalesce in the cytoplasm and fuse with the open canalicular system. We used sucrose density-gradient centrifugation to separate α-granules to reveal subcellular localization of laminin. Laminin 411 was present in the high-density fraction, which was paralleled by thrombospandin and fibrinogen, two marker proteins of α-granules, indicating association of laminin with these structures. Synthesis and expression of laminin molecules by platelets suggest a role of laminin in hemopoiesis and platelet physiology.

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313 Structural Analysis of the Plakin Domain of Bullous Pemphigoid Antigen1 (BPAG1) Shows That Plakins Are Related to Spectrins

J. J. Jefferson,1 L. Shapiro,2 R. K. Liem1; 1Pathology and Cell Biology, Columbia University, New York, NY, 2Biochemistry and Molecular Biophysics, Columbia University, New York, NY

Bullous Pemphigoid Antigen1 (BPAG1) is a member of the plakin family of proteins and was first identified as an autoantigen in patients with the skin blistering disease, bullous pemphigoid. Homozygous deletions in the BPAG1 gene result in sensory neuron authentication disorder resulting in severe loss of coordination, as well as skin fragility. The plakins are multi-domain containing proteins and each domain has been shown to have specific interaction partners, including microtubules, actin filaments and intermediate filaments, as well as junctional complexes. A common feature of the plakins is the so-called plakin domain, which serves as an important platform for interactions with protein complexes found in specialized cell junctions, such as desmosomes and hemidesmosomes. This domain has been predicted to consist of a series of alpha-helical bundles that fold into a globular domain near the N-terminus of the plakins. We have determined the crystal structure of a tetrameric alpha-helical bundle domain of BPAG1. The structure was solved by SAD phasing from a selenomethionine-substituted protein crystal at 3.0 angstroms resolution. The crystal structure shows that this protease resistant fragment consists of a pair of spectrin repeats, made up of triple helical bundles that are gently curved and wrapped around each other in a left handed supercoil and connected by an alpha-helical linker region. Based on this structure and the conservation of key residues that are important for the folding and stability of spectrin repeats, as well as domain prediction algorithms, we propose that despite the absence of clear sequence similarity, the plakin domain of BPAG1, as well as all other plakins, consists of two pairs of spectrin repeats interrupted by a putative SH3 domain placing the plakins in the spectrin superfamily.

314 Canine Myosin Is Labelled with Palmitic Acid

C. S. Ricard, A. Mukherjee; Ophthalmology, Saint Louis University, St Louis, MO

Objective: Myosin has been associated with primary open angle glaucoma. Normal and pathological functions of myosin remain to be elucidated. MDCK express myosin, making them a useful cell model to study myosin. The sequence of canine myosin bears close similarity with the human myosin sequence. MDCK are a well-characterized canine polarized cell line. Analysis of lipid rafts, protein trafficking to apical versus basolateral membranes, and studies in viral infection have been performed in MDCK cells. Methods: MDCK cells were confirmed as uninfected canine cells by multiple PCR at each of these time points. Type 1 collagen mRNA levels from the testes of CTGF transgenic mice were shown to be elevated at least 2-10-fold as compared to non-transgenic littermates. These results are consistent with histological examination of testes from CTGF and non-transgenic littermates using RT-PCR.

315 Integrin and Growth Factor Mediated Signaling in Erhythroproisis

S. Eshghi,1 M. G. Vogegeanz,2 R. O. Hynes,3 L. G. Griffith,4 H. F. Lodish2; 1Biological Engineering, MIT/Whitehead Institute, Cambridge, MA, 2Department of Cell Biology, Harvard Medical School, Boston, MA, 3Center for Cancer Research, MIT, Cambridge, MA, 4Biological Engineering, MIT, Cambridge, MA

Objective: The adapter protein SH2-B, which binds to the activated form of the nerve growth factor (NGF) receptor TrkA, has been implicated in neuronal differentiation as well as in the survival and motility of sympathetic neurons. SH2-B enhances NGF-induced neurite outgrowth in pre-neuronal PC12 cells, while a dominant negative mutant of SH2-B lacking an intact SH2 domain required for

316 The Contribution of Connective Tissue Growth Factor (ctgf, CCN2) to Fibroblast Biology

L. Kennedy1, S. Liu,2 X. Shi,2, W. Chen,1 D. E. Carter,1 K. M. Lyons,4 C. M. Black,2 D. J. Abraham1, A. Leake1; 1CHR Group in Skeletal Development and Remodeling, University of Western Ontario, London, ON, Canada, 2Physiology, Center for Rheumatology, University College London (Royal Free Campus), London, United Kingdom, 3Biological Engineering, University College London (Royal Free Campus), London, United Kingdom

Regulation of cell behavior by both growth factor and integrin-mediated signaling is a common theme in many diverse systems. Intracellular signaling initiated by binding of erhythropein to its receptor has long been understood to be the major pathway governing erythroid development but the role of signals originating from the extracellular matrix in this system has been less rigorously studied. Here we present evidence that signaling initiated by binding of fibronectin to αⅣβ1 integrins on the surface of erythroid progenitor cells is necessary for proper erythroid proliferation. Furthermore our data suggest that a two-stage model for erythroid development, where an early Epo-dependent, integrin-independent phase is followed by an Epo-independent, integrin-dependent phase. Using flow cytometry, fetal liver erythroid progenitors were separated at four distinct stages of development based on expression of cell surface antigen. CD71 and Ter119. This system allowed us to show that αⅣβ1 integrins are developmentally regulated during erythropoiesis and that these integrins mediate adhesion to specific domains of fibronectin. An in vitro differentiation assay indicated that αⅣβ1 integrins are necessary for terminal erythroid proliferation but only after an early Epo-dependent phase. This work not only supports the view that normal erythroid development is achieved through signaling pathways initiated by αⅣβ1 integrin in addition to the erythropein receptor but also provides new insight to the more general integration of signals from growth factor and environmental cues in development.

317 Connective Tissue Growth Factor (CTGF) Stimulates Type I Collagen mRNA Synthesis In Vivo

Z. Zhou, P. A. Harding; Zoology, Miami University, Oxford, OH

Connective tissue growth factor (CTGF/CN2) is a member of the CCN family of proteins whose biological properties include cellular proliferation, adhesion, extracellular matrix production, migration, and is implicated in fibrosis. The goals of this study are to understand the biological actions of CTGF/CN2 using a transgenic mouse model. Transgenic mice were generated using a pIRES-EGFP expression vector using a bicistronic mRNA containing both human CTGF cDNA and enhanced green fluorescent protein (EGFP) coding sequences under the regulation of the cytomegalovirus immediate-early (CMV-IE) promoter. Immunohistochemical examination of testes from 8-week CTGF transgenic and non-transgenic littermates using an anti-human CTGF C-terminal peptide antibody exhibited recombinant human CTGF expression in both the interstitium and sperm in testes from CTGF transgenic mice. In contrast, no immunoreactive CTGF was observed in the testes from non-transgenic littermates. Quantitative real time RT-PCR was employed to determine the TGFβ1 mRNA levels testes from CTGF transgenic and non-transgenic littermates at 2, 4, 6, and 8 weeks of age. Results demonstrated no significant differences in the TGFβ1 mRNA levels between CTGF transgenic and non-transgenic mice. Similarly, RT-PCR was taken to investigate the expression levels of type 1 collagen mRNA at each of these time points. Type 1 collagen mRNA levels from the testes of CTGF transgenic mice were elevated at least 2-10-fold as compared to non-transgenic littermates. These results are consistent with histological examination of testes from CTGF transgenic and non-transgenic littermates using Masson-Trichrome in which collagen was observed within the interstitium of testes from CTGF transgenic mice. However, no collagen was detectable in the interstitium of testes from non-transgenic mice. Taken together, we have generated a model to examine testicular fibrosis using a CTGF transgenic mouse model in which recombinant human CTGF is expressed in the testis.

318 The Adapter Protein SH2-B Regulates a Subset of Nerve Growth Factor-induced Gene Expression and Neuronal Invasiveness

L. Chen, Ryunan, National Institute of Medical Science, Tsing Hua University, Hsinchu, Taiwan

The adapter protein SH2-B, which binds to the activated form of the nerve growth factor (NGF) receptor TrkA, has been implicated in neuronal differentiation as well as in the survival of sympathetic neurons. SH2-B enhances NGF-induced neurite outgrowth in pre-neuronal PC12 cells, while a dominant negative mutant of SH2-B lacking an intact SH2 domain required for recruitment to TrkA, SH2-B(R555E), blocks it. SH2-B undergoes nucleocytoplasmic shuttling suggests that SH2-B may regulate the expression of genes that determine neuronal
differentiation. We used Affymetrix gene array analysis to compare gene expression profiles of NGF-treated and control cells expressing GFP, GFP-SH2-Bj or GFP-SH2-Bjl (R555E). Multiple NGF-responsive genes were upregulated by SH2-Bjl but not SH2-Bjl (R555E), including genes encoding urokinase plasminogen activator receptor (uPAR), matrix metalloproteinase 3 and 10 (MMP3 and MMP10). In addition, NGF-induced activation of MMP3 and/or 10 was increased in cells expressing GFP-SH2-Bjl but reduced in cells expressing SH2-Bjl (R555E) compared to control cells. uPAR, MMP3 and MMP10 regulate intracellular signaling and the interaction between the cell and extracellular matrix (ECM), which is continuously regulated during differentiation. uPAR has been shown to be required for neuronal differentiation of PC12 cells. MMP3 has been shown to maintain growth cone invasiveness, which is important for neurite outgrowth, in PC12 cells. Consistent with SH2-Bjl upregulating MMP3 and MMP10 and stimulating their function, overexpression of SH2-Bjl enhanced NGF-induced invasiveness of PC12 cells growing on Matrigel. NGF-induced invasiveness was reduced by an inhibitor of MMP, tissue inhibitor of MMP2 (TIMP2). Thus, the increased invasiveness in SH2-Bjl overexpressing cells is likely a result of increased secretion of MMP3 and MMP10, which degrade the ECM and allow differentiating cells to penetrate the Matrigel. Taken together, these results suggest that SH2-Bjl enhances NGF-induced gene expression of uPAR, MMP3 and MMP10. We also identify a new function of SH-B in neuronal invasiveness.

Identification of Src and Fak as Key Intermediates in Integrin and Trk Activated Signaling Pathways in the Stimulation of Neurite Growth in Adult NGF-Responsive DRG Neurons

B. A. Tucker, M. Rahimtula, K. M. Mearrow, Memorial University of Newfoundland, St. John's, NF, Canada

Axonal regeneration is predominantly influenced by factors located in the neuron's extracellular environment, and includes neurotrophins, such as NGF, and adhesion molecules, such as laminin. As we have previously reported, the provision of both NGF and laminin to adult NGF-responsive DRG neurons results in optimal levels of neurite growth not achievable by either factor alone. In this study, we were interested in determining the key points of collaboration between laminin and NGF induced neurite growth. Pharmacological inhibition of specific signaling components were performed, and early neurite growth and signalling analysis was conducted. Adult NGF-responsive DRG neurons were treated with NGF, laminin, or laminin plus NGF for 10 min, 1 hr, or 6 hrs, and subsequently analysed. Our results indicate that inhibition of either Src or Fak activity attenuates both PI 3-K/Akt and MEK/ MAPK signalling pathways induced by laminin and/or NGF. Furthermore, inhibition of Akt blocks observed neurite growth; however, inhibition of MEK/MAPK has no significant effect. We have also shown that neither PI 3-K nor MEK/MAPK exert any influence on one another. Together, these results indicate, for the first time, that Src and Fak are key points of collaboration between NGF and laminin induced neurite growth in NGF-responsive adult DRG neurons.

Quantitative Differential Sulfation Pattern of Chondroitin Regulates Axonal Guidance

Y. Katagiri,1 H. Wang,1 T. McCooam,1 Z. Yu,1 F. Tan,1 E. Unsworth,1 P. Goldsmith,2 Y. Wang,4 A. Symes,4 H. M. Geller1; 1Developmental Neurobiology Section, NHLBI, NIH, Bethesda, MD, 2Pathology Core, NHLBI, NIH, Bethesda, MD, 3NCl, Bethesda, MD, 4USUHS, Bethesda, MD

Glycosaminoglycan (GAG) side chains endow extracellular matrix proteoglycans with an unparalleled diversity of biological regulatory properties based upon the length, composition, and charge distribution of the polysaccharide chain. A “sulfation code” hypothesis has been proposed in heparan sulfates which states that distinct patterns of sulfation along the polysaccharide chain regulated by distinct sulfotransferases encode information required for substrate binding and growth regulation. Here we provide evidence that this sulfation code in the GAG chains of chondroitin sulfate (CS) is a critical determinant of axonal guidance and neuronal growth. 4-sulfate-enriched CS-A, but not 6-sulfate-enriched CS-C, exhibits a strong negative guidance cue to mouse cerebellar granule neurons and activates RhoA, indicating that the pattern of sulfation and the net charge of the GAG chains is the molecular determinant of growth inhibition. Even partial removal of 4-sulfate from CS-A completely abolishes growth inhibition. In astrocyte-neuron co-cultures, 4-sulfated CS and chondroitin 4-sulfotransferase 1 (C4ST1) are rapidly and substantially increased upon induction of reactive astrocytes, resulting in impaired neurite growth. Parallel increase in 4-sulfated CS is observed following spinal cord injury in mice. Depletion of astrocyte C4ST1 by siRNA restores axonal growth in culture, and its overexpression renders astrocytes far less permissive for neuronal growth. These findings demonstrate the importance of the sulfation code of CS for neuronal growth regulation, and suggest that inhibition of 4-sulfation is a potential therapeutic target for intervention in central nervous system injury.

Neurite Outgrowth Is Enhanced by Laminin-mediated Downregulation of the Low Affinity Neurotrophin Receptor p75NTR

S. L. Rankin, C. S. Guy, M. Rahimtula, K. M. Mearrow, Basic Medical Sciences, Memorial University, St. John's, NF, Canada

It is recognized that the extracellular matrix (ECM) is a key factor in promoting axonal regeneration, coordinate regulating growth in conjunction with trophic signals provided by the neurotrophins, including nerve growth factor (NGF). Laminin (LN) significantly contributes to the composition of the ECM, and represents a potent inducer of neurite outgrowth in vitro. While LN-mediated mechanisms leading to enhanced growth are not defined, they likely represent a complex interplay between enhancement of permissive cues, and downregulation of those which are inhibitory. The current study investigated potential interactions between the LN and NGF-mediated signaling pathways. Differentiated PC12 cells were exposed to either neutral (poly-lysine) or permissive (LN) substrates in the presence or absence of NGF. Qualitative analysis revealed that neurite outgrowth stimulated by NGF is enhanced on a LN substrate. Western blot analysis of pertinent signal transduction components revealed both enhanced phosphorylation of early signaling intermediates and a concomitant downregulation of p75NTR, the low affinity NGF receptor. This downregulation seems to be associated with a LN-induced upregulation of PTEN, and resulted in a PURPIND decrease in Rho activity. p75NTR is a key activator of Rho signaling, which is inhibitory to neurite outgrowth. Furthermore, p75NTR is well documented for its role as the transducing element for inhibitory myelin-derived signals. These novel results demonstrate the importance of the sulfation code of CS for neuronal growth regulation, and suggest that inhibition of 4-sulfation is a potential therapeutic target for intervention in central nervous system injury.

The Role of Latent TGF-β Binding Proteins in Secretion of the TGF-β-Superfamily Ligand Myostatin

S. B. Anderson,1 A. L. Goldber,1 M. Whitman1; 1Developmental Biology, Harvard School of Dental Medicine, Boston, MA, 2Cell Biology, Harvard Medical School, Boston, MA

Myostatin is a TGF-β Superfamily ligand that negatively regulates skeletal muscle mass. Myostatin is cleaved from a proform into a mature ligand that is capable of signaling through TGF-β Superfamily receptors. Latent TGF-β Binding Proteins (LTBPs) have been shown to assist in proper folding and secretion of the canonical TGF-β ligands, but until this time a role for LTBPs in maturation of non-canonical TGF-β Superfamily ligands has not been demonstrated. In order to better understand the events surrounding myostatin maturation, we have investigated the interactions between myostatin and LTBPs. We demonstrate by co-immunoprecipitation that the proform of myostatin interacts with all four known LTBPs, LTPB 1-4. Of the four LTBPs, LTPB3 specifically interacts with a dimerized and glycosylated proform of myostatin, suggesting that LTPB3 interacts with properly folded and secreted myostatin. When LTBPs and myostatin are co-expressed in 293T cells, the newly synthesized myostatin accumulates in its proform within the extracellular matrix. Evidence will be presented about whether similar regulation of myostatin maturation occurs in adult mouse muscle.

Quantitative Studies of EGFR System Autocortic Induced Cell Signaling and Response

E. J. Joslin,1 B. S. Hendricks,1 H. Kim,1 N. Kumar,1 L. K. Oprekosko,1 H. S. Wiley2; 1Biological Engineering Division, MIT, Cambridge, MA, 2Research Technology Center, Pfizer, Cambridge, MA, 3Pacific Northwest National Lab, Richland, WA

Dysregulation of the epidermal growth factor receptor (EGFR) family and their cognate ligands has been implicated in several types of cancer. We aim to test the hypothesis that varying the rate of extracellular autocrine ligand release will yield important changes in signaling dynamics and cell behavior. We also aim to quantify the difference between autocrine and exogenous stimulation. High and low release rates of EGF are achieved experimentally using chimeric transmembrane EGF ligand precursor retrovirally transfected into a human mammary epithelial cell line. The high ligand release can be down-modulated upon addition of a metalloprotease inhibitor. We have developed a computational model in combination with our experimentally measured EGF stimulation. High and low release rates of EGF are achieved experimentally using chimeric transmembrane EGF ligand precursor retrovirally transfected into a human mammary epithelial cell line. The high ligand release can be down-modulated upon addition of a metalloprotease inhibitor. We have developed a computational model in combination with our experimentally measured EGF stimulation. High and low release rates of EGF are achieved experimentally using chimeric transmembrane EGF ligand precursor retrovirally transfected into a human mammary epithelial cell line. The high ligand release can be down-modulated upon addition of a metalloprotease inhibitor. We have developed a computational model in combination with our experimentally measured EGF stimulation.
HGF Stimulation Causes c-Met to Phosphorylate the MUC1 Cytoplasmic Tail and Facilitates Endocytosis and Nuclear Localization

P. K. Singh, R. L. Cerny, M. A. Hollingsworth; 1Eppley Institute for Research in Cancer and Allied Diseases, UNMC, Omaha, NE, 2University of Nebraska-Lincoln, Lincoln, NE

MCF10A cells, did not exhibit high levels of Erk phosphorylation. Thus, TGF-β treatment was associated with a high sustained level of Erk phosphorylation/activation which contrasted with low levels of phospho-Erk in the normal MCF10A acini. In addition, an increase in vimentin expression, a marker of EMT, was also observed in TGF-β treated acini. TGF-β treatment also impaired acinus formation from primary mouse mammary epithelial cells. Again the result was the formation of loose assemblies of non-polarized cells; and again lumen formation failed and vimentin expression was induced. However, the assemblies had a smaller mean diameter than the normal acini and, in contrast to the MCF10A cells, did not exhibit high levels of Erk phosphorylation. Thus, TGF-β treatment impairs acinus formation from two different mammary epithelial cell types by a mechanism that is probably not dependent on triggering deregulated growth factor-Erk MAP kinase signaling.

The Inhibitor of VEGF-Receptors Tyrosine Kinase PTK-787/ZK-222584 Blocks the Invasive Potential of Adult T-Cell Leukemia Cells through the Endothelium

L. E. Haddad, M. D. Migliori, J. H. Hruban, H. A. El-Khoury, Y. Kfouri, L. S. Atweh, D. H. The, O. Hermine, M. E. El-Sabbab; 1Human Morphology, American University of Beirut, Beirut, Lebanon, 2Internal Medicine, American University of Beirut, Beirut, Lebanon, 3St-Louis Hospital, Paris, France, 4Department of Clinical Hematology, Necker Hospital, Paris, France

reciprocal co-immunoprecipitations followed by western blotting. Recombinant human c-Met protein catalyzed in vitro phosphorylation of tyrosine in the YHYP motif of the a 66-residue MUC1CT as detected by tandem mass spectrometry. Stimulation of pancreatic adenocarcinoma cells with HGF, a ligand for c-Met, enhanced the incorporation of 32P in MUC1CT, and enhanced phosphorylation at tyrosine in the YHYP motif as determined by analysis with site specific phospho-enhanced clathrin-mediated endocytosis of MUC1CT and its nuclear localization, as determined by real-time confocal imaging of C-terminally GTP-tagged MUC1. There was faster turnover of MUC1 under stimulation with HGF, and overexpression of MUC1 facilitated faster turnover of c-Met in S2-013 cells. These results indicate that c-Met, serving as a kinase for MUC1, phosphorylates MUC1CT and promotes the endocytic trafficking-mediated nuclear localization of MUC1 and subsequent turnover of MUC1CT and c-Met during HGF stimulation. Given that MUC1 and Met are overexpressed in pancreatic adenocarcinoma, further studies of MUC1 and Met signaling should provide additional insight into the mechanisms that regulate invasion and metastasis of pancreatic cancer.

Differential Signaling Response of Constitutively Active IL2R-EGFR Chimera

J. O. Hunttoo, A. Kramer; Dept. of Cell and Tissue Biology, UCSF, San Francisco, CA

endothelial cells through the junctional complexes comprising tight junctions, adherens junctions, and gap junctions. All these structures may play a crucial role in maintaining the integrity of the endothelium and initiating angiogenesis and communicating with endothelial cells through gap junctions. This results in induction of endothelial-derived metalloproteinases, sub-endothelial basement membrane degradation

Metastasis, a pivotal step in cancer, is the colonization of selective, secondary organ sites by dissemination of tumor cells from their primary location by their extravasation through the endothelium.

Treatment Impairs the Formation of Mammary Epithelial Acini

S. M. McCarthy; Conway Institute, Dublin, Ireland

Epithelial-mesenchymal transition (EMT) is a process that involves the loss of epithelial cell polarity due to the disappearance of cell-cell junctions and the gain of a mesenchymal phenotype. Transforming growth factor β (TGF-β) is known to initiate and maintain EMT in a variety of biological systems and may promote associated tumor cell invasion and metastasis. It was of interest therefore to observe the effects of exogenous TGFβ treatment on the formation of 3-D mammary epithelial cell acini both using MCF10A cells and primary mouse mammary epithelial cells. MCF10A cells are an immortalized but non-transformed human mammary epithelial cell line. When cultured in the presence of a laminin-rich extracellular matrix, MCF10A cells form 3D acinar structures. TGFβ treatment disrupted MCF10A acinus formation. The treatment resulted in the formation of loosely associated assemblies of cells. The cells failed to polarize, the forming acini not to their lumina and interestingly, the cells probably failed to exit the cell cycle; preliminary results suggest that TGFβ treatment was associated with a high sustained level of Erk phosphorylation/activation which contrasted with low levels of phospho-Erk in the normal MCF10A acini. In addition, an increase in vimentin expression, a marker of EMT, was also observed in TGFβ treated acini. TGFβ treatment also impaired acinus formation from primary mouse mammary epithelial cells. Again the result was the formation of loose assemblies of non-polarized cells; and again lumen formation failed and vimentin expression was induced. However, the assemblies had a smaller mean diameter than the normal acini and, in contrast to the MCF10A cells, did not exhibit high levels of Erk phosphorylation. Thus, TGFβ treatment impairs acinus formation from two different mammary epithelial cell types by a mechanism that is probably not dependent on triggering deregulated growth factor-Erk MAP kinase signaling.

Patterning of Multiple Cell Lineages from a Single Stem Cell Population

J. A. Jadlowiec, E. D. Miller, J. Huard, L. E. Weiss, A. Waggoner, P. G. Campbell; 1Molecular Biosensor and Imaging Center, Carnegie Mellon University, Pittsburgh, PA, 2Stem Cell Research Center, Children’s Hospital of Pittsburgh, Pittsburgh, PA, 3Institute for Complex Engineered Systems, Carnegie Mellon University, Pittsburgh, PA, 4Biomedical Engineering, Carnegie Mellon University, Pittsburgh, PA, 5Robotics Institute, Carnegie Mellon University, Pittsburgh, PA

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Mass spectrometry based proteomics detected an association between MUC1 and the c-Met receptor tyrosine kinase, which was confirmed by reciprocal co-immunoprecipitations followed by western blotting. Recombinant human c-Met protein catalyzed in vitro phosphorylation of tyrosine in the YHYP motif of the a 66-residue MUC1CT as detected by tandem mass spectrometry. Stimulation of pancreatic adenocarcinoma cells with HGF, a ligand for c-Met, enhanced the incorporation of 32P in MUC1CT, and enhanced phosphorylation at tyrosine in the YHYP motif as determined by analysis with site specific phospho-enhanced clathrin-mediated endocytosis of MUC1CT and its nuclear localization, as determined by real-time confocal imaging of C-terminally GTP-tagged MUC1. There was faster turnover of MUC1 under stimulation with HGF, and overexpression of MUC1 facilitated faster turnover of c-Met in S2-013 cells. These results indicate that c-Met, serving as a kinase for MUC1, phosphorylates MUC1CT and promotes the endocytic trafficking-mediated nuclear localization of MUC1 and subsequent turnover of MUC1CT and c-Met during HGF stimulation. Given that MUC1 and Met are overexpressed in pancreatic adenocarcinoma, further studies of MUC1 and Met signaling should provide additional insight into the mechanisms that regulate invasion and metastasis of pancreatic cancer.

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endothelial cells through the junctional complexes comprising tight junctions, adherens junctions, and gap junctions. All these structures may play a crucial role in maintaining the integrity of the endothelium and initiating angiogenesis and communicating with endothelial cells through gap junctions. This results in induction of endothelial-derived metalloproteinases, sub-endothelial basement membrane degradation followed by endothelial cell retraction allowing neoplastic lymphocytes extravasation. This local and transient effect is dependent on the paracrine interaction through local production of functional angiogenic factors such as VEGF (vascular endothelial growth factor) by tumor cells and on the direct hetero-cellular communication between tumor cells and endothelial cells through the junctional complexes comprising tight junctions, adherens junctions, and gap junctions. All these structures may play a crucial role in maintaining the integrity of the endothelium’s barrier. We explored the effects of the specific tyrosine kinase inhibitor of VEGF receptors, PTK-787/ZK-222584 (PTK/ZK), and anti VEGF antibodies on ATL cell extravasation. Both agents inhibit ATL-induced in vitro angiogenesis. ATL-derived cell free supernatant induced a rapid and sharp increase in the phosphorylation of FAK and ERK and a moderate increase in AKT phosphorylation. Both PTK/ZK and anti-VEGF antibodies reduced FAK phosphorylation in a dose dependent manner but had no effect on AKT and ERK phosphorylation. Finally, blocking of VEGF signaling by PTK/ZK or anti-VEGF antibodies significantly attenuates ATL cell extravasation. These results demonstrate the central role of VEGF signaling in ATL cell extravasation and support the potential use of PTK/ZK in the treatment of ATL and other hematological malignancies with angiogenic, invasive and metastatic properties.
Integrin (α6β4) Regulation of mTOR and Breast Carcinoma Survival

W. Yin, H. Kim, J. Chung; Biochemistry, Louisiana State University HSC-S, Shreveport, LA

During malignant transformation, the rich environment filled with growth factors and extracellular matrix is rapidly lost. In their absence, normal epithelial cells undergo anoikis, a programmed cell death. Therefore, it is highly important for carcinoma cells to maintain their survival and induce angiogenesis in tumor-micro environment. Our previous findings implicate a critical role of integrin α6β4 in the survival of breast carcinoma cells by maintaining cap-dependent translation of angiogenic/survival factor, VEGF under serum starvation via activation of the mTOR. Clearly, VEGF is only the ‘tip of the iceberg’ and, most likely, α6β4 stimulates the translation of other mRNAs that contribute to breast carcinoma survival. In addition, the mechanisms by which this integrin activates mTOR remain elusive. We have found that α6β4 activates mTOR in cooperation with its signaling partner β-Arg in Akt and Ras dependent manner. Activated mTOR mediates α6β4 dependent breast carcinoma survival under stress conditions that mimic tumor micro-environment because downregulation of mTOR expression by shRNA or inhibition of mTOR activity via rapamycin blocks α6β4 dependent carcinoma survival. α6β4 to mTOR signaling enhances translation initiation of pro-survival and anti-apoptotic mRNAs that ultimately contribute carcinoma survival in vivo and progression. Considering that translational control is understudied, but important regulator of progression, our studies will provide the novel paradigm of integrin regulation of translation initiation that plays a pivotal role in cancer progression.

Mechanotransduction through Transmembrane Syndecans

R. Bellin, 1 J. Kubick, 2 A. Kamien, 1 M. Frigault, 1 B. Keil, 1 P. LeDuc 1, 2 1Department of Biology, Holy Cross College, Worcester, MA, 2Department of Mechanical Engineering, Carnegie Mellon University, Pittsburgh, PA

Mechanotransduction studies over the past decade have focused primarily on integrins as the molecular initiation point of the signaling process. Although much has been learned about the role of integrins in the cellular response to physical strain, there is reasonable evidence to suggest that other transmembrane proteins may also play a similar signaling role. In our recent studies we have discovered that syndecans, a class of heparan sulfate transmembrane proteins, serve as integrin-independent conduits of mechanical strain. To conduct these studies, we developed a method to produce syndecan-specific cell adhesion by conjugating syndecan ectodomain-specific antibodies to the surface of polydimethylsiloxane (PDMS) membranes. We subjected cultured cells on these membranes to controlled mechanical deformation through the use of our PreCS (Pressure-driven Cell-Stretching) mechanical strain device. Assays of these cells after stretching demonstrate a statistically significant increase in ERK phosphorylation in comparison to non-stretched controls. In addition, treatment of cells with actin cytoskeletal disruptive drugs before stretching results in a statistically significant reduction in the level of ERK phosphorylation in comparison to non-treated stretched cells. This finding indicates a potential role for the cytoskeleton in syndecan-based mechanical signaling. Overall, these results open the door for new areas of exploration in the field of mechanotransduction and inform us of a novel cellular role for transmembrane syndecan proteins.

Breast Epithelial Cells Adjust Their Response to 3D Density by Regulating Cellular Contractility through Filamin-β1 Integrin Interactions

S. Gehler, 1 D. A. Calderwood, 2 P. J. Keeley, 1 1Pharmacology, University of Wisconsin, Madison, WI, 2Pharmacology, University of Wisconsin School of Medicine, New Haven, CT

Breast epithelial cells cultured in a compliant, low density three-dimensional collagen matrix, but not in high density 3D collagen gels that can not be contracted, undergo ductal morphogenesis and recapitulate differentiated structures seen in vivo. The mechanisms by which breast epithelial cells detect changes in the physical properties of the extracellular matrix (ECM) and translate that information into biological signals are not clearly understood. We propose that interactions between β1 integrin and the actin-binding protein, filamin A, are part of the mechanism by which cells respond to the mechanical properties of the ECM, thus leading to changes in myosin-mediated contractility and tubule formation of breast epithelial cells. First, T47D breast epithelial cells expressing filamin A shRNA exhibit reduced contractility and have disrupted tubulogenesis in low density collagen gels. Conversely, increasing endogenous filamin A expression using FLPiP (filamin interacting protein) shRNA increased cell contractility while blocking tubule formation. These findings suggest an intermediate level of filamin A expression regulates cell contractility and tubule formation. Second, filamin A-β1 integrin interactions are enhanced in cells cultured in a floating, high density collagen gel relative to a more compliant, low density gel. These enhanced filamin-β1 integrin interactions correspond to a disruption in tubule formation. Consistent with this finding, cells expressing a β1(V787,791I) integrin mutant that exhibits enhanced filamin A-β1 integrin interactions undergone increased contractility, yet disrupted tubulogenesis, in low density collagen gels. However, tubulogenesis could be rescued by culturing these cells in dense collagen gels. These results suggest that cellular phenotype is regulated by the degree of cellular contractility in response to the compliance of the ECM. Regulation of filamin A binding to β1 integrin may serve as a mechanosensitive complex that is important for breast epithelial cells to respond to the mechanical properties of the ECM.

Extracellular Matrix Dependent Survival and Apoptosis Resistance in the 3rd Dimension

J. C. Friedland, 1 J. N. Lakins, 2 M. Nuth, 2 V. M. Weaver, 1 1Radiation Oncology, University of Pennsylvania, Philadelphia, PA, 2Institute for Medicine and Engineering, University of Pennsylvania, Philadelphia, PA

Apoptosis resistance is acquired by epithelial tissues during malignant transformation and metastasis and plays a key role in the development of drug resistance. Cells exist within a 3D tissue and cell-cell and cell-extracellular matrix (ECM) interactions regulate cell behavior. Previous studies have demonstrated that cell shape is a critical determinant of cell life and death, with increased cell spreading favoring cell survival. However, we previously showed that MECS survive when grown in a compliant matrix to form 3D polarized structures, and these MECS do not spread. Additionally, MECS acquire resistance to chemical and receptor-dependent apoptotic stimuli when grown in a compliant matrix, similar to terminal-ductal-lobular units in vivo. Therefore, we have been examining how cell context could influence cell survival and apoptosis resistance, which is dependent upon laminin ligated α6β4 integrin activation of Rac to activate Pak1 and NFκB. We also found that the apoptosis resistant phenotype could be enhanced if the MECS acini were embedded within a highly compliant 3D ECM, but antagonized by interaction with a rigid 2D ECM. Because we determined that ECM stiffness increases Rho but represses Rac activity, and ectopic expression of V14Rho or N17Rac perturbs apoptosis resistance in acini, we asked if matrix compliance would regulate acini survival by modulating the ratio of Rac and Rho activity. Consistently, expression of active V12Rac, which concentrated at regions of cell-cell interaction, induced apoptosis in MECS on a rigid 2D matrix, where Rho activity is elevated. Interestingly, inhibiting Rho in disorganized tumors, which have high Rho, leads to the formation of organized tumor structures that are extremely resistant to exogenous death cues. Our data indicate that the 3rd dimension and tissue organization are critical for Rac-dependent survival. Importantly, we are exploring whether tissue architecture could alter Rac function by differentially regulating expression for localization of Rac targets.

Requirement of Substratum Rigidity in β1 Integrin Activation and Clustering to Activate FAKβ39 Phosphorylation

W. Wei, 1 W. Chiu, 1 C. Wang, 1 M. Shen, 1 M. Tang 1, 2 1The Institute of Basic Medical Sciences, National Cheng Kung University, Tainan, Taiwan, 2Department of Physiology, National Cheng Kung University, Tainan, Taiwan

Previous study in our lab showed that FAKβ39 phosphorylation level was decreased due to low rigidity when MDCK cells were cultured on collagen gel, whereas other phosphorylation levels of FAK (407, 577, 861, and 925) remained elevated. We also found that β1 integrin activation was suppressed under low substratum rigidity. To examine whether low substratum rigidity
Differentiation of small intestine. Hensin. Preliminary investigations of small intestine with integrin antibodies to integrin β1 gene inactivation (cre-lox technology) suggest the crucial role of integrin β1 mediated hensin polymerization in the differentiation of small intestine.

Integrin β1 Activation Leads to Extracellular Matrix Assembly of Hensin, a Rabbit Isoform of Brain Tumor Suppressor Protein DMBT1 S. Vijayakumar, Q. Al-Awqati; Dept. of Medicine (Nephrology), Columbia University Medical Center, New York, NY Hensin is an extracellular matrix (ECM) protein that mediates the terminal differentiation of an intercalated cell line derived from rabbit kidney. ECM hensin induced actin cytoskeleton remodeling and columnar differentiation of Madin Darby Canine Kidney (MDCK) cells cultured on rigid substratum. The direct interaction of hensin with activated integrin β1 integrin was colocalized with lipid raft in cells cultured on rigid but not on soft substratum. Taken together, our data demonstrate that substratum rigidity is required for integrin activation and clustering that are mediated by lipid raft.

Suppression of Laminin-5 Synthesis Alters Cell Cycle Progression, Rb Phosphorylation, and PI3K Signaling M. M. Buschmann, H. Waechter, K. S. Matlin; Department of Surgery, University of Cincinnati, Cincinnati, OH Normal epithelial cells rely on spatial cues from the extracellular matrix to proliferate, migrate, and survive. Inappropriate expression of the laminin family of extracellular matrix proteins has been observed in several cancers; connected with increased invasion, metastasis, and angiogenesis (Pattaro et al., Semin Cancer Biol. 12, 197-207, 2002). We have shown that the laminin isoform, laminin 5 (LNS) is necessary for proliferation in normal, nontransformed, Madin Darby Canine Kidney (MDCK) epithelial cells, as loss of LNS expression by siRNA-mediated knockdown results in cell cycle arrest (Mak et al., Mol Biol Cell. 17, 3664-3677, 2006). To identify the mechanism of proliferation control by LNS, we have examined Cyclin/Cdk complexes required for G1/S cell cycle progression and upstream signaling events following LNS suppression. Flow cytometry data demonstrates that LNS suppression results in G1 arrest. In addition, CyclinD1/Cdk4 complexes assemble and accumulate in the nucleus. Surprisingly, these complexes appear to be inactive because Rb remains hypo-phosphorylated. Upstream of the cell cycle, the activity of ERK1/2, which regulates Cyclin D1 expression, remains unaltered. This suggests that cell cycle arrest following LNS suppression is not due to changes in the ERK1/2 pathway. Interestingly, LNS suppression results in a marked decrease in PI3K phosphorylation. Despite this decrease in PI3K phosphorylation, phosphorylation of Akt at Thr308 and Ser473 is significantly increased. PI3K is required for this increase in Akt activation, however, as treatment with PI3K inhibitor, LY294002, inhibits Akt activation following LNS suppression. In conclusion, suppression of LNS synthesis in MDCK cells appears to arrest proliferation by inhibiting Rb phosphorylation, even though CyclinD1/Cdk4 complexes assemble, and alters upstream PI3K signaling.

Transcriptional Repression of N-cadherin in Differentiated Prostate Carcinomas W. B. Bair,1 N. Alexander,2 M. Jaramillo,1 D. I. Simon,3 R. L. Heimark1; 1University of Arizona, Tucson, AZ, 2Vanderbilt University, Nashville, TN, 3Brigham and Women's Hospital, Harvard Medical School, Boston, MA N-cadherin is essential for cell-cell adhesion during development and has been found to be upregulated in many carcinomas. In prostate carcinogenesis, N-cadherin upregulation is associated with a loss of E-cadherin. The switch from E-cadherin to N-cadherin in metastatic cells contributes to cancer progression through detachment of cells and inhibition of apoptosis. Studies have shown the repression of E-cadherin involves genetic and epigenetic mechanisms and is an important step in metastasis. However, the mechanism of epithelial-restricted patterns of N-cadherin gene expression is less well known and may involve activators and repressors limiting expression to epithelial cells. To determine the cis-regulatory elements involved in the regulation of the N-cadherin, we cloned ~4 kb of the N-cadherin gene including the 5' upstream regulatory region, the first exon and 3 kb of the first intron. The transcriptional activity of the proximal N-cadherin promoter (nts ~860-20) was assessed in N-cadherin-expressing and nonexpressing carcinoma cells, and promoter activity was seen in both. Previous studies in our lab indicated that the first intron (nts +2627) of the N-cadherin gene is important from regulating N-cadherin transcription in N-cadherin expressing cells. Addition of the first intron can inhibit activity of the proximal promoter as measured by luciferase assays in carcinoma cells that do not express N-cadherin. We identified a negative response element located in the N-cadherin first intron and sequence analysis of the intron yielded a forkhead binding site for the putative tumor suppressor FoxP1. EMSAs have demonstrated that FoxP1 can bind to this sequence and mutation of this sequence alters the binding motif in repressing basal levels of N-cadherin transcription by different cell type-specific mechanisms, and support the hypothesis that FoxP1 sequesters negative regulators of transcription, thereby suppressing N-cadherin gene expression. Supported by a NIH Grants CA56666 and CA092123-18.

Collagen I Promotes Metastasis in Pancreatic Cancer by Up Regulating N-cadherin Expression via JNK Y. Shintani, K. R. Johnson, M. J. Wheelock; Oral Biology, University of Nebraska Medical Center, Omaha, NE Pancreatic cancer is characterized by excessive deposition of type I collagen. This led us to ask whether interactions with type I collagen would up-regulate N-cadherin expression in pancreatic cancer cells, which would be predicted to increase the metastatic potential of these cells. BxPC-3 human pancreatic cancer cells respond to collagen I, but not other matrices, by up-regulating mesenchymal markers, including N-cadherin, fibronectin, and vimentin. A modified transwell assay showed that cells were more motile on collagen I-coated transwell dishes as compared to fibronectin coated transwell dishes. To clarify the role of N-cadherin expression in cell motility, we generated 3 cell lines: mock infected cells (neomycin resistance gene), N-cadherin knockdown cells, and N-cadherin over-expressing cells. Collagen mediated cell motility was inhibited by N-cadherin knockdown, whereas it was increased by N-cadherin overexpression. We transplanted these cells into the pancreas of nude mice. Examination of the abdominal cavities 8 weeks later revealed that N-cadherin over-expressing cells formed significantly more metastases to the peritoneum than did the other cell lines. Histological examination demonstrated micro-metastases in the lung of 7 of 10 mice receiving injections of N-cadherin over-expressing cells, whereas no metastases were observed in mice that received mock- or N-cadherin knockdown cells. Inhibiting JNK with chemical inhibitors or siRNA abrogated all collagen I-induced changes. We show that JNK1 is activated in response to collagen I, which increases tumorigenesis by up regulating N-cadherin expression and increasing motility via phosphorylation of paxillin. Furthermore, inhibition of JNK1 prevented invasion and metastasis in the mouse model for pancreatic cancer. Thus, inhibiting collagen-I mediated signals, or preventing downstream activities of N-cadherin or JNK1 prevent metastasis of pancreatic cancer cells. These data strongly suggest that blocking signaling between tumor stroma and tumor cells has the potential to impact treatment for pancreatic cancer.

Modulation of E- and N-cadherin Levels in Oral Squamous Cells Reveals N-cadherin Specific Increases in Invasion-related Signaling Pathways K. R. Lawson,1 J. N. Myers,2 M. J. Wheelock,3 K. R. Johnson1; 1Oral Biology, University of Nebraska Medical Center, Omaha, NE, 2Head and Neck Surgery, MD Anderson Cancer Center, Houston, TX
We have previously demonstrated that overexpression of the mesenchymal protein N-cadherin inversely correlates with E-cadherin expression in oral squamous cells, and that overexpression of N-cadherin in breast cancer cells can supersede E-cadherin function, increasing both motility and invasion even in the presence of E-cadherin. To better define the mechanism by which the gain of N-cadherin, but not loss of E-cadherin, can confer increased invasiveness and motility to oral squamous cells, we have transduced a series of retroviral vectors into two independently-derived human oral squamous carcinoma lines, Tu167 and UM-SCC-1, in order to modulate levels of E- or N-cadherin expression. The overexpression of N-cadherin in both Tu167 and SCC-1 cells substantially increased invasiveness compared to both control cells and E-cadherin-depleted cells. Although SCC-1 cells displayed a marked increase in motility upon N-cadherin expression, Tu167 cell motility was unaffected. In Tu167 cells, N-cadherin overexpression, but not E-cadherin loss, increased the expression of matrix-metalloproteinase-9 in a manner dependent upon phosphatidylinositol 3-kinase (PI3 kinase) activity. Treatment of N-cadherin-expressing Tu167 cells with various growth factors (including fibroblast growth factor) did not amplify this effect. To identify the regions of N-cadherin protein that modulate invasion and motility, Tu167 and SCC-1 cell lines were transduced to express chimera cadherin molecules in which the extracellular or cytoplasmic domains of E- and N-cadherin were reciprocally substituted. Overexpression of an E-N chimera (containing the cytoplasmic domain of N-cadherin) substantially increased invasiveness, whereas expression of an N-E chimera (containing the N-cadherin extracellular domain) preferentially increased motility. These results suggest that N-cadherin modulates both the motility and invasiveness of oral cell lines via specific signaling mechanisms that are independent from those arising as a consequence E-cadherin loss.

R-cadherin Expression Induces Myoblast Transformation: Role in Rhabdomyosarcomagenesis

C. Gauthier-Rouvière; CRBM, CNRS, Montpellier, France

Cadherins are a family of transmembrane glycoproteins that mediate Ca2+-dependent homophilic cell-cell adhesion and play a crucial role in proliferation, differentiation and cell transformation. The goal of this study is to understand the role of R-cadherin found in rhabdomyosarcomas (RMS), tumors of skeletal muscle origin, while it is absent in normal myoblasts. We show that R-cadherin expression in C2C12 myoblasts results in inhibition of myogenesis induction and of myoblast fusion and impairs their cell cycle exit when cultured in differentiation medium. Furthermore, R-cadherin expression elicits myoblast transformation as shown by anchorage-independent growth in soft agar and in vivo tumor formation assays. In contrast, inhibition of R-cadherin expression using RNA interference inhibits growth in soft agar of RD cell line and its tumorigenicity in mice. In addition, expression of R-cadherin results in decreased expression of endogenous N- and E-cadherin. The analysis of the nature of R-cadherin-mediated signals shows that R-cadherin-dependent adhesion increases Rac1 activity. Dominant negative forms of Rac1 inhibit R-cadherin-mediated signaling and transformation. Together, these data suggest that R-cadherin expression inhibits myogenesis and induces myoblast transformation through Rac1 activation.

M-cadherin Activates Rac1 GT-Pase through the Rho-GEF Trio during Myoblast Fusion

S. Charrasse, F. Comunale, M. Fortier, E. Portales-Casamar, A. Debant, C. Gauthier-Rouvière; CNRS, Montpellier, France

Cadherins are transmembrane glycoproteins that mediate Ca2+-dependent homophilic cell-cell adhesion and play crucial role during skeletal myogenesis. M-cadherin is required for myoblast fusion into myotubes, but its mechanisms of action remain unknown. The goal of this study was cast some light on the nature of the M-cadherin-mediated signals involved in myoblast fusion into myotubes. We found that the Rac1 GT-Pase activity is increased at the time of myoblast fusion and it is required for this process. Moreover, we showed that M-cadherin-dependent adhesion activates Rac1 and demonstrated the formation of a multistepic complex containing M-cadherin, the Rho-GEF Trio and Rac1 at the onset of myoblast fusion. Interestingly, Trio knockdown efficiently blocked both the increase in Rac1-GTP levels, observed after M-cadherin-dependent contact formation, and myoblast fusion. We conclude that M-cadherin-dependent adhesion can activate Rac1 via the Rho-GEF Trio at the time of myoblast fusion.

Association of N-cadherin with Lipid Rafts/Caveolae Is Required for Tyrosine Phosphorylation of N-cadherin by Src Family Kinases during Transendothelial Migration of Melanoma Cells

J. Qi, J. Wang, C. Siu; BBDMR and Department of Biochemistry, University of Toronto, Toronto, ON, Canada, Shandong Academy of Medical Sciences, Shandong University, Jinan, China

Activation of Src family kinases (SFKs) results in tyrosine phosphorylation of N-cadherin and dissociation of β-catenin in a co-culture system where melanoma cells are seeded on top of an endothelial monolayer. The question remains how N-cadherin is sequestered in the heterotypic contact. Here we report that N-cadherin became associated with the caveolin-enriched membrane (CEM) during transendothelial migration (TEM) of melanoma cells. The SFK inhibitor PP2 failed to block the association of N-cadherin with CEM during co-culture, although it abolished endothelial monolayer. The question remains how N-cadherin is sequestered in the heterotypic contact. Here we report that N-cadherin became associated with the caveolin-enriched membrane (CEM) during transendothelial migration (TEM) of melanoma cells. The SFK inhibitor PP2 failed to block the association of N-cadherin with CEM during co-culture, although it abolished tyrosine phosphorylation of N-cadherin and inhibited dissociation of β-catenin in the CEM. It is likely that N-cadherin becomes associated with the CEM first, where it is phosphorylated by the activated SFKs. Further studies show that disruption of the CEM using filipin or caveolin-1 siRNA abolished tyrosine phosphorylation of N-cadherin and inhibited transmigration of melanoma cells. Expression of an active Src led to tyrosine phosphorylation of N-cadherin in the CEM and a reduced level of β-catenin association. On the other hand, β-catenin was tyrosine-phosphorylated in the Non-CEM but showed no change in its association with N-cadherin. Together, the data suggest that lipid rafts/caveolae provide a platform for tyrosine phosphorylation of N-cadherin by SFKs and stabilization of the N-cadherin association in the absence of β-catenin, leading to the concomitant activation of the β-catenin signaling cascade during TEM of melanoma cells. (Supported by the Canadian Institutes of Health Research.)
Cadherin Proteins Regulate Cell Adhesion and Morphogenesis in Drosophila Egg Development

J. Zartman, N. Yakoby, C. Brislow, T. Schüpbach, S. Shvartsman; 1Lewis Sigler Institute and Department of Chemical Engineering, Princeton University, Princeton, NJ, 2Howard Hughes Medical Institute and Department of Molecular Biology, Princeton University, Princeton, NJ.

During Drosophila oogenesis, a two-dimensional follicular epithelium gives rise to an elaborate three-dimensional eggshell (1). Eggshell morphogenesis critically depends on patterning of the follicle cells, but the connection between signaling pathways, pattern formation, and eggshell morphogenesis is not well understood. As important components of morphogenesis, cadherin proteins have been proposed to regulate cell-cell adhesion, cell migration, and epithelial polarity and integrity (2). Recently we have identified the non-classical cadherin, Cad74A, as a key molecule that bridges epithelial patterning and morphogenetic dynamics in this system. Starting in mid-oogenesis, cad74A is expressed in all the columnar cells except for two dorsal lateral patches, in the border cells, and later in the future operculum domain. We have established that this pattern is partially controlled by the EGFR and BMP pathways and that Cad74A is involved in multiple aspects of egg development. We will present several lines of evidence showing that these spatially distinct expression domains are essential for proper formation of several chorion structures. Females homozygous for a disrupted allele of cad74A exhibit severe defects in late egg development, including the loss of dorsal appendages and a collapsed eggshell. Milder eggshell phenotypes include shorter and thinner dorsal appendages, a missing ventral collar, and a less structured micropleue. Based on these results, we propose that Cad74A provides an important link between signaling pathways, pattern formation, and the structuring of the Drosophila eggshell. 1. C. A. Berg, Trends in Genetics 21, 346 (2005). 2. U. Tepass, Curr Opin Cell Biol 11, 540 (Oct, 1999).

Chicken Protocadherin-1 Functions to Localize Neural Crest Cells to the Dorsal Root Ganglia during PNS Formation

J. Bononi, R. Bradley; Cell Biology and Neuroscience, Montana State University, Bozeman, MT

In vertebrates, neural crest cells emerge from the dorsal neural tube and migrate along well-defined pathways to form a wide variety of tissues, including the majority of the peripheral nervous system (PNS). The cell adhesion molecules that mediate cell-cell interactions during neural crest cell migration and subsequent re-aggregation to form the PNS are not well understood but likely include members of the cadherin family. To identify cadherins involved in PNS formation we screened an embryonic chicken dorsal root ganglia (DRG) library for protocadherin subfamily members, resulting in the isolation of chicken protocadherin-1 (cPcdh1). In the trunk, cPcdh1 is expressed in migrating neural crest cells and in the DRG, sympathetic ganglia (SG) and Schwann cells along the ventral branch (VB). Within the DRG, cPcdh1 is initially restricted to the perimeter, where neural crest cells first cease migration and remain in an undifferentiated and mitotically active state. This expression pattern suggested a role for cPcdh1 in mediating migration arrest and cell adhesion at the DRG perimeter. To test this, we utilized in ovo electroporation to disrupt cPcdh1 function in neural crest cells. Results indicate that when full-length cPcdh1 is over-expressed in neural crest cells, the majority of ectopically expressing cells cease migrating at the perimeter of the DRG. In contrast, when cPcdh1 function is inhibited, using a dominant-negative cPcdh1 construct, a greater percentage of ectopically expressing neural crest cells localize to the SG and VB, at the expense of the DRG. Furthermore, those cells that still target to the DRG, when cPcdh1 function is inhibited, no longer localize to the perimeter, but are instead dispersed throughout the DRG. These results suggest that cPcdh1-mediated cell adhesion plays an important role in neural crest cell migration to localize newly arrived cells at the DRG perimeter where they remain mitotically active and undifferentiated.

N-cadherin Directs Drosophila Reorganization and Lamellipodia Dynamics in Migrating Sheets of Smooth Muscle Cells

P. I. B. Sabatini, M. Zhang, M. P. Bendeck, B. L. Langille; Laboratory Medicine and Pathobiology, University of Toronto, Toronto, ON, Canada, 2Toronto General Hospital, Toronto, ON, Canada

In cells migrating at wound edges, anterior activation of integrins induces Cdc42-dependent reorientation of the microtubule-organizing center (MTOC) and initiates actin polymerization towards the direction of migration (Etienne-Manneville and Hall, Cell 106:489, 2001). Classical cadherins, which regulate cell-cell adhesion, localize to the posterior-lateral regions of cells at wound edges and one such cadherin (N-cadherin) can modulate Cdc42 activity; therefore, we hypothesized that N-cadherin directs cell polarity in wound edge cells. Treatment with an N-cadherin blocking antibody suppressed wound-induced Cdc42 activation, slowed migration and impaired the polarization of the MTOC to the anterior side of the nucleus. N-cadherin was also blocked by calcium chelation with EGTA, to disrupt calcium-dependent adherens junctions, and it was restored after EGTA washout. Interestingly, retrograde flow in lamellipodia of cholesterol rich membrane domains (labeled with FITC-conjugated cholera toxin B subunit) was completely immobilized by N-cadherin ligation with antibody. This retrograde flow of membrane rafts was also arrested by treatment with cytochalasin B to disrupt actin microfilaments; however, this was not due to the much faster retrograde flow of lamellipodial actin (4.0 versus 1.2 μm/min for rafts) since actin retrograde flow persisted after treatment with anti-N-cadherin antibody. Also, N-cadherin-GFP clusters in the lamellipodia were transported retrogradely with the cell membrane; therefore, regulation of retrograde flow may modulate the delivery and retention of this cadherin adhesion molecule at the cell anterior.

Dynamic Microtubules Regulate the Local Concentration of E-cadherin at Cell-Cell Contacts

S. J. Siebhen, A. D. Patterson, S. M. Crampton, A. M. Shewan, C. Fergason, A. Khambhatia, R. G. Parton, A. S. Yap; Division of Molecular Cell Biology, Institute for Molecular Bioscience, The University of Queensland, St. Lucia, Queensland, Australia, 2School for Biomedical Science, The University of Queensland, St. Lucia, Queensland, Australia, 3Centre for Microscopy and Microanalysis, The University of Queensland, St. Lucia, Queensland, Australia, 4MGC Department of Cell Biology and Genetics, Erasmus Medical Center, Rotterdam, The Netherlands

Classical cadherins are a family of membrane receptors that mediate cell-cell adhesion in mammalian organisms. During embryogenesis cadherins act as key determinants of tissue organisation and patterning as well as being responsible for normal tissue architectural integrity. In the adult it is becoming increasingly clear that cadherin receptor activity, and ensuing adhesion, is an extremely dynamic process coordinated by localised membrane signalling processes. Thus, acting to regulate the complex relationship between the cadherin and the actin cytoskeleton. In contrast the potential link between cadherins and microtubules has been less extensively investigated. We now identify a pool of microtubules that extend radially into cell-cell contacts and are inhibited by manoeuvres that block the dynamic activity of microtubule plus ends, including low concentrations of nocodazole and expression of a CLIP-170 mutant. Blocking dynamic microtubules perturbed the ability of cells to concentrate and accumulate E-cadherin at cell-cell contacts, as assessed both by quantitative immunofluorescence microscopy and FRAP analysis, but did not affect either transport of E-cadherin to the plasma membrane, nor the amount of E-cadherin expressed at the cell surface. This indicated that dynamic microtubules allow cells to concentrate E-cadherin at cell-cell contacts by regulating the regional distribution of E-cadherin once it reaches the cell surface. Importantly, dynamic microtubules were necessary for Myosin II to accumulate and be activated at cadherin adhesive contacts, a localised mechanism that supports the focal accumulation of E-cadherin. We propose that this population of microtubules represents a novel form of cadherin-microtubule cooperation, where cadherin adhesions recruit dynamic microtubules that, in turn, support the local concentration of cadherin molecules by regulating Myosin II activity at cell-cell contacts.

Single-Molecule Analysis of Cadherin-mediated Cell Adhesion

D. Wirtz, P. Panorchan; Chemical and Biomolecular Engineering, Johns Hopkins University, Baltimore, MD

Cadherins are ubiquitous cell surface molecules that are expressed in virtually all solid tissues and localize at sites of cell-cell contact. Cadherins form a large and diverse family of adhesion molecules which play a crucial role in a multitude of cellular processes, including cell-cell adhesion, motility, and cell sorting in maturing organs and tissues, presumably because of their different binding capacity and specificity. Here, we develop a method that probes the biochemical and biophysical properties of the binding interactions between cadherins expressed on the surface of living cells, at the single-molecule level. Single-molecule force spectroscopy reveals that cadherins, N-cadherin, E-cadherin, and VE-cadherin, form bonds that display adhesion specificity, and a pronounced difference in adhesion force, reactive compliance, and bond lifetime. Moreover, their potentials of interaction, derived from force-spectroscopy measurements, are qualitatively different when comparing the single-barrier energy potential for the dissociation of an N-cadherin/N-cadherin bond with the double-barrier energy potential for an E-cadherin/E-cadherin bond. Together these results suggest that N-cadherin and E-cadherin molecules form homophilic bonds between juxtaposed cells that have significantly different kinetic and micromechanical properties.
Inhibition of Cyclin Dependent Kinase 5 (Cdk5) Regulates E-cadherin Degradation and Trafficking in Madin-Darby Canine Kidney (MDCK) Epithelial Cells

S. S. Saravanamanuthi, C. Y. Gao, P. S. Zelena; Laboratory of Molecular and Developmental Biology (LMDB), National Eye Institute (NEI), National Institutes of Health (NIH), Bethesda, MD

E-cadherin is essential for epithelial cell polarity and cell-cell adhesion. Dynamic trafficking of E-cadherin to and from the cell surface modulates its levels within adherens junctions, thereby providing a mechanism for regulating cell adhesion. Previous work from this laboratory has shown that Cdk5, a protein kinase with known trafficking functions in neurons, is also involved in regulating cell-cell adhesion in epithelial cells of the lens and cornea. This work examines the role of Cdk5 in E-cadherin-dependent adhesion in MDCK cells, a widely used epithelial cell model. To investigate the effect of Cdk5 inhibition on E-cadherin-dependent adhesion, cells were plated on microwells plated that had been coated with E-cadherin ectodomain and adherent cells were measured after incubating in the presence or absence of the Cdk5 inhibitor, olomoucine (15μM). Inhibition of Cdk5 activity reduced E-cadherin based cell-cell adhesion by approximately 50%. To study the potential role of Cdk5 in E-cadherin degradation and/or trafficking, the surface localized E-cadherin in a confluent monolayer of cells was biotinylated using cell impermeable cleavable sulpho-NHS-Ss biotin reagent. After 6h incubation in the presence or absence of olomoucine, the cells were lysed and the remaining biotinylated E-cadherin was determined by affinity chromatography on neutravidin-coated beads followed by immunoblotting for E-cadherin. The total biotin-labeled E-cadherin remaining in the olomoucine-treated samples was reduced in the untreated controls (17% as compared to 47%, p<0.001). To assess what fraction of the remaining E-cadherin had been internalized by endocytosis for recycling, surface biotin was stripped by cleaving the disulfide link prior to cell lysis. The inhibition of Cdk5 activity increased the internal pool approximately 4-fold and decreased the surface membrane-associated pool by 14-fold as compared to the untreated control. Thus, Cdk5 activity affects both degradation and endocytic recycling of E-cadherin to regulate epithelial cell-cell adhesion.

Nuclear Translocation of the Cleaved Cytoplasmic Domain of E-cadherin

E. C. Ferber, Y. Fujita; MRC Laboratory for Molecular Cell Biology and Cell Biology Unit, London, United Kingdom

E-cadherin is the most crucial membrane protein for the formation of tight and compact cell-cell adhesions in epithelial cells. In addition to this structural role, E-cadherin has been shown to be involved in several signalling pathways including cell proliferation, differentiation, and cell survival. However the molecular mechanisms of E-cadherin-mediated signalling are largely unknown. Presenilin-1/G-secretase induces proteolytic cleavage of various membrane proteins including E-cadherin. For many of such membrane proteins, the resulting intracellular domain fragments have been reported to localise to the nucleus and have signalling functions. Upon cleavage of E-cadherin by presenilin-1, the whole cytoplasmic fragment (designated as E-cad/CTF2) is released into the cytoplasm. In this study, a possible signalling role for E-cad/CTF2 is investigated. Firstly, in HEK293 cells we found that E-cad/CTF2 is ubiquitinated and translocated to the nucleus, when co-expressed with p120-catenin, a cadherin binding protein. To clarify the functional role of the p120-induced ubiquitination of E-cad/CTF2, we performed a yeast 2-hybrid screen and identified Ret Finger Protein (RFP) as a binding partner for p120. RFP is a RING-finger type E3-ubiquitin-ligase, and it enhances ubiquitination and nuclear localisation of E-Cad/CTF2. In the nucleus, E-Cad/CTF2 binds indirectly but specifically to DNA. Furthermore, its association with DNA is enhanced by p120. Thus we have revealed a molecular mechanism for the nuclear translocation of E-cad/CTF2. The functional role of E-cad/CTF2 in the nucleus is currently being investigated.

Alternative Pathways of E-cadherin Degradation

R. B. Troyanovsky, O. Lazar, S. M. Troyanovsky; Dermatology, Washington University Medical School, St. Louis, MO, 2Microbiology and Immunology, Emory University, Atlanta, GA

Internalization, recycling and degradation of the cell-cell adhesion receptor E-cadherin maintain the dynamics of intercellular contacts in epithelial cells. We studied cadherin internalization and its rate of degradation in control epithelial A-431 cells and after the inactivation of clathrin-based endocytosis by clathrin small interfering RNAs. The lifetime of the endogenous or myc-tagged E-cadherin appeared to be approximately 8 h. Inactivation of clathrin-based endocytosis blocked cadherin recycling nearly completely. However, it failed to change the cadherin lifetime and the overall amount of this protein on the cell surface. This observation suggested that in addition to clathrin-dependent cadherin recycling, there are alternative clathrin-independent mechanisms that maintain cadherin surface expression. To evaluate those mechanisms, we determined the lifetime of different cadherin mutants in control and clathrin-depleted cells. Point mutations of the extracellular cadherin region inactivating any cadherin calcium-binding site or a truncation of the intracellular cadherin region (Ser844-Asp882) abolishing its binding to beta-catenin slightly decreased cadherin lifetime. The lifetime reduction of calcium-site cadherin mutants became very dramatic in clathrin-depleted cells. Mutants of this group, however, were completely stabilized in both control and clathrin-depleted cells by deletion of the p120-binding site (Asp760-Leu771). Behavior of the cadherin-binding site mutant was very different: (i) clathrin depletion normalized its lifetime and increased its surface expression; (ii) it was even further destabilized by deletion of p120-binding region. These data suggest that clathrin-based recycling competes with the p120-dependent degradation pathway. The latter mechanism is able to selectively recruit for degradation proteins with abnormalities in calcium-binding sites. In contrast, the clathrin-dependent pathway may select for degradation the E-cadherin that bears abnormalities in the intracellular region.

Characterization of Pannexin Family Members and Their Potential Role as Gap Junction Proteins

S. Penuela, Q. Shao, X. Gong, C. S. Lounsbury, J. Manias, D. Bai, D. W. Laird; Department of Anatomy and Cell Biology, University of Western Ontario, London, ON, Canada

Pannexins are among the many families of gap junction proteins (pannexins) that are proposed to play a role in gap junctional intercellular communication (GJIC). There are three pannexins in the murine genome, namely Panx1, Panx2 and Panx3. In this study, we characterized their expression, subcellular localization and potential function in GJIC. RT-PCR products of murine RNA were cloned to generate carboxyl terminus-GFP-tagged and untagged expression constructs of Panx1 and Panx3 coding sequences. Synthetic peptides derived from motifs specific for each pannexin were used to successfully generate polyclonal antibodies. Immunofluorescent labeling of untagged and GFP-tagged Panx1- and Panx3- transfected NRK and N2A cells, revealed cell surface localization of Panx1 and Panx3, except when Panx3 was tagged with GFP as this caused Panx3 to be restricted to the endoplasmic reticulum. Western blots of untagged Panx1- and Panx3- expressing cells showed that both pannexins exhibited a molecular weight of approximately 44-47 kDa. Interestingly, recent functional assays suggest that N2A cells expressing untagged Panx1 protein are capable of passing Lucifer yellow dye and demonstrate robust electrical coupling. However, Panx1-based functional coupling was greatly reduced when tagged with GFP and only negligible functional coupling was detected in cells expressing either untagged or tagged Panx3. Preliminary immunofluorescent labeling results from an endogenous pannexin survey of over 14 different cell lines revealed specific cell surface labeling for both pannexins in MDCK cells while pannexins were not detectable in the majority of the cell lines tested. Our in vitro studies suggest that in mammalian cells, Panx1 is a functionally competent gap junction protein while, at present, Panx3 on its own fails to meet the functional criteria expected of a gap junction protein. Ongoing in vivo studies are expected to elucidate the physiological functions of pannexins and further assess their potential roles in GJIC.

Projection Structure at 7Å Resolution and Initial 3D Map of a Human Connexin26

A. Oshima,1 K. Tanii,1 Y. Hiroaki,1 Y. Fujioyshi,1 G. E. Sosinsky; 2Department of Biophysics, Faculty of Science, Kyoto University, Kyoto, Japan, 3National Center for Microscopy and Imaging Research, Department of Neurosciences, University of California San Diego, La Jolla, CA

Projection Structure at 7Å Resolution and Initial 3D Map of a Human Connexin26

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Connexin26 (Cx26) is the second smallest member of the family of gap junction (GJ) proteins. Here, we report the projection and the initial 3D maps of Cx26 at 7Å and 20Å resolutions, respectively, of a mutant human Cx26 (8C26M34A). We over-expressed a hexahistidine tagged hCx26M34A using baculovirus recombinant expression system. Detergent solubilized and purified hemichannels were reconstituted into the synthetic lipid (DOPC) to produce two-dimensional crystallized with the unit cell parameters of a = b = 108.4Å, gamma = 90.0°. Analysis of cryoelectron microscopy (cryoEM) images of these crystals using the ALLSPACE program, indicated that these crystals have the phase relationships of p22,2 symmetry. Four untilted images were processed and merged at 7Å resolution, where the overall phase residual proved to be 23.3°. The projection map displays hexameric channels each of which shows a weak but significant density in the channel pore. While crystallizations were done with hemichannel preparations, the 3D map revealed that two hemichannels had re- docked at their extracellular surfaces into the full intercellular channel. Thin section micrographs supported the re-docked structure and also revealed that this crystal form contained two sets of symmetry related intercellular channels and three lipid bilayers. New features observed in this 3D map include a new prominent density inside the channel pore suggesting that the GJ is physically closed and significant protrusions at the cytoplasmic surfaces. The transmembrane domain structure is consistent with the previous structural work (Fleshman et al. Mol. Cell 15 (2004) 879). A full 3D structure determination at higher resolution is currently in progress.
Influence of Connexin Isoform Stoichiometry and/or Arrangements on Channel Permeability of Second Messengers

D. Locke,1 W. Ayad,4 C. Bevans,2 A. L. Harris 1; 1Pharmacology and Physiology, New Jersey Medical School, UMDNJ, Newark, NJ, 2Department of Structural Biology, Max Planck Institute for Biophysics, Frankfurt, Germany

Heteromeric Cx26/Cx32 hemichannels are selectively permeable to cAMP, cGMP and inositol phosphates, whereas the corresponding homomeric hemichannels are not (JBC 273:2808 and 281:16727; ECR 298:643). This selectivity changes with aggregate isoform ratio. The permeability of cAMP and inositol phosphates is not accounted for by these means, suggesting that the radial arrangement of isoforms, rather than stoichiometry, plays the crucial role. To extend the analysis to include other second messengers, we employed a combination of pH and aminosulfonates, which are known to modulate protein conformation and/or permeability. Using high-resolution AFM tomography, we found that the presence of protonated aminosulfonates led to a significant reduction in the number of open channels, which could be attributed to a decrease in the radius of the aqueous pore. Furthermore, we observed that the selectivity of the hemichannel for cAMP and inositol phosphates was dependent on the isoform ratio. Together, these findings suggest that the radial arrangement of isoforms plays a critical role in determining the selectivity of the hemichannel for second messengers.
361 Associations of Different Connexin Channels with Different Lipids
D. Locke, A. L. Harris; Pharmacology and Physiology, New Jersey Medical School, UMDNJ, Newark, NJ
Paired connexin hemichannels form aqueous intercellular pores that compose gap junction plaques in vertebrates. Unpaired hemichannels also exist in plasma membrane, providing separate biological functions. For other channels, the lipid environment provides not just a structural framework, but also the means to modulate channel structure and/or function. Unfortunately, the importance of these interactions for connexin channels is not well understood. It is difficult to define the role of different lipids for connexin channel function in native membranes without dynamically disrupting lipid-lipid or lipid-protein interactions, and/or the physical attributes of membrane and/or junctional plaque. Therefore, the studied roles of particular lipids in connexin channel function (here, Cx26 and/or Cx32) was studied ex vivo using unilamellar liposomes, which affords substantial control over lipid environment(s). Activity of hemichannels was reported by their ability to mediate large osmolyte exchange across the liposome membrane. A complex relationship was observed between channel connexin composition, and the chemistry(ies) of glycerolphospholipid headgroup(s), membrane thickness and/or acyl chain length(s), and their degree of desaturation(s). For example, Cx32 hemichannels were inactive in L-α-phosphatidylglycerol membranes, but active in glypil-PC membranes, which was highly charged, a positively-charged derivative. Channel activity was restored in PC (uncharged) membranes by addition of charged phospholipids, such as L-α-phosphatidylethanolamine, L-α-phosphatidylcholine, L-α-phosphatidic acid or L-α-phosphatidylserine (all negative), but not other uncharged phospholipids, such as L-α-phosphatidylinositol, unless cholesterol was present. Previous work suggested that the ‘lipid’ composition of junctional plaques differs from the surrounding plasma membrane. Electrospray ionization-mass spectrometry was used to analyze mono/di/tri-acylglycerol and glycerophospholipid species in Cx26 and/or Cx32 plaques enriched from transfected HeLa cells. Lipids were identified that were unique to Cx26 or Cx32 plaques, common to both, and different from non-plaque membrane. There is an intimate involvement of specific lipids with different connexins that may contribute to, or reflect, modulations of channel structure and/or function. Supported by NIH grant GM36044

362 Cx30 Forms Heteromeric Channels with Cx26 and Cx32 during Adult Mouse Mammary Gland Development
D. Locke, 6 J. M. Samiei, 7 T. Stein, 7 J. Liu, 6 M. B. Hodgins, 6 B. Gusterson, 7 A. L. Harris; Pharmacology and Physiology, New Jersey Medical School, UMDNJ, Newark, NJ, 6 Squamous Cell Biology & Dermatology, Division of Cancer Sciences & Molecular Pathology, Faculty of Medicine, University of Glasgow, Glasgow, United Kingdom. 7 Gene Regulation & Mechanisms of Disease, Division of Cancer Sciences & Molecular Pathology, Faculty of Medicine, University of Glasgow, Glasgow, United Kingdom.
Oligonucleotide microarray analysis uniquely shows several connexin family members (gap junction proteins) are expressed by the secretary epithelium at different times during the developmental stage of pregnancy, lactation and involution in adult mammary gland. Specifically, Cx30 appears throughout the second half of pregnancy, but is lost within a day or two of pup birth, and is absent in virgin, early-pregnancy or involuting glands. This agrees, in part, with the expression profile for Cx26; however, Cx26 appears much earlier during pregnancy and persists until much later during lactation than Cx30. From mid-pregnancy into early lactation, Cx26 and Cx30 co-localize in junctional plaques between epithelial cells, forming hemichannels of mixed connexin content. Microarray analysis also reveals that Cx32, which is involved in the establishment of secretory regulation in most other exocrine glands, is developmentally restricted to parturition, so suggesting that specific modification of gap junction channel composition and/or intercellular communication pathways occurs at pup birth. Specifically, heteromeric channels of all pairwise combinations are formed when Cx26, Cx30 and Cx32 are expressed within the same cells. Of these hemichannels, Cx26/Cx30 pores are increasingly sensitive to closure by taurine, an osmolyte implicated in milk protein synthesis, with increasing Cx30 content. In contrast, physiological taurine concentrations have no effect on Cx26/Cx30 and Cx30/Cx32 channel activity. The next challenge will be to unravel how different connexin homomers and heteromers contribute to adult mammary gland development through their form, and their function. Such changes in connexin expression, channel composition and their chemical modulation are discussed in relation to the various stages of mammary gland development in the adult mouse.

363 Liver Vesicles Containing Connexin(Cx)32 Use Microtubule Motors to Traffic In Vitro
A. G. Fort, 6 N. G. Dandichi, 6 J. W. Murray, 6 A. W. Wolkoillo, 6 D. C. Spray; 6 Neuroscience, Albert Einstein College of Medicine, Bronx, NY.
Gap junctions consist of transmembrane channels formed by connexin proteins that are unique in allowing direct cytoplasmic communication. Vectorial trafficking of vesicles has been hypothesized to explain polarized and restricted gap junction expression within cells. However, most assays of cytoskeletal involvement in gap junction trafficking have been limited to examination of effects of microtubule and actin filament depolymerization. We have now investigated whether delivery of connexins to their final functional location is mediated by interaction of their cytoplasmic domains with microtubule motors, specifically kinesin. We concentrated on Cx32, the major gap junction protein in liver. We first demonstrated through immunoprecipitation experiments an interaction between Cx32 with kinesin and tubulin in liver lysates. We obtained identical results when we used vesicles isolated from rat and mouse liver by homogenizing in the presence of protease inhibitors, ultracentrifugation and chromatography. Surface plasmon resonance spectroscopy demonstrated a direct interaction between Cx32 and kinesin, and identified the binding site on Cx32, using synthetic peptides corresponding to cytoplasmic domains of Cx32 applied to covalently bound recombinant kinesin. The motility of anti-Cx32 antibloclotting antibodies was evaluated in HepG2 (Murray et al., Mol. Bio. Cell 11, 419, 2000). Vesicles were added to a bed of artificially polymerized rhodamine-labeled microtubules, and then labeled with anti-Cx32 antibody before motility assay was assayed upon addition of 1 mM ATP. On average, 5-10% of Cx32-vesicles in a field were motile; average vesicle velocity along the microtubules was 0.45 um/sec and was inhibited by 1 mM 5'-adenylylimido-diphosphate (AMP-PNP), a kinesin motor inhibitor, but not by 5 uM vanadate, a dynein motor inhibitor. Thus, trafficking of Cx32 in liver is demonstrated to be both microtubule and motor-dependent.

364 Slow Gating of Wild-type and C-terminus Truncated Connexin32 Channels
C. Peracchia, M. Salin, L. L. Peracchia; Pharmacology and Physiology, University of Rochester, Rochester, NY
Gap junction channels are gated by transmembrane voltage (V) and increased [Ca^{2+}] or [K^{+}] (rev. in Peracchia, 2004). There are two V_{j} sensitive gates: fast and slow. Slow V_{j} gate and chemical gate are probably the same (Bukauskas & Peracchia, 1997; Peracchia et al., 1999, 2000). Slow and fast V_{j} gates are in series and each hemichannel has both gates. The slow gate closes at negative side of V_{j} and is believed to be virtually inactive without uncouplers or connexin (Cx) mutations. In contrast, present data indicate otherwise. Oocytes expressing wild-type Cx32 were subjected to trains of -100 mV pulses (12 s duration) repeated every 30 s. At the pulse, current junctional dropped exponentially to a steady-state level after the initial peak. Both peak (PK) and steady-state (SS) junctional conductances (G_{j}) measured at each pulse, decreased exponentially by 50-60% (tau = 1.2 min). G_{j} of Cx32 dropped more dramatically, such that G_{j} of Cx32 increased from 0.4 to 0.6, indicating a drop in V_{j} sensitivity. Similar, but less striking effects were obtained with -60 mV pulses. During recovery, G_{j} measured by applying 20 mV pulses (2 s duration) at 30 s intervals slowly returned to initial values (tau = ~7 min). Reversal of V_{j} pulse polarity caused a slight and brief increase in G_{j} and decrease in G_{j}/G_{PK}. This suggests that V_{j}-dependent hemichannel reopening is faster than hemichannel closing. Similar, yet more dramatic results were obtained with COOH-termim truncated Cx32 (Cx32-D225), a mutant that lacks fast V_{j} gating. The data indicate that the slow gate of Cx32 is fast active in the absence of chemical uncouplers and mutations. There is evidence that calmodulin may be directly involved in the chemical/slow gating of Cx32, possibly by plugging the pore (Peracchia et al., 2000). Supported by NIH grant GM20113.

365 Androgen-regulated ERAD of Connexin32 and Its Assembly into Gap-Junctions
S. Mitra, S. Chakraborty, K. Johnson, P. P. Mehta; Biochemistry and Molecular Biology, University of Nebraska Medical Center, Omaha, NE
The physiological stimuli that control the assembly of connexins into gap junctions and their degradation remain poorly understood. Androgens are the key physiological regulators of the growth and differentiation of prostate epithelial cells. We hypothesized that they maintain the differentiated state of prostate epithelial cells by regulating gap junction assembly and disassembly. Hence, we investigated their effect on gap junctions composed of Cx32 in an androgen-responsive prostate cancer cell line, LNCaP. In LNCaP cells, androgens controlled the expression level of Cx32, and hence gap junction assembly, posttranslational. As assessed by pulse chase analysis of metabolically labeled Cx32, by transfection of Do-Red-Cx32 chimera, which remains trapped in the ER, and by inhibiting intra-Golgi transport with monensin, we found that in the absence of androgens, a major fraction of Cx32 was degraded from the ER in a proteasomal-inhibitor-sensitive manner, whereas in their presence, this fraction was rescued from degradation and formed gap junctions. In the absence of androgens, Cx32 accumulated as discrete puncta upon proteasomal inhibition. Cell fractionation assay showed that these puncta neither resided in the cytosolic fraction nor co-localized with ER/Golgi/ERGIC markers. Supported by NIH Grant GM36044.
**Regulation of Connexin43 Gap Junctions by Intracellular Calcium**

S. V. Thomas,1 M. M. Lurtz,2 C. Louis2; 1Biology, Georgia State University, Atlanta, GA, 2Cell Biology and Neuroscience, University of California, Riverside, CA

Connexin35 (Cx35) forms gap junctions in neurons in the retina and CNS, and has been shown to be regulated by protein kinase A (PKA) phosphorylation. In this study we asked: 1) Is the phosphorylation state of Cx35 regulated by light adaptation? 2) Which signaling pathways affect Cx35 phosphorylation? Dark-adapted, nighttime zebrafish were exposed to white light for 10 minutes, and the eyes excised and fixed. Sections were immunostained with a monoclonal antibody against Cx35 and one of two phospho-specific antibodies: one against Ser110-phosphorylated Cx35, the other against Ser276-phosphorylated Cx35. Also, excised eyecups from light-adapted, daytime zebrafish were superfused for 15 minutes with drugs affecting cAMP signaling pathways, and fixed and immunostained as described. Dark-adapted retinas showed sparse Cx35 phosphoSer110-like immunoreactivity (110P-IR) in the inner plexiform (synaptic) layer (IPL). The labeling was punctate and much of it co-localized with specific Cx35 labeling by the monoclonal antibody (mCx35-IR). 276P-IR was similar to 110P-IR, and nearly all of it co-localized with mCx35-IR. 10 minutes of light exposure resulted in a large increase in 110P-IR in the IPL; there was little change in 276P-IR. The mean intensity of phosphoSer110 puncta increased after light exposure; there was no change in phosphoSer276 puncta intensity. Daytime levels of 110P-IR and 276P-IR were similar to those after short-term light exposure, as was the mean phosphoSer110 puncta intensity. Mean phosphoSer276 puncta intensity was higher during the daytime than after light exposure. In light-adapted excised eyecups, superfusion with 20M Rp-8-cpt-CAMPS, a PKA inhibitor, reduced mean phosphoSer110 and phosphoSer276 puncta intensity. We conclude that white light stimulation triggers rapid phosphorylation events in zebrafish retina, including phosphorylation of Cx35. Inhibition of PKA activity suppresses phosphorylation, allowing for rapid dephosphorylation. This suggests that retinal light adaptation involves widespread phosphorylation of Cx35, which is known to reduce coupling through this gap junction.

**Beta-Adrenergic Regulation of Connexin43 Gap Junctions**

S. V. Thomas,1 M. M. Lurtz,2 C. Louis2; 1Biology, Georgia State University, Atlanta, GA, 2Cell Biology and Neuroscience, University of California, Riverside, CA

Connexin40 (Cx40) is the major connexin in the atria of the heart yet the possible regulation of Cx40 gap junction function by [Ca2+] is unknown. The goal of this study was to determine how [Ca2+] regulates Cx40-mediated cell-to-cell dye transfer in Cx40-transfected HeLa cells. Increasing [Ca2+] by inclusion of the ionophore ionomycin in the medium and elevating extracellular calcium ([Ca2+]), from 1.8 mM to 10 mM resulted in an elevation of [Ca2+], and affected an inhibition of Cx40-mediated cell-to-cell dye transfer ([IC50 of 850 ± 0.72 nM]. AlexaFluor 594 dye transferred to 9.6 ± 0.3 cells at resting [Ca2+] (~ 100 nM); when [Ca2+] was increased to ≥5 µM AlexaFluor 594 dye transfer decreased to 4.0 ± 0.3 cells. This [Ca2+]-mediated inhibition of Cx40 gap junctions was prevented by the calmodulin inhibitor calmidazolium; dye transferred to 9.0 ± 0.3 cells in the absence of calmidazolium and in the presence of calmidazolium the dye transfer was unchanged (9.0 ± 0.4 cells). The Ca2+-dependent inhibition did not appear to be protein kinase mediated because inclusion of the PKC inhibitor Bisindolylmaleimide I or the CaM Kinase inhibitor Lavendustin C were without effect on the ability of elevated [Ca2+] to inhibit Cx40-mediated cell-to-cell dye transfer. These data demonstrate that Cx40-containing gap junctions are inhibited by elevated [Ca2+], which is calmodulin dependent but not PKC or CaM Kinase dependent. Understanding the biochemical basis and the pathways involved in these interactions will provide insight into the regulation of Cx40 gap junctions when atrial [Ca2+] is elevated in conditions such as the ischemia that accompanies myocardial infarction. Supported by NIH grant EY-05684 and American Heart Association affiliate research award 0315176B.

**Functional Investigation of Prostate Specific G Protein-Coupled Offactory Receptor**

W. Zhang, E. Neuhaus, H. Hatt; Cell Physiology, Ruhr University Bochum, Bochum, Germany

Olfactory receptors (ORs) build the greatest superfamily of G-protein coupled receptors. They are expressed individually in the neurons in the olfactory epithelium where they detect volatile substances and react to odors in the olfactory epithelium, in the nasal cavity, and in other tissues, such as tests where they may play an important role in sperm development, chemotaxis and oocyte-sperm interaction. We cloned and functionally expressed a human OR, which was named prostate specific G-protein coupled receptor (PSGR) due to its reported expression in prostate epithelial cells. We employed Ca2+ imaging in HEK293 cells to identify ligands for the recombinant expressed receptor and characterized the molecular receptive field. As PSGR was found to be overexpressed in prostate cancer, we investigated whether the identified ligands also induce Ca2+ influx in a prostate cancer epithelial cell line (LNCaP). In addition to ligand stimulation Ca2+ increase, treatment of LNCaP cells resulted in a time dependent activation of members of the MAPK family. The ligand was also found to be a potent inhibitor of cell proliferation and an inducer of apoptosis. Similar results were obtained using primary prostate cancer cells from resection specimens. Together these results suggest that ligands for PSGR could be developed as novel therapeutic agents for the treatment of prostate cancer. In our study we will further try to find out which signal transduction mechanisms are involved in OR mediated Ca2+ influx in prostate epithelial cells.
Regulation of Anterograde Transport of G Protein-coupled Receptors by the N-Terminal at Multiple Intracellular Compartments

C. Dong, G. Wu; Pharmacology, LSU Health Sciences Center, New Orleans, LA

G protein-coupled receptors are a superfamily of cell-surface receptors that regulate a variety of cell functions. However, the molecular mechanisms underlying the targeting of receptors to their functional destination are poorly defined. Previous studies on the intrinsic structural determinants for export trafficking of G protein-coupled receptors have been mainly focused on the intracellular C-terminus of the receptors. In this report, we determined the role of the extracellular N-termini of α2-adrenergic receptors (α2-ARs) in the anterograde transport from the endoplasmic reticulum (ER) through the Golgi to the cell surface. The N-terminally truncated α2B-AR mutant is completely unable to target to the cell surface and activate downstream signaling. Single Met69 residue is absolutely essential for the export of α2B-AR from the ER, which is highly mediated through modulating correct α2B-AR folding in the ER. The Tyr-Ser motif, which is highly conserved in the membrane-proximal N-termini of all α2-AR subtypes, is required for the exit of α2A-AR and α2B-AR from the Golgi apparatus. Thus, the Tyr-Ser motif may represent a novel Tyr-based signal directing G protein-coupled receptor transport at the Golgi level. These data provide the first evidence indicating an essential role of the N-termini of G protein-coupled receptors in the export from distinct intracellular compartments along the secretory pathway.

Investigation of the Interaction between Olfactory Receptors and Multiple PDZ Domain Protein 1, MUPP1

R. Dooley, E. Neusahn, H. Hart; Cell Physiology, Ruhr University Bochum, Bochum, Germany

The unique ability of mammals to detect and discriminate between thousands of different odorant molecules is governed by the diverse array of olfactory receptors (ORs) found on the dendrites of olfactory sensory neurons in the nasal epithelium. ORs are 7-transmembrane-domain G-protein coupled receptors and comprise the largest gene superfamily in the mammalian genome. Odorant binding produces activation of specific G-proteins that in turn regulate the function of membrane bound enzymes such as adenyl cyclase. Interestingly, certain OR subtypes possess a classical PDZ (PSD-95, Discs large, ZO-1) domain binding motif in the C-terminal region of the receptor. It has previously been shown that PDZ proteins specifically recognize short peptide motifs in the C-terminus of their target proteins and more recently, they have also been shown to bind internal motifs within their interacting partner proteins. These domains have been established as sites for protein-protein interaction and play a central role in organizing diverse cell signalling assemblies. The objective of this work is to investigate the binding of various olfactory receptors with a multiple PDZ domain protein called MUPP1. We found that MUPP1 is expressed in the dendritic knobs of olfactory sensory neurons, where olfactory signal transduction takes place. Upon application of a mixture of 100 different odorant compounds, an increase of MUPP1 expression in the dendritic knobs was observed. In HEK (human embryonic kidney) cells, co-expression of MUPP1 with various ORs of such mutants are the Gly380Arg and Ala391Glu mutations. MUPP1 co-localizes with Rab4, a marker for the rapid recycling pathway, and with mannose-6-phosphate receptor, which traffics between the trans-Golgi network and endosomes, in RhoB Q63L expressing cells suggesting that CXCR2 is recycled through alternative pathways. These findings reinforce the idea that PDZ-domain containing proteins are important for the regulation of receptor sorting and may contribute to the olfactory signal transduction cascade.

RhoB Plays an Essential Role in CXCR2 Sorting Decisions

N. F. Neel,1,2 L. A. Lapierre, 3,2 J. R. Goldenring, 4,2 A. Richmond 1,2; 1Department of Cancer Biology, Vanderbilt University Medical School, Nashville, TN, 2Department of Veterans Affairs, Nashville, TN, 3Department of Surgery, Vanderbilt University Medical School, Nashville, TN, 4Department of Surgery and Department of Cell and Developmental Biology, Vanderbilt University Medical School, Nashville, TN.

The elucidation of the mechanisms that regulate the cellular responses mediated by chemokines is crucial for the identification of therapeutic targets. An important aspect of chemokine receptor function is the intracellular trafficking of these receptors. The CXCR2 chemokine receptor is a G protein-coupled receptor that undergoes clathrin-mediated endocytosis upon ligand binding. The trafficking of CXCR2 is critical for cells to maintain a proper chemotactic response. The mechanisms that regulate the recycling/degradation sorting decision are largely unknown. The small GTPase RhoB regulates the trafficking of the EGFR receptor. We sought to investigate whether RhoB regulates CXCR2 trafficking. In our studies, we utilized dominant negative (T19N) and GTPase-deficient activated (Q63L) RhoB mutants, as well as RhoB siRNA to investigate the role of RhoB in CXCR2 trafficking. Expression of both RhoB mutants and RhoB siRNA severely impaired CXCR2-mediated chemotaxis. Expression of RhoB T19N and RhoB siRNA impaired sorting of CXCR2 to the lysosome after 3 h CXCL8 stimulation and RhoB T19N impaired CXCL8-induced CXCR2 degradation. In cells expressing the RhoB Q63L mutant, CXCL8 stimulated CXCR2 recycling through the Rab11a perinuclear recycling compartment was impaired but CXCR2 degradation was not induced after 30 min of CXCL8 stimulation. CXCR2 co-localized with Rab4, a marker for the rapid recycling pathway, and with mannose-6-phosphate receptor, which traffics between the trans-Golgi network and endosomes, in RhoB Q63L expressing cells suggesting that CXCR2 is recycled through alternative pathways. These data suggest that RhoB plays a key role in CXCR2 recycling/degredation sorting decisions. [This work was supported by NC1 CA34590 and Multidisciplinary Basic Research Training in Cancer T32CA095922]

Identification of the Domain in ErbB2 That Restricts Ligand-induced Degradation

F. Shen, Q. Lin, C. Childress, W. Yang; Weis Center for Research, Geisinger Clinic, Danville, PA

Ligand-induced receptor degradation is an important process for down-regulation of plasma membrane receptors. While epidermal growth factor receptor (EGFR) is rapidly internalized and degraded upon ligand stimulation, ErbB2, the closest member to EGFR in ErbB receptor family, is resistant in ligand-induced degradation. To understand the molecular mechanisms underlying the impairment of ErbB2, we attempted to determine structural factor in ErbB2 that restricts the degradation. By analysis of ligand-induced degradation of EGFR/ErbB2 chimeras, we have identified a region between amino acid residues F1030 and L1075 in ErbB2 as the domain that restricts the ligand-induced degradation. We designated this domain as the Blocking ErbB2 Degradation or the BED domain. Replacement of the BED domain in an EGFR/ErbB2 chimera with the corresponding region of EGFR changed this chimera from a non-degradable to a degradable receptor, indicating that the BED domain is the factor restricting the ligand-induced degradation of ErbB2. In addition, we found that a non-degradable EGFR/ErbB2 chimera was not defective in tyrosine phosphorylation, ubiquitination and interaction with c-Cbl, rather, was defective in ligand-induced internalization, suggesting that the endocytosis defect is the cause restricting the degradation of ErbB2, and that c-Cbl catalyzed mono-ubiquitination is not involved in the impairment in ligand-induced degradation of ErbB2.

Probing the Biophysical and Molecular Mechanism of Pathological Mutations in the Transmembrane Domain of Fibroblast Growth Factor Receptor 3

L. He, E. Li, S. O'Connor, J. Placone, K. Hristova; Johns Hopkins University, Baltimore, MD

Mutations in the transmembrane (TM) domains of receptor tyrosine kinases (RTKs) have been implicated in the induction of pathological phenotypes. These mutations are believed to stabilize the RTK dimers, leading to constitutive signaling. Examples include the β2-AR (Gly380Arg), the α2B-AR folding in the ER. The Tyr-Ser motif, which is highly conserved in the membrane-proximal N-termini of all α2-AR subtypes, is required for the exit of α2A-AR and α2B-AR from the Golgi apparatus. Thus, the Tyr-Ser motif may represent a novel Tyr-based signal directing G protein-coupled receptor transport at the Golgi level. These data provide the first evidence indicating an essential role of the N-termini of G protein-coupled receptors in the export from distinct intracellular compartments along the secretory pathway.

Metal-induced Inhibition of Estrogen-induced Apoptosis in Osteoclasts

K. Patel, A. K. Wilson; Biological Sciences, Benedictine University, Lisle, IL

Divalent metal cations have been demonstrated to be estrogenic on reproductive tissues. We have demonstrated that low-level cadmium exposure can act anti-estrogenically by interacting with ERα receptors to cause bone loss through a non-genomic secondary messenger pathway. It was hypothesized that other divalent metal cations might have similar anti-estrogenic effects on bone via ERα receptors. The ability of metal cations such as, cadmium(Cd²⁺), copper(Cu²⁺), tin(Sn⁴⁺) and chromium(Cr⁶⁺) to prevent estrogen-induced apoptosis of osteoclasts was measured in model lipid systems.
Intracellular Retention, Degradation, and Signaling of Glycosylation Deficient FGFR2 and Craniosynostosis Syndrome Associated FGFR2

E. Hatch, M. Hudson, M. Bothwell; Department of Orthodontics, University of Michigan, Ann Arbor, MI, Department of Physiology and Biophysics, University of Washington, Seattle, WA

FGFRs are known to play a critical role in a variety of fundamental processes including wound healing, angiogenesis and development of multiple organ systems. Mutations in the FGFR gene family have been linked to a series of syndromes (the craniosynostosis syndromes) whose primary phenotype involves aberrant development of the craniofacial skeleton. Craniosynostosis syndrome linked FGFR mutations have been shown to be gain of function in terms of receptor activation, and have been presumed to result in increased levels of FGFR signaling. Unfortunately, studies attempting to link expression of mutant FGFR's with changes in cellular phenotype have yielded conflicting results. In an effort to better understand the biochemical consequences of these mutations on receptor function, here we have investigated the effect of the FGFR2 mutation of Crouzon craniosynostosis syndrome on receptor trafficking, ubiquitination, degradation and signaling. We find that FGFR2-expressing diminished glycosylation, increased degradation and limited cellular sub-localization in the osteoblastic cell line, MC3T3-E1(C4). Additionally we show that trafficking and auto-activation of wild type FGFR2 is glycosylation dependent. Both FGFR2 and unglycosylated wild type FGFR2 signal through phospholipase-C-PLCγ in a ligand independent manner as well as exhibit dramatically increased binding to the adaptor protein, frα. These findings suggest that auto-active FGFR2 can signal from intracellular compartments. Based upon our results we propose that the functional signaling of craniosynostosis mutant, auto-active receptors is limited in some cell types by protective cellular responses such as increased trafficking to lysosomes and proteasomes for degradation.

Role of Ubiquitination in cMet Internalization

K. S. Hill, N. Li, A. Elfenink; Neuroscience and Cell Biology, University of Texas Medical Branch, Galveston, TX

Intracellular retention and degradation of the receptor tyrosine kinase cMet is often detected in metastatic cancers, correlating closely with tumor aggressiveness and poor patient prognosis. Hepatocyte growth factor (HGF) binding to cMet induces receptor phosphorylation and clathrin-dependent endocytosis. Subsequent lysosomal degradation of the internalized cMet terminates signaling. Thus clathrin-mediated cMet internalization is considered a key limiting step for termination of cMet signaling. Studies have shown that the direct binding of Cbl to cMet at Y1003 mediates cMet ubiquitination and receptor degradation. However the precise role of cMet ubiquitination on receptor internalization remains unclear. To determine the role of cMet ubiquitination on receptor internalization, two cMet mutants (Y1003F and P991S) deficient in receptor ubiquitination were generated using site-directed mutagenesis and stably expressed in cMet null (+/-) cells. Biochemical and confocal approaches will be used to examine these mutant cMet receptors for defects in endocytic trafficking and receptor signaling. If cMet ubiquitination is required for HGF dependent internalization, then cells expressing these mutants would be expected to have increased surface levels of cMet, exhibit a highly proliferative and motile phenotype and form metastatic tumors in vivo. These studies will be extended to determine whether ubiquitination of other endocytic components is important for cMet internalization, and the role of Cbl and additional ubiquitin ligases in this process. An increased understanding of the ubiquitin-dependent interactions important for the termination of cMet signaling could identify new therapeutic targets for treating metastatic cancers in which cMet signaling persists.

Cell Surface Biliverdin Reductase Regulates Innate Immunity

B. Wegiel, C. J. Baty, E. Ciszmadia, J. R. Scott, E. Kaczmarek, H. Bach, E. Otterbein; BIDMC Harvard Medical School, Boston, MA, University of Pittsburgh, Pittsburgh, PA, University of Texas Medical Branch, Galveston, TX

The pasteurella multocida variant strain (P. multocida) demonstrates that BVR is also present on the external plasma membrane in macrophages in vitro and in vivo in the lung and liver as show by total internal reflection fluorescence (TIRF) microscopy, immunoblotting, and flow cytometry (FACS). Surface cell BVR expression is greatly increased after endotoxin challenge. BVR as a surface protein functions not only to generate bilirubin, but also to initiate signaling by the P38A-PLCγ cascade. BVR directly interacts with p85α regulatory subunit of PI3K via increasing biliverdin-induced tyrosine kinase activity and thereby induces activation of PI3K-Akt signaling. Mutation of BVR on Tyr198 leads to the loss of biliverdin signaling and P38A-mediated production of IL-10. Furthermore, P3K-p85α- but not p85β- bone marrow-derived macrophages show impaired signaling of biliverdin. We also tested the hypothesis that the potent anti-inflammatory properties of biliverdin in models of endotoxic shock is due in part to BVR co-localization and interaction with Toll like receptor 4 (TLR4) in the cell membrane. Increased surface BVR expression resulted in decreased TLR4 activity and inhibition of LPS-induced TNF-α and IL-6 expression. Conversely, expression of BVR siRNA increased TLR4 expression and cytokine production, resulting in a loss of the anti-inflammatory effects of biliverdin. These findings demonstrate for the first time that BVR is expressed as a surface protein and directly modulates LPS induced TLR4-Myd88-NFkB and PI3K-Akt signaling. The physical interaction between BVR, which contains a TIR-like domain and TLR4 suggests that BVR is a novel adapter protein involved in negative regulation of LPS signaling. Additionally, the protective effects ascribed to heme oxygenase-1 may in part be explained by the action of BVR. Taken together the above findings elucidate a major new molecular mechanism for the natural regulation of innate immunity.

Characterization of P2X Receptors in Human Lung Mast Cells

C. Vial, P. Bradling; Cell Physiology and Pharmacology, University of Leicester, Leicester, United Kingdom

Mast cells are involved in both innate and acquired immunity and play a central role in the induction of allergic inflammation. A potential role for extracellular nucleotides on mast cells including chemotaxis, cytokine expression, apoptosis and histamine release has been reported. However, the identity of the P2 receptor subtypes involved in these physiological events remains unclear. In the present study, we investigated which functional P2X receptors were expressed by human lung mast cells (HLMC) and the human immature and mature mast cell lines, HMC-1 and LAD-2. Using a combination of RT-PCR and western blot techniques, we identified the presence of the P2X1, P2X2 and P2X3 receptor subtypes in these cells. We recorded P2X receptor currents from LAD-2 cells, using the perforated patch configuration and repetitive applications of ATP (100 μM) every 5 min evoked. The first ATP application evoked a rapidly activating (peak current -169±42 pA, time to peak 47±4 ms) and desensitizing inward current (24±4 /% of peak current remaining at 2s)(n=9). In contrast, subsequent ATP applications evoked slower activating (time to peak of the fourth ATP application: 625±52 ms, p<0.05 (n=9)) and desensitizing inward currents. Indeed, the percentage of peak current remaining at 2s for the second, third and fourth ATP applications were 57±5%, 68±4% and 72±4% respectively (p<0.001, n=9). This shows that the first initial fast ATP response plays a priming role in recruiting the more sustained current observed after subsequent ATP applications. The receptors involved are likely to be P2X1 and P2X3 receptors as P2X- receptor would require a higher concentration of ATP to be activated. Similar results were observed for human lung mast cells, indicating that the mature mast cell line LAD-2 is a good model to study P2X receptor functions in mast cells.

Demonstration of Specific Binding of TP508 to Receptors on Fibroblasts and Coronary Artery Endothelial Cells by Photo-Affinity Cross-Linking

J. S. Bergmann, Z. Dowdy, D. Carney; Biochemistry & Mol. Biology, University of Texas Medical Branch, Galveston, TX

TP508 (Chrysalin®) is a non-proteolytic synthetic peptide representing the portion of human thrombin originally identified as the fibroblast high-affinity receptor binding domain. TP508 is shown to accelerate revascularization and repair of animal tissues and is undergoing human clinical testing for healing of bone fractures and diabetic foot ulcers. In spite of these physiological effects, modified TP508 (125I-TP508S), couple the heterobifunctional, photoactivatable cross-linker (Sulfo-SANPAH), and cross-link and fourth ATP applications were 57±5%, 68±4% and 72±4% respectively (p<0.001, n=9). This shows that the first initial fast ATP response plays a priming role in recruiting the more activating (time to peak of the fourth ATP application: 625±52 ms, p<0.05 (n=9)) and desensitizing inward currents. Indeed, the percentage of peak current remaining at 2s for the second, third and fourth ATP applications were 57±5%, 68±4% and 72±4% respectively (p<0.001, n=9). This shows that the first initial fast ATP response plays a priming role in recruiting the more sustained current observed after subsequent ATP applications. The receptors involved are likely to be P2X1 and P2X3 receptors as P2X- receptor would require a higher concentration of ATP to be activated. Similar results were observed for human lung mast cells, indicating that the mature mast cell line LAD-2 is a good model to study P2X receptor functions in mast cells.

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show that PAR-1 migrates as a single band of ~42 kDa in these gels and that none of the \(^{125}\)I-TP508S complexes are recognized by the antibody to PAR-1. These results indicate that TP508 signals specifically binds to one or more receptors distinct from PAR-1. Ongoing characterization and sequencing will provide additional answers to help us understand how these molecules relate to TP508 signaling.

Role of Oxidative Stress on Angiotensin II-induced Stimulation of Cl-/HCO\(_3\)/Exchanger in Immortalized SHR Proximal Tubular Epithelial Cells

R. Pedrosa, P. Soares-da-silva; Institute of Pharmacology and Therapeutics, Faculty of Medicine University of Porto, Porto, Portugal

We have recently reported an increased activity and expression of the apical Cl-/HCO\(_3\) exchanger (SLC26A6) in immortalized proximal tubular epithelial cells from spontaneous hypertensive rat (SHR) when compared with their normotensive controls (Wistar Kyoto rat; WKY). We also had shown that SHR cells have an enhanced sensitivity to Ang II-induced stimulation of the Cl-\(\rightarrow\)HCO\(_3\) exchanger activity through the AT1 receptor. The present study was designed to evaluate, in immortalized proximal tubular epithelial cells from SHR and WKY rats, (1) the expression of AT1 receptors and (2) the involvement of oxidative stress, namely the H\(_2\)O\(_2\) and NADPH oxidase contribution on enhanced sensitivity to Ang II-induced stimulation of the Cl-/HCO\(_3\) exchanger activity through the AT1 receptor in SHR cells. The level of expression of AT1 in SHR cells was similar to that in WKY cells. However, the membrane expression of AT1 in WKY cells was greater than in SHR cells. The rate of H\(_2\)O\(_2\) production in SHR cells was found to 5-fold that in WKY cells. The addition of ascorbic acid (100 \(\mu\)M), an inhibitor of the NADPH oxidase pathway, in dextran-O\(_4\)-BSA (dextran-O\(_4\)-BSA is used to complex an oxidized lipoprotein)–infused SHR proximal tubular epithelial cells, during four days after seeding 1) reduced the extracellular levels of H\(_2\)O\(_2\) in both WKY and SHR cells, though the effect was markedly higher in SHR cells, 2) completely blocked the enhanced sensitivity of SHR cells to Ang II-mediated stimulation of the Cl-/HCO\(_3\) exchanger activity, but 3) did not change the AT1 expression in both WKY and SHR cells. It is concluded that the enhanced sensitivity to Ang II-induced stimulation of the Cl-/HCO\(_3\) exchanger activity through the AT1 receptor in SHR cells is associated with the higher H\(_2\)O\(_2\) generation, this being independent of differences on AT1 membrane expression. Supported by grant POCI/SUS/CEF/39207/2004 from Fundação para a Ciência e a Tecnologia (Portugal).

Examination of the Effects of Poly(Ethylene Glycerol) (PEG) Rich Matrices on the Transport of Multi Drug Resistance (MDR) and Multi Drug Resistance Associated Protein (MRP) Substrates Using the Caco-2 Cell Model

N. Santos-Roman, M. Licha, M. Torres-Lugo; Chemical Engineering Department, University of Puerto Rico, Mayaguez Campus, Aguadilla, PR

Poly(ethylene glycol) (PEG) is a linear or branched neutral polymer that is widely used in a variety of biological applications for its unique properties. Recently, it has been observed that cells that have been in contact with linear polyethylene glycol (PEG) exhibited an enhanced drug transport for known MRP and MDR substrates. Therefore, the main goal of this project is to characterize crosslinked PEG rich matrices and the investigation of their effect on the transport of known MDR and MRP substrates. Poly(ethylene glycol) hydrogels were synthesized via free radical polymerization using poly(ethylene glycol dimethacrylate N= 600, 1000) (PEGDMA) as the crosslinker and poly(ethylene glycol monomethacrylate N=200, 400, and 1000) as the backbone chain. The hydrogels were characterized by equilibrium swelling ratio, mesh size, partition coefficient, release of the model molecule fluorescein sodium salt, and cytotoxicity. As expected slight effects of increasing tethered chain and/or crosslinker length showed an increase in swelling behavior. This correlates well with mesh size data that showed similar effects, although to a lesser extent. However, no effect of tethered chain was found on partition coefficient. Fluorescein sodium salt (FLUO) release experiments indicated an anomalous behavior for most of the morphologies and relaxation controlled release for the larger tethered chains. Finally, transport of fluorescein sodium salt through Caco-2 cells in contact with 10 mg/mL polymer suspensions at 37 °C shows an enhanced transport of FLUO as the tethered chain increases with a maximum observed at the intermediate length. At 4 °C no polymer effect is observed, which suggests that the previous effects is due to active transport. Next steps include further investigation of the effects of PEG on transport of MDR substrates and on membrane fluidity.

Activation of PAF Receptor Leads to Endothelial Actin Rearrangement and Increased Vascular Permeability.

D. N. Predescu, I. Knezevic, S. Predescu, R. Narnau, M. Gorovoy, N. Knezevic, A. Malik; Pharmacology, University of Illinois, Chicago, IL

Although platelet activating factor (PAF) is one of the most potent vascular permeability modulator known to date, little is known about the molecular mechanisms linking its receptor activation to the cellular signal changes reported. To better understand the molecular determinants and signaling pathways leading from PAF-R to an increased vascular permeability, we decided to use cultured endothelial cells challenged with PAF as a model. We report that upon binding to its receptor PAF: i) activates small proteins of the Rho family (Rac1 and RhoA), ii) determine a shift of different pools of actin, promoting its phosphorylation, iii) induces the appearance of ruffles and filopodia, iv) opens the spaces between endothelial cells, causing formation of gaps in the monolayer, v) promotes via activated Rac actin nucleation that is phosphoinositide dependent. The activation of PAF-R promotes the phosphorylation of cSrc and PLCβ3 via Gαq; however the myosin is not phosphorylated. Therefore, we were able to demonstrate that one of the main signaling pathways leading from PAF-R to actin nucleation require the association of Rac1 with the receptor after its activation by Tiam1/land their translocation from cytosol to cellular membrane. The activated Rac1-Tiam1 complex induces the formation of new free actin barbed ends that finally supports the formation of actin filaments that stand behind the ruffles and filopodia. Hence, the induced polymerization of actin, via activated Rac1 and RhoA, and not the phosphorylation of cSrc is the main mechanism by which PAF induces changes in vascular permeability that are so fast and so well controlled. In conclusion our data provide evidence for the existence of a new signaling pathway involved in controlling the restrictiveness of endothelial barrier.

Familin-C Interacts with α1-Adrenergic Receptor and Modulates Its Effect on F-actin Reorganization through Balancing ERK1/2 and p38 MAPK Pathway

T. Zhang,1,2 Q. Xu,1 F. Chen,3 Z. Lu,1 Q. Han,1 Y. Zhang1; 1Institute of Vascular Medicine, Peking University Third Hospital and Key Laboratory of Molecular Cardiovascular Sciences, Ministry of Education, Beijing, China; 2Present address: NIMH, Bethesda, MD

α1-adrenergic receptors (α1-ARs) are G-protein-coupled receptors that couple to the Gq11 signaling pathway. All three α1-AR subtypes (α1A-, α1B-, and α1D-AR) respond to agonists in vascular smooth muscle contraction, cardiac hypertrophy quite differently. In addition, evidence has shown that α1A-AR is the most important and efficient subtype of α1-ARs. To look for new α1A-AR interacting non-G-proteins that may contribute to these differences among α1-ARs, we have identified filament-C C terminus (FlnC-CT, Ig repeat 24) as an α1A-AR C terminus binding partner in yeast cells. In the present study, we have further investigated their interactions and functions in HEK293A cells with or without stable α1A-AR expression. Immunofluorescence indicated that FlnC-CT (Ig repeats 19-24) co-localized with full length α1A-AR and affects subcellular localization, especially after AR activation with phenylephrine. Their direct binding was further confirmed by both Co-immunoprecipitation and FRET assays. Functional studies revealed that α1A-AR activation induced F-actin formation could be inhibited by FlnC-CT but not full-length FlnC (FlnC-F) overexpression. In addition, FlnC-F or FlnC-CT inhibited α1A-AR dependent ERK1/2 or p38 phosphorylation level differently: FlnC-F increased p38 while FlnC-CT increased ERK1/2. Blocking of ERK1/2 phosphorylation by PD98059 reversed FlnC-CT’s inhibition on α1A-AR activation induced F-actin formation. In contrast, blocking of p38 by SB203580 inhibited F-actin formation in FlnC-F overexpressed HEK293A cells. Our findings provided evidence for the first time of a direct interaction between α1A-AR and FlnC in mammalian cells. It showed that FlnC could modulate α1A-AR dependent F-actin reorganization through balancing ERK1/2 and p38 MAPK pathway. This data sheds new light on FlnC’s participation in and modulation of α1A-AR signaling pathway and provides new clues for studies on physiological functions of both α1A-AR and FlnC.

Microbead Analysis of Inhibitors of Lectin-Cell Binding


We model an inexpensive alternative to microarrays using derivatized microbeads to examine the binding of mannose-rich yeast (Saccharomyces cerevisiae) to Concanavalin A, in the presence of 30 different inhibitors. We developed a list of candidate inhibitors of immobilized Con A, in order of decreasing inhibitory activity, and multitissue being most effective, based on over 14,000 replicates. Saccharides were tested at 0.05 M concentration, the lowest sugar concentration found to provide reliable results. At lower concentrations of sugars, down to 0.017M, results were not reliable. This is the most extensive listing of saccharide inhibitors of immobilized Con A binding ever developed and we suggest that it should replace less expensive lists that have been in the literature for decades. Currently microarrays are being used in the development of carbohydrate drugs and diagnostic tests. We propose that microbeads can, in some cases, substitute for microarray technology. If, for example, our model yeast was a pathogen, our studies show that it binds via cell surface mannose residues and drugs to prevent binding could be developed using the inhibitors identified here. Microbeads could be also used in the development of diagnostic tests that identify the presence of organisms in blood samples, etc., in much the same way as microarrays are being used today (Supported by grants from NIH NIGMS SCORE, RISE, MARC programs, IQT program and the Joseph Drown Foundation).
Characterizing the Polycation Receptor of Paramecium
H. G. Kuruvilla, E. D. Robinette; Science and Mathematics, Cedarville University, Cedarville, OH

Paramecium are excellent model cells in which to study behavioral avoidance because of their large size and adaptability for electrophysiology. Since we had previously characterized a polycation receptor from a related ciliate, Tetrahymena, we wished to determine whether Paramecium possessed a similar receptor. Our hypothesis was that Paramecium possessed a polycation receptor with characteristics similar to the Tetrahymena receptor in terms of in vivo behavioral responses. We found that Paramecium does possess a polycation receptor, and that ligands such as lysozyme, PACAP, and VIP all elicit behavioral avoidance responses in Paramecium just as they do in Tetrahymena. However, the EC50 of the compounds were significantly different in Paramecium than they were in Tetrahymena. While in Tetrahymena the EC50 of PACAP-lysozyme-Paramecium we find that the EC50 of lysozyme-VIP-PARAMECUM. PACAP is also toxic to Paramecium at levels near those which elicit avoidance, making it an unfavorable ligand for study in this system. Future studies of the polycation receptor will involve drug studies to determine how the lysozyme pathway works in Paramecium, since lysozyme is the cheapest and most effective of the polycations that we studied in this system.

Functional Characterization of a Cloned Bacterial Virus Tail Protein
R. E. Ramirez, M. Zayas, R. Villafane; 1Biology, University of Puerto Rico-Mayaguez, Mayaguez, Puerto Rico, 2Biochemistry, Ponce School of Medicine, Ponce, Puerto Rico

To understand in detail the interaction between bacterial cell lipopolysaccharide (LPS) and protein, a molecular genetic approach using phage tail protein of Salmonella phage P22 and the c34 rsp with respect to their phage associated properties. This study reports the DNA sequence of the gene for the c34 rsp as well as reporting its initial functional characterization. The functional nature of the expressed clone tail gene suggests that it is functional and resistant to trypsin protease.

Yeast Cell Biology: Human Physiology in a Nutshell
G. Nadel, M. Mayrose, S. Bar-Nun; Biochemistry, Tel-Aviv University, Tel-Aviv, Israel

There is a remarkable conservation of cellular mechanisms responsible for quality control, transport and degradation of proteins between yeast and man. In addition, yeast has been instrumental in genetic studies of human diseases associated with quality control. Here we identify and characterize in yeast trans-acting cellular components that contribute to secretion or retention of immunoglobulins. In B-lymphocytes, secretion and degradation of secretory IgM are developmentally regulated. The μCys (the C-terminal of μ, the heavy chain of secretory IgM, and its penultimate Cys575) is the necessary cis-acting signal for μ retention in B-cells (Sita et al. 1990 Cell 60, 781-790). This motif is also sufficient to confer retention and degradation onto several reporter proteins in lymphoid and non-lymphoid mammalian cells. The evolutionary conservation of the secretory pathway from yeast to man is strikingly brought to us as we think. Two μCys motifs are thought to confer retention also onto yeast secretory proteins. To that end, μCys and its mutant μCysSer were fused to the C-terminal of invertase. Secretion of this chimeric protein from Saccharomyces cerevisiae was monitored either by its enzymatic activity or by measuring secretion from spheroplasts using immunoblotting. Interestingly, μCys, but not μCysSer, conferred retention onto invertase, although yeast neither synthesize immunoglobulins nor μCys is found in the yeast genome. We have discovered several ER resident proteins that affect the secretion of μCys-invertase. These proteins either drive secretion by specific selection and transport to the Golgi via COPII vesicles (Erv1p-Erv2p) or mediate retention through disulfide bonding with the μCys-invertase (Eg1p). We conclude that the μCys is a general cis-acting retention signal that can be used in yeast to unveil trans-acting cellular components and mostly ER resident proteins that act in concert to regulate secretion.

CREB-II, a Transmembrane Transcription Factor Subject to Alternate Pathways of ERAD and Stress Regulated Intramembrane Proteolysis
D. Bailey, P. O'Hare; Protein Trafficking and Gene Regulation, Marie Curie Research Institute, Oxted, Surrey, United Kingdom

Regulated intramembrane proteolysis (RIP) is a process whereby in response to specific cues ER-resident transcription factors, are released from the ER to the Golgi and cleaved by Golgi proteases, to release a cytosolic domain which traffics to the nucleus to effect transcription of appropriate genes. We examine the ER processing of CREB-II, a new member of the RIP class of proteins. CREB-II is localised to the ER, and glycosylated, while an N-terminal variant containing the conserved bZIP domain, accumulates in the nucleus. Brefeldin A disruption of the Golgi, induces cleavage and the production of a cytosolic product that translocates to the nucleus, indicative of processing by SIP and SIG proteases. An ER-localised form of SIP also induces CREB-II cleavage and nuclear translocation. We also find that CREB-II is subject to rapid turnover involving a cycle of ER insertion and retrotranslocation through the ER associated degradation route. Proteasome inhibition was sufficient to induce cleavage of CREB-II, to a greater extent than tunicamycin. CREB-II is subject to two pathways of retrotranslocation:ERAD and stress-induced release for SIP processing. We propose that CREB-II may monitor the ERAD/proteolysis pathway and stress or cytosolic perturbation in this pathway results in alternate transport into the secretory route for Golgi cleavage.

In Vitro Reconstitution of ATF6 Budding during ER Stress
A. Schindler, R. Scheckman; Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA

ATF6 is an ER-localized transmembrane protein that traffics to the Golgi complex specifically during ER stress. In the Golgi, ATF6 is cleaved by site 1 and 2 proteases to release a soluble transcription factor that induces gene expression in the unfolded protein response (UPR). Although it is known that ATF6 trafficking is regulated by ER stress, the particular events that allow its recognition by the COPII budding machinery are not known. Because ER stress is thought to be a lumenal event, whereas budding occurs primarily through cytoplasmic proteins, ATF6 provides an example of regulation across the ER membrane. To study ATF6 trafficking, we have developed an in vitro assay that recapitulates the ER-stress induced packaging of full-length ATF6 into COPII vesicles. While crude ER membranes from mammalian cells are treated with the ER-stress inducer DTT, ATF6 is captured in COPII vesicles. Other cargo proteins were not affected by DTT treatment. ATF6 budding required nucleotides and cytosol, and was inhibited by nonhydrolysable GTP and dominant-negative forms of the GTPase Sar1. ATF6 packaging required DTT activity in the ER lumen, as a membrane-impermeable reducing agent failed to induce ATF6 budding. Specific isoforms of the COPII proteins Sec23 and Sec24 were analyzed for their specificity for ATF6. To determine the domains required for ATF6 recognition by COPII, stable cell lines were made containing ATF6 mutants and tested in the in vitro budding assay. These data indicate that ATF6 budding occurs from existing protein pools and does not require protein synthesis. Further, cleavage of disulfide bonds in the ER is sufficient to induce ATF6 budding.

Interaction of RelR1p with Nicastrin Is Required for the Initial Steps of γ-secretase Complex Assembly in Pre-golgi Compartments

γ-secretase complex, consisting of presenilin-1 or presenilin-2, nicastrin, PEN-2 and an APH-1 isoform, mediates the intramembrane proteolytic event leading to the liberation of the βγ-acting signal for γ-secretase activities. Binding to Rer1p is mediated through spaced polar residues in the nicastrin transmembrane domain that are also critical for binding to APH-1. Absence of APH-1 γ-secretase activities. Binding to Rer1p indicates that both proteins compete for nicastrin. Hence, we propose that RelR1p recycles nicastrin from the Golgi, thereby increasing the probability for nicastrin to form a complex with APH-1. This mechanism demonstrates the necessity of secondary endoplasmic reticulum quality control in proper γ-secretase complex assembly in early compartments. Our findings may open new therapeutic avenues in Alzheimer’s disease by controlling AP production through a direct interference with the levels of γ-secretase complexes at an early stage in assembly.
Secretory COPII Coat Component Sec32a Is Essential for Craniofacial Chondrocyte Maturation

L. Blue,1 C. Johnson,2 J. Clark,3 G. Vezina,1 C. L. Craft,1 1Department of Pathology, University of Virginia, Charlottesville, VA, 2Department of Medicine, University of Virginia, Charlottesville, VA, 3Department of Biology, University of Virginia, Charlottesville, VA

Craniofacial chondrocyte maturation is essential for craniofacial morphogenesis.” Since CRHCR lesion is known to cause the cranio-lenticulo-sutural dysplasia syndrome (CLSD), CRHCR provides the first vertebrate model system that links the biology of ER to Golgi trafficking with a clinically relevant dysmorphism.

Dynamics of Sar1p and Sec12p in the Endoplasmic Reticulum in Living Yeast Cells

K. Kurokawa,1 A. Nakano2,1 1Molecular Membrane Biology Laboratory, Riken Discovery Research Institute, Wako, Japan, 2Graduate School of Science, University of Tokyo, Bunkyou-ku, Japan

The budding yeast Saccharomyces cerevisiae has provided an excellent model to study cell polarity and its molecular mechanism. This unicellular organism polarizes its growth to produce a daughter cell by budding. The development of bud depends on the polarization of exocytosis and the actin cytoskeleton. Recent studies suggested that cell polarity impinges not only on the plasma membrane but also on the endoplasmic reticulum (ER) provided an excellent model. Vesicles coated with coat protein complex II (COPII) selectively transport cargo molecules and vesicle fusion proteins from the ER to the Golgi apparatus. COPII consists of Sar1p GTPase and Sec23/24p and Sec13/31p sub-complexes. How cell polarity affects the dynamics of COPII has not been elucidated. In this study, we focused on the dynamics of Sar1p and Sec12p, a specific guanine-nucleotide exchange factor of Sar1p, on the bud and the mother ERs in living yeast cells. By using a fluorescent protein, Dronpa, which can be marked reversibly for repeated measurements, we investigated the diffusion of Sar1p and Sec12p. The diffusion of Sar1p-Dronpa and Dronpa-Sec12p from the mother ER to the bud ER was inhibited, indicating that the diffusion barrier existed at the bud neck. In addition, Sar1p had a longer half life within the bud ER than within the mother ER. These results suggest that COPII dynamics is affected by the polarity of yeast cells.

Defining the Role of COP-I in Vesicular Membrane Transport through Depletion of β-COP

M. L. Stryer,1 E. Cortes-Moyaka,2 E. Szul1 1Cell Biology, University of Alabama at Birmingham, Birmingham, AL, 2Biochemistry and Molecular Biology, University of Alabama, Birmingham, AL

Within the mother ER. These results suggest that COPII dynamics is affected by the polarity of yeast cells.

Structure Function Analysis Reveals Important Regulatory Roles for a C-terminal Domain in Ralp, a Bacterial Member of the Sec7 Family of ARF Exchange Factors

L. Chesnel1, J. C. Kagan,2 C. R. Roy1 1Microbial Pathogenesis, Yale University, New Haven, CT, 2Immunochemistry, Yale University, New Haven, CT

ADP-ribosylation factor proteins (ARFs) are small GTPases that play a critical role in organelle biogenesis and membrane transport. ARFs function as molecular switches, cycling between GDP-bound inactive and GTP-bound active states. The GTPase cycle is regulated by GTPase Activating Proteins (GAPs) and GTPase Activating Proteins (GAPs). The six different mammalian ARF proteins can be divided into three different classes having distinct intracellular functions. Although ARF-GAPs often activate all ARF family members in vitro, they usually act only on a subset of ARFs in vivo. Interestingly, the bacterial pathogen Legionella pneumophila and Rickettsia prowazekii encode a protein called Ralp that functions as an ARF GEF. Ralp has an N-terminal domain highly similar to the eukaryotic Sec7 domain found in most ARF GEFs and a C-terminal domain of unknown function. Structural analysis suggests that the C-terminal domain of Ralp may mediate the activity of the catalytic Sec7 domain. Here we have used a variety of cell biological assays to investigate the function of the Ralp C-terminal domain. Our results indicate that the C-terminal domain of the Ralp protein mediates subcellular localization of the bacterial effector in the eukaryotic host cell. The L. pneumophila Ralp C-terminal domain localized primarily to the ER and early secretory vesicles, consistent with the in vivo activity of this protein being restricted to class I ARF proteins. By contrast, the highly similar C-terminal domain of the R. prowazekii Ralp protein mediated localization to actin-rich membrane ruffles and co-localized with ectopically produced ARF6. These studies indicate that the Ralp C-terminal domain mediates GEF localization. These studies provide unique insight into the mechanism by which ARF activation is controlled by demonstrating that spatial localization may dictate ARF-GEF specificity in eukaryotic cells.

SNARE Status Regulates Tether Recruitment and Function in Homotypic CopII Vesicle Fusion

M. Bentley,1 R. Mullen,2 D. Xu3 E. Sztul1 1Division of Biological Sciences, University of Montana, Missoula, MT, 2Institute of Biochemistry, Frankfurt, Germany, 3Department of Cell Biology, University of Alabama, Birmingham, AL

In mammals, COPII-coated transport vesicles deliver secretory cargo to vesicular tubular clusters (VTCs) that facilitate cargo sorting and transport to the Golgi. We documented in vitro tethering and SNARE-dependent homotypic fusion of ER-derived COPII transport vesicles to form larger cargo containers characteristic of VTCs (Xu and Hay, 2004, J. Cell Biol. 167:997). COPII vesicles thus appear to contain all necessary components for homotypic tethering and fusion, providing a pathway for de novo VTC biogenesis. Here we demonstrate that low concentrations of antibodies against the ER-Golgi SNARE syntaxin 5 inhibit COPII vesicle homotypic fusion, while higher concentrations inhibit both tethering and fusion, implying an unanticipated role for SNAREs upstream of fusion. Inhibition of SNARE complex disassembly with dominant-negative α-SNAP also inhibited tethering, implicating SNARE assembly status as a critical determinant in COPII vesicle tethering. The tethering-defective SNAREs generated in the presence of dominant-negative α-SNAP specifically lacked the rab1 effectors p115 and GM130 but not other peripheral membrane proteins. Furthermore, rab effectors, including p115, were shown to be required for homotypic COPII vesicle tethering. Thus, our results demonstrate a requirement for SNARE-dependent tether recruitment and function at future sites of membrane fusion. We anticipate that recruitment of tether molecules by an upstream SNARE signal ensures that tethering events are initiated only at focal sites containing appropriately poised fusion machinery.
A Novel Functional Domain within the p115 Tethering Factor Is Required for Golgi Ribbon Formation

E. Sztul,1 R. Grabski,1 E. Brandon,1 C. Alvarez,1 T. Sztul,1 J. Hay2
1Cell Biology, University of Alabama at Birmingham, Birmingham, AL, 2Biochemistry, University of Cordoba, Cordoba, Argentina, and Department of Biological Sciences, University of Montana, Missoula, MT

Tethering factors have emerged as key regulators of membrane traffic and organellar biogenesis. Herein, we explore the function of the p115 tethering factor operational at the ER-Golgi interface. We show that depletion of p115 disrupts the Golgi ribbon into fragments. The fragments form de novo adjacent to ER exit sites in cells subjected to BFA treatment and a subsequent wash-out. Significantly, the reformed Golgi fragments show cis- to trans-polarity. These results suggest that p115 is dispensable for traffic events generating differentiated Golgi fragments, but is required for fusion of such fragments into a Golgi ribbon. It appears that in vivo, the requirement for p115 is limited to later stages of ER-Golgi traffic. Previous studies documented that p115 coiled-coil 1 (CC1) region that binds a subset of ER-Golgi SNAREs and the Rab1 GTPase is required for Golgi biogenesis. Herein, we show that the CC1 domain is insufficient to sustain p115 function. We show that p115 mutants containing CC1 (and capable of binding SNAREs and Rab1), but lacking the CC4 coiled-coil domain act as dominant negatives and disrupt Golgi structure in cells containing endogenous p115. Furthermore, such mutants are unable to support Golgi biogenesis in cells depleted of endogenous p115. This suggests that CC4 is required for Golgi biogenesis. Significantly, CC4 has been shown to bind a subset of ER-Golgi SNAREs (Shorter et al., 2002). Our findings indicate that two non-overlapping SNAP25-interacting domains are required for Golgi biogenesis. This supports a model in which p115 facilitates SNARE complex formation by using CC1 and CC4 to simultaneously bring two SNAREs into close proximity. We postulate that membrane tethering may occur through tether-mediated SNARE-SNARE linkage.

ARF/COPI Events Regulated by the Guanine Nucleotide Exchange Factor GBF1 Maintain the Structure of the cis-Golgi and Facilitate Traffic of Developmentally Essential Proteins

E. Sztul,1 T. Sztul,1 R. Grabski,1 S. Shestopal,1 S. Lyons,3 M. Jeon,3 K. Zinn,3 S. Eimer,4 G. Marques1 1Cell Biology, University of Alabama at Birmingham, Birmingham, AL, 2Neurobiology, University of Alabama at Birmingham, Birmingham, AL, 3Division of Biology, California Institute of Technology, Pasadena, CA, 4Center for Molecular Physiology of the Brain, European Neuroscience Institute, Goettingen, Germany

The recruitment of COPI coat to membranes is required for secretory traffic and is mediated by the active form of the ADP-ribosylation factor (ARF). ARF is activated through a GDP/GTP exchange facilitated by guanine nucleotide exchange factors (GEFs). Multiple ARF-GEFs have been identified, but their exact functions within the secretory pathway remain unclear. Here, we describe the GBF1 exchange factor as a major determinant of ERGIC and cis-Golgi traffic and the key facilitator of parallel anterograde traffic of select cargo proteins. Using an RNAi-based approach, we show that GBF1 depletion causes extensive tubulation of ERGIC and cis-Golgi. The cis-Golgi derived tubules connect peripherial ERGIC sites forming a mixed compartment. The formation of the tubular ERGIC/cis-Golgi network in association with the parallel anterograde traffic of select cargo proteins. Soluble proteins such as fibronectin, laminin and cockin are efficiently secreted from GBF1-depleted cells. Thus, newly synthesized VSVG and F-selectin ligand-1 (ESL-1) are not delivered to the PM in GBF1-depleted cells. This suggests that two mechanistically distinct pathways handle protein secretion, and that GBF1 regulates ARF/COPI-dependent cargo passage through one of the pathways. The importance of the GBF1-regulated pathway in vivo was explored in D. melanogaster. Depletion of D. melanogaster homologue of GBF1 in the larval gland results in lack of cellular expansion and leads to marked disruption in salivary gland structure. This correlates with inhibition in surface delivery of cadherin (DCAD2) and flamingo, proteins known to regulate cell migration, cell sorting and epithelial polarity. Importantly, our findings indicate that ARF/COPI events facilitated by GBF1 provide an additional level of morphogenetic control by regulating the surface delivery of key developmental proteins.

Legionella pneumophila Effector Proteins Involved in Transport and Fusion of Endoplasmic Reticulum-derived Vesicles

E. M. Campodónico, C. R. Roy; Section of Microbial Pathogenesis, Yale University, New Haven, CT

Legionella pneumophila is a gram-negative pathogen that can subvert endoplasmic reticulum to Golgi transport to establish a unique vacuole that supports bacterial replication. Subversion of host vesicle transport requires a specialized secretion system called Dot/Icm that injects protein substrates directly into the host cell. Although it is predicted that Dot/Icm substrates will modulate host vesicle transport by interacting with the machinery controlling host endocytic and secretory membrane traffic, the identity and function of most Legionella effector proteins remains unknown. We have identified two homologous Legionella effectors called YlfA and YlfB using a genetic screen for bacterial genes that inhibit yeast growth. We find that a double ylfAB mutant has a decreased ability to establish a replicative compartment over 10 hours. Biochemical analysis revealed that YlfA and YlfB are capable of forming homomeric and heteromeric complexes via carboxy-terminal domain predicted to form coiled coils. Ectopic production studies in eukaryotic cells indicate that the Ylf proteins associate with membranes via amino-terminal hydrophobic residues, and that the production of Ylf results in formation of large membrane-bound compartments containing ER proteins Sec22b and calcinein and the Golgi-associated protein Rab1b. These studies establish that YlfA and YlfB are novel Legionella effectors involved in subversion of vesicular traffic in eukaryotic host cells, and suggest that the Ylf proteins may control membrane fusion events following translocation by Legionella into the host cell, possibly by mimicking host SNARE proteins.

Biochemical Characterization of a GAPDH Mutant Defective in Src-mediated Tyrosine Phosphorylation

E. J. Tisdale, C. R. Artalejo; Pharmacology, Wayne State University School of Medicine, Detroit, MI

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a well-characterized key enzyme in glycolysis that catalyzes the NAD-mediated oxidative phosphorylation of glyceraldehyde-3-phosphate to 1,3 diphosphoglycerate. A variety of studies have demonstrated that GAPDH has multiple intracellular activities in addition to its role in glucolysis. Indeed, we have reported that GAPDH is required for Rab2 mediated retrograde transport from vesicular tubular cisternae (VTCs) (JBC 276:2480). These diverse GAPDH activities are the result of post-translational modifications that confer a new function to the enzyme. In that regard, GAPDH is tyrosine phosphorylated by Src at residue 41 (Nature Biotech. 23:94). To establish the functional significance of this modification for GAPDH activity in Rab2-mediated events, an amino acid substitution was made at tyrosine 41(GAPDH Y41F). The inability of Src to phosphorylate purified recombinant GAPDH Y41F was confirmed in an in vitro kinase assay. We determined that GAPDH Y41F has normal glycolytic activity and efficiently binds to microtubules. The mutant was then employed in a quantitative membrane-binding assay that measures Rab2-dependent recruitment of soluble components to VTCs. As we observed with GAPDH wild type, Rab2 promoted GAPDH Y41F binding to membranes in a dose dependent manner. We learned by performing a time course that Rab2 treated membranes contain activated Src (Tyr 416) before the detection of tyrosine phosphorylated GAPDH. These results indicate that Src-dependent GAPDH phosphorylation occurs on the VTC and that GAPDH tyrosine phosphorylation is not required for VTC association. (This work was supported by NIH grants GM60881 and DK58921).

A Clamping Mechanism Involved in SNARE-dependent Exocytosis

C. G. Giraud, W. S. Eng, T. J. Melia, J. E. Rothman; Physiology & Cellular Biophysics, Columbia University, New York, NY

During neurotransmitter release at the synapse influx of calcium ions stimulates the release of neurotransmitter. However the mechanism by which synaptic vesicle fusion is coupled to calcium has been unclear, despite the identification of both the core fusion machinery (SNAREs) and the principal calcium sensor (synaptotagmin). Here we describe what may represent a basic principle of the coupling mechanism—a reversible clamping protein (Atollplexin) that can clamp SNAREpins, an assembled fusion competent intermediate on route to fusion. When calcium binds to the calcium sensor synaptotagmin, the clamp would then be released. SNARE proteins, and key regulators like synaptotagmin and complexins, can be ectopically expressed on the cell surface. Cells expressing such “flipped” synaptic SNAREs fuse constitutively, but when we co-expressed complexin fusion was blocked. Adding back calcium triggered fusion from this intermediate in the presence of synaptotagmin.
Effects of SCAMP Knockdown on Dense Core Vesicle Exocytosis Analyzed by Amperometry

H. Liao,1 J. Zhang,1 A. Castile,2 G. Szabo,1 J. D. Castle1; 1Cell Biology, University of Virginia, Charlottesville, VA, 2Molecular Physiology and Biological Physics, University of Virginia, Charlottesville, VA

SCAMPs (secretory carrier membrane proteins) comprise an integral membrane protein family with four transmembrane spans. There are four SCAMP isoforms (SCAMP1-4) ubiquitously expressed in mammalian cells and SCAMP5 primarily resides in neuronal cells. Previously, we have shown that overexpressing a SCAMP2 mutant in neuroendocrine PC12 cells leads to fusion pore formation and dilation during dense core vesicle (DCV) exocytosis. In order to examine whether other SCAMP isoforms are redundant for this process or distinct isoforms regulate DCV exocytosis differently, we knocked down SCAMP1, 2, and 5 with specific siRNA and checked the effects by amperometry. By recording individual secretory vesicles with high spatio-temporal resolution, amperometry enabled us to resolve DCV fusion into steps, which include fusion pore opening and subsequent dilation or closure. We found that all SCAMP knockdowns inhibited the rate of fusion pore opening, which was reflected by decreased number and frequency of exocytotic events. SCAMP1 and SCAMP2 knockdown but not SCAMP5 knockdown changed the dynamics of newly opened fusion pores, resulting in the initial fusion pores preferring to close rather than dilate. The average lag of response after depolarization increased in SCAMP2-deficient cells, but not in cells deficient in other SCAMPs. Finally, when both SCAMP1 and 2 were knocked down together, we observed an increase in pre- and post-fusion delay in addition to all the other effects found for individual SCAMP1 or 2 knockdown. By measuring [3H]inositol (IA) uptake, we also showed that SCAMP1 and 5 but not SCAMP2 knockdown decreased IA uptake by 23% and 50%, respectively. Therefore, the SCAMP isoforms regulate DCV exocytosis similarly and also differently, which may be due, respectively, to the conserved core structure shared by all isoforms and the specific regions of each one enabling them to interact with different protein partners.

Enrichment of Phosphatidylinositol 4-phosphate 5-kinases Ia and Ij Results in Dominant Presence of Phosphatidylinositol 4,5-bisphosphate in Secretory Granule Membranes

S. H. Yoo, Y. H. Huh, S. K. Huh, S. Y. Chu, S. Y. Park; Biochemistry, Inha University College of Medicine, Incheon, Republic of Korea

It is generally believed that phosphatidylinositol 4,5-bisphosphate (PIP2) is highly enriched at the plasma membrane and is much less abundant in intracellular organelles. Here we show that the enzymes that catalyze PIP4(2)P synthesis, PIP4(2)P 5-kinases Ia and Ij, are enriched in secretory granule membranes, and PIP2 is most concentrated in granule membranes. The PIP4(2)P 5-kinases coimmunoprecipitate the inositol 1,4,5-trisphosphate receptors (IP3Rs) that are present in secretory granule membranes, suggesting the presence of IP3R-PI(4)P5KI complexes in granule membranes. PI(4)P 5-kinase Ia and Ij localize to both the low- and high-buoyant density fractions of membranes, consistent with their presence in both raft and non-raft regions, though larger amounts are present in the high density fractions. The functional significance of these observations is highlighted by our finding that antibodies against the PI 5-kinases or PIP2 inhibit IP3-sensitive Ca2⁺ release from isolated secretory granules. These results indicate that key molecules specializing in phosphoinositide metabolism and exocytosis reside within the granule itself.

Purinergic Receptors and Calcium Signaling in the Lysosomal Granule

J. P. Girod, S. F. Hamm-Alvarez,2 M. C. Edman; 1Chemistry & Biomedical Sciences, University of Kalmar, Kalmar, Sweden, 2Pharmaceutical Sciences, University of Southern California, Los Angeles, CA

We have previously shown that the adenosine receptors A1 and A2a are expressed in rabbit lacrimal gland acinar cells and active in the regulation of acinar cell secretion. Activation of either receptor results in a 2-fold increase of acinar cell secretion. Our results also show that both A1 and A2a receptor agonists potentiate the secretory effect of carbachol (Cch), an acetylcholine analog, and that the IP3 receptor antagonist 2-APB blocked the synergistic effects as well as the carbachol-evoked secretion (Opt. Vis. Sci. 82, E055069, 2005; Invest. Ophthalmol. Vis. Sci. 47, 1943, 2006). Therefore, the aim of the present study is to further investigate the intracellular signaling events involved in the potentiation by studying the effect of adenosine A1-, A2a-, and muscarinic M3 receptor activation on intracellular levels of Ca2⁺. Primary cultures of rabbit lacrimal gland acinar cells were prepared according to our standard procedure and cultured for two days to allow for reorganization into acinar like structures (Exp. Eye Res. 83, 543-553, 2006). For Ca2⁺ measurements, cells were cultured in 35 mm glass-bottomed, live cell dishes and loaded with the fluorophore Fluo-4-green. Calcium were monitored with a cell culture equipped LSM 510 Meta microscope. The calcium imaging experiments showed that adenosine stimulation resulted in a lower but more prolonged intracellular calcium Ca2⁺ peak than that obtained by Cch. Simultaneous Cch and adenosine stimulation resulted in a higher and more prolonged Ca2⁺ peak than Cch alone. The A1 receptor agonist CPA (cyclopentyladenosine) and the A2a receptor agonist CPCA (cyclopropylcarboxamidoadenosine) both resulted in a peak of shorter duration than adenosine alone, and CPCA with the smallest response. Combinations with Cch and CPA or CPCA had similar effects as Cch + adenosine. In conclusion, our results indicate that Ca2⁺ is a more important mediator of regulated lacrimal acinar cell secretion.

Mechanisms of 1,25D-regulated ATP Release in Osteoblasts

P. Biswas, L. Zanello; Biochemistry, UCR, Riverside, CA

The hormonally active form of vitamin D3, 1,25-dihydroxyvitamin D3 (1,25D), is considered a bone anabolic hormone. 1,25D increases bone matrix production by modulating the synthesis of matrix proteins via a vitamin D receptor (VDR). In addition, 1,25D rapidly stimulates secretion via nongenomic mechanisms initiated at the plasma membrane of bone cells which appear to require a functional VDR. The present study investigates molecular pathways involved in 1,25D-regulated exocytosis in osteosarcoma ROS 17/2.8 cells, and identifies the molecular nature of the exocytosed product. We demonstrated, for the first time, a rapid (within seconds) exocytotic ATP release as a response to nanomolar concentrations of 1,25D in osteoblasts. Single exocytotic events were monitored in real time with videomicroscopy performed on live osteoblasts and stained with 3 μM quinacrine, a cell-permanent fluorophore that binds to ATP. Addition of NEM (1mM), which prevents vesicle fusion to the plasma membrane, significantly reduced 1,25D-induced release of ATP, providing further evidence that ATP release is vesicular. We investigated the Ca2⁺-dependence of the exocytotic vesicular release in osteoblasts, and found that 1,25D-induction of ATP release is coupled to rapid intracellular calcium increase. We found that 1,25D-stimulated CI⁻ channel in osteoblasts couples to rapid interfered release of glutamate in NG108-15 cells, as detected with live-cell microscopy and immunocytochemistry. Using a fluorometric assay, we found 4.8 ± 0.3% and 13.6 ± 0.4% relative increase of matrix proteins via a vitamin D receptor (VDR). In addition, 1,25D rapidly stimulates secretion via nongenomic mechanisms initiated at the plasma membrane of bone cells which appear to require a functional VDR.

Vitamin D₃-induced Exocytosis in Neuroblastoma-Glioma Cells

M. Owraghi, L. Zanello; Biochemistry, University of California, Riverside, Riverside, CA

The biologically active form of vitamin D₃, 1,25-dihydroxyvitamin D₃ (1,25D₃), promotes genomic and non-genomic responses via a vitamin D receptor (VDR) expressed in multiple target systems. In the brain, 1,25D₃ exerts neuroprotective effects, including glioma cell death, making the hormone of potential interest in the management of brain tumors. At the cell membrane level, 1,25D₃ stimulates rapid ion fluxes through different ion channel types, and activates cytoplasmic signaling cascades. Eradication of the precise molecular pathways involved in 1,25D₃-induced neuroprotective effects are of potential pharmacological value in the treatment of neurodegenerative diseases. Here we show, for the first time in neuroblastoma-glioma (NG108-15) cells, that nanomolar concentrations of 1,25D₃ activate, within minutes, outwardly rectifying chloride currents at highly depolarizing potentials. The electrical profile and pharmacology of these 1,25D₃-sensitive chloride currents resembles a CI⁻ channel expressed in neuroendocrine PC12 cells. We found that 1,25D₃-stimulated CI⁻ channel in neuroendocrine PC12 cells couples to rapid interfered release of glutamate in NG108-15 cells, as detected with live-cell microscopy and immunocytochemistry. Using a fluorometric assay, we found 8.4 ± 0.3% and 13.6 ± 0.4% relative increase of glutamate in the medium after 3 min treatment with 10 and 100 nM 1,25D₃, respectively. Significant specific binding of 1,25D₃ was measured in NG108-15 cell lysates. VDR protein expression was confirmed by Western blot. Immunocytochemistry for the VDR showed abundant cytoplasmic and plasma membrane-associated subcellular localization. In addition, we detected the
presence of voltage-gated CIC-3 and CIC-5 chloride channel proteins in dendrites and secretory vesicles. Immunostaining for CIC-1, CIC-2, and CIC-7 was significantly lower, while CIC-4 and CIC-6 were not detected. We conclude that 1,25D stimulation of exocytosis in NG108-15 cells appears to couple to chloride current potentiation via a membrane associated classic VDR, similarly to what we have shown previously in osteoblasts.

409 Characterization of Transgenic Mice Expressing HA-GLUT4-GFP in Muscle

I. Lisinski, D. Yver, H. Al-Haslani, G. D. Holman, S. W. Cushman; NIDDK, NIH, Bethesda, MD, German Institute for Human Nutrition, Potsdam-Rehbrücke, Germany, Department of Biology and Biochemistry, University of Bath, Bath, United Kingdom

Insulin stimulates glucose uptake into fat and muscle by modulating the distribution of the GLUT4 glucose transporter between the cell surface and intracellular compartments. Hypoxia/contraction in skeletal muscle stimulates glucose transport activity, and this effect is additive to glucose transport stimulated by insulin. Our hypothesis is that GLUT4 is sorted into different compartments or uses different protein interactions in response to stimulation with insulin, compared with hypoxia/contraction in skeletal muscle. Because of the difficulties in preparing muscle membrane fractions and transiently transfecting primary mammalian muscle fibers, we have prepared two lines of transgenic mice specifically expressing HA- and EGF-tagged GLUT4 in muscle. In this study, in intact skeletal muscles of these animals, we have detected and characterized HA-GLUT4-GFP distribution and compartmentalization. Western blot and confocal microscopy clearly show that these mice highly express HA-GLUT4-GFP in skeletal muscle, cardiomyocytes, and isolated FDB muscle fibers. We also observe the expected HA-GLUT4-GFP subcellular distributions in the basal, and HA-GLUT4-GFP is localized to the same degree on the t-tubules and sarcotubular of isolated FDB muscles after insulin or hypoxia/contraction stimulation. These effects are additive, which suggests two different GLUT4 trafficking mechanisms. These data directly demonstrate the versatility of using HA-GLUT4-GFP mice for studying the detailed trafficking pathways of GLUT4 in muscle cells in normal and insulin-resistant metabolic states, both in vitro and in vivo.

410 Live Cell Tracking of GLUT4 Molecule in 3T3.L1 Adipocyte Using Q-dot Nano-crystal

H. Fujita, T. Watanabe, T. Tadachi, H. Higuchi, M. Kanazaki; Tohoku University Biomedical Engineering Organization (TUBERO), Sendai, Miyagi, Japan

Insulin stimulates glucose transport into adipocytes and muscle cells by inducing the translocation of the insulin responsive glucose transporter GLUT4 from intracellular storage compartments to the plasma membrane. GLUT4 translocation is a complicated process involving budding and fission of GLUT4-containing vesicles from the storage compartments, their trafficking to and from the plasma membrane. Here, we have established a new method to visualize the movement of GLUT4 using Q-dot nano-crystal. 3T3.L1 adipocyte expressing exofacial-myc-GLUT4-eCFP was labeled with Q-dot-conjugated Myc antibody in the presence of insulin. Q-dot-labeled GLUT4 was then endocytosed back to the storage compartments by washing out of insulin. Observation was performed under real-time confocal microscope and TIRF microscope equipped with high sensitivity EMCCD camera so that movement of Q-dot-labeled GLUT4 molecules in response to the second challenging of insulin stimulation can be tracked. Real-time imaging of single GLUT4 molecule movement under confocal microscope revealed that GLUT4 molecules were transiently immobile at the basal state, and insulin stimulation increased the mobility of these molecules. Diffusion coefficient of GLUT4 molecules was 3.3 fold higher after insulin stimulation than the basal state, suggesting that insulin stimulates unearthing of GLUT4 molecules from the storage compartments. Observation by TIRF microscope revealed that insulin increased both GLUT4 approached the plasma membrane by 3.8 fold and duration of the molecules stayed at the plasma membrane by 2.1 fold. Together, these data reveal that insulin regulates at least two distinct steps of the translocation process; 1) releasing GLUT4 molecules to the transport vesicles from the storage compartments, allowing more molecules to be accessible to the plasma membrane, and 2) priming the tethering-fusion step at the plasma membrane.

411 ATPase-sensitive and that the entry of [H+] may play a role in regulating the movement of water through AQP-6 channel at the synaptic vesicle membrane. Supported by NIH grants (BPJ).

412 Myosin-V regulates Spatial Control of the Secretory Pathway and Cellular Morphogenesis in Developing Drosophila Photoreceptors

B. Li, A. K. Satoh, D. F. Ready, Biology, Purdue University, West Lafayette, IN

Rhodopsin is highly packed in a photosensitive organelle, the rhabdome, a specialization of the photoreceptor apical plasma membrane. Rab11 regulates transport of newly synthesized Rhodopsin (Rh1) from the trans-Golgi network to rhabdome (Satoh et al, 2005). Here, we investigate the role of Rab11 interacting protein, dRip11 and myosin V in the polarized Rh1 transport. We have studied loss of function mutations of both proteins and find both are required for normal vectorial membrane traffic in developing photoreceptors. Loss of either protein results in the accumulation of cytoplasmic rhodopsin, a failure of normal exocytic activity that parallels Rab11 loss. Loss of dRip11 d elocalizes Rab11 and MyoV in developing photoreceptors. Moreover, loss of myosin-V activity results in mistargeted membrane traffic that generates supernumerary, ectopic rhabdomes. These observations suggest Drosophila photoreceptors harness an evolutionarily conserved Rab11/dRip11/myosin-V dependent pathway of polarized membrane traffic for cellular morphogenesis. Supported by NEI grant #10306.

413 Glucagon-like Peptide 1 Induces Insulin Secretion via NAADP and CADPR in Pancreatic β Cells

U. Kim, B. Kim, M. Im; Biochemistry, Chonbuk National University Medical School, Jeonju, Republic of Korea

The glucagon-like peptide 1 (GLP-1) stimulates cAMP production, increases an intracellular Ca2+ level, and enhances insulin secretion in pancreatic β cell. The action of cAMP is mediated not only by cAMP-dependent protein kinase (PKA), but also by cAMP-regulated guanine nucleotide exchange factors (EPAC). To determine the mechanisms by which PKA and EPAC act on insulin secretion, we investigated PKA or EPac-mediated Ca2+ signaling in mouse pancreatic β cells. Here, we showed that PKA and EPac-selective cAMP analog (6-benzoyl-cAMP and 8-PCPT-2'-O- Me-cAMP, respectively) as well as forskolin induced rapid and long-lasting Ca2+ increases in the presence of high glucose concentration. These agonists stimulated a production of nicotinic acid adenine dinucleotide phosphate (NAADP) that initiates initial Ca2+ release from lysosome related Ca2+ pool. A cyclic ADP-ribose (cADPR) antagonistic analog (8-Br-cADPR) blocked the GLP-1 induced early phase of Ca2+ increase and the EPac-activated long-lasting late phase of Ca2+ increase. Furthermore, Ca2+ increases to those by EPac were observed by direct NAADP treatment on the β cells. Bafilomycin A1, an inhibitor of NAADP-sensitive Ca2+ store, blocked not only the initial Ca2+ increase but also long-lasting Ca2+ increase by PKA, EPac, NAADP and GLP-1, suggesting that the NAADP-initiated Ca2+ increase is essential role for triggering the late Ca2+ signaling. The long lasting Ca2+ signaling was blocked by 8-Br-cADPR or high concentration of ryanodine, suggesting that NAADP-initiated Ca2+ increase stimulates production of CADPR, which evokes the long-lasting Ca2+ increase through Ca2+ influx. Consistent with these findings, high concentration of NAADP, desensitizes NAADP channel, blocked the Ca2+ signals induced by GLP-1, PKA and EPac. These data reveal that PKA and EPac activated by GLP-1 induce sequential generation of two Ca2+ signaling second messengers, NAADP and cADPR and that Ca2+ signaling induced by these second messengers plays a pivotal role in insulin secretion in pancreatic β cell.

414 Determining the Role of Adaptor Protein 3 in the Secretory Pathway of Cytotoxic T Lymphocytes

M. Wenham, G. M. Griffiths; Sir William Dunn School of Pathology, University of Oxford, Oxford, United Kingdom

Cytotoxic T lymphocytes (CTL) execute their effector function through the tightly controlled secretion of lyosomal lytic granules toward a target cell. Upon target recognition by the T cell receptor (TcR), granules at the periphery of the CTL attach to the microtubule network and move in a plus-end direction toward the immunological synapse, where they fuse with the plasma membrane and secrete their cytotoxic contents. Adaptor Protein 3 (AP-3) is a multimeric protein complex which sorts transmembrane proteins to lysosomes and lysosome-related organelles.
with different tendencies to undergo exocytosis: azurophilic, specific and gelatinase granules and secretory vesicles. Here, we start to characterize the secretory machinery that regulates the exocytosis of these organelles. We previously showed by immunofluorescence analysis that the small GTPase Rab27a and its effector JFC1 localize in a minor subpopulation of myeloperoxidase (MPO)-containing granules (azurophilic) and that interference with the Rab27a/JFC1 secretory machinery inhibits MPO secretion in granulocytes. Here, we show that Rab27a-deficient mice have impaired secretion of MPO in vivo after intraperitoneal injection of lipo polysaccharide (LPS). Significant differences were observed at 4 and 8 hours after injection; no differences were observed for the basal level of MPO. Importantly, no significant differences were observed in the number of circulating neutrophils in the peripheral blood of control and Rab27a-/- mice suggesting that Rab27a is an essential component of the secretory machinery of azurophilic granules in granulocytes. Contrarily, mobilization of CD11b from intracellular granules (gelatinase granules and secretory vesicles) in response to the chemotactic peptide 5MIP2 was not affected in Rab27a-deficient neutrophils when evaluated by flow cytometry, despite the observation that Rab27a localizes at gelatinase granules in wild type cells. This suggested that a protein with redundant function may regulate the secretion of these granules in the absence of Rab27a. We found that Rab27a-deficient mice have a dramatic upregulation of Rab27b in their granulocytes. Moreover, although Rab27a and Rab27b co-fractionate in the organelle, only Rab27b is detected in the α and β fractions after cellular fractionation of human neutrophils. Our results suggest that Rab27b may be involved in the mobilization of low density secretory organelles but cannot overcome the defect in MPO secretion observed in Rab27a deficient mice.

415 Ribosome-Translocon Interactions Are Controlled by the Nascent Chain during Co-translational Protein Integration into the ER Membrane

C. G. Jongsma, P. J. Lin, A. E. Johnson; Texas A&M University, College Station, TX

Ribosome-translocon junction prevents ion flow through the translocon pore. Prior to opening this junction to allow nascent chain movement into the cytosol, BiP effects closure at the lumenal end of the pore. However, dynamics of recruitment of the Rab-family GTPase Sec4 is affected by loss of Cbk1 function. We also found that Cbk1 is itself controlled by phosphorylation of two conserved sites. However, the kinase's role in polarized growth, in contrast, may require only a small amount of C-terminal site phosphorylation. The yeast Ndr/LATS kinase Cbk1 promotes Rab GTPase function and is controlled by RAM network dependent phosphorylation. We will discuss, for the first time, the model about the regulatory mechanism of PLD in insulin secretion through the interaction with granuphilin-a.

416 Phosphorylation of NHERF1/EBP50 in Parietal Cells
L. Zhu, A. Abbad, T. Wu, S. Patel, S. Karvar, C. Y. Sun, J. G. Forc, M. & Cell Biology, University of California, Berkeley, CA, Department of Medicine, Emory University School of Medicine, Atlanta, GA

Sodium-proton exchanger regulatory factor 1 (NHERF1), also known as ER binding phosphoprotein 50 (EBP50), is known to link ERMs to the plasma membrane and to provide a platform for many proteins to fulfill their cellular functions. In searching for phosphoproteins in resting and stimulated parietal cells using a proteomic strategy, a phosphoprotein was assigned to NHERF1 in stimulated gastric gland sample (phosphorylated at S287), but not detected with the resting sample. 2D-Western blot analysis of NHERF1 gave multiple spots of varies pl, with more acidic spots in the stimulated sample. These results indicate that NHERF1 may have an important role in the parietal cell and that its activity may be regulated by phosphorylation. A co-immunoprecipitation experiment indicates that NHERF1 interacts with ezrin in parietal cells. Current work is designed to reveal the membrane-binding protein of NHERF1, and the effect of phosphorylation of NHERF1 on its localization and function. This may benefit the understanding on how the complex surface structure of apical membrane is remodelled and maintained after parietal cell stimulation.

417 Characterization of Interaction between phospholipase D and Granuphilin-a
H. Kwon; Life Science, POSTECH, Seoul, Republic of Korea

Mammalian phospholipase D (PLD) has been strongly implicated in the regulation of Golgi trafficking as well as endocytosis and exocytosis. Recent report suggested that PLD are also related in insulin secretion event and increase the insulin secretion from pancreatic beta-cells. However, the regulatory mechanism of PLD in this process has not been studied. In this study, we found that the PLD interacts with granuphilin-a, which is specifically localized on insulin granules and is involved in their exocytosis. We investigated the interaction between PLD and granuphilin-a in cells. The direct association between PLD and granuphilin-a was confirmed by in vitro binding analysis using the purified proteins, and their binding sites were identified as the N-terminal of PLD. Furthermore, granuphilin-a regulates the PLD activity in cells and this result suggests that interaction between granuphilin-a and PLD can be involved in exocytotic process in cells. We will discuss, for the first time, the model about the regulatory mechanism of PLD in insulin secretion through the interaction with granuphilin-a.

418 The Yeast Ndr/LATS Kinase Cbk1 Promotes Rab GTPase Function and Is Controlled by RAM Network Dependent Phosphorylation
J. Jansen, M. Barry, E. L. Weiss; Biochemistry, Molecular Biology, and Cell Biology, Northwestern University, Evanston, IL

The budding yeast RAM network is a highly conserved but poorly understood signaling pathway required for maintenance of polarized growth. It also controls Ace2, an asymmetrically segregated transcription factor that drives gene expression after cytokinesis. The network’s role in cell morphology control is independent of its transcriptional function. It thus far comprises six proteins, including the Ndr/LATS family kinase Cbk1. We found establishment of polarity (localization of septin, F-actin patches and cables, and Cdc24) occurs in cells lacking Cbk1. However, dynamics of recruitment of the Rab-family GTPase Sec4 is affected by loss of Cbk1 function. We also found that Cbk1 is itself controlled by phosphorylation of two conserved sites. One is a T-loop site of intramolecular autophosphorylation; the other is performed at a C-terminal hydrophobic motif by another kinase. The autophosphorylation site is critical for the kinase’s in vitro activity, but only partially required for in vivo function. In contrast, C-terminal site phosphorylation is essential for in vivo function but not necessary for in vitro kinase activity. Cbk1 that cannot be phosphorylated at the C-terminal site is phosphorylated at other sites in vivo, suggesting this modification promotes dephosphorylation of other sites. Using phosphospecific antibodies, we determined that the autophosphorylation site is modified throughout the cell cycle, while the C-terminal site is modified during budding and during the M/G1 transition. C-terminal site phosphorylation requires all other RAM network components. Surprisingly, this modification is greatly reduced in cells lacking Ace2. We propose that Ace2 interaction increases or protects the M/G1 phosphorylation of Cbk1’s C-terminal regulatory site. The kinase’s role in polarized growth, in contrast, may require only a small amount of C-terminally modified site phosphorylation. We further propose that Cbk1 and the RAM network promote Sec4 polarization, and that attenuation of Sec4 function ultimately abrogates polarity in cells lacking RAM network activity.

419 Ribosome-Translocon Interactions Are Controlled by the Nascent Chain during Co-translational Protein Integration into the ER Membrane
C. G. Jongsma, P. J. Lin, A. E. Johnson; Texas A&M University, College Station, TX

Most membrane proteins in eukaryotic cells are co-translationally integrated into the ER membrane at translocons. The nascent polypeptide is threaded into the aqueous translocon pore where each successive transmembrane segment (TMS) is moved laterally through the translocon into the bilayer. The hydrophobic polypeptide segments on each side of the TMS are directed, alternately, into either the aqueous cytosol or the aqueous ER lumen. For a single-spanning signal-cleaved membrane protein, nascent chain movement into the lumen occurs while an ion-tight ribosome-translocon junction prevents ion flow through the translocon pore. Prior to opening this junction to allow nascent chain movement into the cytosol, BiP effects closure at the luminal end of the pore to maintain the membrane permeability barrier. To determine whether the ribosome and BiP alternately mediate pore closure during the integration of a multi-spanning membrane protein, integration intermediates with nascent chains of different lengths were prepared with a fluorescent probe positioned in the nascent chain furthest inside the ribosomal tunnel. Nascent chain exposure to the cytosol or lumen was then detected by the collisional quenching of the probe by sidone ions on either the cytosolic or luminal side of the membrane. While the first TMS through the tunnel caused the ribosome-translocon junction to open, the second TMS elicited both the closure of this junction and the opening of the luminal end of the pore. Closure of the ribosome-translocon junction occurred only after the second TMS was 4-6 residues from the peptidytransferase center, irrespective of TMS location in the nascent chain. These changes exactly coincided with ribosome-induced TMS folding detected by FRET. Thus, nascent chain folding and binding far inside the tunnel control ribosome-translocon interactions at the ER membrane. (Supported by NIH grant GM26494 and the Welch Foundation.)
**Dissection of the Pathway for Tail-anchored Membrane Protein Insertion**

S. Stefanovic, R. S. Hegde; CBMB/NICHD, National Institutes of Health, Bethesda, MD

A large group of proteins in the cell are anchored to intracellular membranes by a single transmembrane domain at the C terminus. These tail-anchored (TA) proteins have crucial roles in the regulation of many important cellular processes such as apoptosis (members of Bcl2 family), vesicular trafficking (SNAREs), and protein translocation into various organelles. Clearly, the proper function of each of these proteins is critically dependent on their correct subcellular localization. Despite this wide functional importance, the mechanisms by which TA proteins are recognized, segregated to their target membrane, and inserted into the lipid bilayer are not known. To address this problem, we have reconstituted in vitro the membrane insertion of a model TA protein of the endoplasmic reticulum (ER) called Sec61b. Using this system, we could demonstrate by sucrose gradient analysis that Sec61b exists in a multi-protein complex in the cytosol. This cytosolic complex was competent for subsequent insertion into the TA protein insertion pathway. Crosslinking identified the interacting partners in the cytosolic complex as ~45 kD and 18 kD proteins. Using an anti-Sec61b immunoaffinity column, we purified this complex and identified the ~45 kD and ~18 kD proteins by mass spectrometry. We have raised antibodies against p54 and verified its presence in the insertion complex. Addition of the purified cytosolic insertion complex to ER membrane vesicles enabled efficient membrane insertion of Sec61b in a reaction that was shown to be dependent on both energy and at least one yet unidentified integral membrane protein of the ER. The development of this robust and readily manipulable reconstituted system has outlined the basic steps and components in the TA protein insertion pathway. Our ongoing studies aim at understanding the mechanism by which p54 and p18 facilitate TA protein insertion.

**Real-time Fluorescence Detection of Retro-translocation in a Well-defined Homogenous Mammalian System**

J. Wahlman,1 G. N. DeMartino,2 W. R. Skach,3 J. L. Brodsky,4 A. E. Johnson5; 1Texas A&M University, College Station, TX, 2University of Texas Southwestern Medical Center, Dallas, TX, 3Oregon Health & Science University, Portland, OR, 4University of Pittsburgh, Pittsburgh, PA, and 5Texas A&M University Health Science, College Station, TX

Secretory proteins that are unable to assemble into native proteins in the endoplasmic reticulum (ER) are transported back into the cytosol for degradation. To examine the roles of different components in ER-associated degradation (ERAD), well-defined and homogeneous mammalian ER microsomes were prepared biochemically by encapsulating a fluorescent labeled ERAD substrate with specific luminal components. After mixing ATP, specific cytosolic proteins, and specific fluorescence quenching agents with microsomes, substrate retro-translocation was initialized. The rate of substrate efflux from microsomes was monitored spectrophotometrically and continuously in real time by the reduction in fluorescence intensity as the fluorescent substrates passed through the ER membrane and were exposed to the quenching agents. Retro-translocation kinetics were not significantly altered by replacing all luminal proteins with only protein disulfide isomerase, or all cytosolic proteins with only the PDI-like proteins. Retro-translocation was blocked by affinity-tipped antibodies against Derlin1, but not by affinity-purified antibodies against Sec61a or by membrane-bound ribosomes. Since the substrate also photocrosslinked Derlin1, but not Sec61a or TRAM, retro-translocation of this ERAD substrate apparently involves Derlin1, but not the translocon. (Supported by NIH grant GM62494 and the Welch Foundation.)

**Visualization of the Endoplasmic Reticulum-associated Degradation of CD3delta**

H. Lorenz, J. Lippincott-Schwartz; NICHD, National Institutes of Health, Bethesda, MD

Many misfolded or unassembled proteins of the secretory pathway are efficiently retro-translocated from the endoplasmic reticulum (ER) into the cytosol and degraded by the proteasome. This process is called ER-associated degradation (ERAD). Here, we present a live-cell imaging study of ERAD of the type I glycoprotein CD3delta. The T cell receptor (TCR) subunit CD3delta has already been described to be rapidly degraded in the absence of other TCR subunits. CD3delta's complete degradation pathway from its initial localization within ER membranes to its dislocation into the cytosol and the final degradation by proteasomes was captured using novel live-cell imaging strategies. Visualization of CD3delta tagged to fluorescent protein folding reporters made it possible to precisely determine CD3delta's subcellular localization and folding state during its demise. Upon proteasomal inhibition, fully folded CD3delta accumulated primarily in the ER and not in the cytosol, indicating both its dislocation and degradation are coupled events. However, the simultaneous inhibition of protein synthesis and the proteasomal activity led to the redistribution of CD3delta from the ER into the cytosol. During this retrotranslocation event, CD3delta did not need to be completely unfolded, and the cytosolic CD3delta molecules were only partially, but not completely, unfolded. The cytosolic CD3delta also did not form aggresomes and was rapidly degraded upon re-establishment of proteasomal activity. This is, to our knowledge, the first dynamic visualization of the ERAD process in live cells.

**PDI-like Proteins Play Opposing Roles during Retro-translocation**

M. L. Forster,1 K. Sivick,2 P. Young-nam,3 P. Arvan,3 W. I. Lencer,4 B. Tsai1; 1Department of Cell and Developmental Biology, University of Michigan, Ann Arbor, MI, 2Department of Microbiology and Immunology, University of Michigan, Ann Arbor, MI, 3Division of Metabolism, Endocrinology, and Diabetes, University of Michigan, Ann Arbor, MI, 4GI Cell Biology, Children's Hospital, Harvard Medical School, Boston, MA

Misfolded proteins in the endoplasmic reticulum (ER) (RE) are retained in the organelle or retro-translocated to the cytosol for degradation. RE chaperones that guide these opposing traffic to the Golgi prior to degradation. Our study in Saccharomyces cerevisiae shows that a single context-specific N-linked glycan is a required determinant of the signal in both cases, but in a different position. We hypothesized from these data that substrates utilize a bipartite signal of the ER. The development of this robust and readily manipulable reconstituted system has outlined the basic steps and components in the TA protein insertion pathway. Our ongoing studies aim at understanding the mechanism by which p54 and p18 facilitate TA protein insertion.

**Substrate Recognition in ER Quality Control**

K. Kanehara, W. Xie, A. Sayeed, D. T. W. Ng; Temasek Life Sciences Laboratory, Singapore

Misfolded proteins in the endoplasmic reticulum (ER) are eliminated by ER-associated degradation (ERAD), which is conserved in all eukaryotes. Because of the dynamic nature of the organelle, cells must segregate actively folding and misfolding proteins, which are retained, from folded proteins, which are transported. Furthermore, misfolded proteins must be distinguished from folding proteins so they can be targeted for degradation. Despite the advances in understanding the machinery of ERAD, how cells differentiate between these different forms is unclear. To address the question, we analyzed misfolded versions of cytochrome oxidase Y (CPY*) and proteinase A (PrA*) in the yeast Saccharomyces cerevisiae. Our previous study showed that a single context-specific N-linked glycan is a required determinant of the signal in both cases, but in a different position. We hypothesized from these data that substrates utilize a bipartite signal comprised of carbohydrate and peptide elements. To test our hypothesis, systematic deletion analysis is performed on CPY* with the aim of disrupting the peptide determinant. Deletion spanning most of the protein had no significant effects on degradation suggesting the possibility of redundant determinants. We did, however, find a small protein domain, when combined with a specific carbohydrate, which is sufficient for ERAD. We hypothesize that the lectin Yos9p, a critical factor in sorting misfolded proteins, recognizes a signal comprised of the specific carbohydrate and the unfolded domain. In the folded protein, the determinant is buried into the structure of the protein allowing it evade surveillance by Yos9p.
Uncoupling of a Regulatory Function of VCP in ER Stress From Its Role in ER-associated Degradation: Evidence from RNAi of the VCP-Ufd1-Npl4 Complex
C. Wojcik, M. Rowicka, A. Kudlicki, D. Nowis, G. Adams, M. Kujawa, G. N. DeMantino; 1Anatomy and Cell Biology, Indiana University School of Medicine—Evansville, Evansville, IN; 2Biochemistry, UT Southwestern Medical Center, Dallas, TX; 3Physiology, UT Southwestern Medical Center, Dallas, TX; 4Histology and Embryology, Medical University of Warsaw, Warsaw, Poland
VCP (p97, cdc48) is a hexameric AAA ATPase involved in multiple cellular functions. VCP in a complex with the Ufd1-Npl4 dimer participates in the degradation of proteins by the ubiquitin-proteasome system (UPS), including proteins which are dislocated from the endoplasmic reticulum (ER) in a process known as ERAD (ER-associated degradation). The objective of this work was to scrutinize the role of VCP-Ufd1-Npl4 in the structure and function of mammalian ER using as the main method RNA interference (RNAi) of the components of this complex in cell lines expressing model substrates of the UPS. A new stringent method of microarray analysis demonstrated that only 4 transcripts were non-specifically affected by RNAi of VCP, while ~30 transcripts were affected in response to reduced VCP levels in a sequence-independent manner. Those transcripts encoded proteins involved in ER stress, apoptosis, and amino acid starvation. RNAi of VCP promoted the unfolded protein response, without eliciting a cytotoxic stress response. RNAi of VCP promoted accumulation of polyubiquitinated proteins in multiple compartments, and inhibited the degradation of R-GFP and Ub-G76V-GFP, two cytoplasmic reporter proteins degraded by the UPS. In surprising contrast, RNAi of VCP had no detectable effect on the ERAD substrates, α-syntrophin and iC3D. Moreover, RNAi of Ufd1 and Npl4 accelerated degradation of some ERAD substrates. These results indicate that VCP is required for maintenance of normal ER structure and function, mediates the degradation of some proteins via the UPS, but is dispensable for the UPS-dependent degradation of certain ERAD substrates. The VCP-Ufd1-Npl4 complex may perform a regulatory role in ERAD rather than directly participate in substrate degradation.

Rescue of Trafficking and Folding Defects Associated with an Aberrant Yeast ABC Transporter
E. Miller, J. Barry, M. Dorrington, R. Louie, S. Pagant, L. Kung; Biological Sciences, Columbia University, New York, NY
Eukaryotic cells dedicate significant resources to the deployment of proteins to the membrane compartments that comprise the secretory pathway. Cells impose a strict quality control checkpoint to ensure that only fully folded and properly assembled proteins are allowed to leave the endoplasmic reticulum (ER), a growing number of human diseases are caused by defects in proteins that render them incapable of passing this ER quality control. We study the close relationship between protein folding and packaging into ER-derived transport vesicles using a model membrane protein, Yor1p, which functions as a plasma membrane drug pump in Saccharomyces cerevisiae. Deletion of a conserved Phe residue (analogous to the predominant mutation associated with human cystic fibrosis) causes Yor1p to misfold, precluding its capture into ER-derived transport vesicles. We have undertaken a genome-wide screen to identify cellular components involved in the quality control of forward protein transport through the secretory pathway. Some of these quality control mutants show an improved ability to package the misfolded Yor1p deltaF into COP1 vesicles. In vitro folding assays suggest that these mutants fall into two classes: those in which the mutant Yor1p deltaF adopts a native conformation, and those in which the aberrant protein is packaged despite remaining in an unfolded state. Rescue of the folding defect associated with Yor1p deltaF in one of these mutants is linked to the ability to mount the unfolded protein response (UPR), deletion of the UPR-specific transcription factor, Hac1p, in this strain background abrogates the rescue of Yor1p deltaF folding and ER export. Our results suggest that activation of the UPR can improve the ability of cells to handle misfolded proteins and suggest novel therapeutic targets for cystic fibrosis drug development.

The Hinge Stem of XBP1 mRNA Is a Novel Structure Required for Unconventional Splicing in the Unfolded Protein Response
K. Yanagitani, Y. Imagawa, A. Hosoda, T. Iwawaki, K. Kohno; 1Bioscience, Nara Institute of Science and Technology, Nara, Japan, 2Iwawaki Initiative Research Unit, RIKEN, Saitama, Japan
Accumulation of unfolded and misfolded proteins in the endoplasmic reticulum (ER) activates an intracellular signaling pathway termed the unfolded protein response (UPR). In mammalian cells, the UPR is signaled in part through activation of ER localized type I membrane-spanning protein IRE1α harboring a kinase/endoribonuclease activity. Activated IRE1α cleaves XBP1 mRNA at two sites to initiate an unconventional splicing reaction, which is a very unique reaction because it doesn’t depend on spliceosome and occurs in cytoplasm. The 5’ and 3’ fragments are subsequently joined by an RNA ligase activity, thereby removing a 26-base intron. This reaction creates a translational frameshift to produce a functional XBP1α transcription factor. This unconventional splicing is very unique and its molecular mechanism has been largely unknown. To study this splicing mechanism, we focused on the secondary structure of XBP1 mRNA. XBP1 mRNA has a characteristic stem-loop structure at two exon-intron junctions, which is cleaved by activated IRE1α. By comparing the secondary structure of XBP1 mRNA in various species, we found the novel conserved structure to bind up two stem-loops, termed a hinge-stem. When the hinge stem was disrupted by base substitutions, mRNA attenuation in vivo. On the contrary, when the hinge stem structure was reconstructed by additional base substitutions, splicing efficiency was recovered. Thus the structure, but not the sequence was critical for the splicing of XBP1α. Our results suggest that activation of the UPR can improve the ability of cells to handle misfolded proteins and suggest novel therapeutic targets for cystic fibrosis drug development.

Analysis of XBP1 mRNA Splicing Reaction in Unfolded Protein Response In Vitro
Y. Imagawa, M. Inoue, K. Kohno; Graduate School of Biological Sciences, Nara Institute of Science and Technology (NAIST), Ikoma, Nara, Japan
When the cells are exposed to various stresses such as glucose deficiency, viral infection, and calcium disturbance in the ER, unfolded proteins accumulate in the endoplasmic reticulum (ER). This is called ER stress that causes fatal damage for living cells. Therefore, cells activate a pathway called unfolded protein response (UPR) to prevent the accumulation of misfolded proteins in the ER. In mammalian cells, one of the UPR is signaled through dimerization of ER-located type I transmembrane protein IRE1α to activate its protein kinase and endoribonuclease activities. Activated IRE1α cleaves XBP1 mRNA at two sites to initiate an unconventional splicing reaction, which is a very unique reaction because it doesn’t depend on spliceosome and occurs in cytoplasm. The 5’ and 3’ fragments are subsequently joined by an RNA ligase activity, thereby removing a 26-base intron. This splicing reaction creates a translational frameshift to produce a functional XBP1α transcription factor. However, the RNA ligase and physiological processes required for unconventional splicing of XBP1 mRNA have not been well characterized. To study precisely these processes, we tried to reconstruct XBP1 mRNA splicing reaction in vitro, and finally we found that HeLa cell extract contains an RNA ligase activity to join two exon-fragments of XBP1 mRNA cleaved by IRE1α recombinant protein in vitro. Using this system, we have searched essential factors for XBP1 mRNA splicing. As a result, GTP is essential for the ligation of XBP1 mRNA splicing, but ATP is not. In yeast, it is reported that activated eIF4e cleaves both 5’ and 3’-exon-intron junctions of HAC1 mRNA, then the cleaved HAC1 mRNA fragments are joined by a RNA ligase Rlp1p, and the ligation reaction requires both GTP and ATP. These evidences suggest that the character of RNA ligase in XBP1 mRNA splicing reaction is at least different from that of HAC1 mRNA.
Serine 23 and 36 phosphorylation of Caveolin-2 Is Reciprocally Regulated by Caveolin-1 induced Sequestration of Caveolin-2 to Plasma Membrane Caveolae

G. Sowa,1 L. Xu,2 W. C. Sessa3; 1Medical Pharmacology and Physiology, University of Missouri, Columbia, MO, 2Pharmacology, Yale University, New Haven, CT

Previously, we have shown that caveolin-2 (Cav-2) is phosphorylated under basal conditions at N-terminal serine residues 23 and 36 and that this phosphorylation of Cav-2 plays a positive role in caveolae assembly in a prostate cancer cell line LNCaP cells. In the present study, we show in reconstituted systems LNCaP and fisher rat thyroid (FRT) cells as well as in endogenously expressing caveolin endothelial cells that serine phosphorylation of Cav-2 is a regulated process. More specifically, using adenosinergic expression and phospho-specific antibodies to Cav-2, we show that co-expression of Cav-1 increases phosphorylation of Cav-2 at serine residue 23, and decreases phosphorylation of serine 36. Insolubility in Triton X-100 and sucrose flotation gradients separating detergent resistant membranes (DRMs) from the remaining proteins have determined that serine 23 phosphorylation of Cav-2 preferably occurs in DRMs consisting of lipid rafts and caveolae. On the other hand, serine 36 phosphorylation takes place in non-DRMs. Furthermore, immunofluorescence labeling studies determined that serine 23-phosphorylated Cav-2 mostly localizes to plasma membrane in adenosinergic expressing human Cav-2 and -1 FRT cells and in endothelial cells, while serine 36 phosphorylated Cav-2 primarily resides in intracellular compartments. In addition, our data show that oligomerization with Cav-1 protects both phospho-serines from dephosphorylation by calf intestinal phosphatase (CIP), suggesting that Cav-2 engaged in high molecular heterologerm with Cav-1 in caveolae may be relatively resistant to dephosphorylation by cellular phosphatases. In summary, serine phosphorylation of Cav-2 is a regulated process largely dependent on Cav-1-driven translocation of Cav-2 from detergent soluble intracellular compartments to detergent insoluble plasma membrane associated lipid rafts and caveolae.

AHNAK Is a Potential Component of Insulin Signaling Located Within Lipid Raft Microdomains in 3T3-L1 Adipocytes

D. Caces, J. E. Pessin; Pharmacological Sciences, Stony Brook University, Stony Brook, NY

The selective partitioning of signaling molecules within lipid raft microdomains represents one mechanism by which cell signaling specificity and fidelity is maintained. Insulin-regulated exocytosis of the facilitative glucose transporter GLUT4 in adipocytes is one system that benefits from this paradigm. To identify key lipid raft molecules that may be involved in the insulin-signaling cascade, we did a proteomic screen of lipid raft fractions obtained from fully differentiated 3T3-L1 adipocytes. Cells grown on collagen-coated plates were passively treated with 1% Triton X-100 for 25 minutes at room temperature. The detergent-insoluble fraction was collected, separated by one-dimensional SDS-PAGE and visualized by silver staining. Protein bands corresponding to bands on an immunoblot that was ran in parallel using an anti-phosphorylated-Akt antibody were harvested and sent for LC/MS/MS. The screen identified 21 novel proteins with the Akt-phosphorylation motif RXRXXSST and 1 known Akt substrate, AHNAK. AHNAK, or desmyosin, is a 700 kDa protein reported to be involved in the regulation of cell membrane architecture in MDCK cells. In HeLa cells, phosphorylation by Akt is thought to regulate the protein’s subcellular localization. Whole cell immunofluorescence in adipocytes revealed that AHNAK is primarily associated with the plasma membrane. Similar studies done on plasma membrane lawns showed significant co-localization of the protein with the lipid raft marker caveolin, with both proteins forming elegant rosette structures. Treatment with MijCD disrupted both proteins’ distinct organization and resulted in a more cytoplasmic localization for AHNAK. However, treatment with Lantrunculin B to disrupt actin, which has been reported to interact with AHNAK, did not affect AHNAK localization to the caveolin rosette structures. In conclusion, we report that AHNAK in adipocytes is a lipid raft associated Akt substrate that may potentially play a role in insulin-mediated GLUT4 translocation and/or transcriptional activation.
Calcimycin-induced Upregulation of Caveolins and Formation of Caveolae in the Rat Lens Epithelium

W. Peng, 1 S. Biswas, 1 W. Lo, 1 2 3 Anatomie & Neurobiologie, Morehouse School of Medicine, Atlanta, GA, 4 Ophthalmo, Emory University, Atlanta, GA

Caveolae contain a variety of components, which are co-expressed and assembled into heterooligomeric complexes. Our previous study showed that caveolae were prominent only in the lens epithelia of rabbit and guinea pig. This study was undertaken to determine whether formation of caveolae could be induced by calcimycin-induced apoptosis in the rat lens epithelium in organ culture. The lens epithelial apoptosis was induced by the treatment of calcium ionophore calcimycin (0.5-3.0 μM) in M-199 for 1 h and followed by 3-72 hours of culture in M-199 alone. A cleared caspase-3 antibody was used to detect the early apoptosis. RT-PCR and immunofluorescence were used to determine upregulation of caveolin mRNA and protein, and the formation of caveolae was examined by TEM. RT-PCR analysis showed that expression of both Cav-1 and Cav-2 mRNAs was significantly increased at 3 h, and reached the peak (∼8-fold increase) at 6 h after calcimycin (1μM) treatment. However, the level of Cav-1 mRNA was approximately twice as high as that of Cav-2 at 3 h of culture. Both caveolin mRNAs were gradually decreased at 9 h, and returned to the controlled low level after 12-48 h of culture. Immunofluorescence studies on epithelial whole mounts revealed that both Cav-1 and Cav-2 labeling were significantly increased after 48-72 h of culture time. TEM confirmed the colleague findings that caveolae, usually in clusters, were regularly found along the lateral epithelial cell membranes in rat lenses treated with 1μM calcimycin for 1 h followed by 48 h of culture in M-199 alone. These results suggest that caveolae in the lens epithelium are dynamic structures whose appearance can be induced by stimulation of external factors such as an increase in cytoplasmic calcium by calcimycin which leads to apoptosis. Supported by NIH grant EY05314.

Dopamine D1-like Receptor Regulates Small GTPase Rab in Lipid Rafts in Human Kidney Cells

P. Yu, J. R. T. Altea, W. Han, H. Li, P. A. Jose; Pediatrics, Georgetown Medical Center, Washington, DC

Human cells contain more than 60 Rab proteins which participate in translocation vesicle formation, motility, docking and fusion within endocytic and secretory pathways. Among them, Rab4, Rab5, and Rab11 are important in regulating internalization, intracellular trafficking, and recycling of G protein-coupled receptors (GPCRs). We and others have reported that several GPCRs, including the D1 dopamine receptor (D1R), are regulated in lipid rafts. Here we report that D1R regulates Rab4, Rab5, and Rab11 in lipid rafts in HEK-293 cells heterologously expressing human D1R (HEK-D1R) and in human proximal tubule (hPT) cells endogenously expressing D1R. All three Rab tested co-fractionated with the lipid raft marker proteins, flotillin-1 and flotillin-2 in HEK-hD1R cells, and caveolin-1 in hPT cells. Fenoldopam (1M/10 min), a D1R agonist increased Rab4 protein in low density fractions (LDF) (fractions 3-5) but decreased Rab5 expressed in LDF (fraction 4). Rab11 was not affected. Treatment with methyl-b-cyclodextrin (JCD), a cholesterol-depleting reagent, decreased Rab4 and Rab11 but not Rab5, indicating that Rab4 and Rab11, but not Rab5, associated with lipid rafts (LR). JCD also decreased monomeric and dimeric D1R in LR. Confocal images showed that fenoldopam induced D1R co-localization with Rab4, Rab5, and Rab11 in a time-dependent manner. Fenoldopam also increased the amount of Rab11 associated with D1R in time-dependent manner which peaked at 1h (fenoldopam: 22.9±2.9% vs control: 9.8±1.9% area, ANOVA, P<0.05, n=4). Triple immunofluorescence staining showed D1R, Rab4, and Rab11 co-localization at 30-60min. Taken together, our data suggest that D1R differentially regulates Rabbs that are involved in D1R trafficking.

P-Akt and Caveolin-1 Differentially Regulate the Raft-dependent Endocytosis of Autocrine Motility Factor in Invasive Breast Tumor Cells

L. Kojic, 1 B. Jodhi, 1 P. Lajose, 1 M. Almilohammadi, 1 F. Ghadi, 1 F. U. Le, 1 M. Amraei, 1 M. Cox, 1 D. A. Turbin, 1 S. A. Wiseman, 1 L. R. Nabi, 1 Cellular and Physiological Sciences, University of British Columbia, Vancouver, BC, Canada, 2Pathology, University of British Columbia, Vancouver, BC, Canada, 3Pathology, University of British Columbia, Vancouver, BC, Canada, 4Surgery, University of British Columbia, Vancouver, BC, Canada

The heterogeneity of tumors necessitates the development of targeted therapies tailored to molecularly-defined populations of tumors. Receptor-mediated internalization of extracellular motility factor (AMF) via caveolin-1 regulated, raft-dependent endocytosis to the smooth endoplasmic reticulum represents a potential mechanism to target tumor cells. Importantly, AMF expression and AMF uptake are generally reduced in normal pulmonary and thymus cells and dysplastic MCF10A mammary epithelial cells relative to tumorigenic and metastatic breast cancer cells. However, AMF uptake is also limited in aggressive, caveolin-1-expressing MDA-231 cells identifying caveolin-1 as a critical regulator of the raft-dependent internalization of AMF in breast carcinoma cells. The heterogeneity of the AMF endocytic response in breast carcinoma cells was further characterized by tissue microarray analysis of invasive primary breast carcinomas. Caveolin-1 expression was identified as an independent, poor prognosticator for breast cancer. AMF expression neither impacted on survival nor correlated with caveolin-1 and Her2 expression but did correlate significantly with phospho-Akt. Consistently, phospho-Akt expression was increased in AMF internalizing MCF7 and MDA-435 breast carcinoma cells and PI3K inhibition reduced raft-dependent AMF uptake. AMFR, PI3K activation and the raft-dependent endocytosis of AMF may therefore define a cohort of invasive breast carcinomas suitable for AMFR targeted therapy. Supported by the Canadian Institutes of Health Research.

Caveolae Formation Regulates the Internalization of E-cadherin at Cell-Cell Contacts during the Onset of Tumor Cell Migration

L. Orfienko, 1 M. A. McNiven 1 Center for Basic Research in Digestive Diseases, Mayo Clinic College of Medicine, Rochester, MN, 2Department of Biochemistry and Molecular Biology and Center for Basic Research in Digestive Diseases, Mayo Clinic College of Medicine, Rochester, MN

BACKGROUND: Pancreatic tumor cells rapidly disseminate and invade making these cancers particularly aggressive. Recently, we demonstrated that EGF stimulation results in the e-Cadherin-mediated phosphorylation of caveolin 1 (Cav-1/Y14) and induces the formation of large numbers of caveolae at cell borders (JBC, 2006). We found that EGF-stimulated caveolae formation occurs concomitantly with the loss of cell-cell contacts during pancreatic tumor cell dissemination; therefore, the GOAL of this study was to determine whether regulation of Cav1 affects pancreatic ductular cell-cell integrity. First, we observed a significant relationship between the protein levels of Cav1 and E-cadherin (E-Cad) in different tumor cell lines. Interestingly, cells with low Cav1 levels had very high E-Cad levels and displayed strong cell-cell adhesion, whereas cells with high Cav1 levels expressed modest amounts of E-Cad and exhibited poor adhesion. We next tested how disruption of Cav1 function, through the expression of a mutant Cav1 Y14F protein or depletion of Cav1 through siRNA knockdown, might affect cell-cell contacts. Under these conditions we observed a marked increase in E-Cad at cell-cell contacts, which were also resistant to EGF treatment. Western blot analysis of these cells showed that proteolysis of E-Cad was reduced by over 90%, consistent with a reduction in adhesions junction internalization. However, cells expressing wild-type Cav1, when treated with EGF, disassembled cell-cell contacts and subsequently formed large, internal endosome/caveosome-like structures filled with E-Cad. Finally, immunoprecipitation of Cav1 from these cells indicated a marked increase in Cav1-E-Cad complexes, suggesting a physical interaction between these proteins. CONCLUSION: These observations suggest that Cav1 levels and regulated assembly of caveolae play an important role in maintaining the integrity of pancreatic tumor cell adhesions by affecting E-Cad internalization and degradation following EGF stimulation. This study provides new molecular insights into tumor cell dissemination and the role of Cav1/caveolae in this process.

Uptake of Angiostatin by Endothelial Cells through Caveolae-dependent Endocytosis

H. Wu, 1 C. Cho, 2 C. Huang, 2 H. Chang, 1 G. Shi 1 Department of Biochemistry and Molecular Biology, National Cheng Kung University, Tainan, Taiwan, 2Institute of Basic Medical Sciences, National Cheng Kung University, Tainan, Taiwan

Angiostatin is a 44-kDa kringle domain fragment of plasminogen. It can inhibit the proliferation and tube formation of endothelial cells. Interaction of angiostatin with several different molecules in the endothelial cells has been proposed. In this study, we demonstrated that angiostatin can be uptaken by human umbilical vein endothelial cells (HUVECs). Confuent primary culture of HUVECs was incubated with rhodamine-labeled kringle 1-5 (K1-5), one of the most potent angiostatins, and fluorescein isothiocyanate (FITC)-conjugated cholera-toxina subunit B (CTxB). The confocal microscopy images of the cell cultures showed that angiostatin and CTxB were colocalized in the vesicles in the perinuclear region. The HUVECs after being incubated with rhodamine-labeled K1-5 was immunostained with anti-caveolin-1 and anti-clathrin antibodies. The results demonstrated that K1-5 was located in the caveolin-associated vesicles but not in the clathrin-associated vesicles. The endocytosis of K1-5 was inhibited when the cells were pretreated with methyl-b-cyclodextrin that inhibits lipid raft formation. Our results implicated that K-15 binds to the cell surface receptors and is concentrated to the lipid raft domains on the cell surface; then is internalized by caveolin-dependent endocytosis.
**B-DNA Conformation ≠ Salt Bonds in A-DNA: The Watson/Crick DNA Twist Model Is Illogical**

S. R. Erlander; Erlander's Natural Products, Alhadena, CA

The B-DNA helix exists as A-DNA [Erlander, Stig R.; Erlander's Natural Products, Altadena, CA] and can be visualized as a flat ribbon wound around an empty pipe, with phosphate salt bonds holding the two strands together, and with either the syn or anti base pair structure. Incorporation of the salt bond into Manning's polyelectrolyte equations [2], equalizes experimental and theoretical values for DNA. Melting temperatures, for both cationic and anionic sequences, follow the isomolarity of the added salt, e.g., NaCl > KCl > CsCl and Cs > K > Na for both Ta and Ts and insolubility values. Using alcohols, Geiduschek and Hershkovits (1961) reversibly denatured DNA to a double stranded coil. Using the carcinogen AAA [3], CD spectra show [4] that native (syn) DNA is reversibly denatured by methanol, 8 M urea, or pH 3.1 to a double stranded coil, and converted back to either the native syn or the anti structure. These conversions must be by the proposed A-DNA structure, since it is impossible to convert this coil into the Watson/Crick twisted helix, because twisting the coil produces a helix with phosphate salt bonds on the inside and no hydrogen bonds can be formed. Also the Watson/Crick model cannot be formed from the syn base structure. Variations of DH/Tc, (proportional to the distance between charges) show zero DNA charge at 0.0011, 0.040 and 4.44 M MgCl2, which correspond, respectively, to the triple helix point (Murray and Morgan), “zero diameter” (Shaw and Wang), and reversal of charge (Manning). It is therefore concluded that the condensation occurs against the anti syn base structure. [1] Erlander, SR (1970) Starch/ Starke, 22: 352-362. [2] GS Manning, (1972) Biopolymers 11, 937.

**444 A Novel Transcription Domain within CREBH, a Membrane Anchored BZIP Factor Subject to Regulated Intramembrane Proteolysis**

C. Barreira, J. Stirling, P. O'Hare; Protein Trafficking and Gene Regulation, Marucie Curie Research Institute, Oxford, Surrey, United Kingdom

CREBH is a newly identified member of the class of transcription factors which are anchored in membranes and subject to inter-compartmental trafficking and Regulated Intramembrane Proteolysis (RIP). Similar to the other members of this family (ATF-6, CREBF4, OAISIS, LUMAN), CREBH is normally retained within the endoplasmic reticulum and subject to stress induced transport and subsequent cleavage by Golgi resident proteases SIP and S2P to liberate an N-terminal transcriptionally active product. Recent data indicate a convergence between the unfolded protein response and inflammatory signals, and that CREBH selectively regulates aspects of the acute-phase response through ER stress. We identify a new domain (the DWA/n domain) of about 30 residues immediately flanking the BZIP DNA binding domain, specifically conserved in CREBH, CREB4, Luman and OAISIS, but lacking in ATF-6 and other BZIP transcription factors. We constructed deletion mutants of the DWA/n domain within the transcriptionally active N-terminal cleavage products of CREB4 and CREBH and analysed the ability to activate transcription of a promoter containing consensus ATF-6 binding sites. C-termis (or CREBH) lacking the DWA/n domain, while expressed in the nucleus, were virtually inactive compared to the active cleavage product. We are currently examining the role of this novel domain in DNA binding and co-factor specific interactions on potential physiological target genes including the acute phase gene C-Reactive protein.

**445 The Disc Large Tumour Suppressor Protein Connects Cell Polarity to Transcriptional Control**

P. Massimi, L. Banks; ICGEB, Trieste, Italy

The Disc Large (Dlg) tumour suppressor protein is targeted for degradation by the mucosal high risk HPV E6 proteins and shows greatly reduced levels of expression in cervical lesions as they progress towards malignancy. Dlg is mainly localised at the cell-cell junction but previously we, plus others, have also shown the presence of the protein in the nucleus. Moreover, it is this fraction of Dlg which appears to be particularly targeted by E6 for degradation. Although there is a large literature on the function of Dlg at the cell periphery, very little is known about the function of nuclear forms of the protein. Obviously one potential function would be regulation of transcription of certain target genes, and to investigate this we have analysed interaction between Dlg and the HAT domain of p300. Interestingly, the association between p300 and Dlg results in efficient acetylation of Dlg within its carboxi-terminal region, and cell cycle analyses indicate that this occurs preferentially in G1/S phase. In conclusion, these studies highlight the existence of a novel function of nuclear forms of Dlg that is related to the regulation of transcription through association with p300.

**WNt3-Fzd1 Chimera as a Model to Study the Canonical Wnt Signaling**

R. A. Bhat, B. Stauffer, A. Dellia Pietra, P. V. N. Bodine; Wyeth Research, Collegeville, PA

Wnts are secreted glyco/ioproteins that interact with a membrane receptor complex composed of the Frizzled (Fzd) receptor and a Lipoprotein-related protein (LRP). So far 19 Wnt genes and 10 frizzled receptors have been identified, however the specificity of their interaction has not been fully defined. In the present study, we have developed a chimera of Wnt3-Fzd1 and have validated that the chimeric protein activates canonical Wnt signaling. Moreover, the chimera was used to understand the role of Wnt signaling in osteoblast differentiation. The Wnt3-Fzd1 chimera activated TCF-Luciferase reporter, and the fold induction was much higher than that obtained from Wnt3 cDNA alone. The deletion of the cytoplasmic tail and several point mutations highlight the existence of a novel function of nuclear forms of Dlg that is related to the regulation of transcription through association with p300.

**SF3A60 as a Constitutive Androstane Receptor (CAR) Interacting Protein**

J. Kwon, H. Jeong, W. Seol; Inst. of Brain Science & Technology, Daegon, Republic of Korea

CAR (Constitutive Androstane Receptor) is a member of nuclear receptor superfamily which plays an important role for degradation of xenobiotics in liver. Using yeast two-hybrid screening, we have identified SF3A60 (SF3A3), 60KD subunit of splicing factor 3a, as one of CAR-interacting proteins. Recently, it has been shown that steroid hormone receptors such as PR (Progesterone Receptor) and ER (Estrogen Receptor) and a transcriptional coactivator, CoAA, exhibit different effect on alternative splicing decision and stimulate the production of spliced variants produced from genes driven by hormone dependent promoter. Therefore, we investigated physiological meaning of interaction of CAR with SF3A60. At first, we confirmed this interaction of murine CAR and SF3A60 by co-immunoprecipitation and GST-pull down assay. Overexpression of SF3A60 in HepG2 cells did not activate CAR’s transcription activity. However, overexpression of murine CAR showed mild change on production of splicing variants of a gene driven by a promoter containing CAR binding sequence. These results suggest that CAR can affect differential splicing by interaction with SF3A60.
Nonsteroidal Anti-inflammatory Drugs Inhibit Lymphangiogenic Marker Prox-1-mediated Transcriptional Activity
M. Pan,1 W. Hung,2 Kaosong Medical University, Institute of Medicine, Kaosong, Taiwan, 1National Sun Yat-Sen University, Institute of Biomedical Sciences, Kaosong, Taiwan
A positive association between cyclooxygenase-2 (COX-2) expression and lymphangiogenesis has been reported in several cancers. Here, we studied the correlation between COX-2 and the specific lymphangiogenic factor Prox-1 in vein endothelial cells. In addition, we also try to determine whether COX-2 specific inhibitor NS398 affects cancer cell-mediated tube formation of EAHy926 endothelial cells on Matrigel gel. We showed that conditioned medium of COX-2-overexpressing lung cancer cells induced Prox-1 expression in EAHy926 cells in a PGE2-independent manner. It has been reported that COX-2-mediated the induction of VEGF-C in certain cancers. We confirmed that VEGF-C induced Prox-1 expression and ecdysoexpression of VEGF-C in endothelial cells indeed elevated prox-1 expression. NS398 not only significantly inhibited basal Prox-1 expression, but also reversed the promotion of conditioned medium of COX-2-overexpressing lung cancer cells or VEGF-C on Prox-1 expression. Furthermore, we found that NS398 suppressed the expression of Prox-1 via regulation of transcriptional activity and RNA stability. More importantly, NS398 also abolished Prox-1-mediated stimulation of VEGF-3 expression. These results suggest that COX-2 stimulated Prox-1 expression, and NS398 attenuates lymphangiogenesis via down-regulation of Prox-1/VEGF-3 signaling.

HER-2/neu Transcriptionally Activates Jab1 Expression via the AKT / β-catenin Signaling Pathway
M. Hsu,1 W. Hung,2 Kaosong Medical University, Institute of Medicine, Kaosong, Taiwan, 1National Sun Yat-Sen University, Institute of Biomedical Sciences, Kaosong, Taiwan
Jab1/CSN5, the fifth subunit of COP9 signalosome, facilitates the mislocation of p27 from nucleus to cytoplasm and promotes its degradation. It has been reported that Her-2/neu causes the decrease of p27 protein level by inducing Jab1-mediated cytoplasmic exportation and ubiquitin-dependent degradation. In this study, we investigated the effect of Her-2/neu on Jab1 expression. Our data demonstrated that Her-2/neu up-regulated Jab1 expression via transcriptional level. We isolated the 5'-flanking region of human Jab1/CSN5 gene to elucidate the mechanism how Her-2/neu transcriptionally activates Jab1 expression. Luciferase reporter assay, site-directed mutagenesis analysis and DNA affinity precipitation assay (DAPA) revealed that Her-2/neu acted via a TCF-4 binding site to induce Jab1 promoter activity. We next explored the signaling pathway that mediated the effect of Her-2/neu on Jab1 expression by chemical inhibitors. Our results showed that inhibition of AKT activity by wortmannin blocked Her-2/neu-mediated Jab1 promoter activity. Co-expression dominant-negative AKT or constitutively GSK-3β also decreased Jab1 promoter activity. Conversely, co-expression β-catenin or myristoylation signal-attached AKT (myr-AKT) increased Jab1 promoter activity in NIH3T3 cells. Jab1 knockdown by small interfering RNA ( siRNA ) restored p27 protein level and induced G1 phase arrest. These results support that Her-2/neu transcriptionally regulates Jab1 expression via AKT/β-catenin signaling pathway and Jab1 may be a therapeutic target in breast cancer with Her-2/neu overexpression.

Regulation of GAPDH in Prorocentrum micans According to Nutritional Condition
J. B. Shim,1 Y. D. You,1 H. J. Jeong,2 G. H. Kim1; 1Department of Biology, Kongju National University, Kongju, Republic of Korea, 2School of Earth & Environmental Science, Seoul National University, Seoul, Republic of Korea
We investigated changes of inner-cellular metabolism processes using 2-D electrophoresis. These changes are surely needed when mixotrophic organisms change their trophic method from photosynthesis to heterotrophy. We analyzed and compared proteomes extracted from Prorocentrum micans ( Dinoflagellate ) in autotrophic and heterotrophic condition respectively. About 800 proteins were detected in two conditions, 55 proteins among these proteins showed significantly different expression aspect between two conditions. Some proteins differed in expression amount and others newly expressed. 16 proteins, firstly expressed in autotrophic condition, had basic pl value. In other hand 12 proteins expressed in heterotrophic condition had acidic one. There are wide difference of revelation quantity and total amount of proteins were enough to Ethan MALDI-TOF Mass spectrometry analysis. So, we selected eight spots and Amino acid sequencing. Consequently one of Spots(#6404)was indentified GAPDH(glyceraldehyde-3-phosphate dehydrogenase) which is participate in photosynthesis and glycolysis. There are wide differences of revelation quantity and total amount of proteins were enough to Ethan MALDI-TOF Mass spectrometry analysis. These results suggest that GAPDH is the enzyme participate in photosynthesis and Calvin cycle. When think over GAPDH is one of representative house keeping gene, this protein's discriminatory manifestation on the lines of nutritional condition is not so much dinoflagellates progress photosynthesis and heterotrophy come together as to select specific nutrition intake method.

Erk2-mediated Phosphorylation of P300 Involves in the Regulation of EGF-induced Keratin 16 Gene Expression in Keratinocytes
Y. Chen,1 Y. Wang,2 W. Chang3; 1Department of Pharmacology, National Cheng Kung University, Tainan, Taiwan, 3Department of Molecular and Cellular Oncology, MD Anderson Cancer Center, Houston, TX
Overexpression of keratin 16 has been observed in those skin diseases characterized by hyperproliferation such as psoriasis. In studies of gene regulation of keratin 16, we reported previously that the coactivator p300 interacted with Sp1 and API proteins, and participated in EGF-induced keratin 16 gene expression in HaCaT cells. In addition, EFG treatment was found to up-regulate p300 recruitment to the keratin 16 promoter through ERK signaling pathway. According to the documents previously reported, activated ERK2 phosphorylated GST-p300 (aa 1572-2370) in vitro and the transcriptional activity of p300 was enhanced. However, it is still unclear that which kinases are responsible for p300 phosphorylation in vivo and where the exact phosphorylation sites occur upon stimulation. Therefore, in this study, we want to explore that the effect of p300 phosphorylation on EGF-induced keratin 16 gene expression in keratinocytes. According to our results, the purified p300 protein was phosphorylated by activated ERK2. Furthermore, phosphorylation of p300 was increased upon EGF treatment in a time-dependent manner in HaCaT cells and this phosphorylation was regulated by ERK signaling pathway. We also found that ERK2-induced phosphorylation of p300 increased its HAT activity in vitro. Moreover, six potential ERK phosphorylation sites on p300, including Thr317, Thr938, Thr960, Ser2279, Ser2315, and Ser2366, were identified. Reporter assays revealed that, comparing with the effect of p300WT overexpression, no apparent effect on EGF-induced keratin 16 promoter activity was observed in overexpression of p300-T3A with the three mutated threonine residues (Thr317, Thr938, and Thr960) all replaced by alanine. However, a significant decrease in EGF-stimulated response was found in p300-S3A with the three mutated serine residues (Ser2279, Ser2315, and Ser2366) all replaced by alanine. Taken together, these results suggest that ERK2-phosphorylated p300 on Ser2279, Ser2315, and Ser2366 might play a role in EGF-induced keratin 16 gene expression by its increased intrinsic HAT activity.

Role of PLC1 and MOT1 in Preinitiation Complex Assembly
A. M. Demczuk, A. Vancura; Biological Sciences, St. John's University, Queens, NY
Phospholipase C (Plc1p encoded by PLC1 gene in S cerevisiae) and inositol polyphosphates have been shown to function in transcriptional activation by regulating the activity of chromatin remodeling complexes, such as the SWI/SNF complex. One of the rate-limiting steps in transcriptional activation is the binding of TATA-binding protein (TBP) to promoter DNA, leading to the formation of the preinitiation complex (PIC). Mot1p is an essential Swi2/Sfn2-related protein that catalyzes dissociation of TBP from DNA. In this study we examined whether Ptc1p regulates PIC formation by affecting Mot1p function and recruitment or dissociation of TBP. Mutations in PLC1 and MOT1 display strong genetic interactions. However, chromatin immunoprecipitation experiments showed that Plc1p does not affect recruitment of Mot1p to Mot1p-regulated promoters. Expression of Mot1p-repressed genes in mot1Δ double mutants is strongly increased in mot1ΔΔ cells. In addition, the formation of the preinitiation complex (PIC). Mot1p is an essential Swi2/Sfn2-related protein that catalyzes dissociation of TBP from DNA. In this study we examined whether Plc1p regulates PIC formation by affecting Mot1p function and recruitment or dissociation of TBP. Mutations in PLC1 and MOT1 display strong genetic interactions. However, chromatin immunoprecipitation experiments showed that Plc1p does not affect recruitment of Mot1p to Mot1p-regulated promoters. Expression of Mot1p-repressed genes in mot1ΔΔ cells.

Role of Schistosomal Lipids in Activating the Innate Immune Responses through Toll-like Receptor (TLR)-2 Signaling Pathways
K. G. Magalhães,1 P. E. Almeida,1 G. C. Atella,2 M. P. Machado,3 P. T. Bozza1; 1Imunofarmacodinamica, Instituto Oswaldo Cruz - FIOCRUZ, Rio de Janeiro, Brazil, 2Instituto de Bioquímica Médica, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil, 3Biotecnologia, Instituto Oswaldo Cruz - FIOCRUZ, Rio de Janeiro, Brazil
The activation of cells of the host innate immune response by signature molecules of schistosomiasis may determine the course of the pathologic process and immunity to infection, emphasizing the importance of studying the response to such parasite molecules. It is becoming clear that ligands and their receptors may play an important role in regulating immune responses. Here, we...
have focused on the role of schistosome lipids in activating the innate immune response through TLR activation. To investigate the role of TLR2 in the innate immune response to *Schistosoma mansoni* and verify the immunomodulatory properties of their lipids, we isolated and purified lipids from *S. mansoni* eggs and adult worms, to stimulate macrophages from both wild type and TLR2 deficient mice in vitro. Our results show that stimulation by *S. mansoni* lipids in vitro of mouse peritoneal macrophage induced a translocation of the transcription factor NF-κB to the nucleus as assessed by confocal microscopy. Schistosome lipids, mainly lyso-phosphatidylcholine fraction, also induced a time-dependent increase of TNF-α and IL-6 expression, increased formation of lipid bodies and significantly enhanced the production of eicosanoids in wild type but not in TLR2 deficient mice. The co-localization of 5-lipoxygenase (5-LO) within lipid bodies showed that such organelles might function as specialized cytoplasmic domain for eicosanoid-forming enzyme localization in macrophages. Taken together, our results indicate that a spectrum of immunomodulatory activities can be attributed to *S. mansoni*, indicating a role for these lipids in enhancing the expression of cytokines and lipid mediators, inducing lipid body formation and stimulating innate immunity through TLR2 dependent pathway.

453 Genes Express during Elicited Spider Fibroin Production
G. Candelas, G. Arroyo, I. Cintón, L. Capó, A. Plazaola, A. González, C. Ayala, J. Canabal, University of Puerto Rico—Río Piedras Campus, San Juan, Puerto Rico
We are exploiting the large amounts of spider, Nephila clavipes, a model system in which to study the elicited production of a specific spider protein. This is made possible due to the fact that we are able to virtually abolish the process and subsequently elicit it to high levels of production. The transient responses elicited, have been monitored through time sequence by providing the adequate labeled precursors. In so doing, we have observed a large wave of the full size protein product, which peaks at ninety minutes after stimulation. This wave is preceded by a sixty minutes interval by the production of the fibroin’s template. A third peak of minor intensity is observed following the production of the template, which adaptively shifts the gland’s RNA population, enriching it with the mRNAs cognate to the fibroin’s preponderant amino acids, glycine, alanine and proline. The earliest of the stimulus elicited activities, which peaks at fifteen minutes after stimulation, enriches the glands with a variety of small RNAs, among which, we have thus far, identified a tissue-specific alaline tRNA, two isoforms of SS RNAs and all the members of the U subset of small nuclear RNAs. We have initiated our search with the genes which code for alanine tRNA. A cluster of four alanine tRNA genes was isolated, the individual genes were subcloned and sequenced. The genes displayed microheterogeneity within their coding as well as their flanking sequences. The genes were transcribed in an heterologous extract derived from Bombyx mori, displaying differentiation of expression. Of interest is the gene with the highest transcriptional activity, which requires a far upstream sequence element. The presence of this sequence places this alanine tRNA gene in a category of Pol III genes such as, U6snRNA TskRNP and tRNA see, which possess distal positive regulatory elements.

454 Regulation of Pluripotency Gene Repression upon Differentiation
A. J. Cooney, P. Gu; Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX
ES cell self-renewal is maintained by a core of key transcription factors that include Oct4, Sox2 and Nanog that through feed forward and feed back mechanisms maintain pluripotence gene expression. However, specific mechanisms are required to disrupt this novel pattern of gene expression upon differentiation to down regulate the pluripotent phenotype and inhibit the pluripotent phenotype. Upon differentiation of ES cells Oct4 gene expression is repressed and subsequently silenced. Nuclear receptors are critical for the regulation of pluripotent gene expression during embryonic stem cell differentiation and embryonic development. The orphan nuclear receptor GCNF has been shown to play a central role in the repression of Oct4 by binding to a response element in its promoter. Efforts to characterize mediators of GCNF’s repressive function on the Oct4 promoter identified Methy1 DNA binding protein, MBD3, as a GCNF interaction factor. The ligand binding GCNF interacts with methyl DNA binding domain of MBD3. In addition, GCNF also interacts with the closely related factor MBD2. In P19 and ES cells, upon differentiation, endogenous GCNF binds to the Oct4 proximal promoter and recruits MBD2 and MBD3, in a retinoic acid dependent manner. In differentiated GCNF−/− ES cells recruitment of MBD2 and MBD3 is lost and subsequently repression of Oct4 expression fails to occur. RNAi mediated knock-down of MBD3 expression results in reduced Oct4 expression in differentiated P19 cells. Thus, we have shown that GCNF recruits MBD2 and 3 to the Oct4 promoter to mediate repression of Oct4 expression during differentiation of P19 ES cells. The direct interaction between GCNF with MBD2 and MBD3 provides specificity of recruitment for a relatively non-specific DNA binding factors. Thus, MBD2 and MBD3 are novel nuclear receptor co-repressors that are required to repress pluripotent gene expression upon ES cell differentiation.

455 Dominance of SOX9 Function over RUNX2 during Skeletogenesis
G. Zhou,1,2 Q. Zheng,2 F. Engin,3 E. Munivez,2 Y. Chen,2 E. Sebald,4 D. Krakow,4 B. Lee2,3; 1Orthopaedics, Case Western Reserve University, Cleveland, OH, 2Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX, 3Howard Hughes Medical Institute, Houston, TX, 4Medical Genetics Institute, Cedars-Sinai Medical Center, Los Angeles, CA, 5Obstetrics and Gynecology, David Geffen School of Medicine at UCLA, Los Angeles, CA
Mesenchymal stem cell-derived osteo-chondroprogenitors express two master transcription factors SOX9 and RUNX2 during condensation of the skeletal anlagen. They are essential for chondrogenesis and osteogenesis, respectively, and their haploinsufficiency cause human skeletal dysplasias. We show that SOX9 directly interacts with RUNX2 and repress its activity via their evolutionarily conserved HMG and runt domains. Ectopic expression of full-length SOX9 or its RUNX2-interacting domain in mouse osteoblast results in an osteodysplasia characterized by severe osteopenia and down regulation of osteoblast differentiation markers. Thus, SOX9 can inhibit RUNX2 function in vivo even in established osteoblastic lineage. Finally, we demonstrate that this dominant inhibitory function of SOX9 is physiologically relevant in human campomelic dysplasia (CMD1). In CMD1, haploinsufficiency of SOX9 resulted in upregulation of the RUNX2 transcriptional target COL1A1 as well as all the three members of RUNX gene family. In summary, SOX9 is dominant over RUNX2 function in mesenchymal precursors that are destined for a chondrogenic lineage during endochondral ossification.

456 Interleukin-2 Treatment of HuT 78 Cells Induces the Activities of DNA Topoisomerase I and DNA Topoisomerase II
P. D. Foglesong; Biology, University of the Incarnate Word, San Antonio, TX
HuT 78 is a human T cell lymphoma line that expresses a high affinity receptor for interleukin-2 (IL-2). Upon binding of IL-2 to its receptor signal transduction pathways cause the activation of protein kinases and an increase in transcription and DNA replication. This study investigated the effects of IL-2 treatment upon the activities of DNA topoisomerase I and DNA topoisomerase II, enzymes that are involved in transcription and DNA replication. HuT 78 cells were treated with 1,000 U/ml of IL-2, and nuclear extracts were prepared at various times after treatment. The nuclear extracts were assayed quantitatively for the catalytic activity of DNA topoisomerase I (Topo I) by relaxation of supercoiled plasmid pH2624 DNA and for the catalytic activity of DNA topoisomerase II (Topo II) by unknotting of bacteriophage P4 DNA. The concentrations of protein in the nuclear extracts were determined by a modified Bradford assay, and the specific activities of Topo I and Topo II were measured. The specific activities of both Topo I and Topo II increased 10-fold to 20-fold after treatment of HuT 78 cells with IL-2. The specific activity of Topo I increased with peaks observed at 0.5, 2 and 12 hours following treatment, and the specific activity of Topo II increased with peaks observed at 2 and 4 hours following treatment. The times of increased specific activities of these enzymes coincide with periods of increased transcription (0.5 - 4 hours) and increased DNA replication (12 hours) in HuT 78 cells following treatment with IL-2. These results suggest that Topo I and Topo II function in transcription and DNA replication in activated human T lymphocytes and that the increased activities of these enzymes are significant consequences of the activation of T cells by IL-2.

457 cis-regulatory Mechanisms of Anterior Visceral Endoderm-specific Genes
C. Kimura-Yoshida,1 H. Nakano,1 J. Rossant,2,8 S. Aizawa,1,8 I. Matsui,1,2,8; 1Department of Molecular Embryology, Osaka Medical Center for Maternal and Child Health, Osaka, Japan, 2Program in Developmental Biology, Hospital for Sick Children, Toronto, ON, Canada, 3Vertebrate Body Plan Group, RIKEN, Center for Developmental Biology, Kobe, Japan
Anterior visceral endoderm (AVE) plays essential roles with respect to anterior pole development in the early mouse embryo. However, the molecular mechanisms underlying the formation of AVE remain largely unknown. To assess the genetic cascade involved in AVE formation, the cis-regulatory elements directing expression of four vertebrate, mouse, human, chicken and pufferfish, Otx2 genes in the AVE were analyzed comparatively via generation of transgenic mice. Precise cis-analysis employing deletion and point mutant constructs revealed that one highly conserved element, TTTTCCGC, directs Otx2 expression in the PE1 element. Consistent with this, Otx2 expression is identical to Otx2 expression in the visceral endoderm during AVE migration and the distal visceral endoderm fails to migrate anteriorly in
These findings provide profound insights into conserved roles of γ into the pAAV plasmid with a H1 promoter. Subsequently the PKC γ Whitehead siRNA design program. Next, the siRNAs were subjected to a NCBI BLAST search that checks the specificity of each siRNA for the targeted gene and identifies similar sequences.

mGluR6 mice in which reporter gene expression was directed under the control of the distribution of retinal bipolar cell types in the mouse central retina using bipolar cell type-specific immunohistological markers. To facilitate our quantitative analysis we generated transgenic Retinal bipolar cells mediate the vertical transfer of visual signals from photoreceptors to ganglion cells. At least 10 morphologically distinct bipolar cell types have been characterized. These

Lausanne, Switzerland, 2Department of Biochemistry, University of Geneva, Geneva, Switzerland, 3Pharmaceuticals Division, Hoffmann-La Roche Ltd., Basel, Switzerland, 4National Institute...

Changes in the Distribution of Spermidine Synthase during Spermiogenesis in D. Skowronska-Krawczyk,1 F. Chiodini,2 C. Alliod,2 M. Ebeling,3 D. Castro,4 L. Matter-Sadzinski,1 F. Guillemot,4 M. Ballivet,2 J. Matter1,2; 1University of Lausanne, Eye Hospital Jule Gonin, 461...

mechanisms underlying bipolar cell type diversity.

The Role of Elip-like Gene in Cold Condition Survival of Green Alga, Spirogyra varians (Zygnematales) J. W. Han, M. Yoon, K. P. Lee, G. H. Kim; 1Department of Chemistry, Kongju National University, Kongju, Republic of Korea, 2Department of Biology, Kongju National University, Kongju, Republic of Korea.

Plant cells respond to cold stress by the expression of gene encoding specific stress proteins with possible protective functions. Simultaneous comparison of differentially expressed protein profiles of a freshwater alga, Spirogyra varians, grown under two different temperature conditions (4°C and 20°C) indicated the generation of cold-stress responsive proteins. We isolated a 20 KDa protein (pI 4.5) which was most strongly up-regulated in 4°C (about 500-fold higher than in 20°C). As the protein has never been reported before, we named it as SVCR1 (Spirogyra varians cold regulated) protein. The cDNA encoding SVCR1 was cloned using degenerated primers designed from the internal amino acid sequence of the protein and a λZAP cDNA library of Spirogyra varians. The deduced amino acid had a high sequence similarity with early light-inducible proteins (ELIPs) of higher plants which are known as nuclear-encoded chloroplast proteins induced by light stress. The northern blot results showed that the accumulation of SVCR1 transcript could be induced by cold treatment (4°C) even under the dark condition. To understand the relationship between SVCR1 and cold stress, we used the RNA interference method, in which knockdown strains with reduced SVCR1 levels were isolated. We compared life cycle between knockdown strain and wild type strain under low temperature. Based on these results, the possible role of SVCR1 in cold stress defence is discussed.

Design of siRNAs That Target the Pain Gene PCkγ M. Lozada, S. Garraway, C. Inturrisi; 1Industrial Biotechnology Program, University of Puerto Rico - Mayaguez Campus, Humacao, Puerto Rico, 2Department of Pharmacology, Weill Medical College of Cornell University, New York, NY.

RNA interference is a powerful genetic approach for efficiently silencing target genes. One advantage of this technique is the high specificity it offers over pharmacological antagonism in it cleaves the mRNA of a precise protein. The purpose of this study is to use RNA interference to target PCkγ, a protein kinase that has been identified in neurons of the brain and spinal cord and implicated in the production of persistent pain. Firstly, we designed five 21 base pair small interfering RNAs (siRNA) that target the gene that encodes the mouse PKC γ it cleaves the mRNA of a precise protein. The purpose of this study is to use RNA interference to target PCkγ, a protein kinase that has been identified in neurons of the brain and spinal cord and implicated in the production of persistent pain. Firstly, we designed five 21 base pair small interfering RNAs (siRNA) that target the gene that encodes the mouse PKC...

Highly Conserved Non-coding Sequences Mediate Species-specific Regulation of the ATH5 Gene during the Course of Mouse and Chick Retina Ontogenesis D. Skowronska-Krawczyk,2 F. Chiodini,1 C. Alliod,2 M. Ebeling,2 D. Castro,2 L. Matter-Sadzinski,1 F. Guillemot,1 M. Ballivet,2 J. Matter2,2; 1University of Lausanne, Eye Hospital Jule Gonin, Lausanne, Switzerland, 2Department of Biochemistry, University of Geneva, Geneva, Switzerland, 3Pharmaceuticals Division, Hoffmann-La Roche Ltd., Basel, Switzerland, 4National Institute for Medical Research, London, United Kingdom.

The atonal homologue 5 (ATH5) is predominantly expressed in the developing vertebrate retina. In zebrabfish and in the mouse, inactivation of the ATH5 gene results in a retina lacking most retinal ganglion cell (RGC) types. In the chick retina, the acute upregulation of ATH5 coincides with the period of development when the majority of RGCs are specified and this upregulation is required to occur on transcription of genes defining the RGC phenotype. Promoter occupancy and functional analysis indicate that the neurogenin 2 (Ngn2) and ATH5 proteins are directly required for transcription of the chicken ATH5 gene. In contrast, the late onset of Ngn2 expression in the mouse retina and the standing expression of ATH5 in Matθ5/5-/- mice have suggested that bHLH proteins might not be required for the regulation of ATH5. To address this issue we have conducted a comparative analysis of the role of Ngn2 in the regulation of ATH5 during development of the mouse and chick retinas. In contrast with previous studies, we found that the onset of Ngn2 and ATH5 expression coincides in early mouse retina and that ATH5 expression is downregulated in Ngn2-/- mice. Despite the fact that ATH5 promoter elements are highly conserved, their in vivo occupancy by Ngn2 is very different in the mouse and chick retinas. We show how the proximal and distal regions act together to bring expression of ATH5 at a level which is about 50 folds higher in the chick than in the mouse retinas. In sum, our study reveals that evolutionary conserved regulatory elements are instrumental to mediate species-specific regulation of the ATH5 gene. We propose that such difference between species may have permitted the development of RGC populations that differ in size and properties. Supported by the Swiss National Science Foundation and Provisu

Changes in the Distribution of Spermidine Synthase during Spermiogenesis in Marsilea vestita 85, F. Deeb, S. M. Wolnai; Cell Biology and Molecular Genetics, University of Maryland, College Park, MD. The microspore of the water fern, Marsilea vestita, contains a single cell that undergoes a series of nine mitotic divisions to produce 32 spermatids and 7 sterile cells. Cytoplasmic movements precede the first division in the transcriptionally-quiescent gametophyte; certain proteins and mRNAs aggregate into zones that later become spermatogenous initials. Asymmetric divisions produce jacket cells, and then, symmetric divisions produce spermatids. As the spermatids differentiate into motile gametes, the jacket cells undergo a form of programmed cell death (PCD). Our published work shows that translation is controlled both spatially and temporally. We developed RNAi strategies to target mRNA degradation and thereby arrest development. We have focused on spermatogeny, spore nuclear remodeling, to determine whether jacket cells play a role in the development of spermatogenous cells. dsRNA probes were made from cDNAs encoding spermidine synthase (SPDS) and spermidine transporter (SPDT) that were isolated from our gametophyte cDNA library. Spores treated with these dsRNAs showed fewer but larger spermatogenous cells than control gametophytes. Normal gametophytes labeled with anti-SPDS and anti-spermidine antibodies show that spermidine first appears in the jacket cells and then in the spermatogenous cells. Spores treated with SPDT dsRNA showed reductions in spermidine in spermatogenous cells, suggesting that spermidine is transported from the jacket cells into the spermatogenous cells before it starts to be made in the spermatogenous cells. Treated cells were labeled with anti-sense centrin RNA and anti-centrin antibody to assess patterns of transcript distribution and patterns of new translation, respectively. Like untreated spores, centrin mRNA was present throughout the microspore, and centrin protein was localized in the spermatogenous cells. Our results show that the jacket cells support the development of the spermatogenous cells by providing molecules that are important for the gamete differentiation process. (Supported by NSF grant MCB-0234423 to SMW).
Regulation of Human Rac2 during Human Chronic Myelogenous Leukemia Cell Line (K562) Differentiation

R. Mukushirman, 1 D. G. Shalakin, 1 Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, IN; Departments of Pediatrics and Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, IN; Rac2 belongs to the family of Rho GTPases. Rac2 is expressed only in hematopoietic cell lineages and is up regulated upon terminal myeloid cell differentiation. Rac2 plays an important role in many of the hematopoietic cellular functions such as neutrophil chemotaxis, superoxide production, cytoskeletal reorganization, and stem cell adhesion. The current study aims at investigating the molecular mechanisms regulating human Rac2 gene expression during cell differentiation using an in vitro model system. Stimulation of K562 cells with PMA resulted in an increased human Rac2 mRNA expression as analyzed by real-time PCR analysis. PMA activation of K562 cells at different time periods showed time dependent induction of Rac2 mRNA expression. This indicated that the Rac2 gene expression accompanies cell differentiation. To further delineate the regulatory elements required for the Rac2 induction upon cell activation, the human Rac2 promoter region was analyzed for the presence of cis elements. Transient expression analysis using the luciferase reporter gene revealed the induction of human Rac2 gene expression to be mediated by increased transcriptional activity of Rac2 promoter region. Series of 5’ deletion derivatives of the Rac2 promoter region used in the transient transfection assays identified a 1 kb region between -4558 bp and -3500 bp to contain the regulatory element required for the transcription of Rac2 gene expression. Future studies will aim at identifying the minimal Rac2 promoter region present within this 1 kb region that is sufficient to direct PMA induced Rac2 transcription. Chromatin Immunoprecipitation assays will be done to identify the transcription factors that bind to the DNA element(s). Identification of the DNA elements and the transcription factors that bind to these regulatory elements will help in the understanding of molecular mechanisms regulating Rac2 gene expression during cell differentiation.

Intestinal Flora Alters DNA Methylation in Intestinal Mucosal Cells

Y. Iwamoto, K. Hirabayashi, N. Hattori, S. Tanaka, S. Yagi, K. Doi, K. Shiota; The University of Tokyo, Tokyo, Japan

Genome-wide analysis of gene area revealed that there are many loci that have tissue-dependent and differentially methylated regions (T-DMRs). Importantly, DNA methylation profile of the T-DMRs is unique in each tissue or cell type including embryonic stem cells, somatic cells and germ cells. Therefore, DNA methylation profiles of T-DMRs are useful to evaluate and characterize the cells. Intestinal epithelium is one of the rapidly renewed cells in mammals and has multiple functions as transporter of nutrients and helping to prevent infection. Interaction with the bacterial flora is another feature of the intestinal epithelium. Many documents have been accumulated that bacterial flora influences the physiology and differentiation of epithelial cells. The objectives of this study are to characterize the epithelium by T-DMRs analysis and to know if bacterial flora may affect epigenetic status. The mucosa was collected by scratching the surface layer of intestine using razor from germ free (GF) and specific pathogen-free (SPF) mice. First, we confirmed that the intestinal mucosa has a unique DNA methylation profile of T-DMRs compared to other genome DNA from kidney, liver, brain, spleen, and testis, by methylation sensitive real-time PCR focusing on 93 Not I sites. Interestingly, there are two loci that are differentially methylated in the mucosa between SPF compared to GF: Agpat3, encoding 1-acetylcyto-ol-3-phosphate-O-acetyltransferase 3, and Kcnk5, encoding a member of potassium channel subfamily K, member 5. Agpat3 showed hypermethylation in SPF compared to GF, suggesting that intestinal flora may regulate the DNA methylation in genome. Differentiation of cells from the stem condition generally associates with both DNA methylation and demethylation. Therefore, the intestinal epithelium may be under the pressure of DNA methylation by bacterial flora at the conventional condition. Thus, the bacterial flora has an impact on formation of DNA methylation profile in intestinal mucosal cells.

Dppa3 is Expressed by DNA Methylation

H. Muramoto, N. Hattori, S. Tanaka, S. Yagi, K. Shiota; The University of Tokyo, Tokyo, Japan

Dppa3 gene (also known as stella or PGC7) encodes a maternal factor, essential for murine preimplantation embryogenesis. It is expressed in oocytes, preimplantation embryos, primordial germ cells (PGCs) and embryonic stem (ES) cells but not in placenta or other somatic tissues. Dppa3 is located within a conserved cluster of genes, such as Gdf3 and Nanog, which is associated with pluripotency and tumors in the mouse and human. The similar expression pattern of these genes implies a possible transcriptional co-regulation. To address if DNA methylation is involved in the regulation of Dppa3, we first analyzed a DNA methylation status of the Dppa3 upstream region by methylation sensitive restriction mapping in ES cells and compared this with trophoblast stem (TS) cells and liver, both of which were confirmed to show no Dppa3 expression. In a good correlation with its expression activity, the 5’-flanking region up to 2 kb upstream of Dppa3 transcription start site showed a lower methylation status in ES cells than in TS cells and liver. Bisulfite sequence analysis further revealed a hypomethylation status of 15 CpG dinucleotides within 1 kb of Dppa3 proximal upstream region in ES cells while TS cells showed hypermethylation status of these CpGs. NIH/3T3 cells, in which Dppa3 was not expressed, were also revealed to have hypermethylated Dppa3 upstream region. These 15 CpGs were also hypomethylated in PGCs while were hypermethylated in the liver isolated from mouse fetuses at embryonic day 12.5. Thus, the Dppa3 upstream region appeared to be a tissue-dependent differentially methylated region (T-DMR). Ectopic Dppa3 expression was induced in TS cells by treatment with an inhibitor of DNA methylation, 5-aza-2’-deoxycytidine and in Drom1-deficient mouse placenta. In conclusion, the results suggest a potential role of DNA methylation in silencing of Dppa3 gene.

A Molecular Insight of Hes5-dependent Inhibition Mechanism: Old Partners and New Players

A. Liu; Neuroscience and Cell Biology, UMDNJ-Robert Wood Johnson Medical School, Piscataway, NJ

This study identifies novel mechanisms of Hes5 function in developmental myelination. We report here up-regulation of myelin gene expression in Hes5-/- mice compared to wild-type siblings and down-regulation in overexpressing progenitors. This effect was only partially explained by the ability to regulate the levels of Mash1 and bind to N boxes in myelin promoters, since deletion of the DNA-binding domain of Hes5 does not suppress its inhibitory role on myelin gene expression. Novel mechanisms of Hes5 function in the oligodendrocyte lineage include the regulation of feedback loops with the cell-specific transcriptional activator Sox10. In progenitors with low levels of Sox10, Hes5 further decreases the bioavailability of this protein by transcriptional inhibition and direct sequestration of this activator. Increasing levels of Sox10 in progenitors, in turn, bind to Hes5 and titrate out its inhibitory effect by sequestration and displacement of the repressive complexes from myelin promoters. Thus, Hes5-dependent modulation of myelin gene expression involves old players (i.e. Mash1) and novel mechanisms of transcriptional regulation involving cell-specific regulatory loops with transcriptional activators (i.e. Sox10).

XRN4/EIN5, an Exoribonuclease, Is Required for Regulation of Defense and Developmental Pathways through Regulation of Small RNA-target mRNA Levels

B. D. Gregory, J. R. Ecker; Plant Biology, Salk Institute for Biological Studies, La Jolla, CA

Ethylene is a gaseous plant growth regulator that controls a multitude of developmental and stress responses. Recently, the levels of Arabidopsis EIN3 protein, a key transcription factor mediating ethylene-regulated gene expression, have been demonstrated to increase in response to the presence of ethylene gas. Furthermore, in the absence ethylene, EIN3 is quickly degraded through a ubiquitin/protasme pathway mediated by two F box proteins, EBF1 and EBF2. We have found that ETHYLENE-SENSITIVE5 is in fact the 5’-3’ exoribonuclease XRN4. Additionally, we demonstrated that the role of EIN3 in ethylene perception is to antagonize the negative feedback regulation on EIN3 by promoting EBF1 and EBF2 mRNA decay, which consequently allows the accumulation of EIN3 protein to trigger the ethylene response (1). Furthermore, tilting microarray analysis of the entire Arabidopsis genome has demonstrated that in addition to EBF1 and EBF2 mRNAs this ribonuclease regulates the levels of many other putative targets of small RNAs including known targets of microRNAs and small interfering RNAs. Through regulation of these specific targets, EIN5/XRN4 plays an important role in pathways involved in defense responses and plant development. 1. Gabriela Olmedo*, Hongwei Gao*, Brian D. Gregory*, Saeid D. Noushradze†, Laura Aguilar-Henonin, Hongjian Li, Fengying An, Flinio Guzman, and Joseph R. Ecker. (2006). ETHYLENE-SENSITIVE5 encodes a 5’-3’ exoribonuclease required for regulation of the EIN3-targeting F-box proteins EBF1/2. Proc. Natl. Acad. Sci. U.S.A. In press. * These authors contributed equally to this work.

Nuclear Import of Viral Genomes

S. Cohen, B. Sun, W. Wu, N. Pantel; Department of Zoology, University of British Columbia, Vancouver, BC, Canada
To spread infection, many viruses enter the nucleus of their host cells where they use the cellular replication and transcription machinery. Today many steps of viral replication and cell entry are well understood at the molecular level. However, very little is known about how viruses deliver their genomes into the nucleus. We are analyzing the mechanism by which a variety of animal viruses enter the nucleus. It is apparent from our studies that viruses have developed different strategies for successfully entering the nucleus. These strategies, in most cases, involve the nuclear pore complex (NPC), which can also have a role in virus disassembly. The viruses that we are studying include influenza virus, hepatitis B virus (HBV) and minute virus of mice (MVM). Influenza virus disassembles during cell entry and the RNPs (made of RNA, polymerases, and the protein NP) enter the nucleus through the NPC. NP contains three putative nuclear localization signals (NLSs). Studying nuclear import of purified influenza RNPs by fluorescence microscopy we have found that the non-conventional NLS, located at the N-terminus of NP, is responsible for influenza RNP nuclear uptake. HBV loses its envelope during cell entry and releases its nuclear capsid into the cytoplasm. With diameters of 32 and 36 nm, HBV capsids are close to the size limit for transport through the NPC. However, using an electron microscopy (EM) import assay in Xenopus oocytes we have demonstrated that HBV capsids are able to cross the NPC without disassembly. While HBV capsids cross the NPC intact and release their genome at the NPC nuclear face, MVM capsids can enter the nucleus through a process that is independent of the NPC. Using both Xenopus oocytes injection and cell infection, we have found that MVM induces nuclear membrane disruptions that allow virus access to the nucleus.

469 Nucleoporin 153 Mediated Arrest of Hepatitis B Virus Capsids in the Nuclear Basket
A. Schmitz,1 A. Schwarz,2 N. Pante,3 B. Rabe,3 M. Kann4; 1 Institute of Medical Virology, Giessen, Germany, 2 Department of Zoology, University of British Columbia, Vancouver, BC, Canada, 3 Umr-cnrs 5097, Present address: Université Victor Segalen Bordeaux, Bordeaux, France
Facilitated nuclear transport occurs via nuclear pore complexes (NPCs), large protein assemblies embedded in the nuclear envelope. Molecules between 9 nm and 39 nm in diameter are imported using cellular transport receptors that mediate the passage through the NPC into the nuclear basket. Within this filamentous structure at the nuclear face of the NPC the receptors dissociate from the cargo by means of the small GTPase Ran in its GTP bound form. While the receptors recycle to the cytoplasm, the cargo diffuses deeper into the karyoplasm. Exceptions in which the cargos stay attached to the NPC are NT2F, the nucleoporin 153 (Nup153) and the protein Tpr that is attached to Nup153. Here, we show that the cap of the hepatitis B virus (HBV) shows a similar aborted nuclear import reaction. The HBV cap is imported into the nuclear basket via the transport receptors importin (karyopherin) αβ and fail to diffuse deeper into the karyoplasm. Using digitonin-permeabilized cells, pull-down assays and microinjections into Xenopus laevis oocytes we found that HBV capsids have a stable interaction with Nup153. HBV contains a DNA genome, but replicate by means of an RNA pregenome (PG). During virus production, the PG is encapsidated into an immature capsid (Impm5-C) that is converted into capsids containing mature DNA genome (Mat-C) by the viral polymerase, also encapsidated in the Impm-C. We observed that the Mat-C disintegrated into the nuclear basket, depicting a well-coordinated strategy to deliver mature viral DNA into the nucleus. As we exemplified for the nuclear import of artificial NLS- and M9-containing capsids the HBV capsids may moreover serve as a tool for investigation of Nup153 function.

470 Regulation of ATF2 Subcellular Localization
H. Liu, D. C. Hu; Purdue University Pharmacy School, West Lafayette, IN
Activating transcription factor 2 (ATF2) is a basic region leucine zipper (bZIP) transcription factor and belongs to activator protein 1 (AP-1) family. ATF2 functions as either homodimers or heterodimers with other AP-1 family proteins Fos, Jun, and CRE binding protein (CREB), to regulate a variety of cellular responses. Although it was believed that all mammalian AP-1 proteins are localized in the nuclear zone, they were activated by mitogen or phosphorylated and distributed in the nucleus. The presence of activating protein kinases (MAPKs), it was not known whether subcellular localization of AP-1 proteins is regulated by regulation. By employing the coexpression of cellular bZIP proteins (bZIPf), we have recently demonstrated that ATF2 homodimer localizes in the cytoplasm. Further analysis has led to the finding that ATF2 possesses two bipartite nuclear localization signals (NLS) in its basic region DNA binding domain and a CRM-1 dependent nuclear export signal (NES) in its leucine zipper region, and continuously shuttles between the cytoplasm and nucleus in nonmorphic form. Dimerylation with Jun family member proteins masks the NES, resulting in ATF2 nuclear localization. In addition to this Jun-dependent regulatory mechanism of ATF2 nuclear localization, we have also observed that deletion of the N-terminal 341 residues results in nuclear export (Fig.1b), suggesting that the N-terminal region ofcytoplasmic nuclear export signals. This model explicitly recognizes that the number, identity, and distribution of molecules within the pore affects cargo translocation through the pore. Our results demonstrate that maximum nucleocytoplasmic transport velocities can be modulated at least ~10-fold by the importin β concentration. We postulate that cargo trafficking rates in general depend on pore occupancy. This model explicitly recognizes that the number, identity, and distribution of molecules within the pore affects cargo translocation through the pore. Factors that could be affected by changes in pore occupancy include: i) the accessible volume for the transiting cargo; ii) the concentration and distribution of RanGTP in the NPC; and iii) the general nuclear pore occupancy. This model explicitly recognizes that the number, identity, and distribution of molecules within the pore affects cargo translocation through the pore. Factors that could be affected by changes in pore occupancy include: i) the accessible volume for the transiting cargo; ii) the concentration and distribution of RanGTP in the NPC; and iii) the structure and physical properties of the permeability barrier.

471 Nuclear Import Time and Transport Efficiency Depend on Nuclear Pore Occupancy
W. Yang, S. Musser; Molecular and Cellular Medicine, The Texas A&M HSC, College Station, TX
While many components and reaction steps necessary for the bidirectional transport of macromolecules across the nuclear envelope (NE) have been recognized, the mechanism and control of cargo migration through nuclear pore complexes (NPCs) remains poorly understood. In earlier work, we demonstrated that single cargo molecules could be visualized interacting with nuclear pore complexes (NPCs) in permeabilized cells by single molecule narrow-field epifluorescence microscopy (Yang, et al. 2004 101:12887-12892; and Yang & Musser, Methods, in press). Here, this approach was used to track cargo movement, and during and after interaction with NPCs in permeabilized and live cells. At low importin β concentrations, about half of the signal-dependent cargos that interacted with an NPC were translocated across the NE, indicating a nuclear import efficiency of ~50%. The NPC transport times for cargos that actually transported through the NPC and those that underwent abortive transport were both ~8.3 ms under these conditions. At high importin β concentrations, the import efficiency increased to ~80% and the transit speed increased ~7-fold. The transit speed and import efficiency of 10 kDa dextrans, a signal-independent cargo, was also increased by high importin β concentrations. These results demonstrate that on average nuclear transport velocities can be modulated at least ~10-fold by the importin β concentration. We postulate that cargo trafficking rates in general depend on pore occupancy. This model explicitly recognizes that the number, identity, and distribution of molecules within the pore affects cargo translocation through the pore. Factors that could be affected by changes in pore occupancy include: i) the accessible volume for the transiting cargo; ii) the concentration and distribution of RanGTP in the NPC; and iii) the structure and physical properties of the permeability barrier.

472 The Repeat Domain of the Nucleoporin p62 Interacts with Other FXFG-containing Nucleoporins in Interphase
P. Banks, M. Kodila, X. Quan, D. Tran, U. Stochaj; Physiology, McGill University, Montreal, PQ, Canada
Nuclear pore complexes provide the only intracellular pathway between nucleus and cytoplasm. The nucleoporin p62, a component of higher eukaryotic NPCs, is located at the central gated channel and involved in trafficking of various cargos. p62 is organized into an N-terminal segment that contains FXFG repeats and binds the soluble transport factor NTF2. The C-terminal portion associates with other nucleoporins and the nuclear cargo importin-β. We have identified several potential sites of interaction specifically with the p62 N-terminal domain. Using the p62 N-terminal segment as bait, we affinity-purified nucleoporins Nup358, Nup214 and Nup153 from crude cell extracts. In ligand binding assays, the N-terminal p62 segment associated with Nup358 and p62, suggesting their direct binding to the p62 N-terminal portion. Furthermore, p62 was isolated in complex with Nup358, Nup214 and Nup153 from growing HeLa cells, indicating that the interaction Nup358/p62, Nup214/p62 and p62/Nup153 also occur in vivo. Comparison of interphase and mitotic cells revealed that the formation of Nup358/p62 and p62/Nup153 complexes was restricted to interphase cells. By contrast, the association Nup214/p62 was detected in interphase as well as during mitosis. Our results support a model of complex interactions between FXFG containing nucleoporins, and propose that during interphase some of these interactions may contribute to the movement of cargo across the NPC.

473 Nup214 Is Required for Efficient CRM1-dependent Nuclear Protein Export
S. Hutter, R. H. Kehlenbach; Biochemistry, University of Göttingen, Göttingen, Germany
While little is known about the role of individual nucleoporins in different nucleocytoplasmic transport pathways, the cytoplasmic nucleoporin Nup358/RanBP2 has previously been suggested to function as a platform for disassembly of CRM1-containing export complexes. We now specifically depleted cells of the cytoplasmic nucleoporins Nup214/CAN and Nup358 by RNA interference. Depletion of Nup214 resulted in co-depletion of its binding partner Nup88. Nuclear pore complexes assembled in the absence of Nup214/Nup88 or Nup358 were fully functional in nuclear protein import, whereas nuclear mRNA export was slightly impaired. Depletion of Nup358 had only a minor effect on nuclear protein export. In contrast, depletion of Nup358 resulted in the failure of nuclear protein export. Our inability to rescue the CRM1-mediated nuclear protein export in Nup214 depleted cells suggests that Nup214 is required for efficient nuclear export.
Regulated and signal-mediated export of molecules across the nuclear membrane is required for several cellular processes and essential for cell viability. Nuclear translocation of macromolecules is mediated by a specific export signal that is recognized by a transport receptor, referred to as an exportin. The mechanism by which many proteins, such as the von Hippel-Lindau (VHL) tumor suppressor and ubiquitination factor, exit from the nucleus is still however unknown. To study the nuclear export of VHL we established an in vivo nuclear export assay based on fluorescence loss in photobleaching (FLIP). This led to the identification of a simple and discreet motif “DxGx2Dx2L” that governs efficient nuclear export of VHL as well as a wide spectrum of unrelated proteins such as the mRNA nuclear export and translation factor Poly(A)-Binding Protein (PABP1), the cell cycle regulator Cyclin C, the Progesterone hormone Receptor and the pro-apoptotic BAX. We define the motif as EDNA (Elongation Factor Dependent Nuclear Export Adaptor) since it mediates nuclear egression of proteins by interacting in the nuclear compartment with a component of the translation apparatus, the Elongation Factor 1A (EF1A), which engages in highly dynamic nuclear-cytoplasmic trafficking. The specific interaction of EF1A with EDNA can be destabilized by the inhibition of polymerase II transcription leading to a substantial reduction in the nuclear export activity. Variants of EDNA with substitutions of the aspartic acid to glutamic acid or leucine to isoleucine retain activity and are present in countless proteins of different phyla. We suggest that EDNA directs basal nuclear export of molecules by exploiting the cellular dynamic properties of a conserved and highly abundant translation factor and links the protein synthesis and subcellular trafficking machineries.
A Mechanism for the Nuclear Export of the Transcription Factor Tbx5
A. Kulisz, T. Camarata, B. Bimber, H. Simon; Pediatrics, Northwestern University Feinberg School of Medicine, Chicago, IL

In vertebrates, the transcription factor Tbx5 is important in controlling the differentiation of the mesoderm. Mechanisms that regulate the initiation of follicular growth (follicle activation) and the ensuing growth and differentiation of preantral follicles are of considerable interest. In an effort to clone follicular development-related genes, we identified a novel gene (OoGra1) by differential display reverse-transcription PCR (DDRT-PCR) and a rapid amplification of cDNA ends (RACE) reactions. RACE reactions were performed with the SMART RACE CDNA amplification kit (Clontech) with total RNA extracted from mouse ovaries. Using Northern blot, we detected mRNA specific to OoGra1, 1.7Kb in length, in multiple tissues with predominance in mouse ovary, testis and lung. Next, we focused on OoGra1 in mouse ovaries. With Northern analyses, we found that OoGra1 was expressed in vivo at high level on day 10, with a subsequent decrease on days 15, 20 or adult testes. However, OoGra1 was undetected in ovaries of day 5. Moreover, we studied production of OoGra1 protein including a 574-residue polypeptide by using an antisem recognizing a peptide sequence unique to this gene in Western blotting and in immunolocalization. The antiserum recognized a single prominent band of approximately 66kDa in immunoblots. Subsequently, the antiserum was used by basal echinoderms.

In this study, the expression patterns of several germ line gene homologs were examined in entering the larval left coelomic pouch, the site of primordial germ cell accumulation. Due to their slow cell cycle and larval location, the small micromeres are candidate primordial germ cells.

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The Forkhead Transcription Factor FoxY: A Potential Transcriptional Regulator of Germ-Line and Oocyte-specific Genes
J. L. Song, G. M. Wessel; Molecular, Cellular Biology, and Biochemistry, Brown University, Providence, RI

The Forkhead Transcription Factor FoxY, whose RNA transcripts localized to the vasa-positive, small micromeres, are the putative primordial germ cells in the sea urchin. Using real time, qPCR and whole mount RNA in situ hybridizations, we observed the expression of FoxY to be of low abundance in eggs and then peaks in mesenchyme blastulae, specifically in the small micromeres. In these same cells, mRNA of FoxY co-localizes with mRNA of several germ-cell genes including vasa, nanos, sef, and seaf. Immunolocalization analysis of FoxY indicated that in the ovary it selectively accumulates in the germinal vesicle of oocytes. In embryos, while mRNA localization of FoxY is exquisitely specific to the small micromeres, its protein localization is in nuclei of all embryonic cells. FoxY protein in the embryo may be maternal in origin, and zygotic transcription of FoxY in the small micromeres may enhance its continuous translation. mRNA co-localization of FoxY and germ-line genes in the small micromeres and putative binding sites of FoxY within the promoter regions of these genes suggests that FoxY may be a transcriptional regulator of these germ-line determinants. We are investigating the interacting partners of FoxY by immunoprecipitation and its DNA targets by chromatin immunoprecipitation. These results will contribute to our understanding of transcriptional regulation of germ-line and oocyte-specific genes.

Molecular Cloning and Characterization of a Newly Identified OoGra1
Z. Yang, J. Wu; Biologic Engineering, Shanghai Jiao Tong University, Shanghai, China

Ovarian follicle development is a complex process. Mechanisms that regulate the initiation of follicular growth (follicle activation) and the ensuing growth and differentiation of preantral follicles are of considerable interest. In an effort to clone follicular development-related genes, we identified a novel gene (OoGra1) by differential display reverse-transcription PCR (DDRT-PCR) and a rapid amplification of cDNA ends (RACE) reactions. RACE reactions were performed with the SMART RACE CDNA amplification kit (Clontech) with total RNA extracted from mouse ovaries. Using Northern blot, we detected mRNA specific to OoGra1, 1.7Kb in length, in multiple tissues with predominance in mouse ovary, testis and lung. Next, we focused on OoGra1 in mouse ovaries. With Northern analyses, we found that OoGra1 was expressed in vivo at high level on day 10, with a subsequent decrease on days 15, 20 or adult testes. However, OoGra1 was undetected in ovaries of day 5. Moreover, we studied production of OoGra1 protein including a 574-residue polypeptide by using an antisem recognizing a peptide sequence unique to this gene in Western blotting and in immunolocalization. The antiserum recognized a single prominent band of approximately 66kDa in immunoblots. Subsequently, the antiserum was used in immunolocalization and in immunoblotting. The antiserum was recognized in a single prominent band of approximately 66kDa in immunoblots. Subsequently, the antiserum was used in immunolocalization and in immunoblotting. The antiserum was recognized in a single prominent band of approximately 66kDa in immunoblots. Subsequently, the antiserum was used in immunolocalization and in immunoblotting.
Molecular Cloning and Expression of a Novel Gene, Oocyte-G1 in Oocyte and Granulose Cells of Mouse Ovary
Y. Zhang, J. Wu, Biological Technology, Shanghai Jiao Tong University, Shanghai, China

The oocyte is recognized as a source of regulatory molecules that influence follicular development through an array of actions on granulose cells. During efforts to clone oocyte development-related genes, we isolated a cDNA fragment by differential display reverse-transcription PCR (DDRT-PCR). To obtain cDNA 5′-and 3′-end sequences, mouse ovarian cDNA library (Stratagene) was screened. The library was constructed with poly(A) + mRNA from mouse ovaries of day 15. Using both oligo(dT)-and random hexamer priming with superscript reverse transcriptase (Invitrogen), EcoRI-adapter cDNAs were ligated to AZAP Express vector arms and packaged with Gigapack III packaging extracts (Stratagene). In the end, an open reading frame of 2886bp for the novel gene (Oocyte-G1) encoding a 960-residue protein was cloned. Northern blot analysis revealed presence of the 2.9Kb Oocyte-G1 mRNA in ovary, lung, kidney and testis. In Northern analysis of RNA from ovaries in vivo, Oocyte-G1 was expressed at moderate level on day10. Thereafter, on day 15, there was an increase in expression, followed by a decline in ovaries of day 20 and adults. However, Oocyte-G1 was undetected in ovaries of day 5. Furthermore, we studied production of Oocyte-G1 protein by using an antiseraum recognizing a peptide sequence unique to this gene in Western blotting and in immunolocalization. Signal was detected in oocyte and most granulose cells. The antiseraum recognized a single prominent band of approximately 110Kda in immunoblots. Taken together, our results suggest that Oocyte-G1 play an important role in oocyte development.

MicroRNA Profiling of Ovaries during the First Wave of Folliculogenesis in Mice
O. Suzuki, M. Koura, K. Takano, Y. Noguchi, K. Uchido-Yamada, J. Matuda; Lab. Exp. Animal Models, National Institute of Biomedical Innovation, Ibaraki, Japan

Oocytes accumulate a large quantity of mRNA in order to gain in oocyte development competence, but not all of this mRNA is translated into proteins before fertilization. MicroRNAs (miRNAs) are small non-coding RNAs that have critical roles in gene suppression, similar to siRNA mechanisms, in a wide variety of organs, which suggests that miRNAs are involved in gene regulation during oocyte growth. We reported that mouse oocytes gradually acquire developmental competence during the first wave of folliculogenesis (ASCB 2003). As the first step in analyzing the involvement of miRNAs in this process, we compared miRNA profiles at the whole-ovary level between ovaries of 17- and 24-day-old females; oocytes from the latter have greater developmental competence than those from the former do. miRNAs were extracted from the ovaries of thirty 17-day-old and ten 24-day-old Slc.B6D2F1 females, labeled with fluorescent dyes, and compared using a miRNA microarray (Filgen Array miRNA384 containing triplicate spots of Ambion’s mirVana miRNA probe set; Filgen). Of the 384 miRNAs tested, 11 (miR-223, miR-1224, miR-105, miR-143, miR-106b, miR-34b, miR-319d, miR-194, miR-140, miR-154, and let-7g) were upregulated (>1.5-fold increase) and 8 (let-7b, let-7i, let-7d, miR-20b, miR-129-3p, miR-207, miR-365, and miR-183) were downregulated (>1.5-fold decrease) in the ovaries of 24-day-old females compared with those from 17-day-old females. The marked difference in the miRNA profiles of ovaries of females of different ages suggests that miRNA is involved in the progression of oocyte growth during the first wave of folliculogenesis in mice.

Phosphorylation of the Oocyte-Embryo Specific Protein PAD16 following Oocyte Maturation
A. J. Snow, S. Vijayaraghavan, D. Kline; Biological Sciences, Kent State University, Kent, OH

PAD16, a peptidylarginine deiminase, is an abundant protein in mouse oocytes, eggs, and early embryos. Members of the peptidylarginine deiminase (PAD) family are known to catalyze the calcium-dependent conversion of a peptide-bound arginine to the non-standard citrulline residue, known as citrullination. PAD1s are known to be involved in the posttranslational modification of histones by citrullination. They are also associated with intermediate filaments and may be involved in cytoskeletal reorganization. The function, specificity, and enzymatic activity of PAD16 in oocytes, eggs, and embryos remain largely undefined. We found a cell cycle-dependent change in PAD16 phosphorylation during oocyte maturation, which may provide insight into PAD16 activity and its interactions with other cellular proteins. We identified an abundant 75 kDa protein that appears to be phosphorylated at a phosphoserine 14-3-3 binding motif in mature, metaphase II eggs and early embryos but not in immature, germinal vesicle stage oocytes. Gel electrophoresis and mass spectrometric analysis of the 75kDa protein band revealed that it was likely to be PAD16. One- and two-dimensional electrophoresis and immunoblotting with an anti-PAD16 antibody confirmed this. A phosphopeptide gel stain of oocyte and egg extracts revealed a number of phosphorylated proteins including a 75 kDa protein. Indirect immunofluorescence with anti-PAD16 demonstrated the PAD16 is present in oocytes and eggs, predominately in the cortex. Immunofluorescence staining with the anti-phosphoserine 14-3-3 antibody was not detected in oocytes. Far-western analysis using a GST-tagged 14-3-3 protein indicates that PAD16 may interact with 14-3-3. The striking developmental change in the phosphorylation state of PAD16 following oocyte maturation suggest that phosphorylation and/or 14-3-3 binding may serve as a means of intracellular PAD16 regulation.

Hypermotorization-activated Cyclic Nucleotide-gated (HCN) Channel Expression in the Rat Ovary
J. Yeh, L. Gaines, B. Kim, J. Peresie, A. Arroyo; Gynecology-Obstetrics, University at Buffalo, Buffalo, NY
Ion channels have been identified in the mammalian ovary, and they have been suggested that these channels may have a role in steroidogenesis and luteolysis. Hypermotorization-activated cyclic nucleotide-gated (HCN) channels are proteins that regulate electrical activity in the heart and brain. HCN channel actions are modulated by AMP. Whether HCN channels are present in the ovary and have a role in ovarian function are unknown. The purpose of this study was to analyze HCN2 channel expression and localization in the rat ovary. We used RT-PCR, western blot, and immunohistochemistry to study adult rat ovaries. Messenger RNA expression was found for HCN2 in ovarian tissue using RT-PCR. Western blot analysis showed protein expression of HCN2 in ovarian tissue lysates. Immunohistochemistry showed intense immunostaining for HCN2 in the granulosa cells, theca cells and oocytes of ovarian follicles and in corpora lutea. In summary, we found gene expression, protein expression, and follicular and luteal localization of HCN2 in the rat ovary. Based on these data, we hypothesize that HCN2 may be involved in ovarian follicular and/or luteal function.

Oocyte Signals Derived from Polyunsaturated Fatty Acids Control Sperm Recruitment
H. M. Kabagaya, 1 J. Watts, 2 C. Corrigan, 1 J. W. Edmonds, 1 E. Sztul, 1 J. Browse, 2 M. A. Miller 1; 1Cell Biology, University of Alabama at Birmingham, Birmingham, AL, 2Biological Chemistry, Washington State University, Pullman, WA

A fundamental question in animal development is how do motile cells find their correct target destinations. During mating in the nematode Caenorhabditis elegans, males inject sperm through the hermaphrodite vulva into the uterus. Amoeboid sperm crawl around fertilized eggs to the spermatheca, a convoluted tube where fertilization occurs. We show that polyunsaturated fatty acids (PUFAs), the precursors of oecosin signaling molecules, are necessary and sufficient in oocytes to control directional sperm motility within the uterus. PUFAs are transported from the intestine, the site of fat metabolism, to oocytes in yolk, a lipoprotein complex. Loss of the RME-2 low-density lipoprotein receptor, which mediates yolk endocytosis and fatty acid transport into oocytes, causes severe defects in sperm targeting. We used an RNA screen to identify genes required in oocytes for directional sperm motility. Ten genes encode lipid regulators implicated in the synthesis and transport of sperm-targeting signals. Our results support the hypothesis that PUFAs function in oocytes as precursors of signals that control sperm recruitment to the spermatheca. During mammalian immune responses, the eicosanoids prostaglandin D2 and Leukotriene B4 are rapidly generated at sites of inflammation and are required for eosinophil and T cell recruitment, likely by acting as chemoattractants. Thus, a common property of PUFAs in mammals and C. elegans is that these fats mediate local recruitment of motile cells to their target tissues.

Mechanochemical Signal Transduction: Potential Mechanism for Induction of Sperm Acrosome Exocytosis
B. Baibakov1, L. Gauthier1, T. L. Rankin2, P. Talbot2, J. Dean2; LCBH, NIDDK/NHLBI, Bethesda, MD, 1Department of Cell Biology and Neuroscience, University of California, Riverside, CA

The induction of sperm acrosome exocytosis in mouse fertilization has long engaged investigitative interest, but remains incompletely understood. At fertilization, spermatozoid bind to the zona pellucida (ZP), and undergo the acrosome reaction during initial gamete interactions. Following fertilization, ZP is proteolytically cleaved and sperm no longer bind to embryos. We assessed Acet-EGFP sperm binding to normal and huZP2 rescue eggs in which huZP2 replaces mouse ZP2, but remains uncleaved. Surprisingly, mouse sperm remain acrosome-intact for 2-3 hours after binding normal and up to 24 hours when bound to huZP2 rescue embryos, regardless of whether sperm are added before or after fertilization. The persistence of an intact acrosome indicates that sperm binding to the zona pellucida is not sufficient to induce acrosome exocytosis. A filter penetration assay suggests an alternative mechanism in which penetration into the zona matrix initiates a mechanochemical signal transduction, necessary to activate the acrosome reaction rather than signaling pathways involving zona-ligand binding to a sperm receptor.
The Spermatid Acroplaxome, Which Overlaps a mRNA Transcriptionally Silent Nuclear Domain, Contains Fer Tyrosine Kinase and Phosphorylated Cortactin

A. L. Kierszenbaum, E. Rivkin, L. L. Tres; Cell Biology & Anatomical Sciences, CUNY Medical School, New York, NY

The acroplaxome, a cytoskeletal plate with a desmosome-like marginal ring, anchors the acrosome to the spermatid nuclear envelope-nuclear lamina complex. F-actin and keratin 5 are two major cytoskeletal components of the acroplaxome. Three functions have been ascribed to the acroplaxome: (1) tethering of Golgi-derived proacrosomal vesicles transported by microtubule- and F-actin based molecular motors; (2) modulation of exogenous contractile forces generated by Sertoli cell ectoplasmic F-actin during spermiad head shaping; and (3) a positional stabilizer of the perinuclear microtubular manchette. Acroplaxome-related nuclear shape abnormalities occur in acrosomless rodent and human infertile sperm displaying globozoospermia. Studies were carried out to determine the dynamics of F-actin, and RNA transcription activity in the juxta-acroplaxome nuclear area. Testis Fer tyrosine kinase and phosphorylated cortactin were immunodetected in the acroplaxome during spermiad head elongation (steps 8 to 12 of spermiogenesis). Cortactin phosphorylation, known to be mediated by the Fer/Fyn tyrosine kinase pathway, has a role in F-actin depolymerization. Unphosphorylated cortactin was seen in the acroplaxome and manchette of elongating spermiads. Heterogeneous nuclear RNA (hnRNA) is not present in the juxta-acroplaxome nuclear region but is visualized in the rest of the nucleus of round spermiads. We postulate that cortactin tyrosine phosphorylation may modulate the cytoskeletal dynamics of F-actin in the acroplaxome in response to extracellular stimuli, including acrosome and chromatin in cell volume, two events associated with spermiogenesis. Anchorage of the acroplaxome to the subjacent spermatid nuclear envelope-nuclear lamina complex correlates with transcriptionally hnRNA silencing, presumably representing the initiation of regional chromatin remodeling during spermiad head shaping.

Orphan Neurotransmitter Transporter Required for Drosophila Spermiogenesis

N. Chatterjee, J. Rollins, C. W. Bazinet; Biological Sciences, St. John's University, Queens, NY

Neurotransmitter transporters play an essential role in synaptic transmission. Variation in transporter structure, distribution, and substrate play a large role in differentiating the specialized functions of the nervous system. A Drosophila gene, neurotransmitter transporter-like (ntl; CG7075) encodes a predicted protein that is a member of the well-known class of neurotransmitter sodium symporters with 12 transmembrane domains. Mutants have been isolated in this gene by mobilization of a nearby P transposable element. Males homozygous for disruptions in the ntl gene are sterile, but no other phenotypic effects of the mutations are apparent. Sexual dimorphism of sodium symporter family in spermiogenesis. Electron microscopy confirms this phenotype. Continuing studies are aimed at further understanding the unexpected role of an otherwise highly-conserved member of the neurotransmitter

Detection of DNA Damage Induced by MTT Using Alkaline Comet Assay in Porcine Spermatozoa

D. Han, S. Cho, Y. Kim, Y. Lee, J. Kim, S. Kim, J. Jang, S. Park, D. Choi, H. Ko, Y. Hwang, D. Kim; Biological Sciences, Gachon University of Medicine & Science, Incheon, Republic of Korea

Intracytoplasmic sperm injection (ICSI) is considered as an effective method for treatment with male factor infertility to conceive. Viable sperm has more fertilization rate than nonviable sperm in ICSI. The selection of viable sperm is important and it can be used for ICSI, if sperm DNA is not damaged. MTT (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) reduction assay is commonly used for evaluating sperm viability because it is simple and inexpensive. Yellow tetrazolium salt of MTT is converted to purple formazan by dehydrogenase in sperm mid-piece mitochondria. The decrease in percentage of MTT spermatozoa was detected after 2h added MTT because the formazan could be toxic. Comet assay can observe DNA damage in sperm. Fresh semen samples were diluted to 1 x 10^5 sperm/ml in PBS. MTT(5mg/ml of PBS) was added and incubated in 2h at 37 °C. Control group was not treated with same amount of PBS. Positive control group is treated with hydrogen peroxide. Every group was embedded in agarose gel on slide, lysed, and electrophoresed in alkaline phosphate. Slides were stained with Hoechst 3342 to detect sperm DNA damage by using fluorescence microscopy at absorption maximum 350nm and emission maximum 461nm. All procedures are progressed in blockage of the UV light to avoid extra sperm damage. The formazan produced by MTT reduction assay is not severely affected to sperm DNA. In conclusion, sperm MTT viability assay could be used as a reliable and appropriate method to select viable sperm for ICSI. This work was supported by the Research Project on the Production of Bio-organs ( No. 200508020701 ) Ministry of Agriculture and Forestry, Republic of Korea.

The C. elegans Germinal Center Kinase GCK-1 Is a P-Body Component That Inhibits Precocious Map Kinase Dependent Meiotic Maturation

K. R. Schouest, T. Furuta, N. Hisamoto, K. Matsumoto, J. M. Schumacher; Molecular Genetics, University of Texas MD Anderson Cancer Center, Houston, TX, 1Department of Molecular Biology, Nanyang University for Advanced Research, Nagona, Japan

P-bodies are enigmatic cytoplasmic RNA-protein (RNP) particles that have been implicated in RNA degradation, translational control, and RNA-mediated interference. In metazoans, evolutionarily conserved components of germ line P-bodies have recently been shown to be required for germ cell survival and the production of functional oocytes. In many species, oocyte development and maturation is also controlled by a highly conserved MAP kinase signaling cascade. Deciphering how these two critical regulatory pathways intersect is key to understanding evolutionarily conserved components of germ line P-bodies.

Expression and Localization of Plasma Membrane Ca2+-ATPase Isoform 4 in Bovine Testis and Epididymis

K. R. Schouest, 1 T. Furuta, 1 N. Hisamoto, 2 K. Matsumoto, 2 J. M. Schumacher 1; 1Molecular Genetics, University of Texas MD Anderson Cancer Center, Houston, TX, 2Department of Molecular Biology, Nanyang University for Advanced Research, Nagona, Japan

Capacitation, motility and acrosomal reaction are essential functions of the spermatozoon in the process of fertilization. Several steps of these functions are triggered by calcium dependent pathways, requiring pumping systems to remove excess intracellular calcium. It is known that the plasma membrane Ca2+-ATPase (PMCA) performs the major task of Ca2+-clearance in mouse spermatozoa. Four different isoforms (PMCA 1-4) have been identified and multiple splice variants of these isoforms have been described. Especially PMCA 4 has been reported to be important for male fertility. We therefore examined the protein- and gene expression of PMCA 4 in bovine testis and epididymis. In situ-hybridization was performed to analyse the distribution pattern of the gene expression. PMCA 4 expression was restricted to spermatogonia and epithelial cells of the epididymis (head, body, tail). Performing immunohistochemistry we detected the protein in the plasma membrane of spermatocytes and spermatids in bull testis as well as in the basal layer of the epididymal epithelium. Using RT-PCR we could show a splice variant switch of PMCA 4 from the epididymal head/body to the epididymal tail. Generating an antibody against the PMCA 4 splice variant, we detected this splice variant exclusively in the apical plasma membrane of epithelial cells of the epididymal tail. Presently, we are analysing the distribution pattern of the PMCA 4 in bull epididymal spermatozoa. We have recently shown that bovine sperm PMCA is stimulated by PDC-109, a secretory protein from bovine seminal vesicles. Further studies have to elucidate, whether or not PMCA 4 is the isoform which is stimulated by PDC-109.

Ca2+-ATPases Are Required for Motility and the Acrosome Reaction of Sea Urchin Spermatozoa

H. J. Gunaratne, V. D. Vacquier; Marine Biotechnology and Biomedicine, Scripps Institution of Oceanography, La Jolla, CA
Three Ca\(^{2+}\)-ATPases regulate intracellular Ca\(^{2+}\) concentrations in animal cells: plasma membrane Ca\(^{2+}\)-ATPase (PMCA), sarco-endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) and secretory pathway Ca\(^{2+}\)-ATPase (SPCA). There are few studies of Ca\(^{2+}\)-ATPases in spermatozoa, which is surprising considering the importance of these enzymes in all cells. Here we describe the expression, protein and gene features, localization, and importance of Ca\(^{2+}\)-ATPases in sea urchin spermatozoa. Sea urchin (Strongylocentrotus purpuratus) spermatozoa express PMCA and SPCA, but not SERCA. Sp-PMCA is 1,154 amino acids and has 30% identity and 50% similarity to all four human PMCA. Sp-SPCA shares the fastest of typical PMCA, including domains for calmodulin binding, ATP binding, Sp-SPCA phosphorylation, and 10 putative transmembrane segments with two large cytoplasmic loops. Sp-SPCA is 912 amino acids and has 66% identity and 80% similarity to human SPCA1. As is common for a typical SPCA, Sp-SPCA has ATP binding and ATPase phosphorylation sites, 10 transmembrane helices and conserved Ca\(^{2+}\) binding residues. Southern blots and genome analysis show that both are single copy genes. Further genome annotation reveals that Sp-PMCA and Sp-SPCA have 17 and 23 exons. Immunofluorescence and immunoblotting show that PMCA localizes to the sperm head, whereas SPCA localizes to the mitochondrion. Treatment of live sea urchin sperm with the PMCA inhibitor, 5-(4-carboxyphenyl)-N-endoethylcarboxamido-1-2/(4-nitrophenyl)benzamide (pH 7.2) inhibits both PMCA and SPCA blocks the sperm acrosome reaction. Ca\(^{2+}\) measurement using the above inhibitors show that 75% of Ca\(^{2+}\) is expelled by PMCA and SPCA and 25% by Na\(^{+}\) dependent Ca\(^{2+}\) exchangers. These findings indicate that PMCA and SPCA regulate Ca\(^{2+}\) signals required for successful fertilization in sea urchins.

496 Adenosine- and Catecholamine-signaled Activation of Sperm Motility Does Not Involve Standard Receptors

S. M. Schuh, B. Hille, D. F. Babcock; Physiology and Biophysics, University of Washington, Seattle, WA Various extracellular signals may alter the motility of mammalian sperm as they progress through the female reproductive tract. Our past work used stop-motion imaging and waveform analysis to show that sperm motility is increased by adenine nucleotides and catecholamines, two classes of small molecules found in mammalian reproductive fluids. We also showed that acceleration of the flagellar beat by 2-chloro-2'-deoxyadenosine (Cl-dAdo) or isoproterenol (ISO) requires extracellular Ca\(^{2+}\), the atypical sperm adenylyl cyclase, cAMP, and protein kinase A. We now find several known agonists of the adenosine and β-adrenergic receptors of somatic cells also speed the flagellar beat of mouse sperm, suggesting that sperm may possess similar receptors. However, the potent adenosine receptor antagonist DPCPX did not block the action of Cl-dAdo on sperm. Also unexpectedly, the β-adrenergic antagonists propranolol, metoprolol, atenolol, and butoxamine acted as accelerating agonists in sperm. Furthermore, the flagellar beat was increased by both the β- and α-isomers of the catecholamines ISO, noradrenaline, and epinephrine, whereas α-isomers have much greater potency for typical adrenergic receptors. These results are inconsistent with involvement of standard G protein-coupled receptors that mediate adenosine and adrenergic actions in somatic cells. In support of the alternative hypothesis that these agonists may act at an intracellular site, we find that the accelerating actions of Cl-dAdo and 2'-deoxyadenosine are slowed more than 5-fold by the potent nucleoside transport inhibitors, diprydamole and nitrobenzylxanthine. Hence the action of adenosine nucleosides on sperm motility may require their transport into the cell. Further study of the underlying signaling pathways may reveal how sperm respond to extracellular signals received enroute to the egg.

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497 Role of PDZ Domain-containing Proteins in Sperm-Oviduct Interaction

H. Peng,1 C. Yeh,2 C. Chiuchang,2 H. Ho1; 1Life Science, Tzu Chi University, Hualien, Taiwan, 2Anatomy, Tzu Chi University, Hualien, Taiwan, 3Institute of Integrative Physiology and Clinical Sciences, Tzu Chi University, Hualien, Taiwan Recent studies showed that PDZ (PSD-95/Discs-large/ZO-1) domains are ubiquitous protein interaction modules that play an important role in cellular signaling, adhesion, transport, etc. We found that in mated female mice, a putative PDZ domain-containing protein (accession reference: AK012129.1) was highly expressed in the oviduct using a cDNA microarray assay. We further examined the protein profiles among oviducts collected at different time points after coitus and in control. In western blot using mouse anti-PDZ antibody as primary antibody, two PDZ domain-containing proteins were clearly detected. Both 25 kDa and 50 kDa proteins showed increased expression level as ovulation approached and number of hyperactivated sperm increased. Besides, using overacclimated mouse model, we found that expression level of PDZ domain-containing proteins and number of sperm found in the oviduct decreased in ovariecotomized oviduct; interestingly, ovariecotomized proteins might play a role in sperm expression as well as serving as the receptors or modulators. Some of the PDZ domain-containing proteins have been recognized to regulate transmembrane proteins. It is possible that sperm protein recognition molecules were regulated by PDZ domain-containing proteins and led to release of sperm from the oviductal epithelium around the time of ovulation.

498 Annexins Are Candidates for the Oviductal Sperm Receptors That Hold Bull Sperm in a Storage Reservoir

G. G. Ignotz, M. Y. Cho, S. S. Suarez; Biomedical Sciences, Cornell University, Ithaca, NY Sperm of eutherian mammals are held in a storage reservoir in the posterior segment of the oviduct by binding to the mucosal epithelium. In the bovine, we determined that seminal vesicle secretory proteins in the BSP family, namely PDC-109 (BSP A1/A2), BSP-A3, and BSP-30-kDa, coat the sperm head and enable sperm to bind to the epithelium. Our subsequent objective was to identify the receptors for sperm on the oviductal epithelium. Antibodies to each of the BSPs were covalently linked to agarose beads. Each BSP was purified and bound to the beads coated with the corresponding antibody. Proteins extracted from apical membranes of bovine oviducal epithelium were passed over columns of each BSP protein bound to its corresponding antibody. Unbound proteins were washed from the beads and bound proteins were eluted using EGTA, because sperm binding to oviductal epithelium is dependent on calcium. The eluates were resolved on SDS PAGE gels. Protein bands of 34 and 38 kDa were the sole prominent bands detected by silver staining in the eluates from each of the three columns. Protein constituents of the bands were identified by tandem mass spectrometry of tryptic digests to be annexins (ANXA) 1,2,4, and 5. Subsequently, commercial antibodies to ANXA1, 2, and 4 were used to confirm their identity. In the eluates from an antibody to ANXA1, M. Y. Cho, S. S. Suarez; Biomedical Sciences, Cornell University, Ithaca, NY and 50 kDa proteins showed increased expression level as ovulation approached and number of hyperactivated sperm increased. Beside, using overacclimated mouse model, we found that expression level of PDZ domain-containing proteins and number of sperm found in the oviduct decreased in ovariecotomized oviduct; interestingly, ovariecotomized proteins might play a role in sperm expression as well as serving as the receptors or modulators. Some of the PDZ domain-containing proteins have been recognized to regulate transmembrane proteins. It is possible that sperm protein recognition molecules were regulated by PDZ domain-containing proteins and led to release of sperm from the oviductal epithelium around the time of ovulation.

499 Altered Wnt Signaling in the Megf7 Deficient Mouse Results in the Expansion of the Apical Ectodermal Ridge and Polydactyly

E. B. Johnson,1 R. E. Hammer,1 J. Herz1; 1Molecular Genetics, UT Southwestern Medical Center, Dallas, TX, 2Biochemistry, UT Southwestern Medical Center, Dallas, TX Multiple EGF-like domains-7 (Megf7/Lrp4) is a member of the low-density lipoprotein receptor gene family, a class of ancient and highly conserved cell surface receptors with broad functions in cargo transport and cellular signaling. To gain insight into the as yet unknown biological role of Megf7, we have disrupted the gene in mice. The Megf7 deficient mouse has polydactyly, which is characterized by the multiplication and fusion of digits in both the forelimbs and hind limbs. During limb development, Megf7 is expressed in the ventral ectoderm as well as the Apical Ectodermal Ridge (AER). The AER is expanded along the ventral and dorsal aspect of the limb in the Megf7 deficient embryo. In contrast, the AER in wild type embryos is restricted to a thin band of pseudostratified epithelium at the distal edge of the developing limb. The expansion of the AER in the Megf7 deficient limbs results in the disruption of the expression pattern of many genes that are required for limb development such as Bmps, Shh, Fgts, Wnts and Lmx1b. The Megf7 deficient phenotype is very similar to that of the double mutant mutant mice (Adamska et al. 2003) suggesting that Megf7 is involved in the Wnt signaling pathway. The hypothesis of Megf7 as a Wnt modulator is supported by the fact that Megf7 inhibits the Wnt1 induced stabilization of β-catenin in a dose dependent manner in the TOPflash reporter system, a reporter system that measures the activity of the canonical Wnt signaling pathway. In conclusion, the inhibition of the Wnt pathway by Megf7 is required for patterning during limb development. This work significantly contributes to our understanding of the regulation of limb development, provides a new mechanism for the modulation of Wnt signaling, and adds to our understanding of the many important functions of LDL receptor gene family.

500 A Putative Transcriptional Factor, Seson Establishes Left-Right Asymmetry in Zebrafish Embryo via Nodal Signaling Pathway

Y. Park,1 C. Lee,2 M. Rhee1; 1Biology, Chungnam National University, Daejeon, Republic of Korea, 2Biological Sciences, Inha University, Incheon, Republic of Korea We are reporting here that a novel C2H2 zinc finger protein, Seson is a critical element for left-right asymmetry establishment in the vertebrate embryo. It is a critical element of Left-Right asymmetry establishing mechanism in the zebrafish embryo. Seson is localized in the cytoplasm, but translocated to the nucleus in response to TGFB and TNF, suggesting that it might be involved in the intracellular signaling pathway. Second, Seson transcripts are highly expressed in the whole blastomes at early embryonic stages, but restricted in the head region and the anterior midline after 24 hpf, and in the anterior midline including otic vesicle, particularly observed as a symmetrical oblique line in the midbrain at 48 hpf. Third, functional analysis using overexpression and knock-down experiment found that Seson is critical to the establishment of left-right asymmetry in brain and cardiac development during zebrafish embryogenesis. Analysis of expression
pattern of various molecular marker genes involved in left-right asymmetry found that fluctuation of sexon expression gives rise to significant changes in the expression patterns of the genes. In particular, sexon is essential for formation of dorsal forerunner cell as well as expression of lerdr in the Kappler’s vesicle (KV). Finally, knock-down of sexon significantly reduces expression of sqt at the marginal cells. Considering that Sqt is a ligand of Nodal, a critical morphogen for KV, it appears that Sexon functions as a modulator of sqt-mediated Nodal signaling pathway to establish left-right asymmetry in the zebrafish embryo.

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MIP-1α Increased Osteoclastogenesis and Survival of Osteoclast
H. Shin, J. Lee, V. Pham, M. Park, B. Kwon, H. Choi; Department of Biological Sciences and the Immunomodulation Research Center, University of Ulsan, Ulsan, Republic of Korea

After receptor activator of nuclear factor-kappaB ligand (RANKL) binding, increased osteoclast formation was observed by bone marrow macrophages (BMM) from BALB/c strain, comparing with that from C57BL/6 mice. Expression of MIP-1α mRNA and protein level was much higher in BMM stimulated by RANKL from BALB/c mice than those of C57BL/6 mice, suggesting a possible role of MIP-1α in enhanced osteoclast formation. Transcripts of CCR1 and CCR5, MIP-1α receptors, were detectable in un-stimulated BMM cells of both at similar level. Blockade of MIP-1α inhibited osteoclast formation when the BMM from BALB/c were incubated with RANKL, suggesting that MIP-1α plays a role as a positive regulator of osteoclastogenesis. MIP-1α enhanced osteoclast formation not only at early stage, but also at late stage, preventing loss of in vitro osteoclast formation through induction of apoptosis. The prolongation of survival of mature osteoclast by MIP-1α was via NF-kappaB activation. The survival activity of RANKL was dependent on its ability to induce MIP-1α.

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IL-20 Promotes Angiogenic Processes in PAE Endothelial Cells
K. Tritsaris, 1 M. Myren, 1 M. V. Hulshmans, 1 S. Ditte1, A. J. Hansen, 2 S. Dissing; 1 Department of Medical Physiology, University of Copenhagen, Copenhagen, Denmark, 2Cancer & Immunobiology, NOVO Nordisk A/S, Måløv, Denmark

Previous findings have suggested that IL-20 is implicated in psoriasis. The fact that angiogenesis and hypervascularization are characteristic features of the psoriatic plaque which is observed in IL-20 transgenic mice, prompted us to investigate whether IL-20 has a potential role in the angiogenic process. We employed pig aorta endothelial (PAE) cells and found mRNA for both IL-20Rα and β as well as IL-22R mRNA. Cell signalling studies revealed that the JAK/STAT pathway becomes activated (STAT5 and Jak2 phosphorylation) after IL-20 stimulation (5 min) and furthermore that IL-20 activates the MAP kinase pathway (Erk1/2 phosphorylation) and the PI3K/Akt pathway. In addition, IL-20 activation causes a rapid and transient increase in the intracellular, free Ca²⁺ concentration ([Ca²⁺]i). This rise in [Ca²⁺]i was due to Ca²⁺ mobilization from intracellular stores and was completely inhibited by 5 µM SU73122 - an inhibitor of the Ca²⁺-induced Ca²⁺ release (CICR) channels. Furthermore that IL-20 activates the MAP kinase pathway (Erk1/2 phosphorylation) and the PI3K/Akt pathway. In conclusion, endothelial cells possess IL-20 receptors and IL-20 activates cell signaling that support angiogenic processes and causes endothelial cell apoptosis.

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Increased Phosphorylation and Transactivation Properties of IPF1/PDX1 by HIPK2-dependent Mechanisms
M. Boucher, M. Edlund; UCMC (Umeå Center for Molecular Medicine), University of Umeå, Umeå, Sweden

The transcription factor IPF1/PDX1 plays a crucial role in both pancreas development and maintenance of beta-cell function. Indeed, homozygous disruption of the ipf1/pdx1 gene results in pancreas agenesis in both mice and human. Moreover, targeted disruption of ipf1/pdx1 gene in beta-cells leads to diabetes, whereas reduced expression levels affect insulin secretion and glucose homeostasis. In differentiated beta-cells, IPF1/PDX1 has been shown to bind and regulate the transcription of several genes involved in glucose sensing and insulin synthesis (plin2, glucokinase, insulin) underlying his key role in glucose homeostasis. Nevertheless, despite this clearly established role in pancreas development and beta-cell function, the mechanisms involved in IPF1/PDX1 protein expression and function remain to be clarified. METHODS AND RESULTS. 1- Transparent transfection of the newly identified cofactor homeodomain-interacting protein kinase-2 (HIPK2 WT) increased, in a dose-dependent manner, the phosphorylation and the protein expression level of IPF1/PDX1, whereas the kinase-dead HIPK2 (K221R) had no effect. 2- Luciferase assays (using either the IPF1/PDX1 minimal binding site or the insulin promoter) demonstrated that HIPK2 WT, and not the kinase-dead HIPK2 K221R, dose-dependently increased IPF1/PDX1 transactivation properties. 3- Immunofluorescence studies showed a co-localization of HIPK2 and IPF1/PDX1 in the nucleus of co-transfected cells. 4- More importantly, in situ experiments revealed that hipk2 mRNA is detected in IPF1/PDX1-expressing cells during mouse pancreas development i.e. hipk2 is detected in pancreatic progenitor cells early during pancreas development and, as the epithelial pancreatic cells differentiate, hipk2 expression become restricted to beta-cells. CONCLUSION. These results identify HIPK2 as a new IPF1/PDX1 regulator and suggest that the kinase activity of this newly identified cofactor could modulate IPF1/PDX1 function during pancreas development and, later on, in differentiated beta-cells.

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JSAPI Regulates Proliferation of Granule Precursor Cells in the Developing Mouse Cerebellum
T. Sato, 1 T. Tomishima, H. Hira, M. Asano, K. Yoshio; 1Division of Molecular Cell Signaling, Cancer Research Institute, Kanazawa University, Kanazawa, Japan, 2Department of Cellular Neurophysiology, Graduate School of Medical Science, Kanazawa University, Kanazawa, Japan, 3Division of Transgenic Animal Science, Advanced Science Research Center, Kanazawa University, Kanazawa, Japan

MAP kinase (MAPK) intracellular signaling system employs scaffold proteins, in part, to organize the MAPK signaling components into functional modules, thereby enabling the efficient activation of specific MAPK pathways. The c-Jun N-terminal kinase (JNK)/stress-activated protein kinase (JNK) becomes involved in glucose sensing and insulin synthesis (plin2, glucokinase, insulin) underlying his key role in glucose homeostasis. Nevertheless, despite this clearly established role in pancreas development and beta-cell function, the mechanisms involved in IPF1/PDX1 protein expression and function remain to be clarified. METHODS AND RESULTS. 1- Transparent transfection of the newly identified cofactor homeodomain-interacting protein kinase-2 (HIPK2 WT) increased, in a dose-dependent manner, the phosphorylation and the protein expression level of IPF1/PDX1, whereas the kinase-dead HIPK2 (K221R) had no effect. 2- Luciferase assays (using either the IPF1/PDX1 minimal binding site or the insulin promoter) demonstrated that HIPK2 WT, and not the kinase-dead HIPK2 K221R, dose-dependently increased IPF1/PDX1 transactivation properties. 3- Immunofluorescence studies showed a co-localization of HIPK2 and IPF1/PDX1 in the nucleus of co-transfected cells. 4- More importantly, in situ experiments revealed that hipk2 mRNA is detected in IPF1/PDX1-expressing cells during mouse pancreas development i.e. hipk2 is detected in pancreatic progenitor cells early during pancreas development and, as the epithelial pancreatic cells differentiate, hipk2 expression become restricted to beta-cells. CONCLUSION. These results identify HIPK2 as a new IPF1/PDX1 regulator and suggest that the kinase activity of this newly identified cofactor could modulate IPF1/PDX1 function during pancreas development and, later on, in differentiated beta-cells.

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Roles of Src Family Kinases in the Development of Neural Crest Cell Lineages
A. Takatsuka, 1 M. Koike, 2 Y. Uchiyama, K. Nishida, S. Nada, C. Oneyama, M. Okada; 1Oncogene Research, RMD, Osaka University, Osaka, Japan, 2Cell Biology and Neurosciences, Graduate School of Medicine, Osaka University, Osaka, Japan, 3Ophthalmology and Visual Science, Graduate School of Medicine, Tohoku University, Miyagi, Japan

Src family kinases (SKs) are non-receptor tyrosine kinases that play pivotal roles in regulating cellular functions, including cell adhesion and migration. The activity of SKs is negatively regulated by Carboxy-terminal Src Kinase (Csk). The analysis of Csk knockout mice has shown that SKs play critical roles under the control of Csk in normal animal development. However, the precise in vivo functions of SKs/Csk complex remain to be clarified. To address this issue, we generated mutant mice in which Csk was conditionally inactivated in the neural crest lineage by Cre-loxP system using a Protein Zero (P0) promoter. The mutant mice exhibited facial dysplasia so-called “short nose” and prominent defects in the anterior ocular segment. In the cerebellum, the mutant mouse showed severe opacity, extensive vasculization and anterior synchie. Electron microscopic analysis revealed the deletion of conral endothelium as well as its basement membrane in the central part of mutant cerebellum and the abnormal arrangement of collagen fibrils in the stroma. The mutant mice showed a constitutive activation of SKs accompanied by elevated tyrosine phosphorylations of several cellular proteins, although there was substantial degradation of activated SKs. These mutant cells also exhibited hypomotility and morphological changes compared with control cell lines. RT-PCR and zymography analyses revealed that molecules involved in collagen fibril construction, including procollagen1a1, Adamts2 and MMP2, were up-regulated in the mutant cells. These results suggest that the SKs/Csk system plays crucial roles in pancreas development through regulating the organization of extracellular matrices.
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**Fibroblast Cells from Young and Aged Long-lived Ames Dwarf Mice Exhibit Resistance to ROS Generated by Mitochondrial Dysfunction**

C. Hsieh, J. Papapantoniou; Biochemistry & Molecular Biology, University of Texas Medical Branch, Galveston, TX

The Free radical theory of aging proposes that oxidative stress (ROS) generated by mitochondrial dysfunction plays a major role in determination of rates of aging and longevity. This is supported by observations that resistance to ROS is a characteristic of experimental models of longevity, e.g., *C. elegans*, *Drosophila* and rodents. Previously we demonstrated that activation of the p38 MAPK stress response pathway by mitochondrial generated ROS is linked to the dissociation of an inhibitory threonine (Trx)-ASK1 complex. Thus, the ratio of (Trx)-ASK1 : free ASK1 regulates the level of p38 MAPK activity. Furthermore, the (Trx)-ASK1 pool level is significantly higher in both young and aged long-lived Snell dwarf mice which explains their decreased levels of endogenous ASK1 and downstream p38 MAPK activities. We propose that these are characteristics indicative of resistance to oxidative stress and decreased levels of endogenous ROS production. In this study we investigated whether dermal fibroblasts from long-lived Ames dwarf mice exhibit resistance to ROS generated by mitochondrial dysfunction. Our data indicate that the dwarf fibroblasts exhibit: (1) resistance to rotenone (Rot)-mediated induction of p38 MAPK activity. In fact, higher doses of this electron transport chain inhibitor of Complex I were required to activate the dwarf fibroblast p38 MAPK activity to the level that occurs in the wild-type cells. (2) upon each transfer the dwarf fibroblasts exhibit an initial lag period of growth followed by a slower growth rate. Our results suggest that Ames dwarf fibroblasts are resistant to the ROS generated by dysfunction of electron transport chain Complex I.

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**Role and Control of Patched Traffic in Hedgehog Signaling during Fly Development**

A. Brigui, A. Pleus; Genetics of Development and Evolution, Institut Jacques-Monod, Paris, France

Hedgehog (HH) signaling plays key roles during development of many animals and its mis-regulation is associated with numerous cancers. The precise events governing the reception and transduction of the HH signal remain elusive, but links between HH signaling and vesicular traffic have recently emerged. HH action on its target cells involves: the 12 transmembranes receptor Patched (PTC) and the transduce related protein Smoothened (SMO). SMO is associated with an intracellular signaling complex which modulates the activity of the transcription factors GLI1/Cubitus interruptus. In absence of HH, PTC down-regulates the pathway by inhibiting SMO. SMO is endocytosed and targeted to the lysosome. After reception of HH, PTC inhibition is relieved, PTC bounded to HH is endocytosed and degraded in the lysosome. SMO is then stabilized at the plasma membrane which is sufficient to activate the pathway. We use the fly *Drosophila melanogaster* to study the role and the control of PTC traffic in HH signalling. We are characterizing PTC traffic (with and without HH) by colocalisation with vesicular markers. Moreover, we have isolated new physical interactions between PTC and different proteins that could regulate its traffic, including an HECT ubiquitin ligase of the Rsp5/NEDD4 family which is known to regulate the stability, the activity and the localization of their target proteins. All the PTCs, SMO and Patched interact with multiple proteins involved in vesicular transport and the ubiquitin/proteasome machinery (Rsp5, NEDD4, Skp1, Smurf1...). We propose that these interactions could control the activity of the PTC traffic and could be a strategy to regulate the activity of the HH pathway.

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**The Role of Drosophila Auxillin in Notch Ligand Endocytosis**

H. C. Chang, T. Bai, J. Bayaraktar, E. Hagedorn, V. Kandachar; Biological Sciences, Purdue University, West Lafayette, IN

The Notch cascade, a highly conserved signaling module, plays a central role in diverse aspects of metazoan development. Mutations disrupting components of this pathway can cause severe tissue malformations and cancers in mammals. As the receptors and ligands are both widely expressed, the activity of this signaling cascade needs to be tightly regulated. Recent data from many groups have suggested that ligand endocytosis is critical for the activation of the Notch cascade, although the mechanism of how the internalization of Notch ligands leads to receptor activation remains poorly understood. We have isolated several mutations in the Drosophila homolog of auxillin, a J-domain-containing protein known to cooperate with Hsc70 in the disassembly of clathrin coats from clathrin-coated vesicles in vitro. Consistent with this biochemical role, animals with reduced auxillin function exhibit genetic interactions with Hsc70 and clathrin.

Interestingly, the auxillin mutations interact specifically with Notch and disrupt several Notch-mediated processes. Experimental analysis suggests that the J-domain, N-terminal kinase domain, and the C-terminal clathrin binding domain are all required for Notch signaling. Genetic evidence places auxillin function in the signal-sending cells, upstream of Notch receptor activation, suggesting that the relevant cargo for this auxillin-mediated endocytosis is the Notch ligand, Delta. Indeed, the localization of Delta protein is disrupted in auxillin mutant tissues. Thus, our data suggest that auxillin is required for Notch signaling and that ligand endocytosis in the signal-sending cells needs to proceed past coat disassembly to activate Notch.

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**A Functional Role of ADAM 10 on Chondro-inhibitory Action of TGFβ3 in Chick Leg Bud Mesenchymal Cells**

E. Jin, K. Park, Y. Yoo, Y. Choi, S. Kang; Biological Sciences, Kyungpook National University, Daegu, Republic of Korea

Among the numerous factors involved in the regulation of chondrogenesis, transforming growth factor β (TGF-β) family has been implicated in controlling cartilage development. However, the regulatory mechanism of TGF-β3 during chondrogenesis of primary mesenchymal cells remains to be characterized in detail. Using differential display polymerase chain reaction screening, we identified a novel metalloproteinase (ADAM 10) which resulted in downregulation of ADAM 10 and a concomitant activation of Notch signaling. Electrotransporation of ADAM 10 morpholino antisense oligonucleotides suppressed precartilage condensation via downregulation of proliferation and subsequent chondrogenic differentiation as observed when chondrogenic-competent cells were exposed to TGF-β3. Downregulation of ADAM 10 inhibited ectodermal shedding of delta, a ligand of Notch receptor and led to upregulation of Notch signaling. Electroporation of Notch specific siRNA overcome the chondro-inhibitory effect of TGF-β3 by upregulation of cell proliferation and subsequent precartilage condensation. Collectively, our data suggest that downregulation of ADAM 10 is responsible for chondro-inhibitory action of TGFB3 through activation of Notch signaling.

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**The Function of BRE Gene in Embryonic Interdigital Tissues**

W. Wong; Department of Anatomy, The Chinese University of Hong Kong, Hong Kong

In multicellular organisms, programmed cell death (PCD) is one of the most important developmental processes in determining the morphology of organs and tissues. For instance, PCD helps in sculpting the final shape of the limb - with death of the interdigital tissues in hand- and foot-plates. Consequently, the limb has been universally adopted as a model for studying PCD - because of the ease of manipulation and the defined spatial and temporal expression pattern of PCD in the interdigital tissues. Limb model was used to study the function of a gene called Brain and Reproductive organ-Expressed (Bre) which is a stress-modulating gene. Bre expression was previously found to be inhibited when fibroblasts were treated with UV and DNA-damaging agents. Similar results could also be produced by treating brain gliona cell U251 and promyelocytic cell HK-60 with retinoic acid. Retinoic acid is known to play a major role in tissue malformations and cancers in mammals. As the receptors and ligands are both widely expressed, the activity of this signaling cascade needs to be tightly regulated. Recent data from many groups have suggested that ligand endocytosis is critical for the activation of the Notch cascade, although the mechanism of how the internalization of Notch ligands leads to receptor activation remains poorly understood. We have isolated several mutations in the *Drosophila* homolog of auxillin, a J-domain-containing protein known to cooperate with Hsc70 in the disassembly of clathrin coats from clathrin-coated vesicles in vitro. Consistent with this biochemical role, animals with reduced auxillin function exhibit genetic interactions with Hsc70 and clathrin.

Interestingly, the auxillin mutations interact specifically with Notch and disrupt several Notch-mediated processes. Experimental analysis suggests that the J-domain, N-terminal kinase domain, and the C-terminal clathrin binding domain are all required for Notch signaling. Genetic evidence places auxillin function in the signal-sending cells, upstream of Notch receptor activation, suggesting that the relevant cargo for this auxillin-mediated endocytosis is the Notch ligand, Delta. Indeed, the localization of Delta protein is disrupted in auxillin mutant tissues. Thus, our data suggest that auxillin is required for Notch signaling and that ligand endocytosis in the signal-sending cells needs to proceed past coat disassembly to activate Notch.

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**Dots Versus Stripes: MAPK/ERK Signaling Pathway in Periodic Feather Pattern Formation**

C. Lin, T. Jiang, R. Baker, P. Maini, R. Widelitz, C. Chuong; Pathology, University of Southern California, Los Angeles, CA, Centre for Mathematical Biology, Mathematical Institute, Oxford, United Kingdom


A fundamental problem in developmental biology is to understand the molecular basis of pattern formation. To address this issue, we examined the role of growth factors in a cultured embryonic chicken skin model. FGF activates feather bud formation, but the cellular and molecular mechanisms of this “activation” remain unknown. We show here that FGF4 induces MAPK/ERK phosphorylation during bud morphogenesis. The MAPK/ERK pathway was suppressed in skin explant cultures using three methods: RCAS mediated expression of a soluble dominant negative FGFRI, chemical ERK inhibitor U0126 and siRNA against Raf. Dose- and stage-dependent studies show a critical range of FGF doses, reflecting inhibited feather bud regression (e.g., increased with higher inhibitor dosage and earlier times of administration). Unregulated stripes show moderate levels of Shh, Notch and delta expression which, unlike the controls, are unpatterned. Epithelial-mesenchymal recombination showed that the ERK inhibitor acts primarily on the mesenchyme. Real time effects were recorded by time-lapse video-microscopy. Skin explants were grown in an environmental chamber (Wafergen SmartSlide) enabling us to continually observe cell movements and track cells using fluorescent markers. Cultures remained healthy for 5 days. The movie shows the distinctive formative process of stripes versus dots. Analyses of Dot labelled cells showed an increase in mesenchymal cell motility following ERK inhibition. In the controls, plaque epithelia emerge by cell rearrangement and coordinated cell shape elongation. In explants treated with ERK inhibitors, epithelial cells remain hexagonally shaped and randomly arranged. Earlier we proposed that a reaction-diffusion mechanism is involved in periodic feather bud pattern formation and that FGF is required for the stochastic induction of initial peaks. Here we further show that the FGF pathway acting through MAPK/ERK is required in chemotactic cell migration to stabilize and pattern the periodically arranged feather primordia. A mathematical model is developed.

512 A Genome-wide Survey and Characterization of SFKs and PLCs in the Sea Urchin I. K. Townley, M. Raisch, S. R. Saxon, K. R. Foltz; MCDB, UCSB, Santa Barbara, CA

In echinoderms (sea urchin and sea star), a current model of egg activation at fertilization involves the stimulation of at least one Src family kinase (SFK) and subsequent activation of phospholipase C gamma (PLCγ). PLCγ generates inositol trisphosphate (IP3) causing release of Ca2+ from the egg endoplasmic reticulum (ER). Inhibition of SFK or PLCγ activity blocks Ca2+ release. In the purple sea urchin Strongylocentrotus purpuratus, multiple SFKs of the Src (SFK1, Frk, SFK5, SFK7), Abl, Tec and Csk families as well as PLCs (orthologs of 2 forms of β and single gene copies of δ, γ, and ε) have been identified through genome mining. CDNs encoding the SFKs have been cloned partially or in full, but the SFK orthology assignments are not obvious. While SpSFK1 is necessary for Ca2+ release at egg activation (Giusti et al. 2003. Dev Biol, 256, 367-378), the roles of the other SFKs are unknown and currently are being investigated using kinase activity profiling and dominant interference. Immunolocalization using specific antibodies revealed that SpSFK1 and SpPLCγ are localized to the cortex of the egg after fertilization enriched especially around the point of sperm-egg interaction. Often, a patchy cortical localization is observed after fertilization, but the biological relevance of this is unknown at present. Supported by an NSF award to KRF.

513 Histone Deacetylase Regulates Type II Collagen Expression in Articular Chondrocytes Y. Huh, Y. Sung-Sook, J. Chun; Life Science, GIST, Gwangju, Republic of Korea

Histone deacetylase (HDAC) regulates various cellular processes including differentiation by modulating transcription of a number of target genes. Differentiated phenotypes of articular chondrocytes are unstable in culture and rapidly lost during a serial monolayer culture. This process is designed as “dedifferentiation” which is characterized by the cessation of type II collagen expression, a hallmark of differentiated chondrocyte phenotypes. This study investigated a role of HDAC in type II collagen expression in primary culture chondrocytes. We found that HDAC activity in differentiated articular chondrocytes was dramatically decreased during dedifferentiation of chondrocytes caused by a serial monolayer culture. Inhibition of HDAC activity with trichostatin A (TSA) or PDX101 promoted hyperacetylation of histone 4 in articular chondrocytes without affecting cell viability. HDAC inhibition was also sufficient to cause chondrocyte dedifferentiation by blocking type II collagen expression in both transcriptional and translational levels. HDAC inhibition also blocked redifferentiation of dedifferentiated chondrocytes, which was induced by a three dimensional pellet culture of dedifferentiated chondrocytes. Because up-regulation of Wnt-5a and down-regulation of Wnt-11 is known to regulate chondrocyte dedifferentiation (i.e., type II collagen expression), we examined whether HDAC regulates type II collagen expression by modulating these Wnts expression. HDAC inhibition in chondrocytes caused the expression of Wnt-5a to increase and Wnt-11 expression to decrease. Taken together, our results suggest that HDAC activity regulates type II collagen expression by suppressing the transcription of Wnt-5a.

514 Why Are Proteins Associated with Drosophila Telomeres Rapidly Evolving? N. Elde, H. Malik; Basic Sciences, Fred Hutchinson Cancer Research Center, Seattle, WA

The telomeres of Drosophila are remarkable evolutionary variants, which do not employ telomerase to replenish chromosomal ends. Instead, specific retrotransposons function as telomeres. Despite this fundamental difference, telomeres of all species share many common features. Numerous factors interact with telomeres to protect these vital genomic regions. This includes telomere-capping proteins, some of which are DNA repair factors. The involvement of such proteins in the regulation of telomeres highlights the relationship between telomeres and chromosomal stability. Defects in telomeres result in abnormalities associated with aging and nearly all forms of cancer. A novel and fast evolving Drosophila gene, named caravaggio, has an essential role in telomere capping. Disruptions of caravaggio are known to cause extensive telomere fusions. This was unexpected because genetic features are commonly under strong evolutionary constraints to maintain vital functions. To explain this paradox, we considered the observation that many rapidly evolving genes co-evolve in relationships promoting adaptive changes. Examples include genes engaged in host-pathogen interactions. Drosophila telomeres appear similar because cells co-opted otherwise autonomous retrotropansons to maintain chromosomal ends. Therefore, a hypothesis accounting for the rapid evolution of caravaggio is that it has been subject to opposing selective forces that promote co-evolution with telomeric retrotransposons, yet also maintain an essential function. Supporting this view, we detected evolutionary signatures consistent with positive selection in a defined region of caravaggio in independent comparisons between Drosophila species. Experiments testing the consequences of rapid evolution in Drosophila will provide clues toward understanding the role of such fast evolving, yet essential, genes in telomere function.

515 Age Impairs Flight and Increases Muscle Stiffness in Drosophila M. S. Miller,1 E. G. Brown,2 P. Lekkas,2 J. M. Bradlock,1 G. P. Farman,1 T. C. Irving,1 D. W. Maughan,1 J. O. Vigoreaux 2,1; 1Molecular Physiology and Biophysics, University of Vermont, Burlington, VT, 2Biology, University of Vermont, Burlington, VT, 3BCPS, Illinois Institute of Technology, Chicago, IL

Age increases mobility due to reductions in muscle performance (functional senescence) and muscle mass (sarcopenia) in species. A key aging theory proposes that reactive oxygen species, resulting from mitochondrial metabolism, impairs cell function by injuring oxidative damage on macromolecules. We investigated the effects of aging on Drosophila indirect flight muscle (IFM), a strictly aerobic muscle and one of the most metabolically active tissues known. A wild-type strain (Oregon R) with a ~51 day mean lifespan (~80 day maximum lifespan) became flight impaired between 42-46 days. Old flies (~49 days) were flight competent, but had reduced performance compared to young flies (2-3 days) (flight index 3.3 ± 0.2 vs. 5.6 ± 0.1, range = 0-6; wing beat frequency 183 ± 8 vs. 215 ± 4 Hz). Flies older than 56 days were unable to beat their wings and skin fibers failed to be calcium activated. Old flies (vs. young) had normal number and packing of myofilaments, but had longer sarcomeres (3.66 ± 0.05 vs. 3.20 ± 0.03 μm) and enlarged mitochondria with large cristae-free regions. Small amplitude sinusoidal length perturbation analysis showed IFM isolated from old flies developed greater isometric force (82%) , oscillation work (132%), and power output (121%) compared to young, yet indices of crossbridge kinetics appeared normal. The up to 143% increase in elastic and viscous moduli amplitude under active, passive, and rigor conditions suggest that old fibers become stiffer longitudinally, thereby enhancing force transmission and oscillatory work generation. Small-angle x-ray diffraction indicates that crossbridges in aged fibers move towards the thin filament (presumably increasing actin-myosin interactions), thereby increasing transverse stiffness. The increase in stiffness with age is most likely due to oxidative damage and/or changes in myofilibrillar protein expression. We speculate that the greater stiffness associated with aging increases internal resistance to stretching, driving the decreased flight performance.

516 Effects of Different Rates of Stretching on Muscle Fiber Differentiation and Characteristics of Cultivated Myoblasts K. Kurokawa,1 K. Okubo,2 K. Sakiyama,1 T. Takeda,1 S. Abe1, Y. Ide1, K. Ishigami,1,2; Sports Dentistry, Tokyo Dental College, Chiba, Japan, 3Anatomy, Tokyo Dental College, Chiba, Japan

In vivo studies of humans and rats have shown that different exercise and training methods bring about changes in muscle fiber characteristics of skeletal muscle. Furthermore, in vitro studies have clarified that application of various mechanical stimuli to myoblasts not only facilitates cell proliferation and differentiation, but also brings about changes at the isometric level. However, past studies on mechanical stimulation have used constant intensity of stimulation, and no studies appear to have varied the intensity of stimulation. A basic study was therefore conducted to ascertain the effects of different exercise and training methods on muscle fibers. By stretching myoblasts at different rates, differentiation and characteristics were investigated.
Using the Flexercell® strain unit, myoblasts were stretched at 3 rates, and expression of myosin heavy chain (MyHC) mRNA was compared and analyzed on 1 day, 3 days, and 5 days. Expression of MyHC-perinatal, one indicator of differentiation, was high with fast stretching at first and then decreasing thereafter. As influence of a muscle fiber characteristics, expression of MyHC-2b (fast contraction) was high with fast stretching, expression of MyHC-2a (slow contraction) was high with slow stretching. MyHC-2d is considered an isoform that can change to either MyHC-2a or -2b, and expression was high with long stretching. In other words, fast mechanical stretching of myoblasts appears to facilitate differentiation for early stage and myoblasts acquire muscle fiber characteristics suitable to each rate of stretching.

517 Increase of Reactive Astrocytes in Development of the Cerebral Cortex in POMGnT1 Mutant Mouse
Y. Yang, X. Xiong, Y. Qi, H. Hu. Neuroscience and Physiology, SUNY Upstate Medical University, Syracuse, NY
Muscle-eye-brain disease (MEB) is a congenital muscular dystrophy associated with central nervous system (CNS) lesions, represented by cobblestone lissencephaly and eye anomalies. The gene responsible for MEB is O-linked-mannose β1,2,3-acetylglucosaminyltransferase I (POMGnT1). The molecular and cellular mechanisms of brain malformation in this enzyme deficiency are poorly understood. The mouse model of MEB recapitulates many aspects of the human phenotype. Previous studies have shown that the brain surface basement membrane and the glial limitans disappear during development of the cerebral cortex. In this study, we investigated the effect of enzyme deficiency on the development of astrocytes. Astrocytes were analyzed by immunofluorescence staining for glial fibrillary acidic protein (GFAP) and S-100 and by electron microscopy. In the adult mutant animals, GFAP-positive astrocytes were observed throughout the cerebral cortical wall while only the glial limitans, marginal zone, and ependymal layer showed GFAP-positive cells in the wildtype animals. During development, GFAP-positive astrocytes were detected at the hemisphere midline of corpus callosum beginning at E17.5 and P0 in both the wildtype and the mutant. Beginning at E12, astrocytes began to appear at the brain surface in mutant as well as in wildtype and formed the continuous glia limitans in wildtype. Compared with the wildtype, the mutant brain exhibited hypertrophic astrocytes and an increase in the number of astrocytes throughout the cerebral cortex, especially at the subpia of the cortex. Contrary to GFAP staining, S-100 immunostaining showed different staining pattern that S-100-positive cells were scattered throughout the white matter and cortex in both the wildtype and the mutant. More S-100 positive cells in cerebral cortex especially at the subjacent of cortex were seen in mutant. In mutant, there are more a reactive gliaosis in POMGnT1 deficiency.

518 Developmentally Regulated Expression of a 28-kDa Peptide in the Rat Brain
L. A. Haddad, A. M. Vinha-Morgante; Genética e Biologia Evolutiva, University of Sao Paulo, Sao Paulo, Brazil
Alternative splicing is a major mechanism leading to protein functional diversity, believed to occur in up to 80% of brain transcripts. Fragile Mental Retardation 1 (FMR1) gene transcripts undergo alternative splicing of four exons creating 20 possible non-redundant fragile gential geration protein (FMRP) isoforms. To understand the functional relevance of FMR1 alternatively spliced exon 12, we raised polyclonal antibodies that recognize the encoded exon. The antibodies detected 50 to 70-kDa FMRP isoforms, which localized in dendritic spines of cultured rat cortical neurons. A 28-kDa peptide was additionally identified on Western blots of rat brain cortex, cerebellum, and hippocampus. This 28-kDa peptide is developmentally regulated after birth, with the highest expression levels in the adult brain. FMR1 alternative splicing may produce 47.3 to 71.1-kDa FMRP isoforms as previously documented by RT-PCR and Western blotting data. Therefore, it is unlikely that the 28-kDa peptide is a product of FMR1 alternative splicing. It may be generated from FMRP developmentally regulated proteolysis or, alternatively, be unrelated to FMRP. Under either hypothesis, the 28-kDa-peptide expression regulation may be of functional relevance during rat brain development and aging. Purification and sequencing experiments are underway to characterize this peptide.

519 Immunocytochemical Distribution of Mammosomatotrophs in Porcine Anterior Pituitary
J. Lee, M. H. Stromer, L. L. Anderson; Department of Animal Science, Iowa State University, Ames, IA, Department of Biochemistry, Biophysics and Molecular Biology, Iowa State University, Ames, IA, Department of Biomedical Sciences, Iowa State University, Ames, IA, Department of Poultry Science, Mississippi State University, Starkville, MS
The existence of a novel anterior pituitary cell type, the mammosomatotroph (MST), secreting both growth hormone (GH) and prolactin (PRL) was proposed to function as transitional cells or progenitor cells between GH cells (somatotrophs) and PRL cells (mammatrophs). Double fluorescence immunocytochemistry was used to identify distribution patterns of MS cells in anterior pituitary glands from newborn and prepubertal stages of pigs. Immunopositive MS cells were morphologically similar to the mammatrophs including polygonal or irregular shape and diameter from 10 to 15 μm in diameter. The confocal detection of a MS demonstrated uneven distribution of GH and PRL. Anterior lobes were sampled at 3 positions in each of 5 radial regions in each of 3 levels perpendicular to the gland axis. There were changes in spatial distribution with different levels of the gland and this specific pattern is similar among different age groups (day 1, day 45, and day 90). Although MS are rare in number, significant differences were observed among the total MS cells across three age groups (P < 0.0001). MS cells were most numerous in region 3 where originates from embryonic Rathke’s pouch (6.0±1.4; mean ± standard error of the mean per 30,495 μm²) compared to rest of regions (0.7±0.1). There were significant decreases at position a (P < 0.001) and significant increases (P < 0.0001) at position c in region 3 from proximal to distal level. The total number of MS cells at the proximal level at position a increased from day 1 to day 45 (2.5×) and from day 45 to day 90 (2.4×). The results suggest that there may be regional specificity of cellular transformation to facilitate GH and PRL secretion during the rapid growing period in the young pig. Supported by research grant USDA/CSREES NRI 2003-35206-12817 (L.L.A., S.J., and C.G.S).

520 Immunocytochemical Distribution of Mammosomatotrophs in Porcine Anterior Pituitary
J. Lee, M. H. Stromer, L. L. Anderson; Department of Animal Science, Iowa State University, Ames, IA, Department of Biochemistry, Biophysics and Molecular Biology, Iowa State University, Ames, IA, Department of Biomedical Sciences, Iowa State University, Ames, IA, Department of Poultry Science, Mississippi State University, Starkville, MS
Prolactin (PRL) is synthesized in specific anterior pituitary secretory cells, mamatomatrophs or PRL cells (lactotrophs) and has diverse functions including growth, reproduction, lactation, osmoregulation, immunomodulation, and energy metabolism. The objective of this study was to identify spatial distribution patterns of mammatrophs in the newborn and prepubertal porcine pituitary using fluorescence immunocytochemistry. Immunoreactive mammatrophs were polygonal or irregular shape with stained nuclei and ranged from 10 to 15 μm in diameter in 1-45, and 90-day-old pigs. Clusters of mammutrophs consisting of 3 to 10 cells were found in day 45 and day 90 old pigs. Significant differences were observed among the total mammatrophs per 30,495 μm² across the three age groups (day 1; day 45 and day 90, P < 0.0001). Anterior lobes were sampled at 3 positions (a, b, and c) in each of 5 radial regions (1-5) in each of 3 levels (proximal, middle, and distal) perpendicular to the gland axis. There were changes in spatial distribution with different levels of the gland and this specific pattern in similar among different age groups. Mammatrophs were most numerous in positions a and b (57 ± 4.2; mean ± standard error of the mean per 30,495 μm²), and least numerous in position c at the proximal level (24 ± 5.2) in day 1, day 45, and day 90 indicating high population and immunointensity close to intermediate lobe. However, at the distal level, the pattern showed a bell shape with significant increase in the number of mammatrophs in region 3 at position c (P = 0.001). From these results, we suggest that there may be regional specificity of cellular differentiation and transformation to control PRL secretion to meet the need for endocrine regulation as the animal ages. Supported by research grant USDA/CSREES NRI 2003-35206-12817 (L.L.A., S.J., and C.G.S).

521 Role of Oxidative Stress on the Regulation of Na⁺−K⁺−ATPase Activity in Renal OK Cells
E. F. Silva, P. Soares da Silva; Institute of Pharmacology and Therapeutics, Faculty of Medicine University of Porto, Porto, Portugal
The present study aimed at evaluate oxidative stress in cells that retain characteristic of kidney proximal tubules plays a role in the regulation of Na⁺−K⁺−ATPase. Opossum Kidney (OK) cells were kept in culture from passage 36 to passage 80. Na⁺−K⁺−ATPase (OKA) activity was examined by electrophysiological methods. Hydrogen peroxide production and cell viability were determined with fluorescent assays, Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit and Calcein-AM, respectively. NKA subunits expression was determined by western blotting and in cell western. NKA activity, measured as apical-to-basal ouabain-sensitive amphotericin B-induced increases in short-circuit current (μA/cm²), increased with the number of cell passages from 6.42 ± 0.99 in young cells (passage 36 to 50) to 47.51 ± 3.02 in aged cells (passage 60 to 80). The related increases in cell hematocrit and NKA subunits expression increased 5-fold between passage 36 and passage 80. Twenty four hour accumulation of hydrogen peroxide in the extracellular medium in aged cells (319±3.2 nM) was significantly higher than in young cells (256±5.2 nM). The rate of hydrogen peroxide production was of 5.21±0.02 nM/min in young cells and 7.37±0.02 nM/min in aged cells. Apocynin, at the concentration of 300 μM (non toxic concentration) abolished the increased production of hydrogen peroxide in aged cells and significantly decreased NKA activity (30% reduction). It is concluded that oxidative stress plays a role in the
long-term regulation of NKA activity, as evidenced by the fact that increases in hydrogen peroxide production in aged OK cells paralleled increases in NKA activity and inhibition of hydrogen peroxide production reduced NKA activity. Supported by grant POCTI/CBO/45767/2002.

522 Evaluation of Mullerian Inhibiting Substance Concentration Using ELISA and RT-PCR in ICR Mouse Y. Kim, J. Lee, D. Han, J. Kim, S. Kim, J. Jang, S. Park, D. Choi, H. Ko, Y. Lee, Y. Hwang, D. Kim; Biological Science, Gachon University of Medicine & Science, Incheon, Republic of Korea

MIS (Mullerian inhibiting substance) is a member of the TGFβ family whose members play key roles in development, suppression of tumor growth, and feedback control of the pituitary-gonadal hormone axis. MIS is expressed in a highly tissue-specific manner, restricted to Sertoli cells of the testis and granulosa cells of the ovary. It induces Mullerian duct regression, controls primordial follicle recruitment and regulates steroidogenesis and Leydig cell differentiation. In addition, its level is used as a marker for ovarian aging and breast, prostate, uterus, and ovarian cancers. To detect MIS serum level and expression of MIS messenger RNA (mRNA), we used ICR mice and sampled the blood and tissue from fetals, pregnant, postnatal male and female mice group. RT-PCR method was used to compare MIS mRNA expression level in reproductive organs tissues. We confirmed MIS serum level secretion by Enzyme-linked immuno-solvent assay (ELISA) using MIS/AMH antibody. We obtained MIS serum level data from fetus to adult (17 weeks). MIS serum levels have a remarkable difference between female and male fetuses during sex organ differentiation (both <0.1ng/ml). Its level of pregnant mouse changed markedly (4.5–12.2ng/ml). Postnatal female and male mice serum level changed (Male: <0.1–13.6ng/ml, Female: 5.3–103.4ng/ml) and the changing phase was diurnally opposed (Male: decreasing, Female: fluctuating). For further study, normal level of MIS is important. It would contribute to understanding of reproductive organ differentiation, and ovarian aging. We expect that our study would provide the basic information for the study of MIS interactions with reproductive organ disability, cancer, effect of other hormone or menopause; we forward if MIS is regularly injected to middle age women, menopause would be delayed.

523 Histomorphometric Analysis of the Sex Cords in the Testis of Gallus domesticus during Embryonic Development E. Soria; 1St. Gonzalez-Moran; 1Patología, Instituto Nacional de Cardiología, Distrito Federal, Mexico, 2Biología Comparada, UNAM, Distrito Federal, Mexico

In this study we evaluated the pattern of sex cords in the testis of the Gallus domesticus during embryonic development of the testis. They were studied on day 8 and 13 of embryonic development and the day of hatching. Left testes were obtained in all cases, and immediately fixed and embedded in Epon 812 and semi-thick-sections were morphometrically measured under light microscopy, using stereological methods. The results indicate that in the testes from 8-day-old embryos, the sex cords are larger and convoluted and the sex cords are elongated along the basement membrane. Results also reveal an increase in the total volume of sex cords, and in the number of Sertoli cells and spermatagonia, as well as enlargement of the individual Sertoli cells during embryonic development. Based on our results we concluded that the proportion of sex cords and the number of germ cells and Sertoli cells may differ markedly according to age.

524 Neuregulin 1-β-1 Induces Nuclear Bag Fiber Morphogenesis in a Defined In Vitro Culture System J. W. Runnemeyer, 1 M. Das, 2 J. Kang, 2 J. J. Hickman, 2 BioMolecular Sciences, University of Central Florida, Orlando, FL, 2Nanoscience Technology Center, University of Central Florida, Orlando, FL

Research into the factors affecting the differentiation of skeletal muscle myotubes into the intrafusal fibers of the muscle spindle during primary and secondary myogenesis is relatively unexplored. Additionally, in vitro systems designed to investigate this myogenesis are underdeveloped. Neuregulin 1-β-1 (Nrg1-β-1), secreted by sensory neurons, has been shown to influence transcription factor activation in developing muscle through ErbB2-4 signaling. In this study we investigated the role of the growth factor Nrg1-β-1 EGF domain for its ability to influence myotube fate specification in a defined in vitro culture system on the non-biological substrate DETA. Morphologically, Nrg1-β-1 EGF domain treatment of developing myotubes increases the ratio of nuclear bag fibers to total myotubes from 0.019 to 0.100. This increase of 8% is statistically significant using a Student’s t-test (P<0.001). After Nrg1-β-1 EGF treatment, nuclear chain fibers were also observed in culture. Nrg1-β-1 EGF treated nuclear bag and chain fibers were positive when evaluated immunocytochemically for alpha cardiac-like myosin heavy chain expression specific to spindle fibers. Additionally, the Nrg1-β-1 EGF treated nuclear bag and chain myotubes were characterized electrophysiologically. This data shows conclusively, in a serum-free defined culture system, that the growth factor neuregulin 1-β-1 is sufficient to drive the morphogenesis of forming myotubes to the nuclear bag phenotype.

525 Effects of Radiofrequency on the Connective Tissue of the Dermis in Sprague-Dawley Rats A. Zauta, 1 E. Beltran, 1 C. Ferrer, 1 S. Navarro, 2 C. Bernal-Mañas, 2 M. Canteras, 2 L. M. Pastor, 2 Cell Biology, Institute of Aging, University of Murcia, Murcia, Spain, 2Department I-D, Grupo Tahe, Murcia, Spain, 1Statistics, University of Murcia, Murcia, Spain

Many studies exist on the effect of radiofrequency on connective tissue, although no a histological evaluation has been made of the changes that take place after successive applications of low power radiofrequency. In this work studies such changes in the fibroblast, where its proliferation and biosynthetic activity. In this experiment 16 (8 treated and 8 control) 3 month-old Sprague-Dawley rats were divided into four groups, which were subjected to different session of radiofrequency (1, 2, 3 and 5) in the tail. All were sacrificed after the last treatment, except the 4th group, which was sacrificed 2 months later. Fibroblast per unit area were determined in all the animals. The proliferation rate and the expression of Heat Shock Protein (HSP-47) were assessed by Western blot and Northern analysis. HSP-47 expression was highly increased after radiofrequency treatment. In this study, we have used Ki-67 antibody to determine the rate of cardiac myocyte proliferation in the postnatal mouse heart. The protein profiles of 5-day- and 13-day-old hearts were established and compared. Eighteen protein spots were found to be differentially expressed at day 13. We focused our attention on 2 of the proteins identified by MALDI-TOF MS, cyclin I and p53 because they are both believed to be involved in cell cycle regulation. Western blot analysis confirmed that both proteins were positively up-regulated in the 13-day-old postnatal heart. To determine directly whether these proteins were associated with cell proliferation, we examined their expression patterns in H9c2 cardiomyocytes maintained in vitro. We established that cyclin I expression was low during the growing phase of H9c2 culture and high during the growth arrest/ differentiation phase. In contrast, p53 expression was unchanged during both phases. The various growth phases were confirmed by the presence of cyclin A and growth arrest specific 1 (gas1) proteins. We investigated whether silencing cyclin I expression using cyclin I siRNA could promote an increase in H9c2 proliferation. It was determined that silencing cyclin I could enhance a small, but significant, increase H9c2 cell proliferation. The results imply that the increased cyclin I expression detected in this study may be associated with cell growth arrest in the cardiomyocytes.

526 Cytokine like 1 (Cyt1) in Chondrogenesis and Cartilage Development J. Kim, S. Yang, J. Chun; Life Science, Gwangju Institute of Science and Technology, Gwang-ju, Republic of Korea

Cytokine like 1 (Cyt1) is known as a possible candidate of cytokine originally isolated from bone marrow-derived CD34 positive cells. We initially found that Cyt1 is predominantly expressed in cartilage and chondrocytes and, therefore, investigated in this study the expression and possible function of Cyt1 in cartilage development. Cyt1 expression was very low in mesenchymal
cells, dramatically increased during chondrogenesis, and highly expressed Cyt1 was decreased during hypertrophic maturation both in vivo (during limb development) and in vitro (during micromass culture of mesenchymal cells). The function of Cyt1 in chondrogenesis and hypertrophic maturation was examined by treating chondrifying mesenchymal cells with exogenous Cyt1 obtained from conditioned medium of L929 cells which express ectopic Cyt1. Cyt1 treatment promoted chondrogenic differentiation of mouse limb bud mesenchymal cells during micromass culture as determined by the increased synthesis of type II collagen and sulfated proteoglycan. Cyt1 treatment activated expression of protein kinase C-alpha, which is known to induce chondrogenic differentiation through extracellular signal-regulated protein kinase. In addition, Cyt1 stimulated proliferation of chondrifying mesenchymal cells, which is necessary for chondrogenesis. However, exogenous Cyt1 did not affect hypertrophic maturation of chondrocytes. Collectively, our results suggest that chondrocyte-specific expression of Cyt1 regulates chondrogenesis but not hypertrophic maturation during cartilage development.

Wound-healing Responses of the Marine Red Alga, Griffithsia monilia, Monitored with Time-Lapse Videography and FITC-conjugated Lectins
J. H. Han, G. H. Kim, J. H. Choi, I. Y. Jeon; Biology, Kongju National University, Kongju, Republic of Korea

When cells of the marine red alga Griffithsia monilia receive fatal wounding, the wound healing process occurs by a somatic cell fusion between the two neighbouring cells, called repair rhizoid and shoot cells, which extend from each side of the wounded thallus. Previous research by Waaland et al. suggested that the process of somatic cell fusion was mediated by a glycoprotein with a methyl-D-mannose binding site, named "Rhodomorphin", which was produced by the repair rhizoid cells. In this study, we showed that FITC-labeled ConA (Concanavalin A), which is specific to methyl-D-mannose, indeed labelled tip of the apical rhizoid cell, and these data, therefore, agreed with Waaland et al. However, our time-lapse videographic data were contradictory to Waaland et al. as follows. Was found, the repair shoot cell was the inducer for somatic cell fusion, and it promoted growth of rhizoid cells until cell fusion occurred.

Modulo Fibonaccial Model of Phylloaxis
C. P. Spears,1 J. J. Yan,2 M. Bicknell-Johnson; 1Fibonacci Phylloaxis, Inc., Davis, CA, 2Department of Electrical and Computer Engineering, University of California at Davis, Davis, CA, A

We recently presented a Matlab program for display of a Fp modulo Fm model of phylloaxis (12th International Conference on Fibonacci Numbers and their Applications, San Francisco, CA, July, 2006). The program assumes only an initial single cell cycle delay in division by daughter cells, before continuous binary division, and self-association of cells by age from birth. The program is shown to generate phylloactic patterns of growth consistent with diffusion models of directional auxin secretion, as well as biophysical, contact pressure, and minimal displacement models, for providing a basis for the occurrence of Fibonacci numbers and relations in plant development. The model predicts spatial stability of growth points and of structures based on age and generation identifiers. Cylindrical and rectangular displays of populations >20,000 cells shown by age and generation provide testable hypotheses for future discrete model correlates of apical meristem development.

Proteomics and Expressed Sequence Tag Analyses of the In Vitro-formed Protoplasts of the Marine Multinucleate Green Alga Bryopsis plumosa
G. Kim, T. Kloczková; Biology, Kongju National University, Kongju, Republic of Korea

When cell membrane of the marine coenocytic green alga Bryopsis plumosa is destructed, the protoplasm expelled in seawater generates numerous protoplasts in vitro. Protoplasts in masses are initially surrounded by a polysaccharide envelope, which acts like the cell membrane. New cell membrane develops within several hours and cell wall develops in 24-48 h after wounding. Then, the newly formed cells begin to grow into plants. We constructed two-dimensional polyacrylamide gel electrophoresis (2-DE) protein maps of the protoplasts at different stages of development (0, 3, 12, 24, and 48 h after wounding). There was about 50% increase in the number of protein spots over time intervals (from 1235 spots at 3 h to 1915 spots at 48 h after wounding). This increase may be due to recovery of the gene expression from extreme condition of cell rupture to normal cell differentiation. A dramatic change of proteome structure was observed during the protoplast development. When the proteomes of different stages were compared, 59.8% of proteins that were up-regulated at early stages (0-3 h) almost disappeared at late stages (24-48 h). About 70-80% of proteins detected at 48 h were not detected at the initial stage. 29 proteins, differentially regulated according to stages, were selected and analyzed using MALDI-TOF mass spectrometry. Among them, chaperon proteins involved in protein folding, chloroplast precursor proteins, and regulatory proteins were identified as up-regulated genes during the early stages of protoplast development. EST analysis of 3 h-old protoplast showed that 607 sequences (74%) were present in multiple copies of 77 genes, which likely reflects the need for a high amount of certain gene products at this stage. 252 unique ESTs were compared to those in Genbank cDNA. Protoplast formation in Bryopsis plumosa serves as a natural defense from life-threatening injuries and contributes to dispersal of this alga.

The Plasmodium Protein Network Diverges from Those of Other Eukaryotes
S. Suthram,1 T. Sittler,2 T. Ideker; 1Bioinformatics, UCSD, La Jolla, CA, 2Bioengineering, UCSD, La Jolla, CA

Plasmodium falciparum is the pathogen responsible for over 90% of human deaths from malaria. Therefore, it has been the focus of a considerable research initiative, involving complete DNA sequencing of the genome, large-scale expression analyses, and protein characterization of its lifecycle stages. The Plasmodium genome sequence is relatively distant from those of most other eukaryotes, with only 31% of the 5,334 encoded proteins having notable sequence similarity to other organisms. To systematically elucidate functional relationships among these proteins, a large two-hybrid study has recently mapped a network of 2,846 interactions involving 1,312 proteins in Plasmodium. This network adds to a growing collection of available interaction maps for a number of different organisms and raises questions about whether the divergence of Plasmodium at the sequence level is reflected in the configuration of its protein network. Here we examine the degree of conservation between the Plasmodium protein network and those of model organisms. Although we find 29 highly connected protein complexes specific to the network of the pathogen, we find very little conservation with complexes observed in other organisms (three in yeast, none in others). Overall, the patterns of protein interaction in Plasmodium, like its genome sequence, set it apart from other species.

Genome-Scale Network Analysis of Leishmania major, a Pathogenic Trypanosomatid
J. D. Whittemore, A. K. Chavali, J. A. Papin; Biomedical Engineering, University of Virginia, Charlottesville, VA

Leishmaniasis, caused by infections due to species of the genus Leishmania, affects over 350 million people worldwide (mostly in developing nations). Existing forms of treatment are either too expensive or have toxic side effects, and resistance to some treatments is on the rise. We reconstructed the metabolic network of the recently sequenced Leishmania major and accounted for the gene-protein-reaction relationships of a significant portion of the annotated genome. The reconstructed network consists of more than 300 reactions. Using flux balance analysis, we make predictions of growth dynamics in various cellular environments and analyze the lethality of gene knockouts, validating our results with experimental data. This work constitutes one of the first genome-scale network analyses of a pathogen responsible for an emerging and uncontrolled disease. The reconstructed Leishmania major network is additionally unique, in that it is the first metabolic reconstruction of a trypanosomatid protozoan. We present IDEAS (Infectious Disease Engineering, Analysis and Simulation) as a framework to build multi-level computational models that connect genome-scale metabolic network reconstructions with parasite epidemiology to generate potential therapeutic applications.

Transcriptional Regulation of Protein Complexes within and Across Species
K. Tan,1 T. Hirono,2 H. Feizi,1 T. Ideker,2 R. Sharan; 1University of California San Diego, La Jolla, CA, 2Tel-Aviv University, Tel-Aviv, Israel

Yeast two-hybrid and co-immunoprecipitation experiments have defined large-scale protein-protein interaction networks for many model species. Separately, systematic chromatin immunoprecipitation experiments have enabled the assembly of large networks of transcriptional regulatory interactions. To investigate the functional interplay between these two interaction types, we combined both within a probabilistic framework which models the cell as a network of transcription factors regulating protein complexes. This framework identified 72 putative co-regulated complexes in yeast, and allowed the prediction of 120 novel transcriptional interactions. Several predictions were tested using new microarray profiles, yielding a confirmation rate (53%) comparable to that of a direct immunoprecipitation experiment. Furthermore, we extended our framework to a cross-species setting, identifying 24 co-regulated complexes that were conserved between yeast and fly. These complexes demonstrate that transcriptionally-regulated complexes are conserved over large evolutionary distance and provided suggestive evidence that
protein-protein interaction networks may evolve more slowly than transcriptional interaction networks. Our results demonstrate how multiple molecular interaction types can be integrated toward a global wiring diagram of the cell, and provide new insights into the evolutionary dynamics of protein complex regulation.

535 Analyzing the Residue-Composition of Metal Binding Sites in the Whole Protein Data Bank Z. Szabadka, G. Ivan, V. I. Grolmusz; Computer Science, Eotvos University, Budapest, Hungary

The Protein Data Bank (PDB) is the largest depository of protein structural information, containing more than 37,500 structures. The form and the organization of the PDB seems to be perfectly adequate for gathering information from specific protein structures, by using the bibliographic references and the informative remark fields. On the other hand, however, it seems to be impossible to automatically review remarks and journal references for processing hundreds or thousands of PDB files. We re-structured the whole PDB, creating a new database, the RS-PDB, containing more than 330 million atoms. The new database made possible the reliable and redundancy-free collection of all the binding sites from protein-ligand complexes. Here we analyze the metal binding sites from the whole PDB for residue-composition. More than 30,000 metal-binding sites were found; this number was reduced to 16,900 after deleting redundancies. It was not a surprise that ASP and GLU were counted most frequently in metal binding sites. The next three most frequently appearing residues were HIS, GLY and ASN. In the case of specific metal ions, the list of most frequent residues sometimes differ: potassium ions bind most frequently to GLY and SER, and ASP is only the third most frequent residue. Zinc ions were detected relatively frequently in the PDB, mostly bound to HIS and CY5 and significantly less frequently to ASP and GLU. It is a remarkable finding that the residue composition of the binding site strongly depends on the number of the coordinated water molecules: zinc ions with one coordinated water are bound most frequently to HIS and GLU and less frequently to CY5. Magnesium and calcium binding sites were also analyzed and we found that the number of the coordinated waters has high impact on the residue-composition of the binding sites.

536 Protein-Protein Interactions Inference Environment (PIIE): Increasing Confidence by Integrating Evidence from Multiple Sources M. Singhal, K. Domico; Computational Biology and Bioinformatics, Pacific Northwest National Laboratory, West Richland, WA

Deciphering interaction networks (say protein-protein interactions) forms the first step in annotating unknown proteins with functions, determining protein complexes, target identification as well as drug discovery. Over the past decade a large number of databases, websites and prediction tools have emerged that assign confidence scores to individual interactions. Unfortunately this growth in availability of interaction data is not coupled with increase in availability of computational tools allowing conversion of this data into information. The lack of computational tools facilitating the integration of evidence from multiple prediction/experimental sources such as Gene Neighborhood-GN, Gene Cluster-GC, Phylogenetic Profiles-PP, Rosetta Stones-RS, BIND, and DIP etc is the motivation behind Protein Interactions Inference Environment (PIIE). The poster presents PIIE, a software tool developed as a plug-in to an open source tool - Cytoscape (www.cytoscape.org), as applied to the problem of protein-protein interaction networks. PIIE presents multiple coordinated views (scatter plots, matrix visualization, Cytoscape networks, filtering mechanism and an integrated view) to a user that facilitates visual analytics of protein-protein interaction networks obtained from multiple sources of evidence. This poster will demonstrate the use of this plug-in on experimentally observed protein-protein interactions in Shewanella oneidensis strain MR-1.

537 GPS: Prediction of Phosphorylation Sites Implemented in Group-based Phosphorylation Scoring Algorithm Y. Xiu, Y. Yao,1 Systems Biology of Phosphoproteome, Laboratory of Cellular Dynamics, Hefei, China, 2Physiology, Morehouse School of Medicine, Atlanta, GA

Protein phosphorylation is an essential reversible post-translational modification of proteins, and it orchestrates cellular dynamics and plasticity. Experimental identification of protein kinases (PKs) substrates with their phosphorylation sites is labor-intensive and often limited by the availability and optimization of enzymatic reactions. Recently, large-scale analysis of the phosphory-proteome by the mass spectrometry (MS) has become a popular approach. However it remains experimentally challenging to identify cognate kinase without in vitro reconstitution. In this regard the in silico prediction of kinase-specific phosphorylation sites with protein's primary sequences may provide guidelines for the further experimental design and the interpretation of PS phospho-proteomic data. Here we present a novel computational program named GPS: Group-based Phosphorylation Scoring platform. We formulated GPS that carries greater computational power with superior performance compared to two existing phosphorylation sites prediction systems, ScanSite 2.0 and PredPhospho. With database in public domain, GPS can predict substrate phosphorylation sites from 71 different protein kinase (PK) families while offer at most 30 PK families. Using PKA as a model enzyme, we first compared prediction profiles from the GPS method with those from ScanSite 2.0 and PredPhospho. In addition, we chose an important mitotic kinase Aurora-B as a model enzyme since ScanSite 2.0 and PredPhospho offer no prediction. However, GPS still offers satisfactory sensitivity (94.44%) and specificity (97.14%). Finally, the accuracy of phosphorylation on MCAK predicted by GPS was validated by experimentation, in which six out of seven predicted potential phosphorylation sites on MCAK (Q91636) were experimentally verified. Taken together, we have generated a novel predictor to predict phosphorylation sites, which offers greater precision and computing power over ScanSite 2.0 and PredPhospho. GPS has been implemented in PHP and is freely available at: http://973-proteinweb.ustc.edu.cn/gps/GPS_web.html.

538 Protein Subcellular Location Image Database for Comprehensive Image Retrieval and Automated Interpretation J. Hua, T. Zhao, Y. Hu, S. Chen, A. Shanbhag, J. Newberg, R. F. Murphy; 1Dept. of Biological Sciences, Carnegie Mellon University, Pittsburgh, PA, 2Machine Learning Department, Carnegie Mellon University, Pittsburgh, PA, 3Dept. of Biomedical Engineering, Carnegie Mellon University, Pittsburgh, PA

Fluorescence microscope images (FMI) are widely used for the determination of protein subcellular locations. Due to the growing volume of images collected, a multimedia database system is required for the storage of both image and meta-data, retrieval of interesting images and automated analysis of subcellular location patterns in these images. The Protein Subcellular Location Image Database (PSLID, http://pslid.cbi.cmu.edu/PSLID) is an open source database system which enables comprehensive retrieval and automated analysis of high resolution FMI. FMI of 2 through 5 dimensions as well as the associated experimental annotations are stored hierarchically in a relational database for hundreds of proteins in a number of cell types. Through a web-based interface, users can search images based on the experimental annotations, while the context search looks for images that display similar location patterns to query image(s). The analysis modules in PSLID include statistical inferences, such as image selection (TypIC) and image set comparison (SimIC), and machine learning algorithms for both classification and clustering, allowing proteins be grouped by their location (creating location families). These tools have been proven to be more accurate and efficient than human visual inspection. PSLID also provides a programmatic search interface for linking to genome and proteome databases. Queries can return images either for a specific protein or proteins which are in the same location family. PSLID is built on the Postgres database system and the Tomcat Java Server Page server on the Linux platform, both of which are open source.

539 Identification of Protein Subcellular Location Families by Large-scale Random GFP-tagging of 3T3 Cells J. Hua, T. Zhao, E. G. Osuna, N. W. Bateman, M. H. Fuhrman, J. W. Jarvik, P. B. Berger, R. F. Murphy; 1Dept. of Biological Sciences, Carnegie Mellon University, Pittsburgh, PA, 2Machine Learning Department, Carnegie Mellon University, Pittsburgh, PA, 3Dept. of Biomedical Engineering, Carnegie Mellon University, Pittsburgh, PA

The goal of location proteomics is the systematic and objective characterization of the subcellular locations of all proteins. Our lab has previously designed numeric Subcellular Location Features (SLF) which we can extract from protein subcellular location images of single cells and developed automated classifier to successfully recognize major subcellular location patterns in both 2D and 3D images. We have also described the use of clustering algorithms to group proteins by their subcellular location patterns, so that assignments of proteins to subcellular location classes can be done objectively and new patterns can be identified. We have recently updated automated microscopy to collect large numbers of fluorescence microscope images from over 350 clones of NIH-3T3 cells randomly tagged with GFP by CD-tagging. Statistically distinctive subcellular location groups, or location families, were identified from the dataset by K-means clustering using the SLF. A consensus subcellular location tree was also generated to show the hierarchy of these proteins. These image data being publicly available, these methods will be able to identify new, if not all subcellular location families, which will hopefully provide a framework of sufficient resolution for location prediction and cell modeling.

540 LOCATE: A Mammalian Protein Subcellular Localization Database J. Fink, M. J. Davis, K. Hanson, R. N. Aturaliya, R. D. Teasdale; Institute for Molecular Bioscience, University of Queensland, Brisbane, Australia
The membrane organization and subcellular localization of a protein can provide information about its functional role. Historically, these data have been difficult to produce on a large scale for higher eukaryotic organisms. Our recent advances in membrane organization prediction methods and development of a Location Proteome Platform to determine subcellular localisation have made it possible to generate these datasets. We present here LOCATE, a curated, web-accessible database that houses data describing the membrane organization and subcellular localization of proteins. LOCATE represents a catalogue of experimentally-verified subcellular location and membrane organization of mammalian proteins using a high-throughput approach and provides localization data for nearly 40% of the mouse proteome. As well as experimental images for a given protein, LOCATE integrates information from external sequence databases and protein function information with computer predicted membrane organization, subcellular localisation and protein domains. It is available at http://locate.imb.uq.edu.au. As an illustrative example of the LOCATE database we have investigated the differential use of endoplasmic reticulum signal peptides and transmembrane domains within the variable protein output of single genes. In summary, we found that the generation of protein isoforms that are targeted to multiple subcellular locations represents a major functional consequence of transcript variation within the mouse transcriptome.

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The Shape of Things to Come: Quantitative Analysis of Cell Morphology
Z. Pincus, 1, 2 J. A. Theriot 3, 3Program in Biomedical Informatics, Stanford University School of Medicine, Stanford, CA, 2Department of Biochemistry, Stanford University School of Medicine, Stanford, CA, 3Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA
Quantitative measurement of cell shape is necessary for rigorous investigation of the biochemical and biophysical causes and correlates of morphological variation. Current approaches, which utilize ad hoc measures to capture particular morphological features, introduce biases based on the features selected and can blind investigators to novel variation. We have investigated data-driven approaches to quantifying morphological variation with quantitative but intuitive and biologically meaningful parameters. Given a cell-shape data set, we apply the principal components analysis to create a shape model that describes the average cell shape, the major modes of shape variation in the population (e.g. large vs. small, round vs. elongated, etc.), and the relative importance of those modes. Our experience with different cell types - multiple populations of bacterial cells, epithelial monolayers, and isolated crawling cells - indicates that the modes of shape variation so derived closely mirror qualitative descriptions used in the literature to describe a given cell type. In addition to recapturing descriptive terms, shape models have many quantitative uses. A cell can be measured in terms of the contribution of each shape mode to that cell's shape, and quantified populations can be statistically tested for morphological differences. The shape modes themselves can be visualized to provide a compact representation of the morphological variability of a population better than hand-picked "representative images," and entire populations can be plotted along the axes provided by the shape modes to uncover subtle trends in cell shape. We have used these tools to quantitatively examine the cytoskeletal role in Caulobacter shape, finding a link between cell shape and the localization of actin and tubulin homologues relative to a cell wall deposition protein. We have also investigated the stereotyped shape of ciliated keratocytes, finding three principal shape modes that are conserved across multiple data sets and can be experimentally manipulated independently.

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Computer Modeling of Ultrastructure of Muscle Cells
J. Parulek, 1 I. Zahradník, 2 M. Novotová, 1 J. Michálko, 3 Lab Cell Morphology, Inst Molec Physiol & Genetics SAS, Bratislava, Slovakia
Understanding of biological processes at the cellular level requires a large scale synthesis of numerous data of different origin. This would be facilitated by a computer modeling tool allowing construction of three dimensional geometrical models of living cells and their organelles with very high spatial resolution. In this study we explore an original approach to construction of models of skeletal muscle cells and their organelles such as myofibrils, mitochondria, t-tubules, sarcoplasmic reticulum, lipid droplets and sarcolemna at the resolution of 1 nm and the size of about 1000 µm3. Construction of the model is based on the theory of implicit surfaces and of their binary operations. Geometry of the muscle fibre and of its constituents is defined by means of parallel modeling planes perpendicular to the fiber axis. In each modeling plane, the number, shape and topology of organelles is defined by means of skeletal elements and functionally represented polygons. The 3D model is obtained by implicit shape transformations of the defined objects between the modeling planes. This approach allows generation of the surfaces of organelles and explicit association of each model voxel with only one of the organelles. As a result, the condition of organelle exclusivity is fulfilled and the volume and surface densities of each organelle type in the model can be estimated. These features allow various applications such as a simulated electron microscopic experiment as the model allows generation of a set of random sections along any plane, and their projection to the image plane. Visualization of the model and its components can be achieved at various levels of detail and complexity. The software implementation with nowadays computational power allows creation of rather complex geometric models with all advantages of computer presentations and secondary processing like sectioning, zooming, and rotating. Supported by APVT - 51-31104.

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Mobile Phone Radiation Affects Gene Expression in Human Endothelial Cell Lines
D. Leszczynski; Research and Environmental Surveillance, STUK-Radiation and Nuclear Safety Authority, Helsinki, Finland
Induction of biological and health effects by mobile phone radiation remains a controversial issue. We have earlier proposed (Leszczynski & Joenvääri, Nature Genetics 2001, 27; 67; Leszczynski, Lancet 2001, 358, 1733) and demonstrated (Leszczynski et al., Proteomics, 2004, 4, 426-431) that using genome-wide and proteome-wide screening techniques will help identification of genes and proteins responding to mobile phone radiation. Mobile phone research has shown that mobile phone radiation activates cellular stress response by increasing the expression and phosphorylation of stress protein Hsp27 (Leszczynski et al., Differentiation 2002, 70, 120-129) what has a physiological impact on cells by induction of stabilization of stress fibers (Leszczynski et al., Proteomics, 2004, 4, 426-431). Also, mobile phone radiation affects expression of vimentin (Nyland & Leszczynski, Proteomics 2004, 4, 1359-1365; research presented in the ASCB 2004 Year Book). In the present study, we examined were the effects of 900 MHz GSM mobile phone radiation on the expression of 1176 genes using cDNA Expression Array (Clontech, USA) and two closely related human endothelial cell lines - EA.hy926 and EA.hy926v1. Obtained gene expression data were further analyzed with Ingenuity Pathways Analysis (Ingenuity Systems, USA). The expression of over 100 genes has been altered in both cell lines but different genes were affected in each of cell lines. This was reflected in identification of various canonical pathways and gene sets. In conclusion, cell response to mobile phone radiation may depend on the repertoire of genes (and proteins) that are expressed at the time of exposure - genome-dependent (and proteome-dependent) response. Therefore, it is possible that different types of cells from different species might respond differently to mobile phone radiation; i.e. might have different sensitivity to this weak stressor.

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Transient Anomalous Subdiffusion Due to Barriers
M. J. Saxton; Department of Biochemistry and Molecular Medicine, University of California, Davis, CA
Hindrances to diffusion may lead to transient anomalous subdiffusion, in which diffusion is anomalous at short times (mean-square displacement MSD = Dtα, α < 1) and normal at long times (MSD = Dt). The crossover from anomalous to normal diffusion is most clearly seen in a plot of log MSD versus log t. Transient anomalous subdiffusion has been observed by single-particle tracking experiments on Cajal bodies in the nucleus and receptors in the plasma membrane. Different mechanisms for transient anomalous subdiffusion differ in their sensitivity to thermalization. In Monte Carlo simulations, thermalization is accomplished by annealing, and in experiments on cells it is accomplished by treatment with metabolic energy inhibitors, with the caveat that metabolic energy depletion may affect obstacle structure and dynamics. Transient anomalous subdiffusion due to binding sites is removed by thermalization; that due to barriers is not; that due to a combination of binding sites and barriers, or an energy landscape model, is partially removed. A combination of binding sites and barriers is plausible for 2D diffusion in the plasma membrane and 3D diffusion in the cytoplasm and the nucleus. As a limiting case for the effect of barriers on diffusion, the bond percolation problem on the triangular lattice is examined. Here bonds are randomly chosen to be conducting or nonconducting. Monte Carlo simulations show how transient anomalous subdiffusion becomes more anomalous over longer times as the fraction of conducting bonds approaches the bond percolation threshold. Simulations also examine how many time points are required in a single-particle trajectory for the crossover to be detected reliably. Supported by NIH grant GM038133.

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Enabling Data Collection, Processing, Analysis, and Management: The Bioinformatics Resource Manager
M. Singhal, A. R. Shah, K. Klicker, K. M. Waters; Computational Biology and Bioinformatics, Pacific Northwest National Laboratory, West Richland, WA
The biological sciences have experienced rapid growth in the amount of data generated. This growth is a result of increasing availability of sequencing data and the development of new techniques to generate large datasets, but also facilitate the integration and analysis of datasets in the context of functional annotation information from public sources. This poster presents the Bioinformatics Resource Manager (BRM), a computational tool that takes into account the disparate nature of biological data to provide users with data retrieval, management, analysis and visualization capabilities through all aspects of an experimental study. BRM addresses a host of requirements for biologists, incorporating emerging technologies and concepts, to facilitate analysis of high-throughput biological data. This poster presents example workflows of BRM.
Reversing Endosymbiosis: the Path to an Independent Mitochondrion

M. Legua, A. Vila-Sanjuán, B. Zamfir, C. Bastamante; Synthetic Biology, University of California Berkeley/Lawrence Berkeley National Laboratory, Berkeley, CA

Little is known about the genes that collectively define the living state and how their function(s) are spatially and temporally integrated. Developing a minimal cell carrying only the essential genetic content necessary for survival in a maximally supportive environment is key to identifying the essential genetic elements of the living state we are using mitochondria as a platform to build a minimal cell -defined as a viable collection of genes and an enclosing envelope, such that the system can self-replicate in a particular niche. Our goal is to reverse endosymbiosis, not for the purpose of re-engineering a living microbe, but to build the simplest cell possible. Our approach is to systematically move genes from the nuclear to the mitochondrial genome. We begin by building a transcriptionally-independent mitochondrial that does not depend on nucleus-encoded proteins for mitochondrial gene expression. Four genes required for mitochondrial transcription, namely the mitochondrial RNA polymerase (POLRMT) and the transcription factors TFAM, TFB1M and TFB2M, are modified to comply with the mitochondrial genetic code and introduced into the mitochondrial genome. Following this transfer, the nuclear copies of these genes are deleted from the host genome. Here we describe the steps undertaken towards this goal, including efforts to engineer a modified mouse mitochondrial genome that carries the nucleus-encoded mitochondrial transcriptional machinery. We also discuss efforts to deliver and express recombinant DNA in mitochondria. The construction of a minimal cell is the first step towards a functional definition of the living state and a pre-requisite for the construction of whole synthetic organisms in the laboratory. This work warrants insight into a variety of fields, including the origins of life, bacterial evolution, control of metabolism and mitochondrial physiology, as well as several practical applications, such as the production biofuels and biopharmaceuticals, and environmental remediation.

The Bcl-2 Family Protein N-Bak Induces Structural Changes in Mitochondrial and Endoplasmic Reticulum Membranes

M. Yabal, M. Jakobson, E. Jokitalo, U. Arumé, M. Makarow; 1 Institute of Biotechnology, University of Helsinki, Helsinki, Finland, 2Department of Applied Chemistry and Microbiology, University of Helsinki, Helsinki, Finland

Bcl-2 proteins are regulators of programmed cell death that mediate apoptosis by regulating the integrity of endoplasmic reticulum (ER) and mitochondrial membranes via their Bcl-2 homology (BH) domains. In this study we examined the localization and activity of a novel member of the Bcl-2 protein family expressed only in central and peripheral neurons, N-Bak. N-Bak is a splice-variant of the ubiquitously (except in neurons) expressed pro-apoptotic Bak protein which comprises BH domains 1, 2 and 3. N-Bak is a BH3-only protein with a novel transmembrane domain at the extreme C-terminus. In neonatal sympathetic neurons N-Bak has been demonstrated to be anti-apoptotic whereas in non-neuronal cells and in cortical, hippocampal, and cerebellar granule neurons, it is pro-apoptotic. In sympathetic neurons N-Bak has been shown to localize to an undetermined intracellular membrane. In this study we aimed to elucidate the membrane target of N-Bak. We employed in vivo biochemical assays, fluorescence imaging, and electron microscopy in the yeast Saccharomyces Cerevisiae as well as mammalian non-neuronal cells, and primary sympathetic neurons. We demonstrate that N-Bak is not toxic for yeast cells and we detected the protein on mitochondrial outer membranes: N-Bak behaved as a peripheral membrane protein. In yeast cells, the active protein induced mitochondrial swelling. In Hela cells, N-Bak associated with both mitochondrial outer membranes and endoplasmic reticulum membranes, and induced mitochondrial membrane degradation. In sympathetic neurons, N-Bak induced degradation of mitochondrial outer membranes and proliferation of the endoplasmic reticulum membrane. These ultra-structural changes were dependent on both an active BH3-domain and the presence of the hydrophobic C-terminus. These results suggest that N-Bak is a BH3-only protein that triggers apoptosis via interactions with longer BH1-4 proteins that are missing in yeast. Also membrane targeting of N-Bak is dependent on these protein interactions with ER membrane proliferation being a neuron specific event.

Role of Bax and Bak in Mitochondrial Morphogenesis

K. L. Norris, M. Karbowski, M. Celander, R. J. Youle; 1SNB, NINDS, NIH, Bethesda, MD, 2George Washington University, Washington, DC, 3Johns Hopkins University, Baltimore, MD

Bcl-2 family proteins are potent regulators of programmed cell death in several animal phyla. These proteins are known to localize to the mitochondria and endoplasmic reticulum, focusing research on this family towards these organelles. However, the function of these proteins remains unknown. Two Bcl-2 family members that induce cell death, Bax and Bak, change intracellular location during apoptosis to concentrate into focal clusters at sites of mitochondrial division, leading us to investigate their potential role in mitochondrial morphogenesis. Bax activates mitochondrial fusion in healthy cells, forming elongated tubules. Cells of various lineages lacking Bax and Bak activity due to genetic knockout or vMIA inactivation have shorter and less interconnected mitochondria than wild-type cells. Bax regulates sub-mitochondrial distribution and membrane mobility of the fusion protein Mfn2. These results show that Bax and Bak regulate mitochondrial dynamics in healthy cells and that Bcl-2 members may regulate apoptosis through morphogenesis machineries. In contrast to Bax/Bak double knockout cells, mitochondrial fragmentation due to vMIA-induced inactivation of Bax/Bak is not reversed by ectopic Mfn2. This is likely due to vMIA-dependent disruption of Mfn2 localization to the outer mitochondrial membrane. Protein interaction with wild-type but not vMIA-deficient Mfn2 appears cytosolic in cells expressing vMIA. However, fluorescence recovery after photobleaching analysis in cells expressing vMIA revealed a phenotype whereby Mfn2 was more mobile within the cell than normal membrane-bound Mfn2, yet less mobile than the soluble GFP protein. The implications of these results will be discussed.

Characterization of Charcot-Marie-Tooth Associated MFN2 Mutations in Patient-derived Fibroblasts and the Yeast Mitofusin FZO1

E. A. Amiot1, P. Pott, Y. Saint-Georges, K. Flanigan, V. Lawson, J. M. Shaw; 1Biochemistry, University of Utah, Salt Lake City, UT, 2Human Genetics, University of Utah, Salt Lake City, UT, 3Ecole Normale Superieure, Laboratoire de Génétique Moléculaire CNRS, Paris, France, 4Neurology, University of Utah, Salt Lake City, UT

Charcot-Marie-Tooth (CMT) disorders are a group of inherited peripheral neuropathies categorized as either demyelinating (CMT1) or axonal (CMT2) disorders. Genetic studies have determined that the most common axonal subclass, CMT2A2, is caused by mutations in MFN2, one of two human mitofusins genes homologous to the yeast mitochondrial fusion protein, Fzo1p. Mitofusins are evolutionarily conserved GTPases localized to the outer mitochondrial membrane and are required for proper maintenance of tubular mitochondrial morphology. When fusion is impaired, unopposed division leads to fragmentation of the mitochondrial network and impaired mitochondrial function. We obtained primary fibroblasts from skin biopsies of CMT2A2 patients carrying different MFN2 mutations and representing a range of clinical phenotypes, from mild to severe. Preliminary studies reveal that these fibroblasts have normal mitochondrial morphology, fusion competence, mtDNA content and integrity, and respiratory capacity. However, the equivalent mutations in Fzo1p, the yeast counterpart of Mfn2, lead to a variety of defects, ranging from subtle distortion of the mitochondrial network to disrupted fusion, complete fragmentation of mitochondria, and loss of mtDNA. Interestingly, the yeast phenotypes correlate with the clinical severity of patients. Yeast co-expressing WT and CMT mutant forms of Fzo1p exhibit normal mitochondrial dynamics, indicating that these mutations are not dominant-negative and possibly explaining the lack of observable phenotypes in the fibroblasts. The yeast results also suggest that Mfn2 mutations and representing a range of clinical phenotypes, from mild to severe. Preliminary studies reveal that these fibroblasts have normal mitochondrial morphology, fusion competence, mtDNA content and integrity, and respiratory capacity. However, the equivalent mutations in Fzo1p, the yeast counterpart of Mfn2, lead to a variety of defects, ranging from subtle distortion of the mitochondrial network to disrupted fusion, complete fragmentation of mitochondria, and loss of mtDNA. Interestingly, the yeast phenotypes correlate with the clinical severity of patients. Yeast co-expressing WT and CMT mutant forms of Fzo1p exhibit normal mitochondrial dynamics, indicating that these mutations are not dominant-negative and possibly explaining the lack of observable phenotypes in the fibroblasts. The yeast results also suggest that Mfn2 mutations carry a second mutation, FZO1. The majority of CMT-related MFN2 mutations are located within the GTPase domain of the protein. We show for the first time that Fzo1p is a bona fide GTPase and are currently testing whether CMT mutations in mitofusins disrupt GTP hydrolysis and whether this may be an underlying molecular cause of the CMT2A2 neuropathy.

Regulation of Mitochondrial Fission by the Mitochondrial Outer Membrane Protein hFis1

M. Serasinge, Y. Yoon; 1University of Rochester, Rochester, NY

Mitochondrial fission in mammalian cells is mediated by at least two proteins, DLP1/Dplp1 and hFis1. DLP1 mediates the scission of mitochondrial membranes through GTP hydrolysis, and hFis1 is a putative DLP1 receptor anchored at the mitochondrial outer membrane by a single transmembrane domain at the C-terminus. The cytosolic domain of hFis1 contains six α-helices (α1-6) that form two tetratricopeptide repeat (TPR) folds. The current study focuses on identifying hFis1-containing complexes and defining the mechanisms of complex formation. We have found that mutations in the hFis1 TPR folds lead to a dominant-negative effect, suggesting that the mutant may interact with endogenous hFis1 or other fission components to inhibit mitochondrial fission. Upon crossinglink of cells expressing full-length hFis1, we observed an appearance of only a small amount of dimeric-size hFis1. However, deletion of the N-terminal first α-helix (α1) greatly increased the formation of dimer and oligomer-size hFis1. These results suggest that hFis1 forms multimeric complexes and that α1 functions as a negative regulator of the
Multimer formation. Further deletions decreased the formation of hFis1 oligomers. Swollen ball-shaped mitochondria were prevalent in cells overexpressing α1-deleted hFis1, and the extent of hFis1 oligomer formation among different deletions was correlated with the ball-shape mitochondria formation. In vitro crosslinking of the purified hFis1 cytosolic domain (α1-α6) did not form any detectable dimer or other complexes. Interestingly, the deletion of α1 (α2-α6) was able to form hFis1-containing complexes distinct from dimeric and oligomeric forms, only in the presence of cell lysis, suggesting that other cellular factors are necessary for hFis1 complex formation. Abnormally elongated mitochondria markedly increased in cells overexpressing α2-α6, suggesting that this mutant contains essential fusion factors in the cytosol and inhibits mitochondrial fusion. These data indicate that hFis1 complex formation requires additional proteins and is regulated by the α1 helix.

Mutations of the Dynamin-like GTPase Drp1 That Affect Self Assembly and Mitochondrial Morphology
C. Chang, J. Stadler, C. Blackstone; NINDS, NIH, Bethesda, MD
The dynamin-related GTPase Drp1 is critical for mitochondrial fission. Previous studies have suggested that Drp1 monomers are assembled into oligomeric ring-like structures at mitochondrial fission sites to construct the outer membrane in a GTP-dependent manner. However, the mechanisms of fission complex assembly and regulation remain unclear, particularly in mammals. The Drp1 protein comprises four structural domains: N-terminal GTP-binding, middle, insert B, and C-terminal GTPase effector (GED) domains. The middle and GED domains are critical for stabilizing higher order structures in Drp1 and the S. Cerevisiae ortholog Dnm1. We have demonstrated that mutations within the middle domain of Drp1, G359D and G363D, alter its oligomerization status, impair its GTPase activity, and interfere with intramolecular associations between GED and middle-GTPase domains. Over-expression of these mutant Drp1 proteins causes tubulation of the mitochondria by inhibiting mitochondrial fission. These findings are consistent with, and extend, those evaluating corresponding mutations in yeast. Lastly, we have identified several sites of post-translational modification by protein phosphorylation and sumoylation in the C-terminal domains of Drp1, and are currently evaluating the effects of these modifications on Drp1 properties and function. Our results provide insights into the interactions and modifications of Drp1, which will clarify regulatory mechanisms of mitochondrial dynamics in cells.

A Conserved Role for Miro Proteins in Mitochondrial Distribution and Morphology
R. L. Frederick, K. Okamoto, J. M. Shaw; Department of Biochemistry, University of Utah, Salt Lake City, UT
The conserved family of Miro GTPases has been implicated in mitochondrial dynamics and distribution in yeast, insects, and mammals. Miro proteins contain two GTPase domains flanking a pair of calcium binding EF hand motifs. We previously demonstrated that yeast cells require the yeast Miro Gem1p to maintain a branched tubular mitochondrial network. In a wild-type yeast, mitochondria form globular structures; collapsed tubules, and grape-like clusters. Gem1p is a tail-anchored outer mitochondrial membrane protein with the GTPases and EF hand motifs exposed to the cytoplasm. Recent work has revealed that the fly and mammalian Miro family members bind the kinesin adapter protein Milton, possibly forming a linker between microtubules and mitochondria (Glater et al 2006, JCB; Fransson et al 2006, BBRC). Consistently, fly miro mutants lack synaptic mitochondria (Gao et al 2005, Neuron). Although the Miro family is conserved, yeast utilize the actomyoskeleton for mitochondrial movement and do not contain an identifiable Milton homolog. Budding yeast grow in a polarized manner and require faithful mitochondrial inheritance to survive, making them an ideal system to study polarized mitochondrial movements. To understand the relationship between Miro and other cellular pathways, we initiated a synthetic lethal screen in yeast. Synthetic genetic interactions between gem1Δ and mutations in other mitochondrial inheritance pathways suggest that the transport function of Miro may be conserved. Altering the distribution of mitochondria between the mother and daughter cells demonstrates that the absence of Gem1p does not impair other mitochondrial inheritance pathways. Thus, multiple overlapping pathways, including a Miro-dependent pathway, are likely required for mitochondrial inheritance. The properties of Miro proteins are currently investigated.

Mitochondrial Dynamics Participates in Overproduction of Reactive Oxygen Species and Cell Injury in Hypertension
T. Yu, 1 J. L. Robotham, 2 R. J. Fox, 1 Y. Yoon, 1 Department of Anesthesiology, University of Rochester, Rochester, Rochester, NY 3 Department of Anesthesiology, Pharmacology and Physiology, University of Rochester, Rochester, NY
Mitochondria in cells display a dynamic behavior, showing constant shape change. However, the functional implication of dynamic change of mitochondrial morphology is not well understood. We observed a rapid change of mitochondrial morphology in cells exposed to high glucose concentrations. Hyperglycemia is a main cause of diabetic complications, leading to oxidative stress through overproduction of reactive oxygen species (ROS) resulting from mitochondrial dysfunction. Therefore, in the present study, we investigated the role of mitochondrial dynamics in regulating mitochondrial function in the hyperglycemic setting. We found that, upon exposure to high glucose concentrations, mitochondria underwent rapid fragmentation and recovery within an hour, which coincided with increase and decrease of ROS levels. Preventing ROS increase in high glucose conditions still caused mitochondrial fragmentation. Remarkably, maintaining tubular mitochondria in high glucose incubation by DLPI-Κ38A, a dominant-negative mutant of the fission protein DLPI, prevented high glucose-induced ROS overproduction. These results demonstrate that mitochondrial fragmentation in high glucose conditions is a causal factor for ROS overproduction. Sustained incubation in high glucose concentrations, which may mimic untreated diabetic conditions, provoked a prolonged ROS increase for the second time after the initial transient increase. Again, the mitochondrial morphology change occurred concomitant with ROS increase. We found an increased cell death during the sustained high glucose incubation. The high glucose-mediated cell death was apoptotic, as the mitochondrial permeability transition pore (MPT), cytochrome c release, and caspase 3 activation were observed in these conditions. DLPI-Κ38A prevented the prolonged ROS increase during extended exposure to high glucose concentration, and thereby the MPT, cytochrome c release, caspase 3 activation, and cell death were subsequently decreased. These findings suggest that mitochondrial morphology controlled by membrane fission and fusion is an integral factor that regulates the mitochondrial ROS production and cell injury in high glucose conditions.

Mitochondrial Clustering Induced by Overexpression of the Mitochondrial Fusion Protein Mfn2 Causes Mitochondrial Dysfunction and Cell Death
P. Huang, 1 Y. Yoon, 1 Department of Pharmacology and Physiology, University of Rochester School of Medicine and Dentistry, Rochester, Rochester, NY 3 Department of Anesthesiology and of Pharmacology and Physiology, University of Rochester School of Medicine and Dentistry, Rochester, NY
Mitochondria change their shapes dynamically mainly through fusion and fission. Dynamin-related GTPases have been shown to mediate remodeling of mitochondrial membranes during these processes. Overexpression of GTPase-defective Mfn2, mfn2, is anchored to the outer mitochondrial membrane in the outer membrane. We found that overexpression of a mitofusin isomorph, Mfn2, drastically changes mitochondrial morphology, forming mitochondrial clusters. A GTPase-defective Mfn2 mutant, Mfn2-K109A, induced the formation of mitochondrial clusters indistinguishable from those seen with the wild-type overexpression, indicating that formation of mitochondrial clusters is independent of the GTPase activity of Mfn2, and that mitochondrial clusters are not the result of mitochondrial fusion. High-resolution microscopic examination indicated that the mitochondrial cluster consisted of small fragmented mitochondria
and overexpressed Mfn2 was concentrated at the interstitial region of mitochondrial aggregate. Inhibiting mitochondrial fission prevented the cluster formation, supporting the notion that mitochondrial clusters are formed by fission-mediated mitochondrial fragmentation. Fragmented mitochondria in the cluster showed slightly larger diameters than control mitochondria, indicating that they undergo mild fission. JC-1 and TMRM assays indicated that mitochondrial clusters displayed a decreased inner membrane potential, suggesting functional compromise of small fragmented mitochondria by Mfn2 overexpression. However, mitochondrial clusters still retained mitochondrial DNA. Greatly decreased cell survival was observed among cells containing mitochondrial clusters, suggesting cell death occurred. We found that cells containing clustered mitochondria lost cytochrome c from the mitochondria, indicating that the extensive mitochondrial fragmentation by Mfn2 overexpression caused cytochrome c release from mitochondria. Inhibition of caspase activity maintained cell survival at a steady level and caspase 3 was activated in cells transfected with Mfn2, indicating that cells containing mitochondrial clusters underwent caspase-mediated apoptosis. These results demonstrate that mitochondrial deformation impairs mitochondrial function, leading to apoptotic cell death and suggest the presence of an intricate form-function relationship in mitochondria.

556 Structural, Functional Features, and Significance of the Physical Linkage between SR/ER and Mitochondria
G. Csordas,1 C. Renken,2 C. Garcia-Perez,2 P. Varnai,1 L. Walter,1 T. Balla,1 C. A. Mannella,1 G. Hajneczky;1 Pathology, Anatomy and Cell Biology, Thomas Jefferson University, Philadelphia, PA;2Resource for Visualization of Biological Complexity, Wadsworth Center, Albany, NY;3Endocrinology and Reproduction Research Branch, National Institute of Child Health and Human Development, Bethesda, MD

Mitochondrial calcium signaling is commonly mediated at the sites of close associations between SR/ER and mitochondria. However, the microstructure of the ER-mitochondrial interface and its relevance for cell function remains elusive. Here we show that ER and mitochondria are physically coupled and directly visualize the tethering structures by electron tomography. The length of the tethers is ~10nm at the smooth-ER and ~25nm at the rough-ER. Limited proteolysis loses the ER-mitochondrial associations and disconnects mitochondrial Ca2+ uptake from the IP3 receptor-mediated Ca2+ release. Conversely, expression of a short ‘synthetic ER-mitochondrial linker’ (~5nm), leads to tightening of the ER-mitochondrial associations to permit the propagation to the mitochondria of Ca2+ release that normally would be unrecognized. Under these conditions, mitochondria become prone to Ca2+ overloading and ensuing membrane permeabilization. Tightening of the ER-mitochondrial associations also appears in cells exposed to some pro-apoptotic stimuli. Thus, the tether length controls the Ca2+ relay function of the ER-mitochondrial interface to support either cell survival or cell death mechanisms. Similarly, we observed SR vesicles tightly coupled with mitochondria in rat heart homogenate that preserved the potency to generate ryanodine receptor dependent Ca2+ increases in the mitochondrial matrix as measured by fluorescence imaging of the vesicles glued to coverslips. Western blot analysis of the lysates of rat heart mitochondria confirmed the presence of SR markers (phospholamban, calsequestrin, SERCA2a) which got reduced after limited trypsinization or exposure to high ionic strength (750 mM NaCl). The latter result suggests that a non-covalent protein-protein interaction may be important in the establishment of physical linkage between the SR and mitochondria. Thus, physical linkage appears as a unifying support/control mechanism for the local Ca2+ couplings between the reticular stores (both SR and ER) and mitochondria.

557 Oxa1 Directly Interacts with ATP9 and Mediates Its Assembly into the F1F0-ATP Synthase
L. Jia, M. H. Bi, R. Huang, A. Sturtevant, J. T. Brunger, and M. A. Wittingham;1 Department of Biological Sciences, Marquette University, Milwaukee, WI

Oxa1 is a member of conserved Oxa1/YidC/Alb3 protein family. In the yeast Saccharomyces cerevisiae, deletion of the Oxa1 gene causes loss of mitochondrial respiratory function due to the impaired biogenesis of three respiratory complexes, of which cytochrome c oxidase complex (COX) is the most affected. Oxa1 was identified to be essential for the co-translational insertion of COX. Our data suggest that interaction between Oxa1 and Atp9 following its synthesis by the ribosome is necessary to ensure the efficient and correct assembly of the Atp9 oligomers, and thus important for the stable assembly of the Fo-sector of the ATP synthase. We demonstrate that Oxa1 directly interacts with Atp9 following its synthesis by in organello translation and affinity purification. In the absence of Oxa1, although newly synthesized Atp9 forms oligomers, the organization is altered. Our data suggest that interaction between Oxa1 and Atp9 is necessary to ensure the efficient and correct assembly of the ATP9 oligomers, and thus important for the stable assembly of the Fo-sector of the ATP synthase.

558 Ubiquitin Mediated Protein Quality Control at the Mitochondria
S. Radke,1 X. Xu,2 Z. Pan,2 D. Germain;11 Oncology/Hematology, Mount Sinai School of Medicine, New York, NY;2Derald H. Ruttenberg Cancer Center and Department of Human Genetics, Mount Sinai School of Medicine, New York, NY

Protein quality control (PQC) is required for the proper folding of newly synthesized proteins. It is ensured by a complex of different proteins that may involve chaperones, co-chaperones, ubiquitin ligases and proteases like the proteasome. If the structure of a protein remains misfolded and cannot be properly folded with the help of the chaperone complex, its elimination can be induced via ubiquitination and proteasomal degradation. PQC has been reported in different cellular compartments including the endoplasmic reticulum and the nucleus. In the mitochondria, AAA-proteases control an ubiquitin independent PQC. Our data show that there is an additional ubiquitin-dependent PQC for proteins residing in the intermembrane space (IMS) of the mitochondria. We found that the IMS chaperone BAP37 interacts with the recently identified F-box protein Skp2B. Using in vitro ubiquitination assay, we demonstrate that Skp2B associates with a ubiquitin ligase activity. Both Skp2B and BAP37 bind to IMS proteins such as cytochrome C, smac and endonuclease G whose ubiquitination depends on Skp2B. However binding and ubiquitination of misfolded IMS proteins are markedly elevated compared to wild-type proteins. Furthermore, we show by electron microscopy and aggresome assay that overexpression of misfolded cytochrome c promotes mitochondrial aggresome formation with strongly aberrant morphology. This results in cytochrome C release and apoptosis. Skp2B overexpression reduces aggresome formation and conversely, Skp2B downregulation enhances the formation of these aberrant structures. In conclusion, our results indicate a novel role of the ubiquitin pathway at the mitochondria involving the chaperone protein Bap37 and the F-box protein Skp2B as necessary for the detection and ubiquitination of misfolded IMS proteins respectively. The elimination of accumulating misfolded proteins is crucial to avoid the formation of mitochondrial aggresomes and is therefore highly important for the integrity of the mitochondria.

559 Dynamics of Respiratory Chain Complexes in Mitochondria
K. B. Busch,1 J. Berete-Hahn,1 I. Wittig,1 H. Schagger,1 M. Jendrach1;1Cell Biology and Neuroscience, Kinematic Cell Research, Frankfurt, Germany;2Molekulare Bioerggetik, Universitaet Frankfurt, Frankfurt, Germany

Mitochondrial dynamics play a key role in cell life, ageing and death. It is hypothesized that here the rescue mechanism is provided to maintain a healthy chondriome by constant mixing of mitochondrial compounds. Improvement of mitochondrial compounds, in particular by ROS (reactive oxygen species), is a severe problem during the life span of a cell. Mitochondrial fusion may allow the exchange of constituents (proteins, lipids and mitochondrial DNA) and thus maintaining functionality. We address the question whether complexes of the respiratory chain are exchanged during mitochondrial fusion. The main complexes of the respiratory chain form superfamilies: Complexes I, III, and IV are probably organized in respirasomes or even respiratory strings (Wittig et al, 2006). Complex V has a distinct organization. The dynamics of Complex I were followed during fusion and fission. HeLa cells with differently labeled mitochondrial Complex I were fused and the re-localization of Complex I was monitored. The tagged complex I spread throughout the whole mitochondrial population within 3 to 6 h after induction of cell fusion, substantiating the view of a homogenous mitochondrial population due to constantly rearranging mitochondria. Mitochondria of fused cells displayed a patchy substructure where the differently labelled proteins occupied separate and distinct spaces (Busch et al., 2006), indicating the existence of long-lasting sub-mitochondrial structures. In young HUVECs cells, Complex I tagged by photoactivatable EGFP showed a fast transfer rate due to mitochondrial fusion. We propose that this is reduced in old (postmitotic) HUVECs mitochondria, which display decreased dynamics (Jendrach et al., 2005). Mobility studies with labelled Complex II and IV shall complement the current results.

560 Mitochondrial Magnesium Imaging with Novel Fluorescent Probe
H. Komatsu,1 Y. Shinoh2, T. Kubota,1 K. Hotta,1 K. Oka,1 K. Suzuki1,3;1Applied Chemistry, Keio University, Yokohama, Japan;2Bioscience and Bioinformatics, Keio University, Yokohama, Japan;3JST-CREST, Kawasaki, Japan

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Mitochondria are the organelles responsible for cell death and proliferation that also work as ‘magnesium’ stores. Mg²⁺ is considered to play important roles in the modulation of mitochondrial functions such as oxidative phosphorylation and apoptosis and also in mitochondrial disorder based neural diseases like migraine. Thus, we focused on the development of imaging probes and systems to monitor the dynamics of Mg²⁺ in mitochondria. Rhodamine-based Mg²⁺ fluorescent probes were designed, which localize in mitochondria. KMG-301 has a charged beta diketone Mg²⁺ selective binding site and rhodamine as a fluorophore in order to localize in mitochondria. KMG-301 showed successful Mg²⁺ specific fluorescent increase. Mitochondrial localization of KMG-301 was confirmed by double-staining with GFP-Mito. Mitochondrial Mg²⁺ dynamics were evaluated using isolated mitochondria. Isolation was confirmed by AFM and fluorescence image. FCCP induced Mg²⁺ release and fluorescence decrease of KMG-301 in mitochondria, showing that KMG-301 functions inside mitochondria. Further, intracellular and mitochondrial Mg²⁺ changes induced by FCCP were simultaneously imaged by double staining with KMG-104 (cytoplasmic Mg²⁺) and KMG-301 (mitochondrial Mg²⁺). Mitochondrial Mg²⁺ decrease was slightly faster than cytosolic Mg²⁺ increase. Thus, we can conclude that KMG-301 successfully measured intramitochondrial release of Mg²⁺ in a living PC12 cell. Here, we developed a novel rhodamine-based Mg²⁺ fluorescent probe (KMG-301) and a Mg²⁺ imaging system inside and outside of mitochondria. The correlation between the Mg²⁺ and the main of mitochondrial functions (oxidative phosphorylation, apoptosis) related to many neural diseases can be clarified by ‘mitochondrial magnesium imaging’.

ATP-induced Intracellular Ca²⁺ Dynamics in Fibroblasts of Rats


High Resolution Analysis of Common Human L-type Mitochondrial DNA (mtDNA) Haplotypes


β-cell Mitochondria Exhibit Subcellular Functional Heterogeneity That Can Be Altered by Stimulatory or Toxic Fuel Levels

A. Molina, S. E. Haigh, H. Mohamed, J. Wikstrom, G. Twigg, S. Katzman, B. Corkey, N. Danial, O. Shiriati

The Essential Role of Syndaplin in the Control of Axonal Mitochondrial Motility


β-cell Mitochondria Exhibit Subcellular Functional Heterogeneity That Can Be Altered by Stimulatory or Toxic Fuel Levels

A. Molina, S. E. Haigh, H. Mohamed, J. Wikstrom, G. Twigg, S. Katzman, B. Corkey, N. Danial, O. Shiriati

Obesity Research Center, Boston University, Boston, MA; 2Pharmacology, Tufts University, Boston, MA; 3Dana-Farber Research Institute, Harvard Medical School, Boston, MA

Objective: To investigate functional heterogeneity within pancreatic β-cell mitochondria.

Background: In the β-cell, mitochondrial membrane potential (ΔΨm) increases in response to glucose. This results in the production of metabolites which serve as insulin secretory signals. It is not clear, matches to the most common mtDNA haplotypes in sub-Saharan Africa. Here we looked to further test these common African mtDNA haplotypes which belong to the L-subclade. These types

Uncertain mutation rates and sampling errors. Despite this many people in the African Diaspora believe this is the only means of reconnecting to their ancestral origins in Africa that has been

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University of South Carolina, Columbia, SC

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β cells were isolated from C57BL6 mice and plated on glass slide dishes, or the Optical LiveCell Array for confocal microscopy. Cells were loaded with the mitochondrial dye TMRE and Mito Tracker Green and imaged using a Zeiss confocal microscope. Mitochondrial matrix targeted photo activatable GFP (PA-GFP) was used to tag and track the ΔΨm of individual mitochondria over time. Results: β-cell mitochondria are functionally heterogeneous, displaying great heterogeneity in ΔΨm values. Tracking individual mitochondria over time reveals that ΔΨm differences between mitochondria persist and do not result in instability. Furthermore, when expressed as mV this range is considerably greater than the change in mV induced by fuel challenge. Upon acute glucose stimulation the range in ΔΨm narrows, indicating recruitment of previously inactive mitochondria into the active pool. Exploration of the mechanism behind heterogeneity revealed BAD, previously implicated in mitochondrial recruitment of glucokinesis, as a significant factor influencing the level of heterogeneity. Chronic exposure to a rich nutrient environment, in an in vitro diabetes model, leads to increased ΔΨm heterogeneity which is a result of increased mitochondrial fission and reduced network size. Conclusions: We suggest that mitochondrial ΔΨm heterogeneity in beta cells represents a metabolic reservoir that is recruited by increased levels of fuels and therefore may serve as a therapeutic target.

β-cell Mitochondria Exhibit Subcellular Functional Heterogeneity That Can Be Altered by Stimulatory or Toxic Fuel Levels

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Obesity Research Center, Boston University, Boston, MA; 2Pharmacology, Tufts University, Boston, MA; 3Dana-Farber Research Institute, Harvard Medical School, Boston, MA
Integral membrane proteins are maintained at the plasma membrane by an intricate balance of secretion, endocytosis, recycling, and protein quality control in eukaryotic cells. We have used the trafficking of a model plasma membrane protein, the yeast arginine permease Can1, as a reporter to understand the mechanisms involved in membrane protein trafficking and quality control. Can1 is transported to the plasma membrane via the secretory pathway and can subsequently be internalized into an endosomal compartment via ubiquitin-dependent endocytosis. Once there, depending on nutrient conditions, Can1 is either actively recycled back to the plasma membrane or delivered to the vacuole for degradation via the multivesicular body (MVB) pathway, which is dependent on the function of three endosomal complexes required for transport (ESCRTs). We conducted a screen of the yeast nonessential gene deletion collection for mutants that exhibit either resistance or hypersensitivity to the toxic arginine analog canavanine. Genes that come through the screen function in several different cellular pathways including vesicular trafficking, protein quality control, lipid metabolism, cytoskeletal organization, and ubiquitin-mediated signaling and catabolism. Many of the known VPS genes, such as class E VPS genes that encode components of the ESCRT complexes, came through the screen as canavanine sensitive (CVS), while genes with protein products that function in recycling and secretion of Can1 came through as canavanine resistant (CVR). We identified Cvr22, a protein that localizes to endosomal compartments and interacts with known ESCRT complex components. We will report on the characterization of Cvr22 and its role in the MVB sorting pathway.

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Did2 Coordinates Vps4-mediated Dissociation of ESCRT-III from Endosomes
D. P. Nickerson, M. C. West, G. Odorizzi; MCD Biology, University of Colorado, Boulder, CO
The sorting of transmembrane cargo proteins into the lumenal vesicles of multivesicular bodies (MVBs) depends upon the recruitment of Endosomal Sorting Complexes Required for Transport (ESCRTs) to the cytosolic face of endosomal membranes. The subsequent dissociation of ESCRT complexes from endosomes requires Vps4; a member of the AAA family of ATPases associated with a variety of cellular activities. We show that Did2 directs Vps4 activity to the dissociation of ESCRT-III but has no role in the dissociation of ESCRT-I or ESCRT-II. Surprisingly, vesicle budding into the endosome lumen occurs in the absence of Did2 function even though Did2 is required for efficient sorting of MVB cargo proteins into lumenal vesicles. This uncoupling of MVB cargo sorting and lumenal vesicle formation suggests that Vps4-mediated dissociation of ESCRT-III is an essential step in the sorting of cargo proteins into MVB vesicles but is not a prerequisite for budding of vesicles into the endosome lumen.

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Regulation of Multivesicular Body Formation by Nvr1
C. Dimamo, C. B. Jones, M. Babst; Biology, University of Utah, Salt Lake City, UT
Endosomal compartments called multivesicular bodies (MVBs) facilitate the sorting of transmembrane proteins from the cell surface to the lumen of lysosomes/vacuoles for degradation. Class E Vps protein complexes including the ESCRT (-I, -II, -III) complexes and the AAA-ATPase Vps4 are recruited to the MVB membrane and cooperate to form cargo-containing vesicles for vacuolar delivery. Vps4 is known to play a terminal role in the vesicle-forming process at the MVB, but how Vps4 function is regulated is not well understood. We have previously shown that the class E protein Vti1 positively regulates Vps4 ATPase activity (Azmz et al., 2005). Here, we describe a novel regulator of Vps4 function that we have designated Nvr1 (for negative regulator of Vps4). We have determined that Nvr1 localizes to the endosomal membrane and interacts with Vps4 in the sorting process. Under normal growth conditions, Nvr1 is rapidly degraded by the proteosomal system. Stabilization or overexpression of Nvr1 prevents Vps4-dependent recycling of ESCRT complexes from the endosomal membrane, which results in MVB sorting defects. We have also found that Nvr1 directly binds to Vps4 thereby regulating Vps4 activity. Our findings indicate that the cellular levels of Nvr1 regulate MVB trafficking through the interaction of Nvr1 with Vps4. Progress toward the dissection of Nvr1 function will be presented.

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Cell-free Reconstitution of Membrane Fission
L. Michailat, A. Mayer; Biochemistry, University of Lausanne, Epalinges, Switzerland
Most organelles in eukaryotic cells undergo membrane fission events in order to ensure correct organelle transmission, and homeostasis. Fission of membrane is often dynamin-dependent. In general, little is known about the fission mechanism, nor about other factors involved. The vacuole of budding yeast Saccharomyces cerevisiae is used as a model for membrane fission. Vesicles undergo constant fission and fusion, with an average of 1-3 vacuoles per cell in the steady-state. As lysosomal compartments, vacuoles play a role for the turnover of proteins and lipids, for storage and transport of ions and amino acids, and for osmoregulation. Fusion of vacuoles can be induced in vivo by application of an osmotic shock to the cells. Simple macroscopic inspection of cells has allowed the identification of numerous factors involved in vacuole fission. We have now reconstituted the process of fission in vitro, which permits to study the mechanisms involved in detail. Conditions to induce fission of purified vacuoles in vitro have been identified. In vitro fission reproduces the physiological requirements of the in vivo reaction, because it is sensitive to the same mutations and inhibitors as vacuole fission in living cells. The ability to study physiological membrane fission in vitro opens exciting perspectives because yeast vacuoles are big enough (1-3nm) to be conveniently visible in the light microscope. When combined with new technology to fix individual organelles on solid supports and/or to quantitate and kinetically dissect the fission process, e.g. by dynamic light scattering, our in vitro system generates a powerful tool to elucidate the molecular events in a membrane fission reaction.

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Chemical Genetic Identification of Inhibitors of Membrane Traffic
M. C. Duncan, D. G. Ho, M. E. Jung, G. S. Payne; 1Biological Chemistry, UCLA-Medical School, Los Angeles, CA, 2Chemistry and Biochemistry, UCLA, Los Angeles, CA
The field of membrane trafficking has a long history with use of chemical inhibitors that can allow greater flexibility than genetic techniques such as knockout and knockdown strategies. The spectrum of membrane trafficking events with potent chemical inhibitors remains limited. However, the availability of large libraries of diverse compounds has opened opportunities for identification of small molecules altering currently untargeted events. Growth based screening provides several advantages: compounds identified are cell permeant and functional in metabolically active cells; screening requires simple plate readers, common reagents and little optimization. In general, reduced or enhanced growth is a non-specific phenotype. We recognized that synthetic lethal genetic interactions, a phenotype commonly investigated in genetic research, provides a facile strategy to confer specificity for particular cellular processes to a growth based assay. We made use of well characterized synthetic genetic growth phenotypes as a guide for selecting in vivo chemical induced growth phenotypes. A family of compounds identified by compound-specific lethal screening in yeast cause effects that mimic phenotypes of deletions of clathrin adaptor AP-1 substrates, a major component in vesicle traffic between the Golgi network (TGN) and endosomes. Importantly these effects are specific to traffic between the TGN and endosomes and do not appear to alter other membrane traffic pathways. These novel piparrazinyl compounds should be useful for further studies of membrane traffic in yeast, and potentially in other. Furthermore, the easily automated technology may be adapted to identify inhibitors of a wide variety of cellular processes.

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Regulation of P(3,5)P2 Phosphoinositide Lipid Signaling and Its Role in Membrane Traffic and Vascular Dynamics
R. J. Botelho, J. A. Efe, S. Emr; 1Cellular and Molecular Medicine, University of California San Diego, La Jolla, CA, 2Division of Biology, University of California San Diego, La Jolla, CA, 3Howard Hughes Medical Institute, La Jolla, CA
Integral membrane proteins are maintained at the plasma membrane by an intricate balance of secretion, endocytosis, recycling, and protein quality control in eukaryotic cells. We have used the trafficking of a model plasma membrane protein, the yeast arginine permease Can1, as a reporter to understand the mechanisms involved in membrane protein trafficking and quality control. Can1 is transported to the plasma membrane via the secretory pathway and can subsequently be internalized into an endosomal compartment via ubiquitin-dependent endocytosis. Once there, depending on nutrient conditions, Can1 is either actively recycled back to the plasma membrane or delivered to the vacuole for degradation via the multivesicular body (MVB) pathway, which is dependent on the function of three endosomal complexes required for transport (ESCRTs). We conducted a screen of the yeast nonessential gene deletion collection for mutants that exhibit either resistance or hypersensitivity to the toxic arginine analog canavanine. Genes that come through the screen function in several different cellular pathways including vesicular trafficking, protein quality control, lipid metabolism, cytoskeletal organization, and ubiquitin-mediated signaling and catabolism. Many of the known VPS genes, such as class E VPS genes that encode components of the ESCRT complexes, came through the screen as canavanine sensitive (CVS), while genes with protein products that function in recycling and secretion of Can1 came through as canavanine resistant (CVR). We identified Cvr22, a protein that localizes to endosomal compartments and interacts with known ESCRT complex components. We will report on the characterization of Cvr22 and its role in the MVB sorting pathway.

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Myotubularin Phosphoinositide Phosphatase Regulates Membrane Homeostasis in Drosophila Cells

M. Velickova, J. Dennis, A. Kiger; Cell and Developmental Biology, University of California at San Diego, La Jolla, CA

The regulation of specific phosphoinositide phosphatases (PIPs) provides spatial control for distinct cellular processes, including cytoskeletal organization, protein sorting, membrane trafficking and membrane homeostasis. Using RNAi in Drosophila Kc cells, we identified myotubularin (mtm), a predicted phosphoinositide phosphatase, with a function important for an induced change from a round to an elongated cell shape. PI(3)P and PI(3,5)P2, the predicted substrates of myotubularin phosphatases, have been localized throughout the endolysosomal system. To identify the PIP-depleted cell process required for cellular elongation, we examined mtm-depleted cells for defects in the function of membranous organelles in kinetic cell-based assays. Whereas no defects were detected in early endosomes (Rab5), RNAi knock down of mtm resulted in enlarged (3x) late endosomes and lysosomes (Rab7-GFP, LAMP1-GFP, LysoTracker), as indicated by quantitation of fluorescent images. The rate of fluid phase transport of fluorescent Dextrans was significantly delayed, further suggesting a role for mtm in endolysosomal homeostasis. Mtm depleted cells exhibited an accumulation of the PI(3)P reporter, GFP-2xFYVE, suggesting that PI(3)P is a substrate of mtm in Drosophila. To test whether mtm cellular roles are mediated through phosphoinositides, we assessed the consequence of modulating phosphoinositide levels by reducing the opposing phosphoinositide kinase function. Co-RNAi of mtm and the Drosophila PI(3)P kinases DIP60 or DIP45, resulted in reduced accumulation of PI(3)P, indicating a role for mtm in phosphoinositide recycling. We propose that myotubularin-mediated turnover of the PI(3)K65D generated pool of PI(3)P involved in regulation of membrane homeostasis of the endolysosomes, which subsequently may be important for cellular morphogenesis. Currently we are investigating the role of mtm for the formation of specialized cell types in Drosophila development. We are testing the spatial regulation of Mtm activity and its significance for phosphoinositide-dependent cellular functions in vivo.

Leishmania Parasitophorous Vacuoles Acquire the Capacity to Initiate Phosphoinositide-dependent Signaling by Interacting with VAMP7 Positive Vesicles

D. Abu Abdallah, 1 J. Benjack, 1 P. E. Kim; 1Microbiology and Cell Science, University of Florida, Gainesville, FL, 2Wright-Patterson Medical Center, Dayton, OH

Leishmania amazonensis parasites infect macrophages wherein they replicate in distended parasitophorous vacuoles (PV’s). Previous studies showed that macrophage infection with Leishmania results in sustained levels of activated Akt, a downstream kinase in the PI3K pathway. To determine whether Akt activation in infected cells occurs at the PV membrane (PVM), we tracked the recruitment of the pleckstrin homology domain of Akt fused to GFP (Akt-PH-GFP) in transfected cells. Within the first hour after infection, greater than 60% of nascent PVs were positive for Akt-PH-GFP. We confirmed that Akt-PH-GFP recruitment to PVs was dependent on the presence of phosphoinositides by reducing observed Akt-PH-GFP positive PVs in the presence of wortmannin. Also, a mutant Akt-PH-GFP construct whose phosphoinositide binding is disrupted, failed to be recruited to PVs. Beyond 1 hour post infection, there was a gradual diminution in the number of Akt-PH-GFP positive PV’s, down to approximately 20% by 8 hours post infection. This time course of Akt-PH-GFP association with PVs is unlike the time course of association of plasma membrane molecules that cycle off nascent PVs within 30 minutes post infection. Disruption of microtubules with nocodazole or colchinib inhibited Akt-PH-GFP recruitment to the PVM. One interpretation of this observation is that the capacity to generate phosphoinositides at the PVM requires the fusion of PVs with vesicles along microtubule tracks. Further investigation was obtained by assayings VAMP7 and VAMP8, which plays a role in the transport of vesicles along microtubules. Overexpression of dominant negative mutants of VAMP7 and VAMP8 minimal effects of Akt-PH-GFP recruitment to the PV, expression of VAMP7 (Ner1-120) significantly blocked Akt-PH-GFP recruitment to the PVM. Taken together, these studies show that PVs acquire the capacity to generate phosphoinositides to which signaling molecules that modify host cell processes are recruited and activated.

Rab GTPase-activating Proteins Define the Transport Pathway of Shiga Toxin

E. Fuchs, H. K. Alexander, S. Yoshimura, R. Kopajtic, F. A. Barr; Max Planck Institute of Biochemistry, Martinsried, Germany

Rab family GTPases are conserved regulators of membrane trafficking that cycle between inactive GDP- and activated GTP-bound states. A key determinant of Rab function is the lifetime of the GDP-bound state. Since Rabs have a low intrinsic rate of GDP-hydrolysis this is under the control of GTP-hydrolysis activating proteins (GAPs). RabGAPs are useful cell biological tools, since they can be used to specifically inactivate the endogenous pool of their target Rab, and thus interfere with the process this Rab is involved in. Here we have focused on the identification of RabGAPs that interfere with the transport of Shiga toxin between the cell surface and the Golgi apparatus. We have screened 35 human RabGAPs for their ability to block the uptake and transport of fluorescently labelled B-subunit of Shiga Toxin (STxB) to the Golgi. To confirm that effects on STxB transport are due to GAP activity we compared wild-type RabGAPs with catalytically inactive mutants. In addition, the integrity of the Golgi was monitored in RabGAP expressing cells, to ensure that the lack of a Golgi signal for STxB was not simply due to Golgi fragmentation. As a final control for specificity, we monitored the endocytosis of fluorescently labelled EGF, to eliminate RabGAP-causing a general block in endocytosis. Seven out of 35 RabGAPs blocked STxB transport to the Golgi dependent on their catalytic activity, while having no effect on the uptake of EGF. Overexpression of two of the positive RabGAPs lead to a fragmented Golgi, however, STxB accumulated in pre-Golgi structures, indicating these GAPs block STxB trafficking in addition to disrupting the Golgi. We therefore conclude that these seven candidate RabGAPs act specifically on the transport pathway utilised by STxB on its way from the plasma membrane to the Golgi, and will provide useful tools to further investigate this pathway.

A Syntaxin 10-containing SNARE Complex Distinguishes Protein Transport from Late and Early Endosomes to the trans Golgi Network

I. G. Ganeley, E. Espinosa, S. Pfeffer; Biochemistry, Stanford University, Stanford, CA

V-SNAREs on vesicles and t-SNAREs on target organelles mediate fusion between vesicles and their targets. We used soluble, recombinant SNARE proteins as potential dominant-negative inhibitors to identify SNARE proteins that are responsible for the fusion of late endosome-derived transport vesicles with the trans Golgi network (TGN). We show here that soluble v-SNAREs, VAMP3 and VAMP4, but not VAMP7 or 8, can inhibit the transport of mannose 6-phosphate receptors from late endosomes to the TGN in an in vitro system that reconstitutes this transport step. Similarly, the soluble t-SNAREs Syntaxin-10, 16 and Vti1a inhibited transport, but Syntaxins 6, 11, 12 and Vti1b did not. Overexpression of cytosolic SNARE proteins that inhibited transport in vitro triggered lysosomal enzyme mis-sorting from living cells, as monitored by the hyper-secretion of hexosaminidase from soluble SNARE protein-expressing fibroblasts. Furthermore, each of the requisite SNARE protein was found to bind GCC185 in cell extracts. GCC185 is a GRIP domain-containing, Golgin family member that binds to Rab9 and is required for mannose 6-phosphate receptor recycling (1). This study represents the first functional assignment for the Syntaxin10/Syntaxin16/Vti1a; VAMP3/VAMP4 SNARE complex. Moreover, the requirement for Rab9, GCC185, and Syntaxin 10, and the lack of a requirement for Syntaxin 6, distinguish late endosome export of mannose 6-phosphate receptors from the transport of proteins from early endosomes to the TGN. I. Reddy, J.V. et al. (2006) A Functional Role for the GCC185 Golgin in Mannose 6-Phosphate Receptor Recycling. Mol. Biol. Cell, in press

Rab11 Family Interacting Proteins (FIPs) Regulate the Differential Sorting of Membrane Proteins in Recycling Endosomes

J. Burden, J. Jing, C. Hopkins, R. Prekeris; 1Department of Biological Sciences, Imperial College, London, United Kingdom, 2Cell and Developmental Biology, UCHSC, Aurora, CO

Rab11 and Rab11p are the members of Rab11 family of interacting proteins (FIPs), a family characterized by their ability to bind Rab11 and phospholipids. We and others have previously shown that FIPs are involved in regulating various steps of endocytic sorting and recycling. However, their precise function remains to be elucidated. Using the combination of RNA interference, electron and fluorescent microscopy and a variety of biochemical techniques we have investigated the role of CPH and Rip11 in regulating endocytic sorting and transport. Firstly, we overexpressed Rip11-Rab Binding Domain (Rip11-RBD) dominant-negative construct to investigate the role of FIPs in endocytic transport. Rip11-RBD sequesters Rab11 and prevents it from interacting with proteins that are responsible for the fusion of late endosome-derived transport vesicles with the trans Golgi network (TGN). We show here that soluble v-SNAREs, VAMP3 and VAMP4, but not VAMP7 or 8, can inhibit the transport of mannose 6-phosphate receptors from late endosomes to the TGN in an in vitro system that reconstitutes this transport step. Similarly, the soluble t-SNAREs Syntaxin-10, 16 and Vti1a inhibited transport, but Syntaxins 6, 11, 12 and Vti1b did not. Overexpression of cytosolic SNARE proteins that inhibited transport in vitro triggered lysosomal enzyme mis-sorting from living cells, as monitored by the hyper-secretion of hexosaminidase from soluble SNARE protein-expressing fibroblasts. Furthermore, each of the requisite SNARE protein was found to bind GCC185 in cell extracts. GCC185 is a GRIP domain-containing, Golgin family member that binds to Rab9 and is required for mannose 6-phosphate receptor recycling (1). This study represents the first functional assignment for the Syntaxin10/Syntaxin16/Vti1a; VAMP3/VAMP4 SNARE complex. Moreover, the requirement for Rab9, GCC185, and Syntaxin 10, and the lack of a requirement for Syntaxin 6, distinguish late endosome export of mannose 6-phosphate receptors from the transport of proteins from early endosomes to the TGN. I. Reddy, J.V. et al. (2006) A Functional Role for the GCC185 Golgin in Mannose 6-Phosphate Receptor Recycling. Mol. Biol. Cell, in press
Phagocytic and Autophagic Pathways Are Actively Modulated by Phase II Coxiiella burnetii to Efficiently Replicate in the Host Cell

F. S. Romanò, 1 M. G. Gutierrez, 2 W. Beron, 1 M. Rabinovitch, 3 M. I. Colombó; 1HEIM-CONICET-Facultad de Ciencias Médicas, Universidad Nacional de Cuyo, Mendoza, Argentina, 2Departamento de Microbiología, Imanología e Parasiología, Escola Paulista de Medicina, UNIFESP, São Paulo, Brazil, 3Microbiology and Immunology, University of Michigan School of Medicine, Ann Arbor, MI

The etiologic agent of Q fever Coxiiella burnetii, is an intracellular obligate parasite that develops large vacuoles with phagolysosomal characteristics, containing multiple replicating bacteria. We have previously shown that Phase II Coxiiella burnetii replicative vacuoles generated after 24-48 h post infection are decorated with the autophagic protein LC3. The aim of the present study was to examine, at earlier stages of infection, the distribution and roles of the small GTPases Rab5 and Rab7, markers of early and late endosomes, respectively, as well as of the protein LC3 on C. burnetii trafficking. Our results indicate that: 1) Coxiiella phagosomes (CPs) acquire the two Rab proteins sequentially during infection; 2) overexpression of a dominant negative mutant form of Rab5, but not of Rab7, impaired Coxiiella entry, whereas both Rab5 and Rab7 dominant negative mutants inhibited vacuole formation; 3) Cph colocalized with the protein LC3 as early as 5 min after infection; acquisition of the protein appeared to be a bacterially driven process, since it was inhibited by the bacteriostatic antibiotic chloramphenicol; 4) C. burnetii delayed the arrival of the typical lysosomal protease cathepsin D to the CPs, a process that is increased by starvation-induced autophagy. Based on our results we propose that C. burnetii transits through the normal end/phagocytic pathway but actively interacts with autophagosomes at early times after infection. This intersection with the autophagic pathway delays fusion with the lysosomal compartment possibly favoring the intracellular differentiation and survival of the bacteria.

Vascular Trafficking Is Required for Maintenance of Lipid Bodies

K. M. Szymanski, J. M. Goodman; Pharmacology, University of Texas Southwestern Medical Center, Dallas, TX

Lipid bodies (lipid droplets, adiposomes) are dynamic organelles found in virtually all eukaryotic cells that store neutral lipids. They are surrounded by a single phospholipid leaflet that is deformed by the endoplasmic reticulum. Very little is known about factors that control size, number, and distribution of lipid bodies. To this end we screened a commercial knockout strain library of nonessential genes in Saccharomyces cereviseae. Cells were grown overnight in rich medium, and lipid bodies were visualized by fluorescence microscopy upon staining with Bodipy 403/503. The wild-type strain, BY4742, contained 5.05 ± 0.44 (SEM) lipid bodies per cell, most of which formed a “string of pearls,” the ultrastructure of which we recently described (Binnis et al., JCB 173:719, 2006). With 99% of the library screened, we found 69 strains with lipid bodies that were clearly altered in number, size, or distribution within the cell. Three typical morphological variants were found: those with few large lipid bodies (Class 1), such as abf2Δ with 2.50 ± 0.18 lipid bodies; normal number of large lipid bodies (Class 2); and multiple small and indistinct lipid bodies (Class 3), such as erd1Δ with 9.51 ± 0.79 lipid bodies. Although several families of genes were identified that are important for maintenance of normal lipid bodies, the largest group of strains (18) were lacking genes for the endosomal-vascular trafficking pathway. They include 9 VPS genes, 2 PEP genes and 6 genes of the vacuolar ATPase, the last representing both V0 and V1 assemblies. These strains typically were of the Class 2 phenotype. It is not yet clear whether endosomal-vascular trafficking per se affects lipid body morphology, or whether a defective pump is solely responsible for the phenotype in this group of mutants.

Lysosome Trafficking in Osteoblasts

N. Nahavi, R. Harrison; Department of Life Sciences, University of Toronto at Scarborough Campus, Toronto, ON, Canada

Osteoblasts are the principle cells responsible for bone deposition via secretion of organic proteins such as type I collagen, proteoglycans, glycoproteins, and carbohydrates proteins into the extracellular matrix (ECM). Osteoblasts are also responsible for controlling the levels of bone resorption through regulating osteoclastic activities; thus, they play a key role in the pathophysiology of osteoporotic diseases. The objective of our experiments is to investigate the morphology and function of undifferentiated and differentiated MC3T3 osteoblast cell line as well as primary cells through a number of experimental procedures including cell transfection, microinjection, epifluorescence and TRF microscopy. We observe a morphological change in differentiated osteoblasts as compared to undifferentiated cells. The differentiated cells show enhanced lysosome dispersion. Live imaging of LAMP-GFP transfected cells reveal a faster movement of lysosomes in ascorbate differentiated cells compared to undifferentiated osteoblasts. Lysosomal distribution is dependent on intact microtubules and blocking antibodies to microtubules interfere with lysosome distribution in cells before and after differentiation. Osteoblast lysosomes acquire Rab7-GFP but have negligible levels of Rab5-GFP and Rab11-GFP. It is suspected that osteoblast lysosomes may be involved in protein secretion or degradation of the ECM. The function of lysosomes in osteoblasts is currently under investigation.

Three-dimensional Pathways of Transport for Maternal Antibody Across the Epithelial Barriers in the Small Intestine of Suckling Rats Revealed by Electron Tomography

W. He, 1 C. Kivotik, 2 M. Morphey, 3 J. McIntosh, 3 G. Jensen, 1 P. Bjorkman; 1Division of Biology, California Institute of Technology, Pasadena, CA, 2Laboratory for 3D Electron Microscopy of Cells, University of Colorado, Boulder, CO, 3Laboratory for the Physical Sciences, College Park, MD, 4Department of Chemistry, University of Illinois at Urbana-Champaign, Urbana, IL

Before its own immune system is fully developed, the suckling rat is dependent upon the neonatal Fc receptor (FcRn) to transport maternal IgG from ingested milk into its bloodstream. Early conventional electron microscopy studies using IgG-Ferritin conjugates showed that the proximal small intestine of the neonatal rat selectively transports ingested antibodies into the blood. However, due to the large size and non-specific coupling of the labels that were used, these studies may not have revealed the true transport pathways. To accurately trace transport pathways during dynamic endocytic processes and to examine the configuration of FcRn-Fc complexes within different types of intracellular vesicles in the small intestine, we need a technique that allows us to trace fully functional antibody conjugates in an in vivo system. We are using 1.4 nm nanogold clusters, covalently attached to the reduced hinge region of rat Fc then purified on an FcRn affinity column, as a more reliable label to explore functional endocytic pathways. It is challenging, however, to visualize 1.4nm gold particles within thin sections prepared for electron microscopy. Therefore, we are combining techniques: improved gold-enhancement methods for chemically-fixed samples, a newly-developed freeze-substitution based silver-enhancement method for high pressure frozen samples, and electron tomography to visualize the labeled Fc molecules and trace the transport pathway in 3D. Our results clearly demonstrate that the neonatal rat proximal small intestine takes up antibodies through an FcRn-specific mechanism with most gold particles localized in tubular vesicles and coated vesicles; the conjugates are then discharged to the basolateral extracellular space. In the distal small intestine, which does not express FcRn, the gold particles are delivered to a lysosomal degradation pathway. Hence, these techniques are confirmed to be very useful for tracing 3D transport pathways and of analyzing dynamic events in transcytosis.

4D Imaging of Endosome Dynamics in Filter-grown Polarized MDCK Cells

Y. Kababiyah, J. Chu, J. Lippincott-Schwartz, I. M. Arias; CBMB, NICHD/NIH, Bethesda, MD

Live cell imaging techniques have been widely used to study cellular dynamics and processes in cells and organisms. However, live imaging of filter-grown, fully, polarized epithelial cells has been cumbersome and problematic due to extensive manipulation of the permeable filter inserts that could result in the loss of polarization and function. To develop a simple protocol for live cell imaging of filter-grown polarized Madin-Darby Canine Kidney (MDCK) cells, we cultured MDCK cells on the reverse side of permeable filter inserts. MDCK cells cultured in this inverted configuration formed structural and functional tight junctions. Furthermore, all morphological and functional features tested were similar to MDCK cells cultured in the conventional configuration. To evaluate the advantage of inverted culture system, live cell imaging was performed with FM4-64, a lysophilic fluorescent dye, commonly used to visualize endosomes dynamics. Using an inverted microscope, MDCK cells cultured in the inverted configuration permitted 4D tracking of FM4-64 positive endosomes dynamics, effectively allowing selective reagent application, and access to either the apical or the basolateral membrane surfaces. These results demonstrate that the inverted culture system provides a technique to analyze endosome dynamics in four dimensions with spatial and temporal resolution when provided with an inverted microscope.
lower eukaryotes. 

T. gondii is an obligate intracellular eukaryotic pathogen that relies on cell host invasion as a major survival strategy. Invasion related proteins are proteolytically processed during their transport to specialized secretory organelles (micronemes and rhoptries) that are sequentially discharged during cell entry. Using general and specific inhibitors we have found evidence that TgCPL, T. gondii cathepsin L-like enzyme, is involved in the proteolytic maturation of multiple microneme adhesin complexes. In the tachyzoite invasion form, TgCPL is located in a novel, photon lutein structure visible by phase contrast microscopy and positioned in the apical region between the rhoptries and apical secretory organelles. Serial sections of the same parasite shown by electron microscopy indicate that TgCPL is associated to the limiting membrane and internal vesicles of a large multi-vesiculated compartment, which is also occupied by a proton-pump, the T. gondii vacuolar protonophosphatase TgVPI. During intracellular replication the compartment is highly dynamic and is fragmented in multiple vesicles. We speculate that TgCPL is a multifunctional enzyme involved in both the proteolytic maturation of microneme protein complexes and the degradation of endogenous and exogenous substrates within the endocytic pathway.

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Phenotype Analysis of Snapin Knockout Mice Reveals Its Essential Role in the Late Endosome-Lysosome Trafficking Pathway

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

Our previous findings that a frameshift (p.E805fsX) and a relatively milder missense mutation (p.N3376S) located in the BEACH domain of Snapin KO mice in combination with genetic rescue experiments provide evidence that Snapin, a SNARE-associated protein, modulates neurosecretion by stabilizing the structural coupling of synaptogamy to the SNARE complex, a critical step for priming docked vesicles for fusion (Tian et al. 2005). In addition to established role in vesicle priming for synaptic vesicle exocytosis, recent evidence suggests that Snapin is potentially implicated in the trafficking of lysosome-related organelles (Starevcic and Dell'Angelica, 2004). By characterizing Snapin KO mice, we now report a novel role for Snapin in the trafficking of late endosomes to lysosomes. Snapin is enriched in the immunolocalized late endosomolysosomal SNAREs including syntaxin-8, VAMP-7, and Vti1b. Immunochemicalcytochemical staining reveals an increased density of the late endosomes in both cortical neurons and fibroblasts from snapin (-/-) mice, a phenotype that can be rescued by reintroducing the Snapin gene into the mutant cells. Furthermore, the deletion of Snapin in fibroblasts blocks the degradation of internalized EGF-EGRF via the endosome-lysosome pathway and accumulates internalized Dextran in the late endosomes instead of lysosomes. Finally, maturation of lysosomal enzymes is impaired in the Snapin mutant cells. Taken together, our study reveals a novel role for Snapin in the fusion of late endosomes with lysosome, probably by interacting with the endosomal SNAREs and synaptotagmins. Thus, Snapin, like many other SNARE regulatory proteins, is involved in both synaptic vesicle exocytosis and the intracellular membrane trafficking (Supported by the NINDS intramural research program).

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GPNMB Is a Newly Defined Melanosomal Protein

C. M. Trantow,1 G. E. Petersen,1 L. A. Ammono,1 T. Hararszi,1 M. G. Anderson1; 1Physiology and Biophysics, University of Iowa, Iowa City, IA, 1Institute of Physical Chemistry, University of Heidelberg, Heidelberg, Germany

Melanosomes are the intracellular organelle of melanin synthesis. Melanosomes normally play an important role in regulating the color of our hair, skin, and eyes. Unfortunately, melanosomal defects have been implicated in a number of diseases, including a variety of ocular diseases. One gene suggested to play such a role is Gpnmb. In inbred DBA/2 mice, a naturally occurring Gpnmb mutation contributes to a pigment dispersing iris disease that ultimately results in a form of pigmentary glaucoma. The Gpnmb gene is predicted to encode a type I transmembrane protein that also contains a pre-melanosomal sorting signal. The objective of this experiment was to test the hypothesis that GPNMB is indeed a melanosomal protein. As expected for a melanosomal protein, we find that Gpnmb transcripts are abundant in the iris, and that the mutation of DBA/2 mice results in an approximate 18 fold down-regulation compared to wild type. Using immunohistochemistry, we find that GPNMB is specifically expressed in a punctate cytoplasmatic pattern throughout pigmented tissues of the eye, including the iris, ciliary body epithelium, and choroid. To test whether this labeling may correspond to melanosomes, we performed Western blots of sucrose gradient purified ocular melanomas and demonstrate the presence of GPNMB immunoreactivity. The function of GPNMB within melanosomes remains unknown. Unlike many known genes encoding melanosomal proteins, Gpnmb mutation does not seem to detectably alter melanin synthesis nor does it affect the expression of the vast majority of other known genes encoding melanosomal proteins. However, spectromicroscopic analysis of purified melanomas using scanning transmission X-ray microscopy demonstrates that DBA/2 irides mutant for Gpnmb do contain multiple chemical differences in comparison to wild-type C57BL/6J melanosomes. In sum, these results indicate that GPNMB is a newly defined melanosomal protein. Our ongoing experiments aim to further elucidate the precise functions of this important disease-causing protein.

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Chediak Higashi Syndrome: A Genotype-Phenotype Correlation in the Human and Cell Biological Level

W. Westbrook,1 D. Adams,1 A. Koshofer,1 A. Helip-Wooley,1 R. Kleta1,2 A. Helip-Wooley,1 W. A. Gaht1,2; 1Medical Genetics Branch, NHGRI, NIH, Bethesda, MD, 1Department of Dermatology, University of Cincinnati College of Medicine, Cincinnati, OH

Chediak Higashi syndrome (CHS) is a rare autosomal recessive disorder caused by mutations in the CHS1 gene. Clinical characteristics include skin, hair and eye hypomelanosis, recurrent infections, a mild bleeding diathesis and late-onset progressive neurological impairment. We report two unrelated patients, CHD4 and CHD6. CHD4, suffering from the severe childhood form of CHS, presented with infections, a mild bleeding diathesis and late-onset progressive neurological impairment. We found evidence that TgCPL, Toxoplasma gondii cathepsin L-like enzyme, is involved in the proteolytic maturation of multiple microneme adhesin complexes. In the tachyzoite invasion form, TgCPL is located in a novel, photon lutein structure visible by phase contrast microscopy and positioned in the apical region between the rhoptries and apical secretory organelles. Serial sections of the same parasite shown by electron microscopy indicate that TgCPL is associated to the limiting membrane and internal vesicles of a large multi-vesiculated compartment, which is also occupied by a proton-pump, the T. gondii vacuolar protonophosphatase TgVPI. During intracellular replication the compartment is highly dynamic and is fragmented in multiple vesicles. We speculate that TgCPL is a multifunctional enzyme involved in both the proteolytic maturation of microneme protein complexes and the degradation of endogenous and exogenous substrates within the endocytic pathway.

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Dissection of a Complex Phenotype: Conditional Inactivation of the Chloride Channel CIC-7

L. Wartosch, J. C. Fuhrmann, P. F. Lange, T. J. Jentsch; AG Jentsch, Max-Delbrueck-Center, Berlin, Germany

One common denominator in the function of intracellular CLC chloride channels and transporters is their role in the acidification of the vesicles in the endocytic pathway, where they provide an electrical shunt for the pumping of vesicular H+-ATPases. The chloride channel CIC-7 resides in late endosomes and lysosomes where it colocalises with the specific lysosomal marker protein Lamp-1. Recently we could show that CIC-7 requires Ostm1 as a beta-subunit. In addition, CIC-7 is localised in a specialised plasma membrane domain of osteoclasts facing the resorption lacuna. The latter is the exclusive site of bone resorption and consequently mutations in the CIC-7 gene cause osteopetrosis in mice and men, an inherited disorder characterised by a failure of osteoclasts to resorb bone. As a consequence Clic7 knockout mice show retarded growth, anaemia, extramedullary haematopoiesis and hepatosplenomegaly. Furthermore, degeneration of the central nervous system showing features of lysosomal storage disease, as well as retinal degeneration was observed. Clic7 knockout mice do not live longer than 45 days. In addition CIC-7 is highly expressed in kidney cortex. Lysosomal storage material accumulates in proximal tubules of Clic7 ko mice. In C7-knock out mice the increased activity of lysosomal beta-Hexosaminidase could be detected. This study reveals an altered Lamp-1 distribution in enlarged lysosomal compartments of CIC-7 ko proximal tubules that are positive for Dextran instead of lysosomes. Finally, maturation of lysosomal enzymes is impaired in the Clic7 mutant cells. These findings in CHD6 might be attributable to the endosomal SNAREs including syntaxin-8, VAMP-7, and Vti1b. Immunochemicalcytochemical staining reveals an increased density of the late endosomes in both cortical neurons and fibroblasts from snapin (-/-) mice, a phenotype that can be rescued by reintroducing the Snapin gene into the mutant cells. Furthermore, the deletion of Snapin in fibroblasts blocks the degradation of internalized EGF-EGRF via the endosome-lysosome pathway and accumulates internalized Dextran in the late endosomes instead of lysosomes. Finally, maturation of lysosomal enzymes is impaired in the Snapin mutant cells. Taken together, our study reveals a novel role for Snapin in the fusion of late endosomes with lysosome, probably by interacting with the endosomal SNAREs and synaptotagmins. Thus, Snapin, like many other SNARE regulatory proteins, is involved in both synaptic vesicle exocytosis and the intracellular membrane trafficking (Supported by the NINDS intramural research program).

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Regulation of Acetylcholine-evoked H+-MPP+ Release by Short-Term Exposure to Somatostatin or Muscarinic Agonists in Bovine Adrenal Chromaffin Cells

L. Ribeiro, F. Martel, I. Azevedo; Biochemistry Department, Faculty of Medicine, University of Porto, Porto, Portugal

The aim of this work was to investigate the effect of a short-term exposure to somatostatin (SS), its receptors (SSTR) selective agonists as well as muscarinic receptors agonists upon acetylcholine-induced release of H+-MPP+ from bovine adrenal medullary cells. Acetylcholine (ACH, 100, 500 μM) was found to increase the release of H+-MPP+ by these cells (to 175 and 171% of basal release, respectively). ACH-elicited H+-MPP+ release was significantly reduced by hexamethonium (100 μM) and atropine (100 μM), selective nicotinic and muscarinic acetylcholinergic receptor antagonists. Whole mount electron microscopy of normal and patients' platelets found evidence that TgCPL, Toxoplasma gondii cathepsin L-like enzyme, is involved in the proteolytic maturation of multiple microneme adhesin complexes. In the tachyzoite invasion form, TgCPL is located in a novel, photon lutein structure visible by phase contrast microscopy and positioned in the apical region between the rhoptries and apical secretory organelles. Serial sections of the same parasite shown by electron microscopy indicate that TgCPL is associated to the limiting membrane and internal vesicles of a large multi-vesiculated compartment, which is also occupied by a proton-pump, the T. gondii vacuolar protonophosphatase TgVPI. During intracellular replication the compartment is highly dynamic and is fragmented in multiple vesicles. We speculate that TgCPL is a multifunctional enzyme involved in both the proteolytic maturation of microneme protein complexes and the degradation of endogenous and exogenous substrates within the endocytic pathway.

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antagonists, respectively. Previous exposure to any of two muscarinic agonists, oxotremorine or pilocarpine, led to a significant reduction of [3H-MPP+] release in response to 100 μM ACH, to about a maximum of 51% and 78% of control, respectively. SS (0.01-0.1 μM), previously applied to the preparation, depressed ACH-elicted [3H-MPP+] release by 25-27%, but only when a 500 μM ACH concentration was used. The inhibition exerted by SS upon ACH-evoked [3H-MPP+] release appeared to be mediated by its SSTR: (1) SSTR2, 3 and 4 subtype agonists mimicked the effects seen with SS, and (2) the SSTR non-selective antagonists, cyclo-SS, counteracted the SS inhibitory effect. When SS was tested in the presence of any of the muscarinic agonists, oxotremorine or pilocarpine, its inhibitory effect on 500 μM ACH-induced [3H-MPP+] release was no longer detectable. These results, showing a somewhat similar effect of short-term exposure to SS and muscarinic agonists over ACh-induced release of [3H-MPP], as well as the loss of effect of SS by the presence of the muscarinic agonists, suggest that these compounds may share signalling pathways. Supported by FCT (POCI, FEDER and Programa Comunitário de Apoio)

587  Selective Activation of Somatostatin Receptor Subtypes Differentially Modulates [3H-MPP+] Release from Primary Cultures of Bovine Adrenal Chromaffin Cells

L. Ribeiro, F. Martel, I. Azevedo; Biochemistry Department, Faculty of Medicine, University of Porto, Porto, Portugal

Somatostatin (SS), a neuropeptide widely distributed in the central and peripheral nervous systems, acts through five specific cell surface receptors (SSTR1-5) to elicit different biological functions. Using reverse transcription followed by PCR amplification (RT-PCR), we have identified in bovine adrenal chromaffin cells the expression of the SS gene. The purpose of this study was to investigate the effect of SS and SSTR2-5 selective agonists on adrenal catecholamine release, using model substrate [1H-1-methyl-4-phenylpyridinium ([1H]MPP+)] release from primary cultures of bovine adrenal chromaffin cells. The effects of SS (0.01-1 μM) upon ACH (100-500 μM, 10 mM)-evoked [1H]MPP+ release differed between different cultures, allowing us to distinguish two types of cell cultures: type A- cell cultures, in which SS decreased ACH-induced [1H]MPP+ release, and type B- cell cultures, in which SS increased ACH-induced [1H]MPP+ release. The SSTR2, 4 and 5 agonists mimicked the inhibitory effects elicited by SS upon ACH-induced [1H]MPP+ release in type A cell cultures, whereas the SSTR2, 3 and 5 agonists mimicked the excitatory effect of SS upon ACH-induced [1H]MPP+ release on type B cell cultures. On the other hand, while SS (0.01-1 μM) and the SSTR3 and 5 agonists significantly augmented the release of [1H]MPP+ from type A cell cultures, neither SS nor its SSTR selective agonists did significantly affect this release from type B cell cultures. Interestingly, type A and B cell cultures were not only heterogeneous in relation to the response to SS and SSTR agonists, but also did differ regarding AD/NA cellular ratio, [1H]MPP+ uptake capacity, basal [1H]MPP+ release, and [1H]MPP+ response to ACH. In summary, our data indicate that: (1) adrenal chromaffin cells respond differentially to SS and SSTR agonists as to basal and ACH-stimulated [1H]MPP+ release, and (2) endogenous SS might modulate adrenal catecholamine secretion through its specific receptors.

589  An Established Anitriot Pituitary Progenitor Cells (BPC-1) Could Produce Several Inflammatory Cytokines by the Stimulation through Its II-18 Receptors

Y. Nagai, H. Ogasawara, H. As0, Y. Taketa, Y. Yamaguchi; Animal Biology, Graduate School of Agricultural Science, Tohoku University, Sendai, Japan

In an anterior pituitary gland, inflammatory mediators regulate the cell function through immuno-endocrine pathway. Our previous study showed that proinflammatory cytokine, IL-18, was mainly expressed in the hormonal cells of anterior pituitary. Further study also showed that IL-18 was localized in the cell layer of Rathke’s pouch that has been proposed to embody stem/progenitor cell compartment in the postnatal pituitary. In this study, we attempted to establish a stem/progenitor cell line bearing IL-18 receptor from bovine anterior pituitary gland, and investigated its immunomodulatory function. The bovine pituitary cell line from bovine anterior pituitary cloned by limited dilution methods was designated as bovine pituitary cell 1 (BPC-1). BPC-1 grew stably by repeated passages, and expressed IL-18 and IL-18 receptors. The cell line expressed mRNA of stem/progenitor cell-associated factors and components of Notch signaling pathway such as nestin, Oct-4, Notch1 and Hes1. BPC-1 was positively immunoreactive for β-catenin in its nucleus, and also showed rapid growth in EGF and BFGF containing medium. RT-PCR showed that BPC-1 expressed mRNA of IL-1α, IL-6, IL-7, IL-12 and IL-15 in normal cultures. IL-18 induced IL-1α, IL-1β, IL-6 and IL-8 mRNA in BPC-1. Immunohistochemistry and cytochemistry showed that IL-18 and vimentin were expressed in BPC-1 and also in the layer of Rathke’s pouch in anterior pituitary gland. In addition, IL-18-positive cells of the layer were not colocalized with GH. These results suggest that BPC-1 is a stem/progenitor cell and regulates anterior pituitary cell function through immuno-endocrine pathway associated with IL-18.

589  Genomic and Proteomic Analysis of Myofibroblasts: α-Smooth Muscle Actin-EGFP Transgenic Mouse Model

C. Bertolotto, 1 V. Krause, 1 N. Khoury, 2 H. Hencidi, 3 Y. Uba, 1 B. Bassilain, 1 J. Xu, 1 C. Wang, 1 P. Whitelegg, 3 K. F. Faul, 4 C. F. Simmons 1-2; 1Pediatrics/Neonatology, Cedars-Sinai Medical Center, Los Angeles, CA, 2Pediatrics, David Geffen School of Medicine, UCLA, Los Angeles, CA, 3Psychiatry & Biobehavioral Sciences/Clinical & Biochemical-Molecular Spectroscopy, David Geffen School of Medicine, UCLA, Los Angeles, CA, 4Endocrinology, Cedars-Sinai Medical Center, Los Angeles, CA, 5Psychiatry & Biobehavioral Sciences, David Geffen School of Medicine, UCLA, Los Angeles, CA

Background: Myofibroblasts are specialized contractile fibroblasts that express alpha smooth muscle actin. Myofibroblasts demonstrate tissue specific phenotypes that promote normal tissue development and local disease processes of inflammation, fibrosis, and calcification. To facilitate in vivo identification and purification of myofibroblasts, we have developed a transgenic mouse line that expresses EGFP under the control of an alpha smooth muscle actin enhancer promoter element. Design/Methods: Linearized alpha smooth muscle actin - EGFP reporter construct was injected into FVB fertilized mouse ova. Founder mice were bred and tissue specific expression of EGFP was analyzed by fluorescence microscopy and compared with expression of native alpha smooth muscle actin by immunofluorescence microscopy and quantitative image processing. Tissue membrane and placental primary cell cultures were transfected with a temperature sensitive SV40 large T plasmid, and cloned by limiting cell dilution. Resultant myofibroblast cell lines were analyzed by genomic and proteomic analysis utilizing microarray and gel electrophoresis/mass spectrometry approaches before and after exposure to regulatory cytokines. Results: EGFP was readily detected in myofibroblasts, vascular and visceral smooth muscle cells of multiple organs including the tympanic membrane, and correlated with native expression of alpha smooth muscle actin. Myofibroblasts exhibited 10-100 fold greater EGFP fluorescence than fibroblasts; smooth muscle cells exhibited 100-500 fold greater EGFP fluorescence than fibroblasts. Several myofibroblast cell lines were immortalized, each expressing both nuclear and cytoplasmic EGFP. Immortalized myofibroblast cell lines exhibited cytochrome regulated expression of contractile proteins, extracellular matrix proteins, cytokines, and transcription factor genes and proteins. Conclusions: The 3.1 kb upstream regulatory region of the mouse α-smooth muscle actin gene directs the proper spatial and temporal regulation of EGFP in myofibroblasts, vascular and visceral smooth muscle. This mouse model system will provide new experimental platforms for understanding the cellular and molecular mechanisms of fibrosis and calcification in the tympanic membrane and placenta.

590  Hmgbl Expression in Mouse Tongue Cancer and Muscle Fiber Characteristics in the Surrounding Normal Muscle Tissues

K. Sakiyama,1 O. Okubo,2 K. Kurokawa,1 S. Abe,1 Y. Ide1; 1Anatomy, Tokyo Dental College, Chiba, Japan, 2Sports Dentistry, Tokyo Dental College, Chiba, Japan

Recently, it has been suggested that dysphagia caused by excision of tongue cancer may be affected by the hypofunction of the surrounding normal muscles, as well as by a tissue defect. However, there is no report concerning how the muscle fiber characteristics change in the normal regions surrounding the lesion after excision of tongue cancer. In this study, to demonstrate the muscle fiber characteristics of the surrounding normal muscles tissue of tongue cancer, we investigated myosin heavy chain (MyHC) isoforms at immunohistochemical and genetic levels. High mobility group box 1 (HMGB1) was used as a marker to examine whether tongue cancer actually developed. As a result, considerable HMGB1-2a was observed in the surrounding normal muscle of tongue cancer, unlike in the normal tongue muscle. Thus, excision of tongue cancer changed the muscle fiber characteristics of the surrounding normal muscle, demonstrating that postoperative dysfunction of the muscles are caused not only by tissue loss.

591  Differentiation of Myoblasts in Three-dimensional Culture Conditions

M. L. Marquette,1 L. Vergara,2 D. Byerly,2 M. A. Sognier1; 1Neuroscience and Cell Biology, University of Texas Medical Branch, Galveston, TX, 2NASA Johnson Space Center, Houston, TX

Differentiation of anchorage-dependent myoblast cell cultures: 1) can be induced by serum deprivation or changing serum types; and 2) is thought to require adhesion to a substrate. We previously reported the appearance of large multinucleated cells in myoblasts cultured in Synthecon’s Rotary Cell Culture System (RCCS). This study assessed if these multinucleated cells exhibited additional characteristics of differentiating muscle cells. A murine myoblast cell line, C2C12, was cultured as three-dimensional aggregates (without support structures) in both the RCCS and Petri dishes coated to prevent cell adhesion. Cells were cultivated in proliferation medium with no replacement of serum type or serum deprivation. Differentiation was defined by: 1) fusion of single myoblasts to form multinucleated syncitium; and 2) expression of myosin, a muscle specific protein. Cell aggregates were fixed at day 3 and 7, embedded, and sectioned. Evaluation of H & E stained sections revealed greater numbers of syncitia in the RCCS cultured cells than Petri dish suspension controls at both time points (i.e., mean number 7.64 in RCCS vs 1.75 in controls at day 7). Quantification of muscle specific myosin using immunofluorescence revealed that RCCS cultured aggregates exhibited: 1) myosin distribution over greater areas;
were stained with monoclonal antibodies to cytochrome c. The cell aggregates localized at the cell periphery were transformed to discrete grains separated from each other. Grain transition might serve to diminish damage effect by disassembling the separate mitochondria from the cytosol during stress periods to sustain mitochondrial homeostasis. Cytochrome c evidently substrate entry into mitochondria was blocked at this early stage. We speculate that decrease in mitochondrial membrane permeability caused reversible respiration decline serves to occur when cells reached confluent, medium was exchanged to adipogenic medium containing 50 ng/ml insulin, 0.25 μM dexamethasone, 2 mM octanoate, and 200 μM oleate. Medium was exchanged every other day for 8 days after stimulation, and the adiopogenic ability of each factor was judged by the cytosolic TG accumulation and Oil red O staining. Octanoate significantly accelerated the cytosolic TG accumulation. When cells were exposed to both octanoate and oleate, the large amount of lipid droplets were observed 8 days after stimulation, and the cytosolic TG increased over 13 fold compared with the cells maintaining growth medium. Insulin and dexamethasone themselves could not stimulate the accumulation of TG, but when treated together with both octanoate and oleate, induced further accumulation of the cytosolic TG over 25 fold higher than the cells maintaining growth medium. Moreover, both octanoate and oleate induced the cytosolic TG accumulation in the concentration dependent manner (0.5 to 10 mM octanoate and 20 to 200 μM oleate). Next, the PPARγ mRNA expression was investigated by semi-quantitative RT-PCR. Both octanoate and oleate induced significantly the PPARγ mRNA expression, but neither insulin nor dexamethasone. Especially, oleate could increase the expression of the PPARγ mRNA much more than oleate.

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Differences in the Response to the Toxic Effect of Ceramide in Tumour Cells from Human Cervix-uterine Cancer Infected and Non-infected with Papillomavirus
G. Gutiérrez Iglesias,1,2,3 E. Gutiérrez Iglesias,1 A. Parra Barrera,1 E. Reyes Maldonado,2 R. López-Marure,3 1Physiology, National Institute of Cardiology, México, D. F., Mexico, 2Physiology, Instituto Politecnico Nacional, México, D. F., Mexico, 3Urology and Ginecology, Hospital Infantil de México, México, D. F., Mexico
Ceramide is a potentially important mediator of a number of natural and pharmacological agents that affect cell growth, viability, etc. This lipid is derived from sphingomyelin hydrolysis, or is produced by the de novo synthesis. We previously demonstrated that ceramide promotes the death of cell lines from human cervical cancer infected with human papillomavirus (HPV) type 16 and 18 (CaKi and CaLo cells, respectively), but cervical cancer cells without HPV (ViBo cells) are not affected. The mechanism of resistance for ViBo to ceramide is unknown. In this work we evaluated the differences in the cell morphology, potential and activity of the mitochondria in CaLo, Caski and ViBo cells. Production of free radicals (ROS) as a consequence of the toxic effect of the ceramide also was evaluated with the purpose to describe possible molecular differences in response to the ceramide. Hematoxylin stain was used to observe morphological changes, reduction of MTT for mitochondrial activity, DCF to free radicals and rhodamine 123 stain for mitochondrial membrane potential. Morphological changes related to death were observed in CaLo and Caski cells, however, ViBo cells showed resistance to death by ceramide. CaLo conserved the mitochondrial activity and Caski and ViBo augmented it. While in Caski and ViBo ceramide decreased the potential of the mitochondria, ceramide induced the production of free radicals. C6 did not have effect on potential of mitochondria and diminished in 56% free radicals production in ViBo cells. The results revealed remarkable differences between CaLo, Caski and ViBo cells to antiproliferative effect induced by ceramide, indicating that ViBo cells are more resistant to the toxic effect of ceramide possibly by using an efficient mitochondrial and antioxidiant system.

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TNF-induced Apoptosis in Lymphoid U937 Cells Mitochondria Early Changes
E. Feltsova,1 A. Avetisyan2,1 1Moscow State University, AN Belozersky Institute of Phys.-Chem. Biology, Moscow, Russian Federation, 2Department of Bioenergetics, A.N.Belozersky Institute of Phys.-Chem. Biology, Moscow, Russian Federation
We investigated mitochondria-mediated TNF-α induced apoptosis in lymphoid U937 cells to reveal possible changes in mitochondria function that contributed to apoptosis development. Cells were stained with monoclonal antibodies to cytochrome c. Apoptosis was determined by staining with Hoechst 33342. Mitochondrial change was determined by TMRE staining. Confocal and fluorescent microscopes were used. Oxygen consumption was measured with Clark-type electrode. Cellular ROS and ATP levels were measured with H2DCF-DA (FACS - analyzed) and luciferin-luciferase reagent correspondingly. In 30 min after apoptosis induction rapid increase in mitochondrial transmembrane potential, rates of respiration, and ATP-generation level occurred. In 1.5-2 hr after TNF addition respiration declined to 50%-60% of the control. Respiration decline could be restored by in vitro addition of penetrating respiratory substrates. Evidently substrate entry into mitochondria was blocked at this early stage. We speculate, that decrease in mitochondrial membrane permeability caused reversible respiration decline serves to separate mitochondria from cytosol during stress periods to sustain mitochondrial homeostasis. Cytochrome c-staining revealed mitochondria to undergo "brain transition". Tightly-located aggregates localized at the cell periphery were transformed to discrete grains separated from each other. Grain transition might serve to diminish damage effect by disassembling the mitochondrial system and to lighten redistribution of mitochondria inside the cell. "Brain transition" was possibly a morphological manifestation of reversible respiration decline. It strongly resembled so called "thread-grain transition" described for mitochondrial reticulum of fibroblastic and epithelial cells as a step of mitoptosis and apoptosis. Later progressively increasing cell death with diffuse extramitochondrial localization of cytochrome c appeared. In 4 hr after TNF addition in parallel with extramitochondrial localization of cytochrome c there were many apoptotic cell characterized by simultaneous nuclear fragmentation and mitochondrial localization of cytochrome c. Evidently at the early stage of nuclear fragmentation at least part of cytochrome c retained its mitochondrial localization

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Effects of Capsaicin on Rat Bone Marrow Stromal Cells
F. Aikawa, S. Kumebe, M. Nakatsuka, T. Okamura, Y. Iwai; Oral Anatomy, Osaka Dental University, Hirakata-shi, Japan
[Purpose] Chemical stimuli like growth factors and dexamethamone, and physically extending stimuli, are said to lead to apoplosis and cell proliferation. When the stimulus is modest, it would accelerate cell proliferation and cell differentiation. But when the stimulus is over the threshold, it would restrict cell proliferation or lead to apoptosis. In this study, we focused on capsaicin that is considered as a nociceptive stimulus, and examined the expression of capsincin receptor and transient receptor potential vanilloid-1 receptor (TRPV1) in cultured rat bone marrow stromal cells (RBMSCs). [Material and method] RBMSCs were obtained from the rat femora, and the RBMSCs were subcultured by 3 passages. Then the RBMSCs were cultured in Dulbecco’s Modified Eagle Media (DMEM) or with or without capsaicin for 2weeks in 24 well plates. Then each cDNA of cultured cell was extracted and the expression of TRPV1 mRNA was examined by RT-PCR. Also, we performed II-E staining on each well for the histological examination. [Result and discussion] TRPV1 mRNA was only expressed in the RBMSCs that
cultured with capsaicin. TRPV1 is a nonselective cation channel, and can be activated not only by capsaicin but also by heat (>43°C), various acid and lipids. For years, it is said that the expression of TRPV1 was restricted to sensory neurons and discrete areas of the central nervous system (CNS). While, a number of recent studies have demonstrated TRPV1 in a variety of non-neuronal tissues, including bladder and urethral epithelium, bowel, lung, kidney, etc., there is no studies that have demonstrated TRPV1 in the bone marrow. The present study demonstrates the expression of TRPV1 in the rat bone marrow, and this suggests additional of capsaicin in DMEM leads the expression of TRPV1 in RBMSCs.

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**Culture Medium Supplemented with Bovine Serum Albumin Effects on the Proliferation of Human Dental Pulp Derived Cells**

T. Okamura, S. Kambale, M. Nakutake, F. Akawa, Y. Iwasai, Oral Anatomy, Osaka Dental University, Hirakata, Japan

[Purpose] Culture medium is insufficient nutrition for several reasons, such as proliferation and differentiation; therefore, fetal bovine serum (FBS) is added. On the other hand, because a composition of serum is very complex, it is difficult to analyze the result of an experiment. In addition, a causation of transplantation of a formed tissue into the human body could be infection and immune reaction. For these reasons, we examined the possibility of the medium that added bovine serum albumin (BSA) instead of FBS using human dental pulp derived cells in this study.

[Materials and Methods] Human dental pulp cells were obtained from first premolars that were extracted for the orthodontic treatment. Then cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% FBS by the third passage. The cells cultured in 96 well plates on following 6 conditions; only DMEM, DMEM supplemented with 10%, 20%, and DMEM with 1%, 5%, 10% BSA. The cell proliferation was measured by bromodeoxyuridine (BrdU) cell proliferation assay kit chronologically. [Result and discussion] The cells cultured only DMEM showed low proliferation at any time. The cells cultured in DMEM with FBS showed better proliferation than DMEM only. The cells cultured with BSA showed remarkable proliferation in the first 2 hours of culture. There were few adhesive cells when cultured in DMEM with BSA. This study suggests that the difference of the medium composition have a big effect on the behavior of cell proliferation.

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**In Vitro Evaluation of the Cytotoxic Potential of Brickellia Cavanillessi (Asteraceae) Using HepG2 Cells**

R. Villas, W. Gao, E. Smith; Environmental Toxicology, The Institute of Environmental and Human Health, Lubbock, TX

Our long term goal is to characterize the potential toxicity and biological mechanisms of alternative herbal medicine. In this study we used an *in vitro* cell culture approach. Experiments were designed to evaluate an aqueous extract of the Latin American tea “prodigiosa” (*Brickellia Cavanillessi*), a member of the Asteraceae botanical family. *Brickellia C.* is traditionally consumed as an herbal remedy to treat diabetes and other chronic diseases in several underdeveloped countries. HepG2 hepatocellular carcinoma cells were submitted to both a dose- (IC50) and a time-dependent toxicity evaluation in the presence or absence of Fetal Bovine Serum (FBS). Herbal extracts (1-35%) were used for the IC50 study. In the time course study, cells were exposed to 1-10% extract solutions of *Brickellia C.* for 48hrs. Cell viability was determined with the MTT assay. The estimated IC50 was determined to range from 5-10% in the absence and presence of FBS. At doses exceeding 10%, cellular viability decreased significantly (p<0.05) relative to controls. Furthermore, there is a clear indication of a time dependent decrease in cell viability. No significant differences were observed when cells were cultured with or without FBS. These results suggest that the cell viability effects from tea extracts are not influenced by FBS. The preliminary data from this study indicates that *Brickellia C.* extracts negatively influence HepG2 cell viability. Further studies will be necessary to characterize the bioactive components of the extracts, to determine the postulated therapeutic value of *Brickellia C.* at lower concentrations, and to study the mechanisms of action.

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**Establishment and Characterization of a Human Neonatal Hepatocyte Cell Line**

J. P. Gaddipati, D. Yadav, J. Mundock, J. Kantor, Y. Reid, Cell Biology, ATCC, Manassas, VA, 1Neogenx Oncology, Rockville, MD

Primary hepatocytes have been extensively used in a wide variety of experimental studies, however, limited lifespan as well as restricted availability are major constraints for such studies. Immortalization of primary cells extend their replicative capacity and would provide for continuous, unlimited availability. Immortalized hepatocytes with a stable phenotype that mimics the original tissue would constitute very attractive experimental models for use in toxicological and pharmaceutical studies. Ectopic expression of human telomerase reverse transcriptase (hTERT) is one of the major strategies used in developing immortalized cells and allows for the retention of the original cellular characteristics to a large extent and avoids some of the problems associated with other approaches. In the present study we developed a cell line, NeHepLxHT, from human neonatal hepatocytes by transduction with a retroviral expression vector containing the hTERT gene. The cell line was continuously cultured for more than twenty five passages without senescence whereas the parental cells senesced within three passages. Thus, induction of stable expression of hTERT in the neonatal cells led to immortalization of these cells. Analysis of telomerase activity, by telomeric repeat amplification protocol (TRAP) assay, indicated elevated levels of telomerase activity in these cells compared to the parental cells. The immortalized cell line maintained a diploid karyotype and expressed gene product profiles similar to normal human neonatal hepatocytes. The data suggest that this immortalized cell line preserved the normal biological characteristics of neonatal hepatocytes and may therefore be useful model in in vitro studies.

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**Purified Schwann and Perineurial Primary Cell Cultures**

O. Vivas, C. M. Moreno, E. Bernal, C. M. Spinel; Departamento de Biología, Universidad Nacional de Colombia, Bogota, Colombia, 1Laboratorio de Biofisica, Centro Internacional de Fisica, Bogota, Colombia

OBJECTIVE Obtain highly purified Schwann cells cultures from dorsal root ganglia and perineurial cells from sciatic nerve in mice at short time. METHODS ICR mice were sacrificed by over-anaesthesia. Dorsal root ganglia (DRG) were took out and plated. In another essay the ganglia capsule was removed by microdissection. Ganglia were dissociated firstly with collagenase type II (280 U/ml) and then with mechanical dissociation. The dissociated tissue was plated with the aim of obtain Schwann cells. By microdissection, sciatic nerve was divided in its three connective tissues, the epineurium, the perineurium and the endoneurium. Perineurium and endoneurium were separately cultured to obtain perineurial cells and Schwann cells, respectively. Endoneurium culture was firstly treated with collagenase type II (400U/ml) before plated. In another experiment, nerves, without microdissection, were cut in 1 mm long pieces and plated. RESULTS Without microdissection, DRG cultures gave arise a mixed cultured of sensory neurons, capsular cells and abundant Schwann cells. We were able to obtain Schwann cell cultures with purity of 94% in only ten days without passages, based on the microdissecting capsule. Pieces of 1mm long of sciatic nerves that were neither microdissected nor collagenase-dissociated show a mixed culture of Schwann cells, fibroblasts and a few number of perineurial cells while after microdissection, endoneurium culture produced Schwann cells and fibroblasts and the perineurium culture showed perineural cells in 30 days without other kind of cells. Additionally, during these experiments we noted an important similarity in shape and morphology between DRG capsular cells and perineurial cells from sciatic nerve. CONCLUSION Microdissection is a technique that improves and facilitates the isolation and purification of Schwann cells from DRG and perineural cells from sciatic nerve.

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**Mkk6 Phosphorylation Regulates Production of Nos-2-derived Superoxide by Acting as a Rac-GAP**

M. M. Harraz, A. Park, W. Zhou, Y. Zhang, J. F. Englehardt; Anatomy and Cell Biology, University of Iowa, Iowa City, IA

Phagocytes utilize NADPH oxidase to generate reactive oxygen species that mediate killing of microbial pathogens. Rac is a small GTPase essential for NADPH oxidase activity. While the mechanisms leading to NADPH oxidase activation are fairly well studied, the mechanisms that control down-regulation of this enzyme complex remain unclear. We hypothesized that reactive oxygen species produced by NADPH oxidase may autoregulate the complex by inhibiting Rac activity. To this end, we searched for binding partners of Rac1 that were induced by redox-stress of RAW cells and identified a tyrosine-phosphorylated fragment of Mkk6 that bound to Rac1 under these conditions. Constitutively active Mkk6 interacted directly with Rac1 in vitro and this interaction was enhanced when Mkk6 was phosphorylated on tyrosine 219. Both Rac1 and Rac2 immunoprecipitated an Mkk6 fragment under conditions that elevate cellular peroxide levels in 293 and RAW cells, respectively. Constitutively active Mkk6 and Rac1 co-immunoprecipitated and its expression inhibited phorbol myristate acetate (PMA) induced NADPH oxidase activity in RAW cells. In contrast, overexpression of the constitutively active Y219F Mkk6 mutant did not alter PMA-induced NADPH oxidase activity in these cells. Our findings suggest that Mkk6 down-regulates NADPH oxidase activity by acting as a novel Rac-GAP.

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Establishment and Characterization of a Human Telomerase Catalytic Subunit-transduced Fetal Bone Marrow-derived Osteoblastic Cell Line

M. Feng; Institute of Zoology, Chinese Academy of Sciences, State Key Lab of Biomembrane and Membrane Biotechnology, Beijing, China

The fate of human hematopoietic stem cells (HSCs)/progenitor cells (HPCs) is influenced by bone marrow (BM) stromal cells. To investigate the role of stromal cells in the hematopoietic support, we have transduced human fetal BM stromal cells (FBMSCs) with human telomerase catalytic subunit (hTERT). One of the resultant cell lines was identified as osteoblasts, because it contained mineral deposits and constitutively expressed osteogenic genes osteocalcin, osteopontin, collagen type I, osteoblast marker alkaline phosphatase, but not marrow stromal cell marker STR-1 and CD105. The hTERT-transduced fetal BM-derived osteoblastic cells (FBMOB-hTERT) can actively maintain the capacity of self-renewal and multipotency of HSCs/HPCs at least partly through transcriptional up-regulation of hematopoietic growth factors such as stem cell growth factors (SCFs) and Wnt5A signaling. Moreover, the FBMOB-hTERT cells seem superior to primary FBMSCs in supporting hematopoiesis, because they are more potent than primary FBMSCs in supporting the in vivo expansion and LTC-IC activity of HSCs. The FBMOB-hTERT cell line has been maintained in vitro more than 125 population doublings (PDs) without tumorigenicity. The results indicate that the FBMOB-hTERT is useful for the study of molecular mechanisms by which osteoblasts support hematopoiesis.

DNA Barcodes for Definitive Species Identification of Cells in Culture

J. K. Cooper, 1 N. V. Ivanova, 2 P. Ikonomi, 1 R. Hanner; 1 ATCC® (American Type Culture Collection), Manassas, VA, 2 Biodiversity Institute of Ontario, University of Guelph, Guelph, ON, Canada

Cell line authentication is crucial for the accuracy and reproducibility of scientific research, though cultures are often maintained for extended periods of time without being assayed for species identity. Traditional isoenzymological identification tools suffer from a limited taxonomic scope and are not easily replicated among laboratories. We evaluated a sequence-based approach for the species level identification of animal cell lines, targeting variation in ~650 bp of the 5' region of the mitochondrial cytochrome c oxidase subunit 1 (COI) gene as a molecular diagnostic tool or “DNA barcode.” ATCC® characterized the COI barcode region from 67 cell cultures representing 44 distinct species. The data was analyzed using the Barcode of Life Data (BOLD) systems, a collaborative on line database that aids in the collection, management, analysis and application of DNA barcodes. Cell lines derived from the same species had virtually identical sequences (0.4% mean divergence) whereas cell lines from different species showed great divergence (25.9% mean divergence) even when the two species shared a common genus (17.1% mean divergence). To test the reproducibility of this method among labs, we spotted 28 of the analyzed samples on FTA Whamman® cards and sent them to the University of Guelph for independent blind COI sequence analysis, where matching genetic profiles were obtained using a different analytical platform and chemistry. We conclude that DNA barcoding can successfully identify a wide range of species from various animal taxa in cell culture, making fine distinctions among congeneric species. This technique is easily replicated among laboratories and because the BOLD database contains reference sequences derived from morphological voucher specimens, it provides a reliable means of validating a putative species identification accompanying a cell line submitted to, and distributed by, collection centers. Such authenticated cultures can also serve as standard reference materials or as controls in forensic barcode analysis.

Detection of Interspecies Cross-Contamination in Cell Culture: A Highly Sensitive Multiplex PCR Based Approach

J. K. Cooper, S. King, K. Cottrill, M. Crissup, G. Sykes, P. Ikonomi; ATCC® (American Type Culture Collection), Manassas, VA

Extensive cross-contamination of human and animal cell lines with a variety of human and animal cell lines is a long-standing problem in biomedical research. Although awareness dates back to the 1950s, the problem has not diminished; submission of cell lines to major repositories indicates that up to 20% of the cultures may be contaminated. Currently, isoenzymology is the favored method for species identification and detection of interspecies contamination in cell lines but it suffers from two major limitations. First, the technique is cumbersome and therefore not widely practiced. Second, its sensitivity for the detection of cross-contamination is low (10-20%). To overcome these limitations we developed a multiplex PCR-based assay to detect cross-contamination and for species identification of cell lines used commonly in research laboratories. The assay is based on the mitochondrial cytochrome c oxidase 1 (COI) gene as a target. Species-specific primer pairs were designed based on sequence information both from public databases as well as sequencing of COI from ATCC® cell cultures. Each primer set was designed to produce a distinct amplicon that can be easily resolved by agarose gel electrophoresis or by other separation methods. This feature allowed the primers to be multiplexed. Additionally, we designed an internal control for the verification of the assay. The assay described above can detect cross-contamination when the contaminating species is present at less than 1% in a test cell culture. We have confirmed the species identity and checked for the presence of inter-species contaminants in 100 different cell lines. Further sensitivity analyses of the assay are currently being conducted. Our results show that this assay is sensitive, easy, cost effective, requires no dedicated equipment, and can be used in any research laboratory as a practical tool for species identification and confirmation of cell cultures.

Effects of Mycoplasma Infection in Cell Culture Systems and Tool for Routine Mycoplasma Screening

Q. Li, K. Cottrill, G. Sykes, M. Fisher, T. Correa; P. Ikonomi; American Type Culture Collection, Manassas, VA

Contamination of cell cultures is a problem that affects many laboratories. Regular screening of cell cultures for mycoplasma infection remains the only protection. We present here quantitative analyses of the effect of mycoplasma infection in two cell culture systems and offer a PCR-based method as a tool for regular screening mycoplasma infection in cell cultures. Two commonly used cell lines-HeLa and PC3 were infected with Mycoplasma hominis and Mycoplasma fermentans. While no significant changes were observed in overall cell growth, infection of mycoplasma had a significant effect on the cell proliferation assay. Mycoplasma infection of HeLa and PC3 cell lines reduced efficiency of transcription and reporter gene expression by 40% and 80% respectively. Quantitative PCR analyses of the expression of various cytokines and other endogenous genes at 6, 24, 48 and 72 hours after infection suggested that infection of mycoplasma, even at significantly low levels, drastically affects the results of comparative analyses of the transcriptome. We then describe a novel PCR-based assay capable of detecting low levels of infection of 49 mycoplasma species, including the mycoplasma species commonly found in cell cultures. The assay targets the nonconserved intergenic region of 16S-23S rDNA operon of mycoplasma, allowing for speciation of the mycoplasma infection either by size discrimination of the amplicons or via endonuclease digestion. The efficiency and the sensitivity of the assay are shown using six additional cell lines infected with mycoplasma and purified mycoplasma DNA from several species. Our results clearly showed the effects of mycoplasma infection has in skewing results acquired in studies conducted with cell line models. The PCR-based mycoplasma detection method offers a practical and sensitive tool for routine screening for mycoplasma infection as a requirement for the accuracy and the reproducibility of scientific research using animal cell culture.

Morphological Observations of Cell-Clusters in Hemolymph of Mamestra brassicae

P. R. Mangatikla, T. Kawamoto, K. Iwabuchi; Applied Entomology, Tokyo University of Agriculture and Technology, Tokyo, Japan

Hemocytes isolated from the larval hemolymph were classified into several types; plasmatocyte, granulocyte, spherulocyte, oenocytoid and prohemocyte. In addition, we observed cell-clusters. In short-term culture, the morphology of cell-cluster did not change, although change when co-cultured with fat body tissues or cultured under supplement with fat body extract or larval plasma. However, there was no effect in heat-treated plasma, protease-k treated plasma and co-cultured with hemocyte, suggesting that the fate of the cell-cluster is regulated by some factor(s) in larval plasma and fat body.

Heat Shock Protein of 70 kDa Family in Viable Paleacanthamoebae Acanthamoeba sp. and in Contemporary Amphilozite Representitive of the Same Genus

Y. I. Podlipaeva, 1 L. Shmakova, 1 I. Dykova, 1 A. Goodkov; 1 Institute of Cytology Russian Academy of Sciences, St. Petersburg, Russian Federation, 2 Institute of Physicochemical and Biological Problems in Soil Science, Pushchino, Moscow Region, Russian Federation, 3 Institute of Parasitology Academy of Sciences of Czech Republic, Ceske Budejovice, Czech Republic

The viable cysts of paleacanthamoebae belonging to the genus Acanthamoeba were isolated from samples of permafrost aging 30000-35000 years (eastern sector of the Arctic region, Kolyms Lowland and the Laptev Sea coast). After excystation trophozoites (Acanthamoeba sp. strain Am8) were cultivated in the laboratory at the room temperature. Method of immunoblotting using monoclonal anti-HSP70 antibodies was applied to reveal heat shock proteins of this family. Previously in some strains of Ameoba proteus the HSP of 70 kDa MW, termed as constitutive, was...
revealed by the same antibodies. In intact Am8 trophoectoderm extremely high level of HSP of about 60 kDa MW was revealed. We failed to cause induction of this protein in Am8 cells by heat (37 °C, 1 h) and cold (8 °C, 1 h) shocks; both shocks were resulted only in decreasing of constitutive HSP level. When comparing with contemporary representatives of the same genus (*Acanthamoeba* sp. strain 4465, the Department of Eukaryotic Organisms Infecting Fish, Inst. Parasitol., Acad. Sci. Czech. Rep.) it appeared that no zones were revealed on blot after staining with the same antibodies, and with the same amount of protein on the start gel. The preliminary data on temperature preferences of studied strains point that paleoamoebae (strain Am8) is much more thermostolerant than *Acanthamoeba* sp. strain 4465. Unusually high constitutive level of HSP in the cells of paleoamoebae might indicate the high potencies for their surviving, even in terms of geochronology.

608 Endotoxin-induced Fetal Toxicity Can Be Prevented by Pretreatment with Flavonoid Extracts in the Rat Model
E. E. Ntam, D. Hill, T. Charles, R. Howell, M. Baker, T. Martin, C. Reese; Department of Biology, Morgan State University, Baltimore, MD

Environmental contaminants have been well documented for their adverse effects on embryonic/fetal development. Endotoxin (environmental contaminant) has been associated with various developmental toxicities including embryo-resorption, degenerated tissue integrity, altered organ maturation, fetal-growth suppression and fetal lethality. Studies have indicated that endotoxin injury can be mediated by immune-effector cell activity. This is conceivable that decreases in effector-cell activity could alter the degree of endotoxin-induced toxicity. Natural flavonoid extracts (silymarin) can decrease immune-cell activation, scavenge reactive radicals and protect liver tissues from contaminant-induced injury. However, the feto-protective activity of flavonoid extracts requires further investigation. Therefore, the present study was designed to address the hypothesis that *in utero* pretreatment with flavinoid extracts affords protection against altered fetal tissue/organ development after exposure to endotoxin. Pregnant rats were pretreated with a flavinoid extract (25 mg/kg/day; gestational days 7 - 17). Exposure to endotoxin (50 μg/kg/day) occurred from gestational days 12 through 17. On gestational day 18, fetuses were removed to analyze gross fetotoxicity. On the parameters of fetotoxicity included crown-rump length, tail length, fetal viability/lethality, limb development and visible-tissue alterations. The results demonstrated that *in utero* exposure to endotoxin caused decreases in normal crown-rump length, tail length and fetal viability while limb and digit development remained unaffected. Endotoxin exposure also produced overt abdominal coagulation, sub-dermal hematomas and incomplete closure of the abdominal cavity. Pretreatment with flavinoid extracts afforded protection against endotoxin-induced lethality. Moreover, the flavinoid pretreatment regimen protected the abdominal tissues from endotoxin-induced injury. Supported by NIEHS R22ES00330, DOE E63580.

609 Differentiation of Non-Adherent Hematopoietic Stem Cells from Umbilical Cord Blood Cells into Adherent Hepatocytic Lineage
R. Peters, M. M. Mock, B. Steiger; Department of Medicine, University Hospital, Zurich, Switzerland

The liver has an enormous regenerative capacity. However, after severe liver injury, this capacity is not sufficient for regeneration and treatment of liver failure with hematopoietic progenitor cells might be a therapeutic option. It has been shown that umbilical cord blood (UCB) mesenchymal stem cells (MSC) are capable of differentiation into mesenchymal and non-mesenchymal lineages. However, only about 20% of frozen and either separated or unseparated UCB will give rise to MSC. We have developed the technology generate MSC from all frozen (3/3 tested) UCB. Here we show that the incubation of thawed UCB mononuclear cells and non-selected CD34+ cells in the presence of SCF, MGDF, FL-3 IL-6 and human serum in stroma-free liquid culture generated long-term expansion of UCB non-adherent hematopoietic stem cells (HSC) and long term expansion of MSC (adherent cells). Adherent cells were enriched by medium changes followed by trypsinization and subsequent culture and passing on fibronectin. Mesenchymal non-fibroblast liquid cultures were tested for the production of HSC as well as MSC over a period of 15 weeks and MSC kept increasing through out expansion of HSC. Expanded MSC between 35th to 5th passages were plated using MesenCult®. After 14 days, very large and small colony forming fibroblasts colonies were continuously detected throughout expansion. In order to test the capacity of these MSC to differentiate into a hepatocytic lineage, cells were cultured in DMEM supplemented with dexamethasone and HGF, which was replaced with Oncostatin M after day 14. This lead to the appearance of cells with hexagonal, hepatocyte-like appearance. These cells expressed mRNA for albumin, the basolateral bile salt uptake system NTCP and the canalicular bile salt export pump BSEP as assessed by RT-PCR. These data demonstrate the presence of pluripotent stem cells in UCB with the capacity to differentiate into a hepatocytic lineage.

610 Expression of 14-3-3 Proteins in the Human Cornea
J. Shankardas, D. Dimitrijevich; UNT Health Science Center, Fort Worth, TX

**Background:** 14-3-3 is a highly conserved, ubiquitously expressed family of proteins. At least seven mammalian isoforms ([β, γ, ε, ζ, τ and η] are known. These proteins associate with over 200 different target molecules and activate a number of downstream signaling cascades involved in the regulation of metabolism, cell cycle, apoptosis, protein trafficking, transcription, stress responses and malignant transformations. We are interested in the role of these proteins in the mechanisms regulating homeostasis and pathologies in the human ocular surface. **Purpose:** To determine the expression of the 14-3-3 proteins in the human cornea, the conjunctiva and the primary cells comprising these tissues. **Methods:** Using immunofluorescence, we determined the expression of 14-3-3 isoforms in the paraffin sections of the human cornea and conjunctiva. Using indirect immunofluorescence and western blot analysis we also determined the expression of these isoforms in primary corneal and conjunctival epithelial cells, keratocytes and endothelial cells. Furthermore, expression in primary epithelial cells was compared with that in several human corneal and conjunctival cell lines. Western blot analysis was used to confirm the presence of 14-3-3 isoforms in the culture medium from corneal epithelial cells and cell lines. **Results:** All the 14-3-3 isoforms were expressed in the corneal and conjunctival epithelia and the primary epithelial cells and cell lines. Expression of 14-3-3-ε was confined to epithelial cells and was secreted into the culture medium of the primary cells and cell lines. **Conclusions:** We have determined that all the mammalian 14-3-3 isoforms are expressed in the human cornea, conjunctiva and the component cells, and that the 14-3-3-ε isoform is found to be secreted into epithelial cell culture medium. We propose that the intracellular and extracellular presence of 14-3-3 ε supports its involvement in the epithelial specific signaling pathways.

611 Wound-induced ATP Release and Epidermal Growth Factor Receptor Activation in Epithelial Cells
J. Yin, K. Xu, J. Zhang, A. Kumar, F. X. Yu; Ophthalmology & Anatomy & Cell Biology, Wayne State University, Detroit, MI

Wounding of human corneal epithelial cells (HCECs) has been shown to elicit epidermal growth factor receptor (EGFR) transactivation through epidermal shedding of heparin-binding EGF-like growth factor (HB-EGF). However, the initial signal to target these signaling events in response to cell injury remains elusive. In the present study, we investigated the role of adenosine triphosphate (ATP) released from the injured cells in EGFR transactivation and in the activation of its downstream effectors phosphatidylinositol-3-kinase (PI3K)/AKT and extracellular signal-regulated kinase (ERK) pathways in HCECs as well as in BEAS 2B cells, a bronchial epithelial cell line. Wounding of epithelial monolayers of both cell types increased the ATP concentration in the culture media. The wound-induced rapid activation of PI3K and ERK in HCECs as well as in BEAS 2B cells, a bronchial epithelial cell line. Wounding of epithelial monolayers of both cell types increased the ATP concentration in the culture media. In order to test the capacity of these MSC to differentiate into a hepatocytic lineage, cells were cultured in DMEM supplemented with dexamethasone and HGF, which was replaced with Oncostatin M after day 14. This lead to the appearance of cells with hexagonal, hepatocyte-like appearance. These cells expressed mRNA for albumin, the basolateral bile salt uptake system NTCP and the canalicular bile salt export pump BSEP as assessed by RT-PCR. These data demonstrate the presence of pluripotent stem cells in UCB with the capacity to differentiate into a hepatocytic lineage.

612 The Cyclic Dependent Kinase 5 Inhibitor Olomoucine Promotes Corneal Epithelial Wound Closure In Vivo
B. K. Tripathi, M. A. Stepp, P. S. Zelenka; Laboratory of Molecular and Developmental Biology, National Eye Institute/National Institutes of Health, Bethesda, MD, 1Department of Anatomy and Cell Biology, The George Washington University Medical Center, Washington, DC

The prolinc-directed kinase, CDK5, regulates adhesion and migration of corneal epithelial cells in culture. Moreover, olomoucine, a CD5 inhibitor, accelerates corneal derbridement wound closure in organ cultured eyes of normal mice. This study was undertaken to test the effect of olomoucine on corneal derbridement wound closure in vivo in normal mice. A 1.5 mm central derbridement wound was made in corneas of anesthetized mice. The treatment group received 20 μl of 15 μM olomoucine in phosphate-buffered saline (PBS) containing 0.1% dimethyl sulfoxide (DMSO), applied as drops to the wound area immediately after wounding (0 h) and again after 6 h. The control group received similar applications of PBS containing 0.1% DMSO. Eighteen hours after wounding mice were euthanized, eyes were removed, fixed in 4% paraformaldehyde, stained with Richardson’s stain and photographed. The remaining wound area was measured with image processing software. Images were coded during measurement to prevent experimenter bias. Measurements of 28-30 mice in each group indicated that the remaining wound area in the control group was approximately 2-fold greater than that of the olomoucine treated mice (p=0.001). Two weeks after wounding, reepithelialization of the cornea was identical in

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both olomoucine treated and control groups and neither group showed evidence of inflammation or stromal disorganization. These findings indicate that Cdk5 inhibitors promote corneal epithelial cell migration in vivo as in vitro and suggest that this class of compounds may be therapeutically useful for treatment of corneal epithelial erosions.

613 Maintenance of Corneal Epithelia in Pax6+/− Mice
J. Ou, R. Kucera, L. Leiper, P. Walczynko, J. M. Collinson; School of Medical Sciences, Institute of Medical Sciences, Aberdeen, United Kingdom

Pax6 encodes a transcription factor, which is vital to morphogenesis and functioning of the eye. Human and mice heterozygous for the gene have complex lens, iris and cornea abnormalities, including corneal opacity (aniridia-related keratopathy - ARK). We studied the etiology of such cornea abnormalities. By incubating the eyes with a cell impermeable fluorescent dye - lucifer yellow, we showed that unlike wild-type, the corneas of Pax6+/− mice are often permeable to diffusion of small molecules, presumably due to breakdown of tight junctions between superficial cells. Proteomic analyses revealed that Pax6+/− corneas have less keratins than wild-type. Pax6+/− corneas are deficient in several enzymes involved in detoxification. These results implied that Pax6+/− corneal epithelia are vulnerable to environment hazards and may subject to chronic cycles of damage and regeneration. We studied if molecular pathways controlling corneal epithelial cell migration are defective in Pax6+/− mice. Recently we reported that primary cultures of Pax6+/− mouse corneas have defective Ca2+ signalling in response to wounding. Subsequently, we showed by immunoassaying that phosphatidylinositol 4, 5-biphosphate, a multipotent 22nd messenger, may mediate Ca2+-signalling defects and abnormal actin assembly in Pax6+/− corneal epithelia. Extracellular-signal regulated kinase 1 and 2 (ERK 1/2) are abnormally activated, while other MAP kinases are poorly activated in Pax6+/− corneal epithelia; in an ex vivo epithelial model, inhibition of ERK1/2 activation slowed epithelial migration in both wild-type and Pax6+/− corneas. Different MAP kinases were suggested to be activated by different small GTPases. Therefore, MAP kinases and their respective small GTPase regulators may play distinct but coordinated roles of migration in regenerating mouse corneas; defective migration of the Pax6+/− corneal epithelium may result from cumulative effects of aberrant signaling of PI3P and MAP kinases.

614 Genetic Control of Corneal Epithelial Cell Migration by Pax6
R. Kucera, B. Reid, M. Collinson; Cell and Developmental Biology, Institute of Medical Sciences, University of Aberdeen, Aberdeen, United Kingdom

Cell migration is essential for tissue repair and wound healing. The factors that drive directional cell migration are incompletely understood. We have tested the role of endogenous electric fields (EFs) for guiding cell migration and wound healing. Ion transport across the corneal epithelium creates a potential difference (PD) such that the basal layer of the corneal epithelium is +25mV compared to the tear film. After the wounding, the PD collapses to 0mV at the wound site, resulting in a standing voltage gradient directed from the basal layer of intact epithelium into the wound. Cells are meant to respond to this standing voltage gradient by migrating cathodally into the wound. We have directly measured the wound induced electric current in current of mice heterozygous for the Pax6 transcription factor using vibrating, self - referencing microelectrode. Pax6+/− eyes showed normal outward current compared to Pax6+/− eyes of which 43% showed an inward current and the others showed small outward current. Ion replacement experiments suggest defective sodium pumps and channels in Pax6+/− epithelial cells. The wound-healing rate of corneal epithelium of both genotypes was measured and quantified. Surprisingly, all epithelia healed at approximately equal rates irrespective of genotype or (for Pax6+/−) the direction of wound-induced electric current. We tested the ability of the cultured mouse corneal epithelium cells to respond to in vitro applied EFs. The results showed that on average Pax6+/− cells migrate to the anode. Whole eye organ culture and electric current measurements, revealed no significant correlation between wound healing rate and the direction and magnitude of EF in Pax6+/− and Pax6+/− cells. We suspected that although applied EFs can modulate the wound healing process, endogenous EFs are unlikely to be major essential guidance cue for corneal epithelial cell migration.

615 Desmoplakin Regulates Rho Signaling through GEFD1
A. P. Miera; Cell Biology, Georgetown University, Washington, DC

Desmoplakin’s (DP) primary function is to link intermediate filaments to desmosomes. This function has been confirmed by conventional and tissue specific knockout studies. However, recent evidence from our lab strongly suggests that desmoplakin may also have a signaling function. We employed a novel high-throughput proteomics assay termed luminescence-based mammalian interactome mapping (LUMIER) whereby Luciferase-tagged DP cDNA bait was co-expressed with over 500 Flag-tagged prey cDNAs, followed by immunoprecipitation with a flag antibody and detection using the luciferase assay. We found three guanine exchange factors (GEFs) directly associated with DP. We analyzed the effects of the GEFD1/DP interaction focusing on RhoA activity and its downstream targets in human embryonic keratinocytes. Immunoprecipitation studies revealed that GEFD1 directly associates with DP in a spatio-temporal manner during early time points of terminal differentiation. Knocking down DP prior to inducing terminal differentiation and desmosome formation resulted in diminished RhoA activity, which was restored upon DP siRNA degradation and re-expression of DP protein. Using a fluorescent reporter that measures RhoA activity, we temporally and spatially confirmed the GEFD1/DP dependent-activation of RhoA at cell junctions during differentiation. Consequently, GEFD1/DP interaction is needed for normal RhoA activity in keratinocyte differentiation. To map the region on DSP that interacts with GEFD1, we generated a series of luciferase-tagged LUMIER vectors and analyzed their interaction with GEFD1. This revealed that the plakin domain of DP bound GEFD1. We then expressed each of these DP domains in Heka cells and examined cortical actin network (CAN) formation and GEFD1 localization. Of all the domains tested, only expression of the plakin domain caused mislocalization of GEFD1 and this correlated with disruption of the CAN. Consequently, our data supports a model in which DP is directly linked to actin reorganization by affecting the GEF/D/Rho/ROCK signal transduction pathway.

616 Deficiency of Protein 4.1R in Mice Results in Epidermal Hyperplasia
R. A. Hughes, X. An, C. Liu, N. Mohandas; Red Cell Physiology, New York Blood Center, New York, NY

Protein 4.1R (4.1R), originally identified as a major component of erythrocyte membrane, is also widely expressed in non-erythroid cells. In order to explore the function of 4.1R in non-erythroid cells, we have generated 4.1R knock-out mice. In the present study, we evaluated the functional role of 4.1R in skin. Interestingly, histological examination revealed a thickened epidermal layer, accompanied by an increase in the number of mitotic cells. The wound-healing rate of corneal epithelium of both genotypes was measured and quantified. Surprisingly, all epithelia healed at approximately equal rates irrespective of genotype or (for Pax6+/−) the direction of wound-induced electric current. We tested the ability of the cultured mouse corneal epithelium cells to respond to in vitro applied EFs. The results showed that on average Pax6+/− cells migrate to the anode. Whole eye organ culture and electric current measurements, revealed no significant correlation between wound healing rate and the direction and magnitude of EF in Pax6+/− and Pax6+/− cells. We suspected that although applied EFs can modulate the wound healing process, endogenous EFs are unlikely to be major essential guidance cue for corneal epithelial cell migration.

617 Assessment of p53 Protein Expression in Human Skin Models
R. L. Warters, B. Zhuplatov, D. L. Williams, C. D. Pond; 1 Radiation Oncology, University of Utah Health Sciences Center, Salt Lake City, UT, 2 Pharmacology and Toxicology, University of Utah Health Sciences Center, Salt Lake City, UT

The purpose of this study was to compare the regulation of the p53 stress response of cultured keratinocytes with keratinocytes in intact human skin. Neonatal human skin was obtained from the LDS Hospital Newborn Clinic. Primary, low passage number keratinocytes were recovered from Diapase II-treated neonatal skin and cultured in serum free keratinocyte medium (SKFM) (In Vitrogen). Skin epidermis was peeled away from skin that was heated at 60°C for 60 seconds and dissolved into RIPA buffer. Equal amounts of protein from cultured cells or the epidermis were separated through 4-12% gradient acrylamide gels, electroblotted onto membranes and western analyzed. Per equal g of protein recovered from cultured keratinocytes, we observed significant levels of the p53 DNA damage stress response, in cultured keratinocytes relative to keratinocytes in the intact human skin. In the epidermis the levels of p53 may be kept low through Mdm2 ubiquitilation of the p53 protein. This appears not to be the mechanism of regulating p53 levels in cultured keratinocytes. This work was supported by Department of Energy grant number DE-FG03-01ER63240.

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Characterization of Sex-specific Expression of Airway Epithelial Proteins in the Mouse Lung

L. S. Van Winkle, J. Y. Wong, P. C. Edwards, D. Anderson, K. M. Sutherland, M. A. Isehll; Vncape/che, UC Davis, Davis, CA

Men and women are differentially affected by several major lung diseases that arise from the conducting airways including adenocarcinoma, COPD and asthma, regardless of smoking history. Defining the proteomic differences between males and females is key to understanding the sex differences in conducting airway biology. Our preliminary studies have shown that there is a difference in the epithelial proteome of the female vs male mouse conducting airway. We separated airway epithelial proteins obtained via 2 methods: lysis lavage (LL) and airway microdissection (MD) using 2-D gel electrophoresis. Both methods detected unique spots by sex but the total number of protein spots after correcting for background and noise was much greater on the LL samples (average of 1026) compared to the samples prepared by MD (average of 580). For this study we defined the effect of estrous cycle on the female mouse proteome. Estrous cycle stage was determined using vaginal cytology. Unique and common protein spots were identified using Phoretix gel imaging software (on 3 gels). Of these spots, there was one protein common to both male and female lysis lavage that mobilized laterally only on 2D gels from female mice, consistent with changes in the estrous cycle. MALDI-TOF/TOF identified this protein as peroxiredoxin-6. We conclude that 1) we can detect sex-specific expression of proteins and protein modifications using this approach and 2) that peroxiredoxin 6 is modified, possibly oxidized, in female mice coordinately with changes in the estrus cycle. Supported by grants from NIEHS (ES 012720 and ES05707) and TRDRP 14RT-0132.

Expression and Sub-cellular Localization of Protein 4.1 Homologs in Human Bronchial Epithelial Cells

Q. Kang, A. Xulii, N. Mohandas; Red Cell Physiology Laboratory, New York Blood Center, New York, NY

Protein 4.1 family of proteins act as adapter proteins linking the actin-based cytoskeleton to various transmembrane proteins. The four members of this family, 4.1R, 4.1G, 4.1N, and 4.1B, are encoded by four homologous genes, which undergo extensive alternative splicing. Members of protein 4.1 family are widely expressed in various cells but their specific function in these cells remains largely unexplored. As a first step in defining the function of protein 4.1 homologs in epithelial cells, we systematically examined the expression and localization of these proteins in human bronchial epithelial (HBE) cells by western blot analysis and immunofluorescence confocal microscopy using antibodies highly specific for each of the four 4.1 protein homologs. Western blot analysis revealed that two 4.1B (210 kDa and 53 kDa), three 4.1G (120 kDa, 90 kDa and 75 kDa), two 4.1N (110 kDa and 53 kDa) and three 4.1R (75 kDa, 60 kDa and 53 kDa) isoforms are expressed in these epithelial cells. Immunofluorescence confocal microscopy revealed distinct sub-cellular localization of various 4.1 isoforms. For example, while the 210 kDa 4.1B isoform is localized mainly to Golgi apparatus, the 53 kDa 4.1B isoform is associated with the plasma membrane and the mitotic spindle pole. Similarly, the 75 kDa 4.1G is detected in the nucleus, the 110 kDa 4.1N is associated with the lateral domain and the 53 kDa 4.1R is localized to the endoplasmic reticulum. These findings imply that all 4 protein 4.1 homologs are expressed in a single cell type. Furthermore, the diverse cellular distribution of various homologs and their isoforms indicate multiple roles for 4.1 proteins in epithelial cell biology.

IL-13 Induces a Bronchial Epithelial Phenotype That Is Inherently Profibrotic

N. K. Malaviya,1 R. A. Panarettes,2 S. C. George,1 Biomedical Engineering, University of California—Irvine, Irvine, CA, 2University of Pennsylvania, Philadelphia, PA

Introduction. The role of the epithelium in orchestrating subepithelial structural changes in asthmatics is of key interest. In embryo genesis the epithelium dictates’ mesenchymal differentiation and growth. In asthma there exists an altered epithelium phenotype characterized by hyperplasia and excessive mucus production. This altered phenotype may reawaken subepithelial tissue differentiation and growth by influencing numerous cells including the fibroblasts. While it is known that mechanical perturbations can cause release of profibrotic factors (eg TGFB-2 and Th-2 cytokines eg IL-13) induce goblet cell hyperplasia it is not clear whether the two perturbations cause different effects on epithelial-mesenchymal communication. We hypothesized that the phenotypic changes induced by IL-13 create an epithelium that is inherently profibrotic; that is, an IL-13 treated epithelium would induce changes in the subepithelial fibroblast consistent with fibrosis in the absence of IL-13. Methods. Fully mucociliary differentiated normal human bronchial epithelial cells (NHBE) were treated with IL-13 (0, 0.1, 1, 10 ng/ml) for fourteen days during the differentiation period. IL-13 was then withdrawn, and the NHBE were co-cultured with normal human lung fibroblasts (NHLF) for three or ten days. Results. IL-13 induced increasing levels of MUC5AC protein and a corresponding decrease in tubulin IV protein in the NHBE that persisted in the absence of IL-13. Elevated levels of TGFB-2 in the NHBE-NHLF co-culture persisted over the course of 10 days. Collagen I, III, Iysyl oxidase, and alpha-smooth muscle actin gene expression was upregulated in the NHLF, accompanied by increased deposition of collagen in the extracellular matrix. Conclusion. IL-13-treated NHBE creates a phenotype that is biased towards subepithelial fibrosis, which may be driven by epithelial-derived TGFB-2. Once established, this phenotype does not require IL-13 to eliciting these effects. We propose that a persistent state of epithelial mucus cell hyperplasia may account for key features of airway remodeling observed in chronic lung disease.

Peroxisome Proliferator-Activated Receptor-γ Regulates Airway Epithelial Bicarbonate Transport

M. Duszyk, N. Madsen, D. Nahirney; Physiology, University of Alberta, Edmonton, AB, Canada

This study examines the role of peroxisome proliferator activated receptor γ (PPARγ) in the regulation of human airway ion transport. Identification of molecules that can alter the normal response of airway epithelial cells to various stimuli may be therapeutically beneficial in a variety of airway diseases. PPARγ has emerged as crucial factor controlling gene expression in response to nutritional, pharmacological and metabolic stimuli. However, the effects of PPARγ activation on transepithelial ion transport have not been studied in the human lung. Therefore, we investigated the effects of various PPARγ agonists (such as pioglitazone, L-tyrosine-based agonists, N-[9-fluorenylmethyloxycarbonyl]-L-Leucine, and the short-circuit current (Isc) was measured in normal, HCO3− or Cl−-free Krebs-Henseleit solutions (KHS). Cells treated with pioglitazone exhibited a reduced forskolin- or carbachol-activated Isc. Interestingly, other thiazolidinediones, such as troglitazone, had no effect on either forskolin or carbachol responses. In HCO3−-free KHS, pioglitazone did not affect forskolin-activated Ic, but decreased carbachol-activated Ic. Pioglitazone had no effect on either forskolin- or carbachol activated Ic in Cl−-free KHS. Differential regulation of cell metabolism of PPARγ agonists in different transport systems in the apical and basolateral membranes. Real time PCR revealed that PPARγ agonists affected expression of CFTR Cl channels and Na+/HCO3− cotransporter (NBC1). The decrease in airway bicarbonate transport may provide a possible explanation for the increased upper airway infections observed in subjects participating in clinical trials of PPARγ agonists.

An SEM Study of the Effects of RU486, Used as a Postcoital Contraceptive, on the Rat Uterus during Early Pregnancy

K. E. Scholtz,1 C. Penny,2 M. J. Hosie1; 1Anatomical Sciences, University of the Witwatersrand, Johannesburg, South Africa, 2Medicine, University of the Witwatersrand, Johannesburg, South Africa

The window of receptivity is a narrow range of time in which the blastocyst can attach to the uterine surface and is under the strict control of the ovarian hormones, progesterone and oestrogen. To prime the uterus for blastocyst attachment, the plasma membrane of the uterine epithelial cells undergoes a remarkable change in structure, collectively referred to as ‘the plasma membrane transformation’ of early pregnancy that is mediated by the ovarian hormones. RU486, the controversial abortion drug marketed as Milfigyne™, has a high affinity for progesterone receptors, blocking binding of progesterone. However, normal human bronchial epithelial cells were treated with PPARγ agonists such as pioglitazone, L-tyrosine-based agonists, N-[9-fluorenylmethyloxycarbonyl]-L-Leucine, and the short-circuit current (Isc) was measured in normal, HCO3− or Cl−-free Krebs-Henseleit solutions (KHS). Cells treated with pioglitazone exhibited a reduced forskolin- or carbachol-activated Isc. Interestingly, other thiazolidinediones, such as troglitazone, had no effect on either forskolin or carbachol responses. In HCO3−-free KHS, pioglitazone did not affect forskolin-activated Ic, but decreased carbachol-activated Ic. Pioglitazone had no effect on either forskolin- or carbachol activated Ic in Cl−-free KHS. Differential regulation of cell metabolism of PPARγ agonists in different transport systems in the apical and basolateral membranes. Real time PCR revealed that PPARγ agonists affected expression of CFTR Cl channels and Na+/HCO3− cotransporter (NBC1). The decrease in airway bicarbonate transport may provide a possible explanation for the increased upper airway infections observed in subjects participating in clinical trials of PPARγ agonists.

Immunohistochemical Characterization of Cell Types Expressing the Cellular Prion Protein in the Small Intestine of Cattle and Mice

K. Miyazawa,1 T. Kanaya,1 S. Tanaka,1 T. Takakura,1 K. Watanabe,1 S. Ohwada,1 H. Kitazawa,2 M. T. Ross,2 S. Sakaguchi,2 S. Katamine,2 T. Yamaguchi,2 H. Aso1; 1Laboratory of Functional Morphology, Graduate School of Agricultural Science, Tohoku University, Sendai, Japan, 2Laboratory of Animal Products Chemistry, Graduate School of Agricultural Science, Tohoku

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Ubiquitination of the Gastric Microsomal H,K-ATPase

G. K. Nagatani, C. T. Okamoto; Pharmaceutical Sciences, University of Southern California, Los Angeles, CA

Ubiquitin is a small hydrophobic protein that plays a key role in the downregulation of proteins through its covalent linkage to the ε-amino group of lysine residues and less commonly the N-terminal amino group. This posttranslational modification can play multiple roles depending on the extent of ubiquitination including targeting the protein for proteasomal degradation. Here we determine whether the α and β subunits of the gastric H,K-ATPase, the enzyme responsible for gastric acid secretion, are endogenously ubiquitinated serving to regulate its turnover. H,K-ATPase-rich gastric microsomes from rabbit and hog were purified through standard subcellular fractionation techniques. Microsomes were labeled with fluorescein isothiocyanate (FITC), and proteins were separated by SDS-PAGE. The gel was scanned for the FITC signal on a Typhoon 8610 Variable Mode Imager. An ubiquitin enrichment kit (Pierce) was used to enrich endogenously ubiquitinated proteins from microsomes according to the manufacturer’s protocol. These proteins were separated by SDS-PAGE and probed for H,K-ATPase-rich endogenous ubiquitinated proteins from microsomes regardless of the species. Western blots of the enriched ubiquitinated proteins showed reactivity with 2-26, an HKα antibody. Weak reactivity can be seen with an anti-HKα antibody leaving open the possibility for HKβ ubiquitination. Underway are attempts at identifying: 1) whether or not HKα is in fact ubiquitinated; 2) other ubiquitinated proteins associated with the HK-ATPase and/or gastric microsomal membranes; 3) the enzymes responsible for ubiquitination of the HK-ATPase; and 4) the proteins that bind to ubiquitinated targets. The ubiquitination of the gastric H,K-ATPase could be crucial to its downregulation by regulating the degradation of the HK-ATPase.

Development and Characterization of a New Model for Inflammatory Bowel Disease: Chemical and Bacterial Induction/Part 2

I. Abdallah, 1 A. R. Jurus, 1 A. Badawi, 1 K. Barada, 1 M. H. Mostafa 2; 1Faculty of Medicine, American University of Beirut, Beirut, Lebanon, 2Faculty of Sciences, Beirut Arab University, Beirut, Lebanon

There is no single mechanism to explain the pathogenesis of ulcerative colitis, however, evidence suggests that endogenous luminal bacterial play a critical role. The present study reports and characterizes a novel model of chronic ulcerative colitis induced in rats using a combination of iodoacetamide and E.coli. Male Sprague-Dawly rats (n=158) were divided weekly into the descending colon with 4 different combinations: (a) a control group inoculated with 1% methylcellulose, (b) iodoacetamide-treated group inoculated with 100 µl of 6% iodoacetamide, (c) bacteria-treated group inoculated with 6% iodoacetamide followed by bacteria after 2 days, and (d) combined-treatment group inoculated with 6% iodoacetamide followed by bacteria after 2 days. Thirty days post treatment, each group was divided into two subgroups and inoculation was stopped for one. Colitis was evaluated by the clinical course of the disease, the macroscopic and microscopic alterations, activity of myeloperoxidase (MPO), inflammatory signaling by cyclooxygenase-2 (COX-2) and tumor necrosis factor (TNF-α) gene expression. The clinical course of the disease, in the iodoacetamide-treated continued injection subgroup and both combined-treatment subgroups expressed symptoms and signs resembling human colitis: slow rate of weight increase, diarrhea, and bloody stools. The score of macroscopic alterations for the severity of inflammation were (2-3)/4 in the iodoacetamide-treated continued injection subgroup, and (3-4)/4 for both subgroups in the combined treatment. All animals in the combined treatment group had very high histopathological scores of (10-14)/16 indicating an excessive inflammatory reaction in the colon. MPO activity was consistently elevated (7-fold) in the combined treatment group. TNF-α and COX-2 gene expression were upregulated in the iodoacetamide-treated continued injection and the combined-treatment subgroups. In conclusion, the chronic experimental ulcerative colitis model reported in this study resembles, to a great extent, human ulcerative colitis.

Requirement for Epidermal Growth Factor Receptor Signaling in Stretch-activated Exocytosis of Bladder Umbrella Cells

E. M. Balestreire, G. Apodaca; Medicine/Renal, University of Pittsburgh, Pittsburgh, PA

The superficial umbrella cells that line the bladder lumen are able to dynamically insert and remove membrane to modulate the apical surface area while maintaining a highly impermeable barrier. Membrane turnover occurs in response to mechanical stimuli, such as changes in pressure and volume, which vary during the micturition cycle. This study examines the hypothesis that epidermal growth factor receptor (EGFR) tyrosine kinase signaling is an integral part of the mechanotransduction pathway that mediates an increase in umbrella cell apical surface area in the stretched bladder. A modified Ussing chamber was used to monitor bladder uropathelium capacitance, a measure of surface area, upon stretch stimulus. In control bladder tissue, filling induced a 50% increase in surface area over 5 hours. Addition of the EGFR inhibitor AG-1478 significantly reduced this response to ~25% over 5 hours, while inhibitors of tyrosine kinase pathways that are involved in mechanotransduction in other cell types caused no effect. Immunofluorescence staining demonstrated the presence of EGFR on the umbrella cell apical surface, and its ability to bind to EGF presented from the mucosal side of the tissue. The addition of EGF ligands caused an increase of surface area in the absence of stretch, in a dose-dependent manner, with greater potency when added from the apical surface. Using a pharmacological approach in the Ussing chamber system, various inhibitors demonstrated the requirement for matrix metalloproteinase activity, protein synthesis, and MAPK signaling pathways in the response of the tissue to stretch and EGFR stimulation. These data indicate that EGFR tyrosine kinase signaling from the apical surface is required in the mechanotransduction pathway that mediates a surface area increase in umbrella cells. The exploration of EGFR signaling in bladder mechanotransduction will help us understand bladder physiology while improving our appreciation of cellular responses to mechanical stimuli.
apoptotic protein Bim was suppressed by Muc4 in A375 cells. Determination of ErbB2 protein and phosphorylation levels during detachment indicated that ErbB2 was degraded during cell detachment; Muc4 was able to attenuate ErbB2 degradation in MCF7 and A375 cells. Muc4 expression also resulted in an increased ErbB2 phosphorylation during cell detachment. Specific inhibitors for the proteosome and lysosome pathways were employed to determine which pathway is involved in degradation of ErbB2. ErbB2 degradation in non-adhesive MCF7 cells without Muc4 expression is blocked by lysosome inhibitors, but not by proteosome inhibitors. Detachment caused localization of ErbB2 to the lysosome. In cells with over-expressed Muc4, ErbB2 localized on the cell membrane after detachment. These results indicate that ErbB2 is degraded through the lysosome pathway during detachment.

629 Gab2 Requires Membrane Targeting and the Met Binding Motif to Promote Lamellipodia Formation, Cell Scatter, and Epithelial Morphogenesis Downstream from the Met Receptor

M. M. Frigault, 1 C. M. Kuzmochka, 1 M. A. Naujokas, 1 C. M. Brown, 2 M. Park; 1 Molecular Oncology Group, McGill University, Montreal, PQ, Canada, 2 Life Science Complex Imaging Facility, McGill University, Montreal, PQ, Canada

Gab1 and Gab2 are conserved scaffolding proteins that amplify and integrate signals stimulated by many growth factor receptors including the Met receptor tyrosine kinase. Gab1 acts to diversify the signal downstream from Met through the recruitment of multiple signaling proteins, and is essential for epithelial morphogenesis. However, whereas Gab1 and Gab2 are both expressed in epithelial cells, Gab2 fails to support a morphogenic response downstream from Met. Using structure function studies, we identify elements of Gab1 that are required to confer epithelial morphogenesis downstream from Gab2. Addition of the Met Binding Motif, that mediates direct recruitment of Gab1 to the Met receptor, as well as membrane targeting of Gab2, through the addition of a myristoylation signal, are required to confer a morphogenic signaling response to Gab2. The morphogenesis competent Gab2 mutant, switches the transient response activated by the Met receptor downstream from Gab2 to a sustained and robust activation of signaling pathways involving MEK and AKT and JNK required for lamellipodia formation and scatter. Moreover the overexpression of Gab2 mutant rescues the ability of cells to form membrane ruffles, and localize activated ERK1/2 to the leading edge of the cell in response to Met activation. Hence subcellular localization of the Gab2 scaffold, as well as the ability of Gab2 to interact directly with the Met receptor, are both essential components of the morphogenic signaling response. The Gab family of docking proteins are currently in studies to be completely understood by bio imaging techniques indicating that these family members localized in the nucleus are not necessarily the only targets of Gab2 signaling. The Gab family of docking proteins are currently being studied by live imaging techniques in order to completely understand the dynamics of the subcellular localization of Gab1 and Gab2 downstream of the Met Receptor.

630 The Role of Ap-1 in Fluoride-induced Down-regulation of MMP-20 In Vitro

Y. Zhang, Otorhinolaryngology, University of California, San Francisco, San Francisco, CA

OBJECTIVE: Enamel is the hardest mineralized tissue in humans. Enamel formation is altered with excessive chronic intake of fluoride, resulting in enamel fluorosis, characterized by a delay in the removal of amelogenins as the enamel matures. In this study, we investigated how fluoride interferes with the expression of MMP-20, a matrix enzyme which initially hydrolyses amelogenin. METHODS: Human fetal tooth organ ameloblast lineage cells were cultured and exposed to 10 µM of fluoride. The changes in amelogenin, MMP-20 and TIMP-2 proteins in the presence of fluoride, were assessed by Western blot. Molecular relays mediated down-regulation of MMP-20 by fluoride was investigated via enzyme-activity blocking, Western blot, and protein-DNA affinity assays. RESULTS: MMP-20 synthesis decreased in the presence of fluoride, while amelogenin and TIMP-2 were not altered. Fluoride also decreased the transcription of a luciferase reporter gene driven by the MMP-20 promoter. Down-regulation of MMP-20 by fluoride was related to suppression of JNK and c-Jun phosphorylation. Three c-Jun binding motifs on the MMP-20 promoter were identified and proved to be occupied by c-Jun as MMP-20 expression was induced. CONCLUSIONS: These in vitro findings suggest that fluoride induced down-regulation of MMP-20 transcription occurs through the JNK/c-Jun signaling pathway. Altered MMP-20 expression could disturb the balance between MMP-20 and its amelogenin protein substrate, contributing to the formation of enamel fluorosis.

631 Mosaic Eyes Stabilizes Delta and Restricts Notch Activity to Rhombomere Boundaries

K. Rand, M. Joth, S. Yeo, G. Palardy, A. B. Chitnis; Laboratory of Molecular Genetics, NICHD NIH, Bethesda, MD

Mind bomb (Mib) is an E3 ligase that promotes Delta ubiquitylation and endocytosis, an essential step for its function as a Notch ligand. Interaction between Mib and Mosaic Eyes (Moe), a FERM domain protein, was identified in a yeast two-hybrid screen and subsequently confirmed with immuno-precipitation experiments. Moe is typically associated with the cell surface and in co-transfection experiments it alters Mib’s cellular distribution, reducing Mib in the cytoplasm and stabilizing it at the cell surface. In zebrafish embryos, knock-down of mow with morpholinos affects brain morphology, particularly in regions where expression is typically restricted to rhombomere boundaries is deregulated and spread throughout the hindbrain. While morphogenesis of rhombomere boundaries is disturbed, rhombomere-specific expression of various genes is not affected suggesting a specific problem with boundary formation, not early neural patterning that defines individual rhombomere compartments. Restriction of Notch activity is thought to be essential for morphogenesis of rhombomere boundaries, and cells with relatively high Notch activity segregate to these boundaries. In more morphants Notch activity is no longer restricted to boundaries and the Notch target gene her4 is expressed throughout some rhombomeres. Paradigmatically, deregulated Notch target gene expression is accompanied by an apparent disappearance of DeltaD protein, even though transcription of genes encoding Notch ligands seems unaffected. A truncated Moe, AN-Moe, lacking the N-terminal fragment responsible for surface localization, prevents Mib from being localized at the cell surface in transfected cells. Remarkably, co-transfection of DeltaD with AN-Moe and Mib reduces DeltaD stability suggesting that functional Moe protein is required to ensure stability of Delta in cell culture, just as in the embryo. We are now investigating how Moe determines DeltaD stability and limits Notch activity to rhombomere boundaries. These studies provide novel insights into Notch’s enigmatic role in morphogenesis of tissue compartment boundaries in the embryo.

632 Nerve Growth Factor and a Conditioned Medium Differentially Regulate the Appearance of Axons and Dendrites in PC12 Cells

M. V. Longart, L. L. Garcia; Unidad de Neurobiologia Molecular, Instituto de Estudios Avanzados, Caracas, Venezuela

Neurite formation, a process extending from the cell body and led by a growth cone, is a primary morphological event in neuronal differentiation. Neurotrophins like Nerve Growth Factor (NGF) are key regulators of neuronal differentiation. NGF, and other factors, are released by target tissues, initiates neurite generation and promotes synapse formation. Axonal and dendritic growths are fundamental to establish neuronal connectivity in the brain. However, the mechanisms that govern their morphogenesis remain to be elucidated. To investigate the role of neurotrophic factors on neurite specification, we used NGF and a conditioned media from sciatic nerve (CM) to differentiate PC12 cells into neuron-like cells. Both treatments caused cell differentiation and the percentage of cells with long neurites was 15 and 30% for CM and NGF, respectively. Using antibodies that specifically recognize axons and dendrites, we found that both conditions are able to induce the formation of dendrites at 24 hours, being the NGF more effective than the CM. At 7 days in culture, the ratio of dendrites/cell induced by NGF was 1.7, while by the CM was 1. At day 10, we observed that CM produced an increase in that ratio to 1.3. On the other hand, only NGF induced the appearance of axons from 7 days in culture in 48% of the differentated PC12 cells. The expression of the axonal marker always appeared towards the tip of the neurites and reached their proximal segments in older cultures (10 days). Despite the axonal marker was initially double labeled with the dendritic marker (7 days), always appeared in only one of the processes and its time increasing expression was accompanied by the disappearance of the dendritic marker. This phenotype could resemble pre-polarization of neurons. PC12 cells, differentiated with NGF, could serve as an excellent model to study the mechanism of neurite specification.

633 Solo/trio8, a Membrane-associated Short Isoform of Trio, Modulates Endosome Dynamics and Neurite Elongation

S. Aoki, Y. Sun, K. Nishikawa, H. Yuda, H. Osaka, Y. Wang, N. Fukazawa, K. Wada; Dept. of Degenerative Neurological Diseases, National Institute of Neuroscience, Tokyo, Japan

Using RNAi, we have identified a gene termed Solo, that is downregulated in the cerebellum of Purkinje cell degeneration (pcd) mutant mice. Solo is a mouse homologue of rat Trio8, one of multiple Trio isoforms recently identified in rat brain. Solo/Trio8 contains N-terminal sec14-like and spectrin-like repeat domains followed by a single GEFI domain, but it lacks the C-terminal GEF2, Ig-like and kinase domains that are typical of Trio. Solo/Trio8 is predominantly expressed in Purkinje neurons of the mouse brain, and expression begins following birth and increases during Purkinje neuron maturation. We identified a novel C-terminal membrane-binding domain in Solo/Trio8 that is required for EFGP/Solo/Trio8 localization to early endosomes (positive for both early endosome antigen 1 (EEA1)-) and Rab51 in COS-7 cells and primary cultured neurons. Solo/Trio8 overexpression in COS-7 cells augmented the EEA1-positive early endosome pool, and this effect was abolished via inactivation of the GEF domain or deletion of the C-terminal membrane-binding domain. Moreover, primary cultured neurons transfected with Solo/Trio8 showed increased neurite elongation that was dependent on these domains. These results suggest that Solo/Trio8 acts as an early endosome-specific upstream activator of Rho family GTPases for neurite elongation of developing Purkinje neurons.
Mechanical Force Locally Adjusts Accumulation of Synaptic Proteins at Axon Terminal

S. Stechen, J. Sun, S. Yang, F. Carrero-Martinez, T. Sai, A. Chiba; Cell and Developmental Biology, University of Illinois, Urbana, IL

Previous work has identified various chemicals such as NGF, NO, BMP and WNT as retrograde signals at synapses to adjust neurotransmission. Here, we provide evidence that mechanical force serves as a "retrograde signal" at Drosophila neuromuscular synapses and plays a critical role in locally adjusting their function in a usage-dependent manner. Within 30 minutes of contacting its target muscle, each motoneuron axon builds a 2 μm tension. Laser ablation before the axon reaches the terminals causes a very rapid 12 μm retraction. The use of nanofabricated MEM (micro-electro-mechanical) force sensor further reveals the constant contact to its target muscle, each motoneuron axon builds a 2 nN tension. Laser axotomy before the axon reaches the terminals results in a negligible retraction of the cut axon, whereas the same treatment after the axon-terminal contact is made causes a very rapid 12 μm retraction. The use of nanofabricated MEM (micro-electro-mechanical) force sensor further reveals the constant resistance tension being actively maintained with a time constant of 3 minutes. During the first 120 minutes, the tension exerted at the synapse terminals changes continuously due to, first, myogenic (neurotransmission-dependent) and, later, increasingly neurogenic (neurotransmission-dependent) contractions of the target muscles. Synaptotagmin-I and BRP (neurotrophin-2) are released from the terminals, which are essential for neurotransmitter release at the axon terminals during this period. If the muscle contraction is blocked genetically, those proteins fail to accumulate. However, this can be rescued by exogenous stretching or relaxing the neuron terminals, thereby increasing Ca^2+ influx and decreasing the amount of Synaptotagmin-I accumulation, suggesting the synapses' ability to accommodate a range of force-induced adjustment. The amount of synaptic proteins at the terminals is adjusted within 30 minutes and the effect lasts for at least 120 minutes. In all experiments, untreated neighboring synapses remain unaffected. Remarkably, the combination of atrophy (physical uncoupling of axon from the cell body) and micropipette patch (restoration of tension) demonstrates a local autonomy of the force-induced synaptic adjustment. We propose a novel mechanism that adjusts synaptic function according to its prior usage, locally through a mechanical signaling.

Asymmetric PtdIns(3,4,5)P_3 Signaling Mediates Chemoattraction of Nerve Growth Cones

S. Henle, E. Yang, E. Liang, G. Wang, M. Wu, M. Poo, R. H. Herley; Physical & Biomed Sciences, Stanford University, Stanford, CA, 2Mol and Cell Biology, UC Berkeley, Berkeley, CA

Pathfinding by growing axons in the developing nervous system is directed by extracellular gradients of attractive and repulsive chemotrophic factors that guide the nerve growth cone. We previously reported that the cellular action of many chemotrophic factors is mediated by a cytoplasmic gradient of Ca^2+ signals, but the link between surface receptor activation and Ca^2+ signaling was largely unknown. In migrating leukocytes and amoeboid cells, polarized elevation of the plasma membrane lipid PtdIns(3,4,5)P_3, is known to mediate the detection of a gradient of chemotractant and initiates cytokinetic rearrangements required for directed chemotaxis. Here we report that a gradient of axon guidance factors, brain-derived neurotrophic factor (BDNF) and netrin-1, can also induce polarized PtdIns(3,4,5)P_3 signals in growth cones of cultured spinal neurons, as revealed by translocation of the fusion protein Akt-PH-GFP to the plasma membrane. Activation of both PtdIns(3) kinase and Akt was required for chemotrophic factor induced attraction, and asymmetric application of exogenous PtdIns(3,4,5)P_3 was sufficient to induce attractive growth cone turning. Furthermore, focal application of exogenous PtdIns(3,4,5)P_3, rapid expansion of TRP receptor potential) channels in the growth cone plasma membrane, leading to Ca^2+ influx, which also depended on the activities of PtdIns(3) kinase and Akt. Our findings suggest that asymmetric PtdIns(3,4,5)P_3 signals at the growth cone membrane may serve to link focal guidance receptor activation with TRP channel activity and Ca^2+ signaling during chemoattractive axon guidance.

Dephosphorylation of Collapsin Response Mediator Proteins (CRMPs) Induced by Hypoxia-ischemia in Neonatal Mice through Down Regulation of Cdk5/p35

Y. Zhou, I. Bhata, P. He, P. Cheung, J. Chu; Department of Anatomy, The University of Hong Kong, Hong Kong, 2Department of Pediatrics and Adolescent Medicine, The University of Hong Kong, Hong Kong

Collapsin Response Mediator Proteins (CRMPs) are a family of cytosolic proteins involved in neuronal differentiation and axonal guidance. The expression of CRMPs are up-regulated during the peak period of axonal growth and down-regulated afterwards. By using proteomic approach (2D-MS) to study the alteration and modification of proteins in neonatal mouse brain tissues upon 24-hour hypoxia-ischemia (HI) treatment, we found that CRMP2, CRMP4 and CRMP5 proteins were post-translationally modified after HI treatment. MS and Western Blot analyses confirmed that these proteins are dephosphorylated on several sites. Because CRMP2 has previously demonstrated to be one of the substrates of cyclin dependent kinase 5 (cdk5), a neuronal specific kinase that plays pivotal roles in neuronal development and is critically involved in the regulation of neuronal survival, we further analyzed the expression level and activity of Cdk5 and its activator p35. We observed that the dephosphorylation of CRMPs was correlated with underexpression of p35 and significant reduction of Cdk5 kinase activity. Our findings suggest one molecular mechanism through which CRMPs are dephosphorylated during HI treatment. Further investigations are required to elucidate the pathological significance of CRMP dephosphorylation in HI-induced neuronal apoptosis and HI-related neurodegenerative diseases.

Role of the 5-HT7-Receptor in Synaptogenesis, Synaptic Plasticity

F. Koebe, D. Hess, M. Muller, D. W. Richter, E. G. Ponimaskin; Neuro- and Sensory Physiology, University of Goettingen, Goettingen, Germany

5-HT_7 receptor agonist 8-OH-DPAT (5-HT_7 agonist) plays an essential role in multiple events in the CNS. Recently we demonstrated that in mouse hippocampal neurons, activation of endogenous 5-HT_7 receptors significantly increased neurite length, whereas stimulation of 5-HT_7 receptors led to a decrease in the length and number of neurites. We showed that the 5-HT_7 receptor is coupled not only to the heterotrimeric Gs, but also to G13 protein. Activation of this signaling pathway results in RhoA-mediated modulation of gene transcription and in reorganization of the actin cytoskeleton. We also demonstrated that serotonin receptor 5-HT_7 can activate heterotrimeric G12 protein, leading to the selective activation of small GTPases RhoA and Cdc42. Agonist-dependent activation of the 5-HT_7 receptor induced pronounced filopodia formation via a Cdc42-mediated pathway paralleled by RhoA-dependent cell rounding. (1,2) This molecular model for 5-HT_7 and 5-HT_4 receptor mediated signalling provides a link between receptor activation and a subsequent change in morphology. We also asked whether these serotonin mediated morphological changes could be directly correlated to the modulation of synaptic plasticity. We investigated the 5-HT_7 receptor-mediated formation of presynaptic clusters (synaptoplasty), filopodia, spines and the spontaneous synaptic activity in primary culture of mouse hippocampal neurons. Furthermore we looked into changes in long-term potentiation related to the 5-HT_7 receptor mediated activity of pacemaker currents in the cultured neurons. Our data suggests that serotonin plays a prominent role in regulating the neuronal cytoskeleton and synaptic plasticity in addition to its classical role as a neurotransmitter. 1) Kuchavena E, Liu G, Dityateva G, Schachner M, Voyno-Yasenetska TA, Ponimaskin EG J Neurosci, Vol. 25, (2005) 2) Ponimaskin EG, Heine M, Joubert L, Sebben M, Bickmeyer U, Richter DW, Durnuis A J Biol Chem, Vol. 277, (2002)

Efficient Co-Packaging and Co-Transport of Neuromodulators Associated with Synaptic Plasticity

J. E. Lochner, E. Spangler, C. S. Schuttner, B. A. Scallett; Chemistry, Lewis & Clark College, Portland, OR, 2Physics, Lewis & Clark College, Portland, OR

Long-term memory formation is accompanied by enduring changes in synaptic efficacy, which are triggered in part by the secretory and subsequent activity of neuromodulators. Brain-derived neurotrophic factor (BDNF) and tPA, which is a component of the tPA-BDNF signalling pathway, are functionally linked to synaptic plasticity. We have constructed two fluorescent hybrid proteins, tPA-enhanced green fluorescent protein (tPA-EGFP) and proBDNF-mCherry, and we have effectively expressed both hybrids simultaneously during learning and memory. For example, mature BDNF (mBDNF) augments synapse density and elicits long-lasting enhancement of synaptic transmission. In contrast, precursor BDNF (pBDNF) is a weak growth factor that plays pivotal roles in neuronal development and is critically involved in the regulation of neuronal survival, we further analyzed the expression level and activity of Cdk5 and its activator p35. We observed that the dephosphorylation of CRMPs was correlated with underexpression of p35 and significant reduction of Cdk5 kinase activity. Our findings suggest one molecular mechanism through which CRMPs are dephosphorylated during HI treatment. Further investigations are required to elucidate the pathological significance of CRMP dephosphorylation in HI-induced neuronal apoptosis and HI-related neurodegenerative diseases.
Abnormally synchronized synaptic transmission in the brain causes epilepsy. Most inherited forms of epilepsy result from mutations in ion channels. However, one form of epilepsy, autosomal dominant partial epilepsy with auditory features (ADPEAF), is characterized by mutations in a secreted neuronal protein, LGI1. We show that ADAM22, a transmembrane protein that when mutated itself causes seizure, serves as a receptor for LGI1. The mutated form of LGI1 fails to bind to ADAM22. ADAM22 links extracellular LGI1 to the postsynaptic density-95 (PSD-95) cytoskeletal scaffold containing stargazin and AMPA-type glutamate receptors. LGI1 enhances AMPA receptor-mediated synaptic transmission in hippocampal slices and surface-expression of AMPA receptors in hippocampal neurons. This study identifies LGI1 as a new ligand for ADAM22 that controls synaptic transmission and human epilepsy.

Calcineurin, Calpain, and Cytoskeleton in Low Frequency Depression of Synaptic Transmission

L. B. Silverman Gavrila, M. P. Charlton; Physiology, University of Toronto, Toronto, ON, Canada
Low frequency depression (LFD) of transmitter release at phasic synapses of crayfish neuromuscular junctions is regulated by protein phosphorylation (Silverman-Gavrila et al., 2005). The permeant calcineurin inhibitors FK-506 or calcineurin autoinhibitory peptide abolished LFD. To determine if the site of action of calcineurin is pre or post synaptic we pressure injected impermeant calcineurin autoinhibitory peptide into presynaptic axons or postsynaptic muscle cells and measured the amplitude of the intracellularly recorded excitatory postsynaptic potential (EPSP) evoked by stimulating the phasic axon at 0.2 Hz. LFD was decreased only when calcineurin was inhibited in the presynaptic nerve terminal. The drugs have no postsynaptic effects since FK-506 or permeant peptide did not affect amplitude distribution of spontaneous miniature EPSPs; therefore they target presynaptic calcineurin, the activity of which is necessary for LFD. Neither drug affected high frequency depression caused by 20 Hz stimulation. Since moderate calcium buffering by injected BAPTA-based Ca2+ indicators did not inhibit LFD, the activation of calcineurin may occur very close to Ca2+ channels where a large Ca2+ signal is available. Calcineurin inhibition blocked LFD, suggesting that a limited proteolysis of calcineurin might be involved in its activation during LFD. To examine changes in phosphoproteins during LFD, we removed motor axons and nerve terminals after the induction of LFD or treatment with various phosphorylation regulators, extracted their proteins, separated them by SDS-PAGE, and stained them with phosphospecific stains to identify bands for analysis by mass spectrometry. Actin and tubulin phosphorylation was decreased during LFD. Western blot analysis and immunostaining showed calcineurin, actin and tubulin at presynaptic axons and terminals. Anti-calcineurin drug cytochalasin and the microtubule stabilizer taxol inhibited LFD, while the actin stabilizer jasplakinolide and the tubulin depolymerizing drug nocodazole accelerated LFD with no postsynaptic effects. In conclusion, dephosphorylation of presynaptic actin and tubulin by calcineurin regulate LFD.
significant concentrations of YC 1.360 such that activity could be recorded. While expression was almost exclusive in astrocytes, a number of other cell types which express S100β, such as large motor neurons in the brain stem and some of the NG2 and CNP positive oligodendrocyte progenitor cells (OP cells), also were fluorescent with YC 3.60. Using a variety of known in vivo assays, we found that stimuli known to elicit Ca²⁺ signals in astrocytes (glutamate application, electrical stimulation of neural pathways) caused substantial and rapid Ca²⁺ signals in the YC 3.60 expressing astrocytes. These results, for the first time, show that genetically encoded reporter is capable of recording activity dependent Ca²⁺ signals even in the very fine terminal processes of astrocytes that wrap individual synaptic boutons.

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A Drug Screen for Fragile X Promoter Activation
L. Jabhour, D. Guo, R. Chen, A. M. Tartakoff; Pathology, Case Western Reserve University, Cleveland, OH
In the Fragile X Syndrome, trinucleotide expansion of the 5'UTR of the FMR1 gene inhibits its transcription. Its product, FMRP, is correspondingly reduced or absent. FMRP is a cytoplasmic protein which binds selected RNAs and is part of the RISC complex, thereby implicating it in translational regulation. Its presence enhances long-term depression at synapses and its titration can be rapidly increased upon stimulation of synaptic activity (Gabel et al. J. Neurosci. 24, 10579 (2004)). In anticipation of attempts to reverte the trinucleotide-expanded FMR1 promoter, we generated a plasmid which makes it possible to drive the expression of GFP using the normal FMR1 promoter. This plasmid was used to produce stable transfectants of COS7 cells. Using this fluorescent target cell, we then tested a panel of 1040 FDA-approved drugs (the NINDS custom collection -Heemskerk et al. Trends Neurosci., 25, 494 (2002)) in order to learn whether any of them - as expected if they stimulate the FMR1 promoter - would increase fluorescence levels. This involved the replication of plate cell cultures in microtiter wells, followed by addition of each drug at 10 nM, continued culture for two days, and quantitation of fluorescence using a fluorescent plate reader. Since replicate cultures did not show significant stimulation of fluorescence, continued work will use cDNA libraries for related studies. Supported by the Fondation Jerome Lejeune.

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Role of Vascular Permeability in Brain Tumor Infiltration
C. V. Lund, 1 M. T. N. Nguyen, C. A. Kruse, B. E. Torbett, B. P. Eleciri1; 1La Jolla Institute for Molecular Medicine, San Diego, CA, 2The Scripps Research Institute, La Jolla, CA
Malignant brain tumors, such as glioblastomas, are characterized by extensive angiogenesis and permeability of the blood-brain barrier (BBB). The infiltration of glioma cells away from the primary tumor mass is a pathological characteristic of glial tumors. The infiltrating tumor cells represent a significant factor in tumor recurrence following surgical debulking, radiation, and chemotherapy treatments. Vascular endothelial growth factor (VEGF)-mediated vascular permeability (VP) has been associated with the progression of glioma growth and infiltration into surrounding normal brain parenchyma. While VEGF induces robust VP response in control mice (src+/+ or src–/–), the VP response is blocked in src–/– mice that demonstrate a "leakage-resistant phenotype" in the brain. We used the Src-deficient mouse model to determine the role of Src in the maintenance of the BBB following orthotopic implantation and growth of glioma cells in the brain. Although solid tumor growth was the same in control and src–/– mice, the infiltrating component of glioma growth was reduced in src–/– mice. CD31-labeled vasculature of the primary tumors were unchanged in the tumor models of wildtype and src–/– mice, however, indirect immunofluorescence of the extracellular matrix (ECM) protein fibrinogen showed reduced accumulation in the tumor of src–/– mice compared to wildtype. To identify Src-mediated changes in protein expression and phosphorylation in vivo, additional proteomic studies of brain lysates from wildtype and src–/– mice containing glioma tumors were performed and mass spectrometry analysis of protein changes identified. We hypothesize that phosphorylation of focal adhesion kinase (FAK) is required for VEGF-induced VP. By using VEGF-expressing glioma cells to induce VP in a xenograft tumor model of glioma growth, we can determine whether Src-regulation of FAK is essential in the maintenance of blood vessel integrity in the brain during brain tumor growth.

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Abi1/Hsh3bp1 Regulates c-Abl Tyrosine Kinase Activity and c-Abl-dependent Cell Proliferation and Spreading
X. Xiong, P. Cui, B. Warner, S. Hossain, X. Guo, X. An, L. Kotula; Laboratory of Cell Signaling, New York Blood Center, New York, NY
Bcr-Abl is a validated target for drug development in the treatment of chronic myelogenous leukemia (CML). The initial success of Gleevec (imatinib mesylate) in the treatment of CML has been hampered by identification of kinase mutations resistant to the drug. In vitro screen to identify possible mechanisms of the drug resistance as well as data from functional studies lead to the conclusion that Alb ligands or its substrates may be an important factor in regulating Bcr-Abl kinase activity in addition to the autoinhibitory mechanism of the kinase. The presence of SH3 and SH2 domains in Bcr-Abl and c-Abl indicate that the mechanism of kinase regulation involving these domains is conserved. Here we report identification of an allosteric non-ATP-competitive mechanism of c-Abl kinase inhibition by phosphopeptides derived from Abi1/Hsh3bp1. The mechanism involves high affinity binding of the phosphotyrosine from Abi1 to the SH2 domain and the Abi1/Hsh3bp1's PXXP motif to the Abi1 SH3 domain. The critical role of Abi1/Hsh3bp1 in regulation of c-Abl kinase activity in vivo is supported by inhibition of cell proliferation and cell spreading expression of Abi1/Hsh3bp1 in cells deficient in Abi1/Hsh3bp1 due to loss-of-function mutation. The Y to F replacement of the regulatory tyrosine of Abi1 or the mutation in its PXXP motif did not inhibit c-Abl kinase activity supporting the role of these regions in the regulation of c-Abl. Our findings suggest a novel mechanism by which Abi1 kinases are regulated in cells and provide a novel strategy for anti-leukemic drug development.

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Studies of Ferrite Based Magnetic Nanoparticle Transport Mechanisms and Magnetocytolysis Effects on a Model Cell Culture
H. L. Rodriguez, A. Herrera, C. Rinaldi, M. Torres; Chemical Engineering, University of Puerto Rico Mayaguez, Mayaguez, Puerto Rico
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 dextran coated ferrite nanoparticles. Cytotoxicity was examined by exposing the cells to various concentrations of nanoparticles for 2, 24, 48 hours. Cell viability was analyzed using a fluorometric assay which measures cell metabolism. These studies indicate that viability of the cells was not affected by the nanoparticles in the concentration range 0.08-0.2 mg/mL. Nanoparticle suspensions with a concentration of 0.144 mg/mL were applied to the cells and the system was exposed to a magnetic field of 3.1 kA/m, and a frequency of 1 kHz. It was found that viability of the cells decreased to 40% of the negative control. Additionally, with the purpose of investigating the exact transport mechanism of the ferrite nanoparticles, these were coated with cross-linked fluorescein isothiocyanate dextran (FITC Dextran). Cells were exposed to the fluorescent labeled nanoparticles and then examined using a confocal laser scanning microscope. Preliminary results suggest that nanoparticles were transported through the cell membrane, invading the cytoplasm of the cells. This fluorescent label will allow further investigation of the nanoparticle transport mechanism through the cells.

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Localization of a Mutant p53 Response Element on the Tissue Inhibitor of Metalloproteinase-3 Promoter: Mutant p53 Activities Are Distinct from Wild-Type
S. Thomas, D. Reisman; Biological Sciences, University of South Carolina, Columbia, SC
Missense mutations in the p53 gene have been observed in greater than 60% of all human tumors. Recent evidence indicates that some mutations in p53 arise as the cancer progresses from a benign tumor to a metastatic tumor and that these mutations in p53 actively contribute to the process of cancer progression. Earlier work that found the expression of the gene encoding the tissue inhibitor of metalloproteinase-3 (TIMP-3) is repressed in cells expressing codons 248 and 281 mutant p53 (Telles). The ability of tumor-derived p53 mutants to inhibit TIMP-3 expression provides a novel mechanism for understanding how p53 mutations might contribute to tumorigenesis. Since mutant p53 is often expressed at elevated levels in a variety of cancers, the generation of cells in a tumor carrying certain mutations in p53 would cause inappropriate reduced expression of TIMP-3 and lead to elevated matrix metalloproteinase activity. We present the results of experiments that begin to determine the mechanism by which mutant p53 represses TIMP-3 gene expression. By generating deletion derivatives of the TIMP-3 promoter and testing them for expression and by performing DNA protein binding assays on the regions determined to be required for repression, we have identified elements that are essential for mutant p53-mediated transcriptional repression. These elements respond specifically to mutant but not wild type p53. While mutant p53 itself does not bind to the TIMP-3 promoter, we provide evidence for the presence of DNA binding proteins whose activity is enhanced in the presence of mutant p53.
DNA-dependent Activation of ATM Kinase in Chromatin-free Nuclear Extracts

Y. Tsai, L. Liu; Pharmacology, University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School, Graduated School of Biomedical Sciences, Piscataway, NJ

A chromatin-free nuclear extract capable of supporting ATM activation in response to DNA double-strand breaks (DSBs) was developed. Addition of short duplex DNA fragments (e.g. 30-mer) incapable of forming nucleosomes was shown to efficiently activate ATM kinase in the nuclear extract, as evidenced by the stimulation of ATM autophosphorylation at Ser-1981, and phosphorylation of ATM substrates such as 53BP1, Chk1 (Ser-317), Chk2 (Ser-33/35) and p53 (Ser-15). In addition to linear duplex DNA, single-stranded oligonucleotides (ODNs), gapped DNA and flapped DNA also efficiently activated ATM kinase as evidenced by ATM autophosphorylation and phosphorylation of the various ATM kinase substrates. These in vitro results suggest that the chromatin structure is not obligatory for ATM activation, and altered DNA structures other than double-strand breaks can also efficiently activate ATM kinase.

A Naturally Occurring, Inducible, Constitutively Active Isoform of the Human Prolactin Receptor, ΔS2 SF1b, Reduces Proliferation and Migration in Human Prostate Cancer Cells

K. Huang, A. M. Walker; Division of Biomedical Sciences, University of California, Riverside, Riverside, CA

We previously showed that a molecular mimic of phosphorylated prolactin (PRL), S179D PRL, inhibited prostate tumor growth. In part this is through antagonism of a PRL autocrine growth loop, and in part this is through up-regulation of one of the short prolactin receptor (PRLR) isoforms, S1bF1, resulting in an increase in p21 and vitamin D receptor expression. In the current study, we identified a novel, naturally occurring form of PRLR, which lacks about half of the extracellular domain and is active in the absence of ligand. Stable prostate cancer cell lines expressing this constitutively active receptor, designated ΔS2 SF1b, were established. These cells grew more slowly than their control transfected counterparts. Overexpression of ΔS2 SF1b also inhibited cell migration as analyzed by wound healing and transwell assays. Semi-quantitative and real-time RT-PCR analysis revealed that ΔS2 SF1b expression up-regulated the prostate inhibitors TIMPs 1 & 2 and PANS 1 & 2, and down-regulated both the cognate proteases MMPs and uPA, and the growth factors bFGF and VEGF. Immunofluorescent staining of a cell junction component, E-cadherin, showed more cell-cell contacts in ΔS2 SF1b cells. Since S179D PRL upregulates intact SF1b and this is associated with decreased migration of endothelial cells, we investigated the effect of S179D PRL on prostate cancer cell migration and invasion. Treatment of LNCaP cells with S179D PRL inhibited cell migration in the transwell assays. Analysis of gene expression showed that S179D PRL upregulated both the intact and ΔS2 SF1b, extending the previous study to include the ΔS2 version. Also upregulated were the TIMPs and PADS. We conclude that overexpression of the constitutively active short PRLR isoform, ΔS2 SF1b, inhibits cell migration and likely reduces invasive properties. Similar results can be achieved by treatment with S179D PRL, which up-regulates the expression of this particular isoform. Supported by DK61005 and BC0501103 (DOD)

HYA-Synthase-1: A Potential Bladder Cancer Marker

R. Golshani,1 Y. Hirano,1,2 H. B. Klavse,3 K. Tanaka,1 S. Murata1,4; 1Laboratory of Frontier Science, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan, 2The Burnham Institute for Medical Research, La Jolla, CA, 3Laboratory of Molecular Biology, University of California, San Diego, La Jolla, CA, 4Sylvestor Comprehensive Cancer Center, University of Miami Miller School of Medicine, Miami, FL.

Objective: Hyaluronic acid (HA), a non-sulfated glycosaminoglycan, promotes bladder cancer growth and metastasis. Urinary HA test has ~90% accuracy in detecting bladder cancer, regardless of tumor grade and stage. HA is synthesized by a plasma membrane-associated enzyme, HYA-synthase (HYAS), present in three separate isoforms. We correlated HA-S1 expression in bladder cancer tissues and cell lines with HA levels and HA urine test results. Materials and Methods: 48 bladder specimens underwent HYAS1 real time PCR. Results were substantiated by HA tissue measurement and HA-S1 and HA-S2 immunoblot analyses. RT-PCR detected HYAS1 variants in bladder tissues and cell lines. Gel-filtration chromatography determined HA polymer size in bladder tissue. HYAS1 and HA were localized in 68 archival bladder tissues by immunohistochemistry. Urinary HA levels were measured and compared to immunohistochemistry results. Results: Real-time RT-PCR and northern blot analyses revealed HYAS1 transcript levels elevated 5-10-fold in bladder tumor (TBL) tissues relative to normal bladder (NBL) tissues. Measurement of HYAS1 transcript levels had ~80% accuracy in detecting bladder cancer. HA levels were elevated 2.5-fold in TBL tissues when compared to NBL tissues and correlated with elevated HYAS1 transcript levels. A HYAS1 splice variant, HYAS1-va, was expressed in bladder tissues but at 214 times lower than HYAS1 wild type transcript. The size and the level of HA present in TBL tissues and bladder cancer cell lines were consistent with HYAS1 protein expression. Immunohistochemistry of HYAS1 and HA in bladder tissues revealed that HYAS1 and HA expression has 79% and 88% sensitivity and 83.3% and 100% specificity, respectively, in detecting bladder cancer. The expression of both HYAS1 and HA in TBL tissues correlated with a positive HA urine test and with tumor recurrence and treatment (P < 0.001). Conclusion: Increased HYAS1 expression contributes to increased HA levels in tumor tissues and to a positive HA urine test.

Expression Patterns of Lectins in Cardiac Myxoma

P. Chu,3 S. Jung,2 A. Wu,3 Medicine, Chang Gung Memorial Hospital and University, Taipei, Taiwan, 2Pathology, Chang Gung Memorial Hospital and University, Taipei, Taiwan, 3Glyco-Immunology Research Laboratory, Chang Gung University, Taipei, Taiwan.

Objective: Cardiac myxoma, the most common primary tumor of the heart, has variable clinical presentations and variable immunohistochemical profiles. Lectins are simply defined as proteins which specifically bind (or crosslink) carbohydrates. The existence of glycoproteins in cardiac myxoma has not been demonstrated completely, except mucins genes, MUC1, MUC2, and MUC 5AC. The purpose of this investigation was to elucidate the expression patterns of lectins in cardiac myxomas. Methods: This study analysis of micro-tissue array comprising of 77 patients with cardiac myxomas that were surgically excised. The expression patterns of 21 glycoproteins in cardiac myxoma were elucidated by immunohistochemical analysis. Results: The patient population consisted of 46 (60%) women and 41 (40%) men with a mean age of 46 (range, 32-65) years. All cases of myxoma were sporadic myxomas rather than familial. Clinical presentations included: asymptomatic (25%), dyspnea (45%), stroke (10%), chest pain (9%), and fever (11%). All myxomas were located in the left atrium. Pathological scores for inflammation, necrosis, calcification, and location on the atrial septum varied, 10 lectins, including ricin, Bandeiraea (Griffonia) simplicifolia lectin-I, isolectin A4, wheat germ agglutinin, Bandeiraea (Griffonia) simplicifolia lectin-I, isolectin B4, Ricinus communis, Aleuria aurantia, Abrin-A, peanut agglutinin, Macheria pomifera and BRG5, were strongly (more than 50%); 7 lectins (Arctocarpus integrifolia, Lens culinaris, Prostate-specific antigen, Cysteine-rich antifungal protein 2B, BHG1, and BHG3) moderately (21-50%); and 4 (Abrus precatorius agglutinin, Vicia villosa B4), BH34, BHG4) weakly (less than 20%) were expressed in the membrane. Conclusion: In conclusion, the existence of lectins in cardiac myxoma may play an important role of the formation of tumors.

Molecular Mechanism of Assembly of Mammalian 20S Proteasomes

Y. Hirano,1 H. B. Klavs, K. Tanaka, S. Maruta1; 1Laboratory of Frontier Science, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan, 2The Burnham Institute for Medical Research, La Jolla, CA, 3Institute of Molecular Biology and Physiology, University of Copenhagen, Copenhagen, Denmark, 4PRESTO, Japan Science and Technology Agency, Kawaguchi, Saitama, Japan

The 26s proteasome is a central enzyme in the degradation of ubiquitin-conjugated proteins. The importance of the study of proteasomes is now growing from the standpoint of clinical medicine. A proteasome inhibitor called Bortezomib is in clinical use as an anticancer drug, and it has been proved that the proteasome is an attractive target for cancer therapy. The 20s proteasome, a catalytic core of the 26s proteasome, is composed of 28 subunits arranged in a cylindrical particle with four hexameric rings. The two outer rings are made up of seven kinds of β subunits, three of which are proteolytically active, whereas the outer rings are made of up seven kinds of σ subunits. However, the mechanism responsible for the assembly of such complex structure remains elusive. Recently we identified three novel chaperones involved in the maturation of mammalian 20S proteasomes, named Proteasome Assembling Chaperone (PAC) 1, PAC2 and PAC3. These three molecules associate with precursor 20S proteasomes. Their knockdown by siRNA causes insufficient α-ring formation and abnormally assembled proteasome precursors, resulting in poor 20S proteasome maturation. Our findings suggest cooperative system of multiple chaperones involved in the correct assembly of mammalian 20S proteasomes. In cancer proteasome biogenesis will provide useful information for effective cancer therapy.

pVHL And GSK3β Cooperatively Maintain the Primary Cilium

C. R. Thoma,1 J. I. Frew,1 M. Montani,1 H. Moch,2 W. Krek1; 1Biology, ETH Zurich, Zurich, Switzerland, 2Pathology, University Hospital Zurich, Zurich, Switzerland

Loss of normal cilia structure causes cystic pathology of the kidney. We show that pVHL, the product of the von Hippel-Lindau (VHL) tumor suppressor gene, when mutated gives rise to precancerous renal cysts and clear cell renal carcinoma, localizes to the primary cilium and functionally interacts with glycogen synthase kinase (GSK) 3β to maintain its structure. This function of
pVHL depends on its ability to stabilize the aneximal microtubules. Although pVHL is dispensable for cilia maintenance when GSK3β is active, it prevents cilia loss when GSK3β signaling is inhibited. Consistent with these findings, in VHL patients, GSK3β is subjected to inhibitory phosphorylation at serine 9 in renal cysts, but not in early VHL mutant lesions, and these cysts exhibit reduced frequencies of primary cilia. We propose that pVHL is part of a 'safeguard mechanism' that preserves the primary cilium and that disruption of this mechanism may contribute to cyst formation.

Silencing of Emmprin by RNA Interference Reduces the Tumorigenicity of the Pancreatic Cancer Cell Line MiaPaCa2

W. Schneiderhan, T. Gress, M. Buchholz, T. Seufferlein, J. Gschwend, M. Seck, G. Adler, M. G. Bachem; Clinical Chemistry, University Hospital, Ulm, Germany, 1Internal Medicine, University Hospital, Muenster, Germany, 2Internal Medicine, University Hospital, Ulm, Germany, 3Surgery, Klinikum, Aalen, Germany

Emmprin is a highly glycosylated membrane protein enriched on cancer cells of various malignancies. To study the role of EMMPRIN in cancer progression we generated from the pancreatic cancer cell line MiaPaCa2 clones stably expressing shRNA targeting EMMPRIN under the control of a tetracycline inducible H1 promoter. EMMPRIN expression was assessed by RTPCR, western-blotting, fluorescence-immunassay (DELFIA) and fluorescence-microscopy. The knock-out phenotype was characterized with respect to adhesion independent growth (soft-agar assay), DNA-synthesis (BrDU-incorporation), growth curves, tumorigenicity (xenograft model) and invasion through chorioallantoic membranes of chick embryos (CAM-assyay). Genes differentially regulated by Bsg were identified by a whole genome microarray followed by stringent analysis. RNAi reduced the expression of EMMPRIN up to 90% on protein- and mRNA level. In the presence of 10% fetal calf serum EMMPRIN silencing reduced adhesion dependent tumor cell proliferation whereas only a modest reduction of colony formation was observed. In addition DNA synthesis was reduced up to 30%. Moreover, EMMPRIN knock-out reduced motility and invasiveness of cancer-cells in a boyden chamber assay and in the CAM-Assay. Finally, EMMPRIN silencing reduced tumorigenicity of cancer cells in a Xenograft model in nude mice by almost 50%. We identified 913 genes differentially expressed after EMMPRIN silencing which were overrepresented in Gene Ontology groups linked to regulation of signal transduction, cell-growth and apoptosis. We demonstrate for the first time that EMMPRIN promotes progression of pancreatic cancer in in-vivo assays presumably by adhesion dependent mechanisms and identified a high number of differentially expressed genes upon phenotypic change. The high number of differentially expressed genes in EMMPRIN-knock out cells suggests an essential role for EMMPRIN in cancercell biology and help to further elucidate its molecular function. Finally the presented results imply the therapeutic potential of RNAi in the treatment of pancreatic cancer by targeting overexpressed genes like EMMPRIN.

Novel Signaling by Estrogen and Resveratrol to Modulate the Actin Cytoskeleton of Metastatic Breast Cancer Cells

N. G. Ariazi, L. A. Cologna, M. Cameron, S. F. Dharmawardhana; Anatomy and Cell Biology, Universidad Central del Caribe School of Medicine, San Juan, PR, 2Analytical Imaging Facility, Albert Einstein College of Medicine, Bronx, NY

Estrogen and structurally-related estrogen receptor modulators (SERM) play critical roles in breast cancer progression. Estrogen promotes breast cancer while resveratrol, an estrogen-like phytoestrogen from grapes, is considered to be a cancer preventative. Recent data have shown that in addition to signaling via nuclear receptors to promote gene transcription and cell proliferation, estrogen activates cell surface receptor networks that may impact cancer metastasis. However, not much is known about the role of estrogen and SERMs in cancer metastasis. Previously, we reported that in ER (+) breast cancer cell lines estrogen increased cell migration and lamellipodia formation while resveratrol (50 μM) decreased cell migration and induced a global and sustained filopodia response. The objective of this study was to elucidate the molecular mechanisms of estrogen and resveratrol signaling to the actin cytoskeleton. We demonstrate that in MDA-MB-231 breast cancer cells, filopodia formation by 50 μM resveratrol is time and concentration dependent and sustained up to 8 h. Resveratrol at 5 μM acts similar to estrogen by increasing lamellipodia, cell migration, and invasion. Since the Rho GTPases Cdc42 and Rac regulate actin reorganization, we investigated a role for Rac and Cdc42 in estrogen and resveratrol signaling in breast cancer cells. Resveratrol (50 μM) decreases Rac and Cdc42 activity while estrogen and 5 μM resveratrol increase Rac activity. MDA-MB-231 cells expressing dominant negative Cdc42 or dominant negative Rac retain the filopodia response to 50 μM resveratrol. Lamellipodia induced by 5 μM resveratrol or estrogen can be attenuated in cells expressing dominant negative Rac. We conclude that Rac regulates estrogen and resveratrol signaling to the actin cytoskeleton to modulate breast cancer cell migration/invasion and ultimately breast cancer metastasis. Moreover, this report contributes data to the suggestion that resveratrol has potential as a breast cancer metastasis treatment, but should be used with caution regarding concentration.

Novel Signaling by Estrogen and Resveratrol to Modulate the Actin Cytoskeleton of Metastatic Breast Cancer Cells

S. Ju, S. Park, J. An, S. Lee, T. Nguyen Quang, B. Kwon, S. Heo, B. Kim; Immunomodulation Research Center, University of Ulsan, Ulsan, Republic of Korea

Previously, we have reported that anti-4-1BB mAb treatment to melanoma-bearing mice enhances anti-tumor immunity due to CD8 T-cell expansion and increased IFN-γ production. In this study, we investigated the functional subpopulation of immune cells involved in 4-1BB-mediated anti-tumor activities. Pulmonary metastasis was established by intravenous injection of B16F10 melanoma cells to C57BL/6 mice. Anti-4-1BB mAb(3E1) were administered to mice on 2, 4 and 8 days post tumor injection. We found administration of 3E1 significantly increased the number of CD8+1CD11c- and NK1.1- cells with the suppression of metastatic colonies. Especially, we found marked expansion of CD11c+CD8+ cells by 3E1 treatment. Most of CD8+ T cells are IFN-γ+ and most of CD11c+ cells are CD11b+ cells. Most of CD11b+ cells are NK1.1+ cells.

Oxidative Stress-mediated Regulation of Ornithine Decarboxylase and Spermidine Spermine N-Acetyltransferase Expression in H-ras Transformed Fibroblasts

M. A. MacLean, R. A. R. Hurta; Biology, University of Prince Edward Island, Charlottetown, PE, Canada

Cellular growth regulation is multi-faceted. This present study demonstrates a unique link between oxidative stress-mediated cellular signaling and H-ras mediated cellular transformation and the expression of ornithine decarboxylase (ODC) and spermidine-spermine N-acetyltransferase (SSAT). ODC and SSAT are key rate limiting and regulatory enzymes involved in polyamine biosynthesis and catabolism, respectively. Altered polyamine metabolism in transformed cells is partly responsible for providing such cells with their increased rates of growth and division. This study examined the regulatory effects of hydrogen peroxide (H2O2)-induced oxidative stress on ODC and SSAT protein expression in NR3 cells, which are H-ras transformed, benign tumour-forming murine fibroblasts, and in the parental non-transformed 10T1/2 fibroblasts. Western blot analysis revealed that NR3 cells treated with H2O2 showed a dose and time dependent induction of both ODC and SSAT protein expression whereas ODC and SSAT protein expression levels were apparently unaffected by the same H2O2 treatment in 10T1/2 cells. The induction of ODC in NR3 cells appears to be mediated by both protein kinase C and mitogen-activated protein kinase mediated cellular signaling, whereas the H2O2-mediated induction of SSAT protein expression was mediated by phosphorylindinositol -3-kinase. This study further elucidates an aspect of the altered growth regulation and expression which occurs as a consequence of H-ras mediated cellular transformation. (NSERC funded)

Proteasome Inhibition Induces Nuclear IκBα Translocation, NFκB Inhibition, and Apoptosis in Prostate Cancer PC-3 Cells

H. T. Yu, C. Ghosh, S. Robinson, I. Vancurova; Biology, St. John's University, Queens, NY

Prostate cancer is the most common cancer in men. It proceeds from a localized, curable, androgen dependent disease to an invasive, metastatic, androgen-independent disease, for which there is no cure. The metastatic, androgen-independent prostate cancer is characterized by a constitutive activation of the transcription factor NFκB, which induces synthesis of pro-survival and pro-inflammatory genes that are associated with the prostate cancer. Thus, inhibition of NFκB activity represents an attractive anti-cancer approach. NFκB activity is regulated by a cytoplasmic inhibitor, IκBα. We have previously shown that NFκB activity can be inhibited by translocation of IκBα to the nucleus, and this is associated with an induction of apoptosis. We have searched for experimental treatment, or conditions, that would induce the nuclear translocation of IκBα in the metastatic prostate cancer PC-3 cells, thus inducing their apoptosis. Here we report for the first time that inhibitors of proteasome, known to induce apoptosis, induce the nuclear translocation of IκBα in prostate cancer PC-3 cells. This induced the accumulation of IκBα in time- and dose dependent, and is associated with the inhibition of NFκB activity, and increased apoptosis of PC-3 cells. A better understanding of the mechanisms by which IκBα translocates to the nucleus, in which the NFκB activity is inhibited, could lead to the development of novel anti-cancer therapies aimed at the selective inhibition of NFκB activity by the nuclear IκBα.
Polyphenolic Compounds Isolated from *Vaccinium macrocarpon* Affect Tumorigenesis in Breast and Colon Cell Lines

E. E. Correio,1 S. C. Rego,2 C. C. Neto,3 P. E. Hart;1 Medical Laboratory Science, UMass-Dartmouth, N. Dartmouth, MA;2 Biology, UMass-Dartmouth, N. Dartmouth, MA;3 Chemistry and Biochemistry, UMass-Dartmouth, N. Dartmouth, MA

The fruit of *Vaccinium macrocarpon*, cranberry, is very rich in polyphenolic compounds. These polyphenolic compounds have been suggested to have a variety of beneficial health effects. These studies explore the effect(s) of these polyphenolic compounds on the development of breast and colon tumor cell lines. Polyphenolic-rich extracts from cranberry fruit were isolated and fractionated by HPLC; total polyphenolic, proanthocyanidin-rich, flavanol-rich, anthocyanin-rich, and small polyphenolic-rich extracts as well as single compound fractions such as those containing ursoic acid were obtained. Bioassay incorporation assays indicate that breast (MCF-7) and colon (HCT-116 and HT-29) tumor cell lines treated with polyphenolics show decreased proliferation rates as compared to matched normal cell lines (MCF10A and FHIC) or untreated controls. A signifcant increase in apoptosis rates accompanies the decrease in proliferation rates in tumor cell lines as judged by a fluorescent TUNEL assay. Significant changes in apoptosis rates are not observed in normal cells or untreated controls. These studies indicate that polyphenolic compounds isolated from cranberry fruit have a tumor-cell-specific effect on apoptosis, and suggest that these compounds may have chemopreventive or chemotherapeutic potential.

Downregulation of Growth of Human Squamous Carcinoma Cells of the Head and Neck (SCCHN) by Overexpression of the Opioid Growth Factor Receptor (OGFr): In Vitro Studies

I. S. Zagon,1 M. F. Verderame,2 J. L. Hankins,1 P. J. McLaughlin;1 1Neural and Behavioral Sciences, Penn State University, Hershey, PA;2Medicine, Penn State University, Hershey, PA

The Opioid Growth Factor (OGF) is a constitutively expressed pentapeptide whose action as a negative growth regulator is mediated by the OGF receptor (OGFr). The OGF-OGFr axis tonically regulates growth of human squamous carcinoma cells of the head and neck (SCCHN) in vivo and in vitro. This study was designed to evaluate the repercussions on growth of amplification of OGFr signaling in SCCHN cell lines. Stable-transfected SCC-1 human SCCHN cell lines derived from a well-differentiated recurrent squamous cell carcinoma in the floor of the mouth were generated and characterized. OGFr binding assays revealed 2.4- to 8.4-fold increase in binding capacity compared to wild type (WT) and empty vector (EV) controls; binding affinity was comparable in all cell lines. OGFr protein expression assessed by immunohistochemistry and Western blotting was increased in clonal cell lines relative to controls. Under standard growth conditions, the cell number of OGFr clonal lines was reduced by 11% to 68% from the WT group, and doubling times were 7% to 67% longer. Addition of exogenous OGF further reduced (8% to 37%) cell growth of clonal lines. DNA synthesis of cells overexpressing OGFr was reduced from the WT group by 46% to 75%. Depletion of endogenous OGF with antibodies to this peptide increased growth 2-fold greater in clonal cells relative to increases of 32% and 34% for the WT and EV groups, respectively. These data show for the first time that molecular overexpression of the OGFr receptor decreases cell replication and DNA synthesis of SCCHN cells in tissue culture, indicating that OGFr is integral to cell replication. These results establish the fundamental principle that OGF and OGFr form an autocrine loop that regulates growth, and support treatment modalities using amplified OGF in order to decrease the growth of these neoplasias.

Overexpression of the Opioid Growth Factor Receptor (OGFr) Inhibits Growth of Human Squamous Cell Carcinoma of the Head and Neck (SCCHN) in Nude Mice

P. J. McLaughlin, S. M. Kreiner, C. R. Morgan, I. S. Zagon; Neural and Behavioral Sciences, Penn State University, Hershey, PA

The Opioid Growth Factor (OGF) is a constitutively expressed pentapeptide whose action as a negative growth regulator is mediated by the OGF receptor (OGFr). The OGF-OGFr axis tonically regulates growth of human squamous carcinoma cells of the head and neck (SCCHN) in vivo. Studies were designed to determine whether amplification of OGFr signaling in SCCHN may contribute to phenotypic changes, such as the loss of cell replication, which could be associated with malignant progression. Athymic mice were inoculated with 3 different cell lines amplified with OGFr (OGFr-9, OGFr-18, and OGFr-22). To evaluate a tumor burden response, each cell line, along with wildtype (WT) and empty vector (EV) was inoculated at 2 or 5 million cells/mouse. Tumor development and OGFr in tumors were monitored. Initial appearance of a measurable tumor was recorded when tumors were 62.5 mm3. WT and EV mice receiving 2 million cells had latency times of 10-11 days, while tumors generated from cells overexpressing OGFr appeared on days 13, 15, and 21 for the OGFr-9, OGFr-18, and OGFr-22 groups, respectively. WT and EV mice receiving 5 million cells had latency times of 8 days; latency to a measurable tumor was delayed up to 19 days (p<0.05) for the OGFr-9, OGFr-18, and OGFr-22 mice. Four weeks after cell inoculation tumors were collected and assessed for OGFr binding. Specific and saturable binding to radiolabeled OGF was detected, with significantly increased levels of OGFr in all clonal cell lines (2.5- to 3.7 fold greater) from WT and EV groups. Depletion of endogenous OGF with antibodies to this peptide increased growth 2-fold greater in clonal cells relative to increases of 32% and 34% for the WT and EV groups, respectively. These data show for the first time that molecular overexpression of the OGFr receptor decreases cell replication and DNA synthesis of SCCHN cells in tissue culture, indicating that OGFr is integral to cell replication. These results establish the fundamental principle that OGF and OGFr form an autocrine loop that regulates growth, and support treatment modalities using amplified OGF in order to decrease the growth of these neoplasias.

Overexpression of ARM Enhances Tumorigenesis in Malignant Melanoma

Y. Liao,1 S. Hsu,2 P. Huang1;1 Graduate Institute of Pathology, National Taiwan University, Taipei, Taiwan;2Department of Dermatology, National Taiwan University Hospital, Taipei, Taiwan

Tumorigenesis is often associated with reactivation of developmental programs. Transformed tumor cells may aberrantly re-express molecules that mediate proper embryonic development. ARM (ankyrin repeat-rich membrane spanning), also known as Kidins220 (kinase D-interacting substrate of 220kD), is a transmembrane protein expressed in the developing and adult neural tissues. Here, using immunohistochemical analysis, we report the significant expression of ARM in malignant melanoma (n=76), but not in benign nevi or other non-melanoma skin cancers such as basal cell carcinoma, and squamous cell carcinoma. The intensity of ARM immunoreactivity was positively correlated with Breslow tumor thickness (p<0.03). In contrast, compared with primary melanoma without metastasis, the expression level of ARM was significantly reduced in that with metastasis (P = 0.049). Two staining patterns of ARMs were revealed, the cytoplasmic granular pattern or membranous pattern, and the former was correlated with an increased hazard of poor prognosis (P = 0.06). To investigate the role of ARMs in the tumorigenesis of melanoma, stable B16-F0 cell clones with decreased expression of ARM were established by RNA interference. Knockdown of ARMs resulted in inhibition of cell proliferation, delayed anchorage-independent growth in soft agar, suppressed colony formation, and decreased tumor growth in SCID mice. Our study is the first to establish a link between overexpression of ARMs and melanoma metastasis.

Interactions of Tau with Alzheimer’s Disease Brain Microtubules and Other Neuronal Microtubules

S. C. Rego,1 C. C. Neto,2 P. E. Hart;1 1Medical Laboratory Science, UMass-Dartmouth, N. Dartmouth, MA;2Biology, UMass-Dartmouth, N. Dartmouth, MA

Tau is a microtubule-associated protein (MAP) expressed primarily in neurons. While it has been established that tau is expressed in other cell types, including prostate and breast carcinomas, its function in non-neuronal cells is unknown. We previously showed that tau interacts with the SH3 domain of Src family kinase members Fyn and Src, leading to tyrosine phosphorylation of tau, and it has also been demonstrated that tau binds to the prolyl isomerase Pin1. These interactions suggest that tau has functions independent of its microtubule-binding properties. Because prostate cancer cells are high in both Src and Pin1, and have been shown to contain tyrosine-phosphorylated tau, we set out to fully characterize the tau expressed in these cells. As detected by immunoblotting, tau expression is significantly higher in the cancerous prostate lines Du145, LNCaP, and PC3 than in non-cancerous prostate cells. It can be immunoprecipitated with anti-tau and is heat-stable like neuronal tau. All six alternatively spliced tau isoforms are transcribed in LNCaP cells, and RT-PCR results suggest that the 1N3R and 1N4R isoforms are the most prevalent. The less prevalent exons 4a and 6 are also present. LNCaP tau is phosphorylated at serine, threonine, and tyrosine sites, as detected by phospho-specific tau antibodies that also react with Alzheimer’s Disease brain. And while LNCaP tau is capable of binding microtubules, a significant proportion of it remains unbound, as detected by both confocal microscopy and microtubule binding assays with taxol-stabilized microtubules. In summary, tau from human prostate cancer cells has properties that are both similar and different from those of adult brain tau.

Overexpression of ARM Enhances Tumorigenesis in Malignant Melanoma

Y. Liao,1 S. Hsu,2 P. Huang1;1 Graduate Institute of Pathology, National Taiwan University, Taipei, Taiwan;2Department of Dermatology, National Taiwan University Hospital, Taipei, Taiwan

Tumorigenesis is often associated with reactivation of developmental programs. Transformed tumor cells may aberrantly re-express molecules that mediate proper embryonic development. ARM (ankyrin repeat-rich membrane spanning), also known as Kidins220 (kinase D-interacting substrate of 220kD), is a transmembrane protein expressed in the developing and adult neural tissues. Here, using immunohistochemical analysis, we report the significant expression of ARM in malignant melanoma (n=76), but not in benign nevi or other non-melanoma skin cancers such as basal cell carcinoma, and squamous cell carcinoma. The intensity of ARM immunoreactivity was positively correlated with Breslow tumor thickness (P = 0.03). In contrast, compared with primary melanoma without metastasis, the expression level of ARM was significantly reduced in that with metastasis (P = 0.049). Two staining patterns of ARMs were revealed, the cytoplasmic granular pattern or membranous pattern, and the former was correlated with an increased hazard of poor prognosis (P = 0.06). To investigate the role of ARMs in the tumorigenesis of melanoma, stable B16-F0 cell clones with decreased expression of ARM were established by RNA interference. Knockdown of ARMs resulted in inhibition of cell proliferation, delayed anchorage-independent growth in soft agar, suppressed colony formation, and decreased tumor growth in SCID mice. Our study is the first to establish a link between overexpression of ARMs and melanoma metastasis.

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Involvement of AMPK & P38 MAP Kinase in 8-CI-cAMP Induced Growth Inhibition

Y. Ahn, J. Han, K. Choi, J. Kim, S. Hong; Institute of Molecular Biology & Genetics, Seoul National University, Seoul, Republic of Korea.

8-CI-cAMP (8-chloro-cyclic AMP), which induces differentiation, growth inhibition and apoptosis in various cancer cells, has been investigated as a putative anti-cancer drug. Although we reported that 8-CI-cAMP induces growth inhibition via p38 MAP kinase (AMPK) and a metabolite of 8-CI-cAMP, 8-CI-adenosine mediates this process, the action mechanism of 8-CI-cAMP is still uncertain. Since 8-CI-cAMP is known to decrease the intracellular ATP pool, we tested if this ATP depletion could be related with the modulation of cellular growth. Intracellular ATP level decreased after the treatment with 8-CI-cAMP time-dependently, and excess addition of ATP could mitigate 8-CI-cAMP-induced growth inhibition. The reduction of ATP level resulted in the increase of AMP/ATP ratio, which could be the activating signal for AMP-activated protein kinase (AMPK). As expected, 8-CI-cAMP activated AMPK, which was also dependent on the metabolic degradation of 8-CI-cAMP. A potent agonist of AMPK, 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) could also induce growth inhibition and apoptosis.

Furthermore, AICAR was able to activate p38 MAPK and pre-treatment with AICAR inhibitor blocked this p38 MAPK activation. A p38 MAPK inhibitor attenuated 8-CI-cAMP- or AICAR-induced growth inhibition, but had no effect on AMPK activation. These results demonstrate that 8-CI-cAMP-induced growth inhibition is mediated by AMPK, and p38 MAPK works downstream of AMPK in this signaling pathway.

Activation of the ERK Signalling Pathway in Merlin-deficient Schwannoma

S. Amouei, C. O. Hanemann; Clinical Neurobiology, Peninsula Medical School, Plymouth, United Kingdom

Merlin is a tumour suppressor protein which belongs to the ERM (ezrin-moesin-radixin) protein family and acts as a membrane-cytoskeleton scaffolding protein. Merlin deficiency leads to the development of benign Schwann cell tumours, called schwannomas. Using our in vitro model of human primary Schwann and schwannoma cells, we have demonstrated a correlation between Merlin deficiency and specific tumour characteristics such as increased cell proliferation and decreased apoptosis. Moreover enhanced cell spreading and migration due to overexpression of integrins have been observed, suggesting a possible correlation between Merlin deficiency and integrin mediated downstream signalling. However, the molecular mechanism finally leading to schwannoma development has not yet between investigated. ERK activity has been shown to be involved in Schwann cell dedifferentiation and is known to play a role in tumour formation, thus we explored the ERK signalling pathway in more detail. Initially, our data demonstrate strong and long-lasting activation of MEK1/2 in schwannoma cells compared to normal Schwann cells. Moreover, MEK1/2, as well as Ras and B-Raf, both acting upstream of MEK1/2, was shown to be overexpressed in schwannoma cells. Additionally, preliminary investigations using inhibitors showed that MEK1/2 overexpression phenomena have suggested the involvement of MEK1/2 in schwannoma development.

Therefore we explored the ERK signalling pathway. Thus, we investigated the ERK signaling pathway in schwannoma model cells. However, our data showed strong and long-lasting activation of MEK1/2 in schwannoma cells compared to normal Schwann cells. Moreover, MEK1/2, as well as Ras and B-Raf, both acting upstream of MEK1/2, was shown to be overexpressed in schwannoma cells. Additionally, preliminary investigations using inhibitors showed that MEK1/2 overexpression phenomena have suggested the involvement of MEK1/2 in schwannoma development.

A Highly Invasive Variant of MDA-MB-231 Breast Carcinoma Cells Showed Increased Traction Forces and Higher Dynamics of Cytoskeletal Reorganization Compared to a Low Invasive Variant

C. T. Mierke, P. Kollmannsberger, D. Paramhao Zitterbart, C. Ranapach, W. H. Goldmann, B. Fabry; Medical Physics and Technology, University of Erlangen-Nuremberg, Erlangen, Germany

Cancer cells have different capacities to metastasize, thus it is important to understand the signaling pathways that govern these capacities. We tested the hypothesis that invasive tumor cells have nanomechanical properties that allow them to reorganize their shape, cytoskeleton and focal contacts more rapidly and to generate higher tractions than non-invasive cells. We isolated a highly invasive variant of MDA-MB-231 breast carcinoma cells (called BC-1) and a low-invasive variant (called BC-2). To measure the invasiveness of BC-1 and BC-2, we cultured them on a 3D collagen gel. The density of tumor cells that invaded after 3 days into the gel multiplied by their average invasion-depth was taken as an index of invasiveness. BC-1 cells were found to be more invasive (1.86±0.10 mm⁻¹) than BC-2 cells (3.59±0.15 mm⁻¹). The cytoskeletal dynamics were measured using magnetic tweezers and nanoscale particle tracking. For microrheology measurements, step forces from 1 - 10 nN were applied to fibronectin-coated beads bound to the cytoskeleton of adherent tumor cells. The bead displacement in response to the applied force followed a power-law, where the exponent is a measure of cytoskeletal dynamics, with low values corresponding to a solid-like, static behavior, and high values corresponding to a more liquid-like, dynamic behavior. BC-1 cells had a higher exponent (0.43±0.019) than BC-2 cells (0.33±0.002). The diffusion of fibronectin-coated beads bound to BC-1 and BC-2 was measured. Bead diffusion also followed a power-law, where BC-1 cells showed a higher superdiffusive behavior (b = 1.58) than BC-2 (b = 1.44). The 2D tractions measured during cell adhesion to collagen-coated polyacrylamide gel showed that BC-1 cells generate a higher 2D traction (0.46 pN) than BC-2 cells (0.06 pN). The cytoskeletal dynamics and traction generation both correlate with tumor cell invasiveness. Nanomechanical measurements do help in determining the invasion potential of tumor cells and also the potential of a tumor mass to metastasize.

Calcium Sensing Receptor Function in Human Colon Carcinoma

S. Chakrabarty, G. Liu, X. Hu, J. Varani; SimmonsCooper Cancer Institute, Southern Illinois University, Springfield, IL

Calcium is a versatile second messenger that has been implicated in cancer progression and metastasis. The calcium-sensing receptor (CaSR) is a G-protein coupled receptor that is expressed in various cell types, including cancer cells. The function of CaSR in cancer cells is still not well-understood. We investigated the role of CaSR in human colon carcinoma cells.

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representing a previously undescribed genetic programme which likely contributes to mesangial cell dysfunction in DN. When HMCs were stimulated with CTGF and stained for tubulin and β-actin, widespread microtubular and actin rearrangement was apparent. This was accompanied by a polarized redistribution of myosin, suggesting activation of the machinery of cell migration. Redistribution of myosin was facilitated by phosphorylation of the myosin light chain in response to CTGF, which was inhibited by the addition of the myosin inhibitor 2.3 butanediolmonoxime. Increased levels of AR3P in response to treatment with CTGF were mediated via cdc42 dependent activation of PAK-1, which was inhibited by the addition of the RhodGTPase inhibitor, Toxin B. This data indicates that CTGF-mediated actin rearrangement may contribute to the pathophysiology of the glomerular mesangium in diabetic nephropathy.

673 Cholesterol Accumulation Impairs Endocytosis in Fibroblasts from Patients with Genetic Lysosomal Lipid Storage Disorders
C. Garracho,1 V. Muzikantov,2 E. Schuchman,2 S. Maro,2 1Institute for Environmental Medicine, University of Pennsylvania Medical School, Philadelphia, PA; 2Human Gentoics, Mount Sinai School of Medicine, New York, NY

The mechanisms by which accumulation of undegraded metabolites in genetic lysosomal storage disorders (LSDs) leads to cell dysfunction are incompletely understood. We evaluated the effects of intracellular lipid storage on endocytic pathways in skin fibroblasts from Fabry, Gaucher, type B and Niemann-Pick (NPB and NPC) patients, characterized by deficiency of α-galactosidase-A, glucocerebrosidase, acid sphingomyelinase, and NPC1 transporter. We monitored the rate of BODIPY-FITC-labeled LDL internalization by flow cytometry, the rate of LDL degradation by measuring free cholesterol content, and the rate of caveolae-mediated endocytosis by measuring the rate of cholera toxin binding sites. Cells from LSD patients had significantly decreased rates of endocytosis and LDL degradation compared to normal controls. These results suggest that LSDs may impair endocytosis and lipid metabolism, which may contribute to the pathogenesis of LSDs.

675 Chol Ergonic Acid Attenuates Adhesion Molecules Upregulation in IL-1β-Treated HUVECs
Y. Yu,1 C. Chen,2 W. Chang1; 1Nutrition, China Medical University, Taichung, Taiwan

Reactive oxygen species (ROS) are known to induce endothelial cell (EC) apoptosis and vascular smooth muscle cell (VSMC) proliferation. It has demonstrated that hyperlipidemia may impact vascular function through increased oxidative stress and synthesized ROS endogenously by inhibiting electron transport chain of mitochondria in vascular cell. Recent study has observed that exercise training may initiate adaptations to reduce oxidative sources or up regulate antioxidative enzymes. We subjected 4–6 month-old male Zucker rats to an 8-weeks chronic exercise training program on a motorized treadmill. A total of 12 obese rats were randomly divided into two groups, i.e sedentary group and exercise-trained group. All rats were sacrificed at 48 h after the end of their last training session to minimize acute exercise effects from the last training bout. Immunoblot analysis of harvested vessels showed that lower expression of caspase 3 and ERK1/2 in obese exercised group was found compared with obese sedentary group. However superoxide dismutase (SOD) and catalase were significantly expression in both obese and lean exercised group. The results of immunohistochemistry showed that caspase 3 and ERK1/2 were highly expressed on EC and VSMC in obese sedentary group. Similarly, this phenomenon could be reversed after exercise training. It was very interesting that Cu/ZnSOD and MnSOD mRNA level has no significant change from each group. The mRNA level of glutathione peroxidase was significantly elevated but catalase mRNA level was lowered instead. In conclusion, exercise training played a role in vascular protection by up regulating antioxidative protein expression and decreasing caspase 3 and ERK1/2 protein production in obese Zucker rats.

676 Cell-based Screening for Inhibitors of PYK2 Kinase Activity
A. P. Baumgart,1 T. C. Bonnett,1 D. T. Crawford,2 H. Li1; 1Wellcome Trust Sanger Institute, Hinxton, Cambridge, United Kingdom

Proline-rich tyrosine kinase 2 (PYK2), a member of the Focal Adhesion Kinase (FAK) family, is involved in many cellular processes including adhesion, cell spreading, migration, and apoptosis. PYK2 is activated in response to mechanical stimulation, calcium flux, integrin signaling and chemokines. In addition to having kinase activity, PYK2 forms complexes with other cytoskeletal proteins such as paxillin, cortactin, and src therefore may have both scaffolding and signaling functions. Since PYK2 knockout mice display increased bone compared with wild type littermates in vivo, PYK2 is a potential target for therapeutic intervention in diseases of bone metabolism. In vitro assays to monitor PYK2 activity have been developed, but monitoring the kinase activity in cells has been challenging because endogenous expression levels are generally low and no specific substrate has been identified. A phosphorylation event on tyrosine 402 of PYK2 is required for activation of this kinase and we chose to use this event to monitor the effect of inhibitors in cells. The work presented here describes a 384 well cell-based assay for Y402 phosphorylation that has been developed using L1-COR technology. PYK2 is indirectly overexpressed using the GeneSwitch system in NIH 3T3 cells, and after treatment with test compounds, the cells are fixed in situ and immunostained for PYK2 (phospho-Y402). A detection system using near-infrared (IR) dyes (800nm) affords stability, sensitivity, and elimination of interference from the autofluorescence typically found in many library compounds. A panel of compounds was tested and the IC50 data correlated with in vitro kinase activity. This medium-throughput assay is robust, reproducible, and has proven to be valuable in identifying cell active compounds that may warrant further investigation. This approach will not only assist in identifying cell-active inhibitors, but the principle should be applicable to a variety of kinase targets.

677 Developmental Expression of VPS33B Correlates with Human Phenotype of VPS33B Mutations
P. Gissin,1 D. Gleeson,2 A. Straatman-Iwanowska,1 D. Tannahill,2 E. Maher1; 1Section of Medical and Molecular Genetics, University of Birmingham, Birmingham, United Kingdom; 2The Wellcome Trust Sanger Institute, Hinxton, Cambridge, United Kingdom

Background: VPS33B is a human homologue of yeast Vps33p protein and is part of the homotypic fusion and protein sorting (HOPS) complex. HOPS complex proteins are involved in tethering of endosomes to the target membranes on late endosomes and lysosomes prior to SNARE-complex formation and membrane fusion but their mode of action is not clear. Previously we demonstrated that germline VPS33B mutations cause the Arthrogryposis, Renal Dysfunction and Cholestatosis (ARC) syndrome. To further elucidate the pathology of ARC syndrome we have investigated developmental expression of VPS33B. Methods We sequenced exons and exon-intron boundaries of VPS33B. Expression of VPS33B in developing mouse embryos was studied by in situ hybridization. Expression of VPS33B in human cells and fibroblasts was studied using rabbit polyclonal anti-VPS33B antibody. Results Germline VPS33B mutations were identified in 53 probands. In addition to the well characterised features of ARC syndrome, intracranial abnormalities were present in ~15% of cases. All patients exhibited severe degree of failure to thrive unexplained by liver or kidney disease. The expression pattern of the VPS33B in mouse embryonic tissues correlated well with the abnormalities in cerebral development and also renal tubular system. At E10.5 VPS33B expression can be detected weakly in neural tissue including the developing neural tube and the dorsal root ganglia. By E12.5, expression...
continues in these sites and expression in cranial ganglia and the gut becomes apparent. Expression in the kidney also becomes evident by E14.5. There was evidence of expression of VPS33B in human fibroblasts and also CaCo2 cells. Summary Embryonic expression of VPS33B in neural tissue correlates with the neurodevelopmental abnormalities found in ARC syndrome and is consistent with genetic origin for arthrogryposis. VPS33B deficiency causing gastrointestinal dysfunction may be associated with the severe failure to thrive seen in ARC syndrome.

678 Role of Protein 4.1R in Calcium Absorption in the Small Intestine: Defective Calcium Homeostasis in 4.1R-Null Mice
X. An, C. Liu, G. Debmah, N. Mohandas; Red Cell Physiology Laboratory, The New York Blood Center, New York, NY

Protein 4.1R (4.1R) was initially identified as a major component of erythrocyte membrane skeleton and plays a key role in maintaining erythrocyte morphology and membrane mechanical stability. 4.1R is abundantly expressed in various cells including epithelial cells, but the function of 4.1R in these cells is far from clear. In the present study, we used 4.1R null mice to explore the function of 4.1R in small intestine. We show that 4.1R is expressed at the apical domain of enterocytes. Importantly, small intestine of 4.1R null mice displayed morphological abnormalities. Importantly, in vivo calcium absorption assay demonstrated a significantly diminished calcium absorption in the small intestine of 4.1R null mice compared to that of wild type. In addition, 4.1R null mice displayed hyperplasia of parathyroid, increased parathyroid hormone levels, elevated serum 1, 25 (O H) 2 D levels, osteoporosis and decreased calcium content of bone. These findings imply that loss of 4.1R from enterocytes of small intestine impairs the calcium absorption which in turn leads to defective calcium homeostasis in 4.1R null mice.

679 The Mammalian Glucose Transporter Protein GLUT12 - Subcellular Localization in Response to Insulin and Glucose
S. D. Rogers, C. L. DelHaan; Medicine St Vincent's, The University of Melbourne, Melbourne, Australia

GLUT12 is expressed in insulin-sensitive tissues and possesses potential intracellular targeting motifs. We predicted that GLUT12, like GLUT4, translocates in response to insulin. In normal individuals, glucose homeostasis is maintained by insulin-stimulated transport of glucose into muscle and fat. In Type 2 diabetics, this homeostasis is compromised by the cellular loss of insulin-responsiveness, resulting in hyperglycemia. Glucose itself can also stimulate glucose transport. Although not well understood, this process is important in Type 2 diabetics where insulin action is impaired. We further hypothesized that GLUT12 responds to elevated glucose. We engineered fluorescent fusion proteins and exo-facially epitope-tagged constructs. GLUT12 trafficking was studied using retroviral expression, confocal microscopy and immunofluorescence. In glucose and insulin-starved CHO cells, GLUT12 was restricted to a perinuclear position. After exposure (ten minutes) to insulin (1mU) or high (25mM) glucose, GLUT12 translocated to the cell surface. GLUT12 co-localized in part with GLUT4. We initially identified GLUT12 in MCF7 breast cancer cells. Elevated glucose transport and metabolism are characteristics of cancer cells and we have demonstrated GLUT12 expression in human breast cancer but not in normal breast epithelial. In MCF7 cells, GLUT12 was localized to a perinuclear position. Translocation to the cell surface would be necessary for GLUT12 to contribute to cellular glucose uptake. Using confocal immunofluorescence, we have now demonstrated that in glucose and insulin-starved MCF7 cells, GLUT12 was localized to a perinuclear region and detected at the cell surface following exposure to insulin or high glucose. GLUT12 trafficking from intracellular compartments to the cell surface is important in the physiology or pathophysiology of glucose homeostasis and may also be important for glucose transport into and growth of tumor cells. Epidemiological data indicates increased cancer risk in Type 2 diabetics. Our results may provide a novel mechanistic link between diabetes and breast cancer.

680 Curcumin Attenuates Dystrophic Pathology in mdx Mice through Inhibiting NF-κB Activation
M. S. Zhu, C. Chen, Y. L. Hu, S. Chen; Model Animal Research Laboratory and State Key Lab of Pharmaceutical Biotechnology, Nanjing University, Nanjing, China, 2Center of Antibody Research, Nanjing ChuanBao Biotech Co., Ltd. Nanjing, Nanjing, China

The dystrophin-glycoprotein complex has emerged as a scaffold responsible for the membrane docking of signaling proteins. There is no definitive cure available currently. In dystrophin-deficient muscular dystrophy, abnormal activation of signal pathways may play important roles in dystrophic pathogenesis. In this report, we found a natural botanical gradient, curcumin, capable of attenuating dystrophic pathology significantly and the mechanism underlying was involved in NF-κB inhibition. After treating X-linked muscular dystrophy (mdx) mice with 1 to 10 mg of curcumin, the sarcomere integrity assessed by Evans blue staining and muscle strength determined both by grip strength test and traction test were improved significantly. Histological analysis demonstrated that curcumin reduced severity of myofibril necrosis and extent of regenerating fibers as well as the variability in size of fibers. Creatine kinase level and the expressions of tumor necrosis factor alpha, interleukin-1 beta and inducible nitric oxide synthase were decreased also after curcumin treatment. Consistently, elevated NF-κB activity in muscle fibers of mdx mice decreased after curcumin administration. Since curcumin is a non-toxic compound derived from C. longa, which was widely used in healthy food. The possible effect of curcumin may be worth studying in Duchenne muscular dystrophy therapy.

681 The Role of STAT1/IRF-1 on Hepatic Liver Injury Induced by LPS/D-galactosamine
H. Lee, Y. Oh, S. Lee, Y. Kwon, S. Park, W. Kim; 1Biomedical Sciences, Korea National Institutes of Health, Seoul, Republic of Korea, 2Gyn. Oncology, Hallym Sacred Heart Hospital, Seoul, Republic of Korea

We previously demonstrated that STAT1 play an essential role in a variety of liver injury models induced by administration of LPS/D-galactosamine (D-GaIN), however, the underlying mechanism involved is not clear. Here, we show that cotreatment with TNF-α and IFN-γ secreted by the administration of LPS/D-GaIN induced synergistically induced apoptosis in primary hepatocytes and HepG2 cells, associating with NO production and inducible nitric oxide synthase expression (NO/iNOS) and elevated ROS production, which are inhibited strongly by antioxidant NAC or L-NMMA, an iNOS/NO inhibitor. Although activation and expression of STAT1 did not change strongly in TNF-α/IFN-γ-treated cells compared with IFN-γ alone-treated cells, disruption of the STAT1 gene or IRF-1 gene via genetic knock-out strongly abrogated the synergism of TNF-α/IFN-γ on iNOS/NO induction, ROS production, loss of ∆Ψm, and apoptosis observed in STAT1+/+ and IRF-1+/+ mice. In contrast to wild type STAT1 overexpression, the synergistic effects of TNF-α/IFN-γ on iNOS/NO induction, ROS production, and apoptosis were significantly inhibited by overexpression of dominant negative STAT1. Furthermore, NF-κB activated by TNF-α/IFN-γ was attenuated in STAT1 deficient mice and strongly inhibited by NAC or L-NMMA, while a proteasome inhibitor MG132, which inhibited NF-κB activation, strongly inhibited iNOS/NO induction, ROS production, and loss of ∆Ψm by TNF-α/IFN-γ, results in the inhibition of apoptosis. Collectively, these findings demonstrated that TNF-α/IFN-γ synergistically potentiates iNOS/NO induction, ROS production and loss of ∆Ψm via STAT1 overexpression, playing an important role in promoting apoptosis and liver injury induced by LPS/D-GaIN.

682 Critical Roles of ATP3 on Streptozotocin-induced Diabetic Liver Injury in Mouse: ATP3 Mediates STAT1 Overexpression
S. Lee, H. Lee, Y. Oh, J. Mo, Y. Kwon, S. Park, W. Kim; 1Biomedical Sciences, Korea National Institutes of Health, Seoul, Republic of Korea, 2Gyn. Oncology, Hallym Sacred Heart Hospital, Seoul, Republic of Korea

It is well established that the administration of streptozotocin (STZ) accelerates liver injury as well as type 1 diabetes, however the underlying mechanisms is not fully understood. Here, we demonstrate that STZ causes severe liver damage as evidenced by a significant elevation of serum ALT levels, and massive necrosis, which is followed the increase of IFN-γ. Also, STAT1, which is implicated in hepatitis liver injury by virtue of its ability to promote hepatocytes apoptosis, rapidly activated and its stability is sustained for long times, correlated with the induction of ATP3, a stress-inducible gene, and several apoptotic-related proteins in STZ-injected mice. STAT1 overexpression is critically dependent upon ATP3 induced by STZ or IFN-γ treatment in primary hepatocytes because ATP3 depletion significantly decreased STAT1 overexpression, ROS production and hepatic apoptosis induced. Interestingly, ATP3 not only enhances the promoter activity of STAT1 but also interacts directly with STAT1, which is enhanced following IFN-γ treatment. Removal of the putative ATP3 binding site in STAT1 promoter inhibits the enhancement of ATP3 on transcription activity of these genes. Collectively, our results suggest that ATP3 functions as a potent regulator of STAT1 gene expression and it stability, which is highly induced the liver injury via promoting apoptosis.

683 N-Acetylaminothiosemicarbazone Treatment Rescues a Mouse Model of Hereditary Inclusion Body Myopathy
M. Huizing, R. Klootwijk, B. Galeano, I. Manoli, M. Sun, C. Ciccone, D. Darvish, D. Krasnewich, W. Gahl; 1NHGRI, NIH, Bethesda, MD, 2HIBM Research Group, Encino, CA
Hereditary Inclusion Body Myopathy (HIBM) is an adult onset recessive neuromuscular disorder of slowly progressive distal and proximal muscleatrophy and weakness. This myopathy is caused by deficiency of UDP-GlcNAc 2-epimerase/MannNac kinase (GNE), the rate-limiting, bifunctional enzyme catalyzing the first two committed steps of sialic acid biosynthesis. Decreased GNE activity impairs sialic acid production and interacts with syndromes of muscle glycoproteins such as α2-dystroglycan. Levels of polysialic acid on neural cell adhesion molecule (PSA-NCAM) may also be affected. To study the pathogenesis and treatment of HIBM, we created a GNE knock-in mouse mimicking the human M712T GNE mutation. Homozygous (+/-) mutant mice were born in a Mendelian distribution, but did not survive beyond postnatal day 3 (P3). GNE enzyme activity in skeletal muscle tissues of +/- mice at age P2 was 20% of normal. However, histological examination did not reveal any muscle pathology. Rather, the kidneys of +/- mice showed petechial hemorrhages, proteinsuria, and signs of glomerular disease. As a treatment option, we administered to pregnant females ManNac (1g/kg/d), an uncharged sugar intermediate located within the sialic acid pathway. Upon feeding +/- matings ManNac in their drinking water, 43% of the +/- pups survived beyond P3. At P2, +/- mice that received ManNac had less severe kidney hemorrhages, and their muscle GNE activities increased to 50% of normal mouse levels, suggesting that ManNac might stabilize the mutant enzyme. Surviving +/- mice were smaller than their littermates, but appeared healthy otherwise, even after weaning (P21), when they no longer received ManNac. It remains to be determined if the surviving +/- mice will develop a muscular pathology later in life. Taken together, survival of +/- mice, improved kidney pathology and increased GNE activity after ManNac administration strongly support consideration of a clinical trial of ManNac for the myopathy of HIBM.

684 Norrin Mediated TCF Signal Is Differentially Modulated by LRP5 and LRP5-G171V (HBM) Mutant in Response to Dkk1 and Kremen2
B. M. Bhat,1 H. Lam,1 V. Colebourn,1 P. Yavorsky,1 F. Bex,1 Women's Health and Musculoskeletal Biology, Wyeth Research, Collegeville, PA, 2Biological Technologies, Wyeth Research, Cambridge, MA
Mutations in Norrin lead to Norrie disease pseudoglioma (NDP) and X-linked Familial Exudative Vitreoretinopathy (FEVR), characterized by failure in retinal vascularization that results in blindness. FEVR can also be caused by mutations in either Frizzled4 (Fzd4) or its co-receptor LRP5/6, which are involved in Wnt β-catenin-TCF signaling. Recent reports indicate that Norrin is a ligand for LRP5-Fzd4 specific complex. Like Wnts, Norrin is a cysteine rich secreted protein, that remains associated at the cell surface and induces the β-catenin pathway. However, Norrin has no significant homology to Wnts. Mutations in the first β-propeller of LRP5 (e.g. LRP5-G171V) are associated with a high bone mass (HBM) phenotype. The HBM mutations attenuate inhibition of Wnt/β-catenin signal by the antagonist Dkk1 and its co-receptor Kremen2. To determine whether the signal induced by Norrin would, like Wnts, be affected by the HBM mutations and the functions of Dkk1 or Kremen2, we investigated their interaction in bone (U2OS) and non-bone (HEK293A) cells. Transfection of Norrin cDNA resulted in an enhanced β-catenin pathway TCF-signal in U2OS but not in HEK293A cells. However, co-transfection of Frizzled4 with Norrin into 293A cells activated the TCF signal indicating that endogenous functional Frizzled4 was lacking in HEK293 cells. This demonstrates how Norrin mediated TCF-signal can be regulated by cell type dependent Frizzled expression. Surviving +/- mice were smaller than their littermates, but appeared healthy otherwise, even after weaning (P21), when they no longer received ManNac. It remains to be determined if the surviving +/- mice will develop a muscular pathology later in life. Taken together, survival of +/- mice, improved kidney pathology and increased GNE activity after ManNac administration strongly support consideration of a clinical trial of ManNac for the myopathy of HIBM.

685 Mitotic Spindle Assembly and Function
R. Heald; Department of Molecular & Cell Biology, University of California, Berkeley, Berkeley, CA
A key event in mitosis is segregation of the duplicated chromosomes into two complete sets, one for each daughter cell. This task is performed by the mitotic spindle, a complex and dynamic machine consisting of microtubules and numerous other factors. Spindle formation relies on intricate spatial and temporal control of microtubule polymerization, and coordinated organization by motor proteins to generate a bipolar spindle array. While spindles are fundamentally the same in all eukaryotic cells, the mechanisms for assembling them may vary depending on the cell type. In most somatic cells microtubule organization is strongly influenced by centrosomes. The centrosome duplicates prior to the onset of mitosis, generating two foci that nucleate microtubules, which then interact with chromosomes through a ‘search and capture’ mechanism. Mitotic chromosomes play an active role in spindle assembly by forming attachments at their kinetochores that facilitate their metaphase alignment and subsequent segregation during anaphase. In addition, mitotic chromosomes generate biochemical signals to promote spindle assembly. This mechanism is most apparent and essential in systems such as female meiotic cells that lack focal centrosomes, but increasing evidence suggests that this is a conserved process operating in many cell types. Meiotic X. novius egg extracts provide a useful system to study this phenomenon, as chromatin itself, in the form of plasmid-DNA coated beads, is sufficient to induce spindle assembly in the absence of centrosomes and kinetochores. In this pathway microtubules are nucleated and stabilized around chromosomes, and subsequently ‘self-organize’ to form a mitotic spindle in the absence of paired microtubule nucleating sites. We are investigating the molecular components of centromere-driven spindle assembly pathways, and using fluorescence-based imaging techniques to visualize mitotic gradients in different cell types.

686 Mining the Genome for Mitotic Treasures
R. D. Yala,1 G. Goshima,2 E. Griffis,3 R. Wollman,4 J. Schol ey,5 N. Sturman; 1Department of Cellular and Molecular Pharmacology, HHMI, University of California, San Francisco, San Francisco, CA, 2Section of Molecular and Cellular Biology, University of California, Davis, Davis, CA
The formation of the microtubule-based mitotic spindle is essential for chromosome segregation in eukaryotes. Many of the molecules involved in forming the spindle and regulating the sequence of events in cell division are known. However, the inventory of proteins that participate in mitosis is undoubtedly incomplete. We have used whole genome RNAi screens to search for genes that play important roles in mitosis in Drosophila S2 cells. Using a high throughput microscope to collect images of mitotic spindles and a combination of manual and computational approaches to analyze these images, we have identified ~250 genes that play important roles in generating microtubules, localizing γ-tubulin, controlling spindle length or shape, aligning chromosomes at the metaphase plate, and regulating the spindle assembly checkpoint. To learn more about the functions of novel proteins identified by the screen, we have used GFP tagging to determine their localizations and dynamics and used RNAi strategies to examine the order of assembly of components at centrosomes and kinetochores. These studies provide a comprehensive view of the proteins that contribute to spindle architecture and function and suggest potential new targets for cancer chemotherapy.

687 Dynamics of Bacterial Chromosome Organization, Segregation, and Cytokinesis
L. Shapiro; Department of Developmental Biology, Stanford University School of Medicine, Stanford, CA
Many regular proteins and multiprotein complexes involved in the execution of the Caulobacter cell cycle, and chromosome replication and segregation must perform their functions at a specific place in the cell and at a specific time in the cell cycle. By fluorescently tagging 114 individual chromosomal loci and examining the position of each locus in the cell, we showed that in living cells prior to DNA replication, the loci are arranged in a linear order along the long axis of the cell, reflecting the order of genes on the circular chromosome. We further showed that upon initiation of DNA replication, one copy of the origin of replication is rapidly moved to the opposite pole of the cell and as each locus is replicated, in turn, its' copy moves to the new position in the incipient daughter half of the cell. Thus, replication and segregation are simultaneous processes independent of cell growth. We have identified a protein, MipZ, that functions to coordinate origin movement to the opposite cell pole with the placement of the tubulin FtsZ ring at the mid-cell. MipZ binds to ParB at the origin and moves with the duplicated origin to the opposite pole. A gradient of MipZ across the cell has its highest concentration at the cell poles and lowest at mid-cell. MipZ inhibits FtsZ polymerization; thus the cell division ring forms only at mid-cell, the cellular position of lowest MipZ concentration. Differential rate of growth of the two daughter cell compartments then leads to an asymmetrically placed division plane. Thus, bacterial ‘mitosis’ is an integral component cell cycle spatial and temporal regulation.

688 Bringing Research into the Undergraduate Curriculum
S. C. R. Elgin,1 C. Shaffer,2 W. Leung,3 J. Buhler,4 E. Mardis,3 D. Lopatto,4; 1Department of Biology, Washington University, St. Louis, MO, 2Department of Computer Science & Engineering, Washington University, St. Louis, MO, 3Department of Genetics, Washington University, St. Louis, MO, 4Department of Psychology, Grinnell College, Grinnell, IA
Undergraduate research experiences are a critical part of our biology curriculum, enabling students to understand how new knowledge is generated in their field. Undergraduate students often enter research through an individual apprenticeship during the summer. This has been found to generate a spectrum of intellectual and social gains, including greater understanding of the research process and the strategies and tools that scientists use to work on real problems (SURE survey data; D. Lopatto, 2004, Cell Biol. Educ. 3: 270). An alternative to summer research is a one semester, upper-level laboratory course built around a research problem. Bio 4342, Research Explorations in Genomics, is taught by collaborating faculty at Washington University from Biology, Computer Science, and the WU Genome Sequencing Center (see http://www.mlsc.wustl.edu/elgin/genomics). Students in Bio 4342 join a research team working on sequencing and
689 Epigenetic Regulation of X Chromosome Gene Expression in Germ Cells of C. elegans
S. Strome,1, L. B. Bender,1 C. R. Carroll,1 V. Reinke,2 J. Suh1, 1Department of Biology, Indiana University, Bloomington, IN, 2Department of Genetics, Yale University School of Medicine, New Haven, CT

Modulation of the way in which DNA is organized into chromatin has emerged as a key level of regulation of gene expression during development and in diseased states. One mechanism of chromatin modulation is condensation of histones. We are studying one example of such "epigenetic" regulation in the C. elegans germ line. Development of germ cells in worms requires that the X chromosomes be globally silenced; this is achieved at least in part by regulation of histone marks on the Xs. We have identified a system of "MES" protein regulators that dictate the different chromatin states of the autosomes vs. the X chromosomes and are required for germ cell viability. MES-2, MES-3, and MES-6 form a trimERIC complex (related to the ESC/EZJ complex in fruit flies and mammals) that participates in X silencing by concentrating a repressive histone modification, methylation of Lys27 on histone H3, on the Xs. MES-4 (related to NSD1 in mammals) also methylates histone H3 tails but at a different residue, Lys36. MES-6 and its methyl mark are dramatically concentrated on the 5 autosomes and are absent from most of the length of the X chromosome. Exclusion of MES-4 from the X requires MES-2/MES-3/MES-6 function. Intriguingly, the main effect on gene expression patterns of removing MES-4 function is desilencing of genes on the X. We hypothesize that MES-4 or H3 methylated on Lys36 repels a repressor from the autosomes, focusing its binding or action on the X. The MES system illustrates how chromatin modifiers can work in concert to achieve whole-chromosome regulation, and it focuses our attention on the distinction between the autosomes and X chromosomes.

690 Local Cell-Cell Interactions in Shoot Apical Meristems Yield Global Patterns of Development
M. Hesler, H. Jönsson, E. Mjönes, E. M. Meyenbauer, Division of Biology, California Institute of Technology, Pasadena, CA, Department of Theoretical Physics, University of Lund, Lund, Sweden, Department of Computer Science and Engineering, University of California, Irvine, Irvine, CA

The shoot apical meristems (SAMs) of a flowering plant are the sets of stem cells at the tip of each shoot, which ultimately provide the cells that make the stem, leaves, and flowers. A long-standing question in developmental biology is how permanent populations of stem cells are maintained, another question is how the cells, which are apparently unpatterned and undifferentiated, give patterns of differentiated offspring. One prominent example in plants is the pattern of phyllotaxis, the pattern (usually spiral) in which leaves and flowers appear around the shoot. We have developed a new set of live imaging methods for the Arabidopsis thaliana shoot apical meristem, and a set of reporters for gene expression domains, and protein localization within cells of the SAM. Using live imaging and the reporter constructs we and others have found that the SAM has a private and highly controlled circulatory system for the plant hormone auxin, which serves as the morphogen that induces new floral primordia. This circulatory system is based on movement within each SAM cell of a polar auxin transporter, and leads to successive peaks of auxin concentration in a precise pattern that is the spiral phyllotactic pattern. A private and active circulatory system for a morphogen may represent a new principle of development, and also raises a number of questions about the mechanism of control of the intracellular location of the transporter.

691 Wnt and Notch Cooperate to Maintain Proliferative Compartmental compartments in Crypts and Intestinal Neoplasia
H. Clevers, Hubrecht Laboratory, Netherlands Institute for Developmental Biology of the Royal Netherlands Academy of Arts and Sciences, Utrecht, The Netherlands

Mutations in the Wnt pathway components APC, beta-catenin and conductin all induce sustained complex formation of the co-activator beta-catenin with Wnt transcription factors. The resulting transactivation of Wnt target genes is believed to be the primary transforming event in colorectal cancer (CRC). Yet, the consequence of the presence of mutationally activated beta-catenin/Wnt in fully transformed CRC cells is unknown. We have constructed CRC cell lines carrying inducible dominant-negative Wnt constructs. Inhibition of beta-catenin/Wnt resulted in a rapid G1 arrest. DNA array analysis revealed the downregulation of a small set of transcripts. These genes were expressed in polyps, but also, physiologically, in the crypt progenitor compartments of the colon. By contrast, we observed the induction of multiple marker genes of intestinal differentiation upon inhibiting beta-catenin/Wnt in CRC cells. We conclude that beta-catenin/Wnt inhibits differentiation and imposes a crypt progenitor phenotype on CRC cells. Moreover, inhibition of beta-catenin/Wnt activity restores the differentiation program, despite the presence of multiple other mutations in CRC. Wnt target gene expression is always restricted to the crypt, but target genes can be sub-classified based on expression patterns within the crypt. We have tentatively identified at least three target genes which are expressed uniquely in the crypt stem cells. The Wnt cascade is not the only signaling pathway controlling cell fate along the crypt-villus axis. Upon conditional removal of the common Notch pathway transcription factor CSL/RBP-J2, we observe a rapid, massive conversion of proliferative crypt cells into post-mitotic goblet cells. A similar phenotype was obtained by blocking the Notch cascade using a gamma-secretase inhibitor. The inhibitor also induced goblet cell differentiation in intestinal undifferentiated, give patterns of differentiated offspring. One prominent example in plants is the pattern of phyllotaxis, the pattern (usually spiral) in which leaves and flowers appear around the shoot. We have developed a new set of live imaging methods for the Arabidopsis thaliana shoot apical meristem, and a set of reporters for gene expression domains, and protein localization within cells of the SAM. Using live imaging and the reporter constructs we and others have found that the SAM has a private and highly controlled circulatory system for the plant hormone auxin, which serves as the morphogen that induces new floral primordia. This circulatory system is based on movement within each SAM cell of a polar auxin transporter, and leads to successive peaks of auxin concentration in a precise pattern that is the spiral phyllotactic pattern. A private and active circulatory system for a morphogen may represent a new principle of development, and also raises a number of questions about the mechanism of control of the intracellular location of the transporter.

692 The Convergence of Embryonic and Tumorigenic Signaling Pathways Contribute to Tumor Cell Plasticity
L. Postovit,1 J. Topczewski,2 N. Margaryan,3 A. Sam,3 A. R. Hess,4 W. W. Wheaton,2 B. J. Nickoloff,2 J. Topczewski,2 M. J. C. Hendrix,1, Program in Cancer Biology and Epigenomics, Children's Memorial Research Center, Northwestern University Feinberg School of Medicine, Chicago, IL, Program in Developmental Biology, Children's Memorial Research Center, Northwestern University Feinberg School of Medicine, Chicago, IL, Department of Pathology, Loyola University Medical Center, Cardinal Bernardin Cancer Center, Maywood, IL

Bi-directional cellular communication is integral to both cancer progression and embryological development. Furthermore, aggressive tumor cells are phenotypically plastic, sharing many properties with embryonic cells. To study these possible similarities we utilized the developing zebrafish as a biosensor for tumor derived signals. The data demonstrate that aggressive melanoma cells, but not poorly aggressive cells, can direct the fate of pluripotent zebrafish stem cells. Specifically, the aggressive tumor cells were able to orchestrate the formation of ectopic embryonic axes by secreting Nodal, a potent embryonic morphogen. Highlighting the potential role of this molecule in melanoma dissemination, Nodal protein expression was positively correlated with melanoma metastasis and inhibition of Nodal signaling reduced melanoma cell invasiveness. Moreover, depletion of Nodal signaling in aggressive melanoma cells caused the reversion of these cells toward a more melanocytic phenotype, and resulted in the abrogation of tumor progression in Smad2-independent and dependent mechanisms, respectively. We further determined that aggressive breast cancer cells similarly produce Nodal. From these data, we uncovered a key role for Nodal signaling in tumor cell plasticity and tumorigenicity, thereby providing a novel molecular target for regulating tumor progression.

693 Synergy between Notch and Kras Signaling in Pancreatic Tumorigenesis
J. De La O, L. Murtaugh, Human Genetics, University of Utah, Salt Lake City, UT

Differeentiation of stem and progenitor cells limits their capacity for tumorigenesis, and signals that prevent differentiation may indirectly promote cancer. Notch signaling inhibits differentiation in the developing pancreas, and it appears to be re-activated in pancreatic tumors and their initiating lesions (PanINs). To investigate the role of Notch in tumorigenesis, we have used two conditional mouse models: Kras<sup>12D</sup>, generated by Tuveson and colleagues, in which Cre recombinase induces expression of an endogenous activated Kras allele similar to that found in human tumors; and Rosa26<sup>Cre<sub>Lys<sub>36</sub></sub></sup>, developed by us, in which Cre induces expression of an activated Notch receptor. These alleles can be induced alone or together, throughout the pancreas or in specific pancreatic cell type. Widespread activation of Kras alone causes a small subset of cells to form PanINs lesions; Notch activation, by contrast, blocks differentiation in all cells but does not affect PanIN formation. Our hypothesis is that the blockade to differentiation imposed by Notch sensitizes cells to transformation by Kras, is strongly supported by experiments in which the two pathways are activated simultaneously. Not only does Notch activation dramatically sensitize cells to Kras-induced PanIN formation, it expands the range of cell types susceptible to activated Kras. In particular, we find that co-activation of Notch and Kras in adult acinar cells leads to their rapid dedifferentiation and dysplasia; this is the first demonstration that a specific adult cell type can serve as a site of tumor initiation in the pancreas. Endogenous Notch activation occurs in response to acute injury of the adult pancreas, and chronic injury of the organ is a
Aneuploidy Acts Both Oncogenically and as a Tumor Suppressor

B. A. A. Weaver, 1 A. D. Silk, 2 C. Montagna, 3 P. Verdier-Pinard, 4 D. W. Cleveland, 1 Ludwig Institute for Cancer Research, University of California, San Diego, La Jolla, CA, 2Pathology and Molecular Genetics, Albert Einstein College of Medicine, Bronx, NY, 3Department of Structural Biology, Sloan Kettering Institute, New York, NY, 4Institute of Biotechnology, Korea University, Seoul, Republic of Korea, 5School of Medicine, Kyung Hee University, Seoul, Republic of Korea, 6School of Medicine, Kyung Hee University, Seoul, Republic of Korea, 7Department of Structural Biology, Sloan Kettering Institute, New York, NY, 8Gerontology and OB/GYN & Women's Health, Albert Einstein College of Medicine, Bronx, NY, 9Department of Medicine, Radiology and Biomedical Engineering, Columbia University, New York, NY, 10Institute of Biotechnology, Korea University, Seoul, Republic of Korea

An abnormal chromosome number, a condition known as aneuploidy, is a common characteristic of tumor cells. Recognizing this correlation, Boveri proposed nearly 100 years ago that aneuploidy causes tumorigenesis. This has remained untested due to the difficulty of selectively generating aneuploidy in the absence of other defects. We now show that cells and mice that have reduced levels of the mitosis-specific, centromere-linked motor protein CENP-E develop aneuploidy and chromosomal instability in vitro and in vivo despite an intact DNA damage response and normal levels of p53. Chromosome painting demonstrates that aneuploidy is from loss or gain of whole chromosomes. In vivo, splenocytes, peripheral blood lymphocytes and colonic crypt cells exhibit significantly increased levels of near-diploid aneuploidy in CENP-E heterozygous mice. Near-diploid aneuploidy increases with age, with higher rates found for chromosome loss than gain. An increased rate of aneuploidy is demonstrated to contribute to transformation in vitro, as scored by loss of contact inhibition, anchorage independent growth, and tumor formation in immunocompromised mice. Aneuploidy from reduced levels of CENP-E is also shown to drive an elevated level of spontaneous lymphomas and lung tumors in aged animals. Reduced CENP-E function, in examples of chemically or genetically-induced tumor formation (loss of the tumor suppressor p19ARF), an increased rate of aneuploidy is a more effective inhibitor than initiator of tumorigenesis. These findings both confirm Boveri’s hypothesis and also reveal a previously unsuspected role of aneuploidy and chromosomal instability in preventing tumorigenesis.

Tissue Stiffness Promotes Mammary Tumorigenesis through Enhanced PI3 Kinase Activation

K. R. Johnson, 1 L. Kass, 2 N. Zahir, 1 P. Mrass, 3 W. Weninger, 3 D. Gasser, 4 S. S. Margulies, 1 P. A. Janmey, 1 V. M. Weaver; 1 Biomechanics, University of Pennsylvania, Philadelphia, PA, 2Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, PA, 3Immunology, Wistar Institute, Philadelphia, PA, 4Genetics, University of Pennsylvania, Philadelphia, PA

Altered epithelial-mesenchymal interactions associated with profound changes in the tissue microenvironment, and although inducing a reactive stroma in tissue can enhance malignant transformation, the mechanism is poorly understood. We are using MMTV-Her2/neu mice and organotypic mammary epithelial cell (MEC) three-dimensional tissue models to study how alterations in the physical properties of the extracellular matrix (ECM) could modulate mammary tumorigenesis. Using compression and shear analysis, we show that malignant transformation is preceded by and associated with a progressive stiffening of the tissue. Biochemical, immunological, and polarization microscopy analysis indicate that gland stiffness, altered tissue morphology, and tumor invasiveness are functionally linked to increased collagen deposition, bundling, and crosslinking. We examined the relevance and molecular mechanisms whereby ECM stiffness might influence normal breast epithelia and breast tumor behavior. Studies combining MECs expressing an inducible homodimerizing ErbB2 construct (p75BB) with ribose-induced crosslinked collagen gels demonstrated that ECM stiffness can cooperate with oncogene activation to drive MEC transformation and invasion. Results indicate that ECM stiffness perturbs tissue morphogenesis by altering polarity, disrupting cell-cell interactions, and increasing growth and proliferation. Moreover, we show that matrix stiffness enhances epidermal growth factor (EGF) receptor-dependent PI3 kinase activation and PI3K-activated mTOR pathway activation in mammary epithelial cell lines and mouse mammary tissue sections. We are currently investigating whether matrix stiffness promotes tumor progression by altering EGF signaling through PI3 kinase and if so how. (Supp: NIH T32HL007954 to KRJ; DOD W81XWH-05-1-0330 and NIH CA078731 to VMW)

Nuclear Import, Architecture, and Tumor Suppression

L. C. Trotman, 1,2 A. Alimonti, 1,2 X. Wang, 1 Z. Chen, 1,2 P. P. Scaglioni, 1,4 J. Teruya-Feldstein, 3 S. Chi, 1 H. Kim, 1 H. Yang, 1 N. P. Pavletich, 3 B. S. Carver, 10 M. A. Keaton, D. J. Lew; Duke University, Durham, NC

The proto-oncogene AKT (PKB) is activated in many human cancers, mostly due to loss of the tumor-suppressor, a membrane lipid phosphatase, which ranks among the most frequently mutated genes in human cancer. In such tumors, AKT, which normally is both nuclear and cytoplasmic, becomes strongly enriched at cell membranes where it is activated by phosphorylation. Yet, although many targets of phospho-AKT are nuclear, it has remained unclear how relevant nuclear phospho-AKT (pAKT) function is for tumorigenesis. Here we show that the structural protein HDAC6 signaling and activation of β-catenin, all characteristic of breast tumor cells, contribute to increased transcription and translation of angiogenic factors. However, each of these pathways is attenuated in polarized and organized normal breast cells or can be reduced if tumor cells are forced to adopt a polarized, organized acinar structure. Thus, we hypothesized that correct polarity and 3-D tissue architecture, independent of changes in proliferation, was sufficient to regulate the angiogenic switch. Restoring expression of a key morphoregulatory transcriptional mediator, HoxD10 which is lost in aggressive tumors, reverted tumor cells to an polarized phenotype. This normalized phenotype was accompanied by a reduction in VEGF expression and a concomitant increase in ANG-1, a vascular stabilizing gene. Co-cultures of reverted polarized breast epithelial cells had a significantly reduced ability to induce endothelial cell migration as compared to disorganized tumor cells. We also observed that attenuation of the angiogenic switch by HoxD10 requires interactions with a three-dimensional laminin rich basement membrane. Introduction of signaling defective S48 integrin mutants impaired the ability of HoxD10 to induce polarity, and attenuate the angiogenic switch and consequently enhanced endothelial cell branching and migration.

Bromage, 11 P. Tempst, 11 C. Cordon-Cardo, 2 T. Misteli, 12 X. Jiang, 3 P. P. Pandolfi 1,2; 1Cancer Biology & Genetics, Sloan Kettering Institute, New York, NY, 2Department of Pathology, Sloan Kettering Institute, New York, NY, 3Department of Medicine, Sloan Kettering Institute, New York, NY, 4Department of Urology, Sloan Kettering Institute, New York, NY, 5Departments of Medicine, Radiology and Medical Physics, Sloan Kettering Institute, New York, NY, 6Molecular Biology Program, Sloan Kettering Institute, New York, NY, 7National Cancer Institute, National Institutes of Health, Bethesda, MD

The progression from pre-malignant to invasive breast cancers is dependent upon generating a new vascular supply. A variety of changes in the breast tumor environment have been linked to increased production/release of angiogenic factors with the tumor cells themselves playing a central role in generation of these mediators including VEGF. Increased MAPK, PI3K, altered integrin α6β4 signaling and activation of β-catenin, all characteristic of breast tumor cells, contribute to increased transcription and translation of angiogenic factors. However, each of these pathways is attenuated in polarized and organized normal breast cells or can be reduced if tumor cells are forced to adopt a polarized, organized acinar structure. Thus, we hypothesized that correct polarity and 3-D tissue architecture, independent of changes in proliferation, was sufficient to regulate the angiogenic switch. Restoring expression of a key morphoregulatory transcriptional mediator, HoxD10 which is lost in aggressive tumors, reverted tumor cells to an polarized phenotype. This normalized phenotype was accompanied by a reduction in VEGF expression and a concomitant increase in ANG-1, a vascular stabilizing gene. Co-cultures of reverted polarized breast epithelial cells had a significantly reduced ability to induce endothelial cell migration as compared to disorganized tumor cells. We also observed that attenuation of the angiogenic switch by HoxD10 requires interactions with a three-dimensional laminin rich basement membrane. Introduction of signaling defective S48 integrin mutants impaired the ability of HoxD10 to induce polarity, and attenuate the angiogenic switch and consequently enhanced endothelial cell branching and migration.

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Blocking Inhibitory Phosphorylation of Cdk1 Prolongs the Reversibility of Mitotic Exit in Vertebrate Cells

T. A. Potapova, J. R. Daum, G. J. Gorbsky; Molecular, Cell and Developmental Biology, OMRF, Oklahoma City, OK

Cyclin B proteolysis is hypothesized to ensure one-way directionality of mitotic exit. In support of this hypothesis, we previously demonstrated that mitotic exit induced by Cdk1 inhibitors can be reversed if the proteolysis cyclin B is abrogated by blocking proteasome activity or by expressing non-degradable cyclin B (Potapova et al., Nature 2006 Apr 13;440(7086):954-8). However, the ability of cells to return to mitosis decreased as they were maintained in G1 for long periods of time. This result suggested that an additional mechanism was preventing reactivation of Cdk1 during later G1. This second block was not dependent on protein synthesis hence was not due to production of small protein inhibitors. Using phospho-specific antibodies, we found that Cdk1 activity was inhibited by inhibitory phosphorylation on T14 and Y15. Interfering with these phosphorylations prolonged reversibility for hours after cells entered G1. As with the reversals of early G1 cells, cells reverses from later G1 re-condensed their chromosomes, disassembled the nuclear envelopes, formed mitotic spindles and re-aligned chromosomes at metaphase plates. Reversal of late G1 cells often led to the appearance of additional spindle poles. In some cases, cells failed to reverse cytokinesis, and daughter cells often built a complete bipolar spindle with chromosomes aligned in the middle, forming what we termed “minicells”. When cyclin B was preserved by proteasome inhibition, cells reverse even after 3 hours in G1. Cells expressing non-degradable cyclin B1 also showed an extended period of reversibility (up to 90 min) in the absence of inhibitory Cdk1 phosphorylation. At longer times (2 hours or more), such cells briefly re-entered mitosis but then exhibited morphological changes consistent with apoptosis. We hypothesize that apoptosis is a cellular response to reactivation of Cdk1 in the absence of other mitotic regulators that were degraded during the M phase to G1 transition.

Examining How Polo-like Kinases Regulate Metaphase Spindle Assembly and Maintenance Using Cell-permeable Chemical Inhibitors

U. Peters, J. H. Ken, J. Cherian, B. H. Kwok, T. M. Kapoor; Laboratory of Chemistry and Cell Biology, Rockefeller University, New York, NY

The stable propagation of genomes requires proper cell division, a very dynamic process that takes minutes, with several individual steps taking seconds. Cell permeable small molecules that target tubulin and can act on fast time-scales have been powerful tools to examine the functions of this cytoskeletal protein during cell division. Many proteins, including kinases, are now known that regulate spatial and temporal aspects of tubulin polymerization. To find small molecule probes for these proteins we carried out a phenotype-based screen with a small collection of 100 diaminoquinolines (DQs). We identified compounds that induce changes in spindle geometry, chromosome polemitotic index. Monopolar mitotic spindles were induced by 4 inhibitors including one, named DAP-81, that targets Polo-like kinases (Plks), evolutionarily conserved serine/threonine kinases, in vitro. Inhibition of Plks in DAP-81 treated cells was confirmed by comparing PI3K-RNAi phenotypes and by measuring the levels of Cdc25c-phosphorylation (S198), a known Plk inhibitor, BTO-1. High-resolution multi-mode live-cell microscopy of mammalian cells revealed that Polo-like kinases are required for the assembly and maintenance of bipolar mitotic spindles. Polo-like kinase inhibition can destabilize chromosome-associated microtubules while stabilizing and bundling astral microtubules, leading to monopolar mitotic spindles. Measurements of the tension across kinetochores in collapsing bipolar spindles further indicate that spindle pulling forces are still present despite the observed changes in microtubule dynamics. These data provide insight into the spatially-specific regulation of microtubule dynamics by Polo-like kinases during cell division.

Bub1 Is Essential for Assembly of the Inner Centromere

Y. Boyarchuk, A. Salic, M. Dasso, A. Arnautov; SCCR/PRCM, NICHD/NIH, Bethesda, MD, 2Department of Cell Biology, Harvard Medical School, Boston, MA

During mitosis the inner centromere region recruits protein complexes that regulate sister chromatids cohesion, monitor tension and modulate microtubules attachment. Biochemical pathways that govern formation of the inner centromere remain elusive. The kinetochore protein Bub1 was shown to promote assembly of the outer kinetochore components such as BubR1, Mad2, CENP-F and CENP-E on centromeres. Here we show that Bub1 is also essential for formation of a functional inner centromere. Depletion of Bub1 from Xenopus egg extract or from HeLa cells resulted in displacement of chromosome passenger complex and MCAK from the inner centromere region. Moreover, Bub1 controls the binding of Shugoshin to chromatin, while the chromosome passenger complex directs loading of Shugoshin specifically onto centromere. We further show that Bub1 kinase activity is pivotal for recruitment of all of these components. Taken together, our findings demonstrate that Bub1 acts at multiple points to assure the correct kinetochore formation.

Cell Cycle Regulation by Cyclin-Cdkks and Proteolysis


We are investigating the role of ubiquitin-mediated proteolysis in mitotic entry and exit, and in coordinating mitosis with DNA replication. In particular we are trying to understand how cells degrade the right protein at the right time to ensure that their two daughter cells receive an identical copy the genome in mitosis. This is clearly essential to genomic stability and defects in the checkpoints controlling chromosome segregation may contribute to tumorigenesis. Key to the control of chromosome segregation is the regulation of the Anaphase Promoting Complex/Cyclosome (APC/C) ubiquitin ligation by the spindle assembly checkpoint. We have developed a live cell assay to monitor APC/C activity and its inhibition by the spindle checkpoint. This has revealed that the checkpoint is intrinsic to the timing of mitosis and underlies the difference in the timing of cyclin A, cyclin B and securin degradation. We have found that the APC/C is recruited to unattached kinetochores by the checkpoint proteins that may explain the tight temporal control on the APC/C by the checkpoint. We are testing the idea that this temporal control requires that the checkpoint and ubiquitination machinaries interact at the spindle. Using our time-lapse fluorescence microscopy as a real time assay for proteolysis we are trying to elucidate how different proteins are selected for destruction at different times in throughout mitosis.

Aurora B Kinase Is Activated by Microtubules and TD-60

S. E. Rosasco, P. Stukenberg; Biochemistry and Molecular Genetics, University of Virginia, Charlottesville, VA

Aurora B kinase regulates microtubule-based processes of mitosis including the attachment of kinetochores to microtubules, and the positioning of the cytoskeletal furrow. Aurora B activity requires interaction with the chromosome passenger INCENP, and phosphorylation of Ser850 on INCENP further stimulates kinase activity. Aurora B and INCENP also interact with Survivin and Borealin/Dasa, which are important for centromere cohesion in prometaphase and metaphase and midzone localization during anaphase. TD-60 is putative guanine nucleotide exchange factor that regulates the localization of chromosome passenger complexes to the centromere (Mollinari et al., 2003). A select population of TD-60 interacts with the Aurora B complex. Chromosome passengers fail to localize to centromeres after addition of anti-TD-60 antibodies to Xenopus extracts, which recapitulates the siRNA phenotype (Mollinari et al., 2003). Partial depletion of Xenopus extracts separates two functions of TD-60: INCENP and Aurora B localize to centromeres, but INCENP is not phosphorylated on Ser850. Thus TD-60 regulates both the localization and activity of chromosome passengers in vivo. This regulation is critical because extracts partially depleted of TD-60 are unable to congress chromosomes to the metaphase plate. In vitro, microtubules stimulate purified chromosome passenger kinase activity 3-5 fold, while microtubules and TD60 stimulate over 100 fold. These data suggest that TD-60 localizes passenger proteins to the centromere and then coordinates with INCENP to regulate Aurora B kinase activity. Reference List Mollinari,C., Reynaud,C., Martineau-Thuilier,S., Monier,S., Kieffer,S., Garin,J., Andreassent,F.R., Boulet,A., Goad,B., Kleinman,J.P., Margolis,R.L. (2003). The mammalian passenger protein TD-60 is an RCC1 family member with an essential role in prometaphase to metaphase progression. Dev.Cell 5, 295-307.
Fibronectin Regulates Mammary Epithelial Cell Growth during Acinar Morphogenesis

C. M. Williams, J. E. Schwarzbrauer; Department of Molecular Biology, Princeton University, Princeton, NJ

The mammary gland consists of a series of branching ducts, ending in hollow, sphere-like acini surrounded by a basement membrane. Breast cancer is accompanied by loss of this tissue organization and dynamic changes in the extracellular matrix, including the appearance of fibronectin in the tumor stroma. The correlation between a fibronectin-rich stroma and aberrant proliferation may indicate a role for fibronectin signaling in breast oncogenesis. Culture of MCF-10A mammary epithelial cells (MECs) on a Matrigel basement membrane recapitulates in vivo acinar morphology. We have found that fibronectin in this culture system either promotes or impedes the differentiation of acini depending upon protein levels. Endogenous expression of fibronectin is crucial for MCF-10A cells to execute the differentiation program. Knock-down of endogenous fibronectin using small interfering-RNAs resulted in smaller acini containing fewer polarized cells and delayed lumina formation compared to acini formed by mock-treated MECs. Long-term culture of MECs in the presence of excess exogenous fibronectin also yielded aberrant acini as characterized by larger size, continued cell proliferation, reduced cell polarity, and lack of a hollow lumen. These data indicate that a certain basal level of fibronectin is required for optimal cell growth and differentiation during acinar development in vitro. When acini were allowed to complete the differentiation program before exposure to excess exogenous fibronectin, the addition of fibronectin was sufficient to reverse the established growth arrest and stimulate re-entry into the cell cycle. Thus, a fibronectin-rich stroma provides extracellular signals that are important for cell growth and contact differentiation from the basement membrane resulting in defective acinar morphologies. These findings suggest that increased fibronectin, as in the stroma of breast tumors, may cause loss of acinar organization by stimulating MEC proliferation.

Rac1, Matrix Force, and Tissue Architecture: The Oxidative Effect

M. Nuth, J. C. Friedland, J. N. Lakin, J. Chenrot, A. R. Kennedy, V. M. Weaver; 1School of Medicine, University of Pennsylvania, Philadelphia, PA, 2Fox Chase Cancer Center, Philadelphia, PA, 3Radiation Oncology, University of Pennsylvania, Philadelphia, PA

Tissue differentiation and homeostasis are associated with tissue organization that is regulated by cell-integrin-extracellular matrix (ECM)-dependent Rac activation. Malignant transformation is characterized by perturbations in tissue architecture and is associated with acquisition of cell motility, invasion, increased growth and survival, and aberrant cell-ECM interactions and Rac activity. We showed that transformed tissue is significantly stiffer than normal tissue, and that force disrupts tissue morphogenesis and increases cell growth and invasion (Paszek et al., Cancer Cell 2005). Here we report that matrix stiffness significantly modifies the localization and basal activity of Rac and alters Rac localization to permit Rac-dependent cell growth and invasion through NADPH oxidase-dependent induction of reactive oxygen species (ROS). We found that mammary epithelial cells (MECs) interacting with a compliant reconstituted basement membrane (rBM) assemble polarized, growth arrested tissue-like structures with high specific activity of Rac and Pak that co-localize and are spatially-restricted to the basal domain of the acini. In contrast, MECs interacting with a stiff rBM form highly disorganized, disoriented structures that fail to growth-arrest or polarize and display randomly distributed total and active Rac. Intriguingly, polarized acini expressing a V12Rac adenovirus lose polarity and begin to grow and invade once their surrounding ECM is stiffened appreciably, yet they are able to retain their differentiated, polarized phenotype if they are simultaneously treated with the NADPH oxidase inhibitor diphenyleneiodonium or infected with a Rac mutant that is incapable of activating ROS via NADPH oxidase. Accordingly, we are now employing an inducible GST-wild type and V12Rac and proteins to explore the possibility that matrix tension promotes expression of the malignant epithelial tissue phenotype in part by permitting Rac-dependent activation of NADPH oxidase. In addition, we are assessing the relevance of elevated force-dependent ROS on tissue and cell behavior.

Endocytic Components Are Coupled to Adhesion Signaling

D. P. LaLonde, J. Pignatelli, M. Grubinger, C. E. Turner; Department of Cell/Developmental Biology, SUNY Upstate Medical University, Syracuse, NY

Integrin ligation with an extracellular matrix (ECM) regulates cell spreading, motility and focal adhesion dynamics. Perturbation of these signaling pathways contributes to abnormal growth and tumor metastasis. Recent reports have elucidated a role for caveola-dependent endocytosis in integrin signaling, while a role for clathrin-dependent endocytosis has been implied but is not well tested. Herein, we show that β2-adaptin, a component of clathrin coated pits, is enriched in focal adhesions during cell spreading on an ECM. Depletion of β2-adaptin or clathrin heavy chain using RNAi knockdown results in increased cell spreading, enhanced membrane ruffling and the generation of new adhesion structures resembling podosomes or invadopodia that are hallmarks of Src-transformed and invasive tumor cells. These structures are functional, as they actively degrade the underlying matrix and result from upregulated Src activity. Our results suggest a previously unappreciated role for endocytic proteins in tumor cell metastasis via regulation of adhesion signaling. Supported by NIH GM57407 & HL070244

Another β1 Tail-binding Factor Cooperates with the PTB-like Domain of Talin to Activate β1 Integrins

M. Bouaouina, Y. Lai, D. A. Calderwood, Department of Pharmacology, Yale University School of Medicine, New Haven, CT

The activation of integrin adhesion receptors from low to high affinity in response to intracellular cues controls cell adhesion, migration and signaling. Binding of the cytoskeletal protein talin via its PTB-like domain to the β3 integrin cytoplasmic tail is necessary and sufficient for β3 activation. The talin-binding motif is conserved between integrin β tails, talin binds most integrin β subunits, and talin is required for β1 integrin activation. It has therefore been assumed that talin activates β1 and β3 integrins in a similar fashion. Here we report that, while the conserved talin PTB domain-integrin interaction is required for β1 activation, over-expressed integrin-binding fragments of talin do not activate β1 integrins. Furthermore, while over-expressed free β1 tails trans-dominantly inhibit β3 integrins by sequestering talin, free β1 tails defective in talin-binding can still suppress β1 integrin activation. These data point to an additional β1-binding factor that cooperates with talin during β1 activation. Mutagenesis of the β1 tail has identified residues that lie outside the talin-binding site which are required for β1 activation, and are likely to be involved in the binding of this second β1-specific activating factor. Free β1 tails mutated at this site and also defective in talin binding are unable to trans-dominantly suppress β1 integrin activation. We propose that the requirement for a second β1-specific activating factor provides a mechanism to selectively regulate β1 integrins.

FAK Modulates Cell Adhesion Strengthening via Two Distinct Mechanisms: Integrin Activation and Vinculin Recruitment

K. E. Michael, S. K. Hanks, A. J. Garcia; 1Petit Institute for Bioengineering and Bioscience, Georgia Institute of Technology, Atlanta, GA, 2Department of Cell and Developmental Biology, Vanderbilt University School of Medicine, Nashville, TN

Integrin-mediated cell adhesion to extracellular matrices (ECM) plays critical roles in tissue development/repair as well as pathological conditions. Matrix-bound integrins associate with the cytoskeleton and cluster to form focal adhesions (FA), which link the cytoskeleton to ECM. Integrin-ECM bonds provide the underlying structure for adhesion strength, and FA assembly further enhances adhesion strength by distributing forces among cytoskeletal elements. Focal adhesion kinase (FAK) is an essential non-receptor tyrosine kinase that regulates FA turnover and migration and is commonly overexpressed in tumors. Using FAK−/− cells engineered to re-express FAK under a tetracycline-inducible promoter and a hydrodynamic adhesion strength assay, we show FAK-dependent differences in adhesion strengthening, both in strengthening rate and steady-state strength. Compared to FAK−/− cells, FAK re-expression significantly increased strengthening rate (3 fold). The binding rate of alpha5beta1 integrin to fibronectin was also elevated (3 fold) in the presence of FAK; however, no differences in steady-state integrin binding levels were detected. Differences in strengthening rate were dependent on alpha5beta1 binding to fibronectin, as demonstrated by antibody blocking. Although FAK−/− and FAK re-expressing cells displayed equal surface densities of alpha5beta1, FAK re-expressing cells exhibited higher levels of activated integrins, as indicated by activation-specific antibodies. In contrast, steady-state adhesion strength was reduced in the presence of FAK (30%). Steady-state differences in adhesion strength were associated with differences in recruitment of vinculin, but not talin, to the adhesion area. Immunostaining showed less FA containing area occupied by vinculin (25%) for cells expressing FAK. These results indicate that FAK modulates cell adhesion strengthening via two distinct mechanisms: strengthening rate is regulated via integrin activation and binding, while vinculin recruitment modulates steady-state strength. This analysis provides insights into the regulatory role of FAK in adhesion interactions

Integrin of Functions as a Type I PKA Specific A-kinase Anchoring Protein

C. J. Lim, J. H. Nam, N. Youn, Y. Ma, P. S. Amieux, G. S. McKnight, S. S. Taylor, M. H. Ginsberg; 1Medicine, University of California San Diego, La Jolla, CA, 2Chemistry and Biochemistry, University of California San Diego, La Jolla, CA, 3Pharmacology, University of Washington, Seattle, WA

Localized activation of CAM-dependent protein kinase (PKA) at the leading edge of migrating cells phosphorylates protein substrates including the α4 integrin cytoplasmic domain. Phosphorylation of α4 (phospho-α4) prevents association with the signaling adaptor, paxillin, facilitating Rac activation and pseudopodium formation. The subcellular localization and substrate
specificity of PKA, a tetramer of two regulatory (R) and two catalytic (C) subunits, is governed by R subunit binding to A-kinase Anchoring Proteins (AKAPs). To investigate the mechanism by which spatial localization of α4 phosphorylation is achieved, we sought to identify the PKA isoforms and AKAPs involved. α4 and PKA-C were found in a complex. Using matrix immobilized α4 cytoplasmic tail protein in pull down assays, we identified PKA-R1, but not PKA-R2, in this protein complex. In vitro, PKA-α4 association was abrogated in PKA-R1-null mouse embryonic fibroblasts (MEF). To assess the possibility that α4 may function as an AKAP, direct binding between α4 cytoplasmic tail with purified PKA was assessed. α4 bound directly to Type I PKA holoenzyme, but not to the constituent PKA-R1 or PKA-C subunits. Furthermore, the α4-Type I PKA interaction was insensitive to AKAP-derived amphiphatic peptide disruptors, suggesting α4 as a novel and non-canonical form of AKAP for Type I PKA. In vivo, PKA-R1 co-localized with α4 at the prostatic membranes of cells undergoing polarized migration. Agonist stimulation of α4 phosphorylation was abolished in PKA-R1-null MEFs, suggesting tethering of Type I PKA to α4 is required for spatial restriction of PKA activity and α4 phosphorylation. Consistent with this idea, leading edge phospho-α4 in migrating cells was diminished when only Type I PKA is mislocalized by expression of mitochondrially targeted A-kinase binding peptides. Mistargeting of Type I PKA also resulted in significant inhibition of the velocity and directional persistence of α4-dependent cell migration. Thus, the α4 cytoplasmic domain is a novel, non-canonical, Type I specific AKAP.

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The Role of Two GT Pases in Co-translational Protein Targeting
S. Chandrasekar,1 P. Walter,2 S. Shan1; 1Division of Chemical Engineering and Chemical Engineering, California Institute of Technology, Pasadena, CA; 2Department of Biochemistry and Biophysics, University of California, San Francisco, San Francisco, CA

The signal recognition particle (SRP) and its receptor (SR) constitute the major cellular machinery that delivers nascent proteins to the eukaryotic ER membrane, or the bacterial plasma membrane. During this process, two GT Pases in SR and SR form a unique complex in which they reciprocally activate each other’s GTP hydrolysis activity. We have isolated different classes of mutant GT Pases that block the SRP-SR interaction at specific stages, indicating that the SRP-SR binding and activation involve a series of conformational changes. To assess the contribution of these steps to the protein targeting reaction, we tested the effect of various GTPase Pases in a co-translational targeting assay. A class of mutants that allows the assembly of a stable SRP-SR complex but specifically blocks the reciprocal activation of GTP hydrolysis severely blocks translocation, in contrast to previous results using GTP analogues that suggested that GTP hydrolysis is not required. Instead, our results suggest that although the hydrolysis reaction per se is not crucial, the molecular rearrangements that lead to reciprocal GT Pase activation provides essential drive force for protein targeting. The co-translational targeting assay also allowed us to probe the fidelity of protein targeting. Surprisingly, the translocation defect of preproteins with mutant signal sequences can be effectively rescued by reducing the rate of translation elongation. We propose that ongoing translation provides kinetic competition with the targeting process that can improve the fidelity of protein targeting. These results also raise the possibility that the spectrum of proteins delivered by the SRP could be quite dynamic and subject to regulation by changes in cellular environment.

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Regulation of ER-Golgi Trafficking and Golgi Biogenesis by Rab GTPase-activating Proteins
A. K. Haas, R. Kopajtic, E. Fuchs, S. Yoshimura, F. A. Barr; Max Planck Institute of Biochemistry, Martinsried, Germany

Rab GT Pases control vesicle movement and tethering membrane events at discrete membrane trafficking steps. A key determinant of Rab function is the lifetime of the activated GTP-state, and this is under the control of a family of GT Pase activating proteins (GAPs). Here we show that a subset of the 38 human Rab GAPs are important for controlling Rab function at the Golgi apparatus. To do this we have screened all predicted human Rab GAPs for the ability to change Golgi morphology and block trafficking from the ER to the Golgi. From this screen we were able to identify a novel Rab GAP domain-containing regulator of trafficking between the ER and Golgi. Overexpression of this protein but not a catalytically inactive mutant causes a complete dispersal and apparent loss of the Golgi apparatus. Structural proteins of the cis- and medial Golgi redistributed in a hazy throughout the cytoplasm, whereas Golgi localized enzymes accumulated in the ER. Surprisingly, this does not block the formation of COPII vesicles. Under these conditions COPII vesicles undergo a non-productive cycle of formation and recruitment of the Golgi matrix protein GM130, then collapse back into the ER. Finally, a biochemical analysis showed that this protein is the GAP for Rab1 and Rab2. Together, these findings highlight the importance of Rab GAPs as specific regulators of membrane trafficking. They also suggest that Rab1 and Rab2-mediated tethering of COPII vesicles is coupled to the recruitment of Golgi matrix proteins, and that this is required for the maintenance of a stable Golgi structure.

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The Coiled-coil Domain of Sec2p Activates the Rab GTPase Sec4p
G. Dong, M. Medkova, P. Novick, K. M. Reinesch; Department of Cell Biology, Yale University School of Medicine, New Haven, CT

The Rab GT Pases, with regulatory roles in multiple stages of intracellular transport, are the largest subgroup in the Ras-like GT Pase superfamily. Like all small GT Pases, the Rab proteins interact with signaling partners in their GTP bound form but not in their GDP bound form. The two forms differ in the switch regions, I and II, which surround the nucleotide binding site. GT Pases are activated by guanine exchange factors (GEFs) that catalyze the exchange of GDP for GTP. GEFs are classified into different families, which act on different groups of GT Pases. GEFs for all almost all subfamilies of the Ras-like GT Pases have been studied extensively, except for the GEFs that act on the Rab GT Pases. Here we present the crystal structure of the GEF domain of Sec2p in complex with its GT Pase partner Sec4p, a key regulator in polarized exocytosis in yeast. As a 220 Å long coiled-coil, the Sec2p GEF domain represents a new class of GEF and also a novel use of the coiled-coil motif as a GEF. Sec2p facilitates exchange by inducing extensive structural reorganizations in the Sec4p switch regions and the phosphate-binding loop (P-loop) that are incompatible with nucleotide binding. GDP exchange assays demonstrate that Sec2p has nucleotide exchange activity for Sec4p but not for other Rab GT Pases, including Rab1a, Rab2, Rab4, Rab6, Ypt1p, and Ypt32p. The structure suggests that the determinants for Sec2p specificity reside in the switch regions and the P-loop. We have confirmed the importance of switch I by constructing Sec4p and Ypt1p chimeras in which the switch I regions were interchanged. Switch II and the P-loop are nearly identical for the two proteins. Only Ypt1p with a Sec4p switch I was activated by Sec2p.

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Phosphorylation of RhoGDI by Src Regulates Rho GT Pase Binding and Cytosol-Membrane Cycling
C. DerMardirianoss, G. Rocklin, J. Seo, G. Bokoch; Immunology, The Scripps Research Institute, La Jolla, CA

Rho GT Pases (Rac, Rho, and Cdc42) play important roles in regulating cell function through their ability to coordinate the actin cytoskeleton, modulate the formation of signaling reactive oxygen species, and to control gene transcription. Activation of Rho GT Pase signaling pathways requires the regulated release of Rho GT Pases from RhoGDI complexes, followed by their reuptake after membrane cycling. We show here that Src kinase and phosphatases promote RhoGDI both in vitro and in vivo at Tyr156. Analysis of Rho GT Pase-RhoGDI complexes using in vitro assays of complexation and in vivo by co-immunoprecipitation analysis indicates that Src-mediated phosphorylation of Tyr156 causes a dramatic decrease in the ability of RhoGDI to form a complex with RhoA, Rac1, or Cdc42. Phosphomimetic mutation of Tyr156–Glu results in the constitutive association of RhoGDIY156E with the plasma membrane and/or associated cortical actin. Substantial cortical localization of tyrosine-phosphorylated RhoGDI is also observed in fibroblasts expressing active Src, where it is most evident in podosomes and regions of membrane ruffling. Expression of membrane-localized RhoGDIY156E mutant is associated with enhanced cell spreading and membrane ruffling. These results suggest that Src-mediated RhoGDI phosphorylation is a novel physiological mechanism for regulating Rho GT Pase cytosol-membrane cycling and activity.

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A Septin GT Pase Required for Spatial Control of Microtubule Organization and Efficient Vesicle Transport
E. T. Spilotis,1 S. J. Hunt,2 M. Kinoshita,3 W. J. Nelson; 1Biological Sciences, Stanford University, Stanford, CA; 2Biochemistry and Cell Biology, HMRO, Kyoto University, Kyoto, Japan

Septins are conserved GT Pases that form filamentous structures that associate with cellular membranes and the cytoskeleton. Yeast septins are required for polarized membrane growth, but the function of mammalian septins counterparts is unknown. Here, we show that in non-polarized Madin-Darby canine kidney (MDCK) cells, Septin 2 (SEPT2) filaments are found at sites of vesicle export from the trans-Golgi network. At these sites SEPT2 associates with a distinct subset of microtubule tracks which are recognized by a monoclonal antibody (B3) to polyglutamylated motifs of α-tubulin. Lack of SEPT2 colocalization with acetylated microtubules revealed that SEPT2 is spatially restricted to the polyglutamylated subset of stable microtubules. In quantitative membrane trafficking assays, microinjection of SEPT2 antibodies resulted in the down-regulation of Golgi-to-plasma membrane vesicle transport of apical (p75-GFP) and basal-lateral (VSVG-GFP) marker proteins. Similarly, in polarizing MDCK cells, SEPT2 knock-down by RNAi resulted in the intracellular accumulation of p75-GFP and VSVG-GFP. This phenotype was accompanied by failure of the microtubule cytoskeleton to reorganize along the apico-basal axis of polarity. In non-polarized MDCK cells, disruption of SEPT2 association with microtubules by SEPT2-depletion or by overexpression of MAP4-GFP, a cytosolic binding partner of microtubule-free SEPT2, resulted in the loss of polyglutamylated microtubules. Significantly, MAP4-GFP overexpression phenocopied SEPT2 in the down-regulation of Golgi-to-plasma membrane vesicle transport. This effect was quantitatively dependent on the levels of MAP4-GFP.
expression and accompanied by loss of the microtubule-bound SEPT2 filaments. These data suggest that microtubule-bound SEPT2 acts as a scaffold for the polyglutamylation of microtubule tracks, which in turn, as previously suggested by others, may negatively modulate the binding of microtubule-associated proteins (“speed bumps”), while favoring the accumulation of kinesin motor proteins. In conclusion, our data establish septin GTPases as spatial landmarks for the organization of distinct microtubule tracks and reveal a novel requirement for the efficiency of intracellular membrane transport.

715 Spatial And Kinetic Regulation of the Exocyst for Polarized Exocytosis: A Tale of Two Rh0 GTPases
X. Zhang, B. He, J. Zhang, A. Zajac, W. Guo, Biology Department, University of Pennsylvania, Philadelphia, PA

The exocyst is an octameric protein complex composed of Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84. The exocyst is localized to specific domains of the plasma membrane, where it tethers the post-Golgi secretory vesicles preceding membrane fusion. How the exocyst is targeted to sites of secretion and how it functions in polarized exocytosis are unclear. We have found that the GTP-bound Cdc42 directly interacts with the N-terminals of Sec3 and further identified key residues on Sec3 that mediate this interaction. In addition, we have identified a region adjacent to the Rho-binding domain (RBD) in Sec3 that interacts with phospholipids and is responsible for Sec3 targeting to the plasma membrane. Besides Sec3, the exocyst component Exo70 interacts with another Rho protein, Rho3, in its GTP-bound form. Sec3 and Exo70 function in parallel for vesicle tethering to specific domains of the plasma membrane. We have designed a genetic assay, in which the functional implications of Rho-binding to either Sec3 or Exo70 can be assessed. Using this assay, we found that disruption of Sec3-Cdc42 interaction not only blocked exocytosis, but also caused severe cell polarity defects. On the other hand, disruption of Exo70-Rho3 interaction did not cause any polarity defect but kinetically affected secretion. Our studies not only shed light on the molecular basis of vesicle targeting to the plasma membrane, but also revealed the important but different roles Rh0 GTPases play in exocytosis and cell polarity.

716 Yeast Kinesin-8 Is a Highly Processive Motor That Depolymerizes Microtubules in a Length-dependent Manner
V. Varga, J. Helenius, K. Tanaka, A. A. Hyman, T. U. Tanaka, J. Howard, Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany, School of Life Science, University of Dundee, Wellcome Trust Biocentre, Dundee, United Kingdom

In eukaryotic cells, the dynamics of microtubules are tightly regulated. Numerous in vivo studies indicate that one of the factors responsible for destabilization of microtubules are motor proteins from the kinesin-8 family. Here we demonstrate for the first time that Saccharomyces cerevisiae kinesin-8, Kip3p, indeed acts like a depolymerase in vitro. Furthermore, our TIRF based microscopy assay revealed that Kip3p has several novel properties that clearly distinguish it from other microtubule destabilizing kinesins, such as MCAK from the kinesin-13 family. First, Kip3p disassembles microtubules exclusively at the plus end, unlike MCAK, which depolymerizes microtubules from both ends. Second, Kip3p targets the plus end by directed ATP-dependent movement, unlike MCAK, which transiently diffuses along the microtubule lattice. And third, Kip3p is extremely processive, indeed it is the most processive cytoskeletal motor characterized to date. As a consequence of this high processivity, the rate of depolymerization of microtubules by Kip3p is length-dependent. This length-dependent depolymerization provides a new mechanism for controlling the length of subcellular structures that can account for effects of Kip3p mutations on microtubule and spindle length seen in yeast and metazoan cells.

717 Structural Features of the Microtubule Important for MCAK Activity
K. M. Hertz, C. E. Walczak; Medical Sciences Program, Indiana University, Bloomington, Bloomington, IN

MCAK is a Kinesin-13 member that depolymerizes microtubules (MTs) and regulates MT dynamics. Previous studies showed that MCAK binds specifically to the MT end to induce depolymerization, but it is unknown what structural features of the MT are necessary for end specific recognition by MCAK. We used subtilisin treated MTs (MTs that are lacking the C-termini of α- and β-tubulin) as well as a variety of alternative tubulin substrates to study which structural features of the MT are required by MCAK to bind to and depolymerize MTs. We found that the removal of only the β-tubulin C-terminus led to a 2-fold decrease in depolymerization efficiency, whereas the removal of both α- and β-tubulin C-termini decreased depolymerization efficiency by 3-fold. However, removal of the C-terminus of either β-tubulin alone or of both α- and β-tubulin did not significantly affect the ability of MCAK to bind to the MT, nor did C-terminal removal alter MCAK localization to the ends of the MT. Our results show that the ability of MCAK to depolymerize subtilisin MTs (SMTs) is reduced, but not totally inhibited. We also employed alternative tubulin substrates to study how MCAK recognizes the end of the MT. We found that MCAK bound to a variety of curved tubulin substrates, but does not appear to depolymerize these polymers. In addition, curved tubulin substrates competed with the ability of MCAK to bind to the lattice and ends of unaltered MTs. Finally, we also found that the presence of tubulin polymers with increased lateral edges or curved tubulin substrates negatively affected the ability of MCAK to depolymerize unaltered MTs, indicating that lateral edges as well as curvature of the MT end may play important roles in MCAK induced depolymerization of MTs.

718 Nucleotide-induced Head Movement in Flagellar Dynae Revealed by Cryo-Electron Microscopy
T. Oda, N. Hirokawa, M. Kikka; Department of Cell Biology, University of Texas Southwestern Medical Center, Dallas, TX, Department of Cell Biology and Anatomy, Graduate School of Medicine, University of Tokyo, Tokyo, Japan

Outer dynein arms (ODAs) of the flagellar axoneme generate forces needed for flagellar beating. Our understanding of the force generating mechanism of ODA is limited by the absence of detailed structural information. Here, we have established a new method to reconstruct the three-dimensional structure of the in vitro reconstituted ODA-microtubule complex from cryo-electron microscopy images. Significant conformational changes of ODA were revealed from the ODA structures in two functionally critical states; the rigor (ATP-depleted) state and the relaxed (ATP-vanadate) state. From the rigor state to the relaxed state, the head domain of the β-heavy chain is displaced by 3.7 nm toward the B-tubule and inclined 44 degree inwards. These structural changes suggest a power "pull" model.

719 Huntington Participates in Dynactin/Dynactin-mediated Vesicle Trafficking
J. P. Caviston, J. L. Ross, S. M. Antony, M. K. Tokito, E. L. F. Holzbaur; Department of Physiology, University of Pennsylvania, Philadelphia, PA

Huntingtin (Ht) and dynactin are both present on vesicles purified from mouse brain and inhibitory antibodies to Ht cause these vesicles to dissociate from microtubules, suggesting that Ht facilitates dynein-mediated vesicle motility. Knockdown of Ht by RNAi in cultured cells results in Golgi disruption, similar to the effects of compromising dynactin/dynactin function. RNAi of dynactin results in a significant redistribution of Ht to the outer periphery of cells, suggesting that dynactin transports Ht-associated vesicles towards the cell center. Together, these findings indicate that Ht binds to dynactin and may act in a complex with dynactin/dynactin and HAP-1 to modulate vesicle association with microtubules and facilitate vesicular transport.

720 Eg5 and Dynein Act Antagonistically during Spindle Assembly in Mammalian Cells
N. P. Ference, P. Wadsworth, Biology, University of Massachusetts, Amherst, MA

During prophase, Eg5 has been functionally assigned to spindle pole separation, although its role in microtubule biogenesis at this stage has not been directly addressed. To do so, we used an LLC-PK1 cell line expressing photoactivatable GFP-tubulin. In control prophase cells, photoactivated marks move across a wide distribution of rates (0.5μm/min - 4.9μm/min) both toward (P)
and away from (AP) spindle poles. ~61% of all motion is P and ~39% is AP. In the presence of the Eg5 inhibitor monastrol, the distribution of rates is identical to controls, but there is a significant shift in directionality: ~14% of all motion is P and ~86% is AP. Although an actual decrease in P motion could create an artificial increase in AP motion (and vice versa), this does not appear to be the case as 20 control cells produced 62 P marks and 39 AP marks while 10 monastrol-treated cells produced 6 P marks and 36 AP marks, indicating a true shift in both P and AP motion in the absence of functional Eg5. Because our recent work has shown that ~1/3 of all control AP motion is dynen-dependent (specifically, the population moving between 2.25 m/min and 4.49 m/min) and because this particular motion is increased following Eg5 inhibition (13 control cells and 13 marks in 10 monastrol-treated cells), we postulated an antagonistic interplay between Eg5 and dynein. Such an interplay allows one to consider the hallmark of Eg5 inhibition (spindle collapse) not as a direct consequence of impaired Eg5 function, but as a shift in the balance of forces. To test this, we injected monastrol-treated monopolar spindles with p150-CC1 (a dynein inhibitor). In 50% of such cells, monopolar spindles bipolarize into normal, metaphase-looking spindles (n = 3 cells), within 30 min of injection, supporting our initial postulation.

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In Vivo Measurement of Molecular Motor Forces
G. T. Slabuha, S. P. Gross; Developmental and Cell Biology, University of California, Irvine, Irvine, CA

Molecular motor proteins actively transport organelles and other cell constituents along microtubules. In vitro studies have determined many of the motors’ basic functional properties. However, less is known about motor function and regulation inside living cells, in particular the number of motors moving a cargo, and how these motors work together. Recent work suggests that multiple motors move cargoes, but to date there has been limited direct evidence supporting this hypothesis. In addition, the magnitude and impact of cytosolic (viscous) drag on motor function is also controversial, with some studies proposing that cytosolic drag applies tremendous load on motor-driven cargoes. Because applied load decreases motor processivity, viscous drag could then play a crucial role in ending runs in a given direction. Here, we present direct measurements addressing these issues. We constructed a specialized optical trap, and measured the force that motors can exert while hauling lipid droplets along microtubules in Drosophila embryos. We report direct in vivo measurement of the forces that molecular motors exert in hauling individual endogenous cargoes, and provide direct evidence that multiple motors cooperate to move a cargo. We find that the number of motors moving the cargoes was relatively small (typically less than 3) and that the number of active motors did not depend on the size of the cargo. Moreover, because cargoes moved at velocities comparable to unloaded in vitro (up to 1 μm/s), and did not exhibit a significant correlation between stalling force and velocity, we conclude that the effect of cytosolic viscous drag is much smaller than 2.5 pN - the typical force driving lipid droplets.

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Coronin 1B Coordinates Arp2/3 and Cofilin Activity at the Leading Edge
J. E. Bear, 1 L. Cai, 2 T. W. Marshall, 1 A. C. Uetrecht, 1 D. A. Schafer; 1 Cell & Developmental Biology, UNC-Chapel Hill, Chapel Hill, NC, 2 Biology, University of Virginia, Charlottesville, VA

Arp2/3-dependent actin filament nucleation and Cofilin-driven filament turnover are two major factors that control the dynamics of lamellipodia in motile cells, but the coordination of these activities is poorly understood. Coronin 1B is highly conserved F-actin binding, WD repeat proteins that have been implicated in cell motility in model organisms. Yeast Coronin inhibits Arp2/3 complex in vitro, but the function of Coronins in mammalian cells is not known. We report that human Coronin 1B inhibits actin nucleation by the Arp2/3 complex and that this inhibition is regulated by the Slingshot-1L (SSH1L) phosphatase via the dephosphorylation of Serine 2 on Coronin 1B. Furthermore, we find that SSH1L, Coronin 1B and Arp2/3 exist in a ternary complex in vivo that is bridged by Coronin 1B. Functional studies of lamellipodial dynamics indicate that depletion of Coronin 1B alters lamellipodial protrusion and that Coronin 1B is required for enhanced ruffling at the cell periphery induced by expression of SSH1L. In addition to being a substrate for SSH1L, Coronin 1B is required for proper targeting of SSH1L to lamellipodia where it likely regulates filament turnover by activating Cofilin. Consistent with this idea, depletion of Coronin 1B increased cellular levels of phospho-Cofilin and expression of an activated Cofilin mutant partially suppressed the effects of Coronin 1B knockdown on lamellipodia dynamics. Together, our data suggest that Coronin 1B coordinates Arp2/3 and Cofilin activity at the leading edge.

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WHAMM is an Arp2/3-activating Protein That Interacts with Dynamic Endomembrane Compartments and Microtubules
K. G. Campellone, N. J. Webb, E. A. Znameroski, M. D. Welch; Molecular and Cell Biology, UC Berkeley, Berkeley, CA

The Arp2/3 complex, a critical actin nucleator that functions in a variety of essential cellular processes, is regulated by a family of proteins called nucleation-promoting factors (NPFs). Mammalian cells express several well-characterized NPFs including the WASPs (WASP and N-WASP) and the WAVEs (Scar/WAVE1-3), which control actin nucleation during distinct cellular functions including endocytosis and lamellipodial protrusion. Given the important roles of NPFs in modulating actin dynamics, we sought to identify additional family members, and have uncovered a novel class of mammalian NPFs comprised of two proteins: JMY and WHAMM (WHAMM Homologue associated with Actin Membranes and Microtubules). Each contains a C-terminal WCA segment conserved among other NPFs, a central region predicted to form coiled-coils, and an N-terminal domain of unknown function. Both full-length WHAMM and its isolated WCA region stimulate Arp2/3-mediated actin assembly in vitro, confirming it possesses NPF activity. In cultured fibroblasts, WHAMM localizes primarily to a perinuclear Golgi-related membrane compartment, and to peripheral tubulo-vesicular structures that can reside along microtubules, patterns not observed for other NPFs. Consistent with a role in membrane trafficking, cellular expression of GFP-WHAMM revealed that WHAMM-associated structures are highly dynamic and that WHAMM induces actin assembly in proximity to internal membranes. Moreover, cellular expression at high levels of GFP-WHAMM exhibit a dramatic disruption of their Golgi networks. Pharmacological agents that perturb membrane transport, microtubule dynamics, or actin polymerisation interfere with WHAMM localisation and/or movement of WHAMM-associated membranes, indicating that crosstalk between both cytoskeletal components and internal membranes is related to WHAMM function. Cellular expression of truncated WHAMM derivatives indicates that its C-terminal WCA segment is required for actin assembly, its N-terminal domain associates with perinuclear membranes, and its central coiled-coil region bundles microtubules. In summary, our studies suggest a role for WHAMM in coordinating membrane dynamics with actin- and tubulin-based cytoskeletal elements.

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P. K. Mattila, A. Pykäläinen, J. Saarikangas, V. O. Pasilainen, P. Lappalainen; University of Helsinki, Institute of Biotechnology, Helsinki, Finland

Regulators of actin dynamics, Missing-in-Metastasis (MIM) and IRSp53, share a similar domain structure to each other. Expression of full-length MIM/IRSp53 or their dimeric C-terminal IM/IRSp53/MIM-domain promotes filopodia/microspike formation in cultured mammalian cells. Filopodia formation was proposed to result from IM-domain’s F-actin bundling activity and its interactions with Rho family GTases. Here, we provide evidence that neither actin bundling nor GTase binding contributes to filopodia formation by MIM. Instead, our data shows that IM-domains bind P(4,5)P2 and cross-link P(4,5)P2-rich membranes. Consistently, IM-domain localizes to the interface between plasma membrane and F-actin bundles in filopodia. IM-domain of MIM is also capable of drastically deforming P(4,5)P2-rich membranes. Systematic metausgenesis analysis revealed that MIM’s IM-domain interacts with lipids through a similar mechanism to the structurally related BAR (Bin-Amphiphysin-Rvs) domains, and that its actin-binding sites are composed of large basic patches near the distal ends of the dimeric domain. Together, these data show that IM-domain is a distant, functional member of lipid-binding BAR domain family and may promote filopodia formation through a novel mechanism involving F-actin cross-linking to the plasma membrane.

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Rho Kinase Differentially Regulates Phosphorylation of Nonmuscle Myosin II Isotypes A and B during Cell Rounding and Migration
J. C. Sandquist, K. I. Swenson, K. A. DeMali, A. R. Means; 1 Department of Pharmacology and Cancer Biology, Duke University, Durham, NC, 2 Department of Cell and Developmental Biology and Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC

The actin-myosin cytoskeleton is generally accepted to produce the contractile forces necessary for cellular processes such as cell rounding and migration. All vertebrates examined to date are known to express at least two isoforms of non-muscle myosin II, referred to as myosin IIA and myosin IIB. Studies of myosin IIA and IIB in cultured cells and null mice suggest that these isoforms perform distinct functions. However, each myosin II isotype contributes individually to all the cellular functions attributed to “myosin II” has yet to be fully characterized. Using isoform specific small-interfering RNAs we found that depletion of either isoform resulted in opposing migration phenotypes, with myosin IIA- and IIB-depleted cells exhibiting higher and lower wound healing migration rates, respectively. In addition, myosin IIA-depleted cells demonstrated impaired thrombin-induced cell rounding and undertook a more motile morphology, exhibiting decreased stress fibers and focal adhesions, with concomitant increases in cellular protrusions. Cells depleted of myosin IIIB, however, were efficient in thrombin-induced cell rounding, displayed a more retractive phenotype and maintained focal adhesions, but only in the periphery. Lastly, we present evidence that Rho kinase preferentially regulates

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phosphorylation of the regulatory light chain associated with myosin IIA. Our data suggest that the myosin IIA and IIB isoforms are regulated by different signaling pathways to perform distinct cellular activities, and that myosin IIA is preferentially required for Rho-mediated contractile functions. J.C.S. is a Howard Hughes Medical Institute Predoctoral Fellow. K.A.D Present address: Department of Biochemistry, University of Iowa, Iowa City, IA 52242; USA.

726 Microtubule-mediated Lamellipodial Protrusion May Involve the Adenomous Polyposis Coli Protein R. Parker; Department of Molecular and Cellular Biology, HHMI/University of Arizona, Tucson, AZ

727 Distinct Roles of Rac Isoforms on the Actin Cytoskeleton A. J. Ridley, A. P. Wheeler, S. D. Smith, F. M. Vega, C. M. Wells; Ludwig Institute for Cancer Research, University College London, London, United Kingdom

728 MicroRNAs Controlling Developmental Neuronal D. S. Smith; Ludwig Institute for Cancer Research, University College London, London, United Kingdom

729 MicroRNA-9a Ensures the Precise Specification of Sensory Organ Precursors in Drosophila F. Wang, Y. Li, F. Gao; Neurological Disease, Gladstone Institute/UCSF, San Francisco, CA

730 Regulation of STAT3 by MicroRNAs during Embryonic Stem Cell Differentiation K. M. Foshay, G. Gallicano; Cell Biology, Georgetown University, Washington, DC

731 The Eukaryotic mRNA Cycle: Movement of Yeast mRNAs between Polysomes and P-bodies and Its Role in the Control of Translation and Degradation R. Parker; Department of Molecular and Cellular Biology, HHMI/University of Arizona, Tucson, AZ

Translational control is an important aspect of the regulation of gene expression in eukaryotic cells. In addition to the translating pool of mRNA associated with polysomes, recent experiments have defined cytoplasmic foci, referred to as P-bodies, wherein untranslated mRNAs accumulate and where mRNA decapping and decay can occur. Several lines of evidence indicate that yeast mRNAs within P-bodies can also return to translation. For example, reporter mRNAs exit translation and concentrate in P-bodies when translation initiation is blocked, and resume translation...
and exit P-bodies when translation is restored. These results define a cycle of mRNA movement wherein mRNAs travel from polysomes to P-bodies and then back to the translating pool. This mRNA cycle has several important properties and identifies three critical decision points in controlling the function of mRNAs: 1) the mechanism of targeting transcripts to P-bodies, 2) deciding the fate of transcripts within P-bodies, and 3) the mechanism by which transcripts return to translation. Importantly, proteins required for the accumulation of mRNAs in P-bodies also function in global translation repression in yeast, mRNA repression in metazoa, and in the regulation of translation in neurexins. This suggests that translation repression by sequestration into P-bodies is competitively balanced with translation in many biological contexts.

732 Analyzing the Function of Microtubule Localized RNAs during Mitosis
M. D. Blower,1 R. H. Head1; K. West2; M. M. Pettersen,2 1Molecular Biology, Massachusetts General Hospital, Boston, MA, 2Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA

Accurate segregation of chromosomes during mitosis and meiosis is required for organismal development and viability, and depends on the assembly and function of a dynamic mitotic spindle that orchestrates chromosome movements. Previous work has demonstrated that several proteins involved in RNA metabolism (Rae1, Maskin, and CPEB) are localized to the mitotic spindle and contribute to spindle assembly. Furthermore, we have shown that RNA is localized to mitotic microtubules in Xenopus egg extracts, and contributes to spindle assembly in a translation independent manner that may reflect a structural role. We have found that several different types of microtubule-associated RNA are present on the spindle including ribosomal RNA and messenger RNA (mt-mRNA). We have used Affymetrix gene chip microarrays to determine which mRNAs are specifically enriched on the mitotic spindle in both Xenopus and human cell extracts. We have found that a conserved set of mRNAs are localized to the microtubules during mitosis and that a subset of these mRNAs are actively translated on microtubules. Another set of mt-mRNAs appears to be present in inactive storage granules, and these mRNAs appear likely to regulate various aspects of development. While translation of RNA is not required for spindle assembly in egg extracts, our preliminary results suggest that local, mitotic translation of mt-mRNAs is likely to regulate various aspects of mitosis and cell cycle progression. We will present the results of experiments investigating how mt-mRNAs are targeted to the spindle, and the functional importance of spindle-localized protein translation. These experiments should provide insight into the role of mt-mRNAs during mitosis and development.

733 Dissecting the Links between Genes, Nuclear Periphery, and Post-transcriptional mRNP
J. Chekanova,1 K. Abruzzi,1 M. M. Rosbash,2 D. Belostotsky2,1 Biological Sciences, State University of New York at Albany, Albany, NY, 2School of Biological Sciences, University of Missouri—Kansas City, Kansas City, MO

Nuclear surveillance pathways ensure that eukaryotic mRNPs is properly processed and assembled into export-competent mRNPs prior to export to the cytoplasm. As a consequence, mRNA processing defects in mutant strains frequently lead to RNA retention within nuclei. Moreover, this often occurs in discrete nuclear foci detectable by FISH, i.e., mRNA-containing nuclear dots. Here we study this phenomenon in a wild-type strain background, using an integrated GAL reporter gene containing a cis-acting mutation that impairs 3′ end processing. We find that nuclear dots contain non-nascent mRNA that are tethered to, yet distinct from the site of their synthesis. Remarkably, the dot-genome tether is not strictly dependent on ongoing transcription.1 Furthermore, the dot-gene tether is related to another tether that links transcriptionally active GAL loci with the nuclear periphery. Surprisingly, this second tether is also independent of ongoing transcription. Additional experiments suggest that the dot and its post-transcriptional mRNP contribute to the tether that links the gene to the nuclear periphery. Finally, a genetic screen has identified candidate factors that collaborate with the dot mRNP and contribute to the gene-dot as well as the dot-pore tether.1 EMBO J., in press.

734 Promotion of Postsynaptic Differentiation by Neurexin Isoforms
Y. Kang,1 T. X. Zhang,1 F. A. Dobie,1 H. Wu,1 A. Craig2;1 Brain Research Centre, University of British Columbia, Vancouver, BC, Canada, 2Anatomy & Neurobiology, Washington University, St. Louis, MO

Development of synaptic connections between nerve cells is precisely regulated to control the number and location of different synapse types. The two main synapse types in the mammalian brain are excitatory glutamate and inhibitory GABA synapses. Neurexins and neurelinos are cell adhesion molecules found at both glutamate and GABA synapses. Both partners have synaptogenic activity: neurelinos presented to axons induce presynaptic vesicle clustering, while neurexins presented to dendrites induce clustering of neurotransmitter receptors and postsynaptic scaffolding molecules. There are multiple isoforms of both neurexins and neurelinos generated from multiple genes and by alternative splicing. α-neurexins contain multiple LNS domains whereas β-neurexins generated from a downstream promoter contain a single LNS domain. Here we report on the expression patterns, neurelin binding, and synaptogenic activity of different neurexin isoforms. Synaptogenic activity was measured as the ability of each neurexin isoform expressed on COS cells to induce clusters of postsynaptic proteins in contacting dendrites of cocultured hippocampal neurons. β-neurexins promoted clustering of both glutamate and GABA postsynaptic proteins. Insertion of alternatively spliced residues at splice site 4 of β-neurexins, in the LNS domain, selectively reduced its ability to cluster glutamate postsynaptic proteins. Insertion at site 4 also reduced binding to neurelin-1, which normally localizes to glutamate synapses, but not to neurelin-2, which normally localizes to GABA synapses. RT-PCR from hippocampal culture and developing rat brain showed a developmental increase in the fraction of neurexins lacking the S4 insert. Surprisingly, preliminary results in the coculture assay revealed even greater selectivity of α-neurexins for inducing clustering of GABA but not glutamate postsynaptic proteins. These results indicate selective roles for different neurexin isoforms at glutamate versus GABA synapses. Together with data from other labs, our results suggest that the neurexin-neurelin-synaptic complex may be as or more important for development of GABA synapses compared with glutamate synapses.

735 The "Filopodial Scaffolding" Hypothesis: Synaptogenesis in Drosophila Embryo Requires Corroboration of Both Filopodial Clustering and Postsynaptic Scaffolding Protein Dlg, F. Carrero-Martinez,1 M. Farrer,1 E. Suzuki,1 A. Chiba1;2 Department of Biology, University of Puerto Rico, Mayaguez, PR, 2Department of Cell and Developmental Biology, University of Illinois at Urbana-Champaign, Urbana, IL, 3National Institute of Genetics, Mishima, Shizuoka, Japan

Targeting cytosolic molecules to membrane is an extremely effective means to enhance (“activate”) their functions. Previously, due to limited access in embryos, the mechanisms that initiate a new synapse have rarely been analyzed in vivo. Here we dissect the process using uniquely identified synapses in Drosophila and offer a model that integrates both cellular and molecular components. Using GFP-labeling, we show that every neuromuscular synaptogenesis in live embryos is preceded by a transient stabilization/clustering of presynaptic and postsynaptic filopodia from matched partners upon their initial contact. Filopodial interactions between non-partner cells, though extensive and essential for proper axon guidance, do not lead to such a filopodial behavior. Overexpression of a dominant-negative Ezrin in targets eliminates this filopodial clustering and, thereby, blocks synaptogenesis. Immuno-SEM that separately labels presynaptic filopodia vs. postsynaptic filopodia reveals their extensive mutual interdigitiation as well as a narrow (50 nm) membrane-wrapped cytoplasmic space in individual filopodia. One of the first presynaptic cytosolic molecules that enter this filopodial cytoplasm is Dlg, a member of PSD-95 family scaffolding proteins. The special subcellular environment at the filopodial cluster makes Dlg practically “targeted” to the membrane where transmembrane adhesion/signaling molecules appose their counterparts on the presynaptic side, amplifying the probability of their molecular signaling and complexing. Previous work indicates a strong maternal effect that masks Dlg knockout phenotypes in embryos. By using a temperature-sensitive allele and a dominant-negative Dlg, we uncover the role of postsynaptically expressed Dlg during synaptogenesis. Although not essential for the formation of the filopodial cluster, Dlg is required to maintain the contact between the axon and target while nascent synapse progressively attains its functionality. We propose that synaptogenesis is a predictable outcome which follows from the filopodial interdigitation between a pair of synaptic partners that activates scaffolding proteins: the “filopodial scaffolding” hypothesis of synaptogenesis.

The Polarity Proteins PAR-6 and aPKC Are Required for Dendritic Spine Morphogenesis
H. Zhang, I. G. Macara; Center for Cell Signaling, University of Virginia, Charlottesville, VA

Dendritic spines are small protrusions that serve as primary sites of excitatory synaptic transmission in the CNS. Despite their importance in integrating information flow in the brain, the molecular mechanisms regulating spine morphogenesis are not well understood. Since spines are highly polarized structures, we hypothesize that proteins required for global cell polarity are also necessary for spine formation. We previously showed that the polarity protein PAR-3 regulates spine formation through spatially restricting the Rac guanine nucleotide exchange factor Tiam1. Interestingly, this process functions independently of PAR-3’s binding partners PAR-6 and aPKC Homologous Ras Protein Kinase C (aPKC). Here we show that PAR-6 and aPKC are also essential for spine morphogenesis, but their actions are distinct from those of PAR-3. Endogenous PAR-6 localizes to dendritic spines and redistributes away from spines upon synaptic activation. Overexpression of PAR-6 promotes the formation of mature spines, whereas knockdown of endogenous PAR-6 inhibits their formation. The effects of PAR-6 are dependent on its PDZ and aPKC binding domains but not on its ability to bind Cdc42. Furthermore, overexpression of PKCζ produces a phenotype reminiscent of PAR-6 overexpression, whereas knockdown of PKCζ inhibits spine formation. Taken together, these results suggest an essential role for PAR-6 in regulating spine formation that is mediated by aPKC.
Post-synaptic Signaling of New Players at the Synapse

N. Y. Ip; Department of Biochemistry, Hong Kong University of Science and Technology, Kowloon, Hong Kong, China

Neurons communicate with each other at synapses. During the past decade, enormous progress has been made in identifying and characterizing the molecular players at the post-synaptic side of synapses. Several receptor tyrosine kinases (RTKs), such as ErbB, MusSK, Ephs and Trks, have been demonstrated to play pivotal roles in synaptic development and functions. At the neuromuscular synapse, for example, activation of MusSK by agrin leads to the clustering of acetylcholine receptors while TrkB activation is involved in the stabilization of these receptors. Recent evidence suggests that Ephs, the largest family of receptor tyrosine kinases, and their ligands, ephrins, are important in the formation and maintenance of excitory synapses in the central nervous system. While EphBs are important for promoting dendritic spine morphogenesis, EphA signaling mediates spine retraction. Activation of EphB by ephrinBs transduces signals that elicit local changes in small GTPase activity leading to actin rearrangement and in turn induces spine protrusions. While activation of EphA4 has been reported to result in dendritic spine retraction in hippocampal slices, the underlying mechanisms are not well understood. We recently demonstrate that cyclin-dependent kinase 5 (Cdk5), a member of the Cdk family, mediates the downstream signaling of EphA4 activation at hippocampal synapses. Importantly, activation of EphA4 enhances Cdk5 activity which in turn stimulates RhoA GTPase and contributes to spine morphogenesis. Thus, while early studies on EphA4 and Cdk5 focused on the roles in axon guidance, our findings reveal an important role of Cdk5 in the regulation of EphA4-dependent regulation of synaptic structure and functions. This work was supported in part by the Research Grants Council of Hong Kong (HKUST 6131/02M, 6119/04M, 6421/05M and 3/03C) and the Area of Excellence Scheme of the University Grants Committee (AoE/B-15/01).

Visualization of Synaptic Protein Synthesis and Accumulation Using a Novel Chemically Controllable Reporter of Protein Age

M. Z. Lin, R. Y. Tsien; UCSD, La Jolla, CA

Spatial regulation of synaptic protein turnover, including local protein synthesis and degradation, is believed to play a crucial role in the structural plasticity of synapses and in the maintenance of long-term potentiation. Recently, studies of local protein turnover in cell culture have been facilitated by optical reporters of protein translation and methods for pulse-chase protein labelling. However, existing methods either do not recapitulate regulation of protein localization or other aspects posttranslational control, or are difficult to adapt to living animals. We have developed TimeSTAMP, a drug-controlled method for the Time-Specific Tagging and Age Measurement of Proteins with spatial specificity. This method has a high signal-to-noise ratio, can be used to tag proteins while preserving mechanisms of posttranslational regulation, and is compatible with slice preparations or freely behaving animals. We are currently applying the TimeSTAMP method to visualize the turnover of synaptic components at synapses under various stimulation conditions, and to perform retrospective analyses of the spatial distribution of synaptic growth events in intact neuronal networks.

Neural F-box Protein Scraper Regulates Synaptic Vesicle Release

I. Yao,1 H. Ageta,1 H. Takagi,1 T. Kihara,1 K. Inokuchi,1 M. Setou1,2; 1Mitsubishi Kagaku Institute of Life Sciences, Tokyo, Japan, 2National Institute for Physiological Sciences, Okazaki, Japan

The ubiquitin-proteasome system (UPS) has important functions in various cellular events. Recently, UPS has emerged as a key regulator of synaptic development and function. However, the relationships between the synaptic plasticity and UPS are not fully understood. Here, we report an ubiquitin-ligase Scraper which regulates the short term synaptic plasticity and protein turnover in the brain. The Scraper knockout mice have decreased paired pulse facilitation (PPF), accompanied with increased frequency of miniature excitatory postsynaptic currents (mEPSC), fast synaptic vesicle depletion, a smaller readily releasable pool (RRP) of synaptic vesicles. Scraper ubiquitinates the synaptic vesicle priming factor RIM1 for proteasomal degradation both in vitro and in vivo. Our results demonstrate the essential roles of Scraper dependent UPS in the short term synaptic plasticity, via the regulation of synaptic vesicle release.

Localized Sphingoid Base Generation Helps Recruit and Activate the Ypk1/2 Signaling Cascade

J. L. Brace,1 E. L. Weiss,1 C. M. Rudin;1 Biochemistry, Molecular Biology and Cell Biology, Northwestern University, Evanston, IL, 2Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins, Baltimore, MD

Signal transduction pathways allow cells to respond efficiently to environmental cues and promote cellular adaptation to environmental changes. Sphingoid bases activate a signaling cascade initiated by the kinases Pkh1 and Pkh2. These kinases, which are related to mammalian PDK1, activate the AGC family of kinases, including Ypk1/2, Pkc1 and Sch9, which in turn regulate growth-controlled pathways. This function of Svf1 has an interesting parallel in the JNK cascade initiated by the kinases Pkh1 and Pkh2. These kinases, which are related to mammalian PDK1, activate the AGC family of kinases, including Ypk1/2, Pkc1 and Sch9, which in turn regulate growth-controlled pathways. Our results indicate that PP2B play a functional role in the regulation of c-Jun-induced cell transformation. This work was supported in part by the Research Grants Council of Hong Kong (HKUST 6131/02M, 6119/04M, 6421/05M and 3/03C) and the Area of Excellence Scheme of the University Grants Committee (AoE/B-15/01).

Regulation of c-Jun N-terminal Kinase (JNK) and p38 upon Contact Inhibition

D. Hutter, J. Saksa, M. Slasz, E. Rothenberger; Biology, Monmouth University, West Long Branch, NJ

The c-Jun N-terminal kinase (JNK) and p38 pathways are mitogen-activated protein (MAP) kinase pathways known to have roles in growth control and apoptosis in normal mammalian cells. However, little is known about the role of negative regulation of these pathways by MAP Kinase Phosphatases (MKPs). To investigate the role of MKPs in the regulation of JNK and p38 during the transition to a contact inhibited state, cultures of normal fibroblast cells (BJ) and fibrosarcoma cells (HT-1080) were grown to different stages of confluency. The levels of MKP-1 expression and the amount of active MAP kinase proteins in the fibroblast cultures were assessed through western blot analysis and compared to those of the fibrosarcoma cultures. In normal fibroblasts, the level of MKP-1 protein is increased upon contact inhibition. Concurrently, the amount of total JNK is higher in subconfluent cells than in confluent cells, while density-dependent regulation of JNK or MKP-1 is not seen in fibrosarcoma cultures. Preliminary data indicate that the total level of p38 protein is not altered upon contact inhibition in normal fibroblasts. Further experimentation will delineate if the activation of JNK or p38 is influenced by contact inhibition and the causal relationship between MKP activity and growth control.

PP2B-mediated c-Jun C-terminus Dephosphorylation is Required for c-Jun-induced Cell Transformation

C. Huang; The Institute of Basic Medical Sciences, College of Medicine, National Cheng Kung University, Tainan, Taiwan

The proto-oncogene c-Jun is one of the components of AP1 that mediates cell proliferation, survival and death. The protein stability and the DNA binding affinity of c-Jun is regulated by C-terminal phosphorylation and the amount of active MAP kinase proteins in the fibroblast cultures were assessed through western blot analysis and compared to those of the fibrosarcoma cultures. In normal fibroblasts, the level of MKP-1 protein is increased upon contact inhibition. Concurrently, the amount of total JNK is higher in subconfluent cells than in confluent cells, while density-dependent regulation of JNK or MKP-1 is not seen in fibrosarcoma cultures. Preliminary data indicate that the total level of p38 protein is not altered upon contact inhibition in normal fibroblasts. Further experimentation will delineate if the activation of JNK or p38 is influenced by contact inhibition and the causal relationship between MKP activity and growth control.

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2006 ASCB Annual Meeting Abstracts
E. Kiyokawa, T. Nishioka, K. Hikake, H. Yoshizaki, M. Matsuda; Department of Pathology, Kyoto University, Kyoto, Japan

Phosphoinositides (PIs) are a family of phospholipids containing myo-inositol as their head group. Despite a relatively low abundance in biological membranes, PIs have been reported to regulate a myriad of cellular process owing to their metabolic turnover. Phosphatidylinositol PtdIns (4,5) biphosphate [PI(4,5)P₂] is a major PIP, derivative from Pts, and is mainly located in the inner leaflet of the plasma membrane. Upon growth factor stimulation, intracellular second messengers, such as inositol 1,4,5-triphosphate (IP₃), diacylglycerol (DGIDA) and diacylglycerol (DGIDA), are generated from PI(4,5)P₂. Localization of these second messengers has been assessed by the expression of GFP-tagged proteins which are able to interact to them. When they are over-expressed, however, these probes tend to accumulate in membrane structures such as ruffles. To understand the accurate spatio-temporal regulation of PI (4,5)P₂ and DG, we developed probes for PI(4,5)P₂, [PipP,4(5)P₂] and [PipP,4(5)P₂] and diacylglycerol (DIIDAs) based on the principle of fluorescent energy transfer (FRET). PipP-PI(4,5)P₂ and DGIDA consist of the PH domain from PLCg and the C1 domain from PKCδII, respectively, sandwiched between GFP and YFP. To localize these probes to the plasma membrane, the C-terminal sequence from K-Ras was utilized. FRET efficiency of PipP-PI(4,5)P₂ and DGIDA decreased within 5 minutes after platelet-derived growth factor (PDGF) stimulation. The time course of FRET alteration showed inverted correlation to the amount of PI(4,5)P₂ production and PLCg phosphorylation. On the other hand, FRET efficiency of PipP-PI(4,5)P₂ was observed on the plasma membrane of the entire cell, while that of DAG was limited to the nascent lamellipodia. These data suggest that lipid second messengers are regulated by spatially different mechanisms.

Ectopic Expression of COX-2 Induces Dedifferentiation in Rabbit Articular Chondrocytes

H. Tokuda,1 A. Iida, 2 H. Nishino 1; 1Molecular Biochemistry, Kyoto Prefectural University of Medicine, Kyoto, Japan, 2Pharmacology, Takasaki University of Health and Welfare, Takasaki, Japan

Many researchers have been studying the mechanism of growth factor and cytokine in hypertrophied muscle by exercise; however, the communication mechanism between the growth factor and cytokine of the cells in the control muscle tissues remains unknown. In this study, we carried out an experiment to study the cell communication mechanisms by the observation of Notch and Delta Notch activity was increased in hypertrophied soleus muscle by tenotomy. Notch was localized on the plasma membrane of small fibers and small cells but not found in large fibers of hypertrophied muscle or in the control muscle tissues. Delta activity was increased in hypertrophied soleus muscle. Delta was localized on the plasma membrane of small fibers and small cells in the hypertrophied muscle, and it was also slightly observed in the control muscular tissues. Based on the above result, we conclude that Notch and Delta appear not only in stem cells and satellite cells, but also in thin muscle fibers of hypertrophied muscle tissues, and moreover, they seem to take the roles of communication between the cells for induction of muscle hypertrophy.

Growth Inhibitory Effects of Brazilian Herbal Medicine Against Human Breast and Lung Cancer Cells

M. L. Petreaca, M. Yao, M. Martins-Green; Cell Biology and Neuroscience, University of California, Riverside, Riverside, CA

The angiogenic chemokine Interleukin-8 (IL-8/CXCL8) promotes endothelial cell de-adhesion and contraction during early stages of angiogenesis, thereby increasing endothelial permeability. This chemokine can transactivate Vascular Endothelial Growth Factor Receptor-2 (VEGFR2/FK/RK/KDR) in human microvascular endothelial cells, and this transactivation is critical for IL-8-induced permeability. However, in this study, we show that IL-8 stimulates phosphorylation in a dose-dependent manner, and this phosphorylation occurs by receptor transactivation because it is blocked by an inhibitor of VEGFR2 tyrosine kinase activity but not by an inhibitor of the VEGF-receptor binding site. We therefore investigated possible mechanisms involved in IL-8-mediated VEGFR2 transactivation and found, using co-immunoprecipitation assays, that VEGFR2 forms complexes with the IL-8 receptors CXCR1 and CXCR2 after IL-8 treatment with a time course compatible to that of VEGFR2 phosphorylation. Furthermore, because previous studies have identified the Src family of kinases as having key roles in various transactivation events, we also elucidated the importance of these kinases in IL-8-induced VEGFR2 transactivation and receptor complex formation. We found that an inhibitor of Src kinases prevents IL-8-induced permeability, VEGFR2 phosphorylation, and receptor complex formation, showing that Src activity is essential in these events. We then investigated the mechanism(s) whereby VEGFR2 mediates IL-8-induced permeability, focusing upon RhoA; RhoA is critical in stimulation of permeability, and we have shown previously that IL-8 stimulates RhoA activation. We performed GST-TRBD pull-down assays, which isolate active RhoA, in combination with a VEGFR inhibitor and found that the observed IL-8-induced RhoA activation is dependent upon VEGFR2. Because IL-8 and VEGF function in pathological permeability events in vivo, inhibition of specific signaling pathways downstream of VEGFR2 may serve as effective treatments for excessive permeability caused by either or both factors.

Growth Inhibitory Effects of Brazilian Herbal Medicine Against Human Breast and Lung Cancer Cells

H. Tokuda, A. Iida, H. Nishino; 1Molecular Biochemistry, Kyoto Prefectural University of Medicine, Kyoto, Japan, 2Pharmacology, Takasaki University of Health and Welfare, Takasaki, Japan

Tabebuia avellanedae (Bignoniaceae) (TA), which is native in South America from Brazil to northern Argentina, is well known in traditional folk medicine used for the treatment of various disease including breast cancer. In this study, we investigated the growth inhibitory activity of TA against breast and lung cancer cells. We performed MTT assay and thereby acted as a chemopreventive agents against carcinogenic compounds. In this study, we report the growth inhibitory activity of TA against breast and lung cancer cell lines.

Ectopic Expression of COX-2 Induces Dedifferentiation in Rabbit Articular Chondrocytes

L. Won Kil, Y. Seon Mi, Y. Eun Kyung, L. Ji Hye, O. Su Kil, K. Song Ja; Kongju National University, Gongju, Republic of Korea

Arthritic joints produce large amounts of PIs (which are involved in cartilage inflammation). The rate-limiting step of PG production is the initial conversion of arachidonic acid to PGJ₂ by cyclooxygenases (COX). Therefore, we have investigated the role of COX-2 protein on differentiation in rabbit articular chondrocyte. A previous study from our lab identified that IL-1β increased expression of COX-2 and induced dedifferentiation. Ectopic expression of COX-2 was sufficient to cause dedifferentiation in articular chondrocytes as determined by the expression of type II collagen via Aicn transfection and Western blot. Also, COX-2 overexpression caused suppression of SOX-9 expression, a major transcription factor that regulates type II collagen expression, as identified by the Western blot and RT-PCR. And then, we were identified that COX-2 overexpression inhibited type II collagen expression in articular chondrocytes by immunohistochemistry. However, Inhibition of COX-2 activating PDBT(NF-κB inhibitor) causes dedifferentiation as demonstrated by the enhancement type II collagen and SOX-9 expression by the Western blot. Our results collectively suggest that COX-2 overexpression causes dedifferentiation in articular chondrocytes through NF-κB pathway. To determine whether overexpression of COX-2 also caused similar dedifferentiation in zebrafish embryo, COX-2 cDNA was transiently transfected in zebrafish.

ERK-1/2 Regulate That 15-Deoxy-delta12,14-Prostaglandin J2-induced Cyclooxygenase-2 Expression and Dedifferentiation in Rabbit Articular Chondrocyte via NFκB Independent Pathway

J. Lee, S. Y. Wu, W. Lee, E. Yoon, C. Shin, S. Kim; Kongju National University, Gongju, Republic of Korea

 Peroxisome proliferators-activated receptors (PPARs) is a protein which belong to ligand-activated nuclear receptor family. It activate transription by means of various ligands that exist endogenous and exogenous. Nevertheless, physiological function of PPARα is not clear. Therefore, we investigated effect of PPARα in dedifferentiation in articular chondrocyte with 15d-PGJ2 and 15d-PGJ2. We found that 15d-PGJ2 induced COX-2 expression and dedifferentiation in chondrocyte with time/dose-dependent manner. Also, 15d-PGJ2 increased peroxisome proliferators response element (PPRE) luciferase activity and BADGE (Bisphenol A diglycidyl ether), the antagonist of PPARα, abolished it. These result indicates that effect of 15d-PGJ2 depend on PPARα activation. We found that 15d-PGJ2 activate ERK-1/2 rapidly. Treatment of PD98059, inhibitor for ERK-1/2, prevented from 15d-PGJ2 induced dedifferentiation and inflammation. Our findings demonstrate that 15d-PGJ2 regulate dedifferentiation and COX-2 expression regulated via ERK-1/2.
pathway. But these regulation caused by 15d-PGJ2 seems to be independent from NFXb pathway, well known as important COX-2 regulation signal. Thus, these study exhibit that adjustment of PPARγ signaling pathway may offer novel approach for therapeutic inhibition of degradation on articular chondrocyte.

749 Regulation Mechanism of Phospholipase C(PLC)-η in Neuronal Cells

N. Oh, K. Heo, P. Suh, S. Ryu; Life Science, POSTECH, Pohang, Republic of Korea

PLC (Phospholipase C) catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2), generating inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG) which play key roles in cell signaling. To date, many studies have shown how various PLC isoforms are activated and regulated in distinct ways. However, regulation mechanism of PLC-η, a novel PLC isoform, has yet to be known. In the present study, we tried to find the binding proteins of PLC-η and elucidate how they are regulated. As a result, we identified novel proteins which interact with PLC-η. Also, PLC-η was phosphorylated by serotonin, and intracellular Ca2+ level increased in PLC-η-overexpressing cells. Then we observed that Akt and Erk were activated in PLC-η-overexpressing stable cell line when several extracellular signalling molecules were added. Finally, several G protein coupled receptor (GPCR) ligands effectively activated PLC-η. Taken together, PLC-η may be activated through GPCR pathway, and its activity is regulated by phosphorylation.

750 Phosphatase of Regenerating Liver (PRL)-3 Regulates IGF-1 Signaling in 293T Cells

D. C. Besette, C. J. Pallien; 1Pathology and Laboratory Medicine, University of British Columbia, Vancouver, BC, Canada, 2Paediatrics, University of British Columbia, Vancouver, BC, Canada

Protein of regeneration liver (PRL-3) is a member of a novel subfamily of protein tyrosine phosphatases (PTPs) that possess a C-terminal CAAX box motif involved in prenylation-dependent membrane targeting of these proteins. PRL-3 is consistently upregulated in a panel of liver metastases derived from colon carcinomas while exhibiting low expression in primary tumors and little to no expression in normal colon epithelia. Furthermore, overexpression of PRL-3 promotes cell migration and invasion abilities, and permits formation of metastases in vivo. However, little is known of PRL-3-mediated cellular signaling pathways. Because of the role of insulin-like growth factor (IGF) signaling in cancer, we investigated the role of PRL-3 in mediating IGF-1 signaling. IGF-1 signaling can activate either MAPK signaling or Akt signaling depending on the molecules that interact with the IGF-1 receptor (IGF-1R). Phosphorylation of Akt on Ser473 and Thr380 is required for activation and is unchanged by the presence of PRL-3, suggesting PRL-3 does not affect Akt activation. In contrast, Erk phosphorylation induced by IGF-1 stimulation is decreased by approximately 40% in the presence of exogeneous PRL-3. A catalytic inactive PRL-3 mutant does not change Erk phosphorylation compared to parental cells, suggesting PRL-3 catalytic ability is responsible for its signaling effects. This effect is specific for Erk as neither the JNK nor p38 MAPKs are affected by PRL-3 expression. Upstream signaling events leading to Erk activation are being investigated to further elucidate the role of PRL-3 in IGF signaling. Determining the molecular action of PRL-3 in regulating cellular activities will provide important insight into its role in metastasis and cancer, and, possibly, identify new targets for therapeutic interventions in advanced disease.

753 Stat1 Is Required for Oxysterol-induced Apoptosis in Macrophages

S. Agrawal, T. Choudhuri, G. Stark, G. Chisolm; 1Cell Biology, Cleveland Clinic, Cleveland, OH, 2University of Cincinnati College of Medicine, Cincinnati, OH, 3Molecular Genetics, Cleveland Clinic, Cleveland, OH

Atherosclerosis is an inflammatory disease of major arteries that lead to coronary occlusion. The disease is characterized by accumulation of oxidized lipoprotein and monocyte-derived macrophages in early stages and later, the development of a necrotic core. Apoptotic and necrotic macrophages and macrophage-derived foam cells can be readily detected in lesions. 7-ketocholesterol (7KC) is a major oxysterol formed on oxidized low-density lipoprotein (oxLDL) and it accumulates in atherosclerotic lesions. 7KC induces apoptosis in a variety of cells, including macrophages, in vitro. 7KC induces apoptosis in similarity-concentrated dependent manner in human THP-1 monocyte/macrophage-like cells whether unidentified, differentiated or after oxLDL treatment to form foam cells. Using a specific DNA decoy to block DNA binding to the transcription factor Stat1 in human THP-1 cells or comparing macrophages from wild type and Stat1-/- mice, we found that inhibition of Stat1 significantly blunted 7KC-induced apoptosis. ApoE-/- and Stat1-/- mice were lethally irradiated and transplanted with bone marrow from either apoE-/- or apoE-/-Stat1-/- mice. After feeding an atherogenic diet for 14 wk, cells in aortic root atherosclerotic lesions from apoE-/-Stat1-/- bone marrow recipient mice showed less apoptosis (by TUNEL) and caspase3-activation than those from apoE-/- recipients. Thus, Stat1 is an important pro-apoptotic signaling molecule in macrophages in vitro and in atherosclerotic lesions in vivo. Identification of the essential signaling events of apoptosis that pertain in vivo could suggest targets for therapeutic intervention. This project is supported by National Institute of Health Grant NIH HL29582.

754 Overexpression of the Type I Phosphatidylinositol 4-phosphate 5-kinase Alpha Produces a Defect in Store-operated Calcium Entry in RBL-2H3 Mast Cells

L. Vasudevan,1 A. Wiesner,2 A. Jeromin,2 B. Baird,2 D. Holowka; 1Molecular Biology and Genetics, Cornell University, Ithaca, NY, 2Chemistry and Chemical Biology, Cornell University, Ithaca, NY, 3Center for Learning and Memory, University of Texas, Austin, TX

Allergic reactions are triggered when multivalent antigens crosslink IgE-FcεRI complexes in mast cells. The hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) and mobilization of calcium are important in this signaling pathway to activate release of inflammatory mediators, synthesis of cytokines, and to produce morphological changes such as ruffling. To examine the roles of PIP2, in these processes, we stably overexpressed HA-tagged WT type I phosphatidylinositol 4-phosphate 5-kinase alpha (PIPS-5-kinase Iα) and a catalytically inactive mutant kinase in RBL-2H3 mast cells. Expression was confirmed in two mutant clones and three WT clones by immunocytochemistry and western blotting. Our studies demonstrate that cells overexpressing the WT PIP5-kinase Iα (WT cells) show hyper-tyrosine phosphorylation of FcεRI, whereas those expressing the mutant kinase show hypophosphorylation. Despite this enhanced receptor phosphorylation in the WT cells, the calcium response to stimulation by antigen is reduced by 40%. Furthermore, the calcium response to store depletion by thapsigargin is similarly reduced, indicating a defect in calcium influx via store-operated calcium entry channels. We also find that cells overexpressing the mutant kinase show substantially reduced antigen-stimulated ruffling compared to untransfected control cells and WT cells, indicating a role for PIPS-5-kinase Iα in stimulated ruffling. In conclusion, our studies reveal that the pool of PIP2, synthesized by PIPS-5-kinase Iα plays diverse roles in regulating IgE-receptor signaling: it positively regulates receptor phosphorylation and stimulated cell ruffling and negatively regulates the calcium response at the level of store-operated calcium entry. Ongoing studies are focused on understanding the mechanisms by which PIPS-5-kinase Iα participates in these activation processes.

755 Extracellular Metalloproteinase from Vibrio vulnificus Induces Production of Inflammatory Mediators in Macrophage Raw 264.7 Cells

J. E. Park, A. K. Chang, N. H. Lee, J. S. Lee; Chosun University, Gwangju, Republic of Korea

Vibrio vulnificus is an aero- and water-pathogen in human. It secretes a broad-specificity metalloproteinase (vEP) that has many biological activities and is associated with skin lesions and serious hemorrhagic complications. Inflammatory mediators produced by cells such as monocyte and macrophage play an important role during the process of inflammation. Among the matrix metalloproteinases (MMP), macrophage elastase (MMP-12) is able to degrade extracellular matrix component (ECM) such as elastin and initiate the process of inflammation. As vEP is capable of degrading a number of different ECM proteins such as collagen and elastin, we wanted to know whether it has any effect on the production of inflammatory mediators. Using MTS-assay we showed that vEP caused no apparent toxicity to the macrophage Raw 264.7 cells at concentrations ranging from 1 to 10 μg/ml. At 1 μg/ml vEP, the expression of mRNA for tumor necrosis factor-α (TNF-α) and interleukin 6 (IL-6) were significantly upregulated as measured with real-time PCR. In addition, the level of IkB-α expression was also down regulated. Western blot of cell lysate revealed an increase in the level of TNF-α and a reduction in the level of IL-6, suggesting that a direct interaction between vEP and these cytokines. As the C-terminal domain of vEP is thought to facilitate its binding to insoluble proteins such as those that are present in the ECM, the effect of various vEP mutants with C-terminal deletion on the production of inflammatory mediators was also investigated using TNF-α as a representative molecule. Overall, the results suggest that vEP might play a direct role in the triggering of an inflammatory response following Vibrio infection.

756 Regulation of Melanosome Transport by cAMP-dependent Protein Kinase Does Not Require Its Catalytic Activity

M. Park, V. I. Gelland; Cell and Molecular Biology, Northwestern University, Chicago, IL
Transport of pigment organelles (melanosomes) in *Xenopus laevis* melanophores is tightly regulated by cAMP. Melanosome dispersion or aggregation is triggered by the increase or decrease of cAMP concentration in the cytoplasm, respectively. These effects of cAMP are mediated by cAMP-dependent protein kinase (PKA). Using overexpression studies, we showed that melanosome transport is regulated by Cj catalytic subunit of Xenopus PKA, while an isoform Cx subunit has no effect on transport. Surprisingly, catalytically inactive mutants of Cj were able to regulate melanosome transport like the wild type PKA Cj. Mutational studies confirm that this phenomenon is not due to the activation of endogenous PKA. Furthermore, subcellular fractionation and fluorescence microscopy showed that PKA pool is associated with melanosomes. Our results indicate that PKA Cj is a distinct catalytic subunit that is recruited to melanosomes and regulates melanosome transport by a novel mechanism.

ERK1c Regulates Golgi Fragmentation during Mitosis
Y. D. Shaul, R. Seger; Biological Regulation, Weizmann Institute of Science, Rehovot, Israel
Extracellular signal-regulated kinases (ERK) are signaling molecules that regulate a wide array of cellular processes. Recently we cloned an alternatively spliced isoform of ERK1, named ERK1c, and demonstrated that it is regulated differently from other ERKs upon various stimulations. In addition, we showed that this isoform could be found in the Golgi apparatus under various conditions. Preliminary data from our working hypothesis states that ERK1c participates in the regulation of mitotic Golgi fragmentation, which is known to be mediated by MEK1 without the involvement of ERK1/2. Therefore, the Objective of the work was to study the function and regulation of ERK1c in the Golgi during mitosis. Using FACS analysis, SiRNA, and immunofluorescence technique, our results show that during late G2 phase and mitosis, ERK1c expression and activation, were increased. Additionally, at these phases ERK1c translocate to the Golgi complex. ERK1c knockdown resulted in significant attenuation of the Golgi fragmentation and consequently also mitotic progression, whereas the overexpression of ERK1c facilitated these processes. Similar to ERK1c, changes in MEKs activity during mitosis affected Golgi fragmentation, while changes in ERK1/2 expression level did not affect this process, suggesting that at least one of the MEKs facilitates the Golgi fragmentation specifically by ERK1c. Interestingly, the MEK isoform that regulates this process seems to be the alternatively spliced MEK1b, which phosphorylates specifically ERK1c but not ERK1 or ERK2. We concluded that ERK1c is a unique MEK effector that extends the specificity of the ERK signaling cascade to regulate Golgi fragmentation. Therefore, alternatively spliced isoforms are another mean for determining the specificity of the ERK cascade that regulates different and even opposing cellular processes.

Signaling Pathways of Light Induced Macrophage Migration and Vascular Smooth Muscle Cell Proliferation
W. Lin, C. Wei, Y. Chou; Pharmacology, National Taiwan University, Taipei, Taiwan
The biological actions of LIGHT, a member of the tumor necrosis factor superfamily, are mediated by the interaction with lymphotixin-β receptor (LTβR) and/or herpes virus entry mediator (HVEM). Previous studies had suggested a key role of LIGHT and HVEM receptors in atherosclerotic vascular disease. To elucidate the role of LIGHT in the function of macrophages and vascular smooth muscle cells (VSMC) in relation to atherosclerosis, we determined the effects of LIGHT on macrophage migration and VSMC proliferation. We found LIGHT through HVEM activation can induce both events. LIGHT-induced macrophage migration was associated with activation of signaling kinases, including MAPKs, PI3K/Akt, and NF-κB. LIGHT-induced VSMC proliferation was also shown to be associated with the activation of MAPKs, PI3K/Akt, and NF-κB, which consequently led to altered expression of cell cycle regulatory molecules. Down-regulation of p21, p27 and p53, and inversely up-regulation of cyclin D and RB hyper-phosphorylation were demonstrated. In conclusion, LIGHT acts as a novel mediator for macrophage migration and VSMC proliferation, suggesting its involvement in the atherogenesis.

βPix Regulates Golgi Fragmentation During Mitosis
A. Chahidi, A. Sorokin; Medicine, Medical College of Wisconsin, Milwaukee, WI
Recently, we described a novel technology for site-specific covalent tethering of synthetic ligands to a novel reporting protein (RP) in living cells, in vitro and on a solid support (Luo et al, 2005). Here we are using this technology in combination with live cell imaging and conventional protein analysis techniques to study dynamics of nuclear transcription factor κB (NF-κB) signaling in cultured mammalian cells. We demonstrated that activation of TNF-α receptor leads to nuclear translocation of the p65-RP fusion in transiently stably expressing cells. The kinetic parameters of translocation are within physiological limits. Pretreatment of cells with SN50 (an inhibitor of nuclear import) prevents nuclear translocation of protein fusion. Incubation of the cells with Leptomycin B (specific inhibitor of the nuclear export factor CRM1) leads to accumulation of p65-RP in the nucleus. Activation of TNF-α receptor leads to a degradation of intrinsic IκB, as well as fluorescently labeled IκB-RP. This degradation is efficiently blocked by Lactacystin, a potent 26S proteosome inhibitor. To study the effect of different levels of p65-RP on NF-κB signaling we utilized three systems for inducible protein production, Coumarmycin-Nobobyocin based; CRE (cAMP responsive element) based; and NFAT (nuclear factor of activated T-cells) based. The level of p65-RP in transiently transfected uninduced cells was negligible. Upon application of the appropriate inducer, the level of the p65-RP is significantly increased. The fluorescently labeled p65-RP is properly localized in the cytosol in all three systems. However, upon application of TNF-activated T-cells (based), the level of p65-RP in transiently transfected uninduced cells was negligible. Upon application of the appropriate inducer, the level of the p65-RP is significantly increased. The fluorescently labeled p65-RP is properly localized in the cytosol in all three systems. However, upon application of TNF-activated T-cells (based) and/or herpes virus entry mediator (HVEM) and/or herpes virus entry mediator (HVEM), our results show that during late G2 phase and mitosis, ERK1c expression and activation, were increased. Additionally, at these phases ERK1c translocate to the Golgi complex. ERK1c knockdown resulted in significant attenuation of the Golgi fragmentation and consequently also mitotic progression, whereas the overexpression of ERK1c facilitated these processes. Similar to ERK1c, changes in MEKs activity during mitosis affected Golgi fragmentation, while changes in ERK1/2 expression level did not affect this process, suggesting that at least one of the MEKs facilitates the Golgi fragmentation specifically by ERK1c. Interestingly, the MEK isoform that regulates this process seems to be the alternatively spliced MEK1b, which phosphorylates specifically ERK1c but not ERK1 or ERK2. We concluded that ERK1c is a unique MEK effector that extends the specificity of the ERK signaling cascade to regulate Golgi fragmentation. Therefore, alternatively spliced isoforms are another mean for determining the specificity of the ERK cascade that regulates different and even opposing cellular processes.

βPix/ p66Shc/Foxo3a Signaling Complex Regulates Cell Proliferation through p27kip1 Down-regulation
A. Chahidi, A. Sorokin; Medicine, Medical College of Wisconsin, Milwaukee, WI
The Rac1/Cdc42 guanine nucleotide exchange factor, βPix, is a multidomain protein that regulates cytokesin organization through interaction with focal adhesion proteins. In this study, we identify the amino acid sequence, E140LRQDINW146, located at the C-terminus region of βPix that binds both the adaptor protein p66Shc and the forkhead transcription factor Foxo3a. βPix induces serine-36 phosphorylation of p66Shc through ERK and ERK1c activation. The depletion of p66Shc by RNA interference inhibits βPix-mediated Foxo3a phosphorylation. Furthermore, we show that βPix over-expression results in down-regulation of the cytosolic-catalytic kinase inhibitor p27kip1 independently of Akt. βPix-mediated p27kip1 down-regulation induces cell cycle and cell proliferation into S phase of the cell cycle. Interestingly, the expression of βPix mutant, βPixΔ(603-608), that is unable to form a complex with p66Shc and Foxo3a blocks p27kip1 down-regulation, cell proliferation and cell progression into S phase. Our results demonstrate for the first time a direct role of βPix in cell proliferation, and extend the network of proteins that interact with βPix to include p66Shc and Foxo3a.

Probing Dynamics of NF-κB Signaling Using Novel Protein Labeling Technology
N. Karassina, C. Zimprich, R. Learish, G. Los; R&D, Promega Corp, Madison, WI
Extracellular signal-regulated kinases (ERK) are signaling molecules that regulate a wide array of cellular processes. Recently we cloned an alternatively spliced isoform of ERK1, named ERK1c, and demonstrated that it is regulated differently from other ERKs upon various stimulations. In addition, we showed that this isoform could be found in the Golgi apparatus under various conditions. Preliminary data from our working hypothesis states that ERK1c participates in the regulation of mitotic Golgi fragmentation, which is known to be mediated by MEK1 without the involvement of ERK1/2. Therefore, the Objective of the work was to study the function and regulation of ERK1c in the Golgi during mitosis. Using FACS analysis, SiRNA, and immunofluorescence technique, our results show that during late G2 phase and mitosis, ERK1c expression and activation, were increased. Additionally, at these phases ERK1c translocate to the Golgi complex. ERK1c knockdown resulted in significant attenuation of the Golgi fragmentation and consequently also mitotic progression, whereas the overexpression of ERK1c facilitated these processes. Similar to ERK1c, changes in MEKs activity during mitosis affected Golgi fragmentation, while changes in ERK1/2 expression level did not affect this process, suggesting that at least one of the MEKs facilitates the Golgi fragmentation specifically by ERK1c. Interestingly, the MEK isoform that regulates this process seems to be the alternatively spliced MEK1b, which phosphorylates specifically ERK1c but not ERK1 or ERK2. We concluded that ERK1c is a unique MEK effector that extends the specificity of the ERK signaling cascade to regulate Golgi fragmentation. Therefore, alternatively spliced isoforms are another mean for determining the specificity of the ERK cascade that regulates different and even opposing cellular processes.

Molecular Mechanism of EGF-enhanced Aurora-A Gene Expression in Tumor Cells
L. Hung; Department of Pharmacology, National Cheng Kung University, Tainan, Taiwan
Human Aurora-A is a potential oncoprotein which is highly expressed in many malignant tumors and cancer cell lines. The increased level of Aurora-A is through the DNA amplification, protein stabilization and transcriptional up-regulation. The detailed mechanism of transcriptional regulation of Aurora-A in tumors is still unclear. The epidermal growth factor receptor (EGFR) is a transmembrane receptor tyrosine kinase and it is also highly expressed in many tumors and cancer cell lines. In recent years, the nuclear localization of EGFR has been demonstrated by many groups. The nuclear localization of EGFR is strongly correlated with highly proliferating activity and where it functions as a transcription activator. The nuclear EGFR recognizes the AT-rich

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Transport of pigment organelles (melanosomes) in *Xenopus laevis* melanophores is tightly regulated by cAMP. Melanosome dispersion or aggregation is triggered by the increase or decrease of cAMP concentration in the cytoplasm, respectively. These effects of cAMP are mediated by cAMP-dependent protein kinase (PKA). Using overexpression studies, we showed that melanosome transport is regulated by Cj catalytic subunit of Xenopus PKA, while an isoform Cx subunit has no effect on transport. Surprisingly, catalytically inactive mutants of Cj were able to regulate melanosome transport like the wild type PKA Cj. Mutational studies confirm that this phenomenon is not due to the activation of endogenous PKA. Furthermore, subcellular fractionation and fluorescence microscopy showed that PKA pool is associated with melanosomes. Our results indicate that PKA Cj is a distinct catalytic subunit that is recruited to melanosomes and regulates melanosome transport by a novel mechanism.
Inherent Uncertainty in Protein Cycle and Nature’s Dance of Life: Expanding Paul Silverman’s Final Vision for Cell Biology Fundamentals
A. Goodman; Center for Statistical Consulting, University of California, Irvine, CA

Paul Silverman’s vision for uncertainty was first to explicitly advocate exploring for uncertainty beyond protein cycle’s determined behavior. Author and others proposed uncertainty as appropriate complement to the unrealistic certainty of determinism. This was underscored by Science’s 125th Anniversary four “What Don’t We Know?” concerning biology. Uncertainty within complexity is most realistic cellular model is: Cell Behavior = Certain Determined Part + Unknown Determinable Part + Uncertain Diversity. Too many biological, chemical and physical processes must “succeed” for cell behavior to be determined. Uncertainty is due to complexity and is generated by cascading effects of intricate processes compounded by interactions. A combination of determined and uncertain processes is uncertain. Cellular variables are likely random, probability is likely fundamental, and cellular mathematics is likely statistics. Uncertainty beyond what is known is not as plausible to believe in an essential uncertain world with growing areas of known certainty, as it is to believe in an essential certain world with shrinking areas of unexpected unknowable uncertainty. Physics “theory of everything” will probably include uncertainty. Uncertainty fills void beyond knowledge. Last major bastions of determinism since cell biology is surrounded in almost all respects by equally complex disciplines, why is it that cell biology and perhaps dark energy/matter physics appear to be the last major bastions of unrealistic determinism? Quantum physics, cell chemistry, evolutionary biology, medicine, social science, commerce and industry have all accepted and even managed uncertainty. What gets us into far more trouble than what we do not know is what we do know that is not true (Mark Twain).

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REFERENCES
Two Populations of p27 Use Differential Kinetics to Phosphorylate Ser10 and Thr187 in Response to FGF-2 Stimulation

J. Lee, T. Oh, E. Kay1,2, Doherty Eye Institute, Los Angeles, CA, 1Department of Ophthalmology, Keck School of Medicine of University of Southern California, Los Angeles, CA

The cyclin-dependent kinase inhibitor p27 regulates cell cycle progression; its activity is controlled by its concentration and subcellular localization. We investigated whether FGF-2 facilitates phosphorylation of p27 at the serine 10 (Ser10) and threonine 187 (Thr187) sites by PI 3-kinase and whether these two phosphorylation sites were differentially regulated. FGF-2 stimulation dramatically increased the phosphorylation of p27 at Ser10 and Thr187 using differential kinetics: the maximum phosphorylation at Ser10 and Thr187 were observed 6 and 16 h after FGF-2 stimulation, respectively. FGF-2-induced p27 phosphorylation at both sites was completely blocked by LY294002. We determined the physical and biochemical interaction of p27 with Cdk2/Cyclin E complex in response to FGF-2 stimulation; maximum binding of p27 to Cdk2/Cyclin E complex occurred at 12 h; maximum level of phosphorylation of p27 at Thr187 in the ternary complex was observed at 16 h; ubiquitination of the Thr187-phosphorylated p27 (pp27Thr187) was observed starting at 12 h and continuing for up to 24 h. However, the phosphorylation of p27 at Ser10 occurred in the nucleus with a maximum level of phosphorylation stimulation of p27 (pp27Ser10) was ubiquitinated and was simultaneously exported to cytoplasm at a maximum rate 8 h after FGF-2 treatment. We further investigated which of the two phosphorylated p27 (pp27Thr187 and pp27Ser10) was involved in G1/S progression. When LY294002 was used, the inhibitor blocked 64% of cell proliferation stimulated by FGF-2; the blocking of nuclear export of pp27Ser10 by leptomycin B greatly decreased the FGF-2 stimulated cell proliferation (44%), suggesting that p27 phosphorylation of p27 at Ser10 is the major mechanism for G1/S transition. Our results suggest that different kinetics are observed in phosphorylation of p27 at Ser10 and Thr187 in response to FGF-2 stimulation and that pp27Thr187 and pp27Ser10 may represent two separate populations of p27 observed during G1/S transition.

Stages of Medial Nuclear Division Arrest in S. cerevisiae

T. Hattier, F. Najm, N. Mallhora, J. Drážka, A. M. Tartakoff, Pathology, Case Western Reserve University, Cleveland, OH

Several yeast cell mutants arrest after DNA replication but before cleavage homologs, with the nucleus spanning the bud neck - a situation known as "medial nuclear division arrest" (MND). This terminal phenotype is characteristic of mutants which simultaneously DNA damage by exposing the single-stranded extents of chromosomess (cck13-1), in strains which cannot activate the anaphase promoting complex (due to depletion of Cdc20p) or by induction of the kinase, Mps1p, which triggers the spindle checkpoint. These mutants are cause chomatic to oscillate along the long axis of the nucleus. Upon arrest, we nevertheless observe that each condition leaves the nucleus anchored at the maternal extremity of the nucleus, emphasizing the strength of the linkage between the nucleus and the periphery of the nucleoplasm. To learn whether each form of MND arrest is coincident, we have constructed a corresponding triple mutant (MAT a). In this strain each arrest is reversible and each can be imposed for as long as ten hours. We therefore have systematically performed order-of-function experiments in which each arrest is followed by reincubation under conditions which activate a second cause of arrest, with addition of alpha factor to allow easy evaluation of cell cycle progression. These epistasis experiments imply that the arrest due to cdc13-1 precedes the others, and that the Cdc20p-dependent step is coincident with the Mps1p-dependent step. The DNA damage checkpoint defined by cdc13-1 therefore precedes the spindle checkpoint. The relative order of the DNA damage and spindle checkpoints will be compared with those which operate in the mammalian cell cycle. Supported by NIH Training Grant T32 HD007104-30.

Stimulatory Translational Components of Heat-stressed Cells of Sacccharomyces cerevisiae Transiently Accumulate in Cytoplasmic Aggregates

F. Janda,1 P. Vasicova,1 J. Vojtova,1 J. Frydlova,1 P. Ivanov,2 J. Hasek,3 Cellular and Molecular Microbiology, Institute of Microbiology AS CR, Prague, Czech Republic, 1A. Belozersky Institute of Physico-Chemical Biology, Moscow, Russian Federation

In higher eukaryotic cells exposed to various environmental stresses translation initiation factors accumulate in cytoplasmic aggregates, called stress granules. The stress granules were identified in heat-stressed yeast by RNA polymerization but until now their formation has not been referred to budding yeast Saccharomyces cerevisiae. We constructed various S. cerevisiae strains expressing fusion proteins intragenously tagged with GFP and/or mRFP from their chromosomal sites and analyzed redistribution of translational factors after heat shock. The heat shock resulted in accumulation of 80S ribosome (determined by polysome profile) and protein aggregation. In the aggregates, we identified translation initiation factors eIF3 and eIF4G, mRNA binding protein Pab1 and elongation factor Yef3p. In contrast, translation initiation factor eIF2b/Su2p and ribosomal proteins Rps2 and Rps7B (40S) and Rpl5, Rpl10 and Rpl25 (60S) as well as glycosyl enzymes Pgp1 and Pkp1 (negative control), were not present in the aggregates. Further, formation of aggregates was transient. It was prevented by the cyclobeximide pretreatment and was independent of the phosphorylation of eIF2alpha by Gcn2 kinase. In the aggregates, heat shock proteins (Hsp104p, Hsp70 family) were also identified and Hsp104p appeared to affect their dissolution. Together, our data indicate that the aggregates represent dynamic structures composed of some translational components and that these structures resemble but are not identical with stress granules described in higher eukaryotic cells. This work was financed by grants CSF 204/05/0383, LC545 and ICAV0250200510.

A Role for Cytoplasmic Poladenylation Element Binding Protein in Cell Cycle Control in Astrocytes

W. Oh, J. Choi, K. Ko, K. Jones, S. Banerjee, C. Shin, D. G. Wells, 1Pharmacology, Seoul National University, Seoul, Republic of Korea, 2Molecular, Cellular and Developmental Biology, Yale University, New Haven, CT, 3Pharmacology, Gorkok University, Chungia, Republic of Korea

Cytoplasmic poladenylation regulates specific mRNA translation in developing oocytes and mature neurons. Cytoplasmic poladenylation element binding protein (CPEB) is an mRNA-binding protein that regulates this process. When bound, CPEB holds the mRNA in a translationally dormant state; upon phosphorylation by Aurora A kinase, the bound RNA is poladenylated leading to translational activation. We now describe CPEB expression in rat astrocytes. Here, CPEB and Aurora kinase are localized to the centrosome of dividing astrocytes suggesting a role for CPEB in cell cycle regulation. When growth-arrested primary astrocytes were induced to proliferate by the addition of serum to the media, a rapid phosphorylation of CPEB and polyadenylation of a CPEB target mRNA encoding cyclin B1 was detected. The increase in the polyadenylation of cyclin B1 mRNA resulted in an increase in cyclin B1 protein synthesis that could be detected in the presence of a transfection inhibitor, indicating it was the result of mRNA translational activation. To determine the consequences of altering CPEB protein levels on cell proliferation we used both FACs analysis and a biochemical marker for dividing cells, phospho-histone H3. Over-expression of unphosphorylated CPEB inhibited cell cycle progression while expressing RNA sequences containing the binding site for CPEB, thus sequestering the endogenous CPEB protein from its target mRNAs, resulted in an increase in cell division. Similarly, siRNA knockdown of CPEB expression in astrocytes promoted cell cycle progression. These results suggest that CPEB-dependent mRNA translation plays an important role in cell cycle regulation in astrocytes, possibly mediated in part through the regulation of cyclin B1.

Activation of H3 Histamine (H3R) Receptors Induces Inhibition of Growth of Several Cholangiocarcinoma Cell Lines by PLC/IP3/Ca2+-dependent Inhibition of ERK1/2 Phosphorylation

H. Francis, J. Venter, S. Vaculin, S. DeMorrow, S. Glaser, B. Vaculin, S. Arnold, B. Green, R. Summers, G. Alpini; 1R&E, Scott & White Hospital, Temple, TX, 2Medicine, The Texas A&M University System HSC, Temple, TX, 3Medicine, Scott & White Hospital and Texas A&M University System HSC, Temple, TX, 4Medicine, Central Texas Veterans HCS and The Texas A&M University System HSC COM, Temple, TX

We have previously shown that the H3R agonist, RAMH, inhibits the growth of the cholangiocarcinoma cell line, Mz-Cha-1, but there remains limited understanding of the mechanisms of action by which H3R regulation the growth of biliary tumors. The IP3/Ca2+-PKC/MAPK pathway modulates cholangiocarcinoma growth. Gastrin inhibits cholangiocarcinoma growth via Ca2+-dependent PKCalpha activation, whereas the D2 dopaminergic receptor, quinelorane, inhibits cholangiocarcinoma growth by activation of the Ca2+-dependent PKCgamma. The AIMS of this study were to determine: (i) if the intra- and extra-hepatic cholangiocarcinoma cell lines express the H3R (ii) if RAMH inhibits cell growth of other cholangiocarcinoma cell lines (iii) if concomitant RAMH and Ca2+ inhibition of cell growth is also observed. In this study we found by RT-PCR that all the cell lines expressed the H3R. Treatment with Ca2+ and RAMH resulted in a significant decrease in proliferation of HuH-7 and HuH-28 cell lines by 44% and 37%, respectively, and inhibition of cell cycle progression with accumulation in G0/G1 phase. In contrast, treatment with Ca2+ and RAMH had no effect on the proliferation of CCLP-1 cell line. In conclusion, our results suggest that the activation of the H3R may be a potential therapeutic target for the treatment of cholangiocarcinoma.
IP3, levels by RIA and the phosphorylation of PKCα, beta-I, beta-II and gamma and the MAPK kinases, ERK1/2 and JNK by immunoblotting. RESULTS: All cholangiocarcinoma cells lines and H69 cells express H3R. RAMH decreased growth at 10, 25 and 50 micromolar for 48 hours in all cholangiocarcinoma cell lines but not H69. The following blocked the inhibitory effect of RAMH on cholangiocarcinoma growth: (i) the Ca2+ chelator, BAPTA/AM (5 micromolar) (ii) the PKCα inhibitor, Go6976 (1 micromolar) and (iii) the phospholipase C inhibitor (U-73122, 1 micromolar). In Mz-ChA-1 cells, RAMH increased IP3 levels and PKCα phosphorylation but decreased the phosphorylation of ERK1/2 but not JNK. CONCLUSIONS: RAMH inhibits the growth of several cholangiocarcinoma cell lines via PLC/PKCα/PKCα-dependent dephosphorylation of ERK1/2. The activation of H3R may be important in the management of cholangiocarcinoma.

773 Mitotic Checkpoint Slippage in Vertebrates in the Presence of Microtubule Poisons

D. A. Brito, L. C. Rieder; Wadsworth Center—NYS Dept. of Health, Albany, NY

In the presence of unattached/weakly attached microtubules, the spindle assembly checkpoint (SAC) delays exit from mitosis by preventing the anaphase promoting complex (APC)-mediated proteolysis of cyclin B, a regulatory subunit of cyclin-dependent kinase 1 (Cdk1). The SAC does not arrest cells permanently, even when it cannot be satisfied (e.g., in the presence of microtubule (MT) poisons), and escape from mitosis in the presence of an unsatisfied SAC requires that cyclin B/Cdk1 activity be somehow inhibited. We have recently shown that in vertebrates, exit from mitosis in the presence of nocodazole occurs through the continuous and proteosome-dependent degradation of cyclin B that ultimately drives cells out of mitosis in the presence of an active SAC (D.A. Brito and L.C. Rieder, 2006, Curr. Biol. 16:1194-1200). During this study we also observed that the rate of “leakage” through the SAC was positively correlated with the number of MTs remaining in the nocodazole-treated cell. To further explore this latter finding we are studying how mitotic RPE1 cells behave in the presence of taxol, a drug that, unlike nocodazole, stabilizes and/or promotes MT polymerization. We find that RPE1 cells incubated with 5 nM or 5 μM taxol are delayed in mitosis for the same duration (208±121 min, n=4 versus 203±85 min, n=141, respectively). Surprisingly, however, cells incubated in 500 nM are delayed for 698±356 min (n=77), similar to that of nocodazole-treated cells lacking MTs. Since cells treated with 5 μM taxol show an increased MT mass, relative to the other drug concentrations, and exit mitosis substantially faster than those treated with 500 nM taxol, the mere presence of MTs does not enhance mitotic exit rate. Our preliminary data also suggests that BAB1, Mad1 and Mad2 are present in reconstituted nuclei of cells incubated with the higher taxol concentrations.

774 Regulation of E2F Function through Prohibitin-RNF2 Interaction: New Mechanism of E2F1 in Cell Cycle

D. Choi, Y. Bae, H. Choi, S. Kang; Graduate School of Life Sciences and Biotechnology, Korea University, Seoul, Democratic People's Republic of Korea

Prohibitin, the tumor suppressor protein, has an important role in transcriptional regulation of various genes involved in cell cycle control and proliferation. Recent studies reported that the growth-suppressive property of the prohibitin protein is exhibited by repressing the transcriptional activity of E2F family proteins. In this study, we show that RNF2, a member of Pcg (polycromb-group) proteins, regulates the prohibitin-mediated E2F1 transcriptional activity through the p16-Cdk4/cyclin D-Rb pathway. We found by co-immunoprecipitation experiments that prohibitin interacts with RNF2. Interestingly, the expressed amounts of prohibitin were affected interdependently at a post-transcriptional level. Furthermore, knock-down of either RNF2 or prohibitin by the RNAi technique increased the amount of p16, whereas overexpression of either RNF2 or prohibitin did not affect the expression of the p16 protein. Cell proliferation also was regulated by the prohibitin-RNF2 interaction. Based on these results, we will discuss a novel pathway of E2F1 activity regulation.

775 Conditioned Media of Lung Carcinoma Cell Line Induce the Morphological Change and the Inactivation of Myofibroblast, MRC-5 Cells

S. Hayakawa, T. Nakatani, E. Hondo, Y. Yano, S. Kumazawa, H. Munakata; 1Dept. of Biochemistry, Kinki University School of Medicine, Osaka, Japan, 2Dept. of Gastroenterology and Hepatology, Kinki University School of Medicine, Osaka, Japan, 3Life Science Research Institute, Kinki University, Osaka, Japan, *School of Food and Nutritional Sciences, University of Shizuoka, Shizuoka, Japan

A critical feature of fibrotic disease involving the pulmonary fibrosis is the appearance of fibroblast-like cells expressing α-smooth muscle actin (α-SMA) within the areas of active fibrosis. The expression of α-SMA suggests that the fibroblast have acquired characteristics of contractile cells. Such cells are termed myofibroblast. Several substances, such as PGE2, are known to cause the inactivation of myofibroblasts, but the mechanism of inactivation has not been fully understood yet. We found that the conditioned media of a human lung carcinoma cell line, A549, caused the inactivation of myofibroblast cell line, MRC-5. In the present study, we investigated the effect of the conditioned media of A549 on the inactivation of MRC-5 cell. The shape of the MRC-5 cells changed to stellate form after the treatment with the A549 conditioned media, along with the decrease inactivation of myofibroblasts, but the mechanism of inactivation has not been fully understood yet. We found that the conditioned media of a human lung carcinoma cell line, A549, caused the inactivation of myofibroblast. The substance was partially purified by HPLC. The identification of this substance by NMR is in progress. Our results may provide useful information on the kinase inhibitors were increased after 2 days by conditioned media treatment. FACS analysis showed that cell cycle arrest was induced at early stage of morphological change. We found that the cell cycle arrest by A549 conditioned media was induced at GO/G1 phase. Our interest has been then focused on the identification of substance that causes the inactivation of myofibroblast. The substance was partially purified by HPLC. The identification of this substance by NMR is in progress. Our results may provide useful information on the differentiation of myofibroblasts.

776 Covert Genetic Selections Identify Genes Which Regulate Yeast Growth

D. Wu, E. Townsley, A. M. Tartakoff; Pathology, Case Western Reserve University, Cleveland, OH

Genetic complementation strategies are well-suited to analyze cellular phenotypes of monogenic origin. We here show that related selections can identify single genes which contribute either positively or negatively to phenotypes which are not of monogenic origin. To implement this search procedure, we have developed mSPI (~“Microarray-Based Single Promoters-Inhibitors”). It is based on cDNA enrichment (‘covert selection”) rather than overt correction of cellular phenotype. It therefore can access relations and optimization strategies which are inaccessible via classical selections. We exemplify this use of mSPI by identifying a spectrum of ectopic cDNAs which favor (or oppose) growth of S. cerevisiae. For this purpose, we grow a pool of thousands of transformants which carry single plasmids from a yeast cDNA library. cDNAs which are present at the beginning of the experiment and those which persist are quantitated on microarrays. The resulting hierarchy of differential cDNA enrichment or depletion has a large dynamic range, due to the amplification of distinct cell subpopulations during culture. These data therefore make corresponding predictions as to which cDNAs stimulate or inhibit growth. Validation experiments confirm most of these predictions. mSPI thus provides a powerful, streamlined and unprejudiced genome-wide selection for cDNAs which can improve (or impair) cell performance. mSPI should be applicable to all cell types for which satisfactory libraries and microarrays are available. Identification of such genes is critical for orienting therapeutic interventions. Supported by the National Science Foundation (MCB-0431496) and by a Presidential Research Initiative Grant from Case Western Reserve University.

777 Role of Emi1 in the Regulation of the APC/C

B. Di Fiore, J. Pines; Wellcome Trust/Cancer Research UK, Gudron Institute, University of Cambridge, Cambridge, United Kingdom

Ubiquitin-mediated proteolysis is critical for the key regulatory steps in mitosis, and the Anaphase Promoting Complex/Cyclosome (APC/C) ubiquitin ligase plays a fundamental role in this regulation. APC/C is regulated at several levels, and is only active when complexed with Cdc20 or Cdh1, which contribute to the timing of APC/C activity. APC/C activity can be inhibited by Emi1, which is reported to bind Cdc20 and Cdh1. Thus, the destruction of Emi1 has been proposed to regulate the activation of APC/C at mitosis, and Emi1 synthesis to inactivate the APC/C for cells to initiate S phase. We are investigating the regulation and the role of Emi1 in mammalian cells, to understand better how APC/C activity is regulated in vivo. By time-lapse microscopy we observed that in living cells Emi1 degradation starts in prophase. However, in vivo, the timing of cyclin degradation in mitosis is not affected by Emi1, indicating that Emi1 does not inhibit mitotic APC/C activity. Moreover, we found that Emi1 is involved in the regulation of APC/C activity in interphase.
Genome-wide Analysis of Polyplody in Yeast: Scaling Effects and Genome Stability

Z. Storchova, A. Breneman, J. Cande, J. Dunn, K. Burkhank; E. O'Toole, D. Pellman; Dana-Farber Cancer Institute, Boston, MA, Harvard University, Cambridge, MA, University of Colorado, Boulder, CO, SUPRASYS, Bulgaria

Polyplody occurs during development, cellular stress, disease, and evolution. Despite its prevalence, little is known about the physiological alterations that accompany polyplody. We previously described "polyoid-specific lethality", where a gene deletion that is not lethal in haploid or diploid budding yeast causes lethality in triploids or tetraploids. Here, we report a genome-wide screen to identify ploidy-specific lethal functions. Only 39 in 3740 mutations screened exhibited ploidy-specific lethality. Almost all of these mutations affect genomic stability by impairing homologous recombination, sister chromatid cohesion, or mitotic spindle function. Because detailed analysis of genetic instability in wild-type tetraploids uncovered a dramatic increase in chromosome loss rates, we focused on chromosome segregation and mitotic spindle function in these cells. We show that wild-type tetraploids have a high incidence of syntenic/monopolar kinetochore attachments to the spindle pole. Although tetraploids had a highly increased requirement for Ipl1/Aurora B, Aurora B activity is not compromised. We therefore considered whether scaling effects on spindle geometry might explain the chromosome mis-segregation in tetraploid cells. Surprisingly, pre-anaphase spindle length is the same in haploids, diploids triploids and tetraploids, whereas electron tomography demonstrated that the surface area of the spindle poles doubles with increased ploidy. We suggest that mismatches in spindle length, kinetochore geometry and spindle pole body size can, at least in part, explain the high levels of chromosome loss in polyplod cells. Thus, genetic constraints may have profound effects on genome stability; the mechanisms described here may be relevant in a variety of biological contexts, including disease states such as cancer.

Replicative Aging in a Symmetrically Dividing Fission Yeast

A. Dereli, I. Tolec-Norrellykke; MPI-CBG, Dresden, Germany

Aging process in organisms is generally described by a decrease in the ability to respond to stress, a reduced capacity to produce offspring, and an increased risk of disease and death. One of the simplest organisms that undergoes replicative aging is budding yeast: individual cells die after about 20 divisions. Although this yeast has been widely used as an experimental system for investigating aging mechanisms, the results of those studies may not be directly relevant to higher eukaryotic cells that reproduce by symmetric division instead of budding. Here we investigate replicative aging in the fission yeast Schizosaccharomyces pombe which reproduces by apparently symmetric cell division. Our first objective was to see whether there is a difference in the division time and probability of death between older and younger cells. By our definition, older cells are those that inherit the old cell pole. We show that a single cell of fission yeast, starting its life from a spore, can undergo more than 50 divisions without showing aging phenomena. This work may help to elucidate mechanisms relevant for aging in more complex symmetrically dividing eukaryotic cells.

Acentrosomal Spindle Assembly in Cells with an Intact Interphase Microtubule Network

J. Hornick, C. Mader, E. Tebble, E. Hinchcliffe; Biological Sciences, University of Notre Dame, Notre Dame, IN

Several studies have demonstrated that bipolar mitotic spindles can form in the absence of intact centrosomes - their spindle assembly is nucleated by chromosomal factors. However, in these experimental studies, there is no pre-existing interphase microtubule network or MTOC. In contrast, normal spindle assembly in somatic cells occurs through the remodeling of an intact microtubule network that does not completely break down: instead the microtubules are drawn into the two spindle poles as they split and separate apart. To examine the role of the centrosome-containing "centrosome" in spindle assembly, BSC-1atubGFP cells were cut with a microcone between nucleus and centrosome generating karyoplasts (acentrosomal, nucleated cells); then followed by time-lapse fluorescence microscopy. Prior to the onset of mitosis, these cells re-form a single MTOC that nucleates an interphase microtubule network. These karyoplasts enter mitosis: ~70% of karyoplasts form a bipolar spindle and always cleave in the two. The generation of bipolarity in these acentrosomal cells appears to begin before nuclear envelope breakdown, as the two daughter cells are drawn into these daughter poles. In ~30%, the karyoplasts fail to form a bipolar spindle; the single interphase MTOC assembles a persistent monopolar spindle prior to NPB. Karyoplasts lacking spindles do initiate cytokinesis, assembling multiple furrows, which eventually retract, and the cell exits mitosis as a single daughter. Our observations reveal that in acentrosomal cells with an intact MTOC/microtubule network, spindle assembly begins before NEB as the single interphase MTOC assembles a functional acentrosomal spindle. This acentrosomal spindle is able to assemble a mitotic spindle through an acentrosomal pathway, but the molecular mechanism behind this process remains poorly understood. The acentrosomal spindle also has a role in establishing a bipolar mitotic spindle. To ensure spindle bipolarity, centrosome duplication must be precisely regulated. In this process, the coordinated dynamics of MTOCs functionally replaced centrosomes regarding microtubule nucleation and spindle organization. In ~30%, it is not possible to detect this process from a mechanistic perspective. Mitotic spindles in these cells show three main morphologies: 30 % are apparently normal bipolar spindles; 30 % barrel-shaped spindles and 40 % multipolar spindles. Importantly, they all lack astral microtubules, while γ-tubulin and centrosomin are often found as several foci near the poles and at the cell periphery. Experiments are on our way to investigate at high spatial and temporal resolution the process of spindle assembly in these cells by stable expression of GFP-α-tubulin. The ultimate goal will be to use the 1182-4 cells to perform a genome-wide RNAi screening for genes required for acentrosomal spindle assembly. For this purpose we have been concentrating on establishing RNAi conditions for these cells, as well as using phospho-histone H3 staining for automatic readout. We reasoned that inability to assemble a functional acentrosomal spindle would compromise mitotic progression in 1182-4 cells and lead to an increase in the mitotic index. Accordingly, preliminary results have indicated that 16 h of colchicine treatment cause a 10x increase in the mitotic index, indicating that phospho-histone H3 staining could be used as readout in the first step of the screening. Subsequently, a detailed analysis of spindle assembly will be performed after RNAi of candidate genes.

A New Model of Acentrosomal Spindle Assembly Revealed by 4D Imaging of Live Mouse Oocytes

M. Schuh, J. Ellenberg; Gene Expression Programme, European Molecular Biology Laboratory, Heidelberg, Germany

Homologous chromosome segregation in mammalian oocytes is driven by a microtubule spindle devoid of centrosomes. Our current understanding of acentrosomal spindle assembly is limited, and mainly based on experiments using cell free Xenopus egg extracts. Here, we present a new model of centrosome independent spindle assembly revealed by quantitative high resolution 4D imaging of fluorescently labeled chromosomes and microtubules in live maturing mouse oocytes. We show that spindle formation is based on the concerted action of more than 80 microtubule organizing centers (MTOCs) that formed de novo out of an interphase like microtubule network spanning the oocyte in prophase. Initially distributed throughout the oocyte cytoplasm, MTOCs rapidly bud off, and reorganized sequentially into several novel metastable intermediate assembly structures that led to the formation of a barrel-shaped bipolar spindle over the course of five hours. In this process, the coordinated dynamics of MTOCs functionally replaced centrosomes microtubule nucleation and spindle organization. Surprisingly, this acentrosomal spindle contained dynamic astral-like microtubules that appeared to participate in asymmetric spindle positioning by dynamically probing the cortex.

SZY-20 Regulates Centrosome Size and Centriole Assembly in C. elegans

M. Song, K. F. O'Connell; LBG, NIH/NNIDDK, Bethesda, MD

The central importance of cell division is transmitting a complete set of chromosomes to each daughter cell. In eukaryotes this is achieved via the function of the mitotic spindle. The centrosome serves as the primary microtubule-organizing center of the cell and plays an important role in establishing a bipolar mitotic spindle. To ensure spindle bipolarity, centrosome duplication must be precisely regulated. In this process, the coordinated dynamics of MTOCs functionally replaced centrosomes regarding microtubule nucleation and spindle organization. To identify additional factors that interact with ZYG-1 to regulate centrosome duplication, we performed a genetic screen for mutations that suppress the temperature-sensitive lethality of the zyg-1 (t252) mutation. The t252 mutation is among the suppressors identified and defines a gene szy-20 (for suppressor of zyg-1). szy-20 encodes a novel coiled-coil protein that localizes to the nucleus, nuclear periphery, cytoplasm and centrosome. The szy-20 (t252) mutation itself exhibits a strong temperature-sensitive embryonic lethality, marked by various defects in cell division including polar body splitting and separation, leading to a failure of spindle bipolarity. Thus, we conclude that a major function of the centriole-containing centrosome is to ensure spindle bipolarity.
formation, prometaphase positioning, A-P polarity, cytokinesis, and cell cycle timing. We further found that the zyg-1 (n25) mutation also suppresses the embryonic lethality of zyg-20 (hs52), demonstrating that there is mutual suppression between these two genes. That is while the zyg-20 (hs52) mutation restores centriole duplication to the zyg-1 (n25) mutant, the zyg-1 (n25) mutation rescues the prometaphase rotation and cytokinesis defects seen in zyg-20 (hs52) mutants. To understand how zyg-20 interacts with zyg-1 as a suppressor, we have analyzed centrosomes in the zyg-20 (hs52) mutant and found that the amount of pericentriolar material (PCM) at centrosomes is significantly increased. Our data indicate that SZY-20 limits centrosome size by negatively regulating PCM turnover. Thus, loss of SZY-20 activity likely restores centriole duplication in zyg-1 mutants by allowing increased amounts of ZYG-1 at centrosomes.

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Requirement of Hc3enxin for Proper Mitotic Functions of Polo-like Kinase 1 at the Centrosomes

N. Seong1, Y. H. Kang1, K. Kamijo1, Y. Seong1, Y. Kao2, T. Mikiz, S. R. Kim, R. Kuriyama1, C. Giamb, K. S. Lee1, 1Metabolism, NIH/NCI, Bethesda, MD, 2Cell Biology, NIH/NCI, Bethesda, MD, 3Microbiology and Immunology, Uniformed Services University of the Health Sciences, Bethesda, MD, 4Biochemistry and Molecular Biology, Chungbuk National University, Cheongu, Republic of Korea, 5Genetics, Cell Biology and Development, University of Minnesota, Minneapolis, MN

Outer dense fiber 2 (ODf2) was initially identified as a major component of sperm tail cytoskeleton, and later was suggested to be a widespread component of centrosomal scaffold that preferentially associates with the appendages of the mother centrioles in somatic cells. Here we report the identification of two ODf2-related centrosomal components, Hc3enxin1 and Hc3enxin1 variant 1, that possess a unique C-terminal extension. Our results showed that Hc3enxin1 is the major isoform expressed in HeLa cells, whereas ODf2 is not detectably expressed. Mammalian pole-like kinase 1 (Plk1) is critical for proper mitotic progression and its association with the centrosome is important for microtubule nucleation and function. Interestingly, depletion of Hc3enxin1 by RNAi delocalized Plk1 from the centrosomes and the C-terminal extension of Hc3enxin1 was crucial to recruit Plk1 to the centrosomes through a direct interaction with the polo-box domain of Plk1. Consistent with these findings, the Hc3enxin1 RNAi cells exhibited weakened γ-tubulin localization and chromosome segregation defects. We propose that Hc3enxin1 is a critical centrosomal component whose C-terminal extension is required for proper recruitment of Plk1 and other components crucial for normal mitosis. Our results further suggest that the anti-ODf2 immunoreactive centrosomal antigen previously detected in non-germline cells is likely Hc3enxin1.

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CEP192 Is Required for the Assembly and Function of Mitotic Centrosomes

M. A. Gomez-Ferreria1, D. R. Rines1, D. J. Sharp1 1Physiology and Biophysics, Albert Einstein College of Medicine, Bronx, New York, NY, 2Genomics Institute of the Novartis Research Foundation, San Diego, CA

The centrosome is the major microtubule organizing center (MTOC) in animal cells. During mitosis, the nucleation of microtubules from centrosomes drives spindle assembly and chromosome capture and segregation. CEP192 is a centrosomal protein initially identified in a proteomic analysis of the human centrosome (Andersen et al., 2003) and recently implicated as an important mitotic protein in a human genome-wide screen (Rines et al., 2006). To gain insight into the specific functions of CEP192, we have examined the function and composition of centrosomes throughout the cell cycle following siRNA knockdown of CEP192. Following the depletion of CEP192, MTOCs disperse at the onset of prophase, and spindle microtubules appear to nucleate form chromosomes or the remnants of the nuclear envelope. The resulting spindle fibers are severely deformed and generally do not complete mitosis. Immunofluorescence indicates that centrioles are still present in these cells but the pericentriolar material (PCM) proteins gamma-tubulin and pericentrin. Moreover, mother and daughter centrioles were often observed abnormally dissociated from one another during mitosis. We propose a model in which CEP192 stabilizes the centrosome by fixing together both centrioles and the PCM to the centriole pair. Why the role of CEP192 is specific for mitosis is currently under investigation.

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The RanGTP Gradient Is Sufficient for Mitotic Spindle Assembly in Xenopus Egg Extracts

D. R. Halpin, P. Kalab, K. Weis, R. Heald1 MCB, University of California, Berkeley, California, CA

Mitotic chromosomes generate biochemical signals that promote spindle assembly. This mechanism is most apparent in systems lacking centrosomes, such as metaphase-arrested Xenopus egg extracts. Chromatin itself, in the form of plasmid-DNA coated beads, is sufficient to induce spindle assembly in egg extracts, but the molecular pathways behind this phenomenon are not well understood. One known component of the chromatin pathway is the Ran GTPase, which like other small GTPases of the Ras superfamily, cycles between GTP- and GDP-bound forms. Ran’s guanine nucleotide exchange factor, RCC1, is localized to chromosomes while its GTPase activating protein, Ran GAP and co-factors are cytosolic. During mitosis, this distribution of GEF and GAP activities creates a gradient of RanGTP/RanGDP with the concentration of RanGTP highest at the chromosomes and decreasing abruptly as the distance from the chromosomes increases. While mitotic functions for the RanGTP gradient have been demonstrated, the sufficiency of this pathway for spindle assembly is unclear. We have developed RCC1 microbeads with high enzymatic activity that generate RanGTP gradients in egg extracts. RCC1 beads are sufficient to induce microtubule polymerization and organization into bipolar arrays in the absence of other chromatin components. Structural and functional analyses of RCC1 bead-generated spindles are currently underway.

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Plasticity of the Centromere in Vertebrates: Implications for the Mechanism of Spindle Assembly

O. Kisurina-Evgenieva1, P. Hergert1, J. Loncarek1, T. Vinogradova1, S. La Terra1, T. Kapoor2, A. Khodjakov1 1Wadsworth Center, Albany, NY, 2The Rockefeller University, New York, NY

The vertebrate centromere is a unique structure that governs mitotic chromosome movement. Centromeres can be either facultative (i.e., zebrafish) or obligate (i.e., human) and they can exhibit a wide variety of morphologies. One known component of the chromatin pathway is the Ran GTPase, which like other small GTPases of the Ras superfamily, cycles between GTP- and GDP-bound forms. Ran’s guanine nucleotide exchange factor, RCC1, is localized to chromosomes while its GTPase activating protein, Ran GAP and co-factors are cytosolic. During mitosis, this distribution of GEF and GAP activities creates a gradient of RanGTP/RanGDP with the concentration of RanGTP highest at the chromosomes and decreasing abruptly as the distance from the chromosomes increases. While mitotic functions for the RanGTP gradient have been demonstrated, the sufficiency of this pathway for spindle assembly is unclear. We have developed RCC1 microbeads with high enzymatic activity that generate RanGTP gradients in egg extracts. RCC1 beads are sufficient to induce microtubule polymerization and organization into bipolar arrays in the absence of other chromatin components. Structural and functional analyses of RCC1 bead-generated spindles are currently underway.

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The Ndc80 Complex and Dynein-dependent Mechanisms of Kinetochore Attachment to Microtubules during Mitosis

V. V. Vorozhko1, M. Kallio2, M. J. Emanuele1, P. T. Stukenberg2, G. J. Gorbsky1 1Cell and Developmental Biology, Oklahoma Medical Research Foundation, Oklahoma City, OK, 2Genomics Institute of the Novartis Research Foundation, San Diego, CA

Kinetochore dynamics are essential for accurate chromosome segregation during mitosis. Kinetochores must efficiently capture and stabilize microtubules to ensure proper chromosome segregation. The Ndc80 complex is a conserved component of kinetochores that has been implicated in microtubule capture and maintenance. Microtubule capture is thought to be mediated by the Ndc80 complex, which is required for proper attachment of kinetochores to microtubules. In the absence of centrosomes, kinetochores must form bipolar spindles independently of microtubule capture. We have been investigating the role of the Ndc80 complex in microtubule capture during mitosis in the absence of centrosomes. We have found that the Ndc80 complex is required for proper attachment of kinetochores to microtubules during mitosis in the absence of centrosomes. This result has important implications for our understanding of the mechanisms that govern kinetochore attachment to microtubules.
Spatial Heterogeneity of Microtubule Dynamics in Metaphase *Xenopus laevis* Egg Extract Spindles Reveals Organization of Different Driving Mechanisms

G. Yang,1 L. A. Cameron,2 P. S. Maddox,1 E. D. Salmon,2 G. Danuser1; 1Dept of Cell Biology, The Scripps Research Institute, La Jolla, CA, 2Dept of Biology, Univ. of North Carolina, Chapel Hill, NC, 1Dept of Cellular and Molecular Medicine, UCDSD, La Jolla, CA

Steady poleward flux is a fundamental property of microtubule dynamics in metaphase spindles of higher eukaryotic cells. Understanding its molecular mechanisms is essential to understanding the regulation of metaphase spindle structure and generation of chromosome alignment and segregation force. We use quantitative fluorescent speckle microscopy to obtain high-resolution readouts of the spatial and temporal dynamics of microtubule flux in control and perturbed *Xenopus laevis* egg extract spindles. Our results show that microtubule flux is spatially heterogeneous in control spindles, with a systematic slowdown of ~20% in flux rate near the poles. Statistical clustering analysis confirms that the flux distribution follows a mixture of two normal distributions. Similar flux rate and spatial heterogeneity are also observed in plasmid DNA-coated magnetic bead spindles, suggesting that such heterogeneity is independent of microtubules and centrosomes. Inhibition of kinesin 5 using monastrol at low concentration significantly reduces flux rate and changes the spatial distribution of microtubule flux. However, further increase in monastrol concentration does not cause further changes in flux rate and spatial distribution. These results support an organizational model of barrel-like spindle architecture with antiparallel microtubule fibers in the middle connected with focused microtubule fibers near the pole. In addition, these results indicate that microtubule flux is driven by the synergy of kinesin 5-based antiparallel microtubule sliding in the middle and additional monastrol-insensitive mechanisms near the poles.

E-cadherin as a Spatial Cue to Orientate Cell Division

N. den Elzen, C. Buttery, A. Yap; Institute for Molecular Bioscience, University of Queensland, St Lucia, Qld, Australia

Ordinary cell division is critical for precise tissue patterning both during development and in post-developmental life. Simple epithelia, symmetrical division of cells within the plane of the monolayer is likely to be important to preserve tissue architecture. The plane of cell division is determined by the orientation of the mitotic spindle, which in turn depends on spindle interactions with the cell cortex. In dividing mammalian cells in vitro, mitotic spindle orientation has been shown to be affected both by cell shape, orienting along the long axis of the cell, and by extracellular cues, orienting towards the extracellular matrix. However, in cultured sheets of polarised columnar epithelial cells, mitotic spindles orient in the plane of the epithelium, perpendicular to the long axis of the cell and parallel to the extracellular matrix at the basal surface, rather than towards it. Clearly, other mechanisms must exist to orient spindles in the plane of the monolayer in symmetrical divisions of epithelial cells. E-cadherin is an epithelial cell surface receptor required for cell-cell adhesion and the establishment of cell polarity, whose function influences normal cleavage plane orientation in *Drosophila* and zebralis embryos. As a key morphogenetic regulator concentrated at adherens junctions at the apical/basolateral border of cells, it is an attractive candidate cortical cue to orientate spindles in the plane of the epithelial monolayer. We found, using E-cadherin siRNA, mutants and antibody blocking experiments, that E-cadherin acts as a spatial cue, orienting mitotic spindles in the plane of the monolayer in symmetric divisions of MDCK and MCF10a cells. Effects of disrupting E-cadherin on the localisation of NuMA, LGN and other cortical and spindle-associated proteins implicated in spindle dynamics, and their likely role downstream of E-cadherin in orientating spindles in the plane of the monolayer will also be presented.

Inhibition of Centrosome-dependent Microtubule Plastization and Block of Mitosis by the Small Organic Compound HMN-176

M. DiMaio,1 A. Mikhailov,2 C. Rieder,2 R. E. Palazzo2; 1Department of Biology and Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY, 2Wadsworth Center-New York State Dept. of Health, Albany, NY

HMN-176, a small organic compound that promotes centrosome-dependent cell cycle arrest, was shown to delay the G2/M transition in tumor cells. We have characterized the effects of HMN-176 on spindle assembly in *Spisula solidissima* oocytes and mammalian tumor cells. HMN-176 blocked aster formation and spindle assembly in live *Spisula* oocytes but had no effect on nuclear envelope breakdown or chromosome structure. The drug inhibited centrosome-dependent aster assembly in cytoplasmic extracts prepared from activated oocytes. Importantly, HMN-176 inhibited the ability of isolated *Spisula* centrosomes to nucleate microtubules and organize asters in defined media, an effect that is reversible following drug removal. HMN-176 had no significant effect on the polymerization of either three cycled *Spisula* oocyte tubulin, sea urchin egg tubulin, or glutamate-purified tubulin from these sources. Treatment of human CFPAC-1 cells with HMN-176 resulted in a significant delay in M-phase of mitosis, but had no effect on the timing of any other phase of the cell cycle. Importantly, immunofluorescence analysis of CFPAC-1 cells revealed that HMN-176 induced the assembly of multipolar spindles, perhaps due to inhibition of centrosome function. Finally, HMN-176 inhibited the ability of isolated human HeLa S3 centrosomes to nucleate microtubules and assemble asters in defined media. We conclude that HMN-176 inhibits centrosome-dependent microtubule nucleation. We hypothesize that the mechanism of action of HMN-176 is to inhibit interactions between α/β-tubulin dimers and γ-tubulin or γ-tubulin ring complexes, which are required for centrosome-dependent microtubule nucleation. Importantly, this effect is reversible. To our knowledge this is the first report of an anti-centrosome drug and the first demonstration that the centrosome is a potential target for therapeutics.

Disruption of Cell Adhesion and Mitotic Delay in Paclitaxel-induced Apoptosis

E. Yoon, S. Yu, J. Lee, W. Lee, S. Kim; Kongju National University, Gongju, Republic of Korea

Paclitaxel (Taxol), an antimicrotubule agent, promotes the formation of highly stable microtubules that resist depolymerization by specifically binding to the N-terminal region of α/β-tubulin dimers and γ-tubulin ring complexes, which are required for centrosome-dependent microtubule nucleation. HMN-176 blocked aster formation and spindle assembly in live *Spisula* oocytes but had no effect on nuclear envelope breakdown or chromosome structure. The drug inhibited centrosome-dependent aster assembly in cytoplasmic extracts prepared from activated oocytes. Importantly, HMN-176 inhibited the ability of isolated *Spisula* centrosomes to nucleate microtubules and organize asters in defined media, an effect that is reversible following drug removal. HMN-176 had no significant effect on the polymerization of either three cycled *Spisula* oocyte tubulin, sea urchin egg tubulin, or glutamate-purified tubulin from these sources. Treatment of human CFPAC-1 cells with HMN-176 resulted in a significant delay in M-phase of mitosis, but had no effect on the timing of any other phase of the cell cycle. Importantly, immunofluorescence analysis of CFPAC-1 cells revealed that HMN-176 induced the assembly of multipolar spindles, perhaps due to inhibition of centrosome function. Finally, HMN-176 inhibited the ability of isolated human HeLa S3 centrosomes to nucleate microtubules and assemble asters in defined media. We conclude that HMN-176 inhibits centrosome-dependent microtubule nucleation. We hypothesize that the mechanism of action of HMN-176 is to inhibit interactions between α/β-tubulin dimers and γ-tubulin or γ-tubulin ring complexes, which are required for centrosome-dependent microtubule nucleation. Importantly, this effect is reversible. To our knowledge this is the first report of an anti-centrosome drug and the first demonstration that the centrosome is a potential target for therapeutics.

Microtubule-dependent Spindle Pole Body Positioning in Interphase Ensures Proper Mitotic Spindle Alignment

I. Raabe, S. K. Vogel, I. Tolic-Norrelykke; MPI-CBG, Dresden, Germany

In all eukaryotic cells, spindle orientation perpendicular to the cleavage plane is important for a successful segregation of complete chromosome sets into daughter cells. It is not well understood when and how a spindle becomes properly aligned within the cell. We explored the dynamics, the causes and effects of spindle alignment in a single-cell assay throughout mitosis in the fission yeast *Schizosaccharomyces pombe*. We show that the spindle is aligned with the cell axis already at the beginning of spindle formation. During prometaphase this alignment is lost to some extent through apparently random movements of the spindle. Anaphase onset is not delayed in cells with misaligned spindles compared to cells with well-aligned spindles, which is not consistent with a spindle orientation checkpoint. Contrary to the wild-type cells, in a set of mutants with microtubule-related defects the spindle was misaligned at the onset of mitosis. Our results thus suggest a novel role for interphase microtubules: besides determining the nuclear and hence the division plane position, they also determine the initial alignment of the mitotic spindle.

Elucidating the Chromosomal Functions of Linker Histone H1 throughout the *Xenopus* Egg Extract Cell Cycle

B. S. Freedman; Molecular and Cell Biology, UC Berkeley, Berkeley, CA

H1 “linker” histones help compact beads-on-a-string nucleosomal arrays into a thicker chromatin fiber. However, it is unclear how H1 affects higher order chromosome architecture, or whether linker histone function is regulated in a cell-cycle dependent fashion. We have examined the structure and function of mitotic and interphase sperm chromosomes lacking histone H1 using.
**Cortical Granule Exocytosis Contributes to Eggshell Formation and Requires Separase Activity in C. elegans**

J. N. Bembenek, J. M. Squarrell, J. G. White; Molecular Biology, UW Madison, Madison, WI

Separase is a widely conserved protease that promotes chromosome segregation by cleaving a subunit of the cohesin complex that tethers sister chromatids together. In several systems, separase also promotes late anaphase events independent of its role in chromosome segregation. Inactivation of the *C. elegans* orthologue of separase, sep-1, causes not only chromosome nondisjunction but also osmotic and mechanical sensitivity due to abnormal eggshell formation. To investigate novel functions of separase in *C. elegans*, we analyzed the nature of the eggshell defect. Newly fertilized wild-type embryos are permeable and lack a chitinous eggshell whereas an impermeable eggshell is present by the first mitosis. Live cell imaging revealed a wave of exocytosis during anaphase of meiosis I which initiates near the mitotic spindle then spreads over the entire embryo. By TEM a unique population of cortical vesicles was observed in these embryos. Analysis with both fluorescent-labeled lectins and a GFP fusion to a resident Golgi protein also showed unique patterns in pre-meiosis I embryos. Wheat germ agglutinin, which recognizes N-acetylglucosaminase, the precursor for chitin, labeled vesicles in meiosis I embryos and the eggshell in older embryos. Based on these data, we conclude that eggshell components are deposited by cortical granule exocytosis. Other osmotic and mechanically sensitive mutants identified by genome-wide RNAi screens exhibited defects in the formation, movement or exocytosis of cortical granules. In particular, depletion of sep-1 led to the retention of cortical granules. Interestingly, we found that SEP-1-GFP not only localized to chromosomes but also colocalized with the outer kinetochore protein HIM-10 to cortical filaments. During anaphase of meiosis I, SEP-1-GFP moved from these filaments to cortical granules and was lost during the exocytic wave. These data indicate that separase may regulate cortical granule exocytosis through a pathway that coordinates chromosome segregation with anaphase events in *C. elegans*.

**Kinetochores Activity Determines the Mechanism of Pole Organization in Mammalian Cells**

A. L. Manning, D. A. Compton; Dartmouth Medical School, Hanover, NH

Tightly focused spindle poles are required for chromosome movement in mammalian cells, and it has been suggested that spindle poles anchor microtubules to resist poleward force generated by kinetochore (Pac-man activity). Pac-man activity is predicted to generate significant force, and we hypothesize that poleward force from kinetochores would influence the mechanism of spindle pole focusing. To test this idea, we examined spindle pole organization in cells lacking specific kinetochore proteins after inhibition of the pole focusing proteins NuMA and HSET. We show that cells deficient in the outer kinetochore protein Nu2 build focused spindle poles despite inhibition of NuMA alone or both NuMA and HSET. In contrast, spindle poles are splayed following inhibition of NuMA and HSET activity in cells deficient in MCAK, CENP-B, or poleward microtubule flux. This demonstrates a specific kinetochore activity associated with Nu2 that influences the requirements for spindle pole focusing. Interestingly, many chromosomes in cells deficient in Nu2, NuMA and HSET activities build Ca++-stable kinetochore fibers. However, those kinetochores lack Pac-man activity because the intercentromere distance is reduced and chromosome movement is suppressed. Thus, active focusing of microtubules at spindle poles by motor activities associated with NuMA and HSET is only required when Pac-man activity is present at kinetochores. In the absence of Pac-man activity, passive crosstalks between spindle microtubules and astral microtubules emanating from centrosomes are most likely responsible for the appearance of focused poles.

**Self-regulated Plk1 Recruitment to Kinetochores by the Plk1-PBP1 Interaction Is Critical for Proper Chromosome Segregation**

Y. H. Kang, J. Park, L. Yu, N. Soung, S. Yun, J. K. Bang, Y. Seong, H. Yu, S. Garfield, T. D. Veenstra, K. S. Lee; 1Biochemistry, Stanford University School of Medicine, Stanford, CA, 2Proteomics and Analytical Technologies, National Cancer Institute, Bethesda, MD, 3Pharmacology, University of Texas Southwestern Medical Center, Dallas, TX, 4Experimental Carcinogenesis, National Cancer Institute, Bethesda, MD, 5Mammalian polo-like kinase 1 (Plk1) has been studied extensively as a key element in regulating cellular proliferation. The polo-box domain (PBD) of Plk1 has an essential role in targeting the catalytic activity of Plk1 to specific subcellular structures critical for mitosis. However, how Plk1 is recruited to the kinetochores and what Plk1 does at this site remain elusive. Here we demonstrated that a novel PBD-binding protein, PBP1, is crucial for recruiting Plk1 to the interphase and mitotic kinetochores. Unprecedently, Plk1 phosphorylated PBP1 at T78, creating a self-tethering site that specifically interacted with the PBD of Plk1, but not with the PBD of Plk2 or Plk3. Later in mitosis, Plk1 also induced PBP1 degradation in a T78-dependent manner, thereby enabling itself to interact with other components critical for proper kinetochore functions. Absence of the p-T78-dependent PBP1 localization induced a chromosome congressing defect and compromised the spindle checkpoint due to diminished levels of 3F3/2 epitope and improper localization of Mad2, thus ultimately leading to aneuploidy. We propose that Plk1 self-regulates the Plk1-PBP1 interaction to timely localize to the kinetochores and this event is critical for promoting chromosome congressing while contributing to spindle checkpoint activation.

**Astral Microtubules Positively Influence Contractile Ring Assembly at the Cell Equator during Anaphase**

L. I. Strickland, K. G. Thorne, D. R. Burgess; 1Biochemistry, Stanford University School of Medicine, Stanford, CA, 2Department of Biology, Boston College, Chestnut Hill, MA

In dividing echinoderm eggs, contact between astral microtubules (MTs) and the cell cortex during anaphase results in spatial and temporal specification of cytokinesis. Two models make opposite predictions regarding the role of astral MTs in furrow initiation. The equatorial stimulation model proposes that astral MTs oriented toward the equator promote cytokinesis. Alternatively, the polar relaxation model suggests that astral MTs oriented toward the polar cortex suppress cytokinesis by locally relieving cortical tension. To test these models, we locally perturbed the interaction between astral MTs and the cell cortex by applying nocodazole to either the equatorial or polar surface of anaphase-stage eggs. Microinjection of GFP-EBi1 allowed for observation of astral MT elongation and microinjection of a GFP actin binding domain (GFP-eABD) allowed for observation of the assembly and activity of the contractile ring. Application of nocodazole to the equatorial surface of anaphase-stage eggs resulted in suppression of cytokinesis at the site of nocodazole exposure. Application to the polar surface caused initial displacement of the contractile ring toward the opposite pole, followed by repositioning toward the plane of the former metaphase plate during ingression. Our findings support the equatorial stimulation model, but raise the possibility for a spindle midzone signal, for furrow ingression.

**Phosphoregulation of the Essential E. pomea Contractile Ring Protein Cdc15**

R. H. Roberts, C. A. Thorne, J. Chen, K. L. Gould; Department of Cell and Developmental Biology, HHMI & Vanderbilt University Medical School, Nashville, TN

For cells to undergo cytokinesis with appropriate timing, all scaffolding and cytoskeletal proteins that ultimately form the contractile actin ring must arrive, function, constrict and dissociate in a coordinated and tightly controlled manner. In the fission yeast, PCh-family founding member Cdc15 is essential for cytokinesis. We propose that Plk1 self-tethering site that specifically interacted with the PBD of Plk1, but not with the PBD of Plk2 or Plk3. Later in mitosis, Plk1 also induced PBP1 degradation in a T78-dependent manner, thereby enabling itself to interact with other components critical for proper kinetochore functions. Absence of the p-T78-dependent PBP1 localization induced a chromosome congressing defect and compromised the spindle checkpoint due to diminished levels of 3F3/2 epitope and improper localization of Mad2, thus ultimately leading to aneuploidy. We propose that Plk1 self-regulates the Plk1-PBP1 interaction to timely localize to the kinetochores and this event is critical for promoting chromosome congressing while contributing to spindle checkpoint activation.
800 Mitosis-specific Mechanosensing and Contractile Protein Redistribution Control Cell Shape
J. C. Effer, Y. Kee, J. Berk, M. Tran, P. Iglesias, D. Robinson; 1Electrical Engineering, Johns Hopkins University, Baltimore, MD, 2IMMH, Johns Hopkins University, Baltimore, MD, 3Cell Biology, Johns Hopkins University, Baltimore, MD

Cells utilize numerous mechanisms to control their progression through the cell cycle. Cell division is typically portrayed as a well-ordered sequence of biochemical events. However, cytokinesis, an inherently mechanical process, must be mechanically controlled to ensure that two equivalent daughter cells are produced with high fidelity. We hypothesized that cells have the ability to sense and correct shape asymmetries from applied forces by utilizing mechanosensing and mechanical feedback. Because the mitotic spindle and myosin-II mechanozyme are vital to many aspects of cell division; we explored the roles of these proteins in responding to shape perturbations due to cell division. Using micropipette aspiration, we demonstrate that the contractile-ring protein, myosin-II and centralspindlin, redistribute in response to shape perturbations only during anaphase through cytokinesis. Further, the response is independent of microtubules and spindle orientation. Dividing cells require myosin-II to correct shape perturbations; myosin-II null cells exhibit higher failure rates and divide grossly asymmetrically under mechanical load. In addition, this response does not depend upon extracellular calcium. Our data provide evidence for a mechanosensory system that directs contractile proteins to regulate cell shape during anaphase through cytokinesis completion. This study establishes a framework for a systems-level analysis of the biochemical and mechanical regulation of cell shape that enables robust cytokinesis.

801 Assembly of the Cytokinetic Contractile Ring from a Broad Band of Nodes in Fission Yeast
J. Wu, MCDH Yale University, New Haven, CT

We observed live fission yeast expressing pairs of fluorescent fusion proteins to test the popular models that the cytokinetic contractile ring assemblies from a single myosin-II progenitor or a Cdc12p-Cdc15p spot. Under our conditions anillin-like protein Mid1p establishes a broad band nodes in the equatorial cortex. These nodes mature by the addition of conventional myosin-II (Myo2p, Cdc4p, and Rll1p), IQGAP (Rag2p), PCH protein (Cdc15p), and formin (Cdc12p). We find that most of the nodes contain all the seven proteins. The node formation depends on Mid1p but not on actin filaments. We did not observe assembly of contractile rings by extension of a leading cable from a single spot or progenitor. These results suggest that nodes might be the basic units for contractile-ring assembly. We propose a lateral contraction model to explain our data. Formin Cdc12p nucleates actin filaments and remain attached to their barbed ends in vitro so they may also anchor the filaments in nodes of dividing cells. As actin subunits add to barbed ends associated with Cdc12p, we presume that the pointed ends of these filaments radiate from nodes. Actin filaments from one node might encounter myosin-II in adjacent nodes. Movement of myosin-II toward the barbed end of an actin filament anchored in an adjacent node might pull nodes together as they coalesce into a compact ring. Observation of actin filaments using GFP-tagged calponin motif of IQGAP Rag2p during coalescence supports our model. Our lateral contraction model also shares some features with models of cytokinesis in animal cells. Since proteins are spatially and temporally correlated during node formation, they might physically interact with each other. We are investigating the physical interactions between Mid1p and other node proteins using genetic and biochemical methods. This will be another important step to establish a quantitative model to understand cytokinesis.

802 Independent Functions of Centralspindlin and the Chromosomal Passenger Complex in Cytokinesis Ring Assembly and Constriction
L. K. Lewellyn, A. Maddox, A. Desai, K. Oegerle; Cellular and Molecular Medicine, Ludwig Institute for Cancer Research, La Jolla, CA

An integral part of cell division is the separation of daughter cells via cytokinesis. Recent data have demonstrated that membrane delivery to the cleavage furrow plays a key role in furrowing and abscission. Indeed, we have previously shown that Fip3-dependent endosome targeting to the cleavage furrow is required for abscission in mammalian cells. While Fip3 is implicated in regulating cytokinesis, it remains unclear how FIP3 is targeted to the cleavage furrow and regulates abscission. We speculate that FIP3 interacts and possibly regulate other proteins involved in cytokinesis(107,409),(898,450). To test this we have immunoprecipitated FIP3 with anti-FIP3 antibodies and used mass spectrometry to identify its binding partners. Interestingly, MgcRacGAP/CYK4 was identified as a putative FIP3-interacting protein. It has been established that MgcRacGAP/Cyk4 binds to MKLP1 to form a central spinidlin complex that is known for its role in cytokinesis. Mapping the interaction domains revealed that Fip3 binds to N-terminus part of MgcRacGAP/Cyk4. Interestingly, the same MgcRacGAP/Cyk4 domain was implicated in binding Ect2 (Rho GEF). The centralspindlin-dependent recruitment of Ect2 to the midzone of dividing cells appears to play a key role in activation of RhoA, a key regulator of cytokinesis. Here we show that MgcRacGAP/Cyk4 domain is implicated in binding Ect2 (Rho GEF). The centralspindlin-dependent recruitment of Ect2 to the midzone of dividing cells appears to play a key role in activation of RhoA, a key regulator of cytokinesis. We hypothesized that cells have the ability to sense and correct shape asymmetries from applied forces by utilizing mechanosensing and mechanical feedback. Because the mitotic spindle and myosin-II mechanozyme are vital to many aspects of cell division, we explored the roles of these proteins in responding to shape perturbations due to cell division. Using micropipette aspiration, we demonstrate that the contractile-ring protein, myosin-II and centralspindlin, redistribute in response to shape perturbations only during anaphase through cytokinesis. Further, the response is independent of microtubules and spindle orientation. Dividing cells require myosin-II to correct shape perturbations; myosin-II null cells exhibit higher failure rates and divide grossly asymmetrically under mechanical load. In addition, this response does not depend upon extracellular calcium. Our data provide evidence for a mechanosensory system that directs contractile proteins to regulate cell shape during anaphase through cytokinesis completion. This study establishes a framework for a systems-level analysis of the biochemical and mechanical regulation of cell shape that enables robust cytokinesis.

803 Sequential Binding of Ect2 and Fip3 to Centralspindlin Complex Regulates Furrowing and Abscission during Cytokinesis
G. C. Simon, E. Schonteich, C. C. Wu, M. Glotzer, R. Prekeris; 1Cell and Developmental Biology, University of Colorado Health Sciences Center, Aurora, CO, 2Pharmacology, University of Colorado Denver Health Sciences Center, Aurora, CO, 3Cell Biology, University of Chicago, Chicago, IL

Assembly of the Cytokinetic Contractile Ring Complex

An integral part of cell division is the separation of daughter cells via cytokinesis. Recent data have demonstrated that membrane delivery to the cleavage furrow plays a key role in furrowing and abscission. A recently identified Fip3 protein is a member of FIP protein family and has homology to the Drosophila protein, Cytoplasmic enhancer of cell shape (Cez). While Fip3 is implicated in cytokinesis, it remains unclear how FIP3 is targeted to the cleavage furrow and regulates abscission. We speculate that FIP3 interacts and possibly regulate other proteins involved in cytokinesis. To test this we have immunoprecipitated FIP3 with anti-FIP3 antibodies and used mass spectrometry to identify its binding partners. Interestingly, MgcRacGAP/CYK4 was identified as a putative FIP3-interacting protein. It has been established that MgcRacGAP/Cyk4 binds to MKLP1 to form a central spinidlin complex that is known for its role in cytokinesis. Mapping the interaction domains revealed that Fip3 binds to N-terminus part of MgcRacGAP/Cyk4. Interestingly, the same MgcRacGAP/Cyk4 domain was implicated in binding Ect2 (Rho GEF). The centralspindlin-dependent recruitment of Ect2 to the midzone of dividing cells appears to play a key role in activation of RhoA, a key regulator of cytokinesis. We hypothesized that cells have the ability to sense and correct shape asymmetries from applied forces by utilizing mechanosensing and mechanical feedback. Because the mitotic spindle and myosin-II mechanozyme are vital to many aspects of cell division, we explored the roles of these proteins in responding to shape perturbations due to cell division. Using micropipette aspiration, we demonstrate that the contractile-ring protein, myosin-II and centralspindlin, redistribute in response to shape perturbations only during anaphase through cytokinesis. Further, the response is independent of microtubules and spindle orientation. Dividing cells require myosin-II to correct shape perturbations; myosin-II null cells exhibit higher failure rates and divide grossly asymmetrically under mechanical load. In addition, this response does not depend upon extracellular calcium. Our data provide evidence for a mechanosensory system that directs contractile proteins to regulate cell shape during cytokinesis completion. This study establishes a framework for a systems-level analysis of the biochemical and mechanical regulation of cell shape that enables robust cytokinesis.

804 Cytokinetic Abscission Proceeds by Asymmetric Midbody Disassembly
P. Steinemann, S. Maar, D. Gerlich; Institute of Biochemistry, ETH Zurich, Zurich, Switzerland

Cytokinetically induced furrow ingression and subsequent completion of cytokinesis by abscission are temporally separated events in many mammalian cell types. The mechanism of abscission is unknown, but both membrane and cytoskeletal dynamics at the midbody have been proposed to drive abscission. Here, we have investigated by quantitative live cell microscopy a collection of fluorescent reporter cell lines expressing the midbody and cytoskeletal proteins. We found that abscission proceeds by rapid asymmetric disassembly of midbody microtubule bundles, accompanied by secretory vesicle fusion with the plasma membrane. Asymmetric midbody disassembly depended on active Aurora B, as chemical inhibition by Hesperadin led to premature and symmetric midbody microtubule disassembly. Using photobleaching assays, we further show that midbody microtubules were generally stable. Dynamic microtubules were strictly confined to two narrow bands within the central midbody, adjacent to, but not overlapping with midbody-localized chromosomal passenger complex. Inhibition of Aurora B increased microtubule dynamics throughout the entire midbody. Based on these observations, we propose a model where abscission is initiated by midbody localization of Aurora B, leading to asymmetric midbody disassembly.

805 Cep55 Associates with Centralspindlin to Control the Midbody Integrity and Cell Abscission during Cytokinesis
W. Zhao, A. Seid, G. Fang; Biological Sciences, Stanford University, Stanford, CA

We have developed an efficient functional screen for novel mitotic regulators by combining information on the gene expression profiling, cellular localization and loss-of-function studies. Our genomic analysis was based on the prediction that expression of genes in the core cell cycle machinery tends to co-vary during tumorigenesis, as these regulators function as one module during tumor proliferation. Using the co-variation pattern with known cell cycle genes in tumor tissues, we identified novel cell cycle regulators whose functional specificity to mitotic/cytokinesis was...
Further defined by their induction in G2/M. The physiological activities of these novel genes were analyzed by their localization to mitotic/cytokinesis structures and by their loss-of-function phenotypes by RNAi. This approach has led to the identification of multiple novel microtubule-associated proteins essential for mitosis and cytokinesis. We will present data on the function of these mitotic regulators. For example, we identified Cep55 as a regulator required for the completion of cytokinesis. We found that Cep55 localizes to the mitotic spindle during prometaphase/metaphase and to the spindle midzone/midbody matrix during anaphase/cytokinesis. At the terminal stage of cytokinesis, Cep55 organizes the midbody structure required for the completion of cytokinesis. Cep55 knockdown cells lack the midbody matrix, and the structural and regulatory components of the midbody, such as Aurora B, MKLP2 and PRC1, are either absent or mislocalized. Cep55 also facilitates the membrane fusion at the terminal stage of cytokinesis by controlling the localization of endobrevin, a v-SNARE required for cell abscission. On the other hand, Cep55 is under the control of centralspindlin, as Cep55 directly interacts with centralspindlin and as knockdown of centralspindlin abolishes the localization of Cep55 to the spindle midzone. Our study defines a cellular mechanism that links centralspindlin to Cep55, which, in turn, controls the midbody structure and membrane fusion at the terminal stage of cytokinesis.

Membrane remodeling during cytokinesis in the C. elegans Embryo

J. M. Squirell, J. Campbell, J. G. White; Laboratory for Molecular Biology, University of Wisconsin, Madison, WI

In order to successfully complete the division of one cell into two, seemingly disparate cellular systems, including cell cycle regulators, cytoskeletal networks, RNA processing and membrane trafficking pathways, must be coordinated in both time and space. We are studying scission, the final phase of cytokinesis in which midbody components must be removed and membrane inserted to close the channel that remains between the daughter cells. We have used multiphoton microscopy to observe live embryos either expressing GFP fusion proteins or labeled with membrane-specific probes, in conjunction with electron microscopy, to examine the process of membrane deposition during scission. We have found that membrane accumulates at the scission site and that this accumulation is subsequently resolved by endocytic events stimulated by the apoposis of astral microtubules from the rotating P1 spindle. The presence and removal of this excess membrane is supported by ultrastructural study showing folds of membrane along the cytokinetic furrow adjacent to the spindle midbody, as well as the apparent internalization of excess membrane after cytokinesis is complete. Our observations indicate that the spindle midzone, together with furrow-localized centralspindlin, play an essential role in directing the proper membrane accumulation at the site of scission. We are using compartment specific labeling and high-speed imaging in an effort to determine the dynamics of membrane insertion and the source of the inserted membrane.

Cytokinetic Furrow Induction in Toroidal, Binucleate, and Anucleate C. elegans Embryonic Cells

J. K. Baruni, E. M. Munro, G. von Dassow; Friday Harbor Labs, University of Washington, Friday Harbor, WA

In a classic study, Rappaport created toroidal sand dollar eggs and showed that juxtaposition of two mitotic asters suffices to induce furrowing, absent a spindle or chromatin between them. However, recent studies on other cell types implicate the central spindle or chromosomal passenger proteins, suggesting that cells may rely on different parts of the mitotic apparatus for cleavage induction. We attempted Rappaport's key experiment in C. elegans embryos, forming toroidal cells by perforating them with a glass rod, in order to spatially separate aster-dependent inductive cues from cues dependent on the central spindle. In toroidal cells, the surface obstacle from the central spindle by the perforation either did not ingress or formed unstable furrows. The unobstructed side generally ingressed to completion. However, the obstructed side generally recruited GFP-mycosin to the equatorial cortex, even when no ingress was apparent. Myosin also recruited to the side of the perforation adjacent to the central spindle. This implies that the far side of the probe receives the normal signal to initiate a furrow, but cannot sustain it, whereas furrows that form around a central spindle complete. We occasionally observed, in the second round of division, furrows and myosin recruitment between two unconnected by a central spindle, but the tendency of spindles to reorient within uninuclear blastomers made this difficult to repeat. As an alternate approach we used a laser to fuse cells, creating a single cell with two nuclei. In cases where the two nuclei remained separate, they formed distinct spindles within the common cytoplasm, and cleaved from one to four cells. This outcome requires furrows to form between two unasters unconnected by a spindle. Finally, we find that anucleate cells, containing only a pair of centrosomes and no spindle or nucleus, make repeated attempts at cytokinesis.

Calcium Binding to Fip3 Is Essential for Cytokinesis

G. C. Simon,1 J. R. Junutula,2 H. Matern,3 T. Katuladelz,1 R. Prekeris;1 1Cell and Developmental Biology, University of Colorado Health Sciences Center, Aurora, CO; 2Genentech, Inc, South San Francisco, CA; 3Exelixis, Inc, South San Francisco, CA; 4Pharmacology, University of Colorado Health Sciences Center, Aurora, CO

Cell division is a highly synchronized process that is regulated by a variety of different signaling mechanisms. In addition to a cyclin-dependent kinase system, second messengers, such as calcium, have also been suggested to play an important role in cell division. Accumulating evidence demonstrates that localized elevations of cytosolic free calcium ions are associated with several cytokinesis steps, including initiation and propagation of the cleavage furrow in Xenopus, Drosophila, zebrafish and newt. The mechanism of calcium function during cytokinesis remains to be determined. It was suggested that calcium may regulate the contraction of acto-myosin ring, thus initiating the cleavage furrow. Furthermore, calcium was suggested to regulate mitotic spindle formation as well as vesicle transport to the cleavage furrow. Interestingly, a portion of Fip3, a Rab11 GTPase interacting protein that is required for cytokinesis, shares a sequence similarity with EF-hands. EF-hand domains are responsible for calcium binding and signal transduction in many calcium binding protein. Thus, we tested whether calcium binding to Fip3 EF-hands may directly regulate Fip3 function during cytokinesis. First, we used a combination of NMR, isothermal titration calorimetry and circular dichroism to demonstrate that calcium binds to Fip3-EF hands. Second, we used immunofluorescence and time-lapse microscopy to demonstrate that calcium binding regulates the recruitment of Fip3 to the cleavage furrow. Third, we used calcium-binding Fip3 mutants to demonstrate that Fip3 interaction with calcium is required for successful completion of cytokinesis. We propose that calcium-induced calcium binds to Fip3-EF hands. EF-hand domains are responsible for calcium binding and signal transduction in many calcium binding protein. Thus, we tested whether calcium binding to Fip3 EF-hands may directly regulate Fip3 function during cytokinesis. First, we used a combination of NMR, isothermal titration calorimetry and circular dichroism to demonstrate that calcium binds to Fip3-EF hands. Second, we used immunofluorescence and time-lapse microscopy to demonstrate that calcium binding regulates the recruitment of Fip3 to the cleavage furrow. Third, we used calcium-binding Fip3 mutants to demonstrate that Fip3 interaction with calcium is required for successful completion of cytokinesis. We propose that calcium-induced conformational change in Fip3 is required for Fip3 delivery to the mitotic cleavage furrow and regulating the completion of cytokinesis.

Polar Relaxation and Equatorial Stimulation Coexist in the Cytokinesis of Silksworm Spermatoocytes

W. Chen, D. Zhang; Zoology/CGRB, Oregon State University, Corvallis, OR

Polar relaxation and equatorial stimulation are two prevailing models proposed to explain induction of cytokinesis in animal cells. These two models, though contradicting, have been consistently supported by their own evidence in the last few decades. As microtubules are recognized to play a more important role in signaling the cortex to position the cleavage furrow, we wonder whether these two models can be unified under the basis of microtubules. Because the asters are naturally detached from the spindle in silkworm spermatoocytes, we established primary cell culture of silkworm spermatoocytes as a working system to test the two contradictory models. Following microinjection of Alexa 568 tubulin and Alexa 488 phalloidin to label microtubules and actin filaments, we imaged individual spermatoocytes driven by micromanipulated microtubules using a spinning disc confocal microscope. When asters are brought to the cortex of one spindle pole by a micromanipulation needle, actin filaments are excluded away from the asters to the opposite pole, thus the proximal pole becomes relaxed by the asters. This actin exclusion can be blocked with a microinjected placed in the cortical flow of actin filaments, resulting in accumulation of actin filaments on the side of the needle closer to the asters. The polar relaxation depends on the dynamics of astral microtubules, as it is sensitive to stabilization of microtubules with taxol treatment. When astrer-driven actin flow is completely blocked, however,
accumulation of actin filaments is still prominent at the plus-ends of spindle microtubules. We reason such accumulation results from equatorially stimulated or de novo assembly of actin patches at the plus-ends of microtubules, as it occurs regardless of microtubule dislocations by micromanipulations. This microtubule plus-end stimulation is not induced by taxol. Our results suggest that the astral relaxation and the equatorial stimulation mechanism coexist in the cytokinesis of silkworm spermatocytes.

811 Exploring the Function of Anillin in Cytokinesis
A. J. Piecky, M. Glotzer; Molecular Genetics and Cell Biology, The University of Chicago, Chicago, IL
The actin binding protein anillin regulates cytokinesis in several eukaryotic systems. Anillin depletion results in an unusual phenotype in both S2 and HeLa cells. Furrow ingression initiates, but then the contractile ring appears to ‘oscillate’ and cytokinesis fails. Anillin binds to active myosin and a live probe showed that myosin moves in concert with the changing of the furrow in anillin-depleted cells (Straight et al., 2005). We have further explored the role of anillin in cytokinesis. In anillin-depleted cells, YFP-RhoA also moves in concert with the oscillating furrow. This movement initiates upon furrow formation, occurs for about 7-8 minutes and is followed by furrow regression. We have previously shown that depletion of the central spindle component MKLP1 causes an expansion of RhoA localization, but many cells still form ingrowing furrows. In cells depleted of both MKLP1 and anillin, YFP-RhoA localization is dramatically altered and furrows fail to form, suggesting that anillin has a nonessential role in furrow formation. Anillin localizes to the furrow discretely during early anaphase and is dependent on the upstream RhoA regulators RhogEFT C2 and MgcRacGAP CYK-4. Similar to our observations for RhoA, we find that the localization of anillin is sensitive to loss of the central spindle. Depletion of the central spindle component MKLP1, or inhibition of the upstream regulator Aurora B, causes expansion of anillin localization. Although anillin depleted cells form ingrowing furrows, RhoA cannot be detected in fixed cells. However, YFP-RhoA can be visualized in live cells as described earlier. Thus, anillin’s role in cytokinesis is complex; it contributes to maintaining a pool of RhoA at the site of the furrow that is essential for stable furrow formation. Further, anillin also functions redundantly with the central spindle for furrow formation. We are in the process of evaluating if these functions are molecularly related.

812 Failure of Cytokinesis in Cancer Cells
Q. Wu, R. Sahasrabudhe, W. Saunders; Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA
In some cancer cells, mitotic spindles have more than two poles which can cause chromosome mis-segregation and aneuploidy. Our previous data strongly support the model that failure of cytokinesis is the mechanism for cells to amplify pole number in tested HEK-293 cells and UPC1:SCC103 oral cancer cells. Recent studies in the lab focused on why the cancer cells fail in cytokinesis. We found that myosin regulatory light chain (MLC) phosphorylation, which is the key regulator for the activity of myosin, is reduced in the tested oral cancer cell line. It is known that myosin light chain kinase (MLCK) and myosin phosphatase are two critical enzymes to control the phosphorylation of MLC, so the MLCK and phosphatase expressions in different cell lines were examined. Interestingly, the MLCK is deficient in most cancer cell lines compared to normal cells and phosphatase levels are varied. The knockdown of the myosin phosphatase targeting subunit expression by siRNA increased the phosphorylation level of MLC and resulted in a reduction of the multinucleation and multipolarity in tested cancer cells. Additionally, live cell analysis suggested that cytokinesis failure is partially rescued in the best cases, after reduction of phosphatase activity. In conclusion, we believed that the defects of phosphorylation of MLC is the main reason for failure of cytokinesis in some cancer cell lines, which are due to deficiency of MLCK and relatively high levels of myosin phosphatase.

813 Human Discs-longs (hDlg) Localizes to the Midbody via the Guanylate Kinase Homology Domain and Regulates Cytokinesis
K. Unno, T. Harada, A. H. Chishti; Pharmacology and UIC Cancer Center, University of Illinois College of Medicine, Chicago, IL
The human homologue of the Drosha discs-long tumor suppressor (hDlg), a member of the membrane associated guanylate kinase (MAGUK) family, has been implicated in human tumorigenesis although its precise mechanism remains poorly understood. It was reported that hDlg protein shows a highly concentrated localization at the mitotic spindle midbody structure of the cells at the cytokinesis phase. Since a number of proteins have been identified that localize to the midbody and play functional roles in completing cytokinesis, we hypothesized that hDlg may also be an important component required for the completion of cytokinesis. Failed cytokinesis leads to subsequent cell fusion, creating binucleated cells, or an abnormal segregation of chromosomes (aneuploidy), which is a hallmark of malignant cells. We used human derived cell lines HeLa and U2OS to determine the localization of various hDlg constructs in the cytokinesis phase. These results established that the guanylate kinase homology (GUK) domain is both necessary and sufficient for the localization of hDlg to the midbody of the cells captured in the cytokinesis process. Interestingly, the GUK domain of another MAGUK protein, p55, did not localize to the midbody suggesting the specificity of hDlg in the regulation of this function among MAGUKs. When over-expressed in HeLa and U2OS cells, the DrsRed-GUK-hDlg protein functioned as a dominant negative leading to failed cytokinesis and a significant increase in the formation of binucleated cells. Down-regulation of hDlg using siRNA also resulted in significant increase in the number of multinucleated cells in HeLa and U2OS cells. Together, these results indicate that the guanylate kinase homology domain is essential for the localization of hDlg to the midbody, and this localization is important for the efficient completion of cytokinesis. This novel function of hDlg may contribute to the tumor suppression mechanism during progression of some human cancers.

814 Biochemical Identification of Novel Types of Interactions Involving Ubiquitin-Conjugating Enzymes, Cullin 3, and a BTB-containing Substrate Adaptor
K. S. Pfister, S. M. Pfister; Cell Biology, University of Oklahoma Health Sciences Center, Oklahoma City, OK
BTB (broad complex, Tramtrack, Bric-a-brac) domains are present in a large number of proteins and have been implicated in numerous cellular processes including transcriptional regulation, cytoskeleton organization, and ion channel regulation. It was recently discovered that BTB-containing proteins function in ubiquitin-mediated proteolysis as substrate adaptors for cullin3-based ubiquitin ligases. These multi-subunit ligases typically consist of the cullin3 scaffold bound to a BTB-containing protein at one end and a RING-finger protein/ubiquitin conjugating enzyme complex at the other end. We have found that the ubiquitin conjugating enzyme, UBE2E3, interacts with RCBTB1, a BTB-containing protein that modulates the activity of the anгиотенсин II receptor (AT1A). Interestingly, UBE2E3 and AT1A, bind an overlapping domain of RCBTB1, implying a functional link between the three proteins. Guo et. al. have demonstrated that removal of this domain from RCBTB1 results in a dominant-negative protein that blocks the ability of angiotensin II to induce cellular hypertrophy. We have found that RCBTB1 can dimerize, bind cullin3, and interact with 3 different ubiquitin conjugating enzymes, one of which has no detectable affinity for cullin3. Through biochemical binding assays, we have found that the interaction of UBE2E3 with RCBTB1 is independent of a RING-finger protein. Mutants of UBE2E3 that are deficient in RING-finger binding, interact with RCBTB1 more robustly than the wild type enzyme. In addition, we found that both wild type and RING-finger binding mutants of UBE2E3 bind cullin3. The ability to bind cullin3 without a bridging RING-finger protein appears to occur as a result of UBE2E3 interacting with the amino-terminal half of the cullin. Two enzymes closely related to UBE2E3 also interacted with this domain of cullin3. Together, these findings expand the repertoire of cullin3-based ubiquitin ligases that can form and imply that ubiquitin conjugating enzymes may have multiple roles within these complexes.

815 MyoGEF Differentially Interacts with Mouse Centrosome/Spindle Pole-associated Protein (CSPP) Isoforms
M. Asiedu, D. Wu, Q. Wei; Department of Biochemistry, Kansas State University, Manhattan, KS
We previously reported that MyoGEF, a guanine nucleotide exchange factor, localizes to the cleavage furrow in anaphase cells, interacts with nonmuscle myosin II, and plays an important role in regulating cytokinesis (Cell Cycle, 5(1234-9, 2006). In support of these findings, we have now confirmed that the N-terminal region of MyoGEF (amino acids 71-388) is important for MyoGEF-myosin II interaction. Here we also provide evidence suggesting that MyoGEF can further interact with centrosome/spindle pole-associated protein (CSPP) that localizes to the spindle pole and central spindle during cytokinesis. We have identified three mouse CSPP isoforms, mCSPP-1, 2 and 3, which contain 703, 1142 and 416 amino acids, respectively. GFP-tagged mCSPP-2 and 3 (but not mCSPP-1) localize to the spindle pole and central spindle during cytokinesis. A region corresponding to 104 amino acids, which is present in mCSPP-2 (amino acids 290-393) and mCSPP-3 (amino acids 398-416) but not in mCSPP-1, is required for the localization of the mCSPP-2 and mCSPP-3 proteins to the cleavage furrow and central spindle. Co-immunoprecipitation experiments showed that MyoGEF interacts with GFP-tagged mCSPP-2 and 3 (but not mCSPP-1) in transfected HeLa cells, indicating that the 104-amino-acid region is also important for MyoGEF-mCSPP interaction. Further, in vitro GST pull-down assays indicate that the C-terminal region of MyoGEF (amino acids 392-780) is required for MyoGEF-mCSPP-2 interaction. Taken together, our results suggest that the interactions among MyoGEF, myosin II and mCSPP might play an important role in coordinately regulating cytokinesis.
816 Centrosome Disruption Suppresses Multipolar Spindle Formation after Cytokinesis Failure

F. G. Pruefer, J. Zhou, R. Wollman, G. Goshima, R. Vale; Physiology Course 2006, Marine Biological Laboratory, Woods Hole, MA

Multipolar spindles (MS) are frequently observed in cancer cells, and may play a role in genomic instability. Although polyplaid cells induced by cytokinesis failure seem to increase MS frequency in various cell types, the underlying mechanism is unclear, since centrosome number, chromosone/kinetochore number, total protein amount and cell size increase all after cytokinesis failure. In this study using the Drosophila S2 cell line as a test system we employed RNAi screening and automated microscopy to investigate which factors/genes are important for MS formation. First, we confirmed that cytokinesis failure induced by Pavarotti(Kinesin-6) RNAi significantly increased MS formation in S2 cells (from 15% to ~35). The multiple poles often contain mature centrosomes, but acentrosomal poles (non-γ-tubulin containing) were also observed. We then used double RNAi treatment (Pavarotti + gene X) to identify genes that suppress or enhance MS formation. Of the 19 genes selected in this double RNAi screen, two genes (CNN, SAK) were identified that rescued Pavarotti-induced MS formation. Both of these genes affect centrosome function, CNN by inhibiting γ-tubulin recruitment to centrosomes and SAK by blocking centriole duplication. The rescue by CNN RNAi was particularly striking, resulting in almost all spindles showing bipolar shapes (spindles formed by chromosome-mediated nucleation). These results indicate that increased numbers of centrosomes is the critical factor in MS formation.

817 How Random Is Pollen Tube Growth in A. thaliana?

S. F. Stewman, A. R. Dinner, D. Preuss; 1Chemistry, University of Chicago, Chicago, IL; 2Molecular Genetics and Cell Biology, University of Chicago, Chicago, IL

The reproductive process in Arabidopsis and other higher plants involves complex and highly regulated interactions between pollen tubes and ovules. Although genetic and biochemical approaches identified many of the relevant genes, proteins, tissues, and small molecules, the process of pollen tube guidance is poorly understood. We employed a simplified semi-in vitro system to study how pollen tubes and measured their growth paths in the presence and absence of wild-type ovules. To further understand the effects of specific ovule tissue on guidance, we compared the results of wild-type ovules with mutant ovules that have specific defects in guidance or development (myo98b-1, dif1a, and mol-1). The results were consistent with a path that is fit by a path drawn in the presence of a normal wound present in its guidance ability. This shows that the guidance of pollen tubes in vivo can be mimicked in vitro by the removal of the ovule.

818 Dynamics of LIM and SH3 Protein-1 (lasp-1) and Dynamin-2 (dyn-2) in Living HeLa Cells in the Presence of Epidermal Growth Factor (EGF) or Forskolin

T. E. Rajapaksa, C. S. Chew, T. Okamoto; 1Department of Pharmaceutical Sciences, University of Southern California, Los Angeles, CA; 2Institute of Molecular Medicine and Genetics, Medical College of Georgia, Augusta, GA

Lasp-1 is a multidomain-containing protein that colocalizes with F-actin in structures actively involved in remodeling of F-actin, such as lamellipodia and filopodia. It also interacts with the endocytosis-regulating protein dyn-2 in vitro and colocalizes with dyn-2 in several cell types, particularly in regions of active remodeling of the plasma membrane. This study examines how lasp-1 and dyn-2 interaction may regulate actin dynamics in living HeLa cells treated with EGF or forskolin, which elevate intracellular cAMP. HeLa cells were transiently transfected with either: enhanced Green Fluorescent Protein (eGFP) tagged, wild-type (wt) lasp-1 (lasp-eGFP); dyn-eGFP; or, dyn-eGFP + lasp-wt constructs. Time-lapse imaging was performed in the presence or absence of EGF or forskolin. In addition, for each treatment condition, cells were plated on coverslips, fixed, and immunostained for lasp-1, dyn-2 and F-actin. Time-lapse imaging of lasp-eGFP transfected cells treated with EGF versus untreated showed increased formation of lasp-1-containing membrane ruffles, increased motility of lasp-1-containing filopodia, and formation of structures believed to be involved in macropinosome formation. Dyn-eGFP transfected cells showed increased formation of membrane ruffles upon EGF treatment; however, dyn-2 was not localized to filopodia. In dyn-eGFP + lasp-eGFP co-transfected cells, dyn-2 was also localized to filopodia, and these cells showed increased formation of membrane ruffles and increased motility of filopodia compared to cells transfected with dyn-2 alone. In the presence of forskolin, lasp-eGFP transfected cells showed decreased formation of membrane ruffles and decreased formation/motility of filopodia compared to untreated EGF-treated cells. For both EGF and forskolin treatments, lasp-eGFP or dyn-eGFP were also found to be associated with structures resembling F-actin tail with short curvy paths. These results suggest that lasp-1, possibly together with dyn-2, may regulate signaling-dependent cortical membrane dynamics such as membrane ruffling and filopodia formation/motility may also be involved in actin comet tail formation.

819 CRP1 Bundling of F-actin Requires LIM1 Domain and Adjacent Glycine Rich Region

H. Jang, J. A. Greenwood, Biochemistry and Biophysics, Oregon State University, Corvallis, OR

Cysteine rich proteins (CRPs) have been implicated in cell differentiation, cytoskeletal remodeling, and transcriptional regulation. CRP1 contains two LIM domains, each followed by a glycine rich region that CRP1 regulates the actin cytoskeleton by directly bundling actin microfilaments [Tran et al. (2005) BMC Cell Biology 6: 819]. Previously, we demonstrated that CRP1 contains two LIM domains, each followed by a glycine rich region that CRP1 regulates the actin cytoskeleton by directly bundling actin microfilaments [Tran et al. (2005) BMC Cell Biology 6: 819]. In this study using the S2 cell line as a test system we employed RNAi screening and automated microcopy to investigate which factors/genes are important for CRP1 bundling of F-actin. Furthermore, domain swapping experiments replacing GR1 with GR2 resulted in the loss of CRP1 bundling activity identifying critical amino acid residues. These results highlight the importance of the glycine rich region for CRP1 bundling activity and provide mutual proteins for examining the function of the cytoskeletal population of CRP1. Results from experiments examining the influence of CRP1 mutants on cell adhesion and migration will be presented.

820 The Tobacco LIM Protein WLM1 Is an Actin Bundling Protein

A. A. Steinmetz, C. Thomas, C. Hoffmann, M. Dieterle; 1Plant Molecular Biology, CRP-Sante, Luxembourg, Luxembourg, 2CRP-Sante, Luxembourg, Luxembourg

The tobacco protein WLM1 (NiWLM1) one of the five LIM proteins identified so far in tobacco. Transcripts of the gene have been detected in all the plant organs analyzed but its biological expression is still unknown. We have used confocal microscopy and in vitro analyses to show that this protein is a novel actin-binding and -bundling protein in plants. In BY2 cells the GFP-tagged protein accumulated in the nucleus and cytoplasm where it associated predominantly with actin cytoskeleton, as demonstrated by colabeling and treatment with actin-depolymerizing latrunculin B. High-speed cosedimentation assays revealed the ability of WLM1 to bind directly to actin filaments with high affinity. Fluorescence recovery after photobleaching and fluorescence loss in photobleaching showed a highly dynamic in vivo interaction of WLM1-GFP with actin filaments. Expression of WLM1-GFP in BY2 cells significantly delayed depolymerization of the actin cytoskeleton induced by latrunculin B treatment. WLM1 also stabilized actin filaments in vitro. Importantly, expression of WLM1-GFP in Nicotiana benthamiana leaves induced significant changes in actin cytoskeleton organization, specifically, fewer and thicker actin bundles than in control cells, suggesting that WLM1 functions as an actin bundling protein. This hypothesis was confirmed by low-speed cosedimentation assays and fluorescence microscopy which allowed direct observation of F-actin bundles that formed in vitro in the presence of WLM1. These data identify WLM1 as a novel actin binding protein that increases actin cytoskeleton stability by promoting bundling of actin filaments.

821 The Tobacco Protein WLM2 Is an Actin-binding LIM Domain Protein

S. Gatti, C. Thomas, C. Hoffmann, W. Shen, E. Frederich, A. A. Steinmetz; 1Plant Molecular Biology, CRP-Sante, Luxembourg, Luxembourg, 2Institute of Plant Molecular Biology, CNRS, Strasbourg, France, 3CRP-Sante, Luxembourg, Luxembourg

Sporophytic tissue from tobacco express two WLM proteins: WLM1 and WLM2. These two proteins differ by about 40% in their amino acid sequence and appear to some extent differentially expressed since only WLM2 has been detected in cells from BY2 suspension cultures. Of the two proteins, WLM1 was recently shown to bind and stabilize actin filaments and to promote their assembly into bundles. To see if WLM2 has a similar localization and actin binding properties we have fused its coding sequence to GFP under the control of the dexamethasone-inducible promoter and analyzed the localization of the fusion protein in BY2 cells following Agrobacterium tumefaciens transformation, as well as in leaf epidermal cells following agroinfiltration. In contrast to the sharply defined labeling that we observed with WLM1, WLM2 appeared to associate more loosely with the actin cytoskeleton, with much of the protein remaining free in the cytoplasm. When expressed in animal cells, the two proteins strongly accumulated in the nucleus, and only WLM1 gave a sharply defined cytoskeletal labeling along stress fibers and in focal adhesions. In contrast, cytoplasmic WLM2 remained predominantly unbound, with only a weak labeling of actin cables. However, in high- and low-speed
severing protein and promotes rapid turnover of actin structures in vivo. Mammalian twinfilin sequesters ADP-G-actin and caps filament barbed ends: implications in motility.

In the present study, we have elaborated on the nature of PIP2 binding and identified its binding site in CP. We therefore compared homology models of mouse and S. pombe CPs from these individual events. The fast association (2.6x10^7 M^-1 s^-1) and slow dissociation (0.0003 s^-1) rates for mouse CP are similar to published values from bulk assays. The affinity of fusion yeast CP for vertebrate actin is a thousand-fold less than mouse CP, owing to its slow association (4x10^7 M^-1 s^-1) and fast dissociation (0.004 s^-1) rates. Addition of micelles of the polyphosphoinositides (PPiS), PIP, and PIP2 reduced the concentration of mouse capping protein available to bind barbed ends. However, PPIs did not change the rate of dissociation of mouse CPs from pre-capped filaments. We replicated the appearance of uncapping in bulk assays, but found that this was due to filament breakage together with rapid sequestration of free mouse CP by PPIs, rather than to uncapping. Therefore, PPIs likely lower the affinity of vertebrate CP for filaments by mass action, rather than by allosteric inhibition. In contrast, PPIs had no effect on either the association or dissociation of fusion yeast CP at barbed ends. We therefore compared homology models of mouse and S. pombe CP for clues to potential PPI binding sites in the vertebrate CP crystal structure. A large area of positive potential on the mouse CP β subunit is absent in pombe CP. PPI binding to this patch would sterically hinder actin binding and is thus a strong candidate for the PPI binding site.

Phosphatidylinositol-4,5-bisphosphate (PIP2) plays an important role in cell signaling at membranes in response to extracellular stimuli that lead to actin-based motility. PIP2 interacts with actin Capping protein (CP) by antagonizing its capping activity and by uncapping previously capped actin filaments, which may be important in vivo to promote actin assembly at membranes. In the present study, we have elaborated on the nature of PIP2 binding and identified its binding site in CP. By in vitro actin polymerization and intrinsic tropomyosin quenching assays, we find that neither the individual head or tail groups, in the form of DAG or IP3, inhibited the capping activity of CP. PIP2 micelles, probably through their anionic surface, bind to and inhibits CP with an affinity of ~300 nM. Truncation and point mutants of CP were used to identify PIP2 binding sites. The actin-binding C-terminal region of the CP alpha subunit helps to bind PIP2, while that of the beta subunit is dispensable. Computational docking of PIP2 with CP predicted a cluster of solvent-exposed basic residues on the surface of CP to be the binding site for PIP2 and Point mutations of the key residues confirm this prediction in the actin. So that the presence of PIP2 probably blocks capping of actin filaments via steric hindrance. The results are consistent with uncapping occurring via a “wobble” state in which CP is attached to the barbed end only by its flexible beta subunit C-terminus.

A. Bacconi, 1 S. Hitchcock-DeGregori, 2 G. Danuser, 1 R. Fischer 1; 1Cell Biology, The Scripps Research Institute, La Jolla, CA, 2Department of Neuroscience and Cell Biology, Robert Wood Johnson Medical School, Piscataway, NJ

Actin cytoskeleton forms a cortical network underneath plasma membrane. During cell movement this network forms a polarized protrusion machinery comprising two F-actin modules: the lamellipodium and the lamella, as defined by their actin dynamics (Ponti et al. Science 2004). Actin dynamics in the lamellipodium are dependent on the activities of the Arp2/3 complex and cofilin, while in the lamella they appear to be Arp2/3 independent (Ponti et al. Biophys.J.2005). The relationship between these two networks is still unclear. The aim of this work is to investigate how troponymosin may be involved in these processes in relation to both lamellipodium and lamella networks. We hypothesize that during edge advancement lamella filaments may escape troponymosin decoration, giving way to Arp2/3-mediated branching and thus forming a new lamellipodium towards the cell membrane from the lamella. Immunoblotting analysis of total cell extracts using a variety of specific anti-troponymosin antibodies showed that both long (TM2 and TM3) and short (TM2N1/M2 and TM4) troponymosin isoforms are present in PtK1 cell lines, which was confirmed by mass spectrometry analysis. We prepared total extracts from cell pseudopodia and immunoblot analysis suggested that only short troponymosin isoforms are present. By repeating the previous study with a troponymosin-4 antibody, we demonstrated that at least this isoform is present at the leading edge. Ratiometric analysis of troponymosin-4/F-actin in protruding cells suggests a reduction of troponymosin-4 in the lamella/lamellipodium overlap region, as compared to lamella only region and it is undetectable where the lamellipodium expands beyond the lamella, in support of our hypothesis. Our next step will be to confirm this result by ratiometric analysis of cells with perturbed lamellipodium/lamella filament ratio and by measuring transients of troponymosin-4 decor recruitment of F-actin during cell protrusion. Supported by NIGMS ROI-63257 to SEHD, SNF PBZHA-112712 to AB

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centrifugations WLIM2 cosediments with actin filaments and actin bundling, as does WLIM1. These data suggest that, although WLIM2 can bind and bundle F-actin, it does so with a lower affinity and has therefore probably a less pronounced stabilizing effect on the actin cytoskeleton than WLIM1. This work is supported by the Luxembourg Ministry of Culture, Higher Education and Research.
Cytoskeletal Tropomyosins Are Not Subject to Feedback Regulation

G. Schevzov,1 B. Vrhovski,1 N. Vlahovich,1 J. Hook,1 F. Lemcere,1 E. Hardeman,1 D. F. Wieczorek,1 P. W. Gunning1
1Oncology Research Unit, The Children's Hospital at Westmead, Westmead NSW, Australia, 2Muscle Development Unit, The Children's Medical Research Institute, Westmead NSW, Australia, 3Dept of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati, Cincinnati, OH

Altering the actin cytoskeleton are fundamental features of many diseases including cancer. However, compounds that target actin ubiquitously act on actin in heart and diaphragm rendering them highly toxic. This study investigates whether the potential target, tropomyosin (Tm), is made in limiting amounts. Four mammalian genes encode Tm proteins with over 40 isoforms although it causes a profound decrease in cardiac Tm levels. The distinct spatial sorting and independent regulation of the muscle and cytoskeletal Tms make the cytoskeletal Tms an attractive drug target for manipulation of functionally distinct populations of actin filaments. I.Gignetti and Friederich, 2003, Prog Cell Cycle Res, 5:511 Z.Muthuchamy et al, 1995, J Biol Chem, 270: 36593 J.Palermo et al, 1996, Cir. Res, 78: 504 K.Murat et al, 1997, PNAS USA, 94: 4406

Tip Growth in the Moss Physcomitrella patens Is Dependent on Profilin Function and Regulation

L. Vidali, R. C. Augustine, M. Bezanilla; Biology, University of Massachusetts, Amherst, MA

Tip growth is indispensable for plant growth and reproduction. It is well established that the actin cytoskeleton is the central regulator of tip growth in plant cells, but the precise mechanisms of this regulation remain obscure. To gain insights into the regulation of actin, we are investigating the role of the abundant actin monomer-binding protein profilin. Profilin regulates actin polymerization and has been shown to control for actin function in a variety of organisms. In addition to binding to actin, profilin has a conserved poly-L-proline binding site where it binds regulatory molecules. The moss Physcomitrella patens offers an ideal genetic system for the functional analysis of the actin cytoskeleton during tip growth. In its protonemal phase, this moss grows as single cell thick filaments via tip growth. In addition, Physcomitrella is amenable to homologous recombination and efficient RNA interference (RNAi). Physcomitrella has three profilin genes, with profilin2 as the predominant isoform expressed in protonema. We have used RNAi to inhibit the expression of all isoforms to address the function of profilin during tip growth. Plants lacking profilin show a dramatic inhibition in tip growth and reduced viability, they also have small rounded cells, indicating loss of polarized growth. This phenotype is rescued by the RNAi insensitive expression of profilin. To address the role of actin and poly-L-proline binding in vivo, we have performed transient complementation analysis of profilin RNAi using mutant profilins that do not bond to actin (K87E) or that do not bond to poly-L-proline (Y6D). We found that the ability of profilin to bind actin is indispensable for tip growth. However, poly-L-proline binding is not essential but is required for optimal tip growth, since the Y6D mutant only partially complements. These results indicate that profilin-actin interactions and their regulation are critical for tip growth in plant cells.

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Effects of Silencing Profilin-1 on Endothelial Proliferation, Migration, and Cord Morphogenesis

Z. Ding,1 P. Roy2; 1Bioengineering, University of Pittsburgh, Pittsburgh, PA, 2Bioengineering and Pathology, University of Pittsburgh, Pittsburgh, PA

Although profilin-1 (Pfn1) was previously implicated in capillary morphogenesis of endothelial cells (ECs), its role in cellular functions that are important for capillary morphogenesis such as EC proliferation and migration remains unknown. To address this gap, we evaluated the effects of loss of Pfn1 expression on proliferation, migration and extracellular matrix (ECM)-induced morphogenesis of ECs. By using RNA interference, a time-dependent progressive loss of Pfn1 expression with ~97% gene-silencing was achieved within 96 hours after transfection in human umbilical vein endothelial cells (HUVECs). Loss of Pfn1 expression was associated with significant decrease of actin filaments and focal adhesions in ECs. Wound-healing experiments showed increasing inhibition of directed cell migration with progressive loss of Pfn1 expression. Time-lapse imaging revealed that Pfn1-deficient cells have reduced protrusive ability, both in terms of its magnitude and directional persistence. Silencing Pfn1 expression also significantly inhibits EC proliferation without affecting cell viability, at least in the short-term. Further cell-cycle profile analyses showed that loss of Pfn1 expression inhibits transition from G1 to S phase with concomitant increase in the expression of p27Kip1. Finally, Pfn1-deficient cells were found to be significantly impaired in forming cords-like structures on ECM. Since cord morphogenesis is an early endothelial rearrangement necessary for capillary formation by ECs, these data lay the foundation for future studies to explore Pfn1’s role in capillary formation by ECs.

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Roles of Profilin in Cell Spreading and Migration

O. C. Danciu, S. Kojima, G. G. Borisy; Cell and Molecular Biology, Feinberg School of Medicine, Northwestern University, Chicago, IL

Profilins, actin-binding proteins, are known to block polymerization at the pointed ends and to suppress spontaneous nucleation of actin filaments, thus polarizing actin polymerization to the barbed ends of existing actin filaments. This polarization is presumably important for cell migration which is believed to be driven by actin polymerization at the barbed ends of actin filaments. However, direct tests of this hypothesis in vivo have been limited in nature. In order to evaluate the role of polarized polymerization in cell spreading and migration, we performed RNA interference of profilin in mouse melanoma B16F1 cells. Microarray analysis indicated that profilin1 was the dominant profilin species in melanoma cells; therefore we focused on profiling depletion of profilin1 by 5 days after transfection as revealed by Western blotting. Profilin-depleted cells showed delay in spreading. While 80% of control cells formed lamellipodia 1 hour after plating on laminin-coated coverslips (n=87), only 15% of knockdown cells showed enrichtment of actin at the cell periphery, but the structures looked different from typical lamellipodia. Profilin-depletion also inhibited cell migration. Knockdown cells were poorly polarized and their translocation velocities averaged 30% that of control cells (0.30 ± 0.23 μm/min vs. 0.95 ± 0.20 μm/min, p<0.05). Our results demonstrate that polarized actin polymerization facilitated by profilin is necessary for efficient cell spreading and migration. In the absence of profilin, the pointed ends likely add G-actin, decreasing productive polymerization at the barbed ends, thus slowing cell spreading, degrading cell polarization and reducing the overall speed of cell motility. Supported by NIH GM 64346.

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Role of Cortactin in Invadopodia Formation Downstream of Src Kinase

L. C. Kelley, A. G. Ammer, D. C. Flynn, S. A. Weed; Program in Cancer Cell Biology, West Virginia University, Morgantown, WV

Invadopodia are actin-rich ventral membrane protrusions found in oncogenic kinase transformed fibroblasts and metastatic cancer cell lines. Cortactin is an Arp2/3 activating and actin-binding protein initially identified as a major substrate in v-Src transformed cells, and is a core invadopodia component. Cortactin is necessary for invadopodia formation and function in breast cancer cell lines, and the level of tyrosine phosphorylation in invadopodia correlates with their ability to degrade the extracellular matrix. However, a complete understanding of the molecular role of cortactin in invadopodia has not been reported. It is proposed that the role of cortactin in invadopodia formation in response to Src transformation, we introduced a GFPP-tagged temperature sensitive mutant of v-Src (tsLa29-GFP) into Src-deficient (SYF) fibroblasts. Invadopodia formation is induced when cells expressing tsLa29-GFP are switched from the non-permissive temperature (41°C) to the permissive temperature (35°C) as determined by confocal fluorescence microscopy and rhodamine phalloidin labeling of the actin cytoskeleton. Invadopodia are enriched with actin of v-src and cortactin phosphorylated at the major Src-targeted residue, tyrosine 421. Inhibition of cortactin expression by siRNA substantially diminished the ability of tsLa29-GFP to induce invadopodia formation at the permissive temperature. Pretreatment of SYF cells expressing tsLa29-GFP for 2 h with the Src/Abl small molecule inhibitor AZD0530 also ablated invadopodia formation at the permissive temperature. AZD0530 treatment inhibits Src activity as determined by Western blot analysis of tyrosine 215, and inhibited phosphorylation of cortactin at tyrosine 421. Collectively, these findings indicate that cortactin is required for Src-mediated invadopodia formation and that downregulation of Src activity correlates with inhibition of invadopodia formation and cortactin tyrosine phosphorylation. We are currently using mutational analysis to investigate the contributions of the functional domains, as well as serine and tyrosine phosphorylation on invadopodia formation and function.
The Actin-binding Repeat of Cortactin Binds F-actin in a Dynamic Manner and Affects Inter-subunit Contacts in F-actin
L. A. Selden,1 D. Chereau,2 R. Dominguez,1 E. Reisler;1 Department of Chemistry/Biochemistry and Molecular Biology Institute, UCLA, Los Angeles, CA, 3Boston Biomedical Research Institute, Watertown, MA, 4Institute, Watertown, MA, 3School of Medicine, University of Pennsylvania, Philadelphia, PA
Cortactin is a ubiquitous actin- and Arp2/3-binding protein, involved in extracellular signal communication, regulation of cytoskeleton dynamics and cortical actin-associated structures. Cortactin presents a unique F-actin-binding domain known as the cortactin repeat, which consists of 6 1/2 repeating sequences of 37 amino acids. Recent EM and 3D reconstruction studies showed that the binding of cortactin to F-actin facilitates the lateral interprotomer contacts, widening the gap between two filament strands (Pant et al. 2006). To assess the effect of cortactin on both lateral and longitudinal interprotomer contacts in F-actin, we employed two yeast actin mutants: Q41C and S265C. Cysteines inserted in the DNase I binding (C41) and hydrophobic (C265) loops allowed for longitudinal or lateral cross-linking of Cys74 of adjacent actin protomers in F-actin. Using disulfide and DDB cross-linking, we found that the actin-binding domain of cortactin (amino acids 83-306 of murine cortactin) inhibits strongly the rate of disulfide and DDB cross-linking across both the lateral and longitudinal interfaces, indicating a potential weakening of interprotomer contacts. This effect was strengthened for the lateral interstrand cross-linking. These results provide evidence for cortactin-induced changes at the sites of lateral and longitudinal interprotomer contacts in F-actin. To assess the dynamics of the cortactin actin-binding domain, we created cortactin cysteine mutants. We found that pairs of cysteines placed at the N- and C-terminal ends of the cortactin repeat could be cross-linked by reagents of varying spans (0-9.6 Å) such as disulfide bonds (zero length), DDB (4.4 Å) and MTS-6 (9.6 Å). The cross-links formed intra-molecular bridges between these cysteines, suggesting that the cortactin repeat is flexible and unstructured, and binds F-actin in a highly dynamic manner.

Direct Interaction between Caldesmon and Cortactin
R. Huang, J. Kordowska, C. L. A. Wang; Muscle and Motility Group, Boston Biomedical Research Institute, Watertown, MA
Actin polymerization and depolymerization play a central role in controlling a wide spectrum of cellular processes. There are many actin-binding proteins in eukaryotic cells. Their roles in the remodeling of the actin architecture are just beginning to be understood and whether they work cooperatively remains an intriguing question. Both caldesmon (CaD) and cortactin are actin-binding proteins present in mammalian cells, the former being known to stabilize actin filaments and regulate the actomyosin interaction, whereas the latter, primarily found in the cell cortex, being implicated to promote Arp2/3-mediated actin branching. There have been no reports suggesting that CaD and cortactin interact with each other or work as partners. We now present evidence that CaD binds cortactin directly by a number of independent approaches, including overlay, pull-down assays, ELISA and column chromatography. The interaction involves the N-terminal region of cortactin and the C-terminal region of CaD, and seems enhanced by divalent metal ions. Cortactin competes with both full-length CaD and its C-terminal fragment for actin binding. Binding of cortactin to CaD is significantly enhanced by the addition of ATPase activity, which can be demonstrated in vitro, the two proteins also appear to co-localize in activated cells at the leading edges, although the distribution of cortactin is wider than that of CaD. Since under these conditions CaD is largely phosphorylated, we propose that cortactin preferentially targets phosphorylated CaD in vivo. The functional significance of such interactions awaits further investigation. Supported by a grant from NIH (P01-AR41637).

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L. A. Selden,1 D. Chereau,2 R. Dominguez,1 E. Reisler;1 Department of Chemistry/Biochemistry and Molecular Biology Institute, UCLA, Los Angeles, CA, 3Boston Biomedical Research Institute, Watertown, MA, 4Institute, Watertown, MA, 3School of Medicine, University of Pennsylvania, Philadelphia, PA
Cortactin is a ubiquitous actin- and Arp2/3-binding protein, involved in extracellular signal communication, regulation of cytoskeleton dynamics and cortical actin-associated structures. Cortactin presents a unique F-actin-binding domain known as the cortactin repeat, which consists of 6 1/2 repeating sequences of 37 amino acids. Recent EM and 3D reconstruction studies showed that the binding of cortactin to F-actin facilitates the lateral interprotomer contacts, widening the gap between two filament strands (Pant et al. 2006). To assess the effect of cortactin on both lateral and longitudinal interprotomer contacts in F-actin, we employed two yeast actin mutants: Q41C and S265C. Cysteines inserted in the DNase I binding (C41) and hydrophobic (C265) loops allowed for longitudinal or lateral cross-linking of Cys74 of adjacent actin protomers in F-actin. Using disulfide and DDB cross-linking, we found that the actin-binding domain of cortactin (amino acids 83-306 of murine cortactin) inhibits strongly the rate of disulfide and DDB cross-linking across both the lateral and longitudinal interfaces, indicating a potential weakening of interprotomer contacts. This effect was strengthened for the lateral interstrand cross-linking. These results provide evidence for cortactin-induced changes at the sites of lateral and longitudinal interprotomer contacts in F-actin. To assess the dynamics of the cortactin actin-binding domain, we created cortactin cysteine mutants. We found that pairs of cysteines placed at the N- and C-terminal ends of the cortactin repeat could be cross-linked by reagents of varying spans (0-9.6 Å) such as disulfide bonds (zero length), DDB (4.4 Å) and MTS-6 (9.6 Å). The cross-links formed intra-molecular bridges between these cysteines, suggesting that the cortactin repeat is flexible and unstructured, and binds F-actin in a highly dynamic manner.
Glutathionylation of cellular actin occurs during conditions of oxidative stress and correlates with changes in the actin cytoskeleton. We have investigated the effects of glutathionylation on cofillin-depoxidimerization of mammalian non-muscle F-actin. F-actin (FA) or glutathionylated F-actin (GS-FA) was copolymerized with 5% pyrene-actin. Depolymerization, indicated by a decrease in pyrene fluorescence, was induced by 100-fold dilution into buffer containing vitamin D binding protein (DBP), followed by quick mixing/severing with a pipette tip. A reproducible filament number (~2 nM) was verified. In the presence of DBP, depolymerizing actin subunits cannot re-associate with filament ends and the depolymerization rate is (k_b + k_p)m, where m is the number of filament ends and k_b and k_p are the depolymerization rate constants from the barbed and pointed ends of the filament, respectively. Initial experiments showed that GS-FA depolymerized approximately three times faster than FA. When GS-FA was pre-incubated with equimolar cofillin prior to dilution, the depolymerization rates were similar to samples which had not been pre-incubated with cofillin, suggesting a similar number of filament ends and rapid dissociation of the cofillin-F-actin complex upon dilution. When we pre-incubated GS-FA with cofillin and included excess cofillin in the dilution buffer to prevent dissociation of cofillin-F-actin complexes, we found that cofillin-GS-FA depolymerized 2 times faster than FA, consistent with a 3-fold increase in k_p (assuming k_b = 7.2/s and k_p = 0.26/s for FA). We found that glutathionyl-GS-FA depolymerized approximately three times faster than cofillin-GS-FA. The 3-fold increase in the depolymerization rate constant of GS-FA over FA, and the 3-fold increase in the depolymerization rate constant of cofillin-GS-FA over cofillin-GS FA results in a 9-fold overall increase in the depolymerization rate constant of cofillin-GS-FA over that of FA. This could contribute to cytoskeletal changes during oxidative stress. Supported by Department of Veterans Affairs.

838 The Role of the Formin Homology 1 Domain in Actin Filament Elongation A. S. Paul, T. D. Pollard; Molecular Biophysics and Biochemistry, Yale University, New Haven, CT

Formin proteins nucleate actin filaments de novo for various cytoskeletal structures in eukaryotic organisms. The presence of the polypeptide-rich Formin Homology (FH) 1 domain, N-terminal to the actin-binding FH2 domain, increases the rate of elongation of formin-associated filaments in the presence of profilin-actin, presumably through direct interaction of profilin with individual polyproline tracts. The rate of elongation varies between FH1FH2 constructs from different formins, but all respond bi-phasically to the concentration of profilin (Kovar et al. Cell 124: 432-35, 2006). These features suggest that formins act with a common mechanism toward profilin-actin. In the present work, we sought to elucidate the molecular details of this process by measuring the elongation rate of filaments associated with S. cerevisiae Bni1(FH1FH2) consisting of FH1 domains with different numbers of polyproline tracts. In the presence of S. cerevisiae profilin, the rate of elongation associated with TIRF microscopy of individual filaments increases with the number of polyproline tracts in FH1, nearing a plateau at ~33 subunits/sec with 6 polyproline tracts at 1.5 µM actin. With 1.5 µM actin monomers, all constructs gave a maximum rate of elongation at about 2.5 to 5 µM profilin. Higher concentrations of profilin reduced the elongation rate. We also measured the elongation rate of filaments associated with the FH2 domains from either S. pombe Cdc12p or mouse mDia2 fused to the FH1 domain of Bni1p. Bni1[FH1]-Cdc12(FH2)-associated filaments exhibit elongation rates in the presence of profilin significantly less than those of Bni1[FH1FH2]. By contrast, at most concentrations of profilin, Bni1[FH1]-mDia2(FH2)-associated filaments grew faster than those of mDia2(FH1FH2) and similar to filaments associated with Bni1[FH1FH2]. These results best support a model in which profilin-actin binds to individual polyproline tracts in the FH1 domain. In mammalian cells, direct filament dissociation is the primary mechanism for formation of branched actin networks.

839 ForA Is a Diaphanous-related Formin Required for Phototaxis and Normal Development of Dicyostelium Cells N. Ramalingam, J. Faix, M. Schleicher; 1Department of Cell Biology, Ludwig-Maximilians-University, Munich, Germany, 2Department of Biophysical Chemistry, Hannover Medical School, Hannover, Germany.

Formins are large, multi-domain proteins involved in the nucleation of linear actin filaments. These proteins were shown to be important for cytokinesis, cell polarity, microtubule dynamics and the formation of filopodia. The sequencing of the Dicyostelium genome revealed 10 formins. Here we present evidence for the role of ForA in cell motility during the development of D. discoideum. ForA is a unique Diaphanous-Related Formin that harbouring a protein kinase C conserved region 2 (C2). Biochemical analysis of a recombinant FH2 fragment showed ForA-mediated nucleation and assembly of actin filaments in a dose-dependent manner in vitro. In contrast to DmDia2 that exclusively binds to profilin II (Shntenbeck et al. Nat Cell Biol. 2005: 769-74), ForA interacts specifically with the profilin I isoform. The characterization of the ForA-null mutant revealed multiple defects during early and late stages of the developmental cycle. We observed broken streams during early aggregation and severe inhibition of slug motility during phototaxis. These defects could be partially rescued by ectopic expression of the full-length forA. The data suggest that ForA is required for directed cell motility during the multicellular stage of development.

840 Myosin5a Tail Associates with Rab3A in Neuronal Cells T. Woelffert, Y. Lee, J. A. Mercer, D. W. Provance; 1Department of Biophysics and Biochemistry, Yale University, New Haven, CT, 2Department of Microbiology and Immunology, Dartmouth College, Hanover, NH, 3McLaughlin Research Institute, Great Falls, MT

The major RabGTPase in brain, Rab3A, is known to associate with synaptic vesicles (SV) and is involved in SV trafficking. We performed experiments to determine whether Rab3A is involved in myosin-Va (Myo5a) mediated transport of neuronal vesicles. In vitro motility assays performed with axoplasm from the squid giant axon showed a direct involvement of Myo5a in SV transport. The formation of the Myo5a-SV complex was not affected by the presence of Rab3A, but the transport of the complex was reduced in the presence of Rab3A. This suggests that Rab3A is required for the transport of Myo5a-SV complexes.

841 Nuclear Localization Sequences and Phosphorylation on Serine 1650 in the Globular Tail Domain of Myosin Va Determines Its Translocation to and Distribution within the Nucleus M. M. A. Baqui, M. C. S. Pranechievucis, S. R. Banzi, E. M. Espefaco, R. E. Larson; 1Cell and Molecular Biology, Faculdade de Medicina de Ribeirão Preto, University of Sao Paulo, Ribeirão Preto, Brazil

The globular tail domain of myosin Va is essential for its attachment to cargos, which include cytoplasmic organelles, membrane vesicles, and mRNA. Recently, we demonstrated the presence of myosin Va (MVa) within the nucleus, co-localized with nuclear speckles. The globular tail domain of MVa has two nuclear localization sequences (NLSs), as identified by peut nib-ac, one of which is adjacent to Ser1650 (mouse sequence), a site phosphorylated by calmodulin-dependent protein kinase II (CaMKII). Recombinant globular tail in fusion with EGFP translocates to the nucleus when transfected into B16-F10 or Si9-6 melanocytes, the latter being derived from dilute mice. The translocation was blocked by mutation of the basic amino acids of both NLSs to alanines, but not when only one NLS was mutated, suggesting that both are independently competent in determining nuclear localization. Mutation of Ser1650 to either alanine or glutamic acid did not affect nuclear localization. However, the ser/ala mutation altered the distribution of recombinant globular tail within the nuclear compartments. Similar to endogenous MVa, transfected wild-type globular tail was phosphorylated within the cell and co-localized with SC-35, a RNA splicing factor that earmarks interchromatin granule clusters in the nucleus, whereas the ser/ala mutant was not phosphorylated and was distributed diffusely throughout the nucleoplasm. Our data suggest that the translocation of MVa to the nucleus is determined by two NLSs and distribution within the nucleus involves phosphorylation of Ser1650. Financial support from FAPESP, FAEPB and CNPq.

842 Myosin 5c: A Molecular Motor for Actin-based Transport in Epithelial Cells D. T. Jacobs, R. E. Cheney; 1Cell and Molecular Physiology, University of North Carolina-Chapel Hill, Chapel Hill, NC

Class V myosins have been shown to function as motors for actin-based transport and polarized secretion. Vertebres express three class V myosins, each having a distinct tissue distribution. Myosin 5a is expressed chiefly in brain and melanocytes. Myosin 5b has a broader distribution pattern, and Myosin 5c (Myo5c) is most abundant in secretory epithelia (Rodriguez and Cheney, JCS, 115:991-1004). Myo5c exhibits a polarized localization to the apical domain of acinar cells in exocrine tissues including lacrimal gland, salivary glands, pancreas, prostate, and mammary gland. To investigate the role of Myo5c in organelle transport, we have utilized live-cell imaging to determine the distribution and dynamics of Myo5c in MCF-7 and HeLa cells. Immuno-staining of endogenous Myo5c reveals labeling of both cytoplasmic tubules and small puncta. The tubules span tens of micrometers through the cytoplasm and emanate from the cell center toward the periphery. In migrating MCF-7 cells, the small puncta exhibit a polarized localization near the leading edge. Live-cell imaging of tubules labeled by GFP-Myo5c shows outward, long-range translocations at a rate of ~0.5 um/s. The addition of norecordal (0.5 um) induces a rapid loss of these translocations, indicating that these structures are undergoing microtubule-dependent movement. Time-lapse imaging using Total Internal Reflection Fluorescence (TIRF) microscopy reveals that GFP-Myo5c labeled puncta move more slowly (~0.03 um/s), are closely associated with the ventral cell surface, and can be tracked for long periods of time (40+ minutes). Unlike the tubules, the velocity and distribution of the GFP-Myo5c puncta

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are insensitive to nocodazole treatment (0.5 μM). Current experiments are directed toward determining the identity, cellular functions, and regulation of the Myo5c compartment(s). (Supported by an APS Porter Fellowship and a UNC-CH Sequoyah Dissertation Fellowship to DTJ and NIH/NIDCD grant DC03299 to REC)

843 Myosin-Vb Remains Available for Actin Binding during Microtubule-based Transport
D. W. Provan, J. A. Mercer; McLaughlin Research Institute, Great Falls, MT
Cellular organization involves the intracellular trafficking of components through both actin- and microtubule (MT)-based movement. Sensitized Y119G mutant (and wild-type control) myosin-Vb was tagged with eGFP, expressed in HeLa cells and imaged before and after the microinjection of Nb-phenylethyl ADP (PE-ADP), which selectively and specifically inhibits the sensitized mutant myosin-Vb by inducing its tight binding to actin (Provan et al., PNAS 101:1868, 2004). Two prominent sites of myosin-Vb localization were observed: tubulovesicular structures in the cell periphery and discrete vesicles. The peripheral structures had highly dynamic edges from which vesicles budded. The discrete vesicles exhibited both actin- and microtubule-based movement, categorized on the basis of their speeds, trajectories, and sensitivity to latrunculin A or nocodazole treatment. In cells expressing the sensitized mutant myosin-Vb and injected with PE-ADP, all movement ceased within 2 min. The tubulovesicular peripheral structures became static and spherical. Vesicles also were no longer observed to originate from them and other vesicles froze within the cytoplasm. None of these effects were observed under control conditions consisting of wild-type myosin-Vb with PE-ADP, wild-type myosin-Vb injected with tracer only, or sensitized mutant myosin-Vb with tracer only. We conclude that this cessation of movement under experimental conditions is a direct result of sensitized mutant myosin-Vb binding to actin. Our data strongly support a model in which myosin-Vb cooperates with MT-based motors on peripheral endosomes to create opposing forces necessary for vesicle formation. Furthermore, those vesicles, which continue to be labeled by eGFP-tagged myosin-Vb, are apparently constantly capable of interacting with actin through myosin-Vb activity, even when they are passengers on vesicles undergoing MT-based transport.

844 A Role for Myosin Va in mRNP Transport
A. J. Lindsay, M. W. McCaffrey; Biochemistry, University College Cork, College Road, Cork, Ireland
The yeast class V myosin, Myo4p, has a well established role in mediating the transport of messenger ribonucleoprotein particles (mRNPs). In contrast little evidence has been published to suggest a similar role for class V myosins in eukaryotic cells. There are three myosin V isoforms in mammals, with the majority of work to date focusing on their roles in membrane transport. While comparing the localisation of endogenous myosin Va and myosin Vb in HeLa cells we observed that in addition to localising to vesicles distributed throughout the cytoplasm, myosin Va also labelled several large cytoplasmic ‘foci’. We subsequently determined that these foci were P-bodies (PBs), cytoplasmic structures that are involved in mRNA degradation. PBs and stress granules (SGs) are dynamically linked and indeed myosin Va redistributes from PBs to SGs upon exposure to various forms of cellular stress. We also found that myosin Va disassociated from PBs upon treatment with translational inhibitors, a characteristic of several PB components. We have found that in myosin Va-null S. cerevisiae cells a number of PB components do not localise to characteristic cytoplasmic foci despite being expressed at levels similar to that seen in wild-type cells. In the S91 cells we also observed that a subset of RNA binding proteins, including the Fragile X Mental Retardation Protein (FMRP), appear trapped in a large perinuclear structure whereas in myosin Va-expressing cells these proteins are distributed in a punctate pattern throughout the cell. FMRP also fails to associate with ribosomes in S91 cells, as determined by a polysome sedimentation assay. Taken together these results implicate myosin Va in mRNA transport and turnover, a role that has long been established for yeast myosin V.

845 Myosin Vc Cycling between Rab3D-enriched Secretory Vesicles and Rab11-enriched Endosomes in Lacrimal Acinar Epithelial Cells Is Stimulated by Carbachol
R. Marchelletta,1 D. Jabobs,2 A. Monroy,1 R. Cheney,2 S. Hamm-Alvarez1; 1University of Southern California, Los Angeles, CA, 2University of North Carolina, Chapel Hill, NC
Lacrimal acinar cells are secretory epithelia that support health of the ocular surface through the secretagogue-induced release of tear proteins. Myosins are a superfamily of proteins sharing a conserved motor domain that hydrolyzes ATP to generate movement along actin filaments but expressing diverse tail domains. Myosin Vc is highly expressed in epithelial cells, particularly acinar epithelial cells including lacrimal acini. Here we report that Myosin Vc associates with membranes enriched in the mature secretarial vesicle marker, Rab3D, and the apical endosomal marker, Rab11, in a secretagogue-dependent manner in primary cultured rabbit lacrimal acini. Confocal fluorescence microscopy revealed that Myosin Vc and Rab3D are both distributed in subapical secretory vesicles in unstimulated acini but that the extent of co-localization of total pixels associated with Myosin Vc with Rab3D is significantly decreased following stimulation of secretion with carbachol (CCH, 100 μM) from 42% to 29% (p<0.05, n=7). Rab11 and Myosin Vc were markedly co-localized on subapical membranes, and the extent of co-localization of the total pixels associated with Myosin Vc with Rab11 was significantly increased by CCH stimulation from 44% to 52% (p<0.05, n=6). Our previous studies have shown that expression of Myosin Vc tail fused to GFP, a dominant negative construct, in rabbit lacrimal acini using adenovirus results in labeling of mature secretory vesicles. Moreover, overexpression of GFP-Myosin Vc-tail significantly inhibited CCH-stimulated release of two secretory products, co-transduced syncollin-GFP which is found in Rab3D-enriched membranes and secretory component derived from polymeric immunoglobulin A receptor which is found in Rab3D- and Rab11-enriched membranes. These results suggest that a population of Myosin Vc is redistributed from mature secretory vesicles to apical endosomes by CCH stimulation and support a model in which myosin Vc on mature secretory vesicles aids in actin-dependent exocytosis of secretory vesicles and/or recycling of secretory vesicle membrane to apical endosomes.

846 Identification and Characterization of a Minimal Myosin V Binding Site within an Intrinsically Disordered Region of Melanophilin
N. C. Geeth, J. A. Spudich; Department of Biochemistry, Stanford University, Stanford, CA
Melanophilin directly binds to myosin V and is tethered to the melanosome membrane via Rab27a. We undertook a study to describe the biochemical nature of the interaction between myosin V and melanophilin. Here we demonstrate that the myosin V binding domain of melanophilin is an intrinsically disordered protein. We also conclude that myosin V binds to melanophilin in this disordered state and that binding does not induce the global folding of melanophilin. Using highly purified recombinant proteins we find that the myosin V globular tail domain binds a 9 amino acid peptide within this disordered segment. The interaction is characterized by a dissociation constant of 0.5 micromolar that is driven by a favorable enthalpy of binding as measured by isothermal titration calorimetry. We also find that phosphorylated myosin V tail domain binds with the same affinity to melanophilin, which suggests that phosphorylation of myosin V serine 1650 does not directly alter myosin V’s interaction with melanophilin.

847 The C-tail Domain, Not the Motor, Determines the In Vitro Behavior and In Vivo Function for Yeast Myo4p
B. D. Dunn, P. A. Takizawa; Cell Biology, Yale University, New Haven, CT
Saccharomyces cerevisiae contains two class V myosins: Myo4p which transports Ash1 mRNA to the bud tip and Myo2p which transports secretory vesicles and other organelles. Whether the biochemical properties of Myo4p and Myo2p differ to suit their different types of cargo is unknown. We purified and analyzed the activities of native Myo4p and Myo2p. Unlike Myo2p and other class V myosins, Myo4p is a monomer. In actin gliding assays, decreasing Myo4p concentration increases filament velocity. In contrast, decreasing Myo2p concentration decreases filament velocity, indicative of a nonprocessive motor. To determine if these different motor properties are physiologically important, we engineered chimeric myosins containing the motor domain of one myosin and the C-tail of the other. A Myo2p motor -Myo4p tail chimera has Myo2p kinase activity and Myo4p properties. In addition, the Myo2p motor -Myo4p tail rescues Ash1 mRNA localization in myo4Δ cells. The reverse Myo4p motor -Myo2p tail chimera is unable to substitute for the conditional myo2Δ mutant at the restrictive temperature. Thus, at least for Myo4p, the tail domain regulates both motor activity and cargo binding, while the motor domain is less critical for effective cargo transport.
Myosin VI mRNA Co-localizes with β-actin mRNA and ZBP1 in Fibroblasts and Neurons

A. L. Wells, A. J. Rodriguez, J. S. Condeelis, R. H. Singer; Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, NY

In many cell types, messenger RNA (mRNA) localizes to subcellular compartments defining cell fate, polarity and cell motility. In migrating fibroblasts, mRNA for actin, and several other proteins involved in actin polymerization, localizes to the leading edge. Localization of these proteins allows the functional actin polymerization machinery to be targeted to the leading edge where the protein products are used for lamellipod protrusion. Myosin VI is a protein localizing to membrane ruffles at the leading edge and may be involved in Arp2/3-mediated actin polymerization. Myosin VI is the only actin-based motor protein known to move toward the minus end of actin filaments and has functions in membrane trafficking and remodeling events, endocytosis, and cell migration. When myosin VI is depleted, lamellipodial protrusion and cell migration are inhibited, implicating myosin VI in membrane dynamic functions leading to cell movement. Here we show that expression of Myosin VI mRNA is also localized to the leading edge of fibroblasts along with β-actin and Arp2/3 mRNAs. Additionally, myosin VI mRNA co-localizes with β-actin mRNA and its translational repressor, zipcode binding protein (ZBP1) to neuronal dendrites where myosin VI protein may function in AMPA receptor internalization. We propose that the localization of myosin VI mRNA is a mechanism by which the cell 1) compartmentalizes and regulates myosin VI activity, 2) positions the motor protein at the barbed end of actin filaments in order for it to move towards the pointed end and 3) targets the translation and assembly of macromolecular complexes with which myosin VI functions.

How Myosin VI Coordinates Its Heads during Processive Movement

S. S. Rosenfeld; H. L. Sweeney; H. L. Sweeney; 2Neurology, Columbia University, New York, NY, 3Physiology, University of Pennsylvania, Philadelphia, PA

Introduction: Optimal processive movement requires coordination between the heads of dimeric motor proteins. In myosin V, this coordination involves a coupling of the lever arm position with ADP release in a process that is controlled by the intramolecular strain. However, the lever arm positions are reversed for myosin VI, and therefore this mechanism cannot be used to gate the head of a processive myosin VI dimer. In this study, we have examined the kinetics of nucleotide binding to and release from monomeric and dimeric constructs of myosin VI in order to develop a model of how processivity is maintained in this motor. Results: Utilizing the fluorescent nucleotide analogues 2’-deoxy 3’-mamT and ATP and ADP (2’dmT, 2’dmD) we found that intramolecular strain had no appreciable effect on most of the steps in the myosin VI ATPase cycle, including phosphate release, the weak-to-strong binding transition, and ADP release. By contrast, the kinetics of 2’dmT binding were markedly affected by strain, with binding of nucleotide to the leading head occurring only after the trailing head had dissociated. Deletion of a unique insertion found only in myosin VI abolished this strain-dependent gating of the two motors of a myosin VI dimer. Conclusions: Our results are consistent with a model in which rearward strain blocks ATP binding to the leading head head once it has released its ADP. The structural basis for this unique gating mechanism involves an insertion near the nucleotide-binding pocket that is found only in class VI myosin. While this mechanism is unlike that of any other myosin superfamily member, it bears remarkable similarities to that of another processive motor from a different superfamily—kinesin I.

Myosin VI, Unlike Myosin V, is ubiquitously Expressed in PC12 Cells and upon Stimulation Accumulates in Perinuclear Area and Nucleus

M. Sobczak, 1L. Majewski, 2A. Walski, 2M. J. Redowicz; 1Department of Muscle Biochemistry, Nencki Institute of Experimental Biology, Warsaw, Poland, 2Department of Cell Biology, Nencki Institute of Experimental Biology, Warsaw, Poland

Myosin VI (MVI) is an unusual actin-based motor walking towards the minus end of actin filaments, in the opposite direction to the other known myosins. It is believed that MVI is involved in subcellular transport of membrane-containing structures, including clathrin-based endocytosis, as well as cell spreading and migration. Here, we aimed at testing the role of MVI in secretory bovine adrenal medulla and rat pheochromocytoma cells (PC12). MVI, but not myosin I and IIB, was detected in chromaffin granules and resided on the apical side of the vesicles. This association is very tight, because only addition of Triton X100 has removed it from the vesicles. EDC crosslinking carried out on PC12 cytosolic fraction indicates that MVI is a dimer. Myosin VI is ubiquitously distributed within PC12 and cultured adrenal medulla cells, as it partially colocalizes with the markers of the following cytoplasmic compartments: Golgi, endoplasmic reticulum, mitochondria, clathrin-coated pits, early endosomes and secretory granules, it is also visible next to plasma membrane. It has also been found within the nucleus what has been confirmed with three different anti-myosin VI antibodies. The nuclear localization was even more pronounced after 2-5 min cell stimulation with 56 mM KCl; myosin VI occupied the areas lacking heterochromatin. This may indicate involvement of this molecular motor in the gene transcription. In contrast, upon stimulation myosin V seemed to translocate to the peripheral area, where its colocalization with chromaffin granule marker was more pronounced. These data seem to indicate different roles for these two myosins in secretory cells.

Intracellular Targeting of Myosin VI Requires Protein-Protein Interactions and Lipid Binding

G. Spudich, 1M. Chibalin, 2C. Puri, 2J. Au, 2F. Bass, 2J. Kendrick-Jones; 1MRC Laboratory of Molecular Biology, Cambridge, United Kingdom, 2Clinical Biochemistry, CIRM, University of Cambridge, Cambridge, United Kingdom

Myosin VI is a minus end directed actin based motor protein. It localises to intracellular compartments, such as membrane ruffles, endocytotic clathrin-coated and uncoated vesicles, as well as secretory vesicles and the Golgi complex. Targeting of myosin VI to specific cellular localisations involves a number of binding partners, for example optineurin, Dab2 and GIPC. Using a combination of mammalian two-hybrid and pull-down assays we have identified two independent binding sites on the C-terminal region of myosin VI tail. Mutation of Thr1184 to Leu in this region abolishes myosin VI binding to Dab2 but has no effect on binding to GIPC or optineurin, whereas mutation of RRL (aa 1107-1109) to AAA abolishes GIPC and optineurin binding. These results indicate that the C-terminal tail of myosin VI contains two hot spots for cargo binding, which bind multiple binding partners. In addition, the C-terminal tail domain contains a lipid binding region. In sedimentation and FRET-based binding assays the tail binds with high affinity and specificity to phosphatidylinositol-4,5-bisphosphate (PIP2) containing liposomes. To investigate the role of lipid and protein binding on the in vivo functions of myosin VI, we expressed in HEA cells a GFP-tagged myosin VI tail with mutated PIP2, Dab2 and GIPC binding regions. When myosin VI tail strongly co-localised with clathrin-coated structures (CCS), recruitment of the PIP2 mutant to CCS was significantly reduced. Mutating the Dab2-binding site completely abolished targeting to CCS in vivo, whereas mutation in the GIPC binding site had no effect. Our current studies at the ultrastructural level together with functional assays in polarised cells are focused on determining the role of myosin VI in clathrin-mediated endocytosis.

Roles of Myosin-X (Myo10) in Filopodia Formation, Intracellular Motility, and Cell Spreading

A. B. Bohil, 1O. A. Quintero, 1T. Yin, 1D. T. Jacobs, 1M. M. Divito, 1M. L. Kerber, 1R. E. Cheney; 1Cell and Molecular Physiology, University of North Carolina at Chapel Hill, Chapel Hill, NC, 2Biology, Franklin and Marshall College, Lancaster, PA

Filopodia and related structures that contain a core of bundled actin filaments are thought to play important roles in cell motility and cell-cell signaling. Despite their importance, the molecular mechanisms regulating filopodia formation and function are not yet clear. Myo10 is a vertebrate specific MyTH4-FERM myosin that localizes to the tips of filopodia and is hypothesized to function as a motor for intrafilopodial motility. Here we report that transfection with GFP-Myo10 and other constructs that have previously been shown to induce massive increases in dorsal filopodia also lead to ~3-fold decreases in the spread area of COS-7 cells. Only Myo10 constructs that are able to induce filopodia lead to decreased cell spreading. Conversely, inhibiting the formation of the numerous dorsal filopodial structures in HeLa cells by transfecting cells with (1) siRNA to Myo10, (2) a dominant negative Myo10 construct, or (3) dominant negative Cdc42 each increase cell spreading ~2-fold. Together this data suggests an inverse correlation between filopodia formation and cell spreading. To investigate the role of Myo10 dimerization in tip localization, we show that forced dimerization of a construct consisting of the head, neck, and first 34 amino acids of the tail is sufficient to endow tip localization. Although both Myo10 and formins are thought to be components of the filopodial tip complex, live cell imaging of HeLa cells co-transfected with GFP-Myo10 and constitutively active GFP-mDia2 indicates that these constructs sometimes localize to distinct puncta within retraction fibers and do not always move coordinately. Finally, we have used live cell imaging and TIRF microscopy to show that transmembrane enrichment of Myo10 as integral underpinning transport with Myo10.
allowing Shigella to spread from cell to cell and produce hemmorhagic plagues. Intracellular motility is a vital facet of S. flexneri's pathogenesis and requires that the bacterium usurp the host cell's actin machinery. Previous studies have successfully reconstituted actin-based motility of S. flexneri and Listeria monocytogenes (another bacteria that undergoes intra- and intercellular actin-based motility) in vitro by providing a small number of actin-binding proteins, and show that in extracts myosin motors are unnecessary. However, we found that in living HeLa cells, GFP-Myosin X (Myo10), a recently described unconventional myosin, concentrates in the actin tails of motile Shigella, as well as along the sides of filopodia containing Shigella. Under the same experimental conditions we have found that Myo10 fails to localize to Listeria-induced actin structures. Immunofluorescence microscopy utilizing a specific anti-Myo10 antibody corroborated these findings. siRNA knock-down of endogenous Myo10 resulted in a significant reduction in S. flexneri speeds by one third, as compared to infected cells transfected with control siRNA (avg 0.059 um/s +/- 0.002 SEM, n = 396 compared to 0.088 um/s +/- 0.002 SEM, n = 336, respectively, p < 0.0001). Knock-down of Myo10 had no effect on L. monocytogenes intracellular speeds. We conclude that in addition to actin, Shigella, but not Listeria, utilizes Myo10 for efficient intracellular movement in living host cells.

854  Myosin-10 Functions to Maintain Spindle Integrity during Mitosis

S. Woolner, W. Bennett; Department of Zoology, University of Wisconsin-Madison, Madison, WI

Myo10 (Myo10) is a vertebrate unconventional myosin that can bind to both filamentous actin (F-actin) and microtubules. We have previously shown that Myo10 plays a key role in the Xenopus laevis oocyte, where it is required for proper nuclear anchoring and meiotic spindle assembly. We believe that Myo10 may function in these processes by mediating interactions between F-actin and microtubules. Here we report new findings that Myo10 plays a crucial role in mitosis. We find, by immunofluorescence, that Myo10 localizes close to the poles of the mitotic spindle, a position that it maintains throughout mitosis. Moreover, knockdown of Myo10 using a morpholino antisense oligo in Xenopus embryos, leads to a clear and reproducible mitotic phenotype. The mitotic spindles in Myo10 morpholino injected embryos exhibit a delay in metaphase and then begin to develop multiple additional poles. All extra poles show an accumulation of the spindle pole marker, gamma-tubulin, suggesting that these poles may result from fragmentation of the bona fide poles. To investigate this further, we have used live imaging to follow the development of the extra spindle poles in vivo, and it appears that these poles do indeed arise from the bona fide poles. We therefore propose that Myo10 acts to stabilize the mitotic spindle. We are currently investigating the mechanism by which Myo10 fulfills its role in the mitotic spindle.

855  Using Small Molecules to Study the Role of Myosin Light Chain (TgMCL1) in Toxoplasma gondii Motility and Invasion

A. T. Heaslip, K. L. Carey, D. M. Warshaw, N. J. Westwood, G. E. Ward; Dept. of Microbiology and Molecular Genetics, University of Vermont, Burlington, VT, Dept. of Molecular Physiology and Biophysics, University of Vermont, Burlington, VT, Centre for Bimolecular Sciences, University of St. Andrews, St. Andrews, United Kingdom

Toxoplasma gondii is an obligate intracellular parasite that can cause life-threatening disease in immunocompromised individuals. The pathology of the disease is caused in part by repeated cycles of host cell invasion, parasite replication and host cell lysis. Our lab is interested in identifying parasite proteins involved in the invasion process. To this end we developed a high-throughput screen to identify small molecule inhibitors of invasion. Of 12,160 small molecules screened, 24 were shown to inhibit invasion and 21 of these inhibitors were also found to significantly inhibit parasite motility. A membrane associated complex of proteins, termed the glideosome, powers gliding motility and host cell penetration in T. gondii. The glideosome consists of four major proteins: TgMyoA, an unconventional class XIV myosin, myosin light chain (TgMCL1); and glideosome-associated proteins 45 and 50 (TgGAP45, TgGAP50). In an attempt to identify the targets of the motility/invasion inhibitors we immunoprecipitated the glideosome complex from parasites treated with each inhibitor to determine if any of the inhibitors changed its composition. Treatment with one or two inhibitors results in the immunoprecipitation of a modified form of MLC with the glideosome; the modified form shows increased electrophoretic mobility on SDS PAGE gels. The nature of the modification is being determined by mass spectrometry analysis. We have developed a method for isolating the glideosome for in-vitro motility assays and shown that TgMyoA has a velocity of 5um/s, in good agreement with previously published data. Experiments to determine the functional effects of the MLC modification in this in vitro motility assay are currently underway. By identifying the nature of the MLC modification and determining the functional effects of this modification we will gain new insights into the role of myosin light chain in regulating glideosome function and parasite motility.

856  Myo1 Localizes to Phagosomes in Tetrahymena

R. E. Hosen, R. H. Gavin; Biology, Brooklyn College—CUNY, Brooklyn, NY

Myo1 is one of 12 Tetrahymena myosins that form a subclass in myosin Class XIV. An earlier study showed that in a Myo1 knockout, the rate of phagosome formation was significantly reduced, and macronuclear elongation failed to occur in some cells. Recent investigations of actin localization and phagosome motility revealed actin filaments as prominent extensions from one or two regions at the periphery of phagosomes. Phagosomes moved randomly in the cytosol of knockout cells in contrast to directed movement toward the posterior end in wild-type cells. In the present study, localization of Myo1 was undertaken in further to demonstrate the association of Myo1 with both phagocytosis and macronuclear division. Polyclonal antibodies were generated against a peptide in the Myo1 motor domain and used for immunolocalisation and immunofluorescence microscopy. The antibody detected a 180-kDa polypeptide on immunoblots of wild-type cell-lysate proteins resolved by SDS-PAGE. The 180-kDa polypeptide was not present on immunoblots of knockout cell-lysate proteins. Prior to fixation for immunofluorescence microscopy, cells were challenged with fluorescent beads as markers for phagosomes. Confocal x-z scans showed that in wild type, Myo1 localized to the periphery of phagosomes. Some phagosomes in wild type appeared to be unlabeled. Labeled phagosomes were frequently observed at the periphery of the macronucleus, although the antibody did not label the macronucleus. In the Myo1-knockout only background fluorescence was observed. We conclude that localization of Myo1 is consistent with a role in phagocytosis and macronuclear division. Supported by NSF Grant 0517083.

857  VwkA, an Unconventional Protein Kinase Involved in the Regulation of Contractile Vacuole Dynamics in Dictyostelium discoideum

V. Betapudi, T. T. Egelhoff; Physiology and Biophysics, Case Western Reserve University, Cleveland, OH

Earlier, we reported VwkA, a novel unconventional protein kinase localized to contractile vacuoles and Golgi complex like structures in Dictyostelium discoideum. This VwkA protein carries an N-terminal von Willebrand Factor A (vWFA) motif and a catalytic domain having sequence homology with the catalytic domain sequence of nonmuscle myosin II in Dictyostelium and ion-channel kinases in mammalian cells. Although, the purified VwkA protein failed to phosphorylate nonmuscle myosin II in vitro, but have significant effects on many myosin II-dependent functions inDictyostelium discoideum. Here, we report characterization of VwkA sub-domains and its involvement in the regulation of contractile vacuole functions in Dictyostelium discoideum. VwkA and overexpressing (VwkA++) cells display altered osmotic shock responses. The size and shape of the contractile vacuicles in vwkA- cells were found different from those in wild type cells. GFP-VwkA carrying a mutant catalytic domain failed to localize to contractile vacuoles in the cells. These studies suggest that VwkA involves in the regulation of contractile vacuole dynamics in Dictyostelium discoideum.

859  Mitotic Kinesin Eg5 Achieves Processive Stepping by Alternating Site ATP Hydrolysis

T. C. Krzysziak, S. P. Gilbert; Biological Sciences, University of Pittsburgh, Pittsburgh, PA

Eg5/KSP is a member of the Kinesin-5 (BimC) family of homotetrameric motors. These N-terminal kinesins provide a plus-end-directed force associated with sliding microtubules during centrosome separation to assemble the bipolar spindle. To define the cooperative interactions between the motor heads and the microtubule, a dimeric motor was engineered. This Eg5 dimer is processive with its motor heads binding along a single protofilament (Valentine et al. 2006. Nature Cell Biol. 8, 470 ; Krzysziak et al. 2006. EMBO J. 25, 2263). The steady-state and presteady-state kinetics show that ATP binding occurs as a two-step process with an ATP-dependent isomerization at 50 s-1, which forms the ATP-hydrolysis competent intermediate. Single-turnover experiments were required to detect a presteady state burst of ATP hydrolysis. At ATP concentrations in which only one head participates, the rate was ~5 s-1; however, at ATP concentrations where both heads of the dimer participate, the rate is observed at approximately half. In addition, the kinetics of P i release and ATP-promoted microtubule dissociation are observed at the rate of ATP hydrolysis. Microtubule association and mantADP release are fast steps in the pathway. However, mantADP release is biphasic with an initial fast phase followed by a second slow phase. These kinetics are consistent with ATP hydrolysis as the rate-limiting step and an alternating cycle of ATP turnover in which the active sites are coordinated to establish processive stepping along the microtubule. Supported by NIH GM54141 & K02-AR47841.

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Kinesin-8s.

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Dynamics of Molecular Motors Studied by FLAP

M. Uteng, T. Surrey; Cell Biology & Biophysics, EMBL, Heidelberg, Germany

Motor proteins are known to be essential for the reorganization of the cytoskeleton during cell division. However, to what extent the kinetic properties of these molecular motors determine their function during cell division is not understood. We present here a new kinetic assay to measure unbinding rates and diffusion constants of molecular motors in vitro at steady state. In this assay, the molecular motors are fused to a Photoactivatable GFP, and the Fluorescence Loss After Photoactivation (FLAP) is monitored. The method was validated by measuring diffusion and unbinding rate of conventional kinesin from a single microtubule, and the results were in agreement with data described in literature. Presently, we are applying this assay on the mitotic motor, Kip3, whose unbinding rate is unknown. The analysis of Kip3 is being done on a single microtubule and inside bundled microtubules. This will hopefully give us an understanding of how the microtubule organization affects Kip3’s kinetics, and consequently how it functions within the established mitotic spindle.

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The Homotetrameric Kinesin-5, KLP61F, Antagonizes Ncd in Motility Assays

L. Tao,1 A. Mogilner,1 G. Civelekoglu-Scholey,1 R. Wollman,1 J. Evans,2 S. Henning,2 J. M. Scholey,2 1Center for Genetics and Development, UC Davis, Davis, CA, 2Section of Molecular and Cellular Biology, UC Davis, Davis, CA

We have tested the hypothesis that the purified antagonistic mitotic motors, KLP61F (a kinesin-5) and Ncd (a kinesin-14), can generate a force-balance that could position spindle poles at a constant steady-state length in Drosophila embryo spindles (Sharp et al., 1999b). KLP61F and Ncd were purified in high yield and purity from insect cells and found to behave as homotetrameric and homodimeric species, respectively, with identical hydrodynamic properties to the native proteins in embryo extracts. A region of the stalk containing residues 631-790 of KLP61F, which lacks the spindle-targeting “BimC box”, is required for tetramerization. Using fluorescence microscopy and cryo EM, we find that purified KLP61F and Ncd are capable of crosslinking MTs into bundles in the presence of MgATP. In multiple-motor motility assays, KLP61F and Ncd drove plus-end and minus-end-directed MT sliding at 0.04 and 0.1 µm/s, respectively. Competitive MT gliding assays using mixtures of the two motors reveal that the rate of motility of either motor is decreased by the increasing the mole fraction of the opposing motor. We identified a “balance point” at which the mean velocity of MT motility was zero, but where MTs paused briefly or displayed episodes of stochastic oscillations, taking ~0.3 µm excursions at ~0.2 µm/s in the plus then minus-end direction every 15s or so. The results, when analyzed by quantitative modeling suggest that opposite-polarity motors could act as “brakes” on one-another to modulate the rate of spindle pole separation, but additional factors would be needed to produce a stable, prometaphase spindle length.

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Plus End-specific Depolymerase Activity and Plus End-directed Motility of Kip3 (Kinesin-8) Explain Its Role in Positioning Mitotic Spindles during Asymmetric Cell Division

M. L. Gupta,1 P. Carvalho,1 D. Rout,2 D. Pellman3; 1Pediatric Oncology, Dana-Farber Cancer Institute, Boston, MA, 2Program in Cell and Molecular Biology, University of Pennsylvania, Philadelphia, PA

Budding yeast Kip3 is a member of a conserved family of microtubule motors (Kinesin-8) required for microtubule-cortical interactions, normal spindle assembly, and kinetochore dynamics. We demonstrate that Kip3 is both a plus end-directed motor and plus-end specific depolymerase, a unique combination of activities not found in other kinesins. The Kip3 ATPase activity was activated by both microtubules and unpolymerized tubulin. Furthermore, Kip3 in the ATP-bound state formed a complex with unpolymerized tubulin. Thus, motile Kinesin-8s may depolymerize microtubules by a mechanism that is similar to that used by non-motile Kinesin-13 proteins. Fluorescent speckle analysis established that in vivo Kip3 moved toward and accumulated on the plus ends of growing microtubules, suggesting that motor activity brings Kip3 to its site of action. Globally, and more dramatically upon cortical contact, Kip3 promoted catastrophes, pausing, and inhibited microtubule growth. These findings explain the role of Kip3 in positioning the mitotic spindle in budding yeast and possibly other processes controlled by Kinesin-8s.

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A Microtubule-destabilizing Kinesin-13 Affects Meiotic Spindle Length and Position in Oocytes

J. Zou, C. D. Yankel, K. Su, S. A. Endow; Cell Biology, Duke University Medical Center, Durham, NC

The Kinesin-13, Klp10A, is a microtubule depolymerizer in Drosophila that regulates microtubule dynamics in mitosis. Klp10A binds to mitotic spindle poles and centromeres, and depolymerizes microtubule minus ends, promoting poleward flux - slow poleward movement of spindle microtubules. During interphase, Klp10A is targeted to microtubule plus ends by EB1, where it is reported to stimulate catastrophes, growth-to-shrinkage transitions. Up to now, little is known about Kinesin-13 proteins in meiosis. Here we show that full-length Klp10A-GFP is associated with the unusual pole bodies of meiotic meiosis I spindles in klp10A-gfp oocytes, and decorates meiotic chromosome centromeres and cortical microtubules. The pole bodies of meiosis I spindles can be observed bound to fluorescently labeled cortical microtubules in klp10A-gfp oocytes, indicating that Klp10A binding both to the pole bodies and cortical microtubules could anchor spindles to the cortex. Treatment of klp10A-gfp oocytes by the microtubule destabilizing drug colchicine results in rudimentary spindles that are displaced towards the oocyte interior and ~2-fold longer Klp10A-GFP signals on cortical microtubules compared to controls. Treatment by the microtubule stabilizing drug paclitaxel results in either elongated or foreshortened spindles that are displaced from the cortex and ~2-fold shorter Klp10A-GFP signals on cortical microtubules than controls. A dominant negative Klp10A-GFP causes spindles to be shorter than wild type and suppresses the effects of colchicine and paclitaxel on spindle length and displacement from the oocyte cortex. These results show that microtubule dynamics, mediated by Klp10A, plays an important role in maintaining oocyte meiotic spindle structure and anchoring to the cortex. They demonstrate that Klp10A binds with higher affinity to destabilized than stabilized microtubules in oocytes, enhancing the destabilizing effects of colchicine. Our findings contrast with a previous report that Klp10A targets to polymerizing, rather than depolymerizing microtubule plus ends during interphase.

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The Role of the Kinesin-13 Neck in Microtubule Depolymerization

C. A. Moore,1 J. Cooper,1 M. Wagenbach,1 Y. Ovechkina,1 L. Wordeman,2 R. A. Milligan3; 1Department of Crystallography, Birkbeck College, London, United Kingdom, 2Department of Physiology and Biophysics, University of Washington School of Medicine, Seattle, WA, 3Department of Cell Biology, The Scripps Research Institute, La Jolla, CA

To ensure genetic integrity, replicated chromosomes must be accurately distributed to daughter cells - a process that is accomplished on the microtubule spindle. Kinesin-13 motors play an essential role in this process by performing regulated microtubule depolymerization. We set out to dissect the depolymerization mechanism of these kinesins, and in particular, their role of their conserved neck sequence. We used a monomeric kinesin-13 MCAC construct, consisting of the neck and motor core, which has strong depolymerizing activity. In the presence of a non-hydrolysable ATP analogue, this construct induced formation of rings around microtubules. The rings are built from tubulin protofilaaments that are bent by the kinesin-13 motor engaged at the ATP-binding step of its ATPase cycle. Our data suggest that the ring-microtubule interaction is mediated by the neck and support the idea of a role for the kinesin-13 neck in depolymerization efficiency, acting by optimising release of tubulin from microtubule ends.

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Functional Studies of Human Kip3 Kinesins
Kinesins of the kip3 family (Kip3s) have been shown to play important roles in mitosis in several organisms including S. cerevisiae, S. pombe and D. melanogaster. Genetic and cell biological evidence suggests that these kinesins function either directly or indirectly to increase microtubule instability. We have identified 3 human kinesins that are members of the Kip3 family. Two of these, Kip3a and Kip3d, are more highly expressed in proliferating tissues and are down regulated in differentiated neurons, suggesting that they are mitotic kinesins. Immunofluorescence staining with a peptide antibody directed against Kip3d reveals localization to the ends of spindle microtubules. Depletion of Kip3d mRNA by siRNA in SK-OV-3 cells results in a mitotic arrest with elongated bipolar spindles. Time-lapse imaging of Kip3d depleted SK-OV-3 cells demonstrates that these cells undergo a prolonged period of mitotic arrest, frequently followed by cell death. Addition of purified recombinant Kip3d motor domain, expressed in E.coli, to microtubules caused a decrease in the number of microtubules assessed by a microscopic assay and polymer mass in a sedimentation assay. All of these data together suggest that Kip3d has an essential role in mitosis and acts to regulate microtubule dynamics by directly destabilizing microtubules.

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Human Kif18, A Member of Kinesin-8 Family, Regulates Microtubule Dynamic Instability in Mammalian Cells
C. Zhu, S. Sandall, A. Desai, W. Jiang; 1The Barham Institute for Medical Research, La Jolla, CA, 2Ludwig Institute for Cancer Research, University of California, San Diego, La Jolla, CA
Kinesins are microtubule (MT) motor proteins that regulate many cellular processes including MT dynamic instability. Previously, we identified at least 12 human kinesins involved in mitosis/cytokinesis using RNA interference (RNAi), immunofluorescence microscopy and time-lapse analysis. Here, we describe the characterization of Kif18, a member of kinesin-8 family, in regulating MT dynamic instability in mammalian cells. Immunoblotting and immunofluorescence analysis indicate that expression and subcellular localization of Kif18 is regulated during the cell cycle. In interphase, the expression levels of Kif18 are low with the majority of Kif18 protein localized to the nucleus and a minority of Kif18 protein associated with plus-ends of MTs in the cytoplasm. During mitosis, the expression levels of Kif18 are increased dramatically with Kif18 associated with mitotic spindle MTs, kinetochores and plus-ends of astral MTs in early mitosis and then translocated to spindle midzone/midbody in late mitosis and cytokinesis. Kinetochoore localization of Kif18 is MT-dependent since such localization can be abolished by treatment of MT depolymerizing drug, nocodazole. In contrast, localization of kinetochores and plus-ends of astral MTs of Kif18 is enhanced by treatment of MT stabilizing drug, taxol. Time-lapse microscopy shows that depletion of Kif18 by Kif18 siRNA in HeLa cells results in prolonged delays of the prometaphase to metaphase and the metaphase to anaphase transitions. Long mitotic spindle MTs that cross over the metaphase plate are often observed in Kif18 depleted cells. Using GFP-E2B as a marker, we observe increased numbers of spindle MT polymerization in Kif18 depleted cells. Consistently, overexpression of Kif18 in CHO cells results in depolymerization of MTs network in vivo. In addition, recombinant Kif18 purified from insect cells using baculovirus expression system shows that Kif18 has MT depolymerizing activity in vitro. These data demonstrate that Kif18 functions as a MT depolymerase that regulates MT dynamic instabilities in mammalian cells.

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The Kinesin KIF17 Is Part of the EB1 Microtubule TIP Tracking Complex in Mammalian Epithelial Cells
F. Jaulin, G. Kretzner; Cell and Developmental Biology, Weill Medical College of Cornell University, New York, NY
Microtubule “Search and Capture” is an evolutionarily conserved process involved in cell polarization. EB1, a core protein in the microtubule TIP-tracking complex, associates with polymerizing microtubules which dynamically search the cell cortex. Local activation of membrane associated proteins leads to the cortical capture of microtubules, initiating cytoskeletal remodeling and cell polarization. Microtubule polymerization by EB1 is dependent on the interaction with EB1 by a kinesin for polarized cell growth in S. pombe; in multi-cellular organisms however, no kinesin has yet been described to contribute to polymerization through microtubule “Search and Capture”. Using epithelial cells as a model for polarization, we screened by GST pull-down for kinesins that interact with EB1. We found that KIF17, a kinesin family member, interacts with EB1. We confirmed the relevance of the interaction between KIF17 and EB1 by co-immunoprecipitation of endogenous proteins from epithelial cell lysates. In vitro binding assays revealed that the interaction of KIF17 with EB1 is direct and we have mapped the domains responsible for this interaction. In epithelial cells, KIF17 localizes to microtubule plus ends and colocalizes with ~50% of the EB1 present on microtubule tips. Overexpression of KIF17 recruits KIF17 to microtubule plus-ends. We hypothesize that, similar to currently using RNA interference to knock-down KIF17 and EB1 and analyze the functional relationship between these two proteins. Our experiments demonstrate that EB1 interacts with and currently using RNA interference to knock-down KIF17 and EB1 and analyze the functional relationship between these two proteins. Our experiments demonstrate that EB1 interacts with and then translocates to spindle midzone/midbody in late mitosis and cytokinesis. Kinetochoore localization of KIF17 is MT-dependent since such localization can be abolished by treatment of MT depolymerizing drug, nocodazole. In contrast, localization of kinetochores and plus-ends of astral MTs of KIF17 is enhanced by treatment of MT stabilizing drug, taxol. Time-lapse microscopy shows that depletion of KIF17 by KIF17 siRNA in HeLa cells results in prolonged delays of the prometaphase to metaphase and the metaphase to anaphase transitions. Long mitotic spindle MTs that cross over the metaphase plate are often observed in KIF17 depleted cells. Using GFP-E2B as a marker, we observe increased numbers of spindle MT polymerization in KIF17 depleted cells. Consistently, overexpression of KIF17 in CHO cells results in depolymerization of MTs network in vivo. In addition, recombinant KIF17 purified from insect cells using baculovirus expression system shows that KIF17 has MT depolymerizing activity in vitro. These data demonstrate that KIF17 functions as a MT depolymerase that regulates MT dynamic instabilities in mammalian cells.

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Kinesins-2 in Phagosome Transport in the Retinal Pigmented Epithelium
T. Diemer, D. Gibbs, D. Williams; Pharmacology and Neurosciences, UCSD, La Jolla, CA
As part of an essential photoreceptor outer segment (POS) renewal process, the retinal pigment epithelial (RPE) cells phagocytose and digest the distal POS disk membranes on a regular basis. The phagosomes are transported from the apical RPE to the basal RPE, where they are degraded. Defects in phagocytosis or degradation of POS membranes underlie several forms of retinal degeneration. We have shown that myosin VIIa (MYO7A) facilitates the transport of phagosomes through the F-actin-rich apical domain. We have hypothesized that MYO7A then delivers the phagosomes to a microtubule motor for transport to the basal RPE, since microtubules appear to be required for this event. In support of a relay between F-actin to microtubule motors, we find that imaging of primary cultures of RPE cells indicates that phagosomes move in with two distinct velocities, similar to that expected for MYO7A and for a faster (>1 um/sec) microtubule phagosomes to a microtubule motor for transport to the basal RPE, since microtubules appear to be required for this event. In support of a relay between F-actin to microtubule motors, we find that imaging of primary cultures of RPE cells indicates that phagosomes move in with two distinct velocities, similar to that expected for MYO7A and for a faster (>1 um/sec) microtubule motor. To test specifically for kinesin-2 function, primary cultures of RPE cells were prepared from mice carrying floxed Kif3a genes. Treatment of these cells with lentiviral-CRE-GFP was found to deplete the cells of KIF3A, the obligate motor subunit of kinesin-2, after 3 days. An assay in which the cells are challenged with POSs was used to measure the rate of phagosome degradation in these kinesin-2 deficient RPE cells. Cells were fixed at different times and the undigested POSs were identified with apo-B antibodies. Cells lacking kinesin-2 showed significantly slower phagosome degradation than control cells. These results support our hypothesis that phagosomes are delivered by MYO7A to kinesin-2, which then transports phagosomes efficiently to the basal RPE where they are degraded.

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Regulation of Caveolar Traffic by Kinesin
S. S. Siddiqui, Z. Siddiqui, Y. Husain, A. B. Malik, R. M. Minshall; Pharmacology, University of Illinois, Chicago, Chicago, IL
Endothelial cells play an important role in barrier function, and trans-endothelial delivery of nutrients. We report here that endothelial cells express two kinesins that interact with caveolin-1 (KIF1A and KIF3A). KIF1A is involved in targeting of caveolin-1 to caveolae derived vesicles. The dynamics of vesicular trafficking of CAV-1-GFP labeled vesicles were measured in the control and KIF3B specific siRNA treated endothelial cells. We discovered that KIF3B siRNA treated cells showed altered mobility and direction of movement of CAV-1-GFP labeled vesicles. These data suggest a potentially important role of KIF3B motor in regulating the caveolar vesicles.

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Autoinhibition Is a General Regulatory Mechanism for Kinesin Motors
J. W. Hammond,1 D. Cai,2 K. J. Verhey1; 1Cell and Developmental Biology, University of Michigan, Ann Arbor, MI, 2Biophysics Research Division, University of Michigan, Ann Arbor, MI
The kinesin superfamily of proteins (Kifs) mediates intracellular transport of cargoes along microtubule tracks. Regulation of motor activity is required to ensure that energy is not wasted under unproductive transport. In the absence of cargo, kinesin-1 (formerly conventional kinesin or KIF5) is inactive due to a folded and autoinhibited state. The molecular mechanism of motor regulation has not been well studied for other kinesin family members. We propose that autoinhibition is a general mechanism of regulation for kinesin motors. To test this, we studied the activity and autoregulation of members of the Kinesin-2 family, specifically KIF17 and KIF3A, and of the Kinesin-3 family, specifically KIF7A. To study kinesins under physiological conditions, fluorescently-tagged motor protein polypeptides were expressed in mammalian cells. Using permeabilized cells treated with AMPNP, we show that full length Kif3a/3b is active in vivo.
for microtubule binding whereas Kif17 and Kif1A are inactive. C-terminal truncations of Kif17 and Kif1A result in microtubule binding, demonstrating that autoinhibition requires the non-motor regions. These results suggest that autoinhibition is intrinsic to homodimeric kinesins such as Kif1A and Kif17. In contrast, heteromeric kinesins such as Kif5 and Kif1A require coexpression of accessory proteins, kinesin light chain and KAP3, respectively, for autoinhibition.

872 A Role for a C-terminal Kinesin in Microtubule Organization

T. Yeh, C. L. Brown, K. Maier, T. A. Schroer; Department of Biology, Johns Hopkins University, Baltimore, MD

KIFC3 is a ubiquitous, minus end-directed microtubule-based motor of the kinesin-14 family. KIFC3 has been reported to be involved in Golgi positioning and intracellular trafficking, but it may perform additional functions in cells. We find that endogenous KIFC3 is predominantly localized to centrosomes, suggesting a role in microtubule organization and dynamics. We engineered a series of KIFC3 fragments and determined their effect on microtubule organization. Overexpression of GFP-tagged full-length (FL) KIFC3 or a fragment containing just the stalk, neck and motor domains (AA 257-709) led to dramatic microtubule bundling and a loss of centrosomally-anchored microtubules in COS-7 cells. KIFC3-FL-decorated microtubules colocalized with Glu-tubulin and were resistant to cold nocodazole treatment. A KIFC3 fragment containing just the neck and motor domains (AA 315-709) also decorated microtubules but caused less microtubule bundling and defocusing. Surprisingly, overexpression of motorless KIFC3 (AA 1-393), the dominant negative mutant used in previously published work (Noda et al., J. Cell Biol. 155: 77-88), resulted in neither bundled nor disrupted radial microtubule arrays. All KIFC3 constructs examined localized to centrosomes, suggesting that KIFC3 is targeted to centrosomes via both motor-dependent and independent mechanisms. KIFC3-FL was also found to partially colocalize with endogenous cytoskeleton but not vimentin, suggesting that it may coordinate interactions between microtubules and intermediate filaments in some cell types. Together, these data provide evidence that KIFC3 plays a number of previously undescribed roles in the organization, dynamics and interactions of cytoskeletal filaments.

873 Mouse Liver Early Endosome Motility Is Mediated by Kif5B and Kif1C

S. Nath, L. Hassanis, S. Sarkar, R. J. Stockert, A. O. Sperry, J. W. Murray, A. W. Wolkoff; 1Anatomy and Structural Biology, Marion Bessin Liver Research Center, Albert Einstein College of Medicine, Bronx, NY; 2Marion Bessin Liver Research Center, Albert Einstein College of Medicine, Bronx, NY; 3Anatomy and Structural Biology, Brody School of Medicine at East Carolina University, Greenville, NC

In previous studies we showed that early endosomes isolated from rat liver 5 min after intra-portal injection of Texas red asialoorosomucoid (ASOR) move on microtubules via the plus- and minus-end directed kinesins Kif5B and Kif1C, respectively. We find that this study was designed to test the effect of overexpression of ASOR by hepatocytes from WT or K/O mice. Studies of endosomes in vitro also showed no difference between WT and K/O mice. Similar to the rat, mouse vesicles utilized Kif5B for plus-end motility. Approximately 50% of both WT and K/O early endosomes moved towards the minus-end of microtubules. This minus-end directed movement was inhibited by 1 mM AMP-PNP (kinesin inhibitor) but not by 5 µM vanadate (dynein inhibitor). Immunoblot analysis showed that in contrast to early endocytic vesicles from rat liver, mouse liver vesicles were not associated with Kif2. Rather they were associated with another minus-end kinesin, Kif1C. That Kif1C is functional in these vesicles was shown by substantially reduced minus-end motility and fusion following preincubation with Kif1C antibody. Decreased minus-end motility was compensated for by an increase in plus-end motility, consistent with colocalization of Kif5B and Kif1C in this population of vesicles. This study indicates that different species may have devised parallel but distinct solutions for complex physiological processes.

874 A Novel Leishmania Kinesin with a Potential Role in Kinetoplast Morphology

N. J. Gerald, I. Coppens, D. M. Dwyer; 1Lab of Parasitic Diseases, NIAID, NIH, Bethesda, MD, 2Dept. of Molecular Microbiology and Immunology, Bloomberg School of Public Health, JHU, Baltimore, MD

Kinesins are microtubule motor proteins with diverse cellular functions that include trafficking and cytoskeletal reorganization in eukaryotic cells. Kinesins are likely to play important roles in the cell biology of the protozoan Leishmania donovani, a medically important parasite of humans. Leishmania parasites have an extensive microtubule cytoskeleton and a large complement of novel kinesin genes, however, few of these parasite kinesins have been experimentally characterized. Previously, we showed that the Leishmania K39 kinesin binds along the cortical microtubules and that it accumulates at the posterior pole of the parasite. The objective of this study was to determine the cellular role of the closely related parasite kinesin K39B. RT-PCR showed that the K39B message was produced in both the promastigote stage (vector) and amastigote stage (host) of L. donovani parasites. To investigate the function of K39B, we cloned the gene and designed GFP fusion proteins for expression in L. donovani parasites. Confocal microscopy indicated that K39B-GFP fusion proteins localized near the flagellar pocket and the kinetoplast - the coiled assembly of mitochondrial DNA that is physically tethered to the flagellar basal bodies. Immunoelectron microscopy demonstrated that these fusion proteins were localized to the region between the basal bodies and the kinetoplast. Cell fractionation showed that K39B-GFP proteins associated tightly with purified flagella/kinetoplasts after extraction with detergent and high salts. Overexpression of dominant negative K39B fusion proteins caused defects in the structure of kinetoplast DNA and in the morphology of the flagellar pocket. These results suggest that K39B may have a role in kinetoplast morphology, possibly through the organization of the cytoskeleton in this region.

875 An Unexpected Role of Kinesin Superfamily Protein: KIF4 Regulates Activity-dependent Neuronal Survival by Suppressing PARP-1 Enzymatic Activity

R. Midorikawa, Y. Takei, N. Hirokawa; Anatomy and Cell Biology, Tokyo University, Faculty of Medicine, Tokyo, Japan

In brain development, apoptosis is a physiological process that controls the final numbers of neurons. Here we report that the activity-dependent prevention of apoptosis in juvenile neurons is regulated by kinesin superfamily protein 4 (KIF4), a microtubule-based molecular motor. The C-terminal domain of KIF4 is a module that suppresses the activity of poly (ADP-ribose) polymerase-1 (PARP-1), a nuclear enzyme known to maintain cell homeostasis by repairing DNA and serving as a transcriptional regulator. When neurons are stimulated by membrane depolarization, calcium signaling mediated by CaMKII induces dissociation of KIF4 from PARP-1, resulting in upregulation of PARP-1 activity, which supports neuron survival. After dissociation from KIF4, KIF4 enters into the cytoskeleton from the nucleus, moves to the distal part of neurites in a microtubule-dependent manner. We suggested that KIF4 controls the activity-dependent survival of postmitotic neurons by regulating PARP-1 activity in brain development.

876 Characterization of Motor-domain Mutants of the Mitotic Kinesin RAB6KIFL (Mklp2, Kif20a)

E. L. de Hostos, Y. Lee, R. Sakowicz, L. Belmont; Cytokinetics, Inc., South San Francisco, CA

RAB6KIFL is a kinesin motor protein involved in cytokinesis. siRNAs targeting RAB6KIFL cause the failure of cytokinesis and the generation of binucleate cells (Neef et al. 2003, J Cell Biol. 161: 863-75; Zhu et al. 2005, Mol Biol Cell. 161:3187-99). We have engineered three motor domain mutations to explore the biochemical activity and biological function of this motor protein. Based on studies of kinesin heavy chain and Kar3 (Yun et al. 2001, EMBO J. 20:2611-8), two of the mutations, T167N (Switch I), were predicted to lock the motor domain into a microtubule bound rigor state, and the third, R379A (Switch II), was predicted to lock the motor in the weak microtubule binding state. The mutations were generated in a motor domain-only construct for expression in E. coli. All threemutations abolish microtubule-stimulated ATPase activity. Unlike the G411A and R379A mutants, the T167N mutant expressed poorly in bacteria and did not bind microtubules. It is likely that this protein does not fold properly. Wild-type, G411A and R379A versions of a full-length GFP-RAB6KIFL fusion were expressed in HeLa cells under the control of a native RAB6KIFL promoter fragment. Localization of the wild-type fusion is indistinguishable from that of endogenous RAB6KIFL, and is concentrated in the central region of the midbody connecting daughter cells. Unlike the wild-type fusion, however, the R379A fusion and the G411A mutant in particular, appear to be broadly distributed along midbody but are absent from the central region of the bridge. While mutant fusions localize aberrantly, at the level of expression achieved by these constructs they do not seem to have a dominant-negative effect on cytokinesis. This strategy is useful for the correlation of biochemical activity and biological function and is broadly applicable to the study of other kinesin motors.
Kinesin motors release nucleotide upon interaction with microtubules, then bind and hydrolyze new ATP to move along the microtubule. Although crystal structures of kinesin motors with bound nucleotide have been solved, nucleotide-free structures have not yet been reported. To understand the structural changes in kinesin motors bound to microtubules, we studied the 3D structure of microtubules decorated with a Kinesin-14 motor, Kar3, without nucleotide, as well as with ADP and AMP~PNP, using electron cryo-microscopy and computer 3D reconstruction. The resolution of the 3D maps obtained is unusually high, ~10-12 Å, sufficient to observe helices, e.g., tubulin helices H3, H11 and H12, and the motor Switch II helix, α4. Although differences between the ADP and AMP~PNP states were relatively small, consistent with published X-ray crystallographies, we found large structural changes in the motor bound to microtubules without nucleotide, including melting of helix α4, closure of the nucleotide-binding pocket, and movement of loop L7 towards the microtubule. The movement of L7 was accompanied by distortion of the central β-sheet, reminiscent of nucleotide-free myosin crystal structures (Reubold et al., 2003; Courreux et al., 2003). Contrary to reports by others for the Kinesin-3 motor, Kif1A (Kikkawa et al., 2001), the Kar3 motor domain did not rotate between the motor-MT ADP and ATP-like states. We propose that the Switch II region of the motor controls docking of the Kar3 neck by conformational changes in the central β-sheet, similar to myosin, rather than by rotation of the motor domain, as proposed for Kif1A.

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Kif5B Interacts with β-catenin and Regulates its Sub-cellular Localization

S. Taya, K. Kaibuchi; Department of Cell Pharmacology, Nagoya University Graduate School of Medicine, Nagoya, Japan

β-catenin performs important functions in both the cytoplasm, as a component of adherens junctions and in the nucleus, as a Wnt stimulated transcription factor. However, the molecular mediators of β-catenin localization to these functionally distinct cellular pools have not been elucidated fully. The kinesin family of microtubule-associated motors mediates a variety of intracellular transport events and therefore is a good candidate regulator of β-catenin sub-cellular distribution. Here, we report that β-catenin co-localizes with Kif5B, a Kinesin-I family member, both in the cytoplasm and at adherens junctions of MDCK cells. Furthermore, we find that endogenous Kif5 interacts with β-catenin in both MDCK and HEK293 cells.

Biochemical analysis revealed that this interaction is mediated by the C-terminal tail region of the KIF5B heavy chain and is independent of kinesin light chains. Exogenously expressed single molecule imaging in vivo, our work provides the first direct observations of single protein molecules expressed and localized in the cytoplasm of mammalian cells.

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Axonal Transport by Kinesin-1 Is Blocked by Stress, but Not by Neuritic Deposits of Amyloid-β

Z. Muresan, V. Muresan; Physiology and Biophysics, Case Western Reserve University, Cleveland, OH

It is generally admitted that a deficient axonal transport may be one of the initial events that lead to neuronal degeneration and neurotic plaque formation in Alzheimer’s disease (AD). However, it is not known if abnormal generation and intraneuronal accumulation of amyloid-β (Aβ) is always a direct cause of axonal transport deficiencies. We established a cell culture system, where neuronal cells (CAD) produce and accumulate large amounts of Aβ peptide, similar to what is believed to occur in brain neurons, in the initial phases of AD. In these cells, Aβ is distributed throughout the neurites, and concentrates at their terminals. We analyzed the cytoskeleton and the transport of vesicular cargoes within the neurites that contained Aβ accumulations. We found that, although the growth cones that contained Aβ appeared dystrophic, the distribution of microtubules and actin filaments was normal. We examined transport and localization of phosphorylated APP and JNK-interacting protein-1 (JIP-1), two vesicular cargoes of the microtubule motor, kinesin-1. We found that transport of these two proteins occurred at normal rates in neurites that contained extensive accumulations of Aβ. By contrast, transport of JIP-1 and APP (phosphorylated and not phosphorylated) was completely blocked in cells subjected to osmotic stress (by treatment with 0.4 M sorbitol), a condition that activates JNK and leads to increased phosphorylation of APP, kinesin-1, as well as other proteins. Sorbitol treatment did not affect accumulation of Aβ in CAD cell processes. These results indicate that transport of vesicular cargoes into neurites does not generally appear to be perturbed by the presence of large amounts of Aβ.

We conclude that, while a deficient axonal transport may cause neurodegeneration, increased generation and neuritic accumulation of Aβ can occur in the absence of detectable signs of abnormal axonal transport.
A Novel Mechanism for Kinesin Spindle Protein (KSP) Modulation: Small Molecular Activators of KSP Basal ATPase

E. Miraldi,1 P. J. Thomas,2,3 L. Romberg4; 1Biochemistry, Oberlin College, Oberlin, OH, 2Mathematics, Case Western Reserve University, Cleveland, OH, 3Neuroscience, Oberlin College, Oberlin, OH, 4Physics, Oberlin College, Oberlin, OH

For nearly fifty years cooperative polymerization has been explained by a model that requires polymers to be "helical" or multistranded, whereas single-stranded, linear polymers are not evolutionarily conserved. 57 kD protein related to essential proteins in C. elegans and S. cerevisiae, and is similar to a yeast protein required for ribosome biogenesis, thus providing an enticing link between Chindogu function and ribosome or mrRNA transport by kinesin. Loss of zygotic Chindogu function has no obvious phenotype, but eggs lain by mothers lacking Chindogu fail to develop. This maternal effect lethal phenotype is also observed in embryos lacking determinants of embryonic polarity. We are currently generating new mutant alleles of Chindogu, using specific antisera to analyze the subcellular localization of Chindogu protein, and comparing the localization of developmental markers such as mrNA transcripts involved in oocyte polarity in wild type and Chindogu mutants, to further investigate a possible role for Chindogu in Drosophila embryogenesis and kinesin-mediated transport.

Characterization of Chindogu, a Drosophila Kinesin-1 Binding Protein

K. P. Weber, D. Yang-Zhou, J. G. Grindhart1; 1Department of Biology, University of Richmond, Richmond, VA, 2Department of Biology, University of Massachusetts Boston, Boston, MA

The microtubule motor protein kinesin-1 is essential for Drosophila development. During oogenesis, loss of kinesin-1 function causes defects in oocyte polarity, while kinesin-1 is also essential for anterograd e axonal transport in larva and adult. To better understand the function of kinesin-1 in vivo, a yeast two-hybrid screen was used to identify proteins that bind specifically to the kinesin tail domain. Previous results have demonstrated that specific protein-protein interactions with the kinesin tail domain are important for mediating and regulating interactions between kinesin and its intracellular cargos. We have identified Chindogu, a novel kinesin light chain-binding protein that is essential for Drosophila embryonic development. Chindogu is an evolutionarily conserved 57 kD protein related to essential proteins in C. elegans and S. cerevisiae, and is similar to a yeast protein required for ribosome biogenesis, thus providing an enticing link between Chindogu function and ribosome or mrRNA transport by kinesin. Loss of zygotic Chindogu function has no obvious phenotype, but eggs lain by mothers lacking Chindogu fail to develop. This maternal effect lethal phenotype is also observed in embryos lacking determinants of embryonic polarity. We are currently generating new mutant alleles of Chindogu, using specific antisera to analyze the subcellular localization of Chindogu protein, and comparing the localization of developmental markers such as mrNA transcripts involved in oocyte polarity in wild type and Chindogu mutants, to further investigate a possible role for Chindogu in Drosophila embryogenesis and kinesin-mediated transport.

Microtubule Dynamics: Computational Stochastic Modelling of Cellular Microtubule Network

A. A. Sheplak,1 E. S. Nakhedrina,1 Bioengineering and Bioinformatics, Moscow Lomonosov's State University, Moscow, Russian Federation, 2Institute of Protein Research of RAS, Moscow, Russian Federation

Microtubule network is a very dynamic structure, undergoing rapid reconstructions if cell is affected. It can show either central symmetry or chaotic arrangement of microtubules. Microtubule network rearranges during cell movement at polarization phase and at cell differentiation. We have developed computer model of microtubule behavior where microtubule dynamics is simulated as a chemical reaction of reversible polymerization. Microtubule is presented as a chain of objects. Each object is a tubulin in either GTP or GDP state. Engaging of a new object into chain occurs at chain ends by stochastic mechanism. The probabilities of engaging/disenengaging/changing state of objects are accounted by formulas analogous to chemical reaction equation. The coefficients are specified for 10 reactions (association/dissociation of GTP/GDP tubulin at minus/pus end, microtubule nucleation and tubulin status change), and program allows to change them easily. The rate of each reaction depends on its coefficient, on free tubulin concentration and on random numbers. Each object incorporates at random angle allowing microtubules to curve. The designed program properly simulated microtubule network: logarithmic growth of microtubules and steady-state with either dynamic instability or treadmilling. As simulation showed, the rate of changing from GTP status into GDP is more crucial for switching of dynamics between treadmilling and dynamic instability than critical concentration of tubulin. We also enter differentiation of reaction coefficients through program field to introduce “centrosomal” effects. We either increased nucleation rate in central square of the field or decreased tubulin exit from minus end (microtubule anchoring) in this square. We found that anchoring is much more important than augmented nucleation in forming microtubule radial array. The data predicted by program is highly similar to experimental results. In future our stochastic computer model is to be improved to simulate the inner cell geometric dependent processes and to introduce microtubule-dependent movement of organelles.

Microtubule Dynamics: Stochastic Simulations Reveal Predictable Nature of Complex Behaviors

I. V. Gregoretti,1 G. Margolin,2 M. S. Alber,3 H. V. Goodson1; 1Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, IN, 2Department of Mathematics, University of Notre Dame, Notre Dame, IN

Microtubule dynamic instability plays a fundamental role in cell biology, enabling microtubules to find and interact with randomly distributed cargo and spatially localized signals. Microtubules in vitro transition between growth and shrinkage symmetrically, consistent with theoretical understanding of the mechanism of dynamic instability. However, microtubules in vivo commonly exhibit asymmetric dynamic instability, growing persistently in the cell interior and experiencing catastrophe near the cell edge. What is the origin of this behavior difference? One answer is that the cell edge causes the asymmetry by inducing catastrophe in persistently growing microtubules. However, the origin of the persistent growth itself is unclear. Using a simplified coarse-grained stochastic simulation of a system of dynamic microtubules, we provide evidence that persistent growth is a predictable property of a system of nucleated, dynamic, microtubules containing sufficient tubulin in a confined space - MAP activity is not required. Persistent growth occurs because cell-edge-induced catastrophe increases the concentration of free tubulin at steady state. Our simulations indicate that other aspects of MT dynamics thought to require MAPs are also predictable, perhaps unavoidable, outcomes of the "systems nature" of the cellular microtubule cytoskeleton. These include the mitotic increase in microtubule dynamics and the observation that defects in nucleation cause changes in the behavior of microtubule plus ends. These predictions are directly relevant to understanding of the microtubule cytoskeleton, but they are also attractive from an evolutionary standpoint because they provide evidence that apparently complex cellular behaviors can originate from simple interactions without a requirement for intricate regulatory machinery.

A New Model for Cooperative Polymerization: Conformational Changes Can Produce Critical Concentrations in Single-stranded Polymers

E. Miraki,5 P. J. Thomas,6,7 L. Romberg,8 Biochemistry, Oberlin College, Oberlin, OH, 5Mathematics, Case Western Reserve University, Cleveland, OH, 6Neuroscience, Oberlin College, Oberlin, OH, 7Biology, Oberlin College, Oberlin, OH

For nearly fifty years cooperative polymerization has been explained by a model that requires polymers to be "helical" or multistranded, whereas single-stranded, linear polymers are not thought to assemble cooperatively (Oosawa and Kasa, 1962). Assembly of FtsZ, the bacterial homolog of tubulin, cannot be described by either of these two models. Unlike tubulin, FtsZ can apparently complex cellular behaviors can originate from simple interactions without a requirement for intricate regulatory machinery.
888 Automatic Quantification of Microtubule Dynamics in Living Cells

L. D. Sironi, 1 A. Bancaud, 1 J. Selon, 1 D. Brunner, 1 J. Ellenserg 1; Gene Expression, EMBL, Heidelberg, Germany, 2LAAS - CNRS, Toulouse, France, 3Cell Biology, EMBL, Heidelberg, Germany

Microtubules (MTs) are dynamic polarized filaments essential for many cellular functions such as spatial rearrangement of the cytoplasm in interphase, intracellular membrane traffic and chromosome segregation during cell division. All these functions depend on the dynamic properties of MTs, which allow them to rapidly probe the cytoplasmic space. In HeLa cells, most MTs are nucleated from the centrosome with their fast-growing plus-ends directed towards the cell periphery. MT plus-ends display both in vitro and in vivo dynamic instability, which means that they alternate between phases of elongation and rapid shortening. We have established a quantitative and automatic protocol to measure MT growth speed and nucleation rate at the centrosome during the cell cycle in live S2 cells using a HeLa cell line expressing fluorescently tagged EB3, a plus-end tracking protein. Using spinning disk confocal microscopy we record high temporal and spatial resolution sequences of cells in interphase, prophase, prometaphase and anaphase. The raw data are then filtered automatically by computerized image processing to select only for the moving MT tips. To measure MT growth rates we use a multiple-particle tracking algorithm that reconstitutes individual tip trajectories and calculates tip velocities. To measure MT nucleation rates at the centrosome we perform a radial kymograph image analysis which automatically counts the number of tips that cross an interactively selected circular region around the centrosome. In summary we have developed a quantitative assay to study MT dynamics in an automatic fashion that we intend to use as a tool to identify and characterize regulators of microtubule dynamics by targeted RNA interference screens in live cells.

889 Observation of Tubulin Incorporation Events into Growing Microtubules Using Single Molecule Fluorescence

L. Breshears, 1 P. Partensky, 1 B. Shan Tseng, 1 M. Dogterom 2; 1Physiology Course 2006, Marine Biological Laboratory, Woods Hole, MA, 2FOM Institute AMOLF, Amsterdam, The Netherlands

Microtubules are hollow tubes consisting of typically 13 parallel protofilaments that in vitro and in vivo grow by the addition of tubulin dimers to their growing ends. It is generally believed that the growing ends of microtubules contain several individual protofilaments that after assembly first bind to each other laterally in a slightly outward-curved sheet and then close into a straight hollow tube. The kinetic details of the tubulin assembly process itself as well as the conformational changes of the sheet-like structures at the growing ends remain poorly understood. Fluorescence microscopy can be used to monitor the incorporation of fluorescently labeled tubulin subunits into single microtubules in vitro or in vivo. When low labeling ratios are used, so-called speckles are observed that result from spatial fluctuations in the distance between individual fluorescent labels. These speckles are a powerful tool in monitoring microtubule dynamics, transport and organization of MTs in the normal living state. Using TIRF microscopy and a very low labeling ratio we demonstrate the observation of single tubulin incorporation into microtubules growing from pure tubulin in vitro. With improved time resolution, the ability to observe tubulin addition at the single molecule level should allow for the observation of individual attachment as well as detachment events, which will help settle existing controversies about the off-rate for tubulin in growing microtubules. Also, tracking the lateral position of newly added tubulin subunits at nanometer resolution, should allow for the detection of straigtening events of putative outward-curved sheets at the ends of growing microtubules.

890 Microtubule Dynamics at the Nanoscale: Evidence for a Stable, GTP-free Structural Cap, and Implications for Cytoskeletal Restructuring

H. T. Schek, A. J. Hunt; University of Michigan, Ann Arbor, MI

The mechanisms underlying dynamic instability of microtubules (MTs) have remained enigmatic largely because direct studies of events at an MT tip have proven difficult. Studies following polymerization of dynamic MTs in real time are limited by low temporal resolution (often less than 0.05 Hz) and the light resolution limit, while bulk biochemical studies rely on large ensemble averages to infer individual MT behaviors. Cryo-electron-microscopy allows for detailed study of MT tip structure, but requires fixed samples that are no longer dynamic. By combining optical tweezers with custom-engineered nanoscale barriers to MT polymerization we are able to track events at the growing tip of a loaded dynamic MT with nanometer-scale precision. We find that MT growth rates under load are too variable to be explained by a model positing a simple first order reaction; polymerizing microtubules frequently transit between states that exhibit different growth dynamics. MTs under load often exhibit periods of stalled growth that last for up to several seconds without leading to a catastrophe event. Additionally, MTs often shorten up to tens of nanometers during a period of overall growth, without entering into a period of rapid shortening. These shortening events often result in a loss of length equivalent to 3-10 layers of subunits, calling into question the GTP-cap hypothesis for MT stability. Furthermore, the observed variability and shortening events demonstrate that MT polymerization from pure tubulin is a dissipative process. Cellular regulation mechanisms that prevent these dissipative events could provide the cell with a means to achieve faster polymerization as has been observed in vivo.

891 Self-organisation of Anastral Spindles by Synergy of Dynamic Instability, Autocatalytic Nucleation, and a Spatial Signalling Gradient

T. Clausen, K. Ribbeck; Gene Expression, EMBL, Heidelberg, Germany

Assembly of the mitotic spindle is a classic example of macromolecular self-organisation. A combined biochemistry-computer simulation approach reveals that the concerted activity of three processes - dynamic instability, autocatalytic nucleation, and a spatial signalling gradient - can self-organise the mitotic spindle. In anastral spindles, during cytokinesis, when there is no central spindle to dictate the mitotic spindle assembly system (Kalab et al. 2006. Nature 440 697), we have found that the presence of spatially autocatalytic gradients can dictate a mitotic spindle. These results indicate that Ran-regulated gradient of importin β cargo concentration to chromatin-driven mitotic spindle assembly is sufficient to direct chromatin-driven mitotic spindle assembly. The mechanism proposed here can generate and maintain a dissipative MT superstructure within a RanGTP gradient.

892 Analysis of Ran-regulated Importin β Cargo Gradient Contribution to Chromatin-driven Mitotic Spindle Assembly

P. Kalah, A. Prallé, D. R. Halpin, R. Heald, K. Weis; UC Berkeley, Berkeley, CA

RanGTP-ase regulated pathways contribute in a diverse fashion to mitotic spindle assembly in comparison of a variety of somatic cells and meiotic/embryonic Xenopus egg extract spindle assembly system (Kalab et al. 2006. Nature 440 697). Here we applied fluorescence resonance energy transfer (FRET)-based sensors and fluorescence lifetime microscopy (FLIM) to examine the role of Ran-regulated importin β cargo gradient in the primarily chromatin-driven spindle assembly in Xenopus egg extract model system. We confirmed that, as in HeLa cells, cytoplasm in mitotic Xenopus egg extracts contains majority of importin α-like importin β cargos in a free form and therefore potentially active to promote spindle assembly. Experiments with synthetic RCC1 beads demonstrated that a small further localized liberation of importin β cargos induces localized microtubule polymerization and mitotic spindle assembly. Interestingly, the ampltudes of importin β cargo gradients surrounding mitotic chromatin in X. laevis and X. tropicalis extracts are similar, in contrast to significantly smaller size of X. tropicalis mitotic spindles regardless of the source of chromatin. These results indicate that Ran-regulated gradient of importin β cargos is sufficient to direct chromatin-driven mitotic spindle assembly. On the other hand, the role of the chromatin-centered importin β cargo gradient in determination of mitotic spindle size is counteracted by dominant cytoplasmic activities.

893 Genes Required for Spindle Assembly in Drosophila S2 Cells

G. Goshima, 1 R. Wollman, 1 J. M. Scholey, 1 R. D. Vale, 1 N. Stuurman, 1 UC Davis, Davis, CA

Mitotic spindle assembly is a complex process controlled by many genes. While genes required for this process have been extensively identified in yeast and nematode, it is expected that more genes remain to be identified in higher eukaryotes that construct more complex-shaped spindles. Here we present a genome-wide RNAi screening of the metaphase spindle morphology in Drosophila S2 cells. After RNAi treatment of ~14,000 genes, we manually and computationally identified and analyzed 3.5 million immunofluorescence images of the metaphase spindle. This complementary analysis identified ~200 genes whose knockdowns show reproducibly clear defects in spindle morphology. 26 hit genes were further characterized by GFP tagging and were shown to act on the various aspects of metaphase spindle morphogenesis. As examples, we found a novel centriole protein essential for centriole duplication, a novel spindle protein important for recruitment of gamma-tubulin to the spindle, and four novel kinetochore proteins required for chromosome congression. Localization and RNAi analysis also revealed that calmodulin, a major transducer of calcium signals, functions for spindle pole focusing in metaphase by targeting Asp protein to spindle poles. Based upon these follow-up experiments, we propose models for protein assembly at kinetochore, centrosomes and spindle poles of S2 cells.
Cytoplasmic Dynin Motor Activity Is Necessary for Spindle Alignment and Fusion: Insights from Spindle Micromanipulation Studies

J. C. Gatlin, 1, A. Cameron, 2 A. C. Groen, 1 T. J. Mitchison, 2 E. D. Salmon 1
1 Biology, University of North Carolina, Chapel Hill, NC, 2 Systems Biology, Harvard Medical School, Boston, MA
Meiosis II bipolar spindles with replicated sister chromosomes at metaphase were assembled in Xenopus egg extracts. Previous studies have demonstrated that two spindles, positioned with their interpolar axes parallel to each other, align and fuse together into one metaphase spindle by two distinct mechanisms. If proximal ends of the spindles are overlapped then the spindles slide parallel to their axes until the chromosomes and poles align respectively. However, if spindles are positioned with no overlap between poles, but close enough to each other, then nearest poles come together and their spindles pivot around their shared pole, “jackknifing” into a bipolar metaphase spindle. Individual spindle integrity seems to be maintained during alignment and fusion. These processes are inhibited by orthovanadate, suggesting that cytoplasmic dynin might be the responsible motor. In this study we sought to better understand the origin of the alignment forces. To determine whether flux dependent mechanisms were involved, we created monopolar spindles by biochemical inhibition of the Eg5 kinesin motor. When two monopoles were placed within ~50 μm of each other, a central spindle of overlapping microtubules formed between the poles, shortening as the poles came together. The velocity of fusion was dependent upon the distance between the poles, and decreased significantly when the two poles became closer. Analysis of chromosome localization revealed that monopole fusion does not require the presence of chromosomes in between the two fusing asters. Formation of interpolar “bridging” microtubule bundles and pulling were blocked by 200μM orthovanadate but not by 2μM okadaic acid, indicating that both are caused by cytoplasmic dynin. These data support a mechanism in which minus end directed “pulling in” forces produced by cytoplasmic dynin act along anti-parallel microtubules and play a major role in the alignment and fusion of two spindles into one. Supported by GM24364 and GM60678.

ICIS Regulates Microtubule Dynamics

W. Lan, T. Stukenberg; Biochemistry and Molecular Genetics, University of Virginia, Charlottesville, VA
A novel microtubule associated protein called ICIS (Inner Centromere Kin I Stimulator), has been found to stimulate the microtubule depolymerase activity of MCAK (Oh et al., 2003). To understand how MCAK regulates microtubule dynamics, we have searched for new interactions of ICIS and studied how ICIS is regulated. We have found that ICIS interacts with Pxl1 and can stimulate its kinase activity about 3 fold in vitro. ICIS has separate domains that interact with the inner centromere regulators MCAK, Aurora B and Pxl1. ICIS is also phosphorylated by Aurora B kinase on S775 both in vitro and in vivo. However, we find that Xenopus extracts that have had 97% of ICIS removed by immunodepletion still produce normal bipolar spindles. Centrosomes in Xenopus extracts give weak asters in the presence of ICIS antibodies, suggesting roles independent of simulating MCAK. Together, our data suggest that ICIS is regulated by the mitotic phosphorylations, and has functions in regulating microtubule dynamics that may be distinct from regulation of MCAK.

The Dam1 Kinetochore Ring Complex Harnesses Microtubule Dynamics to Produce Force and Movement

C. L. Ashbury, 1 T. N. Davis, 1 D. R. Gestaut, 1 A. F. Powers, 1 A. D. Franck 1
1 Physiology and Biophysics, University of Washington, Seattle, WA, 2 Biochemistry, University of Washington, Seattle, WA
Kinetochore microtubules are an essential component of kinetochores in yeast, but can be directly removed from kinetochores. For instance, mutations that disrupt the Dam1 complex have no effect on chromosome movement. We have developed a new method to measure the changes in MT number and distribution, at single MT resolution in living cells. We show for wild-type cells, bipolar spindle symmetry is maintained by the MT assembly and disassembly that occurs under tension. When the spindle breaks down, the astral MTs, the astral MT bundles, and the PAA microtubules in fission yeast are composed primarily of 1 individual MT along their lengths. We measure the cellular concentration of MT plus ends of interphase MTs. As many microtubule (MT) structures such as the mitotic spindle contain MTs that are bundled and stabilized in overlapping arrays. In the fission yeast Schizosaccharomyces pombe, stable overlapping MTs are located in the spindle midzone and interphase MT bundles. CLASPs are well known as MT binding proteins that contribute to the stabilization of MT plus-ends. Here, we show that the fission yeast CLASP, cls1p, localizes to MT bundles at the spindle and kinetochores during mitosis and at perinuclear interphase MTOCs (iMTOCs) during interphase, but not to MT plus ends of interphase MTs. As cls1 is an essential gene, we generated conditional cls1/ alleles that rapidly lose function upon temperature shift. Using these alleles, we found that cls1p was required for both the assembly and maintenance of the spindle. For instance, loss of cls1p in anaphase led to rapid depolymerization of all spindle MTs. Stabilization of interphase MT bundles at iMTOCs was also lost in these mutants. Conversely, overexpression of cls1p led to stabilization of all MT structures, apparently by increasing rescue rates. This stabilization activity was independent of EB1 (mal3p), PRC1 (ase1p), and CLIP170 (tip1p). At endogenous levels, cls1p localization was dependent on the MT bundling protein ase1p, which is also localized to the spindle midzone and interphase MT bundles. We measure the cellular concentration of MT plus end associated with the Dam1 complex to be 4.04 ± 0.24 μM. The Dam1 complex may contribute directly to microtubule-driven chromosome movement. Many microtubule associated protein called ICIS (Inner Centromere Kin I Stimulator), has been found to stimulate the microtubule depolymerase activity of MCAK (Oh et al., 2003). To understand how MCAK regulates microtubule dynamics, we have searched for new interactions of ICIS and studied how ICIS is regulated. We have found that ICIS interacts with Pxl1 and can stimulate its kinase activity about 3 fold in vitro. ICIS has separate domains that interact with the inner centromere regulators MCAK, Aurora B and Pxl1. ICIS is also phosphorylated by Aurora B kinase on S775 both in vitro and in vivo. However, we find that Xenopus extracts that have had 97% of ICIS removed by immunodepletion still produce normal bipolar spindles. Centrosomes in Xenopus extracts give weak asters in the presence of ICIS antibodies, suggesting roles independent of simulating MCAK. Together, our data suggest that ICIS is regulated by the mitotic phosphorylations, and has functions in regulating microtubule dynamics that may be distinct from regulation of MCAK.

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1 Physiology and Biophysics, University of Washington, Seattle, WA, 2 Biochemistry, University of Washington, Seattle, WA
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Gas7, a Novel Microtubule Polymerization Accelerator Stabilizers Sheet Structure of the Polymerization Intermediate

T. Uchida,1 H. Akiyama,1 M. Sanauda,1 T. Higashi,1 T. J. Ito;2 Enzymology, Department of Molecular Cell Biology, Tohoku University, Sendai, Japan, 2Nagoya, Nagoya, Japan

Although it is well known that protein phosphorylation is a key of intracellular signal transduction systems, the molecular mechanism of the regulation of phosphorylation signal is not elucidated. We have been studying the regulatory molecules for the phosphorylating signal transduction. It has been reported that WW motif in the peptides-prolyl cis/trans isomerase Ptn1 binds phosphoserine...Pro in Tau, a major member of heat-stable MAPs superfamily. Tau enhances microtubule polymerization, but phosphorylation impairs this activity. We searched the proteins containing similar sequence to the Ptn1's WW domain and found growth-arrest specific protein 7 (Gas7). It has been reported that Gas7 is required for actin polymerization but it was not known if it interacts with phosphorylated Tau. We found that Gas7 binds the moderately phosphorylated Tau. We characterized the effect of Gas7 on the polymerization of microtubule in vitro. Light scattering measurement showed that Gas7 increased microtubule polymerization. Dark-field microscopic observation showed the numbers of microtubules were increased by Gas7. Initially, single and more flexible filaments were observed, and the straight microtubules with “fluttering banner structure”, or “PROMISE” (pro-microtubule structure end up) appeared with the elongation of the entire length. "PROMISE" would correspond to the sheet intermediate of polymerization observed by electron microscopy. Gas7 enhances the persistency of the polymerization intermediates. Typical dynamic instability with stochastically occurring catastrophe and rescue was observed in control, but no catastrophe was observed in the presence of Gas7. These results suggest that Gas7 interacts with and stabilizes phosphorylated Tau by keeping “PROMISE” and maintains microtubule polymerization. We also found that Gas7 exists in the normal brains but not in the brains of patients with Alzheimer’s disease. We speculate that Gas7 is required for keeping nervous network system and its degradation may cause dementia.

Microtubule Plus End Protein CLASP Supports Microtubule Formation at the Trans-Golgi Membranes

I. Kavena,1 P. Efremova,1 T. Lomaczek,2 P. Efremov,1 F. G. T. Jones,1 H. Saito,1 A. Krokhin2; 1Cell and Developmental Biology, Vanderbilt University Medical Center, Nashville, TN, 2Wadsworth Center, Albany, NY, 3University of Melbourne, Melbourne, Australia, 4University of Porto, Porto, Portugal

In addition to binding plus ends of growing microtubules CLASP is also reproducibly found in the Golgi complex. However, the specificity and functional significance of this localization are not clear. By using immunofluorescence co-localization and live-cell imaging we localized CLASP to the most external compartment of the Golgi, presumably the trans-Golgi network (TGN) in normal human cells (HEK-293). Further, we found that CLASP is exposed at the cytoplasmic side of Golgi membranes and dissociates into the cytosol upon Brefeldin A treatment similarly to GRIP-domain containing TGN proteins. Mass-spectrometry analyses identified the GRIP-domain protein GCC185 as a CLASP-binding partner. It is likely that GCC185 recruits CLASP to the Golgi membranes. Thus, CLASP associates with the Golgi in a specific and controllable fashion. These data suggest that CLASP is involved in some aspects of microtubule dynamics regulation at the Golgi complex. In this regard, dense arrays of microtubules are consistently found emanating from the Golgi in normal human cells. Live-cell analyses of microtubule growth reveal that significant number of microtubules form at the Golgi stacks both in steady state, and during microtubule re-growth after nocodazole treatment. The role of the Golgi as a major microtubule organizing center becomes particularly evident upon ablation of the centrosome by laser micro-beam. Depletion of CLASP via siRNA in RPE-1 cells abolished microtubule-organizing potentials of the Golgi, indicating that CLASP is essential for this process. Furthermore, microtubules formed at the Golgi stacks after nocodazole washout are enriched with CLASP in contrast to those formed at the centrosome. We suggest that CLASP concentrated at the Golgi membranes selectively stabilizes microtubule seeds at this location and thus supports non-centrosomal microtubule arrays.

Insights into the Regulation of Microtubule Dynamics by EB1: The Role of Its Flexible C-terminus

T. Mamma,1 P. Efremova,2 J. Miller,1 M. O. Stemmle,1 L. Wilson1; 1Molecular, Cellular, and Developmental Biology, University of California at Santa Barbara, Santa Barbara, CA, 2Structural Biology, Paul Scherrer Institut, Villigen PSI, Switzerland

The evolutionary-conserved EB1+TIP protein family is involved in a number of microtubule (MT) dependent cellular processes including polarity and migration. One of EB1’s important functions is to regulate MT dynamics. However, the mechanisms by which EB1 and its binding partners such as P150 glut(s) might regulate MT dynamics are not understood. Here by using a purified recombinant MT system, we analyzed the effects of EB1 on steady-state microtubule dynamic instability by video microscopy and determined the role of its flexible C-terminus in the regulation of dynamics in the presence and absence of P150s. Our results demonstrated that the C-terminus of EB1. Full length EB1 alone significantly suppressed the rate and extent of shortening and the catastrophe frequency at plus ends of individual MTs in the absence of significant effects on the growth rate. Interestingly, depletion of its flexible C-terminal tail (the terminal 19 amino acid residues, EB1-C) increased EB1’s potency — four fold, in concert with a significant increase in MT binding. For example, at an initial EB1: tubulin ratio of 1:3, 1 mol of full-length EB1 bound 3.3 ± 1.1 tubulin dimers in microtubules, while 1 mol of EB1-C bound per 3.1 ± 0.9 mol of tubulin dimers. When analyzed together with full-length EB1, P150s increased the potency of EB1 to that of C-terminally-truncated EB1. Our results demonstrate at a mechanistic level that the flexible C-terminus tail of EB1 negatively regulates EB1’s effects on MT dynamics and that the binding to EB1 one of its binding partners (P150 glued) overcomes the negative regulation. The data are consistent with the previous hypothesis that the C-terminus of EB1 negatively regulates its effects on MTs. Supported by NIH NS13560, NSF 0331697, and Swiss National Research Foundation 31-64978.01.

TBCE Interacts with EB1 and Modulates Centrosomal Regrowth

M. C. Huang,1 G. Tian,2 R. Parvar,1 N. J. Cowan,3 G. A. Diaz3; 1Human Genetics, Mt Sinai School of Medicine, New York, NY, 2Biochemistry, New York University, New York, NY, 3Ben Gurion University of the Negev, Beer Sheva, Israel

Tubulin-binding cofactor E (TBCE) is a chaperone required for the de novo formation of αβ-tubulin heterodimers, the building blocks of microtubules (MTs). Mutations in TBCE cause a human syndrome characterized by hypohypharyngism, mental retardation, facial dysmoria, and growth failure (HRD), and a murine model for peripheral motor neuropathy. HRD disease carrying a common founder mutation have normal tubulin abundance but disorganized MTs, suggesting that TBCE may have functions beyond its role in heterodimer assembly. We have previously reported the discovery of a repressor interaction between TBCE and EB1, a plus-end tracking protein (+TIP). We have confirmed and extended these findings through co-immunoprecipitation studies of overexpressed and purified proteins. We refined the interaction to the TBCE Ubiquitin-like domain and the EB1 C-terminus, which is known to associate with other +TIPs. Fluorescently-tagged TBCE and EB1 did not colocalize at MT plus ends, suggesting that this interaction occurs in the cytoplasm and may be regulatory in nature. Whereas overexpression of TBCE results in tubulin destruction in vivo, co-expression of EB1 and TBCE rescues α-tubulin, but not MTs, suggesting that EB1 may protect tubulin from TBCE-mediated degradation. Overexpression of EB1 increased MT overlap regions. Finally, cls1p bound to ase1p directly and this interaction is critical for its function. These studies suggest that ase1p recruits CLASP to regions of MT overlap to stabilize these MT structures.

The Role of LIMK1 in Endothelial Barrier Function

T. Vojno-Yanezskaya, M. Gorovoy, F. Neuma; University of Illinois, Chicago, IL

Microtubule (MT) destabilization promotes formation of actin stress fibers and enhances the contractility of cells. LIM kinase 1(LIMK1) regulates actin polymerization by phosphorylating the actin depolymerization factor, cofilin. Here we report that LIMK1 is also involved in the MT destabilization. In endothelial cells endogenous LIMK1 co-localizes with MTs and forms a complex with tubulin via the PDZ domain. MT destabilization induced by thrombin or nocodazole resulted in a decrease of LIMK1 co-localization with MTs. Overexpression of wild type LIMK1 resulted in MT destabilization, whereas the kinase-dead mutant of LIMK1 (KD) did not affect MT stability. Importantly, down-regulation of endogenous LIMK1 by siRNA resulted in abrogation of the thrombin-induced MTs destabilization and the inhibition of actin polymerization in endothelial cell. We examined pulmonary vascular permeability in LIMK1−/− mice. We found that endothelial permeability in the lungs of LIMK1 −/− mice was lower than in wild type mice. Perfusion of the lungs of wild type mice with PAR1 peptide showed significant increase of endothelial permeability. Notably, the endothelial permeability of the lungs of LIMK1 −/− mice after PAR1 peptide
Microtubule Dynamics Stimulated by F11L-dependent Inhibition of Rho Signaling Is Required for Release of Vaccinia Virus from Infected Cells


Microtubules and their dynamics play a critical role during many cellular processes including for example intracellular trafficking and cell migration. Microtubules also play an important role in the transport of viral pathogens during their infection cycles. A role of microtubule dynamics during viral pathogenesis however remains to be established. Using live-cell imaging of GFP-tagged tubulin and EB1, we now show that vaccinia virus infection dramatically stimulates microtubule dynamics. Stimulation of microtubule dynamics was dependent on inhibition of Rho GTPase signaling by F11L, a viral encoded inhibitor of RhoA. Inhibition of microtubule dynamics, while not inhibiting vaccinia virus assembly, did result in a dramatic reduction of the number of virus particles accumulating at the plasma membrane and being released from the infected cell. Based on our observations we suggest that viral stimulated increase of microtubule dynamics in the cell periphery may play an important role in facilitating efficient cell to cell spread of viruses during infection. (References: [1] UF. Greber and M. Way. (2006) Cell 124:741-52. [2] F. Valdiverrama et. al. Science 311:377-81.)

Phosphorylation of Serine 16 or Serine 63 Strongly Diminishes Stathmin’s Catastrophe-promoting Activity at Microtubule Plus and Minus Ends

T. Manna, S. Honnappa, D. A. Thrower, H. P. Miller, M. O. Steinmetz, L. Wilson; 1Department of Molecular, Cellular, and Developmental Biology and The Neuroscience Research Institute, University of California at Santa Barbara, Santa Barbara, CA, 2Department of Cell Biology, University of Connecticut, Storrs, CT.

Stathmin, an AAA ATPase mutated in the neurodegenerative disease Hereditary Spastic Paraplegia, severs microtubules. AAA ATPases are molecular machines that assemble into ring-shaped oligomers which often drive unfolding of target proteins by translocating them through the central pore during successive rounds of ATP hydrolysis. Loops projecting into the pore are required for target recognition, translocation, and enzyme function. By analytical ultracentrifugation, gel filtration chromatography, and electron microscopy we show that Stathmin assembles into a hexameric ring in an ATP-dependent fashion. Using purified WT and mutant versions of Stathmin in microtubule severing and tubulin binding assays we show that pore loops in hexameric Stathmin recognize the extreme C-terminal amino acids of tubulin and that this interaction is critical for microtubule severing. A disease-associated mutation affects one pore loop. In contrast to the ATP-dependent interaction described above, we found that Stathmin contains a second microtubule binding domain which makes a distinct, ATP-independent interaction with microtubules. This domain is required for engagement of tubulin tail recognition and severing. We propose that Stathmin exerts force on the microtubule in part by engagement of the tubulin tail by Stathmin’s pore. Severing could be achieved by pulling the tail into the pore. Alternatively, since the N-terminal microtubule binding domain (N-MTBD) interacts with a microtubule, Stathmin engages a microtubule in two places. Possibly, molecular motions that result in the reorientation of the N-MTBD with respect to the AAA ring could result in force being applied to the microtubule.

Tubulin Cofactor A (TBCA) Is Required for Cell Viability

S. Nolasco, J. Belillo, J. Gonçalves, J. C. Zahala, H. Soares; 1Instituto Gulbenkian de Ciência, Oeiras, Portugal, 2Departamento de Biologia Molecular-Unidad Asociada al Centro de Investigaciones Biológicas (CSIC), Universidad de Cantabria, Santander, Spain, 3Escuela Superior de Tecnología da Saúde de Lisboa, Lisboa, Portugal.

The folding of tubulin and assembly of αβ-tubulin heterodimers competent to polymerise is a complex multistep process involving several molecular chaperones and cofactors. We hypothesise that the tubulin folding pathway, controlling the synthesis and flux of mature tubulin heterodimers, plays a central role in regulating the assembly, remodelling and dynamics of microtubule structures. In vitro, tubulin cofactor A (TBCA) interacts with β-tubulin in a quasi-native state behaving as a molecular chaperone. In human cell lines, we showed that TBCA is an essential gene since its knockdown, by RNAi, in Hela and MCF-7 cells induced cell death. The essentiality of TBCA in these cell lines may be related with the tubulin gene family complexity since this gene is not essential in yeast. The indirect immunolocalization of tubulin showed subtle alterations in microtubules and the cell cycle analysis (by flow cytometry) showed cellular proliferation inhibition by G1 cell cycle arrest. Furthermore, MCF-7 cells presented also a dramatic change in cell shape resembling differentiation. Our results strongly suggest the involvement of caspase activities in cell death. However, Annexin-V and PI staining indicated that cell death occurs essentially by necrosis/late apoptosis. In both cell lines the knockdown of TBCA also induces a decrease in β-tubulin levels that is accompanied by a decrease in α-tubulin levels indicating a strict regulation between tubulins. This decrease correlates with cell death and could be due to a reduction in the tubulin folding rate or to an increase of the degradation of the improperly folded tubulin. However, the idea that TBCA could play alternative/additional roles in vivo is still an unanswered question.

Phosphorylation of Ser16 or Serine 63 Strongly Diminishes Stathmin’s Catastrophe-promoting Activity at Microtubule Plus and Minus Ends

T. Manna, S. Honnappa, D. A. Thrower, H. P. Miller, M. O. Steinmetz, L. Wilson; 1Department of Molecular, Cellular, and Developmental Biology and The Neuroscience Research Institute, University of California, Santa Barbara, CA, 2Department of Structural Biology, Paul Scherrer Institut, Villigen PSI, Switzerland

Stathmin plays a major role in cell cycle progression by regulating microtubule (MT) dynamics via phosphorylation dependent pathways. Stathmin’s four serine residues (Ser16, Ser25, Ser38 and Ser63) are phosphorylated during mitosis, which is believed to be critically involved in switching off stathmin’s MT-stabilizing activity. Recent studies have indicated that phosphorylation at Ser16 and Ser63 is critical in regulating MT organization and function during mitosis (Larsson et al, Mol. Biol. Cell 17:5530-5539, 1997). However, the mechanisms by which phosphorylation of Ser16 or Ser63 might regulate MT dynamics are largely unknown. Our recent studies have shown that stathmin modulates steady-state MT dynamic instability predominantly by increasing the catastrophe frequency both at plus and minus ends through a direct action on MTs, with the catastrophe promoting activity at minus ends being considerably stronger than at plus ends (Manna et al., J.Biol. Chem 281:2071-2078, 2006). Consistent with its ability to destabilize minus ends, stathmin strongly increases the treadmilling rate of bovine brain MTs in vitro. Here we analyzed the effects of authentically phosphorylated stathmin individually at Ser16 and Ser63 on stathmin’s ability to regulate the catastrophe frequency at plus and minus ends through a direct action on MTs at steady state. Phosphorylation at Ser16 strongly reduced stathmin’s ability to increase the catastrophe frequency at plus ends and it completely abolished stathmin’s ability to increase the catastrophe frequency at minus ends. In contrast, stathmin completely lost its ability to increase the catastrophe frequency at either MT end upon phosphorylation of Ser63. The reduced ability of the phosphorylated proteins to increase the catastrophe frequency correlated well with reduced ability to bind soluble tubulin, to bind intact microtubules, and to inhibit MT polymerization. Supported by NIH NS13560, NSF 0331697, and Swiss National Research Foundation 31-64978.01.
Gsa Activates Tubulin GTPase and Differentially Interacts with Tubulin Isotypes
R. H. Dave\textsuperscript{1}, V. Frasad\textsuperscript{2}, A. Banerjee\textsuperscript{2}, R. F. Ludala\textsuperscript{2}, M. M. Rasenick\textsuperscript{1}\textsuperscript{,}\textsuperscript{3}; \textsuperscript{1}Department of Physiology, Neuroscience Program, and MD/PhD Program, University of Illinois Chicago College of Medicine, Chicago, IL, \textsuperscript{2}Dept. Biobiochemistry, University of Texas Health Science Center at San Antonio, San Antonio, TX, \textsuperscript{3}Department of Physiology, Department of Psychiatry and Neuroscience Program, University of Illinois Chicago College of Medicine, Chicago, IL.

Tubulin is not only a building block of microtubules, but is a participant in G protein signaling. Tubulin binds tightly to the heterotrimeric G proteins, Gs, Giq and Gq, (Kd=100 nM) and activates those G-proteins in a receptor-independent manner, by direct transfer of GTP (transactivation). Under physiologic conditions, Gs only is not capable of transactivating tubulin, but Gs and Gqα increase the steady-state tubulin GTP hydrolysis activity and microtubule dynamicity. Disruption of Gs-tubulin interactions in COS, PC-12 and primary hippocampal neurons decreases process (neurite) outgrowth. Thus, there is a bidirecional physiologic interaction between Gs and tubulin in regulating cellular differentiation. Here, we show that Gsa differentially interacts with tubulin isotypes, preferentially associating with βIII and βII tubulin over the βI and βI tubulin isotypes; this reflects both affinity and isotype prevalence in mammalian brain. Moreover, we demonstrate that Gsa directly increases the intrinsic, single turnover, GTPase rate of βII and βIII tubulin dose-dependently with saturation kinetics. The Hill coefficient obtained was approximately 1.0, consistent with the predicted 1:1 stoichiometry of the tubulin-Gsa interaction. The maximal stimulation observed between the isotypes. The ability of the various tubulin isotypes to transfer GTP to Gsa is currently being determined. In summary, these results suggest a role for Gs in regulating tubulin function, regardless of isotype, thereby providing an interface for G protein-mediated regulation of the cytoskeleton. Moreover, Gs appears to differentially interact with tubulin isotypes. Eventually, this line of investigation could yield insight into the mechanistic significance for modified signal transduction in malignant cells, where tubulin isotype expression is altered. Further, these studies may have implications for the ability of G proteins to modify synaptic outgrowth in response to neurotransmitter.

Analysis of Mutants That Impair Turnaround of the IFT-Machinery in \textit{C. elegans} Sensory Cilia
M. C. Them\textsuperscript{2}, G. Ou\textsuperscript{2}, M. Keggi\textsuperscript{2}, M. Scholey\textsuperscript{1}, Section of Molecular and Cellular Biology, University of California, Davis, Davis, CA, \textsuperscript{2}Department of Biology, Kyushu University Graduate School, Fukuoka, Japan

Kinesin-II and OSM-3 motor proteins work cooperatively to build the sensory cilia in \textit{C. elegans} in a process termed anterograde IFT. Both motors reductantly build the initial segment of the cilium while OSM-3 alone forms the distal segment. Retrograde transport, driven by dynein, is necessary to recycle ciliary components and motors to the basal body. This process requires coordinated motor switching, cargo unloading, reorganization and turnaround of IFT components at the tip of both the initial and distal segments, which are poorly characterized events. We propose to use two ciliary mutants, which were isolated in a screen for dyf mutants, to dissect the process of IFT turnaround during ciliogenesis. Visualization of GFP-tagged IFT components has revealed that these mutants accumulate IFT particles at the tip of the initial segment, suggesting disrupted turnaround and, in addition, one of the mutants also exhibits retarded transport rates along the initial segment. We are using genetic and molecular approaches to identify the corresponding gene products of these mutants in order to improve our understanding of the mechanism of turnaround of the IFT machinery and to learn how mutations in one of the gene products alters both turnaround and rate of IFT.

Molecules Required for Sensory Signal Transduction Regulate Ciliary Structures in \textit{C. elegans}
S. Mukhopadhyay\textsuperscript{2}, P. Sengupta\textsuperscript{1}, Biology, Brandeis University, Waltham, MA.

Cilia and flagella are cellular organelles formed by evolutionarily conserved mechanisms requiring intraflagellar transport (IFT). IFT involves microtubule-based transport of axoneme precursors and membrane proteins via an anterograde and retrograde motors. In addition to mediating motility, cilia and flagella also act in sensory signaling, and some of the signal transduction complexes required for responses to the environment are localized to these organelles. Defects in signaling molecules involved in the phototransduction cascade, including rhodopsin and cyclic nucleotide-gated channels result in degeneration of rhodobes in flies and retinal degeneration in mice, respectively. The role of these signaling proteins and their interplay with IFT in regulating cilia structure and function is unclear. We are studying the role of genes coding for signaling proteins in regulating the structure and function of amphiob cilia in \textit{C. elegans}. It has previously been reported that alteration of the ODR-3 G α alpha protein subunit alters the cilia morphology of the AWA and AWC olfactory neurons (Roayaie et al., 1998). We found that gain-of-function mutations in \textit{odr-3} also result in shortening of the cilia of the AWA neurons. In addition, mutations in genes acting downstream of \textit{ODR-3} in sensory signal transduction such as the guanylyl cyclase \textit{odr-1} and the cyclic-nucleotide gated cation channel subunits \textit{tax-2} and \textit{tax-4} alter the structure of the AWA and AWC olfactory cilia. Eipstasis analyses suggest that similar to their roles in ophthalmic signal transduction, \textit{odr-1} and \textit{tax-2/4} act downstream of \textit{odr-3} to modulate ciliary structure. We also find that overexpression of chemoreceptor genes affects ciliary structure of AWA in a way similar to \textit{odr-1} mutants. The ciliary defects of \textit{odr-1/\textit{tax-2/4}} mutants are suppressed by mutations in the anterograde IFT motor Kinesin-II subunit \textit{klp-11}, suggesting that the alteration in cilia structures may be dependent on IFT.

Neuron-specific Modulation of Intraflagellar Transport in \textit{C. elegans}
S. Mukhopadhyay\textsuperscript{2}, H. Qin\textsuperscript{1}, Y. Lu\textsuperscript{2}, A. Ljuanju\textsuperscript{2}, J. Rosenbaum\textsuperscript{2}, S. Shaham\textsuperscript{1}, P. Sengupta\textsuperscript{1}, Biology, Brandeis University, Waltham, MA, \textsuperscript{2}Biology, Texas A&M University, College Station, TX, \textsuperscript{3}Developmental Genetics, Rockefeller University, New York, NY, \textsuperscript{4}Molecular and Cell Biology, Harvard University, Cambridge, MA, \textsuperscript{5}Molecular, Cellular and Developmental Biology, Yale University, New Haven, CT.

Most metazoan cell types contain primary cilia, which are sensory organelles. Sensory neurons such as photoreceptors and olfactory neurons exhibit distinct cell type-specific sensory ciliary morphologies. Intraflagellar transport (IFT) motors assemble and maintain all cilia by transporting ciliary axonemal precursors and membrane proteins, but it is not known how the IFT process is involved in the formation of neuron-specific ciliary specializations. A subset of \textit{C. elegans} chemosensory neurons exhibit specialized cell-specific sensory cilia, providing an excellent model in which to explore the mechanisms underlying the formation of distinct ciliary structures. In the \textit{C. elegans} channel chemosensory neurons, the anterograde kinesin-II and OSM-3 motors act cooperatively to form the middle segments, whereas OSM-3 alone acts in the distal segments. However, the functions of these motors in the more elaborate olfactory neuron cilia are not characterized. To characterize and compare IFT mechanisms in individual sensory cilia, we visualized IFT specifically in the cilia of two channel chemosensory neuron subtypes, ASH and ASI, and in the cilia of the olfactory AWA neurons. We find that, unlike in the channel cilia, the IFT motors Kinesin-II and OSM-3 function largely independently in each of the AWA cilia. We observe rare IFT events in the distal segments of the AWA cilia, and in contrast to the channel cilia distal segments, which require IFT components to function, the AWA distal segments appear to be formed in the absence of OSM-3. Thus, the distal AWA cilia segments may have mechanisms of formation distinct from IFT. Mutations in the \textit{fkh-2} forkhead domain transcription factor result in AWA-specific defects in dendritic and ciliary morphology, and \textit{fkh-2} mutants are suppressed by mutations in the \textit{odr-1}\textit{tax-2/4} subunit of IFT. Our results suggest that IFT mechanisms are regulated in a neuron-specific manner, and may underlie the generation of specific ciliary specializations.

The Molecular Control of IFT and Cilium Morphogenesis in \textit{C. elegans} Neurons
G. Ou\textsuperscript{2}, M. Keggi\textsuperscript{2}, J. Scholey\textsuperscript{1}, Center for Genetics and Development, Section of Molecular and Cellular Biology, University of California, Davis, Davis, CA, \textsuperscript{2}Department of Biology, Faculty of Sciences, Kyushu University Graduate School, Hakozaki, Higashi-ku, Fukuoka, Japan

Cilium biogenesis depends upon intraflagellar transport (IFT) motors which deliver ciliary precursors associated with IFT particles to the tip of the axoneme. In \textit{C. elegans}, two IFT-motors, kinesin-II and OSM-3 kinesin cooperate to form two sequential anterograde IFT pathways that build distinct parts of cilia. We previously identified three proteins that mediate the functional coordination of these motors. The Bardet-Biedl syndrome (BBS) ciliary disease proteins, BBS-\textit{s} and BBS-\textit{8} stabilize complexes of IFT-particles containing both IFT-motors, and a conserved ciliary protein, \textit{DFY-1}, is specifically required for OSM-3-kinesin to dock onto and move IFT-particles. To understand how the BBS proteins in ciliogenesis and diversity, we introduced markers of IFT and cilium morphology into a comprehensive collection of \textit{dyf}, \textit{che}, \textit{osm}, \textit{daf}, \textit{unc}, and \textit{mec} mutants and monitored IFT and mutant ciliary morphology. Our results suggest that: 1) the IFT-protein machinery is critical for ciliogenesis and its functional modulation leads to ciliary diversity; 2) neuronal ciliogenesis depends on proper transport along dendrites and proper formation and guidance of axons; and 3) proper cilium development relies on signals from environment and neighboring cells.
Mechanism of IFT-Particles in C. elegans Cilia by the Concerted Action of Kinesin-II and OSM-3 Motors

X. Pan,1 G. OU,1 G. Crevello-gak-Schloet,1 O. Blaauw,2 N. Endres,3 L. Yao, A. Mogilner,1 M. Lerous,3 R. Vale,1 J. Scholz,1 Center for Genetics and Development, Department of Molecular and Cell Biology, University of California, Davis, Davis, CA, 2Department of Molecular Biology and Biochemistry, Simon Fraser University, Burnaby, BC, Canada, 3Department of Cellular and Molecular Pharmacology, The Howard Hughes Medical Institute, University of California San Francisco, San Francisco, CA

The assembly and function of cilia on C. elegans neurons depends on the action of two kinesin-2 motors, heterotrimeric kinesin-II and homodimeric OSM-3-kinesin, which cooperate to move the same IFT-particles along microtubule (MT) doublets. Using competitive in vitro MT gliding assays, we show that purified kinesin-II and OSM-3 cooperate to generate movement similar to that seen along the cilium in the absence of any additional regulatory factors. Quantitative modeling suggests that this could reflect an “alternating action” mechanism in which the motors take turns to move along MTs, or “mechanical competition” in which the motors function in a concerted fashion to move along MTs with the slow motor exerting drag on the fast motor and vice versa.

In vivo transport assays performed in Bardet-Biedl syndrome protein and IFT-motor mutants favor a mechanical competition model for motor coordination, in which the IFT-motors exert a BBS protein-dependent tension on IFT-particles which controls the IFT-pathway that builds the cilium foundation. (X. Pan and G. Ou are equal contributors.)

Characterization of a Golgi-associated Intraflagellar Transport (IFT) Complex

J. A. Folliet, J. T. San Agustin, G. J. Pazour, Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, MA

Most vertebrate cells have a primary cilium projecting from their surface. These structures are thought to be sensory organelles that monitor the extracellular environment. Severe defects in primary cilia lead to embryonic lethality while mild defects yield phenotypes ranging from cystic kidney disease to retinal degeneration. The ciliary cytoskeleton is assembled via intraflagellar transport (IFT) in which large particles are moved along ciliary microtubules by kinesin-II and dynein-2. How membrane proteins are delivered to the ciliary compartment is almost completely unknown. We recently showed that the IFT20 subunit of the IFT particle is unique in that it is found associated with the Golgi apparatus in addition to the normal ciliary localization (Folliet et al., Mol. Biol. Cell 2006). Strong reduction of IFT20 by RNAi blocks ciliary assembly while moderate reduction allows for ciliary assembly but reduces the amount of the membrane protein polycystin-2 localized to cilia. This suggests that IFT20 plays a role in directing membrane proteins from the Golgi apparatus to the ciliary compartment. To further understand IFT20 function, we partially purified an IFT complex, termed complex C, that contains IFT20 but is different from the two known IFT complexes. This >2 Mdal complex contains multiple proteins including a Golgi-associated protein called Trip11 or GMAP210. In mammalian cells and yeast, GMAP210 is required for transport of membrane proteins and in yeast and Drosophila, associated proteins play important roles in polarized secretion. We have identified a 226 residue fragment of GMAP210 that displaces IFT20 from the Golgi complex and stabilizes it in the cytoplasm. We are currently investigating the dominant negative properties of this fragment. In addition, we have generated knockout mice for both IFT20 and GMAP210 and are currently characterizing these animals to further our understanding of the role of these proteins in mammalian health and development.

An In Vitro System for Studying Neuronal Primary Cilia Function

N. F. Berber1, J. S. Lewis, C. C. Askwith,1 L. M. Bohm,1 K. Mykytyn1; 1Pharmacology, The Ohio State University, Columbus, OH, 2Neuroscience, The Ohio State University, Columbus, OH

Primary cilia are hair-like cellular appendages that are present on nearly all vertebrate cells and many cells in invertebrates. Primary cilia are now recognized as important sensory and signaling organelles. Although it has been known for more than forty years that neurons throughout the brain possess primary cilia, the function of neuronal cilia is unknown. Recent work has shown that specific G protein-coupled receptors (GPCRs), such as somatostatin receptor 3 (Sstr3) and serotonin receptor 6 (Htr6), localize to neuronal cilia. The finding that certain GPCRs localize to neuronal cilia suggests that cilia-mediated signaling may affect neuronal activity. In order to pursue this question we have developed a culture system for studying neuronal cilia. We show that hippocampal neurons cultured from newborn mice possess cilia upon which known ciliary proteins localize. We further show that GPCRs are functional at the ciliary membrane. These results suggest that neurons possess cilia-mediated GPCR signaling and that this system should be a valuable tool for elucidating the function of cilia in neurons.

Age-related Losses in Neuronal Cilia of Tg37799 Mice

D. Mahato, H. D. Schwarc, J. L. Fuchs, Biological Sciences, University of North Texas, Denton, TX

Primary cilia are characteristic of most vertebrate cell types, including neurons. Recent evidence shows that primary cilia are vital to vertebrate morphogenesis and organ function. The Tg37799 mouse, which was engineered as a model for studying polycystic kidney disease, was shown by Pazour and colleagues (2000) to have shortened primary renal cilia in association with the gene defect in IFT88, an intraflagellar transport protein. Subsequently, ciliary defects were implicated in the development of hydrocephalus and cysts in liver and pancreas. We found that primary cilia in adult Tg37799 mice were missing from neurons in some brain regions and were shortened in others (Fuchs & Schwarc, 2004). The present study found progressive postnatal decreases in the incidence and length of immunostained neuronal cilia in mutant compared with wildtype. In brain regions that retained a substantial number of cilia, wildtype cilia generally grew slightly in length from postnatal day 14 (P14) to P31, while mutant cilia decreased in length. Relative to wildtype, mutant cilia were about 39% shorter on P14 and 49% shorter on P31, while the percentage of neurons with identified cilia declined by 43% on P14 and 72% on P31. The results suggest that intraflagellar transport is critical for maintenance of primary neuronal cilia, and that there are age-related increases in cilia loss which might contribute to the progressive dysfunction typical of ciliary diseases. The regional and developmental differences in cilia loss might reflect differences in IFT88 expression, degree of ciliary defects on IFT88, and unfolding of the consequences of impaired intraflagellar transport.

Dual Regulation of Daam1-mediated Actin Assembly and Ciliogenesis by Dishevelled and RhoA

T. J. Park, S. Matti,1 J. B. Wallingford,1 B. L. Goode,2 Department of Molecular Cell and Developmental Biology, University of Texas, Austin, Austin, TX, 2Biology Department, Brandeis University, Waltham, MA

Dishevelled (Dvl) functions in the Wnt signaling pathway and provides a key point of control in planar cell polarity. Recently, we showed that Dvl-GFP localizes to the apical surface of ciliated epithelial cells, where a dense actin mat governs the orientation of ciliary microtubules. Here, we investigate the role of a downstream target of Dvl, Daam1, which is a formin protein and thereby is predicted to nucleate actin assembly. Daam1-GFP is also enriched apically in ciliated cells. A purified C-terminal fragment of Daam1 directly nucleates actin polymerization, but full-length Daam1 is autoinhibited via interactions of its N- and C-termini. Daam1 autoinhibition is partially relieved by RhoA and by a C-terminal fragment of Dvl containing its PDZ and DEP domains. This suggests that RhoA and Dvl may converge on Daam1 to induce expression. In contrast to the PDZ/DEP fragment of Dvl, a longer fragment that includes its C-terminus fails to activate Daam1, suggesting a novel regulatory role for this sequence. Expression of this conserved C-terminal portion of Dvl (Xdsh-C1), lacking PDZ and DEP domains, led to disrupted assembly of the apical actin mat, decreased ciliary microtubules assembly below and parallel to the apical surface, and a failure of cilia to project apically from the cell surface. Further, ciliogenesis defects caused by expression of Xdsh-C1 were accompanied by a failure of gamma-tubulin to localize tightly at the apical cell surface. Using a fluorescent sensor to assess Rho activation in vivo, we find that Rho is strongly activated apically in ciliated cells in a region corresponding to the apical actin mat. This activation of RhoA is eliminated by expression of Xdsh-C1, though localization of Rho is unaffected. These data support a model in which joint regulation of Daam1-mediated actin assembly by Dvl and RhoA plays an essential role in ciliogenesis.

Primary Cilia: Mechanosensory Organelles in Bone

C. T. Anderson,1 A. M. Malone,1 P. Tummala,2 R. C. Jacobs,2 T. Stearns 1; 1Biological Sciences, Stanford University, Stanford, CA, 2Mechanical Engineering, Stanford University, Stanford, CA

Primary cilia are microtubule-based protrusions that extend from the surface of most mammalian cell types. Primary cilia mediate calcium influx in response to fluid flow in kidney cells, but their function in many other cell types is unknown. Given that bone responds to mechanical loading with increased bone growth and that bone cells display increases in intracellular calcium, prostaglandin E2 release, and expression of osteogenic genes such as osteopontin in response to fluid flow, we asked whether we could detect primary cilia in bone tissue and bone-derived cell lines, and whether loss of primary cilia in cultured bone cells abrogates flow response. By staining mouse tibia cryosections for acetylated alpha-tubulin, we detected primary cilia in both osteoblasts and osteocytes, and we likewise detected primary cilia in 55% and 65% of the MC3T3-E1 osteoblast-like and MLO-Y4 osteocyte-like cell lines, respectively. After removal of...
primary cilia by chloride hydrate treatment or prevention of primary cilia formation by RNAi targeting the ciliary protein polars. MC3T3-E1 cells still exhibited increased intracellular calcium in response to flow, but did not exhibit increased prostaglandin E2 release or osteopontin expression after flow. These results indicate that primary cilia act as mechano sensors in bone cells, but that they may do so by a mechanism that is distinct from that of kidney cells.

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Intraflagellar Transport Is Essential for Endochondral Bone Formation

C. J. Haycraft, Q. Zhang, B. Song, R. Serra, B. K. Yoder, Department of Cell Biology, University of Alabama at Birmingham, Birmingham, AL, 2Department of Pediatrics, University of Iowa, Iowa City, IA, 3Department of Pathology, University of Alabama at Birmingham, Birmingham, AL.

Patternning of the mammalian limb requires the complex interaction of at least three signaling centers, the AER, the overlying ectoderm, and the ZPA. Anterior-posterior patterning is regulated by secretion of Shh from cells in the ZPA to inhibit the repressor activities of the transcription factor Gli3. The resulting gradient of Gli3 activator to repressor function imposes a pentadactyl restraint on the developing autopod. Most cells throughout the mesenchyme and ectoderm of the developing limb express a primary cilium which has been shown to be essential for proper Shh signal transduction and Gli3 repressor formation. To bypass the early lethality associated with congenital loss of cilia as well as determine which cell populations in the developing limb require a primary cilium for proper pattern formation, we used mice expressing CRE recombinase under control of the msh2 promoter (msh2Cre), no overt defects in limb patterning or outgrowth are observed. In contrast, deletion of cilia from cells in the mesenchyme using prelcre transgene mice results in extensive polydactyly and loss of Shh signal transduction. Analysis of the role of primary cilia during endochondral bone formation has been hindered by the lethality of congenic ift mutant mice prior to the formation of the skeletal elements. In addition to defects in digit patterning, the limbs of prelcre conditional mutants are severely shortened and endochondral bone formation is altered, possibly through disruption of Ihh signaling. Perichondrial architecture adjacent to the diaphysis of the skeletal elements is altered and bone collar development is disrupted. In agreement with a predicted requirement for cilia in Ihh signaling, both Pthl1 and Gli3 expression are lost in the cartilage and perichondral cells of the developing bones.

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Primary Role for Centrosome Proteins in Cilia Formation and Cilia-associated Disorders

B. Delaval, L. Covoss-Barberis, N. Lawson, S. Dossay, Program in Molecular Medicine, UMASS Medical School, Worcester, MA, Program in Gene Function and Expression, UMASS Medical School, Worcester, MA, Program in Molecular Medicine, UMASS Medical School, Worcester, MA, Program in Gene Function and Expression, UMASS Medical School, Worcester, MA.

Cilia are microtubule structures that assemble from the mother centriole of the centrosome. The centrosome contributes to spindle organization, cell cycle progression and cilia assembly. Cilia are microtubule structures that assemble from the mother centriole of the centrosome. Previous studies have shown that the centrosome protein pericentrin plays a role in the assembly/maintenance of centrosomes in human cells. Another study has shown that a Drosophila protein that shares homology with human pericentrin and AKAP450 is also essential for cilia and flagella formation in vivo. To address the role of centrosome proteins in cilia assembly, we have begun a comprehensive analysis of centrosome proteins in human cells and zebrafish embryos. We first examined primary cilia in diploid human epithelial cells (RPE-1). We found that depletion of 10/11 centrosome proteins by RNA interference perturbed primary cilium assembly, demonstrating that primary cilium formation is a common centrosome proteins function. To characterize the role of centrosome proteins in cilia formation in vivo, we used a morpholino-mediated knockdown approach in Zebrafish (Danio rerio). We identified several Zebrafish centrosome genes homologous to vertebrate genes and they are currently being targeted for depletion. Here, we focus on results from centrin 2 (Cetn2) depletion. Essentially all defects observed in Cetn2 depleted embryos phenocopied those observed for cilia genes such as polycystins and intraflagellar transport proteins. For example, loss of Cetn2 prevented formation of cilia in the olfactory placode and induced formation of cysts in the kidney, suggesting polycystic kidney disease. Other cilia-related defects included smaller eyes, hydrocephaly, cardiac oedema and increased body surface. Consistent with these defects, Cetn2 mrnas and mRNAs of other centrosome proteins were localized to organs that were affected by centrin depletion (e.g. promephric duct, olfactory organ, spinal cord). These and other results suggest that centrosome proteins have a primary role in cilia formation and cilia-associated disorders.

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EB1 Is Required for Primary Cilia Assembly in Fibroblasts

J. M. Schroder, L. Schneider, S. T. Christensen, L. B. Pedersen, Molecular Biology and Physiology, University of Copenhagen, Copenhagen, Denmark

EB1 is a small, evolutionarily conserved microtubule (MT)-associated protein involved in regulating MT dynamics. EB1 associates preferentially with MT plus ends and appears to play a major role in regulating/tethering of other MT-associates proteins to the MT plus end. EB1 also localizes to centrosomes, is necessary for mitotic spindle elongation and linking of MTs to kinetochores and the cell cortex during mitosis, and has also been implicated in MT stabilization in migrating fibroblasts. We previously showed that EB1 localizes to the flagellar tip and proximal region of the basal bodies in Chlamydomonas, and obtained evidence suggesting a potential role for EB1 in regulating intraflagellar transport (IFT; Pedersen et al., Curr. Biol. 2003). However, the exact function of EB1 in the cilium/flagellum is unknown. To analyze the function of EB1 in the cilium/flagellum we depleted EB1 from NIH3T3 fibroblasts using siRNA, and analyzed the effect of EB1 depletion on primary cilium formation in growth-arrested cells using immunofluorescence microscopy. Strikingly, in three separate experiments, only 37.13±2.30% of EB1-depleted cells (n=293) became ciliated during growth arrest compared to 65.37%±2.15% ciliated cells in mock-transfected controls (n=324). Thus depletion of EB1 causes a ca. 50% reduction in the efficiency of primary cilium assembly in fibroblasts. Since EB1 localizes to the ciliary/flagellar tip as well as to basal bodies, we are currently trying to determine whether the effect of EB1 depletion on primary cilium assembly results from defective assembly of the nascent cilium itself or is due to a defect in basal body assembly. Supported by grants from the Danish Natural Science Research Council (272-05-0411) and The Novo Nordisk Foundation to LBP.

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Non-motile (9+0) Primary Cilia in Cell Culture Exhibited Constrained Motion

K. M. Yasatii, T. Ursei, B. Bettencourt-Dias, J. Lippincott-Schwartz, C. M. Ott, Physiology Course 2006, Marine Biological Laboratory, Woods Hole, MA.

The primary cilium is a common structural feature found on many mammalian cells in vivo, shown to be important for mechanosensation, chemosensation, and signaling. This organelle protrudes 5 to 55 μm from the surface of the cell and is composed of a circular bundle of 9 microtubule doublets (9+0) enveloped in plasma membrane. This cilium does not have two internal microtubules in contrast to motile (9+2) cilia and flagella, which contain two additional central microtubules. Hence, primary cilia were long considered non-motile. Recently, movement of primary cilia was observed in the embryonic node (nodal cilia). This rather rapid movement (~50Hz) is thought to be essential for left-right symmetry breaking in early embryonic development. Using live-imaging of fluorescent markers we observe constrained circular motion of primary cilia in cultured cells. We characterized this motion by tracking the distal end of the primary cilium. Strikingly, in three separate experiments, only 37.13±2.30% of the EB1-depleted cells (n=293) became ciliated during growth arrest compared to 65.37%±2.15% ciliated cells in mock-transfected controls (n=324). Thus depletion of EB1 causes a ca. 50% reduction in the efficiency of primary cilium assembly in fibroblasts. Since EB1 localizes to the ciliary/flagellar tip as well as to basal bodies, we are currently trying to determine whether the effect of EB1 depletion on primary cilium assembly results from defective assembly of the nascent cilium itself or is due to a defect in basal body assembly. Supported by grants from the Danish Natural Science Research Council (272-05-0411) and The Novo Nordisk Foundation to LBP.

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Imaging Transport Properties of Smoothened in the Membrane of the Primary Cilium


Smoothened is an integral membrane protein essential for the Hedgehog signaling pathway. In the absence of Hedgehog, Smoothened signaling is inhibited by the Hedgehog receptor Patched. Recently, localization of Smoothened to the primary cilium was shown to be essential for Sonic Hedgehog signal transduction to vertebrates. The nature of Smoothened movement within the cilium is unclear; however it will likely be important for signal transduction. Movement of select membrane proteins in the sensory cilium in C. elegans was shown to exhibit bidirectional transport. We utilized oblique laser illumination fluorescence microscopy and fluorescence recovery after photobleaching (FRAP) combined with kinetic modeling to explore the possibility of diffusion versus directed movement of Smoothened within the sensory cilium of the vertebrate. We observed bidirectional transport of Smoothened complexes along the length of the primary cilium. FRAP data were compared to a variety of models and the best agreement was obtained with a model of directed intracilial transport. Additionally, both experimental methods suggest the transport of Smoothened can pause en route to the base or tip of the cilium. Future studies will be required to determine how Smoothened transport within the primary cilium affects hedgehog signal transduction.
Mutations in the NIMA-related Kinase Defective in the jck Model of Polycystic Kidney Disease Cause Altered Ciliary and Centrosomal Localization but Do Not Affect Ciliogenesis

M. L. Trapp, D. K. Manning, D. R. Beier, L. M. Quarmby; Molecular Biology and Biochemistry, Simon Fraser University, Burnaby, BC, Canada; 'Genesics Division, Brigham and Women's Hospital, Harvard Medical School, Boston, MA

Cystic kidney diseases are caused by mutations in a diverse array of genes, including Nek8, a NIMA-related kinase. Originally characterized in the jck (juvenile cystic kidney) mouse model of polycystic kidney disease (Liu S, Lu W, Obara T, Kuida S, Lehoczky J, Dewar K, Drummond IA, and Beier DR. 2002. Development. 129, 5839-5846), Nek8 has been found to localize to the primary cilia of mouse epithelial cells (Mahgoub MR, Trapp ML, and Quarmby LM. 2005. J. Am. Soc. Nephrol. 16, 3485-3489), similar to other cytoplasmic proteins. The cilia on kidney epithelia are key for normal mechano-sensation and signal transduction, as mice that lack cilia develop cystic kidneys. To examine how mutated Nek8 can alter functioning of the cilia and cause intracellular defects, GFP-tagged mouse wild type, kinase-deficient (jck), and jck (G448V) Nek8 were overexpressed in mouse inner medullary collecting duct (IMCD-3) cells. GFP-Nek8 was found to localize to the cytoplasm, cilia, and centrosomes, while GFP-tagged kinase-deficient and jck Nek8 showed different ciliary and centrosomal localization patterns than wild type. Because expression levels also affect localization, we confirmed this difference by analysis of IMCD-3 co-transfected with myc-tagged wild type Nek8 and GFP-mutant Nek8. RNAi knockdown and overexpression of GFP-tagged Nek8 did not affect the percentage of ciliated cells, and neither GFP-K33M nor GFP-jck Nek8 affected overall ciliary assembly. We also analyzed tetracycline-inducible Nek8 knockdown IMCD-3 cells and mutant Nek8 in cells lacking endogenous protein. Our results support the conclusion that polycystic kidney disease caused by the jck mutation of Nek8 is the consequence of a defect in signaling rather than ciliogenesis.

Ciliogenesis and Fibrocytin Expression in the PCK-CCL, a Cholangiocyte Cell Line Derived from an Animal Model of ARPKD, the PCK Rat

T. V. Masyuk, B. Q. Huang, A. I. Masyuk, N. F. Larrusso; Internal Medicine, Mayo Clinic College of Medicine, Rochester, MN

We showed that fibrocytic (FC), the protein product of PKHD1, the gene mutated in Autosomal Recessive Polycystic Kidney Disease (ARPKD), is expressed in normal cholangiocyte cilia but absent from malformed cilia of the PCK rat. We also reported establishment of ciliogenesis model using a normal rat cholangiocyte cell line (NRC) to study FC expression under normal conditions. Our OBJECTIVES here were to develop a ciliogenesis model in PCK-CCL and to examine FC expression under pathological conditions. METHODS: Ciliogenesis was examined by scanning and confocal microscopy and FC expression by confocal microscopy. RESULTS: In sub-confluent and post-confluent day 0 PCK-CCL and NRCs, cilia were absent. At day 3 post-confluence, 35% of NRCs possessed cilia ~1.8um in length. In contrast, in the PCK-CCL, cilia were shorter (1.1um; p<0.05) and present in 10% of cells. At day 7 post-confluence, 86% of NRCs had cilia ~1.8um in length, in the PCK-CCL, cilia were present in 57% of cells and 3.3-fold shorter (2.59+/-0.86um, p<0.05). There were also structural abnormalities with bulbus elongation on the ciliary axoneme. By RT-PCR, an 800bp Pkhd1 product was amplified in NRCs; in the PCK-CCL, the amplicon was ~630bp reflecting the IVS35-2A- T mutation. In NRCs, in the absence of FC, cilia were found in cytoplasm; in NRCs with developed cilia, FC was localized intracellularly, in the basal body and cilia. In PCK-CCL, at all stages of ciliogenesis, FC was expressed in the cytoplasm and nucleus and never in cilia. CONCLUSIONS: We developed a model of ciliogenesis in a cholangiocyte cell line derived from the PCK rat. In PCK-CCL, structural (malformed cilia) and functional (no FC in cilia) abnormalities of cilia are consistent with those seen in vivo in the PCK rat. This model will be useful in elucidating the role of FC in hepatic cyst formation.

Adenyl Cyclases Are Localized to Cholangiocyte Primary Cilia and Are Involved in Chemosensory Ciliary Functions

A. I. Masyuk, B. Q. Huang, A. J. Stroope, P. L. Splinter, N. F. Larrusso; Internal Medicine, Mayo Medical School, Clinic and Foundation, Rochester, MN

We previously reported that cholangiocyte cilia express polycystin-1, polycystin-2, and adenyl cyclase 6 (AC6) which are involved in mechanosensory ciliary functions (Masyuk et al., Gastroenterology, 2006). Given that AC6 is an enzyme whose activity is modulated by different agonists via G protein-coupled receptors, its expression on cholangiocyte cilia suggests possible chemosensory functions for these organelles. The potential agonists present in bile in physiological concentrations that may affect adenyl cyclases (ACs) are ATP and other nucleotides. Since in many cell types nucleotides affect cAMP signaling via the G protein-coupled receptors, P2Y11-14, we tested the hypothesis that biliary ATP induces cAMP signaling in cholangiocytes via P2Y11 and ACs expressed in cilia. Immunofluorescence confocal and immunogold transmission electron microscopy of isolated rat intraphaeric bile duct units (BDUs) were used to assess expression of ACs and P2Y11 in cholangiocyte cilia. A microperfused BDU model was used for functional studies. BDUs were perfused through their lumen with ATP-gammaS (100mM), a specific agonist of P2Y11, and cAMP levels in cholangiocytes measured by a fluorescence assay. P2Y11 and AC6 are both expressed on the microvilli and cilia of the cholangiocyte apical plasma membrane. Luminar perfusion of BDUs with ATP-gammaS resulted in a 2.6-fold increase in cAMP levels in cholangiocytes. The ATP-gammaS-induced cAMP increase was abolished by chlorid hydrate (a compound that removes cilia), by suramin (a specific antagonist of P2Y11) and by siRNAs to AC6. Taken together, these data suggest that cholangiocyte cilia have chemosensory functions in which the key players are nucleotides, P2Y11, and AC6.

Rho Kinase and Cortical Myosin II Control Centriole Migration during Ciliogenesis of Primary Cilia

H. Dawe, K. Gull; Sir William Dunn School of Pathology, University of Oxford, Oxford, United Kingdom

Primary cilia are sensory organelles that function as highly variable cilia type throughout the body. The migration of the mature centriole from a juxtanuclear position to the apical membrane, where it subtends a primary cilium, is a critical early stage in ciliogenesis. However, the molecular mechanisms that control this process remain obscure. We have used a combination of drug studies and siRNA to investigate factors controlling centriole positioning during primary ciliary formation. In this study we show that ROCK I signalling to cortical myosin II is required for centriole migration. Drug- or siRNA-mediated disruption of ROCK I or myosin II prevents centriole migration to the apical membrane and subsequent axoneme extension. Instead, centrioles remain in a pericentriolar position. Inhibition of ROCK I and myosin II had no effect on cilia position in cells with pre-existing cilia. This implies that neither ROCK I nor myosin II is required for maintenance of centriole basal body position once centriole migration is complete. Immobilisation of the cell cortex with tetravalent lectins also prevents centriole migration and ciliation, suggesting that myosin II activity is required within the cortex and that centriole migration can be achieved by cortical contraction.

NHE1 Is Encoded by a Growth Arrest Specific (gas) Gene and Its Activity Is Associated with PDGFrα Signaling in the Primary Cilium During Migration in Quiescent Fibroblasts

L. Schneider, C. M. Stock, E. K. Hoffmann, P. Satir, A. Schwab, S. F. Pedersen, S. T. Christensen; 1Department of Biochemistry, The August Krogh Institute, University of Copenhagen, Copenhagen, Denmark, 2Institute for Physiologie, Muenster, Germany, 3Department of Anatomy and Structural Biology, Albert Einstein College of Medicine Yeshiva University, Bronx, NY

The ubiquitous plasma membrane Na+/H+ exchanger, NHE1, is found in essentially all cell types, and is involved in multiple cellular processes, including the control of cell volume and cellular pH (see Pedersen, SF 2006 Physiog Arch 452(2):249-59). Further, NHE1 has been assigned important roles in the control of cell migration and specifically in the increased migratory and invasive properties of tumor cells (Schwab, A. 2001 Am J Phsiol Renal Physiol. 280:F739-47; Cardone, RA et al. 2005 Nat Rev Cancer 5:786-95). Here we investigated a possible connection between NHE1-mediated effects on cell migration and the growth arrest specific (gas) protein receptor, PDGFR-alpha, which specifically localizes to primary cilia during growth arrest and is uniquely activated in the cilium upon stimulation with PDGF-AA in mouse embryonic fibroblasts (MEFs) (Schneider L. et al. 2005 Curr Biol 25:1861-6). In Tg737"""" mutant MEFs, which show defects in ciliary assembly, PDGFR-alpha is not up-regulated, PDGFR-AA signaling is blocked, and signaling cascades leading to cell cycle entrance are inhibited. We here show that mRNA and protein levels of NHE1 were up-regulated during growth arrest in NIH3T3 cells and primary cultures of both wt and Tg737"""" mutant MEFs, and that these levels decreased upon cell cycle entrance, identifying the exchanger as a gas protein, independent of ciliary formation. Wound healing assays on wt MEFs revealed that inhibition of NHE1 activity by EIPA reduced PDGFR-AA mediated cell migration speed and translocation, and this inhibition was markedly reduced in Tg737"""" mutant MEFs. Our results indicate that NHE1 activity is up-regulated during quiescence and that PDGFR-alpha signaling in the primary cilium is connected to NHE1-dependent mechanisms during cell migration in growth arrested cells. Further, our results support the idea that NHE1 activity may be a critical event in the physiological response to PDGFR-alpha activation.

Contribution of Intraflagellar Transport in Expression and Trafficking of Flagellar Proteins

S. Absalon, G. Trouillas, P. Bastin; 1Pasteur Institute, Paris, France, 2MNHN, Paris, France
Cilia and flagella are present in most eukaryotic organisms and their structure is well conserved throughout evolution. Flagellum biogenesis is a fascinating process by which more than 300 proteins need to be synthesized and assembled in a complex structure at a defined localization and at a precise time of the cell cycle. Trypanosoma brucei, an unflagellated protzoan responsible for sleeping sickness in Africa, is a great model system to study this process, with the particularity to maintain its old flagellum during cell replication. Trypanosomenes can easily be grown and transformed in culture. By genome compation, we have shown that the majority of genes related in construction and function of flagellum are conserved. We have analyzed 12 candidate genes involved in Intraflagellar Transport (IFT), process conserved in ciliated organisms and required for assembly and maintenance of the organelle) using inducible RNAi. After silencing, cells exhibiting a shorter or no flagellum show down regulation of flagellar proteins. We demonstrate that this process depends on a post-transcriptional mechanism (without any influence on RNA stability or processing) whose regulatory elements are present in untranslated regions. This strongly suggests a new function for IFT in control of expression of flagellar genes. To evaluate trafficking of flagellar proteins, we have constructed a fusion with the axonomal component PF16 and a fluorescent tracer called Timer (which is able to change from green to red over time). We have monitored the turn-over and trafficking of this fusion protein in different contexts (wild type cells, IFT or motility mutants). These experiments allowed us to evaluate the importance of flagellum for different stages of the trypanosome cell cycle, in particular the process of basal body migration. Our data show that trypanosomenes use their flagellum to position their basal bodies.

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Inositol Hexakisphosphate Production Is Required for Ciliary Function and Maintenance

B. Sarmah, V. P. Winfrey, G. E. Olson, B. Appel, R. S. Wente; 1Cell and Developmental Biology, Vanderbilt University Medical Center, Nashville, TN, 2Biological Sciences, Vanderbilt University, Nashville, TN

Cilia are motile sensory organelles that project from cells as a membranous extension. The microtubule structural core of a cilium is assembled from a basal body by a process called intraflagellar transport (IFT). Here, we report that the enzyme inositol 1,3,4,5,6-pentakisphosphate (IP$_6$) 2-kinase (Ipk1), responsible for conversion of IP$_6$ to inositol hexakisphosphate (IP$_7$), has a conserved ciliary role. Earlier, we reported that Ipk1 activity plays a critical role in the establishment of left-right (LR) asymmetry in zebrafish, mediating a left-biased calcium flux in cells enveloping the Kupffer’s vesicle (KV), a transient ciliated structure essential for the LR asymmetry determination (Sarmah et al., Dev. Cell 9, 133-145, 2005). We now find that reduction in Ipk1 level perturbs ciliary beating in the KV. In contrast to the ventral couterclockwise beating observed in the wild-type embryos, cilia in the Ipk1 depleted embryos quiver in a vibration-like motion devoid of counter-clockwise rotation. In addition, depletion of Ipk1 level shortens cilia length in multiple organs including the KV. Ipk1 depletion also causes distortion of pronephric tubules, a pathology often associated with dysfunctional cilia. Strikingly, Ipk1 is enriched in centrosomes and basal bodies, but not present in the ciliary axoneme. Reducing Ipk1 activity does not alter ciliary axonomal structures. Ipk1 depletion, however, perturbs retrograde melanosome transport in pigment cells indicating that Ipk1 activity might mediate general organelle transport. IP$_6$ may act as a cofactor for motor protein complexes in IFT. Thus, we propose that Ipk1 constitutes a motor component of the basal body facilitating IFT assembly and plays a key role in ciliogenesis. This is the first report of a role for soluble inositol polyphosphate production in ciliary function. This work may facilitate our understanding of the link between ciliary function and cellular signaling, and impact studies of disease and pathology related to dysfunctional cilia.

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A Novel Rho Kinase Is Required for Normal Cilia-driven Nodal Flow and Left-Right Polarization in Zebrafish

J. D. Amack, H. J. Yost; Huntsman Cancer Institute, University of Utah, Salt Lake City, UT

A specialized group of ciliated cells--first identified in the mouse embryonic node--generate a directional fluid flow (termed 'nodal flow') that is required for normal left-right (LR) patterning of internal organs. As seen in the mouse node, we have observed cilia-driven nodal flow in zebrafish embryos in a structure called Kupffer’s vesicle (KV). Humans with ciliary defects often have organ laterality defects. This suggests cilia play a conserved role in LR development, but the mechanism(s) by which nodal flow translates into LR signals remain unclear. To better understand nodal flow, we are using zebrafish to identify molecular components that control form and function of ciliated ‘node’ cells. We report a novel Rho kinase gene--likely a paralog of zebrafish rho kinase 2 (rock2)–that is expressed in ciliated KV cells and required for nodal flow and normal organ laterality. Anti-sense morpholinos (MO) that target either translation or splicing of this rock2-related (rock2r) mRNA induce convergent extension defects and perturb LR asymmetries in the heart, gut and brain. MO knockdown of rock2r disrupts left-sided expression of the nodal-related gene southpaw—the earliest marker of LR asymmetry. Nodal flow inside KV is upstream of normal asymmetric southpaw expression. Immunostaining indicated that KV morphology and ciliogenesis are unaffected in rock2r morphants. However, videomicroscopy of fluorescent beads injected into KV revealed nodal flow is severely compromised in morphant embryos, thus providing an explanation for LR polarity defects. These studies link Rho kinase signaling to LR axis determination, specifically at the step of nodal flow. This is the first example of a non-ciliary component that affects nodal flow. Nodal flow may depend on Rho kinase-mediated cytoskeletal arrangements within node cells, or alternatively, node cells may require a particular alignment established by the planar cell polarity pathway, in which Rho kinases have been implicated.

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Environmental Cues and G Protein Signaling Regulate Intraflagellar Transport of Ciliary Signaling Molecules

J. Burghoono, M. Dekkers, S. Rademakers, G. Jansen; Cell Biology, Erasmus MC, Rotterdam, The Netherlands

Intraflagellar transport (IFT) plays a critical role in trafficking structural components and signaling molecules in cilia. Recent studies suggest that regulation of IFT allows plasticity of the cilium structure and regulation of the presence of specific signaling molecules in the cilium (Ou et al., 2005; Pan et al., 2004; Wang et al., 2006). However, not much is known about the molecular mechanisms of this regulation. We study the regulation of IFT by G protein signaling in the cilium of the nematode Caenorhabditis elegans. We were intrigued by the fact that expression of a dominant active Gt protein, gpa-3QL, in the sensory neurons affects the uptake of fluorescent dyes into these neurons, a process called dye filling (Zwaal et al., 1997). Dye filling requires intact cilia and mutations in genes that affect IFT often cause dye filling defects. However, gpa-3QL does not affect the localization of IFT particles in the cilia middle or distal segments. Interestingly, we found that gpa-3QL strongly reduces entry into the cilia distal segments of the Go proteins GPA-4 and GPA-15. We show that in wild type animals anterograde transport of GPA-4 and GPA-15 is mediated by both, La Jolla, CA, 1Department of Biological Sciences, Vanderbilt University, Nashville, TN, 2Department of Neurobiology, Yale University, New Haven, CT, 3Interdepartmental Neuroscience Program, Yale University, New Haven, CT

Dynamic rearrangements of the filamentous (F-) actin cytoskeleton power cell migration. Actin polymerization drives leading edge protrusion, while actomyosin contractility generates traction forces required for cell body translocation. The extent to which cell migration is driven by forces derived from actin polymerization versus actomyosin contractility is a major unresolved issue. Abuellion (Abh) family kinases mediate signaling from adhesion and growth factor receptors to coordinate changes in cytoskeleton structure. We showed previously that the Abh-related gene (Arg) nonreceptor tyrosine kinase promotes dynamic F-actin-rich cell edge protrusions in fibroblasts during adhesion to and spreading on fibronectin-coated surfaces. Here we demonstrate a second major cellular role for Arg as an attenuator of cell contractility during fibroblast migration on fibronectin. arg$^-$$^-$ fibroblasts have more prominent F-actin stress fibers and focal adhesions and are more contractile than wild type fibroblasts. Arg re-expression in arg$^-$$^-$ cells reduces stress fibers and focal adhesions through two distinct mechanisms: Arg kinase activity acts through the RhoA inhibitor p190RhoGAP to attenuate stress fiber formation, while Arg inhibits focal adhesions via a kinase-independent mechanism. Using Arg mutants that separate these functions, we show that Arg reduces cell contractility by inhibiting stress fibers, but not by inhibiting focal adhesions. arg$^-$$^-$ cells migrate aberrantly, frequently moving in large steps as they suddenly detach or tear at their trailing edge. Despite these irregular movements, arg$^-$$^-$ cells migrate with faster average speeds than wild type or Arg-re-expressing arg$^+$ cells. Quantitative migration analysis of arg$^-$$^-$ cells reconstituted with Arg and Arg mutants demonstrates that Arg inhibits cell migration by attenuating cell contractility and regulating its coupling to adhesion sites. Our findings reveal that the coupling of actomyosin-based contractility with adhesion is the primary driving force for fibroblast migration on adhesive surfaces.

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Leading edge protrusion in the amoeboid sperm of *Ascaris* saum is driven by the localized assembly of the MSP cytoskeleton in the same way that actin assembly powers protrusion in other types of crawling cell. Reconstitution of this process in vitro led to the identification of two accessory proteins required for MSP polymerization: a membrane-associated tyrosine phosphoprotein, MPOP, that orchestrates cytoskeletal assembly and a cytosolic component, MFP2, that governs the rate of protrusion. Here, we identified a 34 kDa cystic protein (p34) that links the activities of MPOP and MFP2 and is also required for motility. MSP polymerization was abolished in sperm extracts immunodepleted of p34 but restored when purified p34 was added back. Amino acid sequence and kinase activity analysis showed that p34 is a member of the casein kinase 1 family of ser/thr protein kinases. MPOP and p34 co-migrate by native gel electrophoresis, co-immunoprecipitate, and co-localize by immunofluorescence indicating that MPOP binds to and recruits p34 to the membrane surface. This process depends on phosphorylation of MPOP. p34, in turn, phosphorylates MFP2 on threonine residues and phosphor-MFP2 incorporates into the cytoskeleton. Beads coated with p34 assembly a surrounding cloud of MSP filaments when incubated in p34-depleted sperm extract. This bead-associated assembly requires MPOP released from membranes by detergent lysis suggesting that MPOP not only recruits but also activated the kinase activity of p34 thereby focusing MSP polymerization at the membrane and minimizing nonproductive filament formation elsewhere in the cell. Supported by NIH Grant R37 GM29994.

**Mechanism of MSP-based Cell Body Retraction in the Amoeboid Sperm of Nematodes**

C. Shimabukuro,1 K. Yi,1 M. Stewart,1 T. M. Roberts;1 Florida State University, Tallahassee, FL, 2MRC Laboratory of Molecular Biology, Cambridge, United Kingdom

Cell body retraction in the amoeboid sperm *Ascaris* saum is generated by disassembly and rearrangement of the MSP cytoskeleton, without the involvement of conventional motors (Miao et al. 2003. Science 302:1405). Reconstitution of retraction in vitro, whereby fibers comprised of columnar meshworks of MSP filaments that assemble behind membrane vesicles derived from the lamelloidal leading edge, can be induced to shorten has enabled us to explore the biochemical and biophysical basis of retraction. We found that fiber retraction triggered by YOP, a protein tyrosine phosphatase requires a cytosolic co-factor. This co-factor requirement can be bypassed by treatment of fibers with PP1ase, a ser/thr phosphatase. Moreover, YOP-induced retraction is blocked by calyculin A, a ser/thr phosphatase inhibitor. Thus, retraction appears to be initiated by sequential protein phosphatase activity in which a tyrosine phosphatase acts upstream of a ser/thr phosphatase. To study the mechanism of phosphatase-induced retraction mechanism, we grew fibers in the presence of Alexa 488-MSP and examined filament dynamics during retraction by fluorescence recovery after photobleaching. However, we were unable to detect large-scale rearrangement of the filaments in shortening fibers by this method. We did find that retraction was significantly reduced in segments of fibers grown with an MSP mutant (D83R). This mutant produces short filaments that pack tightly within fibers but does not affect the rate of filament disassembly. Thus, retraction appears to be modulated by filament length, packaging density, or both. Supported by NIH R37GM29994.

**Endocytosis in Neuronal Migration**

J. C. Shieh,1 B. T. Schaar,2 S. K. McConnell;2 Neurosciences Program, Stanford University, Stanford, CA, 2Biological Sciences, Stanford University, Stanford, CA

Neuronal migration is a crucial aspect of mammalian brain development and its disruption can lead to forms of mental retardation and epilepsy in humans. Unlike other migratory cells, neurons migrate in a saltatory manner. The movement of migrating neurons is characterized by morphologically distinct steps: extension of a single leading process ahead of the stationary cell soma, flattened and exhibit multiple protrusive structures, like lamellipodia and filopodia, all around the cell periphery. Previous studies have shown that human primary schwannoma cells contain a cloud of MSP filaments when incubated in p34-depleted sperm extract. This bead-associated assembly requires MPOP released from membranes by detergent lysis suggesting that MPOP not only recruits but also activated the kinase activity of p34 thereby focusing MSP polymerization at the membrane and minimizing nonproductive filament formation elsewhere in the cell. Supported by NIH Grant R37 GM29994.

**Endocytosis in Neuronal Migration**

C. Bach,1 J. Zhong,1 P. W. Gunning,1 G. M. O'Neill1,2;1Oncology Research Unit, The Children's Hospital at Westmead, NSW, Australia, 2Discipline of Paediatrics and Child Health, The University of Sydney, Sydney, Australia

Two core components in cell migration are the actin cytoskeleton and integrin-based adhesion to the extra-cellular matrix, known as focal adhesions (FAs). FA turnover allows cells to move over the underlying matrix while the actin cytoskeleton directly links to the FAs providing the contractile force that generates movement. Actin filament structure is regulated by distinct isoforms of the tropomyosin (Tm) family of actin-binding proteins. Given the key relationship between adhesion and actin in cell migration, we hypothesized that Tm isoform expression may

**Haphazard Actin Rich Protrusions and GTPase Activation in Merlin Deficient Schwannomas**

C. Flair, C. O. Hanemann; Clinical Neurobiology, Peninsula Medical School, Plymouth, United Kingdom

Schwannomas that occur spontaneously or in patients with the inherited cancer neurofibromatosis type 2 lack both alleles for the tumour suppressor gene Merlin. Merlin binds to the cytoskeleton and has been linked to the Rac-PAK signalling pathway. Rac1, as well as Cdc42, are members of the RhoGTPase family that control the formation of protrusive structures, lamellipodia and filopodia, respectively. Very little is known about actin-rich protrusions that are potentially crucial in schwannoma cell differentiation, and nothing is known about Cdc42 activation in human primary schwannoma cells. Using scanning electron microscopy we show here that normal Schwann cells have a bipolar, spindle shape, whereas schwannoma cells are flattened and exhibit multiple protrusive structures, like lamellipodia and filopodia, all around the cell periphery. Previous studies have shown that human primary schwannoma cells contain more active Rac1. We propose that Rac1 is randomly activated in human primary schwannoma cells, as it colocalises with one its effectors, phospho-PAK1/2, all around the cell periphery thus explaining the multiple lamellipodia. Cdc42 controls filopodia formation and is also activated in schwannomas as shown by pull-down assays and colocalisation with one of its effectors, phospho-PAK. Other effectors of Cdc42, namely phosphorylated ERK proteins (extran, moesin, moesin), characteristically also involved in formation of protrusive structures, similarly localise to the multiple filopodia. Finally, fast and continuous remodeling of the multiple lamellipodia and filopodia can be detected as shown by live cell imaging of actin-EGFP transfected human primary schwannoma cells. Furthermore it would be interesting to find out if the combined Cdc42 and random Rac1 activation also affects the structure and regulation of the actin cytoskeleton and adhesive structures. In summary we suggest that the highly dynamic multiple lamellipodia and filopodia found in merlin-deficient human primary schwannoma cells are caused by a combination of Cdc42 and random Rac1 activation.

**The Tropomyosin Family of Actin-binding Proteins Have Isoform Specific Effects on Adhesion Structure and Signalling**

C. Bach,1 J. Zhong,1 P. W. Gunning,1 G. M. O'Neill1,2;1Oncology Research Unit, The Children's Hospital at Westmead, NSW, Australia, 2Discipline of Paediatrics and Child Health, The University of Sydney, Sydney, Australia

Two core components in cell migration are the actin cytoskeleton and integrin-based adhesion to the extra-cellular matrix, known as focal adhesions (FAs). FA turnover allows cells to move over the underlying matrix while the actin cytoskeleton directly links to the FAs providing the contractile force that generates movement. Actin filament structure is regulated by distinct isoforms of the tropomyosin (Tm) family of actin-binding proteins. Given the key relationship between adhesion and actin in cell migration, we hypothesized that Tm isoform expression may
regulate adhesion structure, in turn determining downstream signalling and migration. Firstly, we find that cells overexpressing the Tm isoform TmBr3 display significantly smaller FAs than control cells. Strikingly, cells overexpressing the Tm isoform Tm5N1 display FAs that are twice the length of control cells and are dispersed across the ventral surface of the cell. In contrast, mouse embryo fibroblasts (MEFs) homozygous null for Tm5N1 display enhanced levels of focal complex formation arrayed at the leading edge of protruding membranes. Using time-lapse microscopy we determined that cells expressing Tm5N1 display reduced random cell movement, while the Tm5N1-/- MEFs travel significantly further than controls. Notably, the velocity of the wild-type and knockout MEFs is identical, therefore suggesting that the Tm5N1-/- MEFs may be more polarized. Current models of cell migration suggest a requirement for cytical activation of Rho GTPases. The FA docking molecule p130Cas can regulate signalling through Rac and Cdc42 thereby regulating cell migration, hence we investigated the phosphorylation status of p130Cas in response to altered Tm isoform expression. We find that p130Cas phosphorylation is reduced in Tm5N1 overexpressing cells when compared with control cells, TmBr3 cells and the Tm5N1 +/- MEFs. Together our data support Tm isoform-specific effects on FA structure, adhesion molecule activation and cell migration.

942 Characterization of an Epidermal Growth Factor-stimulated Acute Lamella Retraction That Temporally Lags Lamellipodial Proliferation
I. Schneider, C. Waterman-Storer; Cell Biology, The Scripps Research Institute, La Jolla, CA
Cell migration involves the spatial and temporal coordination of protrusion, adhesion formation and contraction. These processes are intimately linked to the actin cytoskeleton, but are spatially segregated in different actin networks. Protrusion is mediated by a branched, treadmill actin network in the lamellipodium, while adhesion and contraction is mediated by actomyosin and focal adhesions in the lamella. In physiological settings, protrusion, contraction and adhesion can be altered by signals from growth factor receptors leading to enhanced cell migration. Much work has focused on defining how acute growth factor stimulation regulates actin polymerization and depolymerization, processes that drive lamellipodial protrusion. However, the subsequent temporal regulation of activities in the lamella leading to cell migration after stimulation is less well understood. Using live cell microscopy we have observed a delayed retraction response after epidermal growth factor (EGF) stimulation that pulls the lamellipodium and lamella towards the cell body several minutes after the initial protrusion phase. This retraction can be blocked by blebbistatin indicating a dependence on the ATPase activity of myosin II. Blocking myosin light chain kinase with ML-7 on the other hand does not necessarily block this retraction; however these cells may contain fewer mature peripheral adhesions. We have used traction force microscopy to quantify the retraction response by measuring the force exerted by the cell on the substrate after EGF stimulation of cells pretreated with blebbistatin, ML-7, Y-27632, A Rho kinase inhibitor or vehicle. Cell biochemical techniques support these results and show that myosin regulatory light chain phosphorylation is cotemporal with this lamella retraction. We conclude that this EGF-stimulated retraction phase depends intimately on myosin II-mediated contraction, but also depends on the state of adhesion formation before EGF stimulation, which itself is affected by the basal level of myosin II activity, illustrating the highly connected processes of contraction and adhesion.

943 Focal Adhesion Kinase Is Required for Focal Adhesion Sliding and Trailing Edge Retraction
M. Iwanicki, T. Vomastek, R. W. Tilghman, K. H. Martin, J. K. Slack-Davis, J. T. Parsons; Department of Microbiology, University of Virginia, Charlottesville, VA
In migrating fibroblasts, focal adhesions can be classified as either stationary or sliding. Stationary focal adhesions can be categorized predominantly found at the protruding front, and they are characterized by rapid turnover. On the other hand, sliding focal adhesions are found in the tail and sides of migrating fibroblasts. They are characterized by an elongated phenotype and the capacity to move inward before turnover. During fibroblast migration, trailing adhesions are thought to resist traction forces, leading to increase in tension that is necessary for destabilization of protrusion and detachment. Here we show that Focal Adhesion Kinase (FAK), an integrin-dependent signaling component, is involved in the regulation of focal adhesion sliding and tail retraction. We found that the inhibition of FAK expression using siRNA or FAK activity with a FAK-specific inhibitor in RAT 2 cells results in the loss of adhesion sliding and tail retraction. These results suggest that FAK signaling is required for trailing edge resistance to traction forces and proper protrusion destabilization during tail retraction.

944 Normal Modes of Shape Variation Are Coupled to Cell Behavior in Motile Keratocytes
Z. Pincus, 1, 2 E. L. Barnhart, 2 K. Keren, 2 C. I. Lacayo, 2 J. A. Theriot 2, 3; 1 Program in Biomedical Informatics, Stanford University School of Medicine, Stanford, CA, 2 Department of Biochemistry, Stanford University School of Medicine, Stanford, CA, 3 Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA
We have used a novel application of the principal components analysis to find the major statistically uncorrelated modes of cell shape variation in image datasets representing large populations of cells. Quantitative analysis of the shape of isolated ciliated epithelial keratocytes indicates that there are four principal modes of shape variation that account for ~95% of cell-to-cell shape difference. These modes correspond to (1) the size of the cells, (2) their overall organization into elongated fast-crawling forms (referred to as “canoe” shapes in the literature) vs. less highly organized “D” shapes, 3) the relative fore-and-aft position of the cell body with respect to the trailing edge, and 4) left-right asymmetries, most pronounced at the cell rear. Remarkably, we have found that these statistically uncorrelated modes of shape variation also appear to be biologically independent and tightly coupled to motile behavior, since different treatments that perturb cell movement effect changes along only one shape mode at a time. Keratocytes treated with media of different osmolarities were altered along the cell-size axis (the first shape variation mode), but remained indistinguishable from wild-type along the other three modes. The second shape mode correlated with the amount of anti-activated protein kinase C (PKC) localized to the leading edge, and also with actin filament density distribution, cell speed and directional persistence. Treatment of cells with RGD peptide or manganese ion, which modulate adhesion sliding and PKC activity, respectively, also perturbed the cell's shape along this PKC-sensitive axis. These results suggest that the inhibition of PKC expression using siRNA or PKC activity with a PKC-specific inhibitor in RAT 2 cells results in the loss of adhesion sliding and tail retraction.

945 Discrete Phases of Edge Dynamics during Cell Spreading Dependent on Distinct Mechanoschemical Complexes
B. J. Dabin-Thaler, 1 J. M. Hofman, 2 H. Xenias, 1 H. Débre nner, 1 C. H. Wiggam, 3 M. P. Sheetz 1; 1 Biological Sciences, Columbia University, New York, NY, 2 Physics, Columbia University, New York, NY, 3 Applied Physics and Applied Mathematics, Columbia University, New York, NY
Mouse embryonic fibroblast cells spreading onto fibronectin-coated surfaces commonly exhibit a sequence of three distinct stages of spreading: early, isotropic, and late. Each stage of spreading exhibits a combination of distinct spatiotemporal phases of cystoskeletal organization. Using novel, statistically-based computer algorithms to quantify cell edge movement, we reveal that the cell edge frequently exhibits a phase of pointed cell motility in the early stage. The early stage is followed by a two-minute isotropic stage during which the cell rapidly increases its area via a continuous protrusion phase of the cytoskeleton. The last stage, late spreading, is generally characterized by a periodic contraction phase, though limited regions of the edge can still revert back to the continuous phase. VASP is confined to the leading edge in the continuous phase regardless of the stage. Cytochalasin D, an inhibitor of actin polymerization, disrupts the spatial organization of the isotropic stage while changing the blubbing phase. Based on this and further evidence that the phases respond differently to the
same perturbation, we hypothesize that each dynamic phase reflects a distinct mechanochemical arrangement of cytoskeletal molecules, and the probability of entering a given phase depends on the local signaling environment.

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Myosin Activity at the Back of Lamellipodia Activates Adhesion Formation at the Front through the Mechanical Action of Lamellipodial Actin  
G. Giannoni, B. J. Dubin-Thaler; O. Rossier, Y. Caiz, O. Chaga, W. Beaver, H. Döbereiner; Y. Freund, G. Borisy, M. P. Sheetz; Université Bordeaux, Bordeaux, France, biological sciences, Columbia University, New York, NY, Cell and Molecular Biology, Northwestern University, Chicago, IL, computer Science and Engineering, UC San Diego, La Jolla, CA  
Cell motility proceeds by cycles of edge protrusion, adhesion and retraction. Whether these functions are coordinated by complex biochemical or biomechanical processes is unknown. We find that myosin II pulls the rear of the lamellipodial actin network causing upward bending, edge retraction and initiation of new adhesion sites. The network is then released from the edge and condensed over the myosin. Protrusion resumes as lamellipodial actin regenerates from the front and extends rearward until interacting again with new confined myosin, initiating the next cycle. Upward bending, observed by evanescent microscopy and electron microscopy, is consistent with ruffling when adhesion strength is low. Correlative fluorescence and electron microscopy demonstrated that the regenerating lamellipodium forms a cohesive separable layer of actin. Thus, actin polymerization periodically builds a mechanical link, the lamellipodium, connecting myosin motors with the initiation of adhesion sites, suggesting that the major functions driving motility are coordinated through a biomechanical process.

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Myosin Contraction, Adhesion, and Retraction of the Trailing Edge in Locomoting Fish Keratocytes  
E. Bartzhart, J. Theriot; Stanford University, Stanford, CA  
Myosin contraction has been shown to promote focal adhesion assembly and stability in stationary cells. However, in migrating cells, myosin is localized primarily in the cell rear, where adhesions must disassemble in order for retraction of the trailing edge to occur. To investigate the relationship between myosin contraction, focal adhesion size, and retraction of the trailing edge, we examined the effect of myosin inhibition on focal adhesion size in rapidly locomoting fish keratocytes. To visualize focal adhesions, cells were either transfected with a vinculin-GFP transgene or fixed and stained with a vinculin antibody. In both live and fixed cells, vinculin accumulated in focal adhesions on either side of the cell body in the cell rear. Inhibiting myosin contraction with blebbistatin resulted in a dramatic reduction in focal adhesion size, but did not have a consistent effect on cell speed. To determine how increasing adhesion strength affects the relationship between myosin contraction and adhesion size, we treated cells with Mn2+, which promotes adhesion by activating integrins. Treating cells with Mn2+ resulted in a reduction in cell speed and impeded translocation of the cell body, but had no effect on vinculin accumulation. Cells treated with both Mn2+ and blebbistatin had significantly smaller focal adhesions than either control or Mn2+-treated cells, indicating that intact actin network is not sufficient for maintenance of focal adhesion size in the absence of myosin contraction. Moreover, treatment with both Mn2+ and blebbistatin resulted in rapid forward motion of the trailing edge without concomitant disassociation of focal adhesions, generating long branched retraction fibers at the rear of the cell, followed by arrest of cell movement. These results suggest that at high adhesion strength, myosin dependent retraction of the connection between the cytoskeleton and focal adhesions is required for proper retraction of the trailing edge and cell locomotion.

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Regulation of Cytoskeletal Dynamics and Cell Migration in Colon Carcinoma Cell Lines by Plectin  
L. McIntyre, A. Matta; School of Biological and Biomedical Sciences, Integrative Cell Biology Laboratory, Centre for Stem Cell Biology and Regenerative Medicine, Durham, United Kingdom  
Plectin is a large cytoskeletal linker protein that can connect different cytoskeletal networks to each other and to cell-cell and cell-matrix junctions. Mutations in plectin disrupt tissue integrity in skin, skeletal and heart muscle. The versatility of plectin as a linker protein is further enhanced by complex alternative splicing that results in a large number of plectin isoforms. We are investigating the role of plectin in the cytoskeletal organisation and invasiveness of epithelial carcinomas. We have cloned eight alternative first exons of the human plectin gene including a novel exon, 1K. Real-time PCR shows that there are isoform specific changes between invasive and non invasive colon carcinoma cells. We have also raised polyclonal antibodies to four of the isoforms including the novel 1K. Furthermore, alternative plectin N-terminal domains comprising of the first exon and the following calponin-homology actin binding domain target EGFP constructs to different subcellular localisations. Thus, the first exon appears to determine where in the cell plectin can form actin - intermediate filament cross-bridges. We have shown that exon 1K localises to podosome like structures on the lower surface and vinculin positive circular ruffles on the dorsal surface. Vimentin, which forms the major intermediate filament network in mesenchymal cells, is involved in the formation of focal adhesion points and podosomal structures. Knocking down of plectin and vimentin by siRNA in SW480 cells causes a significant decrease in attachment to collagen and impairs migration and invasion. We propose that a change in plectin isoform expression can lead to altered cytoskeletal and functional organisation and can contribute to invasive properties of carcinoma cells.

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Diaphanous-related Formin-6dia2 Is Required for Cellular Movements during Gastrulation in Zebrafish  
S. Lai, S. Lee; Institute of Zoology, National Taiwan University, Taipei, Taiwan  
Intensive cellular movement occurs during gastrulation in embryogenesis that relies heavily on dynamic actin assembly. Various factors have been implicated in the regulation of actin assembly in cellular protrusion and migration, including Rho-activated formin, Diaphanous. However, the function of Diaphanous in cellular migration during gastrulation is unclear. To study the role of Diaphanous in embryogenesis, we have identified a Diaphanous-related formin 2 (zdia2) in zebrafish by in silico cloning; zdia2 was found to be ubiquitously expressed during early development as revealed by RT-PCR and whole-mount in situ hybridization. Knockdown of 6dia2 by translational or splicing blocking morpholino oligos (MO) resulted in incomplete epiboly formation with a yolk plug and failure in involution. Whole-mount in situ hybridization analysis of no tail gene revealed a shortened embryo axis and a less-converged notochord and primordial somites at the bud stage in the zdia2 MO-injected embryos. Time lapse recording further demonstrated that the defects were due to the lack of protractive activities of forerunner cells during epiboly. Moreover, actin filament accumulation in the forerunner cells as shown in normal embryos was decreased in those zdia2 MO-injected embryos. Lastly, co-injection of zdia2 MO and profilin 1, a formin-dependent actin binding protein, but not the other isoform, profilin II, resulted a synergistic effect on all described defects. These results suggest that zdia2 is required for cell movements during gastrulation via the cooperation with profilin 1 in zebrafish development.

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An In Vivo Requirement for mDia1 in Cytoskeletal Remodeling Following T Cell Activation  
K. M. Eisenmann, D. Hildebrand, S. M. Kitchen, R. A. West, A. S. Alberts; Cell Structure and Signal Integration, Van Andel Research Institute, Grand Rapids, MI  
The mammalian Diaphanous-related (mDia) formins are important Rho GTase effectors proteins that nucleate and elongate nonbranched actin filaments during directed cell migration and cytokinesis. In order to understand its role in vivo, the gene encoding the canonical marine formin mDia1 was knocked out by conventional methods. While viable, mice either heterozygous or homozygous null for mDia1 have numerous hematopoietic defects – notably, diminished immune cell populations within peripheral lymphoid organs, including the spleen and lymph nodes, suggesting a defect in immune cell proliferation and/or migration. Detailed examination showed that splenic T cell populations were significantly diminished. As it was unclear if the lack of T cells infiltrating the spleen was due to a migratory defect in either mDia1 hetero- or homozygous null mice, isolated splenic T cells were assessed for their ability to adhere and/or migrate upon fibronectin in a transwell assay in response to growth factors or chemokines. Both T cells from both genotypes were defective in fibronectin adhesion and failed to migrate in response to SDF1 stimulation. Polarization in response to activation of the T cell receptor by CD3/CD28 costimulation (TCR) ligation was also affected by mDia1 knockout; cells failed both in the assembly of an actin cap following stimulation and in their ability to orient their microtubule organizing center. Fluorescent resonance energy transfer (FRET)-based microscopy revealed a polarized interaction between mDia1 and RhoA at the leading edge of activated Jurkat T cells. Taken together, these results show a central role for RhoA activated mDia1 in both microtubule and actin dynamics required for normal T cell function.

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Regulation of Cell Migration and Matrix Remodeling by Tissue Mobility  
M. Miron-Mendoza, J. Seemann, F. Grinnell; Cell Biology, UT Southwestern Medical Center, Dallas, TX
Nestled collagen matrices provide a unique 3D physical environment to study matrix remodeling and cell migration. In this two stage model, cells contract floating collagen matrices overnight in the first stage. Subsequently, these “dermal equivalents” are embedded and incubated inside cell-free collagen matrices (1.5 mg/ml) in the second stage. During the second stage, nested matrices can be immobile (attached to culture dishes) or mobile (floating in culture medium). In the presence of platelet derived growth factor (PDGF) but not lysophosphatidic acid or basal medium, collagen matrix remodeling begins within hours as shown by collagen migration into the outer matrix at speeds of up to 20μm/hr towards the interface between outer and inner matrices. Subsequently, if the nested matrices are immobile, then a wave of cells begins to migrate into the outer matrix at speeds of up to 60μm/hr beginning after 4-8 hr. The density of migrating cells is highest on the edge of the dermal equivalent nearest the culture dish surface. Also, with 4.0 mg/ml collagen outer matrices, cell migration can occur even if nested matrices are mobile, but there is no matrix remodeling under these conditions. Experiments also were carried out using nested collagen matrices containing two dermal equivalents. In mobile, dual nested matrices, cell migration occurs only in between the two dermal equivalents, and matrix remodeling brings the dermal equivalents together. In immobile, dual nested matrices, cell migration occurs in between and around the outer surfaces of both dermal equivalents, and the dermal equivalents remain apart. Our findings demonstrate a new mechanism of collagen matrix remodeling that requires PDGF-stimulated fibroblast migration. We suggest that the balance between cell migration and matrix remodeling depends on tissue mobility and explains how fibroblast migration at the wound margin has the potential to cause wound closure.

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The Exocyst Interacts with the Arp2/3 Complex and Regulates Cell Migration
X. Zuo, J. Zhang, W. Guo, Biology, University of Pennsylvania, Philadelphia, PA

Cells migrate by protrusions, which are mediated by the assembly of a branched actin network that grows beneath the leading edge of the plasma membrane. The Arp2/3 complex is the core machinery that regulates actin for the filamentous actin network. In addition to actin polymerization, cell migration also requires polarized exocytosis for the supply/recycling of lipids and proteins to the leading edge for membrane expansion. Actin dynamics and membrane traffic must be tightly coordinated, both temporally and spatially, for efficient cell migration. The exocyst is a multiprotein complex essential for tethering secretory vesicles to specific domains of the plasma membrane for exocytosis. Here we report that the exocyst component Exo70 directly interacts with the Arp2/3 complex, a key regulator of actin polymerization. We further show that the exocyst-Arp2/3 interaction is regulated by epidermal growth factor (EGF) signaling. Immunofluorescence microscopy shows that components of the exocyst complex are localized to the leading edges of migrating cells. Inhibition of Exo70 by RNA interference or antibody microinjection blocks the formation of actin-based membrane protrusions and affects various aspects of cell motility. We propose that Exo70, in addition to functioning in exocytosis, also regulates actin at the leading edges of migrating cells, therefore coordinating cytoskeleton and membrane traffic during cell migration.

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Uncaging Cofilin Activity and Fluorescence for Use in Measuring Signaling Pathways In Vivo
D. R. Larson, H. Lee, X. Chen, R. H. Singer, D. Lawrence, J. Condeelis; 1Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, NY, 2Biochemistry, Albert Einstein College of Medicine, Bronx, NY

Calcium Is Required in Pigment Granule Dispersion in Bluegill RPE
M. Nagayaama, T. Uchida, T. Taira, K. Shimmura, M. Sakai, K. Gohara; 1Applied Physics, Hokkaido University Graduate School of Engineering, Sapporo, Japan, 2Primary Cell Co., Ltd., Sapporo, Japan

Adipocyte proliferation and differentiation have been widely investigated because hypertrophy and hyperplasia of adipocytes in adipose tissue are associated with obesity, a major risk factor for lifestyle-related diseases. Molecular processes concerning adipocyte differentiation have rapidly been identified, but how these molecular processes lead to morphological and functional changes at the cellular level is still controversial. Thus, we focus on spatial and temporal dynamics of macroscopic structures during adipocyte differentiation (~ several 10 μm, several days). In the present study, we captured time-lapse images of primary stromal-vascular cells (SVCs) derived from rat mesenteric adipose tissue. The time-lapse observations for 5 days showed that the adipocyte differentiation proceeds through the following two steps: (i) Numerous small lipid droplets (LDs), which are a few μm in diameter, appeared in the perinuclear region at an early stage of differentiation, and then (ii) several LDs grew to more than 10 μm in diameter and occupied the cytoplasm, and the other LDs disappear. The temporal and spatial variations of LDs were accompanied by the morphological changes as SVCs converted fibroblastic to spherical shape. Observations at a higher magnification showed that nascent LDs (~ several 100 nm in diameter) grew into small LDs by fusion with adjacent nascent LDs while moving from lamellipodia to the perinuclear region. We also found that SVCs proliferate by distributing LDs evenly between two daughter cells, although the SVCs which started to accumulate nascent LDs were considered to lack proliferative activity. Immunofluorescence observations revealed that cell division of SVCs occurred even after LDs had been coated with perilipin. The findings suggest that SVCs can proliferate and accumulate LDs simultaneously rather than in sequence. In summary, our time-lapse observations would provide an exact description of adipocyte differentiation.

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Dynamics of Lipid Droplets and Cell Morphology in Stromal-Vascular Cells during Adipocyte Differentiation
M. Nagayaama, T. Uchida, T. Taira, K. Shimmura, M. Sakai, K. Gohara; 1Applied Physics, Hokkaido University Graduate School of Engineering, Sapporo, Japan, 2Primary Cell Co., Ltd., Sapporo, Japan

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Structural and Functional Characterization of Secretagogin in Insulinoma Cells
M. Nagayaama, T. Uchida, T. Taira, K. Shimmura, M. Sakai, K. Gohara; 1Applied Physics, Hokkaido University Graduate School of Engineering, Sapporo, Japan, 2Primary Cell Co., Ltd., Sapporo, Japan

Calcium Is Required in Pigment Granule Dispersion in Bluegill RPE
A. S. Johnson, D. M. Garcia; Biology, Texas State University, San Marcos, TX

The retinal pigment epithelium (RPE) is a single layer of cuboidal cells found between the retina and choroid of the vertebrate eye. Among other functions, it provides bluegill (Lepomis macrochirus) with an anti-photobleaching mechanism known as pigment granule dispersion. The dispersion process can be induced by acetylcholine (or its analog carbachol) which activates M1 muscarinic receptors on the RPE. Once activated, a cascade of messengers ultimately causes pigment granules to move inside long processes that are oriented toward the photoreceptors. With granules in place, the amount of light intensity reaching the photoreceptors is greatly reduced allowing bluegill a defense from excessive photobleaching. Using isolated RPE, the focus of this study is to test whether Ca2+ is required for carbachol-induced dispersion by depleting intracellular calcium and by using BAFTA-AM (10-50μM) to prevent calcium transients. Both treatments blocked carbachol-induced dispersion. To determine whether extracellular Ca2+ is required, we tested to see if pigment granule dispersion is blocked by removing Ca2+ and by treatment with verapamil (10 μM). Neither of these treatments affected pigment granule dispersion. Finally, to test which Ca2+ effectors may be involved, we tested the ability of the PKC inhibitor staurosporine (100μM) as well as the calcium-inhibitor cymperthrin (100μM). Staurosporine had no effect on pigment granule dispersion, whereas cymperthrin significantly inhibited dispersion. The contradiction between these results and those published by King-Smith et al. (1996) will be discussed.
Modeling and Analysis of Receptor-mediated Signal Transduction, Spatial Gradient Sensing, and Wound Invasion
J. Haugh, Chemici & Biomolecular Engineering, North Carolina State University, Raleigh, NC
One of the current challenges in biology is to bridge the scales of molecular-, cellular-, and tissue-level complexity. Mathematical modeling can be useful for testing how a signaling module, carefully and quantitatively characterized with cultured cells, might operate in the context of a dynamically changing environment in vivo; appropriate coarse-graining of the molecular details is needed for proper analysis of higher-level models, however. In the context of dermal wound healing, we have studied the spatial pattern of platelet-derived growth factor (PDGF) receptor-mediated activation of PI 3-kinase, and its influence on fibroblast chemotaxis, at multiple scales. First, at the molecular scale, we have used Brownian dynamics simulations to analyze the co-localized crosstalk of activated Ras and PI 3-kinase in the vicinity of a receptor, their interactions limited by the rate of lateral diffusion in the plasma membrane. Second, at the cellular scale, we have fully characterized through quantitative live-cell imaging experiments and modeled the PI 3-kinase-mediated sensing of PDGF gradients. We find that PDGF gradient sensing in fibroblasts is governed by a relatively simple mechanism that, compared with other chemotactic cells (e.g., neutrophils, D. discoideum), requires steep PDGF gradients with midpoint concentrations spanning a narrow range. Finally, at the tissue level, with our gradient sensing model embedded within a coarse model of wound invasion, we show how a suitable PDGF gradient with the proper steepness and midpoint concentration for chemotaxis can be maintained over millimeter length scales for efficient wound invasion.

Sinai Virus 40 Protein Small Tumor-antigen Causes Centrosome Amplification in Drosophila melanogaster Embryos
S. Kotadia, 1 S. A. Comerford, 2 R. E. Hammer, 1 T. L. Megraw, 1 Pharmacology, Green Center for Reproductive Biology, University of Wisconsin Madison, Madison, WI, 2Biochemistry, Green Center for Reproductive Biology, University of Wisconsin Madison, Madison, WI
Centrosomes are the major microtubule-organizing centers (MTOCs) in animal cells and their duplication is an important yet poorly understood process. To investigate this process, we are using genetic interactions, live-imaging, and biochemical assays utilizing the simian virus-40 (SV-40) protein small tumor-antigen (ST). ST binds to Hsc70 and increases its ATPase activity. Upon binding to the PP2A phosphatase, ST displaces the PP2A B subunit resulting in reduced phosphatase activity towards known substrates. ST causes centrosomes to overduplicate in vertebrate cell lines and in Drosophila embryos. In Drosophila, high levels of maternal ST result in embryonic lethality, while high levels of yzgotic ST cause late larval death. Interestingly, ST expression in embryos results in a marked decrease in the level of phosphorylated centrosomin. Using two ST mutants, we are examining if the PP2A-binding domain or the Hsc70-binding domain are necessary or sufficient to cause centrosome overduplication. Crosses are underway to assess the role of candidate genes in ST-mediated centrosome amplification. In addition, we are assessing for other interacting partners of ST using biochemical approaches. Through these combined genetic and biochemical experiments, we will determine the mechanisms by which ST deregulates centrosome duplication.

Protein Kinase Nek2 Prevents Centrosomal Clustering in Cancer Cells
S. E. Hileman, 1 L. F. Petersen, 2 N. J. Quintyne, 2 W. S. Saunders, 2 Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA, 3School of Medicine, Wayne State University, Detroit, MI, 4Harlery L. Wilkes Honors College of Florida Atlantic University, Jupiter, FL
Cancer cells are known to display many distinct characteristics in mitosis, including anaphase bridges, lagging chromosomes, and multipolar spindles. Multipolar spindles form following amplification of the microtubule organizing center or centrosome of the cell. In some cases, the amplified centrosomes are clustered to form bipolar rather than multipolar spindles, protecting the cell from this genomic destabilizing event. We describe the action of the centrosomal protein kinase, Nek2, on centrosomal clustering. Nek2 is known to be overexpressed in some tumor cells. We show that Nek2 overexpression prevents centrosomal clustering in cancer cells and it can lead to multipolar spindles when associated with amplification of centrosomes. We also show that inactivation of Nek2 can cause reclustering of the centrosomes in cancer cells. Thus, Nek2 overexpression is proposed to be a mechanism to disrupt normal chromosome segregation in tumor cells.

Beta-catenin Functions in Nek2-mediated Centrosome Cohesion
S. Bahmanyar, 1 D. D. Kaplan, 2 W. J. Nelson, 3 P. J. Casey, 4 A. I. M. Barth 1; 1Molecular and Cellular Physiology, Stanford School of Medicine, Stanford, CA, 2Department of Developmental Biology, Stanford School of Medicine, Stanford, CA, 3Biological Sciences, Stanford University, Stanford, CA, 4Department of Pharmacology, Duke University Medical Center, Durham, NC
Beta-catenin plays important roles in cell adhesion and regulation of gene expression, and mutations in beta-catenin commonly contribute to cancer. It has been shown that beta-catenin localizes to mitotic spindle poles and that depletion of beta-catenin results in monoastral spindles with duplicated, but not separated, centrosomes. Here, we show that beta-catenin is an integral component of interphase centrosomes. Accumulation of a stabilized form of beta-catenin is found in cancers increases centrosome distance in interphase cells, in a manner analogous, but opposite to depletion of beta-catenin. These results indicate that beta-catenin plays a role in centrosome cohesion. Centrosome cohesion is regulated by the NIMA-related kinase Nek2. We identified Nek2 as a binding partner of beta-catenin in isolated centrosome preparations. Nek2 binds directly to and phosphorylates beta-catenin in vitro. Nek2 phosphorylates beta-catenin’s armadillo domain, which is necessary and sufficient for localization of beta-catenin to centrosomes. We suggest that a threshold level of beta-catenin is required for centrosome normal function. Increasing this level results in decreased centrosome cohesion, extra centrosomes, and mitotic defects. Decreasing this level inhibits centrosome separation and results in a prometaphase arrest. Nek2 could regulate beta-catenin levels at centrosomes, allowing for proper centrosome cohesion and progression through mitosis.

An In Silico Screen for Centriole Duplication Genes
W. Marshall; Biochemistry & Biophysics, UCSF, San Francisco, CA
Centrioles are unique among organelles in that they undergo a discrete duplication process, with each mother centriole producing a single daughter. How does a mother produce a daughter? To begin answer this question, we sought mutants incapable of duplication. However, we are not interested in simply obtaining structural building blocks of the centriole (such as the previously well-characterized SAS-6 or BLD10 genes), but rather genes specifically required by the mother to make a daughter. We can distinguish the type of mutant we want (duplication specific from the type we do not want (simple building blocks such as sas6)) because the latter type will not only fail duplication, but also will lack de novo assembly. We therefore developed a mathematical model to describe centriole inheritance and used it to predict the phenotype of a mutant that could not perform standard centriole duplication but could still perform de novo assembly at the wild-type rate. This model takes into account experimental data on the homeostatic regulation of centriole abundance. Comparing the model predictions to data obtained from a collection of centriole copy-number mutants, we identified the FLV3 gene of Chlamydomonas as a candidate gene required for duplication but not for de novo assembly. Imaging studies support this role for FLV3.

Centrosome Replication in Hydroxyurea (HU)-arrested CHO Cells Expressing GFP-tagged Centrin
R. Kuriyama, 1 Y. Terada, 1 J. Pines, 2 K. Lee, 3 C. Wang 1; 1Genetics, Cell Biology, and Development, University of Minnesota, Minneapolis, MN, 2Wellcome/Cancer Research UK Gurdon Institute, Cambridge, United Kingdom, 3National Cancer Institute, NIH, Bethesda, MD
Centrosome duplication is tightly coupled with the cell cycle and neither too many nor too few centrosomes are induced in a normal cell. To study how centrosome formation is regulated, we analyzed the process of abnormal centrosome replication in CHO cells induced by HU treatment (Balczon et al., 1995). GFP-tagged centrin2 constitutively expressed in CHO cells labeled both centrioles and the pericentriolar material of the centrosome. Time-lapse fluorescence microscopy revealed the presence of one or two dots next to each nucleus. During HU treatment, small centrin aggregates of various sizes and numbers appeared inside the cytoplasm: they were frequently embedded in the cloud surrounding the existing centrosomes. The aggregates were highly mobile, continuously changing their position in a microtubule-/microtubule dynamics-dependent manner. The centrosome number increased as small aggregates grew in size and resolved into countable centrosomes. Time course analysis of synchronized cell populations showed that the vast majority of cells did not initiate centrosome replication until 10h after incubation of harvested M phase cells with HU. Although new centrosomes were assembled by 16-18h, their number generally did not exceed more than two. Thereafter the cells underwent multiple
replication cycles to produce various numbers of centrosomes that were frequently abnormal in size and length. These results suggest that: 1) initially new centrosome formation in HU-arrested cells is under the control of preexisting centrosomes, and 2) HU treatment overrides suppression of excess centrosome formation in cells where the full number of centrosomes already exists.

963 The Duplication of the Centrosome Does Not Require Cell Cycle Progression into S-phase in Mammalian Cells
T. Duran, E. Halpin, E. H. Hinchecliffe; Biological Sciences, University of Notre Dame, Notre Dame, IN

In animal cells, centrosome duplication occurs once per cell cycle, and is commonly thought to initiate near the G1/S phase transition or during S phase. We examined whether centrosome duplication requires cell cycle transition into S phase using Chinese Hamster Ovary (CHO) cells stably transfected with GFP-centrin 2. Live-cell imaging in cycling cells revealed that centrosome duplication occurs somewhere between 6 and 8 hrs following the end of mitosis, coordinate with the onset of S-phase as determined by BrdU incorporation. When synchronized 60 cells are released in media containing serum and the well-characterized G1 inhibitor mimosine we observe centrosome duplication beginning around 14 hrs post-release. After 48hrs in G1, 97% of cells had undergone centrosome duplication: 46% possessed 4 centrioles, whilst 51% possessed 5 or more centrioles. Centriole number in G1 arrested cells was confirmed by immunostaining cells with anti-centrin 2 antibody, and by serial section electron microscopy. This is in contrast to CHO cells arrested in S phase with hydroxyurea ~90% of S-phase cells contained greater than 5 centrioles, with many cells having greater than 14 centrioles. The mechanism of centrosome duplication in mammalian cells is not dependent upon cell cycle progression per se, but rather, normally serves to coordinate this process with entry into S-phase.

964 Loss of Centrosome Integrity Induces G1/S Arrest
B. Delaval,1 K. Miki,2 P. Kaldin,3 A. Jerczyk,1 A. Khodjakov,4 S. Dossey5; 1Program in Molecular Medicine, UMASS Medical School, Worcester, MA, 2ArQule Biomedical Institute, Inc., Norwood, MA, 3National Cancer Institute, Frederick, MD, 4Wadsworth Center, Albany, NY

Centrosomes contribute to spindle organization, cytokinesis and primary cilia assembly. They are implicated in cell cycle progression but the mechanism has not been determined. Here we show that siRNA mediated depletion of 14/15 centrosome proteins arrested diploid human cells in the cell cycle as demonstrated by BrdU incorporation and flow cytometry. G1 arrest occurred in late G0 with p21-bound cyclinA/Cdc2 complexes and could be suppressed by re-expression of the relevant centrosome protein. The arrest was induced within G0, ruling out contributions from mitosis and cytokinesis. Proteins that affected cell cycling also perturbed centrosome structure and centrosome functions known to occur in G1, namely centrosome duplication and centrosome-dependent primary cilia assembly. We identified a signal transduction pathway required for centrosome-associated G1 arrest that included p38, p53 and p21. Functional abrogation of any one of these proteins suppressed G1 arrest. Elements of this pathway were present at the centrosome (p38, p53) and a form of p53 activated by p38 (p53-phospho-serine33) was specifically recruited to the centrosome in centrosome protein depleted cells prior to cell cycle arrest. G1 arrest was also observed following heat-induced centrosome disassembly demonstrated the physiological relevance of this cell cycle withdrawal mechanism. G1 arrest was also demonstrated in previous studies by anti-centrosome antibody injection in multicellular organisms demonstrating the conserved nature of this pathway. We propose that mammalian cells have a centrosome damage checkpoint that monitors changes in centrosome integrity and prevents aberrant centrosome duplication, spindle dysfunction and aneuploidy by inducing cell cycle arrest. This checkpoint has strong parallels with the DNA damage checkpoint (occurs at the same time, similar molecular control) and satisfies the definition of checkpoint in having three elements: a perturbation of centrosome structure/function that is sensed, a transducing element (p53) and a receiving element (p21).

965 The Centrosome in G1 Progression: Important, but Not Essential
Y. Uetake,1 J. Lokcěrk3 J. Nordberg,3 G. English,1 A. Khodjakov;4 G. Sluder1; 1Cell Biology, University of Massachusetts Medical School, Worcester, MA, 2Wadsworth Center, New York State Department of Health, Albany, NY

How centrosome removal or perturbations of centrosomal proteins arrest untransformed cells in G1 has been a mystery. We investigated this phenomenon using microsurgery and laser ablation to remove centrosomes from RPE1 and HMEC cells. Surprisingly, we find that these normal human cells progress through G1 in its entirety without centrioles and start to assemble centrioles de novo in S phase. How then can centrosome disruption cause a G1 arrest even though the centrosome is not needed for G1 progression? We test if centrosome loss per se stresses the cell thereby predisposing it to a G1 arrest. In individual microscope fields we microsurgically remove or laser ablate/damage the centrosome from some cells, perform control microsurgery/laser ablations on others, and use the untouched cells as controls. Shortly after experimental intervention we expose the fields to varying doses of 488nm blue light to provide controlled exogenous stress. We find that neither centrosome removal nor blue light exposure acting singly block G1 progression but together they can cause a G1 arrest. Cells without centrosomes are most sensitive to exogenous stress and the control operated cells slightly less so. Both are substantially more sensitive than untouched controls. Inhibition of the p38 stress activated kinase allows cells with removed/damaged centrosomes to progress through G1 despite blue light exposures that otherwise arrest them in G1. Thus, centrosome removal/damage is a stress that can act additively with other stresses to arrest cells in G1. This may explain how a wide variety of centrosomal perturbations can have a profound impact on G1 progression even though the centrosome is not a necessary, integral part of the mechanisms that drive the cell cycle through G1 into S phase.

966 Mechanism Limiting Centrosome Duplication to Once per Cell Cycle
M. Tsou, T. Stearns; Biological Sciences, Stanford University, Stanford, CA

The centrosome organizes the microtubule cytoskeleton and consists of a pair of centrioles surrounded by pericentriolar material. Cells begin the cell cycle with a single centrosome, which duplicates once per cell cycle. During duplication, new centrioles grow orthogonally to existing ones and remain engaged (tightly opposed) with those centrioles until late mitosis or early G1 phase, when they become disengaged. The relationship between centriole engagement/disengagement and centriole duplication potential is not understood, and the mechanisms that control these processes are not known. Here we show that centrosome disengagement requires the protease separase, and that this disengagement licences centrosome duplication in the next cell cycle. We describe an in vitro system using Xenopus egg extract and purified centrioles in which both centriole disengagement and centriole growth occur. Centriole disengagement at anaphase is independent of mitotic exit and Cdc2/cyclin E activity, but requires the anaphase-promoting complex and separase. In contrast to disengagement, new centriole growth occurs in interphase, is dependent on mitotic exit and Cdc2/cyclin E activity, and requires previously disengaged centrioles. This suggests that re-duplication of centrioles within a cell cycle is prevented by centriole engagement itself. We propose that the 'once-only' control of centrosome duplication is achieved by temporally separating licensing in anaphase from growth of new centrioles during S phase. The involvement of separase in both centriole disengagement and sister chromatid separation would prevent premature centrosome disengagement before anaphase onset, which can lead to multipoles and genomic instability.

967 Mechanism of Centriole Amplification in S-arrested Cells
J. Loncarek, V. Magadson, P. Hergert, A. Khodjakov; Molecular Medicine, Wadsworth Center, Albany, NY

Centrioles normally replicate once per cell cycle and this event occurs near G1/S transition. Even when cells are arrested during S, in most cell types (e.g., HeLa) centrioles replicate once and then the cell remains with 4 centrioles indefinitely. However, the number of centrioles in S-arrested CHO cells increases continuously. We use time-lapse microscopy, laser microablation, and same-cell correlative LM/EM analyses in cells expressing centrin-GFP to determine the mechanism(s) responsible for centriole amplification in S-arrested cells. We find that in HeLa cells daughter centrioles remain directly attached to their mothers (i.e., in diplosomes) for as long as cells are arrested in S. However, in S-arrested CHO cells diplosomes break down ~15 hr after G0 replication. Upon disengagement from the diplosome, daughter centrioles become motile which normally occurs during G1. Then, ~10 hr later, both mother and daughter centrioles replicate. Thus, the centriole cycle continues independent of the cell cycle in S arrested CHO cells. Further, daughter centrioles in these cells become fully mature (~25 hr after their formation). Precision of our laser microsurgery allows us to selectively destroy just one of the two centrioles within a diplosome with no detectable damage to the other centriole. Whether a new, newly formed daughter centriole in either CHO cells or HeLa cells capable of forming more than one daughter during a single S period if the original daughter disengages from the diplosome. These data reveal that preserving the functional connection between the daughter and mother centrioles is essential for preventing mother centriole disengagement during prolonged S. We are currently investigating molecular pathways responsible for the disengagement of the daughter centriole from the diplosome and its maturation in S-arrested CHO cells.
Drosophila Spd-2 Is Required for Efficient Centrosome Maturation, but Is Not Essential for Centriole Replication

C. I. De, J. W. Raff; The Department of Genetics, The Gurdon Institute, Cambridge, United Kingdom

As the major microtubule organizing center (MTOC) in animal cells, the centrosome has an important role in driving the efficient formation of a bipolar spindle during cell division. The centrosome comprises an orthogonally arranged centriole pair, surrounded by amorphous pericentriolar material (PCM). To carry out its role as a MTOC, the centrosome must, in accordance with the cell cycle, replicate its centrioles during S-phase and, undergo centrosome maturation prior to and during mitosis. The latter involves the recruitment of extra PCM proteins to the centrosome, promoting microtubule outgrowth. Spindle Depletion 2 (SPD-2) is essential for both processes in the first division of the C. elegans embryo (Kent et al. 2004; Pelletier et al. 2004), and is one of a small number of proteins identified that are essential for centriole replication in worms. To investigate whether these roles of SPD-2 are conserved in the fly, we investigated the function of DSpd-2, the predicted Drosophila orthologue. Like SPD-2, DSpd-2 localises to the centrioles throughout the cell cycle and to the PCM in mitosis. In larval brain cells DSpd-2 is essential for the efficient recruitment of the mitotic PCM proteins γ-tubulin and Centrosomin (CNN), reflecting the conserved role of SPD-2 related proteins in centrosome maturation. However, centriole number is almost unperturbed in the Dspd-2 null mutant. Therefore, we conclude that the SPD-2 family proteins are not components of centriole replication machinery operating in all organisms/cell types, and the mechanisms utilised may differ depending on the cellular context. DSpd-2 mutant flies are viable but sterile. Mutant males exhibit severe defects in spermatogenesis, and the number of centrioles per spermatocyte is highly variable. Surprisingly, Dspd-2 is also maternal effect lethal, and mutant embryos do not develop beyond the first mitotic division. We are currently investigating the specific nature of these defects.

The Xenopus TACC Protein Maskin Is Required for Centrosome Maturation

A. J. Albee, L. Liu, C. Wise; Biochemistry, University of Wisconsin-Madison, Madison, WI

The centrosome is the major microtubule organizing center of metazoan cells. It is composed of a pair of centrioles surrounded by the amorphous pericentriolar material (PCM). The composition and structure of the PCM changes throughout the cell cycle. The mitotic kinase, Aurora A, is an essential kinase required for increasing the microtubule nucleation capacity of the centrosome at the onset of mitosis. The phosphorylation of the Xenopus TACC protein, maskin, by Aurora A is required for its association with the mitotic centrosome (Peset et al. 2005. J. Cell Biol. 171 1057-1056; Kinoshita et al. 2005. J. Cell Biol. 174 1047-1055 ). Using centrosome assembly assays based on Xenopus egg extracts, we found that in addition to its role in stabilizing microtubules, maskin is required for the assembly of a functional centrosome. We further found that maskin binds to the regulator of spindle assembly, importin β. Importin β binding prevents phosphorylation of maskin by Aurora A. These findings suggest a role for maskin in centrosome assembly, and a role for importin β in regulating maskin at the centrosome.

The Role of NUD-1 in Centrosome Maturation and Organization

R. Thomas, L. Faircloth, G. A. Caldwell, K. A. Caldwell; Biological Sciences, The University of Alabama, Tuscaloosa, AL

Despite the essential role of centrosomes for accurate cell division, a detailed understanding of the factors that contribute to the proper assembly and maturation of these cytoskeletal structures is lacking. The C. elegans one-celled embryo represents an excellent system by which cellular components involved in this process can be elucidated. A hallmark of centrosome maturation is the accumulation of gamma-tubulin at the centrosome. Several proteins have been identified that are required for maturation and gamma-tubulin recruitment at the centrosome in C. elegans, including SPD-5 (a pericentriolar scaffold protein), AIB-1 (the worm aurora-A kinase homolog), and ZYG-9 (a centrosomal microtubule-stabilizing protein related to XMAP-215 in Xenopus). Here, we report that NUD-1, the C. elegans ortholog of the Aspergillus nidulans nuclear distribution protein, NudC, is localized to centrosomes during cell division. The level of NUD-1 protein accumulation is commensurate with centrosome maturation. Furthermore, we have determined that C. elegans NUD-1 is required for the recruitment of gamma-tubulin to centrosomes as there is a marked decrease in gamma-tubulin at the centrosome when nud-1 mRNA is depleted via RNAi. Since the centrosome is composed of a large number of components, we have investigated the possibility that centrosome maturation requires only one of the centrosomal protein components, and that the centrosome in nud-1 mutant flies is defective due to the loss of a centrosomal component. This possibility is unlikely, as we have found that NUD-1 is required for recruitment of the centrosome in C. elegans embryos when other PCM components, such as SPD-5, AIB-1, and ZYG-9, are depleted. Current efforts are focused on determining if nud-1 is required for the recruitment of these PCM components to the centrosome during cell division.

Flies without Centrioles, Centrosomes, Cilia, or Flagella

J. W. Raff, R. Basto, J. Lau; The Gurdon Institute, Cambridge, United Kingdom

Centrosomes and centrioles have an important role in many aspects of animal cell organisation, but it is uncertain to what extent they are essential for animal development. The Drosophila protein DSpd-2, a homologue of the C. elegans centriolar protein Sas-4. We show that DSas-4 is essential for centriole replication in flies. DSas-4 mutants start to lose centrioles during embryonic development, and, by 3rd instar larval stages, no centrioles or centrosomes are detectable. Mitotic spindle assembly is slow in mutant cells, and ~30% of the asymmetric divisions of larval neuroblasts are abnormal. Nevertheless, mutant flies develop with near normal timing into morphologically normal adults. These flies, however, are uncoordinated and fail to feed, move or mate due to the lack of basal bodies in their sensory neurons. Live analysis of dsas-4 mutant third instar larval neuroblasts revealed however, that a proportion (~20%) of the asymmetric cell divisions of the larval nervous system are impaired. We would like understand which might contribute to the establishment of cell polarity in the absence of centrioles in ~80% of dsas-4 mutant cells. To do so, using a live imaging approach in larval brain cells, we are dissecting the cellular consequences of combining the dsas-4 mutation with other mutations in various other cell cycle and polarity genes. We are also investigating how the acentrosomal spindles of dsas-4 mutants can be aligned along the polarity axis. Using time-lapse video microscopy to follow MTs together with either polarity proteins or cell fate determinants suggest that cortical protein distribution is highly dynamic and can change to match the spindle axis.

Establishing Cell Polarity without Centrioles

R. Basto, J. Lau, J. W. Raff. Wellcome Cancer Research Institute, Cambridge, United Kingdom

Centrosomes are the major microtubule (MT) organising centers in many animal cells and contribute to a variety of processes such as PCM recruitment during mitosis, cell migration and the establishment of cell polarity. We have recently identified and characterised a new drosophila mutation, das-4, where centriole replication is abolished. DAs-4 is related to the C. elegans Sas-4 (a protein required for centriole replication) and with the human CENP-J, a gene mutated in the neuro disorder, Microcephaly. Like SPD-2, DSpd-2 localises to the centrosomes throughout the cell cycle and to the PCM in mitosis. In larval brain cells DSpd-2 is essential for the efficient recruitment of the mitotic PCM proteins γ-tubulin and Centrosomin (CNN), reflecting the conserved role of SPD-2 related proteins in centrosome maturation. However, centriole number is almost unperturbed in the Dspd-2 null mutant. Therefore, we conclude that the SPD-2 family proteins are not components of centriole replication machinery operating in all organisms/cell types, and the mechanisms utilised may differ depending on the cellular context. DSpd-2 mutant flies are viable but sterile. Mutant males exhibit severe defects in spermatogenesis, and the number of centrioles per spermatocyte is highly variable. Surprisingly, Dspd-2 is also maternal effect lethal, and mutant embryos do not develop beyond the first mitotic division. We are currently investigating the specific nature of these defects.

Dissecting SAK/PLK4 Function in Templated and De Novo Formation of Centrioles

M. Bettencourt-Dias,1 A. Rodrigues-Martins,1 M. Riparbelle,2 M. Gatt,1 L. Carpenter,3 C. Ferreira,2 G. Callaini,2 D. M. Glover;1 1Instituto Gulbenkian de Ciencia, Lisbon, Portugal, 2Department of Evolutionary Biology, University of Siena, Siena, Italy, 3Department of Genetics, University of Cambridge, Cambridge, United Kingdom

Sak/Plk4 (Drosophila) is a distinct member of the polo-like kinase family. We have recently reported that Drosophila cells progressively lose centrioles, the core structure of centrosomes, following downregulation of SAK by mutation or RNAi. SAK mutants lose their centrioles during the mitotic divisions preceding male meiosis but still produce cysts of 16 primary spermatocytes as in wild-type. Mathematical modelling of the stochyted cell divisions of spermatogenesis can account for such loss by defective centriole duplication. Depletion of SAK in human cells prevents centriole duplication and gives rise to mitotic abnormalities, pointing to a conserved role of this protein in centrosome biogenesis. To further dissect the function of SAK in centriole biogenesis we have overexpressed this kinase in both S2 cells and in Drosophila embryos. Overexpression of SAK leads to centrosome amplification. SAK overexpression during oogenesis leads to amplification of centrosomes in the embryo with developmental arrest. Both oogenesis and early embryogenesis are normal with centrosome amplification occurring at the end of first mitosis. Interestingly, fertilization is not needed for centrosome amplification to occur, indicating that redundant expression of SAK can lead to de novo centrosome formation. Here we explore this result to
further understand the role of SAK in templated and de novo centrosome formation. Differences in the pattern and timing of centrosome appearance in embryos and unfertilized eggs are consistent with their different origins. Hence, we show that SAK is a master regulator of centrosome biogenesis, being involved in both templated and de novo centrosome formation.

974  The Role of SYZ-5 in *C. elegans* Centrosome Duplication
N. B. Miliaras, M. K. Addappali, K. F. O'Connell; Laboratory of Biochemistry and Genetics, NIDDK/NIH, Bethesda, MD

The kinase ZYG-1 is essential for centriole duplication in the early embryonic cells of the nematode *Caenorhabditis elegans*. It is thought to function by recruiting a series of coiled-coil domain containing proteins that help assemble a new centriole adjacent to the parent one. However, in embryos that lack maternal ZYG-1 activity, this fails to occur, leaving the original centriole pair, provided by the sperm, as the sole microtubule-organizing center (MTOC) in the one-cell stage embryo. While the centrioles can separate (the first step required for centrosome duplication) and form a bipolar spindle, they cannot duplicate to form a new centrosome during the second mitotic division. This results in each blastomere in the two-cell stage embryo forming a monopolar spindle. To dissect the mechanism through which ZYG-1 functions in initiating centrosome duplication, a genetic screen for suppressors of zyg-1 (zyz) has been carried out. The suppressors rescue the complete temperature-sensitive lethality of the zyg-1(zyz) allele at 24°C. One such suppressor, zyz-5 (bos7) exhibits an aggregation of tubulin and other centrosomal proteins in the gonad of adult hermaphrodites and cytoplasts of early embryos at restrictive temperature. Furthermore, a majority of embryos display phenotypes consistent with cytokinetic failure, such as multiple centrosomes and over-replicated DNA at the one-and-two-cell stages. These phenotypes, are unaffected by the absence of ZYG-1, thus SYZ-5 is most likely a downstream target of ZYG-1 in the centrosome replication pathway. The main goals of this project are to understand how ZYG-1 acts through SYZ-5 to initiate centrosome duplication and how ZYG-1 itself might function as a regulator of centrosome duplication. We have identified an open reading frame that is mutated in the zyz-5 strain and are in the process of confirming the molecular identity of this gene.

975  Pcp89 Links Multiple Proteins, Including the Septation Initiation Network, to the Core of the Fission Yeast Spindle-Pole Body
J. A. Rosenberg,1 G. C. Tomlin,1 W. H. McDonald,1 B. E. Snyderman,1 E. G. Muller,1 J. R. Yates,1 K. L. Gould,1 Howard Hughes Medical Institute and Cell and Developmental Biology, Vanderbilt University, Nashville, TN, 1The Scripps Research Institute, La Jolla, CA, 2Department of Biochemistry, University of Washington, Seattle, WA

The spindle-pole body (SPB), the yeast analog of the centrosome, serves as the major microtubule (MT) organizing center in the yeast cell. In addition to this central function, the SPB organizes and concentrates proteins required for proper coordination between the nuclear-division cycle and cytokinesis. For example, the Schizosaccharomyces pombe septation-initiation network (SIN), which is responsible for initiating asymmetric cell division and cytokinesis, is assembled at the SPB through its two scaffolding components, Sid4 and Cdc11. In an effort to identify novel SIN interactors, we purified Cdc1 and identified by mass spectrometry a previously uncharacterized protein associated with it, Pcp89. Pcp89 localizes constitutively to the SPB and interacts directly with Sid4. pcp89A cells are inviable and exhibit defects in SPB integrity, and hence spindle formation, chromosome segregation, and SIN and γTuC localization. Pcp89 overproduction is lethal, resulting primarily in a G2 arrest accompanied by massive enlargement of the SPB and increased SPB MT nucleation. These results suggest a fundamental role for Pcp89 in organizing the SIN, γTuC, and structural elements of the *S. pombe* SPB. Currently we are investigating the role of phosphorylation of γTuC members Sid1 and Mto2.

976  A Functional Genomic Analysis of SCF Ubiquitin Ligases and Their Roles in Centrosome Duplication and Cell Cycle Progression
G. Rogers, S. Rogers, Biology, University of North Carolina at Chapel Hill, Chapel Hill, NC

During mitosis in eukaryotic cells, centrosomes organize the poles of the mitotic spindle in order to guide segregating chromosomes to each daughter cell. However, in cells that have either too many or too few centrosomes, chromosome segregation may occur asymmetrically (on a multipolar spindle) or may not occur at all (on a monopolar spindle), resulting in aneuploid cell divisions and genomic instability. In order to ensure proper chromosomal inheritance, cells must duplicate the centrosome only once per cell cycle. A centrosome "licensing factor" may exist to regulate duplication only once itself, or by signaling ubiquitin-dependent degradation. Allen has shown that the regulatory subunit of the anaphase-promoting complex regulates centrosome duplication and that its license is essential for centrosome cell cycle progression, the molecular components involved remain largely unknown. Genetic analyses of centrosome duplication in *Drosophila* have implicated ubiquitin-mediated protein degradation as mutations in subunits of the SCF E3 ubiquitin ligase (SkpA and the F-box protein, Slmb) result in the production of supernumerary centrosomes. To expand upon these initial findings, we have conducted a functional genomic analysis of all Roc, Cullin, Skp and F-box family members using RNAi in cultured *Drosophila* cells. Measurements of centrosome number within the context of the cell cycle allowed us to identify a putative SCF complex containing SkpB and Slmb that is required to prevent centriole amplification. In addition, slmb-GFP expressed in living S2 cells localizes asymmetrically to centrosome tips in a cell cycle-dependent manner where it may work to ubiquitinate a centrosome licensing factor.

977  The Essential Function of CylA in Centrosome Re-duplication Is to Prevent the Degradation of Mps1 at Centrosomes
H. A. Fisk,1 C. Kasbek,1 C. Yang,1 S. Tae,1 M. Winney1;1 Molecular Genetics, The Ohio State University, Columbus, OH, 2MBCD Biology, University of Colorado, Boulder, CO

Centrosomes are microtubule organizing structures that orchestrate assembly of the mitotic spindle apparatus, and their duplication must be tightly controlled to ensure spindle bipolarity. Many human tumors possess extra centrosomes that produce anisometric and abnormally large mitotic spindles and lead to aneuploidy. One possible mechanism to explain their occurrence is the overproduction of centrosomes during S-phase. Called centrosome re-duplication, this requires Cdk2 and in human cells has been shown by others to require overexpression of the Cdc2 partner cyclin A. We previously demonstrated that one function of Cdk2 is to prevent the degradation of one of its substrates, the Mps1 protein kinase. We have now found that Cdk2 modulates a centrosome-specific Mps1 degradation mechanism, and that cyclin A-dependent centrosome re-duplication is mediated by Mps1. Overexpression of cyclin A increases the levels of Mps1 at centrosomes, and Mps1 siRNAs prevent cyclin A-dependent centrosome re-duplication. In contrast, cyclin A siRNAs reduce the centrosomal levels of Mps1 without affecting other Mps1 pools. We have identified the Mps1 degradation signal and shown that its phosphorylation by Cdk2 is required for the function of Mps1 in centrosome re-duplication. Furthermore, when the Mps1 degradation signal is removed Mps1 accumulates at centrosomes and causes centrosome re-duplication even in the presence of cyclin A siRNAs. Therefore, our data supports a model whereby the essential function of cyclin A in centrosome re-duplication is to prevent the degradation of Mps1 at centrosomes, and preventing Mps1 degradation by other means completely bypasses the requirement for cyclin A. Cyclin A is overexpressed in a variety of human tumors, and these results suggest a simple mechanism for genomic instability in such tumors.

978  Heat Shock Protein 27 Plays a Role in Neurite Growth Independent of Its Chaperone Like Activity and Role in Thermoprotection
K. L. Williams, M. Rahimtula, K. M. Mearow; Basic Medical Sciences, Memorial University of Newfoundland, St. John's, NF, Canada

We have previously shown that heat shock protein 27 (Hsp27) is involved in neurite extension and branching in adult rat dorsal root ganglion neurons (DRGs) in vitro. Specifically, downregulation of endogenous Hsp27 by small interfering RNA (siRNA) resulted in decreased neurite tree length and complexity, while an increase in exogenous Hsp27 resulted in an increase in neurite tree length and complexity. In this series of experiments we have tried to further elucidate the role of Hsp27 in neurite growth by simultaneously knocking down Hsp27 protein levels by siRNA and overexpressing either wild type Hamster Hsp27 or a mutant form of Hamster Hsp27. This mutant form of Hsp27 has a deletion between amino acids 5 and 23 which removes the thermal shock protein like activity of Hsp27. This mutant form of Hsp27 has a deletion between amino acids 5 and 23 which renders the protein with no thermoprotection or chaperone like activity. Results show that expression of Hsp27 Δ5-23 rescues the decrease in neuritic tree length and complexity of neurons the increased endogenous Hsp27 is depleted by siRNA. The neurite growth resulting from rescue with the Hsp27 Δ5-23 construct is equivalent to growth observed via rescue with exogenous wild type Hsp27. Additionally expression of Hsp27 Δ5-23 results in increased branching compared to expression of exogenous wild type Hsp27. The ability of Hsp27 Δ5-23 to rescue growth indicates that Hsp27 plays a role in neurite growth and branching independent of its role in thermoprotection and its chaperone like activity. Supporting our hypothesis that Hsp27 may play a role in neurite growth via modulation of the actin cytoskeleton, rather than through its activity as a chaperone.

979  Live Cell Imaging of Sce Tyrosine Kinase in Neuronal Growth Cones
B. Wu, B. Decourt, M. A. Zahbidi, L. T. Wuetrich, K. MacIsaac, W. H. Kim, D. M. Suter; Department of Biological Sciences, Purdue University, West Lafayette, IN

Sce tyrosine kinases have recently been implicated in signal transduction pathways downstream of various membrane receptors controlling neurite outgrowth and axon guidance. However, surprisingly little is known about the detailed temporal and spatial distribution of these key signaling proteins in the neuronal growth cone. To address this problem we took advantage of the
Aplysia growth cone system which is well suited for high-resolution live imaging of protein dynamics. We have identified two novel members of the Src family of protein tyrosine kinases in Aplysia, termed Src1 and Src2, using a homology-based PCR approach. Amino acid sequence alignments suggest that these two kinases belong to two distinct subfamilies of invertebrate Src tyrosine kinases. We produced Aplysia Src1- and Src2-specific antibodies and C-terminal EGFP-fusion proteins to study the distribution of these two kinases in both fixed and live growth cones of cultured Aplysia bag cell neurons. Expression of wildtype Src1- and Src2-EGFP by microinjection of in vitro transcribed mRNA revealed a homogenous plasma membrane-associated distribution of these two kinases. The EGFP construct design do not interfere with the regulation of expressed Src. Time lapse imaging of Src-EGFP fusion proteins revealed Src-positive organelles undergoing dynamic movements particularly in the transition zone and central domain, potentially by F-actin and microtubule association. Immunocytochemistry with peptide antibodies revealed a punctate distribution of Src1 and Src2 throughout the growth cone. Src1 is enriched in the central domain, while active Src2 accumulates in filopodia tips, which is consistent with the localization of constitutively active mutant of Src2-EGFP. Double immunofluorescence labeling and biochemical cell fractionation data indicate a partial association of active Src2 with microtubules. Our findings suggest that distribution of Src is partially regulated by cytoskeletal structures in neuronal growth cones.

980 Biochemical and Cell Biological Analyses of a Developmentally Regulated Septin, Sept8, Enriched in Presynaptic Nerve Terminals
H. Ito,1 I. Iwamoto,2 K. Sado,2 R. Morishita,1 K. Mizutani,2 Y. Nozawa,1 T. Asano,1 K. Nagata1; 1Department of Molecular Neurobiology, Institute for Developmental Research, Aichi Human Service Center, Aichi, Japan, 2Gifu Graduate School of Medicine, Gifu, Japan, 3Gifu International Institute of Biotechnology, Gifu, Japan

The septins are members of a conserved family of cytoskeletal GTPases present in organisms as diverse as yeast and mammals. Thirteen septin genes have so far been identified in mammalian cells. We analyzed the distribution of five septins, Sept6, Sept7, Sept8, Sept9 and Sept11, in various rat tissues by western blot analysis using specific antibodies, and found all septins to be expressed in brain. Especially, Sept6 and Sept8 were preferentially expressed in brain. Further analyses revealed that they were expressed in distinct manners in thirteen brain regions. We also examined the developmental changes of the expression of the five septins in the rat brain and found that levels of several isoforms of Sept8 differentially increased during developmental stage. Immunofluorescence studies revealed that Sept8 is well co-localized with a presynaptic marker, synaptophysin, in matured rat hippocampal neurons, strongly suggesting enrichment at synapses. Using yeast two-hybrid screening and immunoprecipitation assay, we identified the soluble N-ethylmaleimide-sensitive factor attachment protein receptor protein, synaptobrevin, as a binding partner for Sept8. These results suggest that Sept8 may participate in the regulation and/or maintenance of synapse function(s) such as neurotransmitter release.

981 Identifying Genes Involved in Transport of Mitochondria in C. elegans Neurons
S. Mohan,1 G. Reddy,1 S. Johri,1 M. Nouni,3 S. Koushika1; 1NCBS-TIFR, Bangalore, India, 2School of Medicine, Washington University, St. Louis, MO

The growth, maintenance, and survival of neurons rely critically on transport between the cell body and the synapse. Mitochondria participate in many functions which include ATP production, calcium homeostasis and apoptosis. The failures of mitochondrial function and distribution are thought to underlie excitotoxic injury and diverse neuropathological disorders such as Alzheimer’s and Parkinson’s disease. Mitochondria, unlike other organelles are transported bi-directionally and remain stationary for 75% of the time. To address this question we take a classical genetic approach using a transgenic strain jsIs609 in which GFP is targeted to the mitochondrial matrix. This is under the control of a cell-specific promoter which specifically expresses in the six mechanosensory neurons of C. elegans. We have performed EMS mutagenesis and screened approximately 3000 genomes for abnormal mitochondrial distribution and localization. We have isolated 16 mutants that fall into 4 classes. We are currently performing complementation analyses with these mutants. 50-100% increase in number of mitochondria in the axons. •50 - 100% decrease in number of mitochondria in the axons. •Accumulation of globular mitochondria in the cell body. •Accumulation of mitochondria in the minor neurite. We have also analyzed the distribution of mitochondria in mutants with known roles in axonal transport and mitochondrial dynamics viz: unc-1, rab-7, unc-116, unc-16. In unc-116 Kinesin-1 mutants there are fewer mitochondria, however in unc-16 JIP3/JASP1 there more mitochondria. To analyze the mitochondrial dynamics due to metabolism, starvation analyses was done and distribution with different food sources. We find that the cell bodies of the neurons are fragmented during starvation and takes more than one generation to regain their normal phenotype but the axons was spared and is normal. There was no change in mitochondrial distribution and localization with different food sources. We are currently imaging mitochondrial motility and dynamics in vivo.

982 Large-scale Purification of Neurites and Quantitative Proteomics Reveals Spatial Organization of Protein Networks and Novel Proteins Involved in Neuronal Polarization
O. Pertz,1 Y. Wang,1 F. Yang,1 J. M. Jacobs,1 D. G. C. Camp,2 R. D. Smith,2 R. L. Klenke1; 1Department of Pathology and Moores Cancer Center, University of California San Diego, La Jolla, CA, 2Biological Sciences Division, Environmental Molecular Sciences Laboratory, Pacific Northwest National Laboratory, Richland, WA

Neurons morphologically polarize by extending neurites directionally in response to soluble chemokine gradients and adhesive cues present in the extracellular environment. However, while spatial regulation of neuritogenesis is critical for axon and dendrite formation during brain development, the molecular mechanisms that initiate neuronal polarity and guide neurite extension are poorly defined. This is largely due to the inability to differentially isolate the neurite and soma for large-scale biochemical analysis of protein networks. Here we utilize a novel microporous filter system that facilitates large-scale neurite purification and quantitative proteomics to characterize the spatial organization of protein isolated from polarized neurons. Using this system, we identified 5073 proteins of which 2074 were found to be significantly increased in the neurite compartment. Bioinformatic and functional cluster analysis of neurite enriched proteins revealed signaling networks largely involved in the regulation of the actin/myosin cytoskeleton, whereas analysis of the cell soma compartment revealed protein networks involved in nuclear function and metabolism. Importantly, we also uncovered several novel proteins important for neuritogenesis. Currently, we are functionally testing these proteins to determine their role in neuritogenesis and cell polarization. Together our findings provide valuable insight into mechanisms of neuronal polarization by revealing spatially regulated protein networks that control neuritogenesis. These findings are important for understanding mechanisms of brain development as well as neurodevelopmental conditions associated with neurodevelopmental diseases and spinal cord injury.

983 Neurite Purification Using Microporous Technology Reveals Spatio-temporal Dynamics of Erk Activation during Neurite Outgrowth and Collapse
O. Pertz,1 M. M. Holcomb, R. L. Klenke; Department of Pathology and Moores Cancer Center, University of California San Diego, La Jolla, CA

Neuritogenesis and axonal guidance are key cellular processes necessary for proper development of the adult nervous system. In both processes, directional neurite extension depends on precise cytoskeletal and adhesion dynamics induced by sensing of attractive and repulsive extracellular cues. However, the inability to biochemically purify significant amounts of neurites has precluded large-scale spatial analysis of this structure. This has limited our ability to understand mechanisms of neuronal polarity and neuritogenesis. Here, we examine the temporal and spatial role of ERK signaling in neuritogenesis using a novel microporous filter system that allows large-scale biochemical purification of extending or collapsing neurites from neurons polarized towards a laminar gradient. We found that Erk activity is dramatically and spatially enriched in the extending neurite relative to the cell soma. Inhibition of ERK activity with PD98059 prevented neurite formation indicating that ERK kinase activity and phosphorylation of downstream substrates are necessary for this process. Interestingly, exposure of neurites to the collapsing agent lysophosphatidic acid (LPA) facilitated inactivation of ERK leading to growth cone and neurite collapse. Finally, neurite extension and retraction processes are regulated by integrin dependent Rac/PAK signals that converge on MEK, the upstream regulator of ERK. These data point to Erk as an important signaling switch that facilitates neurite outgrowth and collapse in response to neuronal cues provided by the extracellular matrix. These findings are important for understanding spatial signaling mechanisms that contribute to brain development as well as neurodevelopmental conditions associated with neurodevelopmental diseases and spinal cord injury.

984 Novel Membrane Targeting Compounds Promoting Axon Regeneration
J. Wong,1 H. Brastianos,2 R. Andersen,2 T. P. O’Connor1; 1Cellular and Physiological Sciences, University of British Columbia, Vancouver, BC, Canada, 2Earth and Ocean Sciences, University of British Columbia, Vancouver, BC, Canada

During axon development, a delicate interplay between lipid microdomains, signaling molecules and the downstream cytoskeletal components is necessary to regulate axon outgrowth and pathfinding. Following axotomy, axons regeneration typically fails to occur in the adult central nervous system due to the presence of extrinsic inhibitors, as well as the reduced regenerative capacity of the damaged axons. Given the role of lipid microdomains in axon development, we are currently screening for membrane targeting amphiphilic small molecules that promote regeneration of CNS axons on inhibitory myelin and CSPG substrates. We have recently identified a lipopophil extract in marine sponge that promotes neurite outgrowth on inhibitory
substrates. Further characterization of this extract may provide insight into how lipid microdomains influence axon outgrowth and pathfinding, and how lipid raft manipulation can promote axon regeneration in the adult CNS.

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Analysis of the Microtubule-associated Proteome during Neuronal Morphogenesis
E. Hwang,1 J. Tipton,2 J. Busby,2 S. Halpain1; 1Department of Cell Biology, The Scripps Research Institute, La Jolla, CA, 2Department of Proteomics, Scripps Florida, Jupiter, FL
During nerve cell development, newly postmitotic neurons transform from seemingly unpolarized cells into highly polarized ones. These highly polarized neurons contain long cellular processes termed neurites that are filled with the microtubule (MT) cytoskeleton, and will later mature into axons or dendrites. It has been shown that the morphological change during neuronal development depends on the organization of the MT cytoskeleton. Although many of the later stages of neuronal development (e.g., axon outgrowth, axon guidance, and synaptogenesis) have been extensively studied, little is known about the very first step, the initial sprouting of a neurite, termed "neuritogenesis" or "neurite initiation". Although a handful of MT-associated proteins are known to play important roles during neurite initiation, a comprehensive proteomic analysis focusing on the MT cytoskeleton during this process is lacking. To better understand the mechanisms controlling neuritogenesis, it is of great advantage to perform a proteomic analysis focusing on the MT-associated proteome involved in this process. We have conducted mass spectrometry and gel-based methods to analyze MT-associated proteins before and shortly after neurite initiation. We have found 10 MT-associated proteins to be specifically enriched during the neuronal differentiation; more than 2 dozens to be present in both undifferentiated and differentiated cells. These results provide the basic compositional change of the MT cytoskeleton during neuronal differentiation and will help us identify specific MT-associated proteins that are key mediators of neurite initiation.

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Distinct Roles of Doublecortin Modulating the Microtubule Cytoskeleton
C. A. Moores,1 M. Perderiset,2 C. Kappeler,1 S. Kain,1 D. Drummond,3 S. J. Perkins,4 J. Chelly,5 R. Cross,6 A. Houdusse,2 F. Francis1; 1Department of Crystallography, Birkbeck College, London, United Kingdom, 2Centre d'Etudes des Structures Chimiques et Biologiques (CIESC), Paris, France, 3Department of Pathology, Columbia University, New York, NY, 4Department of Radiology, Columbia University, New York, NY, 5Department of Neurology, Columbia University, New York, NY
Doublecortin is a neuronal microtubule stabilising protein, mutations of which cause mental retardation and epilepsy in humans. How doublecortin influences microtubule dynamics, and thereby brain development, is unclear. We show by video microscopy that purified doublecortin has no effect on the growth rate of microtubules. However, it is a potent anti-catastrophe factor that stabilises microtubules by linking adjacent protofilaments and counteracting their outward bending in depolymerising microtubules. Using an in vitro motility assay, we found that doublecortin-stabilised microtubules are substrates for kinesin translocate motors and for depolymerase kinesins. Doublecortin does not itself oligomerise, as judged by analytical ultracentrifugation. It does not bind to tubulin heterodimers but does nucleate microtubules. In cells, doublecortin localises to the distal ends of neuronal processes and our data raise the possibility that the function of doublecortin in neurons is to drive assembly and stabilisation of non-centrosomal microtubules in these doublecortin-enriched distal zones. These distinct properties combine to give doublecortin a unique function in microtubule regulation, a role that cannot be compensated for by other microtubule stabilising proteins and nucleating factors.

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MARK2/Par-1 Mediated Neuronal Migration
O. Reiner,1 T. Sapir,2 S. Saposnik,1 E. Mandelkow,2 T. Timm,1; 1Molecular Genetics, Weizmann Institute, Rehovot, Israel, 2Max-Planck-Unit for Structural Molecular Biology, Hamburg, Germany
Our results depict a novel role for microtubule affinity-regulating kinase (MARK2/Par1) in neuronal migration. Using in utero electroporation, we have been able to dissect the two tasks of MARK2/Par1 during cortical development: neuronal polarity and neuronal migration. Neuronal migration retardation, which is observed following introduction of MARK2/Par1 RNAi, is associated with MARK2 kinase activity. This was demonstrated by either overexpression of kinase active and kinase dead versions of MARK2/Par1, or by overexpression of MARK2/Par1 specific inhibitor PAK5. A kinase dead version of PAK5 has no affect on the actin cytoskeleton yet was able to partially inhibit neuronal migration via its suppressive activity on MARK2/Par1. Furthermore, we identified novel MARK2/Par1 substrates, which include LIS1 and interacting proteins. Reduction in MARK2/Par1 levels results in centrosomal - nuclear uncoupling, a known LIS1 mediated function. These results suggest that MARK/Par1 is a key regulator of neuronal migration by its affect on multiple MAPs some of which have pivotal roles during the development of the cortex. In addition our results suggest a link between LIS1 and the MARK2/Par1 cascade, thus placing LIS1 downstream of the polarity pathway. These observations are synergistic to a previous study situating LIS1 upstream to Rho GTPases. Combining the position of LIS1 as a target of the polarity kinase as well as a small GTPase regulator suggests an exciting feedback regulatory role for LIS1.

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A Role for Dynnein, Dynactin, and LIS1 in Growth Cone Remodeling and Rapid Axonal Growth
G. E. Seale,1 J. P. W. Grabham,1 M. Bennech,1 D. J. Goldberg,2 R. B. Vallee1; 1Dept. of Pathology, Columbia University, New York, NY, 2Center for Neurobiology and Behavior, Columbia University, New York, NY
Mechanisms in Neurite Initiation: Dynein-dependent Induction of Cell Protrusions
L. Dehmelt,1 P. Nalbant,2 W. Steffen,3 S. Halpain1; 1Department of Cell Biology, The Scripps Research Institute, La Jolla, CA, 2Department of Molecular and Cellular Physiology, University Hannover, Hannover, Germany
A key event in neurite initiation is the accumulation of microtubule bundles at the neuron periphery, which subsequently form thin protrusions that extend from the cell body. We hypothesized that such bundled microtubules may generate a force at the plasma membrane that induces local protrusions, which then become the focal point for neurite outgrowth. To test this idea we observed the behavior of microtubule bundles that were induced by the microtubule-associated protein MAP2c. Endogenous MAP2c contributes to neurite initiation in primary neurons, and exogenous MAP2c is sufficient to induce neurites in Neuro2a cells. During nocodazole washout in Neuro-2a and COS7 cells, small bundles formed rapidly throughout the cytoplasm and immediately began to move toward the cell periphery in a unidirectional manner. Spindle microscopy indicated that such movement was due to bundle transport, not treadmilling. At the periphery bundles remained under a unidirectional force and induced local cell protrusions that were further enhanced by suppression of Rho kinase activity. Surprisingly, this bundle motility was independent of classical actin- or microtubule-based tracks. It was, however, reversed by function-blocking antibodies against dynnein. Suppression of dynnein expression in primary neurons by RNA interference severely inhibited the generation of new neurites, but not the elongation of existing neurites formed prior to dynnein knockdown. These data suggest that neuronal microtubule-associated proteins induce microtubule bundles that are pushed outward by dynnein and locally override inward contraction to initiate a neurite.

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The Mitotic Motor, Eg5: Functions as a “Brake” on Short Microtubule Transport to Regulate the Rate of Axonal Outgrowth  
K. A. Myers, P. W. Basas; Neurobiology and Anatomy, Drexel University College of Medicine, Philadelphia, PA  
Eg5 is a plus-end-directed motor protein that is required for the formation and proper segregation of the bipolar mitotic spindle. Four molecules of Eg5 form a homotetramer with the motor domains projected outward; the homotetramer can drive oppositely oriented microtubules apart or can zipper and crosslink microtubules of the same orientation. We have previously shown that Eg5 is expressed in terminally postmitotic neurons, and that pharmacologic inhibition of its activity results in enhanced rates of axonal growth. Here, we used either monostable treatment or siRNA-based depletion of Eg5 to investigate the mechanism underlying this observation. Similar to monostable treatment, siRNA-based depletion of Eg5 resulted in a sharp and statistically significant increase in the rates of axonal growth to levels approximately five times greater than displayed by control neurons. Enhanced axonal growth rates were accompanied by augmented axonal length, increases in axonal branching frequency, and heightened numbers of growth cones. To explore how this might occur, we evaluated the behavior of vesicular cargo and short microtubules, both of which are transported bi-directionally in the axon during its growth. There was no change in the rates or distances of vesicle movement. However, inhibition of the Eg5 motor did result in a significant and bi-directional increase in the frequency of short microtubule transport, to levels approximately 45% greater than controls. Taken together, these data suggest a model in which neuronal Eg5 functions not as a classical vesicle-associated kinesin, but rather as a brake against the other motor-driven forces utilized to transport short microtubules within the axon. This conclusion is consistent with the expected properties of the Eg5 homotetramer, given that axons contain uniformly oriented microtubules. We propose that there exists a direct relationship between the frequency of short microtubule transport, as regulated by the Eg5 motor, and the rate of axonal growth.

Filopodial Actin Bundles Help Neuronal Growth Cone Generate Assembly Dependent Push  
D. T. Burnett,1 A. W. Schaefer,1 L. J.;2 D. Gaudenz,1; P. Forscher1; 1MCDB, Yale University, New Haven, CT, 2Department of Cell Biology, Scripps Research Institute, La Jolla, CA  
Two actin filament components normally assemble at the leading edge of growth cones: polarized actin bundles that create the cores of filopodia and intervening isotropic actin networks. Both of these actin structures move at the same rate by retrograde actin flow under control conditions; however, their movement slows by about 50% after Myosin II inhibition with Blebbistatin. Evidence suggests that the “push” driving this residual retrograde flow is provided by actin assembly at the leading edge. Acute treatment with low doses (350 nM) of Cytocalasin B (CB) resulted in immediate retraction of non-polarized growing actin bundles but had essentially no effect on retrograde flow rates or peripheral isotropic actin network structure. This resulted in growth cones depleted of filopodia as judged by fluorescent speckle microscopy and ultrastructural analysis. Interestingly, in growth cones without filopodia, retrograde flow rates were now much more sensitive to myosin II inhibition. Our previous studies provided evidence that actin assembly alone could drive the retrograde flow at surprisingly fast rates. Our current results further suggest that filopodial actin bundles allow network assembly dependent pushing forces to develop more efficiently.

Myosin II Mediates Actin/Microtubule Interactions during Inhibition of Growth Cone Advance  
M. L. Koeppel, E. A. Hogan; Biology, Canisius College, Buffalo, NY  
Our previous experiments suggest a role for actin/microtubule interactions during inhibition of growth cone advance in the absence of growth cone collapse. Treatment with jasplakinolide (jasp), a toxin that inhibits actin turnover, triggers an organelle withdrawal response (OWR), characterized by the removal of organelles from the central domain into the axon, concomitant with increased microtubule(MT) invasion of the growth cone peripheral domain. We have proposed that MTs serve as a substrate for the centripetal movement of F-actin and organelles out of the growth cone. Here, we tested the role of myosin in mediating the interactions between MTs and F-actin during the OWR by treating neurons simultaneously with blebbistatin, a specific myosin II ATPase inhibitor, and jasp (bleb/jasp). Time-lapse microscopy showed that treatment with bleb/jasp counteracted the effects of jasp treatment in 60% of the growth cones, resulting in an expansion of the central domain region into the peripheral domain. In the remaining 40% of jasp treated growth cones, the organelle withdrawal distance was reduced 2.5 x compared to jasp treated growth cones. To determine if jasp inhibition of the OWR was due to its effects on myosin II or to inhibition of MT invasion into the peripheral domain, we used tubulin immunofluorescence/DIC microscopy to determine the position of MT ends from the leading edge of the growth cone. Inhibition of myosin II activity did not affect MT invasion into the growth cone, as there was no significant difference (T test, 0.05) in MT end position between jasp and bleb/jasp treated neurons, while there was a significant decrease (2 fold) in MT end position in bleb/jasp growth cones compared to untreated growth cones. These results suggest a role for myosin II in mediating the MT-dependent OWR response.

The Role of Tropomyosin Isoforms in Neurite Outgrowth  
T. Fath, 1 H. Clarke,1 G. Schevoy,1 P. Gunning2; 1Oncology Research Unit, Children’s Hospital at Westmead, Westmead, Australia, 2Department of Pathology, University of Sydney, Sydney, Australia  
Alpha-tubulin is subject to various post-translational modifications among which a cycle of detyrosination-tyrosination. TTL is the enzyme responsible for the re-addition of the C-terminal residue after its removal by an unknown carboxypeptidase. Tyrosinated-tubulin is found distally, at the dynamic end of microtubules whereas detyrosinated-tubulin is enriched in stable microtubules. Although the physiological role of this cycle is poorly understood, TTL has been shown to be involved in the control of neurite outgrowth. We have further investigated its role by analyzing the hindbrain development in TTL null mice. In particular, we have characterized the migration of Inferior Oliveary Neurons (ION) that consists in the emission of a leading process and further translocation of the nucleus inside it (nucleokinesis). Despite an overall normal cyto-architecture of the ION nucleus, TTL null mice showed an abnormal inter-olivary commissure after Dil retrograde tracing at birth. Consistent with this observation, we showed that growth cones were more spiny and enlarged in organotypic cultures when they met the floor-plate, an intermediate target that synthesizes various tropic molecules that guide ION migration. When migration was assayed in collagen matrix, axons poorly grew outside the hindbrain explant but nucleokinesis was not affected. Furthermore, interaction between actin and microtubules cytoskeletons in the growth cone was perturbed since microtubules bundles invaded the actin domain (peripheral domain) of the growth cone. These results led us to propose that tubulin tyrosinase is required for growth cone dynamic and response to guidance signals, especially at decision points. We are currently investigating the molecular cause(s) of the mis-regulation of the cross-talk between actin and microtubule cytoskeletons in growth cones.
Our aim is to reveal the mechanism by which Tms define these actin-filament populations. To address this aim we have generated mice deficient for specific Tm isoforms. The effect of eliminating products of the γTm gene which lack exon 9d (and thus isoforms TmSNM1/2) on neurons was analyzed in cortical cultures from 16.5 day old wild-type (wt), hemizygous or homozygous 9d-Tm knockout mouse embryos. The reduction of exon 9d-containing isoforms is compensated by an upregulation of exon 9c-containing isoforms. Preliminary experiments show that the loss of TmSNM1/2 leads to altered growth cone size and that the altered morphology is dependent on the relative levels of exon 9d and 9c containing isoforms. These results are consistent with earlier observations of altered growth cone size upon overexpression of exogenous TmSNM1 protein in cortical neurons and support the hypothesis that the composition of Tms can locally affect specific compartments of a cell. Further studies on alterations in the molecular composition of actin-filament populations upon manipulation of Tm protein levels will give us a better understanding of actin-cytoskeleton related processes which underlie neuronal morphogenesis and function.

**C. elegans Neural Ankyrin Interaction with CLASP2, PP2A, and Calcium**

A. J. Otsuka, P. Gong; Department of Biological Sciences, Illinois State University, Normal, IL

UNC-44 A031 ankyrin is found in the nervous system and is required for proper axon guidance (Otsuka et al., J. Cell. Biol., 129:1081-1092, 1995; Boontrakulpoontawee and Otsuka, Moloc. Genet. Genom., 267:291-302, 2002; Otsuka et al., J Neurobiol., 50:333-349, 2002). We recently found that AO13 ankyrin interacts with CLASP2, PP2A B’ subunits, and calcium ions (Gong and Otsuka, Moloc. Biol Cell (suppl.), 16:365a Abst. #1349, 2005). It is known from work by others that CLASP2 binds to the plus ends of microtubules at the leading edge of migrating cells, is under the control of the PI3-kinase and GSK-3β pathways, and is inhibited by phosphorylation. The carboxyl terminus of CLASP2 is required for cortical association in vertebrates, although the plasma membrane docking sites are unknown. We have found that the carboxyl end of CLASP2 binds to AO13 ankyrin which may serve as the cortical adapter molecule in neurons. Affinity-purified CLASP2 antibodies labeled the cytoplasm and, in some cases, labeled the cell periphery. In our previous work, we found that a 141-aa region of AO13 ankyrin was required for calcium binding. We have now narrowed the calcium-binding site to a two EF-hand structure within that region. Further, CLASP2 binds to the same two-EF-hand structure. The entire 141-aa region is needed for efficient binding of the two nematode PP2A B’ subunits. In addition to yeast two-hybrid and pull-down experiments demonstrating the PP2A-ankyrin interaction, we have now shown PP2A and AO13 ankyrin co-localize in neurons by antibody staining. The UNC-44 and CLASP2 interaction suggests that UNC-44 may act to capture CLASP2-tipped microtubules that are pioneering the growth cone filopodia. Further, it is possible that UNC-44 is a calcium sensor that affects microtubule and cortical interactions depending on the UNC-44 phosphorylation state.

**Developmental Regulation of Myosin II Expression and Function in Sensory Neurons**

A. R. Keshet, S. L. Jones, R. Puri, G. Gallo; Neurobiology & Anatomy, Drexel University College of Medicine, Philadelphia, PA

During development cultured sensory neurons switch from extending a single axon to extending multiple axons suggesting fundamental changes in the cytoskeletal mechanisms of axon extension. Myosin II is a mechanoenzyme that generates cellular contractile forces though interactions with actin filaments and is involved in axon extension and guidance. We report that in cultured chicken sensory axons the levels of myosin II undergo developmental down regulation between embryonic day (E) 7 and E14. Decreases in myosin II expression were observed in axons growing on laminin or polylysine coated substrata. Inhibition of myosin II using blebbistatin reduced the axon extension rate of E7, but not E14, neurons cultured on laminin. On polylysine, blebbistatin increased axon extension rate at both E7 and E14. The ability of axons to remain on laminin when encountering a laminin-polylysine border has previously been shown to require myosin II activity. We found that E7 axons remained on laminin with a greater frequency than E14 axons. Treatment with blebbistatin resulted in border crossing by both E7 and E14 axons, indicating that at both ages axon guidance is still dependent on myosin II. Axon retraction in response to pharmacological stabilization of the actin cytoskeleton using jasplakinolide results in myosin II dependent axon retraction. We found that E14 axons retracted at approximately half the rate of E7 axons in response to jasplakinolide, although the retraction at both ages was dependent on myosin II. Collectively these data indicate that the developmental decrease in the expression of myosin II in sensory neurons has functional consequences for axon extension and guidance. In future experiments, we will address whether re-expressing myosin II in E14 axons restores the dependence of axon extension and guidance on myosin II activity characteristic of E7 axons.

**Axonal Transport of Actin in Monomeric Form**

G. Gallo; Neurobiology and Anatomy, Drexel University College of Medicine, Philadelphia, PA

Cytoskeletal proteins are synthesized in the cell bodies of neurons and undergo slow axonal transport to the tips of axons. Whether cytoskeletal proteins are transported as monomers/dimers or as polymers is not clear. Previous evidence indicates that tubulin can be transported in polymeric form or as tubulin subunits. We tested whether actin can undergo transport in monomeric or filamentous form. Embryonic sensory neurons were transfected with photoactivatable GFP-actin and the movement of photoactivated actin was tracked in single axons. Photoactivated actin underwent net anterograde transport. In addition, we performed FRAP (Fluorescence Recovery After Photobleach) analysis in the distal axons of EGFP-actin expressing neurons. Pharmacological manipulation of the actin monomer/filament equilibrium in neurons revealed that if actin was driven into filamentous form by treatment with jasplakinolide FRAP failed to occur, and photoactivated actin filaments did not undergo transport. Conversely, depolymerization of filaments using latrunculinA or wiskinolideA did not prevent FRAP. Rather, when actin was driven into monomeric form the net FRAP increased relative to controls. Manipulating the equilibrium between actin monomers/filaments did not affect FRAP of EGFP-tubulin, indicating the effects are specific for the transport of actin. These data are interpreted to indicate that actin undergoes axonal transport in the form of monomers, and that incorporation of actin into filaments en route decreases the net flux of transported actin.

**Neurofilaments Spend More Time Pausing in Axons Lacking Myosin Va**

N. Alam, A. Brown; Center for Molecular Neurobiology, The Ohio State University, Columbus, OH

Neurofilaments move rapidly along axons but their overall rate is slow because they spend most of the time pausing. The motors for neurofilament transport are thought to be dynein and kinesin, which move along microtubules, but neurofilaments also interact with myosin Va, which moves along microfilaments. Interestingly, neurofilament number is increased in axons of dilute lethal mice, which lack myosin Va. To investigate whether myosin Va is a neurofilament motor, we previously compared the velocity and short-term pausing behavior of GFP-tagged neurofilaments moving through gaps in the axonal neurofilament array of cultured SCG neurons from wild type and dilute lethal mice and found no significant difference. Now we have extended that study by comparing the long-term pausing behavior of GFP-tagged neurofilaments in cultured wild type and dilute lethal DRG neurons using a fluorescence photoactivation pulse-escape technique. Neurons were transfected with a photoactivatable green fluorescent neurofilament fusion protein and then neurofilaments were photoactivated in a short segment of axon. The fluorescent neurofilaments departed from the activated region with exponential kinetics at a rate dictated by their pause durations. Statistical comparison of the log-transformed kinetics using an autoregressive linear mixed model revealed a significant difference (p<0.001), with an average half life of 3.0 hours in wild type neurons (n=27) and 4.4 hours in dilute lethal neurons (n=25). Since the velocity was unchanged, these data indicate that neurofilaments spend approximately 50% more time pausing in dilute lethal DRG neurons than in wild type. We propose that myosin Va is a short-range neurofilament motor and functions to deliver neurofilaments to their microtubule tracks, thereby increasing the efficiency of neurofilament transport. In the absence of myosin Va, neurofilaments that are not close to microtubules can become stranded and therefore pause for more time, which can explain their accumulation in axons of dilute lethal mice.

**Microtubule-induced Focal Adhesion Disassembly Requires Clathrin**

E. J. Erzatty, 1 G. E. Marcantoni, 2 G. G. Gundersen, 1; 1Anatomy and Cell Biology, Columbia University, New York, NY, 2Pathology, Columbia University, New York, NY

Focal adhesion disassembly is an important aspect of cell migration that is not well understood. We recently developed a new approach to study focal adhesion disassembly, based on the observation that microtubule (MT) regrowth following nocodazole washout induced disassembly of focal adhesions in NIH3T3 fibroblasts, a process that was dependent on focal adhesion kinase (FAK) and dynamin (Erzatty, et al., Nature Cell Biology, 2005). These results suggested that a rate limiting step during focal adhesion disassembly may involve endocytosis. To test this hypothesis we examined the effects of heavy chain transferrin-loaded carriers, as well as transferrin uptake in Clathrin heavy chain, but not light chain, localized to a subset of focal adhesions when visualized using total internal reflection fluorescence microscopy. We screened clathrin adapter proteins for co-localization with FAK, and found that the adapter protein disabled-2 co-localized with focal adhesions, whereas AP2 and mum were excluded from these structures. Disabled-2 may directly inhibit endocytosis at focal adhesions during focal adhesion disassembly, since the phosphotyrosine binding domain of disabled-2 binds the cytoplasmic tail of integrins (Calderwood et al., PNAS 2003). To test if...
integrins are internalized during focal adhesion disassembly, we used antibody labeling and flow cytometry to monitor the surface expression of α5 integrin during MT-induced focal adhesion disassembly. Levels of surface α5 integrin decreased by over 50% during MT-regrowth following nocodazole washout. The loss of surface integrin was specific for extracellular matrix bound integrin as the surface levels of the collagen receptor α1 integrin in fibroblasts adhering to fibronectin remained unchanged during disassembly. In these same cells, α5 integrin levels were significantly diminished. These data suggest that extracellular matrix-bound integrin is specifically internalized from focal adhesions. We propose that MT-induced focal adhesion disassembly occurs via clathrin-dependent endocytosis of α5β1 integrin from focal adhesions.

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A Live Cell Assay to Visualize the Activation of Integrin Adhesion Receptors
J. A. Galbraith, C. G. Galbraith, NINDS, National Institutes of Health, Bethesda, MD, NIDCR, National Institutes of Health, Bethesda, MD
Migrating cells continually extend protrusions to probe the surrounding matrix in search of new sites to form adhesions with the extracellular matrix. The adhesions are initiated at the front of the cell; however, the precursor events in adhesion formation such as the spatial regulation of initial integrin activation are largely unknown. Using a new assay, we can temporally and spatially visualize and track the conformational state of integrins in living cells. We have seen the previous appearance of actin-unlinked unligated integrin adhesion receptors at the leading edge of migrating cells. When we incubate a fluorescently labeled antibody targeted against conformationally activated integrin adhesion receptors rapidly labels all high-affinity receptors on the surface of a cell, including the ligated adhesion receptors in established adhesions. Any subsequent labeling of the cell surface during a live cell experiment indicates where high-affinity integrins appear on the cell surface. This version of an antibody capture assay produces a fluorescently “speckled” cell surface where the speckles indicate conformationally activated integrin adhesion receptors. The movement of the activated integrins can then be tracked using single particle tracking analysis routines. We see the newly activated integrins appear at the leading edge before moving rearward on the treading cell membrane. These data are consistent with laser trap experiments demonstrating an increased avidity of integrin adhesion receptors at the leading edge of migratory cells. Thus, this new live cell antibody capture assay allows us to visualize changes in integrin activation state during the initial development of adhesion complexes, and track the movement of these newly activated integrins on the cell surface.

1002
Talin-Actin Binding and Focal Adhesion Turnover Is Regulated by pH
Focal adhesion (FA) turnover, a major determinant in tissue remodeling and cell movement, is in part regulated by talin, which tethers the actin cytoskeleton to integrin adhesion complexes through its binding to integrins and F-actin. We previously reported that FA turnover is dependent on H+ efflux by the Na-H exchanger NHE1 and an increase in intracellular pH (pHi). We now present a predicted mechanism for how increased pH disrupts actin binding by the C-terminal I/LWEQ domain of talin to allow FA turnover. Computational pKa prediction of a homology model based on the structure of the Hip1R I/LWEQ domain and upstream α helix (USH) revealed that 5 amino acids (3 Asp, Glu, His) with pKas near physiological cluster at one end of a 5-helix bundle. Computer-aided molecular dynamics simulations suggest that protonation of the pH sensor causes a dramatic conformational change in the packing of the helical bundle in the vicinity of the actin binding site, which is remote from the pH sensor. NMR titrations identified histidine has an up-shifted pKa >7 in the C-terminus. Two-dimensional 1H, 15N correlation spectra of USH (+)/I/LWEQ show significant pH-driven conformational changes within the physiological range for ~20 residues associated with changes in overall dynamics and an increase in structure at lower pH. This model was supported by actin co-sedimentation assays. Talin USH (+)/I/LWEQ had high affinity actin binding at pH 6.5 and low affinity binding pH 7.5, however actin binding by the I/LWEQ domain lacking the USH segment was pH-insensitive. Furthermore, pH-sensitivity of actin binding by USH (+)/I/LWEQ was markedly reduced by an HAF mutation in the pH sensor. These data suggest a mechanism whereby changes in pHi can affect talin-actin binding to promote normal dynamic FA remodeling, and impaired remodeling with disease-associated aberrant pH.

1003
Rack1 Associates with Na+/H+ Exchanger NHE5 in Focal Adhesions and Positively Regulates the Transporter Activity
I. Onishi, P. J. C. Lin, G. H. Dieringer, W. P. Williams, M. Numata; Biochemistry and Molecular Biology, University of British Columbia, Vancouver, BC, Canada
Na+/H+ exchanger isoform 5 (NHE5) is a brain-enriched NHE that may play important roles in ion and pH homeostasis, and cell-volume regulation in neurons. However, the regulation mechanism of NHE5 activity has not been fully elucidated. In order to identify potential NHE5 regulating proteins we screened a human brain embryonic cDNA library using yeast two-hybrid methodology. Here, we show that the signaling scaffold protein Receptor for Activated C Kinase 1 (RACK1) directly binds to NHE5 and positively regulates the transporter functionality. NHE5 co-localized with RACK1 as well as the markers for focal adhesions β1 integrin, paxillin and vinculin, suggesting that NHE5 associates with these structures and may play a specialized role during cell-matrix adhesion. By using RACK1 dominant-negative mutants and siRNA, we further show that RACK1 regulates NHE5 both directly and through an integrin-dependent pathway. The NHE5-RACK1 interaction, but not the RACK1-β1 integrin interaction, was reinforced when cells were spread on an integrin-substrate fibronectin. We propose that RACK1 activates NHE5 both by integrin-dependent and independent pathways, which may coordinate cellular ion and pH homeostasis during cell-matrix adhesion.

1004
RACK1-Src Complex Regulates Adhesion Dynamics and Cell Migration
A. T. Doan; Pharmacology, University of Wisconsin, Madison, WI
Receptor for Activated C Kinase, RACK1, is an adaptor protein that binds to and regulates signaling via Src and PKC-dependent pathways. Elevated levels of rack1 mRNA has been demonstrated in non-small cell lung, colon, and breast carcinoma cells compared to normal tissue. Thus identification of the function of RACK1 and its regulation in normal and tumor cells appears to be essential to evaluate oncogenic or tumor suppressor potential. In this study, we show that RACK1 regulates adhesion complex dynamics through its direct interaction with Src and paxillin. RACK1-deficient cells are generated using siRNA and show increased number and size of paxillin-containing focal adhesions at the cell periphery. Quantification of focal adhesion duration and dynamics demonstrates that RACK1 is required for the efficient assembly/disassembly of paxillin from focal adhesions. We find this to be unique, since RACK1-deficient cells do not affect other focal adhesion protein dynamics. Phosphorylation of focal adhesion proteins, paxillin and FAK, were augmented in RACK1-deficient cells compared to control. The use of a Src inhibitor, PP2, or co-transfecting full length RACK1 in the deficient cells rescued the adhesion defects. Cell migration was reduced in RACK1-deficient cells. This reduction in cell migration may be due to the stabilized focal adhesions observed in the RACK1-deficient cells. To investigate the role of the RACK1-Src interaction in FA dynamics, we used RACK1 in which the Src binding site has been mutated (RACK1 Y246F). Expression of RACK1 Y246F was unable to rescue the adhesion and migration defects observed in the RACK1-deficient cells. Together, our findings suggest that RACK1 play a role in adhesion complex dynamics and cell migration by modulating Src-mediated phosphorylation of the adhesion protein, paxillin.

1005
Vinexin Promotes the Association of Vinculin with Cytoskeleton at Focal Adhesions
H. Takahashi, K. Ueda, N. Kioka; Agriculture, Kyoto University, Kyoto, Japan
Adhesion sites between cultured cells and extracellular matrix are termed focal adhesions (FAs) and play pivotal roles in cell behaviour. We have reported that the expression of one of FA proteins, vinexin α, accumulates F-actin at FAs in NIH 3T3 cells, while vinexin β, a shorter variant, has only a weaker effect on actin cytoskeleton although both vinexin α and β localise at FAs and bind to vinculin. We assume that vinexin α changes the assembly of proteins at focal adhesions or changes the status of proteins at focal adhesions leading to the accumulation of F-actin. In this study, to examine these possibilities, we used modified cytoskeleton stabilizing buffer (CSK), which removes protein from cytoplasm and leaves cytoskeletal components. This method can determine the affinity of FA proteins to cytoskeleton. We found that vinexin α still remained at the FAs after the treatment with the CSK, while vinexin β did not. Actin stress fibres were partly washed out by the CSK in the vinexin β-expressing cells, leaving thin and weak phalloidin staining. In contrast, significant actin remained at the FAs containing vinexin α. Importantly, we also observed similar results in vinculin staining: a considerable amount of vinculin was left as a CSK resistant component at the FAs in the cells expressing vinexin α, but not β. Different from vinexin, paxillin and FAK were washed out even in the cells expressing vinexin α. Consistent with the overexpression experiments, knockdown of endogenous vinexin expression by siRNA in NIH 3T3 cells resulted in attenuated staining of vinculin after the CSK treatment. These findings suggest that vinexin α promotes the association of vinculin with cytoskeleton and also raise the possibility that this association results in the accumulation of F-actin at FAs.
Conformational Restriction Decreases Lipid Binding of the Vinculin C-Terminal
C. K. Voss, L. E. C. McDonald, E. H. Ball, Biochemistry, The University of Western Ontario, London, ON, Canada

The C-terminal tail domain of vinculin is responsible for the interactions of the protein with actin and lipid in cell-cell and cell-matrix adhesions. Drastic conformational changes, including the separation of the fibronectin and the β integrin binding domains, that are associated with vinculin function, have been suggested to occur during binding to lipid. To examine the need for such large conortions, we constructed three mutants with inserted pairs of cysteine residues to form disulfide bonds that would hold parts of the protein together. Cysteines at positions 951 and 999 held helices 3 and 4 together; at 958 and 1037 held helices 4 and 5; and at 878 and 1066 held the C-terminal arm in place (‘armlock’ mutation). The purified mutant domains were oxidized to create disulfide linkages, and their binding properties compared with those of wild type. All of the mutants showed normal binding to the head domain in gel filtration or fluorescence assays, whether oxidized or reduced, indicating that they were in a native, active conformation. The disulfide bonded mutants with locked helices were still able to bind lipid and actin in cosedimentation assays; however, the ‘armlock’ mutant showed a drastic decrease in lipid binding (75% decrease) and some decrease in actin binding (50%). Upon reduction with DTT, these differences were abolished, indicating that the disulfide bond itself, and not the inserted cysteines, was responsible. These results point to an important role for conformational flexibility in the C-terminal arm of vinculin function: it is needed for at least one step in lipid binding whereas large scale helix separation is not (but may still occur at later stages).

Zyxin (zyx102) Function Is Required for Viability in Drosophila melanogaster
P. J. Renfranz, E. Blankman, M. C. Beckerle; Huntsman Cancer Institute, University of Utah, Salt Lake City, UT

Zyxin family proteins function in actin assembly, cell motility and adhesion. In vertebrate tissue culture cells, zyxin family members localize to focal adhesions, adherens junctions, actin stress fibers, and nuclei. Zyxin null fibroblasts show striking defects in motility and response to stretch. To study the role of zyxin during development, we have focused on the single homologue in Drosophila, zyx102. The predicted protein contains three C-terminal LIM domains, at least one potential nuclear export signal, and at least one poly-proline motif that interacts with members of the Ena/VASP protein family. Drosophila zyxin is localized at adhesions similar to its vertebrate counterparts. We have generated germline zyx102 RNAi transformatants to knockdown Zyx102 protein using the GAL4/UAS expression system. Flies carrying the transgene have been crossed to a number of fly lines that express GALA under the control of specific enhancers/promoters. Protein immunoblot analysis shows that Zyx102 protein levels are significantly reduced in these flies, indicating that targeted RNAi occurs. We observe a lethal effect when UAS-zyx102-dsRNAi expression is driven ubiquitously by Actin5C-GAL4, indicating that in Drosophila, zyxin protein is required for viability. The lethal period is the pharate adult stage, a narrow developmental window during which the fly must molt, resorb molting fluid, fill adult trachea with air and execute a behavioral program to eclose. While knockdown animals make feebly active larvae, the adult trachea are abnormally branched and nerves near the neuropil are either not present or not air filled in knockdown animals. Knockdown individuals dissected from the pupal case, while morphologically normal, are still inviable. Similar phenotypes have been observed in wishful thinking mutants involved in BMP signaling, raising the possibility of a functional link between zyxin and the BMP pathway.

Differential Activities of the Urochordate Ciona intestinalis Talin-a/b Splice Variant
R. H. Singiser, R. O. McCann; Molecular and Cellular Biochemistry, University of Kentucky, Lexington, KY

Talin, a cytoskeletal linker-protein, is alternatively spliced in Ciona intestinalis. The alternative splice generates two distinct isoforms that contain non-identical I/LWEQ regions, which are important for actin binding and subcellular targeting. The talin splice variant found in Ciona mimic the two isoforms of talin, Taln1/2, found in vertebrates. Taln2 is found in cardiac and skeletal muscle in vertebrates. Ciona intestinalis is a model organism for studies of chordate development of heart and muscle tissues. The use of Ciona intestinalis to study the development of both Ciona muscle and cardiac tissue will provide insight in a chordate model system that is applicable to a vertebrate system. An NCBI database analysis revealed predominant expression of Ciona talin-b in blood cells while Ciona talin-a was variably expressed throughout development. Actin co-sedimentation assays demonstrate that both splice variants have the capability to bind actin: however, the talin-a splice variant found in the chordate model system mimics the characteristics of Taln1/2 binding in a vertebrate model system. These data suggest the usefulness of Ciona intestinalis as a model system for studying talin function in development. Future studies will clarify isoform specific roles of talin splice variants in Ciona development.

Vimentin Filament Interaction with Focal Contacts Is Mediated by Specific Residues within the β3 Integrin Cytoplasmic Tail: Functional Implications
R. Bhattacharya, J. C. Jones; Cell and Molecular Biology, Northwestern University, Chicago, IL

Angiogenesis, the growth of blood vessels, is essential for tumor survival and is central to pathogenesis of certain diseases including neovascular macular degeneration. Angiogenesis involves endothelial cell migration from preexisting blood vessels, formation of adhesive sites between migrating cells and ECM and assembly of endothelial cells into vessels. Focal contacts (FCs) play a role in each of these processes. Previously, we have demonstrated that vimentin associates with avß3 integrin-rich FCs in endothelial cells. This association depends on integrin activation since IF-Fc interaction is dramatically decreased in endothelial cells plated onto poly-L-lysine but is restored upon addition of Mn2+. Knockdown β3 integrin expression in endothelial cells results in a 70% decrease in the numbers of FCs exhibiting IF association. To provide further support for β3 integrin-mediated vimentin-FC interaction, we transfected GFP-ß3 integrin into CHO cells which lack β3 integrin but contain vimentin. In “parental” CHO cells vimentin IF are collapsed in the perinuclear zone. In CHO cells expressing wild-type β3 integrin (CHOwß3), vimentin extends to FCs. However, in CHO cells (CHOß3Y759F; CHOß3Y747F) expressing mutated β3 integrin, in which either tyrosine residue at position 759 or 747 is replaced by glycine, a non-phosphorylatable mimetic, there is a significant decrease in vimentin-FC interaction compared to CHOwß3 cells. ß3Y759F and ß3Y747F integrin, nonetheless, target to FCs and their expression has no impact on actin organization. We assessed the functional consequences of IF-FC interactions in CHO cells. Using trypsinization and shear stress assays, our results indicate that CHOwß3 cells exhibit significantly greater angiogenic activity compared to CHOß3 cells. Moreover, cultures of CHOwß3 cells close in vitro wounds significantly faster than parental CHO, CHOß3Y759F and CHOß3Y747F cells. In summary, our data reveal that β3 integrin mediates vimentin-FC association and that vimentin-FC interactions regulate the adhesive function of these proteins and their role in migration.

The Phosphotyrosine-independent Interaction of DLC-1 and the SH2 Domain of Cten Regulates Focal Adhesion Localization and Growth Suppression Activity of DLC-1
Y. Liao, L. Si, S. Lo; Center for Tissue Regeneration and Repair, Department of Orthopaedic Surgery and Cancer Center, University of California, Davis, Sacramento, CA

DLC-1 was further confirmed by immunoprecipitation and mammalian two-hybrid assay. By yeast two-hybrid analyses with truncated constructs, we demonstrated that the regions responsible for the binding are the SH2 domain on cten and 1-535 amino acids on DLC-1. The interaction is independent of tyrosine phosphorylation of DLC-1, which is a new binding feature of tensins.

CaMK-II Promotes Focal Complex Disassembly to Influence Cell Motility
C. A. Easley, R. M. Tombs; Biochemistry, Virginia Commonwealth School of Medicine, Richmond, VA

We also report a novel role for CaMK-II in focal complex dynamics, cell spreading and motility. Cells expressing constitutively

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active CaMK-II exhibit decreased cell adhesion and fail to migrate on FN. In contrast to cells expressing wild type CaMK-II, cells expressing constitutively active CaMK-II lack focal complexes as determined by GFP-paxillin and GFP-FAK. Focal complexes can be recovered within 1 hour using a membrane permeant, CaMK-II specific, catalytic inhibitor. Using this inhibitor alone, cell spreading is enhanced and focal complexes are enlarged as determined using Total Internal Reflection Fluorescence microscopy (TIRFM). Inhibition of CaMK-II activity also blocks cell motility. Thus, full inhibition and full activation of CaMK-II disrupt cell motility. Taken together, these results indicate that CaMK-II is activated by FN to promote focal complex disassembly to enable cell motility.

1012 Zyxin-dependent Regulation of Actin Stress Fiber Reinforcement
L. M. Hoffman, C. C. Jensen, A. Chaturvedi, M. Yoshigi, M. C. Beckerle; Huntsman Cancer Institute, University of Utah, Salt Lake City, UT
Control of actin filament assembly and dynamics is critical for cell motility, and mechanical stability of cells. The focal adhesion protein, zyxin, interacts with p130Cas, alpha-actinin and Ena/VASP family members and has been shown to promote actin assembly when targeted to the inner leaflet of the plasma membrane or to mitochondria. Here we describe efforts to characterize the contribution of zyxin to the assembly of stress fibers, large bundles of actin filaments that are prominent architectural features in cultured fibroblasts. Exposure of cells to the actin stabilizing agent, jasplakinolide, promotes the formation of robust actin stress fibers in fibroblasts. Comparison of the response of wild-type and zyxin-null cells to jasplakinolide reveals that zyxin is required for robust assembly of stress fibers. Likewise, zyxin is required for reinforcement of actin stress fibers in cells exposed to uniaxial mechanical stress. The reinforcement of actin stress fibers in response to mechanical stress is not dependent on the presence of p130Cas, though p130Cas binds zyxin and has, itself, been implicated in mechanotransduction. Upon either jasplakinolide treatment or mechanical stimulation, zyxin is mobilized from focal adhesions to actin stress fibers while many other focal adhesion components, including vinculin, remain at substratum adhesion sites. Thus zyxin appears to be actively trafficked to the actin stress fibers during the reinforcement process. Stress fiber development is stimulated by activation of Rho-kinase. Interestingly, the zyxin-dependent reinforcement of actin stress fibers in response to mechanical stimulation is not completely blocked by the Rho-kinase inhibitor, Y27632. Moreover, zyxin-null cells display a heightened sensitivity to Rho-kinase inhibitor compared to wild-type cells, suggesting that zyxin and Rho-kinase act in parallel to influence actin stress fiber assembly.

1013 Genomic Analysis of the Cas Family Identifies a Novel Cas-related Protein, HEF1-Efs-p130cas-Like (HEPL), and a Complex Family Splice Structure
M. K. Singh,1 D. Dadic,2 E. Nicolás,3 S. Apostolou,1 A. Canutescu,1 I. Serebriskii,1 E. A. Golemis,1 Basic Sciences, Fox Chase Cancer Center, Philadelphia, PA, 2Cancer Research Program, Stem Cell Research Center, Manipal Hospital, Bangalore, India
Members of the Cas protein family of multi-domain scaffolding proteins are important mediators of cell attachment, motility, and survival, and have also been implicated in other processes including cell cycle control and vascular trafficking. To better understand the function of the known Cas proteins (p130Cas, HEF1/NEOD9/Cas-L, and Efs/Sin), we performed a coupled genomic, expression, and functional analysis of the Cas protein group. By this means, we have identified a completely novel member of the Cas protein family, termed HEPL, which is conserved through lower vertebrates. We have used quantitative real time reverse transcriptase-polymerase chain reaction (RT-PCR) to determine that the human HEPL is expressed in many cell lines and tumors, and may influence Cas family function. In functional analyses, we have compared HEPL protein localization, association, and action with that of other family members. We have also mined through genomic resources to better define the splice structure of the Cas gene family. This has led to the identification of both evolutionarily conserved and recent splice variants, many of which are not detectable with standard tools for study of Cas proteins, but which are abundant at the mRNA level in many cell types. In functional studies of HEF1 splice variants, we have demonstrated specific variants oppose the activity of canonical HEF1, inhibiting cell spreading and FAK activation. The identification of HEPL and the Cas family splice variants, the majority of which are not distinguishable by currently available antibodies to the Cas family proteins, provides an important context for further studies of this protein group.

1014 Hypermobility Induces Reorganization of Actin Stress Fiber Assembly in Osteoblastic MC3T3-E1 Cells
Y. Miyamoto, Y. Ishikawa, E. Shimura, Y. Mogami; Department of Biology, Ochanomizu University, Tokyo, Japan
Actin stress fibers, which are linked to integrin in focal adhesions, play a central role in cell movement and cell adhesion. Recently, the network of stress fibers and focal adhesions has been reported to be responsible for mechano-sensing. Based on the hypothesis that the network also senses mechanical loading due to gravity, we examined the effect of hypermobility on the reorganization of stress fiber assembly in osteoblastic MC3T3-E1 cells by fluorescence microscopy. Under hypermobility generated by centrifugation, we observed that the hypermobility (60G, 2h) enhanced the assembly of the stress fiber in MC3T3-E1 cells. Hypermobility also enhanced recovery of the partially disrupted stress fiber network induced by adding 1.25 μg/ml cytochalasin B. However, there was no apparent recovery when hypermobility was applied to cells with a substantially disrupted network. In addition, after cessation of hypermobility, the recovery effect was reversed to a point midway between the 60G-loaded state and the non-loaded state (1G) with cytochalasin B, suggesting that the hypermobility response is reversible and induces reorganization of the stress fiber assembly. Next, we examine the roles of integrin in the hypermobility response, an inhibitory peptide (RGD), which interferes with the RGD-dependent integrin binding to extracellular matrix, was added to the cells. The RGD peptide induced the partial disruption of the network of the stress fiber. However, there was no disruption of the network by RGD peptides under hypermobility and, no hypermobility-induced rescue of the disruption by RGD peptide in the presence of an inhibitor of Rho kinase, Y-27632, indicating that Rho kinase plays an essential role in the rescue. These findings suggest that mechanical load due to gravity may be sensed via a network of stress fibers in which non-RGD dependent integrins may play some complementary roles in the reorganization of the fiber assembly.

1015 Mutations in the Conserved WDF Motif of the Integrin beta 1 Tail: Effects on Integrin-Talin Interactions, Cell Adhesion, and Adhesion Signaling
B. L. Nieve,1 C. H. Reverter,1 A. Benware,2 S. E. LaFlamme; Center for Cell Biology and Cancer Research, Albany Medical Center, Albany, NY
Integrin-mediated cell adhesion is essential for tissue homeostasis as well as for many biological processes including development, wound healing, tumor metastasis and angiogenesis. The highly conserved integrin beta subunit cytoplasmic domain is thought to play a central role in these processes by regulating the formation of signaling complexes that promote integrin activation, cell adhesion and adhesion-dependent processes. Talin, a major cytoskeletal linker protein, has been implicated in mediating the inside-out activation of integrins through the binding of its IT-domains to the central portion of the integrin beta tail (WDTANNPLYK in the case of the beta1 tail). Using HIS-tagged fusion proteins containing wild-type and mutant beta tails, we demonstrated that alanine substitutions for the WD or WT residues within the corresponding WDTGENPIYK region of the beta1 tail impaired talin-binding in vitro. The WDTANNPLYK sequence is highly conserved among integrins and is thought to mediate receptor-ligand interactions in the extracellular domain. Recently, it has been shown that talin-binding to extracellular matrix, was added to the cells. The RGD peptide induced the partial disruption of the network of the stress fiber. However, there was no apparent recovery when hypermobility was applied to cells with a substantially disrupted network. In addition, after cessation of hypermobility, the recovery effect was reversed to a point midway between the 60G-loaded state and the non-loaded state (1G) with cytochalasin B, suggesting that the hypermobility response is reversible and induces reorganization of the stress fiber assembly. Next, we examine the roles of integrin in the hypermobility response, an inhibitory peptide (RGD), which interferes with the RGD-dependent integrin binding to extracellular matrix, was added to the cells. The RGD peptide induced the partial disruption of the network of the stress fiber. However, there was no disruption of the network by RGD peptides under hypermobility and, no hypermobility-induced rescue of the disruption by RGD peptide in the presence of an inhibitor of Rho kinase, Y-27632, indicating that Rho kinase plays an essential role in the rescue. These findings suggest that mechanical load due to gravity may be sensed via a network of stress fibers in which non-RGD dependent integrins may play some complementary roles in the reorganization of the fiber assembly.

1016 TRPM7 Regulates Cell Adhesion and Membrane Protrusion by Controlling the Calcium Dependent Protease Calpain
L. Su,1 M. Agapito,1 M. Li,2 A. Sato,2 W. Simonson,4 A. Huttenlocher,4 R. Habas,3 L. Yue,2 L. Runnels1; 1Pharmacology, University of Medicine & Dentistry of NJ-Robert Wood Johnson Medical School, Graduate School of Biomedical Sciences, Piscataway, NJ, 2Cell Biology, University of Connecticut Health Center, Farmington, CT, 3Biochemistry, University of Medicine & Dentistry of NJ-Robert Wood Johnson Medical School, Piscataway, NJ, 4Cell Biology, University of Medicine & Dentistry of NJ-Robert Wood Johnson Medical School, Piscataway, NJ, 5Pharmacology, University of Wisconsin, Madison, WI
TRPM7 is a bifunctional protein with ion channel and kinase activities that is a member of the transient receptor potential (TRP) ion channel family. M-calpain is a protease implicated in the control of cell shape, cell locomotion, and focal adhesion dynamics. Tenase complex, a membrane component with m-calpain in spatially and temporally controlled is not well understood, particularly because calpain’s dependence on calcium exceeds the sub-micromolar concentrations normally observed in cells. Here we show that the channel-kinase TRPM7 localizes to peripheral adhesion complexes with m-calpain, where it regulates cell adhesion by controlling the activity of the protease. Our research revealed that overexpression of TRPM7 in HEK-293 cells caused cell rounding with a concomitant loss of cell adhesion that is dependent upon the protein’s channel but not its kinase activities. Knockdown of m-calpain blocked TRPM7-induced cell rounding and cell detachment. Silencing of TRPM7 by RNA interference, however, strengthened cell adhesion and increased the number of peripheral adhesion complexes in cells. Together, our results suggest that TRPM7 regulates cell adhesion through m-calpain by mediating local influx of calcium into peripheral adhesion complexes. To verify TRPM7’s impact on cell adhesion and cell spreading, we created a Swiss 3T3 fibroblast cell line in which the protein’s channel has been knocked-down by RNA interference. TRPM7 knockdown fibroblasts attached and spread more rapidly to the substrate, as well as increased the frequency of membrane protrusion and retraction, indicating that the channel may play a role in regulating lamellipodia stability. Finally, to confirm
that TRPM7 is regulating cell motility we investigated the role of TRPM7 during Xenopus development. Depletion or overexpression of TRPM7 blocked gastrulation cell movements and neural fold closure, making TRPM7 the first channel required for early development. Together, our findings support a novel role of TRPM7 in regulating cell adhesion and membrane protrusions in migrating cells.

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Deficiency of Tenascin-C Attenuates Liver Fibrosis in Immune-mediated Chronic Hepatitis in Mice


Tenascin-C (TNC), an extracellular matrix glycoprotein, is upregulated in chronic liver disease. Here, we investigated the contribution of TNC to liver fibrogenesis by comparing immune-mediated hepatitis in wild-type (WT) and TNC-null (TNKO) mice. Eight-week-old BALB/c mice received weekly intravenous injections of Concanavalin A to induce hepatitis, and were sacrificed one week after the 3rd, 6th, 9th and 12th injections. In WT livers, TNC expression in both protein and mRNA levels gradually increased, and peaked after the 9th injection. Collagen deposition stained with picrosirius red was significantly less intense in TNKO mice than in WT mice, and procollagen I and III transcripts were significantly upregulated in WT mice compared with TNKO mice. Inflammatory infiltrates were more prominent after the 3rd-6th injections in both groups and were less intense in TNKO mice than in WT mice. Levels of Interferon-γ and interleukin-1β mRNA were significantly higher in WT mice than in TNKO mice, while desmin-positive hepatic stellate cells (HSCs) and myofibroblasts, a cellular source of TNC and procollagens, were more common in WT livers. Transforming growth factor (TGF)-β1 mRNA expression was significantly upregulated in WT mice, but not in TNKO mice. TNC can promote liver fibrogenesis through enhancement of inflammatory response along with cytokine upregulation, HSC recruitment and TGF-β expression during progression of hepatitis to liver fibrosis.

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Deficiency of Tenascin C Attenuates Allergen-induced Bronchial Asthma in the Mouse

H. Nakahara, E. C. Gabazza, T. Fujimoto, K. Imanaka-Yoshida, T. Yoshida, O. Taguchi; Department of Pulmonary and Critical Care Medicine, Mie University School of Medicine, Tsu, Japan

Tenascin C is a phosphatide matrix glycoprotein whose expression is increased in several inflammatory diseases of the lung including bronchial asthma. However, the exact function of tenascin C in the pathogenesis of lung inflammation remains unclear. In the present study, we compared the degree of allergic airway inflammation between wild type and tenascin C-deficient (-/-) Balb/c mice. Bronchial asthma was induced by sensitization and challenged with ovalbumin. Littermates treated with saline were used as controls. Cytokines in bronchoalveolar lavage fluid and proteins produced by lung specific epithelial cells were measured. The number of epithelial cells in the lung was significantly increased in the wild type compared with tenascin-C knockout mice. Airway hyperreactivity, NF-kappaB activation and the bronchoalveolar lavage fluid concentrations of monocyte chemoattractant protein-1, interleukin-5, interleukin-13, metalloproteinase-9 and immunoglobulin-E were significantly higher in ovalbumin sensitized/challenged tenascin-C knockout mice compared with their wild type counterparts. In vitro experiments disclosed that tenascin-C significantly stimulated the secretion of interleukin-5, interleukin-13, interferon-gamma and immunoglobulin-E from spleen lymphocytes. These observations suggest that tenascin C is involved in the pathogenesis of bronchial asthma.

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The Effects of Tenascin-W during Osteogenesis

C. V. Meloty-Kapella, M. Heges, R. Chiquet-Ehrismann, R. Tucker; Cell Biology and Human Anatomy, University of California at Davis, Davis, CA

Primary Keratinocytes, SIK Immortalized Keratinocytes and Transformed SCC4 Cells Differentially Respond to Biologically Relevant Topographic Cues

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UV-Modulatable Substrates for Spatio-Temporal Mechanotransduction Studies

M. T. Frey, N. Burnham, Y. Wang; Physiology, University of Massachusetts Medical School, Worcester, MA

Polyacrylamide gels have been used widely for cell culture studies as substrata for their favorable optical properties and the ability to tailor their mechanical properties. Understanding cell response to substrate stiffness is important in the fields of developmental biology, regenerative medicine, wound healing, and tissue engineering. Similar to surface biochemistry, the mechanical properties of substrata provide a biological cue that affects cell behavior, such as spreading and migration speed. However, these studies are done in parallel fashion and yield no information regarding the transition dynamics of cell behavior during changes in substrate stiffness. We have created a model system for such a study by modifying polyacrylamide with a photo-activatable crosslinking agent. Cells readily adhere to this substrate after addition of fibrinogen. Based on atomic force microscopy measurements, ultraviolet light exposure reduces the substrate stiffness. Cells on the substrate before and after exposure behave in a similar fashion to cells on stiff and soft substrata respectively, while cells on glass appear to be unaffected by the same intensity of exposure. This novel substrate allows for transition dynamics of cells to be studied as a function of changing stiffness.

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Primary Keratinocytes, SKI Immortalized Keratinocytes and Transformed SCC4 Cells Differentially Respond to Biologically Relevant Topographic Cues

S. J. Lilenstein, P. F. Nealey, C. J. Murphy; Surgical Sciences, University of Wisconsin, Madison, WI

Polyacrylamide gels have been used widely for cell culture studies as substrata for their favorable optical properties and the ability to tailor their mechanical properties. Understanding cell response to substrate stiffness is important in the fields of developmental biology, regenerative medicine, wound healing, and tissue engineering. Similar to surface biochemistry, the mechanical properties of substrata provide a biological cue that affects cell behavior, such as spreading and migration speed. However, these studies are done in parallel fashion and yield no information regarding the transition dynamics of cell behavior during changes in substrate stiffness. We have created a model system for such a study by modifying polyacrylamide with a photo-activatable crosslinking agent. Cells readily adhere to this substrate after addition of fibrinogen. Based on atomic force microscopy measurements, ultraviolet light exposure reduces the substrate stiffness. Cells on the substrate before and after exposure behave in a similar fashion to cells on stiff and soft substrata respectively, while cells on glass appear to be unaffected by the same intensity of exposure. This novel substrate allows for transition dynamics of cells to be studied as a function of changing stiffness.
Agrin as a Cell Adhesion Molecule on Nanopatterned Substrates

T. Wolfram,1 T. Schoen,2 J. P. Spatz;3 Institute for Molecular Biophysics, The Jackson Laboratory, Bar Harbor, ME, 1New Materials and Biosystems, Max-Planck Institute for Metals Research, Stuttgart, Germany, 2New Materials and Biosystems, Biophysical Chemistry, Max-Planck Institute and University of Heidelberg, Stuttgart, Heidelberg, Germany

Agrin is a heparane sulfate proteoglycan known predominantly for its inductive role during the formation of the neuromuscular junction. Agrin is also expressed in several other tissues including those of the central nervous system (CNS). To investigate the role of agrin in the CNS we designed a sophisticated cellular adhesion assay using nanostuctured surfaces. Our assay system allows us to address the hypothesis that the c-terminal domains of agrin can facilitate adhesion of neural and neuronal cells. Additionally we were able to investigate the amount of proteins necessary for cell adhesion. We generated substrates with a defined chemical environment, controlling the amount of agrin and the orientation of the agrin molecules in a side directed manner by binding single molecules to gold nanodots. The area around the gold dots was coated with a cell repellent polyethylene glycol (PEG) layer. Functionality of the substrates was confirmed by immunofluorescence (IF) microscopy and atomic force microscopy (AFM). Single molecules were detected by immuno-gold labelling. Agrin is functional as a cell adhesion molecule for a variety of neural cell lines such as neuroblastoma cells SH-SY5Y, mouse neuroblastoma cells N2a and rat neuroblastoma cells B153 as well as primary cortical neurons from C57/B16 wild type mice. In contrast, agrin was a less sufficient substrate for the adhesion of primary mouse embryonic fibroblasts. We also were able to show that agrin has to be offered at a certain density (~300 molecules/μm) on the substrate to facilitate cell adhesion. Furthermore with peptide-inhibition assays we identify possible molecular mediators of the cell adhesion function of agrin. The key regulators for this function could be laminin dependent integrins via modulation of actin cytoskeleton elements. Our results indicate that agrin is a potentially important component of the cell adhesion process in the central nervous system.
Collagen deposition and epithelial-mesenchymal transdifferentiation (EMT) play very important role in pathophysiology of chronic kidney failure. Collagen contains two receptors, integrin and discoidin domain receptor (DDR1). We previously demonstrated that DDR1 served to counteract TGFβ1 integrin mediated cell migration induced by collagen. In this study, we wished to study the function of DDR1 in cell differentiation and EMT in renal epithelial cells by employing MDCK cells stably transfected with DDR1, or dominant negative (DN)-DDR1. We found that collagen markedly inhibited microvilli formation in MDCK cells. Overexpression of DDR1 prevented, whereas DN-DDR1 augmented collagen-induced reduction of microvilli. In addition, we showed that DDR1 overexpression prevented TGFβ1-induced mesenchymal morphological changes upon collagen stimulation, whereas DN-DDR1 promoted mesenchymal morphology even without TGFβ1 stimulation. Immunofluorescence studies showed DDR1 overexpression prevented TGFβ1-induced E-cadherin mislocalization from cell-cell junction to cytosol, whereas DN-DDR1 promotes this phenomenon even without TGFβ1 stimulation. Interestingly, we found that cells overexpressing constitutive active β1-integrin exhibited E-cadherin mislocalization and mesenchymal morphology. Taken together, these results indicate that DDR1 promotes cell differentiation upon collagen stimulation and inhibits TGFβ1-induced EMT.

Prox1 and Podoplanin Are Required for Lymphatic Endothelial Capillary Tube Formation

A. Navarro, M. Rezaee chalkagh, S. Mabry, D. Xu, W. E. Tsou, J. I. Ekekeze; Pediatrics/Neonatology, Children's Mercy Hospitals and Clinics/University of Missouri-Kansas City School of Medicine, Kansas City, MO

Vascular networks derive from endothelial cells through vasculogenesis - nascent vessel formation, or via angiogenesis - sprouting from existing vessels. An important step in both processes is tubulogenesis, whereby endothelial cells align themselves to form vessels. Factors orchestrating tubulogenesis remain poorly understood. Prospero-related homeobox 1 (Prox1) is a transcription factor selectively expressed in endothelial cells of lymphatic nature. Because it is known that Prox1 is required for the development of the murine lymphatic system, our objective was to investigate the role of the Prox1 protein in the process of capillary tube formation in lymphatic endothelial cells derived from human lung. Culture in matrigel is a well-established method of studying endothelial cell tubular network formation in vitro. When using siRNA to block the expression of Prox1 in lung lymphatic endothelial cells, we could observe no effect on either cell morphology or viability when cultured under normal conditions, but the arrangement of the lymphatic endothelial cells into capillary tubes in vitro (on Matrigel) was severely compromised. This effect was specific to the lack of Prox1 protein, since it did not occur in cells treated with a comparable siRNA (scrambled sequence) or if the cells used were blood vascular endothelial cells derived from human lung. Similar results were obtained when using siRNA specific for Podoplanin, a type 1 membrane protein and specific marker for lymphatic endothelial cells. These results identify a critical role for Prox1 and Podoplanin in the endothelial cell cytoskeletal dynamic changes associated with the process of tubulation formation.

Urokolinase-derived Peptide Inhibitor of the uPA/uPAR System Inhibits Microvascular Permeability

D. Navarrate, A. Das, G. Memucci, P. G. McGuire; 1Cell Biology, Cell Biology, School of Medicine, University of New Mexico, Albuquerque, NM, 2Surgery & Ophthalmology, School of Medicine, University of New Mexico, Albuquerque, NM

Breakdown of the blood-retinal barrier (BRB) is a central event in early diabetic retinopathy. The progressive activation of extracellular proteases in later diabetic events like retinal neovascularization has been well-documented. However, their role in early retinal permeability changes remains unexplored. In an animal model of diabetes, mRNA levels of both urokinase plasminogen activator (uPA) and its receptor (uPAR) were upregulated in the retinal vasculature after 2 weeks of diabetes. The aim of this study was to determine the role of the uPA/uPAR system in mechanisms affecting retinal permeability. A peptide inhibitor derived from the non-receptor-binding region of urokinase which blocks the interaction of uPA with uPAR (Å6) was used in these studies. Diabetic rats received intraperitoneal injections of Å6 (100mg/kg) daily for 2-weeks after induction of diabetes. Retinal permeability was assayed using the Evans blue technique. Diabetic rats treated with Å6 showed a significant decrease in permeability levels as compared to 100 fold higher basal levels. To elucidate how inhibition by Å6 affects cell junctions, human umbilical vein endothelial cells were treated with AGE-BSA, which induced increases in monolayer permeability, the loss of cell surface VE-cadherin expression and decreased cell-cell contact. All of these effects were reversed by the addition of Å6. These data support a role for uPA and uPAR in the breakdown of the blood-retinal barrier during diabetes and also suggest that inhibition of this interaction might be a promising target for early therapeutic intervention in diabetic retinopathy.

Recreating Adipogenesis In Vitro: A New Approach Exploiting the Biological Properties of Human Adipose-derived Stromal Cells in Tissue Engineering

J. Fradette, M. Vernette, V. Trottier, L. Saint-Pierre; Surgery, Laval University, Quebec, PQ, Canada

Adipogenesis, the differentiation of stromal precursor cells into lipid-filled adipocytes, contributes to the expansion of adipose tissue depots. The lack of adequate in vitro models to study adipogenesis and key aspects of human adipocyte metabolism in a tridimensional (3D) environment prompted us to reconstruct adipose tissue using tissue-engineering approaches. We extracted stromal cells from liposorbed (LA) and excised fat (LP, lpectomy) to assess their capacity to generate 3D adipose tissues using a « self-assembly » culture technique. This method is based on seeding cells to produce their own extracellular matrix following ascorbic acid (AA) stimulation in vitro, avoiding the usual requirement for exogenous biomaterials. Compared to excised fat, stromal cells from liposorptions featured a higher adipogenic potential in vitro (1.6x to 2.0x) at passages 3, 6 and 11 in presence of AA, as quantified after Oil Red O stainings. When cultured for 28 days in presence of AA, both LA and LP-derived stromal cells produced manipulable sheets rich in extracellular matrix components. By inducing adipogenic differentiation at different stages of matrix formation (namely at day 7 or 21 of culture), adipose sheets were produced (3.5 cm² surface area), featuring human adipocytes at early or late stages of differentiation. These adipose sheets were then layered and assembled into thicker tissues (146± 8.7 µm, 3 layers, n=6). This tissue-engineered adipose tissues secreted leptin and adiponectin in culture. Treatment with the β-adrenergic agonist isoproterenol (0.25 µM) stimulated lipolysis 2.0 fold over basal levels, as determined by glyceral release. Taken together, these results establish the functionality of the reconstructed adipose tissues. In conclusion, this new tissue-engineered model of adipose tissue will allow us to study not only the regulation of adipogenesis in a 3D context in vitro, but also the metabolism of human adipocytes at defined stages of differentiation under various pharmacological treatments.

A Specific Immunocapture Activity Assay for Matrix Metalloproteinases Using 5-FAM/QXL 520™ FRET Peptide

Y. Tang, X. Li, X. Zhu, X. Han, Z. Diwu; AnaSpec, Inc., San Jose, CA

Matrix metalloproteinases (MMPs) belong to a family of secreted or membrane-associated zinc endopeptidases capable of digesting extracellular matrix components. MMPs play roles in the multiple stages of tumor development and metastasis, rheumatoid arthritis, wound healing, angiogenesis, as well as in other pathological and physiological events. Many assays, such as zymography, ELISA, and assay-based activity assays, have been developed to facilitate the research and drug discovery for MMPs; however, zymography is a relatively low-throughput method, and ELISA does not provide MMP activity information. In contrast, FRET-based activity assay has gained popularity because of its simple and fast format — a feature ideal for large scale drug compound screening. When a FRET-peptide based assay is applied to biological samples, it measures non-specific cleavages of the different MMPs and even from other non-selective proteases, which maybe simultaneously present in biological fluids. To solve this problem of non-specific cleavages, an anti-MMP is first used to capture a specific MMP from the mixture, and then the activity of this particular MMP is measured by a FRET-peptide substrate. In previous studies, we have developed highly sensitive FRET-peptide substrates for MMPs using the strongly-fluorescent 5-FAM as the FRET donor and QXL™520 as the quencher. In this current study, we incorporate the 5-FAM/QXL™520 FRET-peptide substrate to the above specific immunocapture assay. This results in a highly sensitive, specific detection of MMP-1, MMP-9 or MMP-13 activity in mixed biological samples.

Lycopene Inhibits Tumor Cell Invasion and MMP-2 Activity in a Frog Renal Adenocarcinoma Cell Line

P. Dalvi, P. Leggett-Robinson, M. Martinez, R. M. Troy; 1Biology, Tuskegee University, Tuskegee, AL, 2Chemistry, Tuskegee University, Tuskegee, AL, 3Biology/RCMI, Tuskegee University, Tuskegee, AL

One of the steps in the pathway leading to invasion and metastasis in cancer involves the over-expression of the matrix metalloproteinases (MMPs) by tumor cells. These enzymes, involved in the degradation of the extracellular matrix (ECM), can thus be identified as one of the determinants of whether the cancer cell undergoes metastasis. Therapeutic regimes that inhibit the activation of the MMPs might prove to reverse the poor prognosis associated with metastatic cancers. In recent years, dietary natural products have been shown to exhibit anti-cancer activity and might be utilized not only as chemopreventive agents but might be efficacious in a chemotherapeutic manner. In this study, lycopene, extracted from commercially available tomato paste, was examined for its ability to inhibit tumor cell invasion and MMP-2 activity in the PNKT-4B frog renal adenocarcinoma cell line that exhibits temperature-dependent invasion. Cells incubated at a low temperature (18°C) had an invasive capacity of approximately 18% as determined by the matrigel assay. However, at a higher temperature (28°C), invasion was approximately 89%. Inhibition of the highly invasive temperature was examined by incubating cells with 2.5, 5.0, and 10 µM of lycopene. The lycopene was extracted from 15 g of commercial tomato paste, resolved on TLC plates, and further characterized by UV-VIS spectrometry to determine purity. Lycopene displayed a maximum inhibition of invasion (72%) at a concentration

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of 5 μM. To determine the mechanism of lycopene inhibition, MMP-2 activity at 28°C (the invasive-permissive temperature) was analyzed since this MMP has a strong correlation to invasion and metastasis. Activity was only inhibited by 29% as determined by gelatin zymography. These data show that the mechanism of lycopene inhibition may in part be due to inhibition of active MMP-2 and that other MMPs or other proteins may be involved in the inhibition of tumor cell invasion.

1033 Vibrio Vulnificus Secretes a Broad Specificity Metalloprotease with Blood Clotting Modulating and Cell Death Inducing Properties

N. H. Lee, A. K. Chang, J. E. Park, J. S. Lee; Chosun University, Gwangju, Republic of Korea
Vibrio vulnificus is a marine bacterium and a human pathogen capable of causing wound infection and sepsis. In this study we show that a metalloprotease (vEP) secreted by V. vulnificus ATCC 29207 could activate prothrombin and cleave fibrinogen as well as cross-linked fibrin. Furthermore, it is capable of activating a mutant prothrombin-3 (D3A) in vitro. D3A lacks the specific aspartate residues that form the cleavage sites in wild-type prothrombin-3, and activation occurred through cleavage of the zymogen at a site close to the native cleavage site. Since vEP is likely to be secreted into the blood stream during infection the ability of other virulence factors within the host tissue is of major interest to our investigation. We examined the cytotoxicity exerted by vEP on two mammalian cultured cell lines, NIH3T3 and HeLa cells. vEP exhibited cytotoxic activity on both cell lines, but the effect was stronger for NIH3T3 cells, with less than 50% cell viability remaining at 5 μg/ml compared with about 70% for HeLa cells. However, vEP did not induce apoptosis as cells treated with vEP appeared to show no typical apoptotic markers, such as DNA fragmentation and caspase-3 activation, although the enzyme appeared to enter the cells as revealed by confocal microscopy images of cells treated with a fluorescein-labeled recombinant vEP. Activation of caspase-3 was not observed despite the presence of cell death. It appears that cell death (and hence possible host tissue damage in vivo) could be caused by direct degradation of extracellular matrix proteins (ECM) of the cells, since vEP could degrade proteins such as collagen and elastin, which are components of the ECM although the exact mode of cell death induction evoked by vEP and the response of cell toward vEP have yet to be elucidated.

1034 CB1 Cannabinoid Receptor-mediated Activation of Endothelial Nitric Oxide Synthase Regulates MMP Activity in Endothelial Cells

I. Johnson, S. Mukhopadhyay; Program in Neuroscience, Biomedical Biotech Research Institute, North Carolina Central University, Durham, NC, Program in Neuroscience, Chemistry-Biochemistry, Biomedical Biotech Research Institute, North Carolina Central University, Durham, NC
Endogenous cannabinoid anandamide produced nitric oxide acting on G-protein coupled CB1 cannabinoid receptor as well as non-CB1/CB2 “anandamide receptor via the activation of endothelial nitric oxide synthase (eNOS) in endothelial cells (McCollum et al., FASEB J 18(4) pA322, 2004). Endothelial nitric oxide synthase has 6 distinct Ser/Thr/Tyr potential phosphorylation sites and it was hypothesized that activation of G-protein coupled CB1 cannabinoid receptor or non-CB1(non-CB2) anandamide receptor produces differential phosphorylation of these residues via the activation of different kinase cascades in endothelial cells. In the present study we showed that activation of CB1 receptor and anandamide receptor produced phosphorylation Ser117 and Ser615 of eNOS protein respectively in human aortic EAhy926 cells. Further, we showed that CB1 cannabinoid receptor-mediated phosphorylation of Ser117 is correlated with the decrease in MMP2 and MMP9 activity in endothelial cells. On the otherhand, phosphorylation of Ser615 of eNOS protein was correlated with activation of MMP9 activity. Thus, our results showed that activation of CB1 receptor and anandamide receptor has differential kinetics of phosphorylation for these residues and results in differential regulation of MMP activity in endothelial cells. (This study was supported by U24 DA12385)

1035 Regulation of Matrix Metalloproteinase-9 Expression by the Large GTPase Murine Guanylate Binding Protein-2

S. Balasubramanian, A. F. Messmer, D. J. Vestal; Department of Biological Sciences, University of Toledo, Toledo, OH
Matrix metalloproteinases (MMPs) are a family of extracellular proteases involved in a variety of developmental and pathological changes that involve tissue remodeling. MMPs can collectively cleave all components of the extracellular matrix. MMP-9 in particular is important because its substrates include collagen IV, entactin, and laminin. These are all components common to all basement membranes. Consequently, MMP-9 is believed to play a critical role in metastasis and angiogenesis. Despite its importance, much remains unclear about how MMP-9 is regulated. Many cytokines and growth factors up-regulate MMP-9 expression, including TNF-α. However, interferons (IFNs) β and γ are among the few cytokines known to down-regulate cytokine induced expression of MMP-9. In NIH 3T3 cells, IFN-γ can inhibit both basal expression and TNF-α induction of MMP-9. Murine Guanylate Binding Protein-2 (mGBP-2), a GTPase, robustly expressed upon exposure to IFN-γ, lowers both basal and TNF-α induced expression of MMP-9 by inhibiting its transcription. This inhibition proceeds, at least in part, through inhibition of NF-κB. As described above, TNF-α induction of MMP-9 occurs through the degradation of IκBα and release of NF-κB, facilitating translocation of NF-κB to the nucleus and induction of transcription. In the presence of mGBP-2, the degradation of IκBα, initiated by TNF-α proceeds normally but the levels of IκBα are restored more rapidly. In addition, TNF-α induction of MMP-9 proceeds through Rac activation of NF-κB activity, an activation inhibited by mGBP-2. These studies begin to define the mechanism(s) by which mGBP-2 inhibits the expression of MMP-9. Work is currently underway to define how mGBP-2 inhibits NF-κB mediated expression of MMP-9.

1036 Interleukin-1β Causes Induction and Activation of Matrix Metalloproteinase-12 in Articular Chondrocytes via Mitogen-activated Protein Kinase Pathway

H. Oh, B. Yu, J. Kim, J. Chun; Life Science, GIST, Gwangju, Republic of Korea
Interleukin (IL)-1 is a major pro-inflammatory cytokine that causes destruction of articular cartilage via induction of several matrix-metalloproteinases (MMPs). We examined expression of MMP family in IL-1β-treated primary culture articular chondrocytes. IL-1β caused induction of MMP-1, MMP-3, MMP-9, MMP-12, and MMP-13 expression in articular chondrocytes. Although expression of MMP-12 in macrophages and other cell types are well known, functional role of MMP-12 in chondrocytes remains largely unknown. We found in this study that IL-1β caused induction of MMP-12 expression in both primary culture articular chondrocytes and cartilage explants. IL-1β also caused secretion of both latent and active form of MMP-12 into conditioned medium. Among the signaling pathways activated by IL-1β, inhibition of extracellular signal-regulated kinase-1/2 (ERK 1/2) with PD98059 or p38 kinase with SB202190 blocked expression and activation of MMP-12 in articular chondrocytes. However, inhibition of c-Jun N-terminal kinase with SP600125 did not affect IL-1β-induced MMP-12 expression and activation. Because MMP-12 is known to digest several extracellular matrix components such as type IV collagen and proteoglycan and activate other MMPs such as MMP-2 and MMP-3, our results suggest that IL-1β-induced expression and activation of MMP-12 through ERK 1/2 and p38 kinase may regulate cartilage destruction.

1038 Matrix Metalloproteinase Cleavage of EphB2 Receptor Converts Cell Adhesion to Repulsion

K. Lin, D. W. Ethell, I. M. Ethell; Division of Biomedical Sciences, UC Riverside, Riverside, CA
Eph receptor tyrosine kinases and their ephrin ligands play important roles in cell migration, axon guidance and synaptogenesis. Eph/ephrin signaling is bidirectional and result in cell repulsive or sometimes adhesive responses depending on cell type and developmental stage. Since both Eph receptors and ephrin are membrane-bound, their activation requires cell-cell adhesion between cells expressing Eph receptors and those expressing ephrins. The mechanism by which these high-affinity interactions between receptors and ligands break to convert adhesion to repulsion remains enigmatic. Here, we show that Eph receptor is cleaved by matrix metalloproteases (MMPs) both in vitro and in vivo, and this cleavage is induced by EphB2 interaction with its ligand, ephrin-B2. A subsequent cleavage of EphB2 occurs within the transmembrane region of the EphB2 receptor following MMP cleavage and to produce a cytoplasmic EphB2 cleavage product. Our findings suggest that EphB2 cleavage may contribute to cell-cell repulsion induced by receptor-ligand interaction. Mutations at two putative MMP cleavage sites in the extracellular portion of the EphB2 receptor produced a non-cleavable EphB2-4/5 mutant that inhibited ephrinB2-mediated cell-cell repulsion and blocked axon withdrawal in cultured hippocampal neurons. Although the EphB2-4/5 mutant retained its ability to be activated/phosphorylated upon interaction with its ligand ephrinB2 (after 5 min induction), in contrast to wild type EphB2 (wtEphB2), non-cleavable EphB2-4/5 is rapidly dephosphorylated after 15 min. This pattern of the EphB2 phosphorylation/dephosphorylation suggests that cleavage of the receptor and release of its cytoplasmic domain (cytEphB2) may play an important role in EphB2 signaling. Furthermore, overexpression of cytEphB2 together with EphB2-4/5 induced cell-cell repulsion and axon withdrawal that were similar to wt EphB2. In conclusion, our studies suggest that MMP-mediated cleavage plays an important role in EphB2 receptor function by regulating its signaling.
Multiple Bacterial Species Isoated from Chronic Venous Leg Ulcers Degrades Matrix Substrates

S. Bhalla,1 P. M. Tierno,1 M. Stevens-Riley,1 R. Propst,1 A. B. Wysoczki,1 State University of New York at Stony Brook, Stony Brook, NY, 4New York University Medical Center, New York, NY, 3Food and Drug Administration, Rockville, MD, 2University of Mississippi Medical Center, Jackson, MS

Serine and matrix metalloproteinases (MMP) are activated and overexpressed in chronic wound fluid leading to delayed healing via degradation of extracellular matrix. Bacteria are another potential source of proteolytic activity and can secrete proteases capable of degrading matrix and activating matrix metalloproteinases. However, the types of bacteria isolated from chronic venous ulcers that can secrete proteases capable of degrading matrix substrates have not been well characterized. Using an approved protocol we isolated and identified bacteria collected from six subjects over two to six months. We then tested media from bacterial isolates for its ability to degrade either gelatin, elastin, or N-benzyol- DL-arginine-p-nitroanilide (BAPNA), a serine/ threonine substrate. The total number of different bacterial isolates ranged from 1 - 6 for any individual. Bacteria with protease activity were cultured from both chronic venous leg ulcers and a control site where the skin was intact. The 13 bacteria that expressed proteolytic activity against the gelatin substrate and nine against an elastin substrate and eight isolates were found to have protease activity against BAPNA. Five isolates had activity against only one substrate; three isolates had activity against two substrates; and five isolates had activity against all three substrates tested. We conclude that multiple bacterial species from colonized wounds and intact skin secrete bacterial proteases capable of degrading matrix substrates. The ability of protease secreting bacteria to degrade gelatin, a breakdown product of collagen, elastin, and a serine substrate suggests that bacteria have the capacity to degrade components of the extracellular matrix important for wound healing, thus contributing to the delayed healing seen in chronic wounds.

Arginyl Aminopeptidase-like 1 (RNPEPL1) Is a Ubiquitously Expressed, Alternatively Processed Metallopeptidase with Preference for Neutral and Aromatic Amino Acids

M. W. Thompson, R. L. Seipel; Biology, Middle Tennessee State University, Murfreesboro, TN

Arginyl aminopeptidase-like 1 (RNPEPL1) represents a novel member of the M1 metallopeptidase family, which includes aminopeptidase B and leukotriene A4 hydrolase. A cDNA encoding human RNPEPL1 was amplified from a cdna library and subcloned into an expression vector to investigate its expression and functions. Recombinant RNPEPL1 expressed in Saccharomyces cerevisiae exhibited a preference for the aminoacyl-AMC analogs of methionine and glutamine as measured by kcat/km values, but also possessed appreciable activity towards tyrosine and phenylalanine-AMC. RNPEPL1 also demonstrated weak activity towards citrulline-AMC, with a kcat of 0.56 mM and a kcat of 26.5 min-1, indicating its possible involvement in processing citrulline-containing peptides. In addition to exhibiting a distinct substrate specificity pattern, the catalytic activity of RNPEPL1 was maximal at a pH between 6.8 and 7.8 and was insensitive to chloride activation, in contrast to most other members of the M1 metallopeptidase family. The catalytic activity of RNPEPL1 was strongly inhibited by EDTA, amastatin and arginine hydroxamate, but relatively insensitive to captopril. The expression of RNPEPL1 was investigated in a panel of normal human tissues by RT-PCR, and found to be expressed in all tissues. The investigation of four potential splice variants was also examined, revealing that three of the four alternative choices were utilized, including intron inclusion/exon exclusion, exon skipping, and alternative 3’ splice site choice. This demonstrates the potential for at least 8 different protein isoforms. In addition, a minor alternative mRNA species present in bladder tissue was revealed.

Analysis of Gene Expression Following Disruption of Gap Junction Mediated Cell-Cell Communication by Ouabain

D. C. Barnard, D. H. Hafer, P. L. Jones, P. Lavoie, D. Sammartino, M. L. S. Ledbetter; Biology, College of the Holy Cross, Worcester, MA

In an effort to understand the consequences of gap-junction-mediated cell-cell communication that stems from inhibition of the sodium pump by ouabain, we have used microarray technology to analyze global gene expression over three hours of treatment with ouabain and another three hours after removal of the drug. Using human 293T/17 cells (ATCC, CRL-11268), we confirmed that cells exposed to 1 µM ouabain lost their ability to low-molecular-weight dye during that time, but recovered substantially in ouabain-free medium. To examine changes in gene expression during this time course, we isolated total cellular RNA from five populations of cells: untreated (control), exposed to ouabain for 1 hour or 3 hours, and allowed to recover for 1 hour or 3 hours. With the assistance of the Genome Consortium for Active Teaching, we obtained microarrays printed with the HEEBO set of oligonucleotides representing complete genomic coverage. This allowed comparison of the expressed RNA levels between untreated control cells and the treated experimental cells. Data from the microarrays were analyzed using a combination of computer programs and genes whose expression patterns showed similar changes over the course of the experiment were clustered. Several families of genes displayed enhanced expression during ouabain treatment, reverting to normal levels during recovery. Among these genes were genes associated with responses to heat-shock and oxidative stress, genes associated with vesicle trafficking, genes associated with components of the mitochondrial electron transport chain, and genes associated with several signaling cascades. Connexin genes are not among them. From these preliminary results we conclude that the response of cell-to-cell communication to treatment with ouabain is likely to be an indirect outcome of other more immediate cellular responses, which are consistent with generalized stress. Supported by the National Science Foundation (DUE 0308559) and the Holy Cross Summer Undergraduate Research Program.
The Role of Clathrin in Gap Junction Internalization
L. Chambers,1 B. M. Nickel,1 B. H. Achete,2 Y. Arain,1 S. A. Murray1;1Department of Natural Sciences, Virginia Union University, Richmond, VA; 2Cell Biology and Physiology, University of Pittsburgh, Pittsburgh, PA, 3San Diego State University, San Diego, CA

Gap junction-mediated intercellular communication has been implicated in the development, differentiation and function of most tissues. The rates of plaque removal as well as formation are thought to be critical to gap junction function, but the mechanisms that are involved in the disassembly of gap junction plaques are not well understood. In this study, the role of clathrin in gap junction plaque invagination, internalization and annular gap junction formation was investigated with transmission electron microscopy, immunocytochemical localization and computer reconstruction techniques. In addition, a fluorescently-tagged gap junction protein Connexin43(Cx43), was expressed in adrenal cells. In these cells, and the frequency of gap junction internalization was analyzed with time lapse live imaging techniques to monitor annular gap junction vesicles. In some cases, both clathrin and its adaptor protein, AP-2 were observed, with immunocytochemical techniques, to colocalize with Cx43 annular gap junction vesicles and gap junction plaques. The AP-2 was concentrated and preferentially localized on only one side of the gap junction plaque while the other side was sometimes devoid of AP-2 staining. Three dimensional imaging techniques were used to explore the association between Cx43 (gap junction plaques or annular gap junctions) and clathrin, AP-2, and lysosomes. Based on observations of the fusion of cytoplasmic vesicles and lysosomes and the association of clathrin and AP-2, it is suggested that gap junction endocytosis is one step in the process of connexin degradation and that clathrin is involved in the gap junction internalization. NSRF MCB-0444398

FGF-1 Increases the Plasma Membrane Permeability through Connexin43 Hemichannels: Involvement of Connexin C-Terminus and Intracellular Free Ca2+ Concentration
K. A. Schalper,1 M. A. Retamal,1 K. F. Shoji,2 A. D. Matinez,1 J. C. Sáez1;1Departamento Fisiología, Pontificia Universidad Católica de Chile, Santiago, Chile, 2Núcleo Milenio de Inmunología e Inmunoterapia, Santiago, Chile, Instituto de Neurociencias, Universidad de Valparaiso, Valparaíso, Chile

Connexin hemichannels (Cxs-HCs) are plasma membrane channels permeable to ions and small cations molecules. They are expressed by most cell types and remain preferentially close under resting conditions. Physiological stimuli that induce opening of HCs in the presence of extracellular divalent cations remain unknown. To show that the latter may occur, we studied the effect of FGF-1 on the cellular permeability mediated by HCs in the presence of extracellular Ca2+/Mg2+. We used HeLa cells stably transfected with wild type Cx26 (HeLa-Cx26WT), Cx43 (HeLa-Cx43WT), Cx45 (HeLa-Cx45WT) or Cx43 truncated in its C-terminus at aa 257 (HeLa-Cx43Δ257). Cells were treated with FGF-1 for different periods of time in medium containing Ca2+ (1.8mM) and Mg2+ (1mM). The plasma membrane permeability was assessed by time-lapse imaging of ethidium bromide (EtBr) uptake. The amount of HCs in the cellular surface was evaluated by protein biotynilation, followed by pull-down and immunoblotting. Intracellular [Ca2+] was measured using Fura2-AM and image processing. After 7h of FGF-1 addiction, the EtBr uptake rate and [Ca2+] increased significantly only in HeLa-Cx43WT and HeLa-Cx45WT but not in HeLa-Cx43Δ257. The FGF-1-induced increase in rate of EtBr uptake was rapidly blocked (<30 sec) by La3+ (200 μM). In HeLa-Cx43WT, BAPTA-AM (5μM) prevented the FGF-1-induced increase in rate of EtBr uptake and 4Br-A23187 (5μM, 40 min) mimicked all the FGF-1-induced effects studied. The participation of P2X and TRP channels was ruled out using pharmacological criteria. Thus, FGF-1 increases the plasma membrane permeability mediated by HCs under physiological conditions. The effect requires amino acid residues present in connexins 43 and 45 but not in connexin26 C-terminus that in conjunction with enhanced [Ca2+], increases the levels of HCs in the cellular surface.

PDGF Induces a Transient Increase in the Permeability of Cx43 Juncitons
J. P. Elkoy,1, H. Nelson,1 A. M. Burt; Physiology, University of Arizona, Tucson, AZ

In Cx43 expressing cells growth factors that result in PKC activation typically lead to reduced junctional dye permeability (p_j) and electrical conductance (g_j). These decreases require phosphorylation of Cx43 at S368 and involve reduction of channel conductance (γ_j). Accordingly, Cx43-S368A comprised junctions are unresponsive to PDGF application and direct PKC activation, and Cx43-S368A channels are less likely to adopt the 50-60pS subconductance state typically induced by these agents. Thus, PDGF stimulation is linked to Cx43 phosphorylation (at S368) and decline of p_j, g_j and γ_j. However, recent evidence have shown that phosphorylation of Cx43 at Y204 (Biophys J 2006, 91:565) is for Cx43-S368A junctions (CircRes 2006, 98:1498). Importantly, junctions with many 50-60pS channel events were better permeated by the small, cationic dye NBD-M-TMA (high p_j) than junctions without such events. To reconcile these apparently discrepant results, we determined the dye transfer rate constant (k_j) and conductance of Cx43 junctions as a function of time following PDGF application. As expected, ko increased during the first 10 min (to 44±6.6 % of initial value) and the relative contribution of 50-60pS channels increased. Over this same time period, modest shifts toward higher k_j were observed although continued PDGF exposure resulted in complete uncoupling. These results suggest that junctional permeability (k_j/g_j) increases during the first 10-15 minutes of PDGF exposure, remains elevated for a short period (10-15 minutes) and then decreases to pre-treatment or lower levels. The transient nature of the increase in k_j/g_j likely involves reconfiguration of individual, low permeability channels to a high permeability configuration with ongoing decreases in channel number (i.e. Cx43 channel turnover). This transient increase in permeability may constitute a regulatory trigger within tissues that primes the cells for subsequent growth when both p_j and g_j have decreased.

Purinergic Regulation of Connexin43 Gap Junctions Is Dependent on Serine368
A. W. Hunter, R. Gourdie; Medical University of South Carolina, Charleston, SC

Catheterization is thought to be required for gap junction function. Conversely, there is evidence that connexins, the subunits of gap junctions, play an important role in the formation of cadherin-based cell-cell contacts. However, the molecular mechanisms that provide the functional linkage between cadherins and connexins at sites of cell contact are largely undefined. ZO-1, an actin-binding MAGUK protein, localizes to both gap junctions and cadherin-based junctions, and thus is a good candidate to mediate interplay between cadherins and connexins during junction formation and maintenance. To test this idea we used a spinning disk confocal microscope to image live HeLa cells that transiently co-expressed connexin43-CFP with either N-cadherin-YFP or YFP-ZO-1, in a background of stably expressed untagged connexin43. Consistent with our previous findings, YFP-ZO-1 colocalized with connexin43 was confined mainly to the periphery of gap junction plaques. Peripherally associated YFP-ZO-1 manifested as highly dynamic punctae that moved predominately along plaque edges, although some punctae appeared to traverse plaques from edge to edge. The movement of YFP-ZO-1 at plaque edges was associated with dramatic undulations in plaque morphology, which gave the qualitative impression that tension generated at sites of edge-localized YFP-ZO-1 might influence gap junction dynamics. In support of this idea, fixed cell analysis showed that ZO-1 at plate edges also colocalized with actin filaments. Interestingly, like YFP-ZO-1, N-cadherin-YFP associated with gap junctions in living cells localized preferentially to plaque edges. Furthermore, the dynamic behavior of edge-localized N-cadherin-YFP mimicked that of YFP-ZO-1. Extensive colocalization of N-cadherin and ZO-1, including at the periphery of connexin43 gap junctions, was confirmed by confocal analysis in fixed cells. These observations support the hypothesis that ZO-1 integrates N-cadherin adhesion and connexin43 membrane dynamics at specialized junctional interfaces, possibly by modulating linkages to the actin cytoskeleton.
Src Utilizes Cas to Block Gap Junctional Communication Mediated by Connexin43

Y. Shen,1 P. R. Khushi,1 A. P. Moreno,2 G. S. Goldbergl; 1UMDNJ, Stratford, NJ, 2University of Utah, Salt Lake City, UT

Malignant and metastatic tumor cells often display less gap junctional communication than their benign or normal counterparts. The Src tyrosine kinase phosphorylates Cas to confer metastatic growth potential to transformed cells. The gap junction protein Cx43 is a tumor suppressor that can inhibit tumor cell growth. Src can phosphorylate Cx43 to block gap junctional communication between transformed cells. However, mechanisms by which this event actually closes intercellular channels have not been clearly defined. Here, we report that Src utilizes Cas to close gap junction channels formed by Cx43. This observation may help explain how gap junctional communication can be suppressed between malignant and metastatic tumor cells.

Cx43 Phosphorylation: Connecting Specific Phosphorylation Events to Changes in Trafficking, Assembly, and Electrophoretic Mobility

J. Solan, P. Lampe; Fred Hutchinson Cancer Research Center, Seattle, WA

Cx43 has been shown to have different phosphorylation which can be distinguished by their electrophoretic mobility, yet the actual phosphorylation events which lead to these conformational changes and their functional significance have not been linked. Using antibodies that are targeted to specific phosphorylation sites, we have found two phospho-epitopes, residing around S364/S365 and S325/S328/S330, which contribute to the mobility phenotypes of Cx43 and are analyzing the functional significance of these sites. We have found that phosphorylation at a single residue, S365, leads to the P1 isoform of Cx43. We have also found that a phosphopeptide which resides around S325/S328/S330 contributes to formation of the Triton X-100 insoluble P2 isoform of Cx43. In MDCK cells stably expressing serine to alanine mutations at all of these sites (S325/S328/S330A), the P2 isoform of Cx43 is not formed nor is Cx43 efficiently trafficked to the plasma membrane. Mutation of these sites individually did not recapitulate the S325/S328/S330A phenotype, indicating that it is some combination of these sites that are involved in gap junction formation. Interestingly, the S325/S328/S330A cells also do not appear to be phosphorylated at S364/S365. This may be one of the first site specific examples showing a hierarchy of phosphorylation events involved in Cx43 transport to the plasma membrane and inclusion in the Triton X-100 insoluble gap junction plaque.

The Effects of Intracellular Reactive Oxygen Species on Cx43 Expression and Phosphorylation in Enterocytes

G. Valdimarsson,2 R. Szajkowski,3 J. K. Friel,4 W. Dichi-Jones; 2Zoology, University of Manitoba, Winnipeg, MB, Canada, 3Human Nutritional Sciences, University of Manitoba, Winnipeg, MB, Canada, 4Nursing, University of Manitoba, Winnipeg, MB, Canada

Several studies have shown that increased intracellular reactive oxygen species (ROS) alter the expression of gap junction proteins and influences gap junctional intercellular communication (GJIC). We have previously shown that iron supplements in human breast milk increase lipid peroxidation, generate ROS in cultured enterocytes, and induce apoptosis. In the present study, we demonstrate the effects of oxidative stress and iron supplemented breast milk on the expression and phosphorylation patterns of connexin 43 (Cx43) in CaCo-2BBE cells. Preliminary semi-quantitative RT-PCR data suggest that known inducers of ROS, including hydrogen peroxide and xanthine-xanthine oxidase (XOX), decrease Cx43 transcript levels. Western blot analysis indicates that, in untreated cells, the non-phosphorylated isoform of Cx43 is predominant. Treatment with either hydrogen peroxide or XOX induced formation of phosphorylated isoforms of Cx43, as well as increased overall protein expression of both phosphorylated and non-phosphorylated isoforms. Furthermore, iron treatments also induced expression of phosphorylated Cx43. XOX, hydrogen peroxide and iron treatments also decrease transepithelial electrical resistance across CaCo-2BBE confluent monolayers. In scrape-load experiments treatment with glycerophosphoric acid, as well as iron and peroxide decreased GJIC. Taken together, these results suggest that ROS and exogenous iron increase Cx43 protein expression and phosphorylation, likely via translational control. Furthermore, there is a correlation between increased Cx43 protein expression and phosphorylation and decreased epithelial GJIC and membrane integrity.

Altered Distribution of Connexin43 in Oubain-treated MDCK Cells

C. M. Fanning, R. Korkel, M. H. A. R. K. van den Berg, B. Wipke; 1Biology, College of the Holy Cross, Worcester, MA

Many types of cultured mammalian cells communicate among themselves through gap junctions. This communication is reversibly inhibited by exposure of the cells to the cardiac glycoside ouabain, known to inhibit the cells’ sodium pump. We have found that MDCK cells (ATCC CCL-34) treated with 1 μM ouabain reduce their ability to transfer tracer dye molecules over the course of 3 hours. Replacement of ouabain with drug-free medium allows recovery over the next 3 hours. Here we report that the distribution of Connexin43 (Cx43) immunofluorescence changes during treatment and recovery. Untreated cells show punctate chains of fluorescence at cell-to-cell junctions and diffuse staining in a perinuclear region, as reported previously (Berthoud, V.M., et al. 1992 Eur. J. Cell Biol. 57: 40-50). Within the first hour of ouabain treatment, the surface fluorescence disappears, and is replaced by fluorescence in cortical vesicles; after 3 hours very little fluorescence can be detected. During recovery surface fluorescence reappears at cell-to-cell appositions. Amiloride treatment protects the cells from ouabain-induced loss of junctional staining, presumably because amiloride block Na+ channels and prevents Na+ from entering. Treatment of these kidney-derived cells with the mineralocorticoid hormone aldosterone along with ouabain likewise protects them from loss of junctional staining. However, addition of epinephrine or spinonolactone, both antagonists of the aldosterone receptor, counteracts this effect, and junctional staining is lost even in the presence of aldosterone. This study supports a model in which sodium homeostasis is somehow required for the maintenance of Cx43 at junctions, a prerequisite to cell communication. We propose that aldosterone protects cells from the effects of ouabain by acting through its intracellular receptor to normalize cytoplasmic conditions. Thanks to Pfizer Inc. for a sample of eplerenone, to the National Science Foundation (DUE 0308559), and to the Summer Undergraduate Research Program at College of the Holy Cross.

Identification of Phosphorylation Sites in Lens Fiber Cell Connexins by Matrix Assisted Laser Desorption/Ionization Mass Spectrometry and Tandem Mass Spectrometry

D. B. Shearer,1 W. Ens,2 K. G. Standing,3 G. Valdimarsson; 1Zoology, University of Manitoba, Winnipeg, MB, Canada, 2Physics, University of Manitoba, Winnipeg, MB, Canada

Lens junctional complexes are composed of connexins. The lens is an avascular tissue that is dependent on gap junctions for the distributions of nutrients as well as the removal of waste products. Two connexins, Cx44 and Cx49, are highly expressed in bovine lens fiber cells. Phosphorylation of connexins is believed to be involved in the regulation of several processes, such as the assembly and disassembly of connexons, and the gating and permeability properties of the intercellular pore. To begin to characterize the post translational regulation of the connexins expressed in the lens we have used crude membrane preparations from bovine lens and analyzed the proteins present by proteolytic digestion, followed by HPLC separation of the resulting peptides and analysis by MALDI mass spectrometry and tandem mass spectrometry. We were able to obtain significant coverage of the cytoplasmic portions of Cx44 and Cx49, and to identify several phosphorylated residues from each protein. All of the phosphorylated residues in Cx44, thr237, thr299 and thr302 and ser240 and ser244, were located in the carboxy terminal. Cx49 was phosphorylated on ser114, ser117, and ser133 from the cytoplasmic loop domain and at ser257, ser260, ser264, ser265, ser296, and ser299 from the carboxy terminal. In addition, we identified other posttranslational modifications including a deamidation at asn120 in Cx44, and potential cleavage sites in each protein. The extensive coverage of lens connexin proteins obtained by MALDI mass spectrometry and tandem mass spectrometry in this study provide the foundation for the lens to become a useful system for studying the effects of kinase activation on connexin phosphorylation.

Connexin43 Gap Junction Proteins Are Up-Regulated in Remyelinating Spinal Cord

W. A. Roscoe,1 E. Messersmith,2 A. Meyer-Franke,2 B. Wipke,3 S. Karlik1; 1Physiology, University of Western Ontario, London, ON, Canada, 2Elan Pharmaceuticals, South San Francisco, CA

Alterations in the expression of gap junction proteins have previously been observed in several diseases affecting the central nervous system; however, the status of Connexin43 (Cx43) has not yet been reported in demyelination and remyelination. We used a chronic guinea pig model of EAE treated on days 40-60 post immunization (p.i.), with an adenosine amine congener (ADAC, 100 μg/kg), an anti-alpha 4 integrin blocker (CT301, ELN 69299, 30 mg/kg bid) or both CT301 and ADAC, to investigate the expression of Cx43 in demyelinating and remyelinating spinal cord lesions. Hartley guinea pigs were immunized with homogenized whole CNS and CFA. Animals became chronically ill by day 40 p.i. and animals with paralysis were entered into the study. Clinically, the combination treatment was not as effective as CT301 alone (p<0.05), but significantly better than ADAC alone or saline-treated animals (p<0.05). Kruskal-Wallis ANOVA on Ranks, Tukey HSD: Cx43 was effectively (1.7-fold) higher in the saline control (p<0.01). Cx43 expression was virtually absent in demyelinated lesions of saline-treated controls compared to healthy tissue and normal appearing white matter (NAWM) (ANOVA, p<0.001), whereas, Cx43 was considerably increased in remyelinating lesions of all treatment groups (ANOVA, p<0.001), most notably in CT301-treated animals. These changes in Cx43 expression indicate altered astrocyte function in neuroinflammation and recovery.
Mouse connexin30.2 (Cx30.2) is a recently discovered connexin that is expressed in the heart in the sinoatrial and atrioventricular nodes. Most cells in the heart co-express multiple connexins, and we have been studying the interactions between these connexins, including Cx40, Cx43 and Cx45. We have studied the possible heteromeric interactions between Cx30.2 and other cardiac connexins (such as the nodes or the cells that connect the specialized conducting system to the working myocardium).

These connexins (such as the nodes or the cells that connect the specialized conducting system to the working myocardium). A necessary prerequisite for separation is that cells resolve cell-cell junctions that bind them. However, the mechanisms by which junctions are resolved and reestablished are not well known. We have found that gap junctions (GJs) are internalized to form cytoplasmic double-membrane vesicles, termed annular gap junctions (AGJs). In some instances, AGJs appear to spontaneously reinsert into the plasma membrane, suggesting a possible mechanism for sequestration and re-use of GJs. We hypothesize that, utilizing such a mechanism, gap junction intercellular communication (GJIC) could be rapidly modulated without requiring energetically costly transcription and translation events.

In the present study, we developed a model system to test this hypothesis using the inflammatory mediator, thrombin, which has been described to induce transient, reversible cellular separation in primary pulmonary artery and microvascular endothelial cells (PAECs and PMVECs). We are studying the effects of thrombin on the localization of three components of cell-cell junctions: Connexin 43 (Cx43) (GJs), E-cadherin (adherens junctions), and occludin (tight junctions). Immunofluorescence imaging revealed that thrombin induced cellular uncoupling within 20 to 90 minutes. As compared to controls, treated cells showed a significant decrease in Cx43 GJs at the plasma membrane accompanied by a significant increase in intracellular vesicles containing Cx43. When thrombin was removed from media, re-coupling in both PAECs and PMVECs began within 10 minutes. These results suggest that inflammatory mediator-induced separation of endothelial cells is a suitable model system for studying cellular uncoupling and re-coupling events.

Expression of Cx43 in vivo and in cardiac-derived cells harboring an autosomal dominant Gja1 (Cx43) gene mutation. J. W. Ansley, D. W. Laird1, 2; 1Physiology and Pharmacology, University of Western Ontario, London, ON, Canada; 2Anatomy and Cell Biology, University of Western Ontario, London, ON, Canada

More than 30 mutations in the GJA1 gene encoding Cx43 have been linked to ocudentodigital dysplasia (ODDD), a pleiotropic, autosomal dominant disorder. In the present study we employed a mouse model of ODDD, containing a unique Cx43 mutation, to examine the localization and fate of mutant and wild-type Cx43 in vivo and in isolated cells. Western blot assessment revealed differential regulation of total Cx43 protein in various tissues from mutant mice, when compared to wild-type littermates. Concentrating primarily on the heart, where Cx43 expression is critical for proper function, we observed a four-fold decrease in Cx43 level, with a preferential reduction in highly phosphorylated species of Cx43. Other cardiac gap junction proteins, Cx40 and Cx45, did not exhibit any evidence of up-regulation in mutant mouse hearts. The severe reduction in total Cx43 level in mutant mouse hearts suggests a destabilization of co-expressed wild-type Cx43, possibly through premature targeting to degradative pathways. Preliminary confocal microscopy data supports possible Cx43 trafficking defects in primary cardiomyocytes isolated from neonatal mutant mice due to the presence of the Cx43 mutant. These cells also exhibit a faster rate of cell beating compared to parallel cells prepared from wild-type mice. To further study the expression and fate of Cx43 in the heart, cardiac fibroblast cell lines capable of repeated passage have been derived from wild-type and mutant littermates. These cultured cell lines represent a valuable model system for elucidating the mechanisms of assembly and function of Cx43-containing junctions.

Expression and Fate of Cx43 In Vivo and in Cardiac-derived Cells Harboring an Autosomal Dominant Gja1 (Cx43) Gene Mutation

Heteromorphic Compatibility of Cx30.2 with Other Cardiac Connexins

J. Mencel, J. Neiman, J. Poss, R. Collins, R. D. Veenstra, E. C. Beyer, Pediatrics, University of Chicago, Chicago, IL, Pharmacology, SUNY Upstate Medical University, Syracuse, NY

Mouse connexin30.2 (Cx30.2) is a recently discovered connexin that is expressed in the heart in the sinoatrial and atrioventricular nodes. Most cells in the heart co-express multiple connexins, and we have been studying the interactions between these connexins, including Cx40, Cx43 and Cx45. We have studied the possible heteromeric interactions between Cx30.2 and other cardiac connexins. Cx30.2 was amplified from mouse genomic DNA by PCR, yielding a sequence that showed 100% identity with GeneBank AJ441561. Cx30.2 was transiently transfected into HeLa cells or HaCaT cells that had previously been stably transduced with other cardiac connexins. Cx30.2 was detected with anti-Cx30.2 antibodies or with anti-HA antibodies that recognized an HA epitope appended to the C-terminus. Immunoblotting showed that the transfected cells abundantly produced the introduced connexin proteins. Double label immunofluorescence microscopy showed that Cx30.2 was extensively co-localized with co-expressed Cx43 or Cx45 at appositional membranes. Connexins from cells co-expressing Cx30.2 and other connexins were solubilized with 1% Triton X-100 and purified according to their affinity for anti-HA beads. Co-expressed Cx30.2 or Cx45 co-purified with the Cx30.2-HA protein. These results suggest that Cx30.2 can form heteromeric connexons with other cardiac connexins (Cx43 and Cx45). Heteromeric channel formation might affect the properties of gap junction in regions that co-express these connexons (such as the nodes or the cells that connect the specialized conducting system to the working myocardium).
Adrenergic Receptor Regulation of Cardiac Connexins
A. Froger,1 H. Gerami,1 J. Hall,1 M. M. Lurtz;2 Department of Physiology and Biophysics, University of California, Irvine, CA, 3Department of Cell Biology and Neuroscience, University of California, Irvine, CA

Catecholamines are physiologic cardiac modulators acting through adrenergic receptors (AR). Using H9c2 cells, an embryonic rat cardiomyocyte cell line expressing multiple connexin (Cx) proteins including Cx43 and Cx40, it was determined that AR activation by the catecholamine noradrenaline (NE, 100 μM) results in a ~50% reduction in AlexaFluor594 dye transfer 2 min after NE addition that is sustained for at least 30 min [transfer reduced from 23.3±1.2 cells prior to NE addition to 11.3±0.3 cells and 13.0±1.5 cells 2 min and 30 min post-NE addition, respectively]. In contrast, ~90% of connexin-mediated biological coupling in the H9c2 cells was inhibited following the addition of the gap junction inhibitor carbenoxolone (35 μM, 20 min). Parallel experiments were performed on HEK293 cells stably transfected with either Cx43 or Cx46. Cx43-mediated biological coupling was significantly inhibited within 2 min of NE addition [transfer reduced to 1.0±0.6 cells from 19.3±0.9 cells] and remained ~95% inhibited through the duration of the experiments [30 min, 0.6±0.3 cells]. Similar to the results obtained in H9c2 cells, Cx46-mediated biological coupling was significantly, but incompletely inhibited following NE addition [transfer reduced to 6.0±1.0 cells and 5.3±2.1 cells 2 min and 30 min post-NE addition from 12.8±0.5 cells prior to NE addition]. Since NE activates multiple AR isoforms, the contribution of the effect of α- or β-AR isoforms on Cx43 biological coupling was determined. Cx43 biochemical coupling was significantly reduced following activation of α-AR by xylazine (100 μM, transfer decreased from 21.5±0.5 cells to 2.5±0.5 cells) and α-AR with phenylephrine (100 μM, transfer decreased from 22.0±0.9 cells to 1.3±0.3 cells). These data suggest that the regulation of cardiac connexin biological coupling following AR activation is dependent on both the connexin protein(s) and the AR isoform(s) expressed.

Autophagy is a cellular mechanism for degradation of proteins and organelles. Several disease-associated mutant proteins have impaired degradation. One such mutant is a proline-to-serine mutation at position 88 in a gap junction protein, human connexin 50 (CX50P88S), which accumulates in cytoplasmic multilamellar structures. To study the mechanism of formation of CX50P88S accumulations and its possible association with autophagy, a DNA construct encoding CX50P88S with a tetracysteine tag appended at its carboxy terminal was produced by PCR and expressed by stable transfection of HEK293 cells. Fluorescence-labeled autophagosomal and lysosomal markers (MDC, LC3 and LAMP1) associated with the CX50P88S accumulations. ERGIC-53 immunoreactivity co-localized with GFP-LC3 or with CX50 immunoreactivity, suggesting that the autophagosomal membrane and CX50P88S may originate from the ER or Golgi. Sequential labeling with bionsecoidal compounds that fluoresce at different wavelengths (FITC, green, and RhoX, red) demonstrated that CX50P88S was long-lived and that newly synthesized protein could either be added at the periphery of previously formed accumulations or start the formation of new ones. To investigate whether wild type (wt) connexins could be degraded by autophagy, HEK293 cells stably transfected with wt CX50 and NRK cells which endogenously express wt Cx50 were subjected to starvation. Under these conditions, cells showed overlap between GFP-LC3 fluorescence and immunoreactivity to anti-connexin antibodies. Moreover, treatment of cells expressing wt CX50 or Cx43 with chloroquine, a lysosomal inhibitor, increased the extent of overlap between LC3-labeled and anti-CX50 or anti-Cx43 immunoreactive structures under normal growth conditions and more substantially under starvation conditions. Based on these results, we conclude that autophagy is an important pathway for degradation of both wild type and mutant connexins.

Subcellular Localization of GFP-tagged Rat Eag K+ Channels in Neurons
C. Tang,1 H. Lin,1 C. Jeng;2 Department of Physiology, National Taiwan University, College of Medicine, Taipei, Taiwan, 2Institute of Anatomy & Cell Biology, National Yang-Ming University, College of Medicine, Taipei, Taiwan

The eag potassium (K+) channel is a member of the superfamily of voltage-gated potassium channels. The mammalian eag subfamily includes eag1 and eag2 K+ channels, both of which have been shown to be expressed exclusively in the central nervous system. Previous studies from our lab have demonstrated that native rat Eag1 and Eag2 channels displayed differential subcellular localization patterns in hippocampal neurons. In the present study, we aim to study the subcellular localization of GFP-tagged Eag1 and Eag2 K+ channels in neurons. GFP fusion constructs were created by subcloning rat Eag1 and Eag2 cDNAs into pEGFP vectors, respectively. Upon whole-cell patch clamp recording, HEK293T cells transfected with the GFP-tagged cDNAs displayed outward rectifying K+ currents that were characteristic of rat Eag channels. We next transfected cultured rat neurons with GFP-Eag1 or GFPEag2 cDNA to study the subcellular localization pattern of GFP fluorescence by using confocal microscopy. We found that the localization patterns of GFP-Eag fusion proteins were reminiscent of those for their native counterpart proteins. Our present findings provide further evidence supporting the idea that rat Eag K+ channels are localized in the dendrosomatic compartment of neurons.

Identification of a Critical Threonine in the Early Biogenesis of Voltage-gated Potassium Channels
L. McKeown, M. Burnham, G. Edwards, O. T. Jones; Faculty of Life Sciences, University of Manchester, Manchester, United Kingdom

The dynamic expression of voltage-gated ion channels in discrete areas of the cell surface is a fundamental factor in controlling membrane excitability. In neurons, voltage-gated potassium channels affect the shape, size and duration of action potentials and, thereby, act as potent modulators of excitatory events. Unfortunately, how such channels are trafficked to and from the cell surface is poorly understood although there is evidence that regulation of their export from the endoplasmatic reticulum serves as an important checkpoint in regulating channel assembly and surface density. Based upon studies of the ‘simple’ Kv1 family of voltage-gated potassium channels (VGKs) it has been proposed that topogenesis involves the co-operative insertion of the second (S2) of six (S1-S6) transmembrane spanning domains by virtue of its propensity to act as a cryptic signal peptide. In exploring possible ER control mechanisms, we identified a highly conserved motif in the extracellular S1-S2 linker which we hypothesise may be critical for Kv biogenesis. Using immunofluorescence microscopy, patch-clamp electrophysiology and site-directed mutagenesis we have confirmed that a single threonine residue is necessary for the correct trafficking of Kv1.4 to the cell surface. Mutation of this residue to alanine or to a phosphominic aspartic acid prevented cell surface expression by retaining channels in the ER. However, any ER retention is abrogated by conservative mutation of threonine to a serine residue. Significantly, the threonine residue is conserved in all VGK family and the corresponding mutations in both Kv2.1 and Kv3.1 reveal similar patterns of ER-retention. Consequently, we have identified a critical threonine residue that appears to play a general and pivotal role in the biogenesis and/or trafficking of voltage-gated potassium channels to the cell surface that, we suggest, acts by coordinating hydrogen bonding interactions with S1-S2 residues early in Kv biosynthesis. Supported by BBSRC(UK).

The Molecular Mechanism Underlying Axon-dendrite Targeting of Kv3 (Shaw) Voltage-gated Potassium Channels
M. Xu, R. Xiao, S. Qin, M. Zhu, C. Gu; Neuroscience, The Ohio State University, Columbus, OH
Kv3 (Shaw) channels, with rapid activation and deactivation kinetics, are involved in fast-spiking of GABAergic interneurons and neurotransmitter release in nerve terminals. The physiological significance is underscored by behavior abnormalities and neurodegenerative disease in mice and human, carrying mutations in their Kv3 channel genes. It is known that the placement of ion channels on the plasma membrane is critical for proper neuronal functions. Kv3 channels target to axons and/or dendrites in neurons, depending on isoforms, splicing variants and brain regions. However, little is known regarding the underlying mechanism. Here we report that C-terminal alternative splicing controls Kv3.1 axon-dendrite targeting through a highly conserved motif. First, HA-tagged two spliced variants of Kv3.3 channels, Kv3.1a and Kv3.1b, differentially localize to somatodendritic and axonal surfaces of hippocampal neurons. Next, with two reporter membrane proteins, TIR-GFP and CD4, the axonal targeting motif (ATM) was surprisingly mapped to a region, which is not only present in both splicing variants but also highly conserved in all Kv3 channels. We further discovered that the ATM binds to ankyrin G, which is previously known as critical to target voltage-gated sodium and KCNQ channels to axon initial segment. Finally, the splicing domain, likely through regulating the ATM-ankyrin G binding, controls the axon-dendrite targeting of Kv3.1 channels.

Membrane Trafficking of K-Cl Cotransporter KCC4 Is Important for Ovarian Cancer Cell Invasiveness
Y. Chen,1 M. Shen;2 1Institute of Basic Medical Sciences, National Cheng Kung University, College of Medicine, Tainan, Taiwan, 2Department of Pharmacology, National Cheng Kung University, Tainan, Taiwan

The cellular function of membrane electroneutral K-Cl cotransporter (KCC) is to regulate epithelial ion transport and osmotic homeostasis. KCC activation by IGF-1 plays an important role in IGF-1 receptor signaling to promote growth and spread of breast cancer, cervical cancer and ovarian cancer cells. Among different KCC isoforms, KCC4 is highly associated with cancer invasion and metastasis in human cervical and ovarian cancer. Here we investigate the regulatory mechanisms of KCC4 membrane trafficking and its functional significance in tumor biology.
Evidence for Anion Channel-mediated Superoxide Permeability in Endosomal Membranes

D. R. Mumbengegwi, Q. Li, C. Li, E. Bear, J. F. Engerlhardt, T. Chang, F. J. McDonald; Department of Physiology, University of Otago, Dunedin, New Zealand

The epithelial sodium channel (ENaC) is a key regulator of extracellular fluid homeostasis and blood pressure. The classic ENaC consists of three subunits: α, β, and γ, which are highly expressed in epithelial tissues such as kidney, colon, and pancreas. Despite close similarity, a fourth ENaC subunit called Murr1/COMMD1 is downregulated by proinflammatory modulators phorbol myristate acetate (PMA) and tumor necrosis factor alpha (TNF-α) blocked the membrane trafficking of KCC4. Thus we conclude that IGF-1 stimulates the membrane trafficking of KCC4 through lipid rafts-associated vesicles and PI3-K pathway. Moreover, the IGF-1-stimulated KCC4 membrane trafficking may be important for ovarian cancer cell invasiveness.

Identification of RhoA Effector Signals Involved in the Suppression of the Voltage-gated Potassium Channel Kv1.2

L. Stirling, A. D. Morielli; Cell and Molecular Biology Program, University of Vermont College of Medicine, Burlington, VT, Department of Pharmacology, University of Vermont College of Medicine, Burlington, VT

Kv1.2 is a Shaker family voltage-gated potassium channel expressed in neurons, cardiac muscle, vascular smooth muscle, and epithelial cells. Previous research has shown that the small GTPase RhoA interacts with the N-terminal of the channel and that increased RhoA activity leads to a reduction of Kv1.2 current. Other studies have shown that Kv1.2 regulation by tyrosine kinases involves trafficking of the channel away from the cell surface. The objective of this study is to investigate the RhoA signals involved in Kv1.2 trafficking. Flow cytometry was used to measure the amount of Kv1.2 on the surface of an HEK293 cell line that stably expresses the channel. The RhoA inhibitor, C3 exoenzyme, significantly upregulates the channel at the cell surface. Conversely, activation of RhoA with Lysoosphatidic acid (LPA) results in a dose-dependent suppression of cell surface Kv1.2. Pharmacological inhibition of Rho kinase, a downstream effector of RhoA, with either Y27632 or HA1077 significantly upregulates Kv1.2 at the cell surface. When combined, Y27632 and HA1077 produce an additive effect. This suggests that more than one signaling pathway acts to regulate cell surface Kv1.2 downregulation. Pre-treatment with Y27632 also prevents the LPA-induced loss of cell surface Kv1.2. From these data we conclude that activation of RhoA by LPA leads to loss of Kv1.2 from the cell surface and that Rho kinase signaling is at least partially responsible for this suppression. Other candidate RhoA effectors involved in this signaling pathway are currently under investigation, including Protein kinase C-related kinase (PKR).

Endosomes Luminal Chloride Ions Regulate Calcium Permeable Channels in SKD1/vps4B (E253Q) Induced Large Endosomes - Direct Patch-Clamp Study

M. Saito, P. Hanson, P. Schlesinger; Cell Biology & Physiology, Washington University, St. Louis, MO

Endocytosis plays important roles in nutrient uptake, regulation of proteins and lipids in the plasma membrane and various intracellular membranes. Endocytosed molecules travel to the early endosome (EE), where they are sorted for recycling to the plasma membrane or degradation by lysosomes. In this process, a series of vesicular fusion events take place that require calcium ions. The EE lumen is thought to serve as a source for these calcium channels. However the identity of calcium conducting channels that execute this function in EE remains speculative. The characterization of endosome calcium channels is important in understanding the endocytic pathway. The objective of this study was to identify EE calcium permeable non-selective calcium channels (ECC). Methods: Enlarged EE (3–6um in diameter) were produced by expressing a dominant-negative mutant, E235Q, of SKD1/vps4B (AAA-ATPase) in HEK293 cells. The EE-lumen calcium channel (ECC) method was modified to allow patch-clamping of the native endosome membrane, and ii) To identify the Endosome calcium permeable non-selective Calcium Channels (ECC). Procedures: Enlarged EE (3–6um in diameter) were produced by expressing a dominant-negative mutant, E235Q, of SKD1/vps4B (AAA-ATPase) in HEK293 cells. Manually isolated enlarged endosomes were subjected to whole-endosome and inside-out patch-clamp study. Results: The ECC was identified in EE membrane. The ECC was equally permeable to both Na⁺ and K⁺, and also to Ca²⁺ (1:3 ratio to K⁺). ECC was activated by >1mM amiloride and by >300mM La³⁺. The most striking aspect of ECC gating was strong dependence on the luminal chloride concentration. ECC was activated when CF was lowered from 150mM to 30mM. Conclusion: We have identified a Ca²⁺ regulated calcium permeable cation channel in SKD1/vps4B (E253Q) induced large EE membrane. Since the activation of CF in the EE lumen is critical for effective acidification of endosomes by vacuolar type H⁺-ATPase, the discovery of ECC suggests that EE luminal chloride ions may play an important role in regulating endosome function. The presence of functional expression of several TRP channels on human synovial cells and proposed a significant role in mediation of calcium dependent proliferative and secretory responses in joint inflammation. The present study further supports our hypothesis comparing changes in synovioocyte response to thermal sensitive TRP channel activation with proinflammatory modulators phorbol myristate acetate (PMA) and tumor necrosis factor alpha (TNF-α). Fluorescent imaging of SW982 synovial cells loaded with Fura-2 reveals immediate and delayed cytotoxic calcium (Ca²⁺;Ca⁺⁺) oscillations elicited by TRPV1 agonists capsaicin and reserpinatoxin in 20 - 40 % of cells recorded. Pretreatment with TNF-α (1 - 50 ng/ml for 4 - 12 hours) significantly increased numbers of cells responding as well as the frequency of capsaicin induced [Ca²⁺]c spikes, while minimally affecting basal oscillation rate. No changes in capsaicin and reserpinatoxin mediated [Ca²⁺]c responses were observed after pretreatment with PMA (25 nM, 5 min - 12 hours). Next, heat (43 °C) induced [Ca²⁺]c changes in synovioocytes

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were recorded using a temperature controlled chamber. The two-peaked response to heat with rapid transient spikes and a subsequent slow rise reflected TRPV1 and TRPV3 channel activations, respectively. Pretreatment with TNF-α increased average amplitude of heat induced [Ca2+]c responses and accentuated the second rising phase. Five minute pretreatment with PMA caused disappearance of the [Ca2+]c response declining phase which appeared as a single calcium wave with fast and slow rising phases. Thus, TNF-α and a PKC activator provide differentially enhanced calcium responses in syneurocytes during inflammation depending on the TRP stimuli. Supported by the Dana Foundation and NIH P01 NS011255-31.

1071 The Role of TRP Channels in Mechanotransduction of HUVEC
Y. Katunouchi, T. Suemori, K. Naruse; Cardiovascular Physiology, Okayama University, Okayama, Japan
Human umbilical vein endothelial cells (HUVEC) at the interface between the bloodstream and the vessel wall are continuously subjected to mechanical stimulations in vivo, such as shear stress, hydrostatic pressure and stretch, and it is widely recognized that they are playing an important physiological role in cardiovascular system. In response to stretch, we reported that intracellular Ca2+ increased transiently through the activation of mechanosensitive (MS) cation channel in HUVEC. However, the molecular entities that form the MS cation channel in the cell remain unknown. Recent papers report some members of the transient receptor potential (TRP) channel have been suggested as one of components of MS channels. To identify the molecules forming MS in HUVEC, we investigated the expression and localization of TRP channels in this cell. We observed the expression of several isoforms of TRP channels in HUVEC using semi-quantitative RT-PCR and immunoblotting method. Among these channels, immunocytochemistry using anti-hTRP2 antibody revealed that hTRP2 was localized very close to the mesh-structure of the cortical-actin, and the cyskeleton spectrin network tethered by ankyrin, in semi-intact plasma membrane of HUVEC. Moreover, hTRP2 co-immunoprecipitated the cyskeleton proteins and several components of focal adhesion molecules. These interactions were reduced by the deletion of cytoplasmic domain in TRP2. It was noteworthy that stretch-induced change in [Ca2+]c measured by fura2 was significantly higher in hTRP2 transfected HEK293 cells and that the stretch-induced change in [Ca2+]c was significantly inhibited in HUVEC treated with siRNA against hTRP2. From these results, we would like to discuss molecular complex that forms MS channel, above all the possible involvement of TRP2 in HUVEC.

1072 Pkd1l3 and Pkd2l1, Two Members of the TRP Family of Ion Channels, Are Co-expressed in a Subset of Taste Receptor Cells
N. D. Lopez-Jimenez, M. M. Cavenagh, E. Sainz, M. A. Cruz-Ithier, J. F. Battey, S. L. Sullivan; Laboratory of Molecular Biology, NIDCD/NIH, Rockville, MD
Taste receptor cells (TRCs) are responsible for detecting incoming tastants. The detection of sweet, bitter, and savory tasting compounds is mediated through G protein coupled receptors, whereas sour (protons) and salty (sodium ions) stimuli are thought to act by directly passing through or modulating ion channels. To better define taste signal transduction pathways, we constructed and sample sequenced a TRC-enriched library. One of the clones in this library encoded PKD1L3, a largely uncharacterized member of the transient receptor potential (TRP) family of ion channels. Here we report the genomic structure, polymorphic variations, and expression patterns of Pkd1l3. In order to determine the expression of Pkd1l3 in taste receptor tissues, we performed cDNA microarray analysis of RNA isolated from mouse tongue-derived taste bud cells and human TRC-enriched libraries. In addition, we performed in situ hybridization and immunohistochemical studies that Pkd1l3 is specifically expressed in a subset of TRCs. Furthermore, we show that in TRCs Pkd1l3 is co-expressed with Pkd2l1, also known as Trpp3. Given the precedence for PKD/TRP channels to function as heteromers, these results suggest that within TRCs Pkd1l3 and PKD2L1 may function as a heterodimer. The TRCs expressing Pkd1l3 and Trpp3 are distinct from those that lack co-expression, lending support to the hypothesis that Pkd1l3 and TRPP2 play a role in sour or salty taste signal transduction. These results provide the first evidence for a role of PKD/TRP channels in taste receptor cell function.

1073 A Role for Reactive Oxygen Species in Activation of Store-operated Ca2+ Channels in RBL-1 Cells
Y. Wang, R. Bloom, V. Nguyen, J. Schroder; Division of Biological Sciences, University of California, San Diego, La Jolla, CA
Store-operated Ca2+ entry plays important roles in intracellular Ca2+ store replenishment and Ca2+ signaling in excitable cells. However, the mechanisms linking Ca2+-store depletion to the ensuing Ca2+ channel activation in the plasma membrane remain elusive. Here we show that depletion of intracellular Ca2+ stores results in the production of reactive oxygen species (ROS), and that the ensuing Ca2+ influx requires ROS production in RBL-1 cells. Both store depletion-induced ROS production and Ca2+ influx were inhibited by two independent ROS scavengers, MCI-186 (edaravone) and BH(2)(3-s-tetra-4-hydroxyniosene). Application of hydrogen peroxide activated inward Ca2+ currents, and also caused cytosolic Ca2+ elevation in RBL-1 cells. The iPLA2 inhibitor, 2-APB and the two ROS scavengers, MCI-186 and BH4 inhibited both H2O2-induced responses as well. Together, our results suggest that ROS play a pivotal role in signaling the depletion of Ca2+ stores to the activation of store-operated Ca2+ channels in the plasma membrane.

1074 Reducing Agents Enhance Hemichannel Permeability by Increasing Open Probability of Connexin43 Hemichannels, a Cysteine Dependent Action
M. A. Retamal, K. A. Schalper, K. F. Shoji, F. F. Bukauskas, J. C. Saez, M. V. L. Bennett; 1Departamento de Ciencias Fisiologicas, Pontificia Universidad Catolica de Chile, Nucleo Milenio de Immunologia e Inmunoterapia, Santiago, Chile, 2Neuroscience, Albert Einstein College of Medicine, Bronx, NY
In astrocytes, connexin43-hemichannels in non-junctional membrane (Cx43-HCs) are opened by metabolic inhibition and can then be closed by reducing agents (see accompanying abstract). Here, we examined whether reducing agents affect HCs formed of different connexins under normoxic conditions. We used HeLa cells stably transfected with mouse Cx43 or Cx43-EGFP (Cx43 with EGFP on the C-terminal), both of which have cytoplasmic (MS) cation channel in the cell. Here we show that depletion of intracellular Ca2+ stores results in the production of reactive oxygen species (ROS), and that the ensuing Ca2+ influx requires ROS production in RBL-1 cells. Both store depletion-induced ROS production and Ca2+ influx were inhibited by two independent ROS scavengers, MCI-186 (edaravone) and BH(2)(3-s-tetra-4-hydroxyniosene). Application of hydrogen peroxide activated inward Ca2+ currents, and also caused cytosolic Ca2+ elevation in RBL-1 cells. The iPLA2 inhibitor, 2-APB and the two ROS scavengers, MCI-186 and BH4 inhibited both H2O2-induced responses as well. Together, our results suggest that ROS play a pivotal role in signaling the depletion of Ca2+ stores to the activation of store-operated Ca2+ channels in the plasma membrane.

1075 How Metabolic Inhibition Increases Membrane Permeability through Connexin Hemichannels
M. A. Retamal, K. Schalper, K. Shoji, L. Reuss, G. Ahlstein, M. V. L. Bennett, J. C. Saez; 1Departamento de Ciencias Fisiologicas, Pontificia Universidad Catolica de Chile, Nucleo Milenio de Immunologia e Inmunoterapia, Santiago, Chile, 2Neuroscience, Cell Biology, University of Texas Medical Branch, Galveston, TX, 2Neuroscience, Albert Einstein College of Medicine, Bronx, NY
Connexin-based hemichannels (HCs) form cell-cell channels at gap junctions and are also found in non-junctional membranes, where their open probability is low. In rat cortical astrocytes, metabolic inhibition (MI) increases permeation through non-junctional HCs by insertion of new HCs into the surface with little change in open probability. HC opening may contribute to cell death by a role for cystines in the Cx43, Cx43-257stop, Cx43-EGFP truncated at aa 257 (Cx43-257), Cx43-EGFP truncated at aa 257 (Cx43-257EGFP), Cx43-EGFP truncated at aa 257 (Cx43-257EGFP). The amount of surface Cx43 and Cx43-EGFP increased only ~15% after 10 min DTT treatment, markedly less than the increase in dye uptake or single channel activity at positive potentials. Thus, DTT increased opening of HCs in single channel activity at positive potentials. This action appears to require cytoplasmic cysteines, which are present in the Cx43 and Cx43-EGFP C-terminus, but not in other connexins tested.

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1076 Contribution of Aquaporins on Cellular Water Transport Observed Using Microfluidic Cell Volume Sensor

S. P. O'Hara, B. Huang, P. Splinter, J. Nelson, X. Chen, N. LaRusso; Center for Basic Research in Digestive Diseases, Mayo Clinic, Rochester, MN

Internalization of the obligate intracellular apicomplexan parasite, Cryptosporidium parvum, results in the formation of a unique intramembranous yet extracellulosic nodule on the apical surface of susceptible host epithelial cells, a process that depends on host cell membrane extension. Recently, we showed that C. parvum infection of biliary epithelial cells (cholangiocytes) induces localized membrane translocation/insertion of SGLT1, a Na+/glucose cotransporter, and aquaporin 1 (AQP1), a water channel, at the attachment site (PNAS, 2005; 102:6338). The expression of AQP1 was localized in the basolateral membrane of the proximal tubules. In the present study, we have used the yeast two-hybrid approach to investigate the putative TAT1-associated proteins in the rat small intestine. TAT1 message was detected in intestine, placenta and liver in rats. In the small intestine, TAT1 immunoreactivity was detected in the basolateral membrane of enterocytes. Inhibition of AQP3 with HgCl₂ reduced the swelling rate by 60%, but it had almost no effect on cells transfected with AQP4, consistent with previous reports. The cell volume cytometer appears able to serve as a simple quantitative tool to rapidly assay AQP3 activity. References: [1] A.S. Verkman. J. Cell Biol. 118, 3225 (2005). [2] D. A. Ayala, F. Sachs, S. Besch, P. Gottlieb, and S. Z. Hua. Analys. Chem. 77, 1290 (2005).

1077 Aquaglyceroporin AQP9 Is the Major Glycerol Channel in Mouse Erythrocytes

Y. Liu,1,3 D. Promenez,1 A. Rojek,1 N. Kumar,1 S. Nielsen,1 P. Agre,1 J. Carbrey1; 1Cell Biology, Duke Medical Center, Durham, NC, 2Biological Chemistry, Johns Hopkins University School of Medicine, Baltimore, MD, 3Water and Salt Research Center, University of Aarhus, Aarhus, Denmark, 4Malariology Research Institute and Department of Molecular Microbiology and Immunology, Johns Hopkins University Bloomberg School of Public Health, Baltimore, MD

It has been observed for many years that human and rodent erythrocytes are highly permeable to glycerol, which can be inhibited by HgCl₂. Recently, aquaglyceroporin AQP3 was discovered and proposed to be the major glycerol channel in human and rat erythrocytes. However, AQP3 was not found in mouse erythrocytes. We revisited this question and identified another aquaglyceroporin, AQP9, instead of AQP3, in mouse erythrocytes. The expression level of AQP9 in mouse erythrocytes increases as reticulocytes mature into erythrocytes, and as neonatal pups develop into adult mice. Compared to wild-type mice, those from AQP9 deficient mice are defective in the rapid transport of glycerol across the cell membrane. The expression of AQP1 and the urea transporter-B in the AQP9 null erythrocytes is the same as in wild type cells, and as a consequence, the water and urea permeabilities are intact in AQP9 null erythrocytes. In an osmotic fragility test, AQP9 null erythrocytes are much more resistant to lysis by 0.3 M glycerol than wild type and heterozygous erythrocytes. In vitro experiments reveal that mature erythrocytes are much more sensitive to hypotonic shock. Here we report that mature erythrocytes produce glycerol. However, plasma glycerol is an important substrate of lipid biosynthesis for intraerythrocytic malarial parasites. AQP9 null mice that are infected with Plasmodium berghei, the mouse malarial parasite, survive longer during the initial phase of infection compared to wild type mice. We conclude that AQP9, but not AQP3, is the glycerol channel expressed in mouse erythrocytes. The higher expression of AQP9 in mature mouse erythrocytes indicates that AQP9 maybe an important channel for small neutral molecules other than glycerol or urea under specific physiological conditions. During malarial infection, AQP9 affects the pathogenesis of malarial parasites and may be an important part of a pathway that provides Plasmodium with glycerol for lipid biogenesis for maximal growth.

1078 Myosin II-dependent Membrane Translocation of SGLT1 and AQP1 Is Required for Efficient Host-Cell Membrane Protrusion during C. parvum Cellular Invasion

S. P. O'Hara, B. Huang, P. Splinter, J. Nelson, X. Chen, N. LaRusso; Center for Basic Research in Digestive Diseases, Mayo Clinic, Rochester, Rochester, MN

The obligate intracellular apicomplexan parasite, Cryptosporidium parvum, results in the formation of a unique intramembranous yet extracellulosic nodule on the apical surface of susceptible host epithelial cells, a process that depends on host cell membrane extension. Recently, we showed that C. parvum infection of biliary epithelial cells (cholangiocytes) induces localized membrane translocation/insertion of SGLT1, a Na+/glucose cotransporter, and aquaporin 1 (AQP1), a water channel, at the attachment site (PNAS, 2005; 102:6338). The resultant localized water influx facilitates parasite cellular invasion by promoting host-cell membrane protrusion. However, the molecular mechanisms by which C. parvum induces membrane translocation/insertion of SGLT1/AQP1 are obscure. It is known that non-muscle myosins localized to the cortical actin meshwork of epithelial cells are involved in vesicle trafficking. Thus, we report here that cultured human cholangiocytes express several non-muscle myosins including myosin Ia, Ic, IIa, IIb, Va, Vc and X. Moreover, translocation/insertion of SGLT1/AQP1 are obscure. It is known that non-muscle myosins localized to the cortical actin meshwork of epithelial cells are involved in vesicle trafficking. Thus, we report here that cultured human cholangiocytes express several non-muscle myosins including myosin Ia, Ic, Ila, Ib, Va, Vc and X. Moreover, C. parvum infection of cultured cholangiocytes results in the localized selective aggregation of two host-cell myosins: Ib and X at the region of parasite attachment as assessed by both dual-label immunofluorescent confocal microscopy and transfection of cells with GFP-tagged constructs. Furthermore, inhibition of myosin light chain kinase with ML-7, or specific myosin II inhibition by blebbistatin, significantly decreases (p < 0.02) C. parvum-induced accumulation of SGLT1 at infection sites. Finally, treatment of cells with either ML-7 or blebbistatin significantly inhibits (p < 0.05) C. parvum cellular invasion. Thus, localized actomyosin-dependent membrane translocation of transporters/channels initiated by C. parvum promotes membrane extension and parasite internalization, a phenomenon that may also be relevant to the mechanisms of cell membrane protrusion in general.

1079 Identification of the Intracellular Binding Protein with T-type Amino Acid Transporter TAT1 Using the Yeast Two-Hybrid Screening

N. Anzai,1 S. Khamdang,2 D. Kim,1,4 Y. Kanai,1 H. Endo2; 1Department of Pharmacology and Toxicology, Kyorin University School of Medicine, Tokyo, Japan, 2Chouun University College of Dentistry, Department of Oral Physiology, Republic of Korea

TAT1 (T-type amino acid transporter 1) is a Na⁺/amino acid transporter that is expressed in the kidney and intestine. It is involved in the transport of amino acids and is thought to be a candidate protein for the TAT1-mediated amino acid transporters. In this study, we screened a mouse kidney cDNA library using TAT1 as a bait. We found a cDNA clone that encodes a protein of 104 amino acids. We designated this clone as MASP-1. The sequence of MASP-1 is highly homologous to human MASP-1. We then examined the expression of MASP-1 in various tissues and found that it is highly expressed in the kidney and intestine. We also found that MASP-1 is localized to the basolateral membrane of kidney epithelial cells. In conclusion, MASP-1 is a candidate protein for the TAT1-mediated amino acid transporters.

1080 Formation of a P2X4/7 Heteromeric Receptor

C. Guo, O. S. Qureshi, R. D. Murrell-Lagirdo; Pharmacology, Cambridge University, Cambridge, United Kingdom

Extracellular ATP affects many different biological processes in cells by activating plasma membrane P2 receptors. The ionotrophic P2X receptors are non-selective cation channels and 7 transmembrane domain receptors. They are activated by extracellular ATP and are involved in many physiological processes, including neurotransmission, inflammation, and pain transmission. In this study, we investigated the formation of a P2X4/7 heteromeric receptor. We found that P2X4 and P2X7 form a functional heteromeric receptor, which is different from the homomeric receptors. The heteromeric receptor has unique properties, such as increased sensitivity to agonists and decreased desensitization. We also found that the heteromeric receptor is involved in many physiological processes, including pain transmission and inflammation. In conclusion, the P2X4/7 heteromeric receptor is a novel and important receptor type that is involved in many physiological processes.
Nonosecond Electroporation and Electrophoretic Phosphatidylserine Translocation - In Vitro and In Silico

P. Vernier, M. J. Ziegler, Y. Sun, M. A. Gundersen; MOSIS, Information Sciences Institute, University of Southern California, Marina del Rey, CA, Mork Family Department of Chemical Engineering and Materials Science, University of Southern California, Los Angeles, CA, Department of Electrical Engineering-Electrophysics, University of Southern California, Los Angeles, CA
Electroporation technology is widely used to facilitate transport of normally excluded substances --- nucleic acids, pharmaceutical compounds, and other materials --- into biological cells. Microsecond, kilovolt-per-meter pulses produce conductive pores in the cytoplasmic membrane, permitting migration of charged species and large molecules across the compromised membrane barrier presented by the membrane lipid bilayer. Nanosecond, megavolt-per-meter pulses --- much higher power but lower total energy than electroporative pulses --- produce large intracellular electric fields without destructively charging the plasma membrane. Nanoelectroporation perturbation of human lymphoid cancer cells (and other cell types) results in two immediate physiologically and biophysically significant events: intracellular calcium release and externalization of phosphatidylserine (PS) from the inner leaflet of the plasma membrane to the outer face of the cell. Nanoelectropulsed cells subsequently display evidence of a progression to apoptosis (programmed cell death), including chromatin condensation, caspase activation, and characteristic changes in cell morphology. Experimental observations and molecular dynamics simulations of membrane perturbations driven by nanosecond, high-field electric pulses support the hypothesis that nanoelectropulse-induced phosphatidylserine (PS) externalization is associated directly with nanoelectropulse exposure, is driven by the electric potential that develops across the lipid bilayer during an electric pulse, and is facilitated by the poration of the membrane that occurs even during pulses as brief as 3 ns. Molecular dynamics simulations of phospholipid bilayers in supraphysiological electric fields show a tight association between PS externalization and membrane pore formation on a nanosecond time scale, are consistent with experimental evidence for confinement of PS translocation to the anode-facing pole of the cell, and provide theoretical support for details of a developing biophysical model for nanoelectroporation and nanosecond PS externalization.

Diazoxide Acts More as a PKC-ε Activator, and Indirectly Activates MitoKATP Channel in Cardiomyocytes

M. Kim, I. Ahn, I. Yoon, S. Lee, E. Baik, C. Moon, Y. Jung; Department of Physiology, School of Medicine, Ajou University, Suwon, Republic of Korea
Diazoxide (DZX), a well-known mitoKATP channel opener, has been demonstrated to exert cardioprotective effect against ischemic injury through mitochondrial ATP-sensitive potassium (mitoKATP) channel and protein kinase C (PKC). The aim of this study was to clarify the role of PKC isoforms and the relationship between the PKC isoforms and the mitoKATP channel in DZX-induced cardioprotection. PKC-ε activation was examined by Western blotting and kinase assay. Flavoprotein fluorescence, mitochondrial Ca²⁺ and mitochondrial membrane potential were measured by confocal microscopy. Cell death was determined by TUNEL assay. Treatment with 100 μM DZX induced translocation of PKC-ε from the cytosolic to the mitochondrial fraction, and a specific blockade of PKC-ε by either εV1-2 or dominant negative mutant PKC-ε (PKC-ε:KT) abrogated the anti-apoptotic effect of DZX. DZX-induced flavoprotein oxidation was remarkably inhibited by either εV1-2 or PKC-ε KR, while DZX-induced translocation and activation of PKC-ε remained unaltered after treatment with 5-hydroxydecanoyl (5-HD). A direct PKC activation by transfection of wild type PKC-ε mimicked flavoprotein-oxidizing effect of DZX, and this effect was completely blocked by either εV1-2 or 5-HD. During hypoxia, DZX prevented the decrease of mitochondrial Ca²⁺, mitochondrial depolarization and cytochrome c release induced by hypoxia, and all these effects of DZX were blocked by εV1-2 as well as 5-HD. In summary, these results suggest that DZX induces isoform-specific translocation of PKC-ε as an upstream signaling molecule for mitoKATP-channel, rendering cardiomyocytes resistant to hypoxic injury through inhibition of mitochondrial death pathway.

Global Elevation of Cytosolic Ca²⁺ Concentration Activates Ca²⁺-induced Ca²⁺ Release, but Is Not Essential for Ca²⁺ Store Refilling in Jurkat T Lymphocytes

S. Dadsetan, A. Forina; UC Davis, Davis, CA
Precise control of many T cell functions relies on release of Ca²⁺ from the intracellular store. Refilling of the store is vital for maintaining prolonged Ca²⁺ signaling; however this process remains poorly understood. We investigated the relationship between the elevation of cytosolic Ca²⁺ concentration ([Ca²⁺]c) triggered by store depletion and the extent of store refilling which was assessed by [Ca²⁺]c measurements during sequential application of cyclopiazonic acid (30μM), a reversible SERCA blocker. We found that in Jurkat T cells the [Ca²⁺]c levels measured following activation of store-operated calcium entry (SOCE) inversely correlated with the extent of store refilling. Moreover, pretreatment of cells with blocking concentrations of ryanodine (400μM) facilitated store refilling while reducing SOCE. These results indicate that global cytosolic Ca²⁺ elevation activates a Ca²⁺-Induced Ca²⁺ Release (CICR) via ryanodine receptors, which prevents Ca²⁺ accumulation within the store. In addition, elevation in global [Ca²⁺]c, was efficiently blocked by extracellular La³⁺ with an IC₅₀ ~ 50 μM. However, store refilling persisted even when global cytosolic Ca²⁺ elevation was abolished with 0.1-1 μM La³⁺. Higher concentrations of La³⁺ (>10 μM) were required to prevent store refilling. These data indicate that a small number of active plasma membrane Ca²⁺ channels may support global cytosolic Ca²⁺ elevation, whereas activation of a large number of channels stimulates CICR to further amplify cytosolic Ca²⁺ signaling. Research described in this abstract was supported by Philip Morris USA Inc. and Philip Morris International.

TGN to Plasma Membrane Transport of HSV-1 Capsids Requires PKD

C. Mihai, J. Duron, R. Lippe; Pathology & Cell Biology, University of Montreal, Montreal, PQ, Canada
The TGN plays a central role in sorting proteins for delivery to various organelles, secretion or expression at the cell surface. Work from other laboratories has shown that PKD is an important regulator at the TGN for the transport of cargo destined for the basolateral membrane. PKD recruitment from cytosol to the TGN depends on diacyl glycerol (DAG), whose depletion develops across the lipid bilayer during an electric pulse, and is facilitated by the poration of the membrane that occurs even during pulses as brief as 3 ns. Molecular dynamics simulations of phospholipid bilayers in supraphysiological electric fields show a tight association between PS externalization and membrane pore formation on a nanosecond time scale, are consistent with experimental evidence for confinement of PS translocation to the anode-facing pole of the cell, and provide theoretical support for details of a developing biophysical model for nanoelectroporation and nanosecond PS externalization.

Arf1p Is Involved in Transport of the GPI-anchored Protein Gas1p from the Late Golgi to the Plasma Membrane

Y. Liu, S. Lee, F. Lee; Institute of Molecular Medicine, National Taiwan University, Taipei, Taiwan
Arl1p Is Involved in Transport of the GPI-anchored Protein Gas1p from the Late Golgi to the Plasma Membrane

C. Mihai, J. Duron, R. Lippe; Pathology & Cell Biology, University of Montreal, Montreal, PQ, Canada
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Bioisynthetic Trafficking and Sorting of Caveolin-1 Visualized by Multicolor Imaging

A. Hayer, C. Bissig, A. Helenius; Biochemistry, ETH Zurich, Zurich, Switzerland
Caveolae are flask-shaped membrane invaginations involved in endocytosis, transcytosis, lipid homeostasis, and modulation of signaling. Their functional principle is distinct from other known vesicular transport mechanisms in that once assembled, they are stable, and do not undergo cycles of assembly and disassembly. Our previous results suggested that caveolar domains are initially formed in the Golgi complex. Using various fluorescence microscopy techniques in cells expressing a GFP-tagged version of caveolin-1 (Cav1), the major structural component of caveolae, we further investigated how caveolar domains assemble and how they are transported from the Golgi complex to the cell surface. FRAP analysis of the Golgi pool of Cav1-mGFP revealed that the protein is highly immobile in this compartment. Mutants of Cav1 incapable of forming caveolae were highly mobile in the Golgi complex, suggesting that loss of diffusion...
mobility correlates with caveolar domain formation. We then tested the molecular requirements for efficient Golgi-exit of Cav1 by overexpressing FAPP1-PH and dominant negative versions of Arf1 and Dynamin-2 in cells. Comparing the effect of these constructs on transport of Cav1-mGFP, GFP-GPI and VSVG-GFP suggested that Cav1-mGFP and GFP-GPI are transported to the surface by a common mechanism that is distinct from transport of VSVG-GFP. Finally, two-color live-cell imaging in cells coexpressing Cav1-mCherry with either GFP-GPI or VSVG-GFP showed that Cav1-mCherry and GFP-GPI left the Golgi in common vesicles, while there was no overlap between Cav1-mCherry and VSVG-GFP. When vesicles positive for both GFP-GPI and Cav1 arrived at the cell surface, the GFP-GPI was overexpressed, while the caveolar domain remained stable. Together, these results are consistent with a model in which caveolar biogenesis occurs in the Golgi complex. These newly assembled caveolar domains can incorporate lipid-raft components such as GFP-GPI during transit to the cell surface, but exclude non-raft proteins such as VSVG.

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Phosphatidylinositol 5-Kinase Stimulates Apical Biosynthetic Traffic via Arp 2/3-dependent Actin Comets
C. J. Guerriero, K. M. Weisel, J. R. Bruns, O. A. Weisz; Cell Biology and Molecular Physiology, University of Pittsburgh, Pittsburgh, PA
Polarized epithelial cell function relies on the proper sorting and distribution of newly synthesized proteins to the apical and basolateral membrane domains. We are interested in how these delivery pathways are regulated by phosphatidylinositol 5-kinase. Overexpression of murine phosphatidylinositol 4-phosphate 5-kinase alpha (PISK) in Madin-Darby canine kidney (MDCK) cells stimulated the rate of surface delivery of the apical marker influenza hemagglutinin (HA) but had no effect on the traffic of another apical protein (p75) that is sorted via a distinct mechanism. PI(3K)-mediated increases in phosphatidylinositol 4,5-bisphosphate (PI(3,5)P2) levels result in activation of Wiskott-Aldrich syndrome protein (WASP)/Scar family members and lead to cytoskeletal rearrangements, including Arp2/3-dependent formation of actin comets. To test the potential role of N-WASP in HA delivery, we inhibited Arp2/3 function by expressing the HA domain from Scar1, another WASP family member. Expression of the HA domain significantly inhibited activated actin comet formation and slowed the rate of HA delivery, while having no effect on p75 delivery. Similarly, pharmacological inhibition or stimulation of actin comet formation correlated with parallel effects on HA delivery. PI(3K) and HA (but not p75) were visualized at the tips of actin comets in fixed MDCK cells, consistent with a role for actin comets in delivery of this marker. Our data suggest that multiple classes of apically destined transport carriers exist in polarized epithelial cells, and implicate a role for PI(3,5)P2-stimulated actin comets in the transport of a subset of these carriers.

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Drab6 and the Secretory Pathway Organize Microtubule-based Transport of Osk mRNA to the Posterior Pole of the Drosophila Oocyte
In the Drosophila oocyte, polarized secretion occurs towards restricted neighboring cells and asymmetric transport controls the localization of several mRNAs to distinct cortical compartments. How the differential sorting of the axis determining mRNAs is coordinated during Drosophila oogenesis is at present poorly understood. mRNAs are in part properly localized by the orchestrated action of Microtubule-based motors such as Kinesin, Dynemin, and Dynemin-interacting proteins such as Bicaudal D (BicD). In this study, we have characterized the function of Drab6, the Rab6 GTPase ortholog, during oogenesis. Rab proteins organize membrane exchanges between intracellular compartments and specify their identities, suggesting that they may also play an important role in the establishment of intracellular polarity. We show that Drab6 interacts with BicD, indicating that, as in mammalian cells, Drab6 and BicD likely co-ordinate Dynenei powered movements of transport vesicles along Microtubules (MTs). In the absence of Drab6, a severe effect on oocyte polarity was observed. First, polarized transport and delivery of components to the plasma membrane, such as the TGF-ε homolog Gurken, were affected. Second, osk mRNA transport was impaired. We also provide evidence that Drab6 is required for the organization of part of the MT network, possibly by focusing MT plus ends to the posterior. Our results point to an interesting connection between Rab protein-mediated secretion, mRNA transport and the organization of the cytoskeleton.

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Apical Biosynthetic Trafficking Itineraries in Polarized Epithelial Cells
K. O. Cressawn, B. A. Potter, A. Ozcan, G. Apodaca, O. A. Weisz; Medicine, University of Pittsburgh, Pittsburgh, PA
The crucial function of polarized epithelial cells is to sort proteins to distinct membrane domains along both the biosynthetic and endocytic pathways. Sorting signals in basolaterally-destined proteins have been well characterized and generally involve specific cytoplasmic peptide motifs. In contrast, proposed apical sorting signals include associations with lipid rafts, cytoplasmic peptide motifs, and N- or O-glycans. Our lab has previously shown that biosynthetic apical delivery of the sialomucin endolyn is dependent on a subset of its N-glycans. A similar N-glycan-dependent sorting signal is also required for apical delivery along the endocytic pathway, suggesting that sorting along both pathways may occur at a single site. Traditionally it has been thought that biosynthetic sorting occurs in the trans-Golgi network, however endolyn internalized from the cell surface does not access this compartment. Recently it has been shown that basolateral proteins can access common recycling endosomes en route to the plasma membrane and that biosynthetic sorting may occur at this site. Because internalized endolyn can access common recycling endosomes, we determined whether this might represent a common sorting site for newly synthesized and postendocytic endolyn. However, selective inactivation of common recycling endosomes after internalization of horseradish peroxidase-conjugated transferrin did not perturb apical delivery of newly-synthesized endolyn. Similar results were obtained for other apical proteins including influenza hemagglutinin and p75(TNF)
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. Our data suggest that unlike basolateral proteins, apical proteins do not transit common recycling endosomes en route to the plasma membrane.

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The Arf GAP Centaurin Alpha-1 Is Implicated in Immature Secretory Granule Trafficking and Maturation in PC-12 Cells
J. L. Laramore, C. Chapman, A. Smith, A. Theibert; Neurobiology, University of Alabama at Birmingham, Birmingham, AL, Natural Sciences, Longwood University, Farmville, VA
Centaurin alpha-1 is a high affinity PtdIns(3,4,5)P3 binding protein that is a candidate transducer in the neuronal receptor-activated PI 3-kinase cascade. Centaurin alpha-1 has a GAP homology domain and overexpression reduces cellular levels of Arf6-GTP, supporting its function as an Arf GAP. In addition to other binding partners such as actin, casein kinase, PKC, nucleolin, and KIF13b, the clathrin adaptor protein AP-3 has been identified as a potential centaurin alpha-1 interacting protein. AP-3 localizes to endosomes and secretory granules. Endogenous centaurin alpha-1 localizes to punctate structures in the PC12 neurosecretory cell line and cultured hippocampal neurons. Using immunocytochemistry with markers of vesicular compartments, our studies show that endogenous centaurin alpha-1 does not colocalize significantly with transferrin, the transferrin receptor or EEA1, suggesting that it does not associate specifically with endocytic vesicles. In contrast, we observe substantial co-localization (25-50%) between centaurin alpha-1 and markers of dense core vesicles including furin and the chromogranin, and between centaurin alpha and AP-3. Manipulation of centaurin alpha-1 expression by overexpression or siRNA-mediated knock-down leads to changes in the localization of several secretory granule proteins. Treatment of PC-12 cells with brefeldin A, a known inhibitor of secretory granule maturation, results in a change of centaurin alpha localization similar to changes in furin localization resulting from brefeldin A. These data support the hypothesis that centaurin alpha-1 is an Arf GAP that functions in the regulated secretory pathway and is likely to be involved in the trafficking and maturation of immature secretory granules.

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N-terminus of Naked2 Directs TGF-containing Exosome Vesicles to the Lower Lateral Membrane of Polarized Mkdck Cells
C. Li, M. Hao, Z. Cao, W. Ding, R. Graves-Deal, J. Hu, D. Piston, R. Coffey; Dept. of Medicine, Vanderbilt University, Nashville, TN, Dept. of Molecular Physiology and Biophysics, Vanderbilt University, Nashville, TN, Dept. of Cell and Developmental Biology, Vanderbilt University, Nashville, TN, Dept. of Medicine and Cell and Development Biology and Dept. of Veterans Affairs Medical Center, Vanderbilt University, Nashville, TN
TGFα is the major autocrine EGF receptor ligand in vivo. In polarized epithelial cells, proTGFα is synthesized and delivered to the basolateral cell surface. We previously reported that Naked2 interacts with basolateral sorting determinants in the cytoplasmic tail of a Golgi-processed form of TGFα and that TGFα is not detected at the basolateral surface of Mkdck cells expressing myristoylation-deficient (GA2) Naked2 (Proc Natl Acad Sci 101:5571-5576, 2004). Herein we demonstrate that wild type, but not GA2, Naked2-associated vesicles fuse at the plasma membrane by total internal reflection fluorescence microscopy. These vesicular exports are delivered to the lower lateral membrane of polarized Mkdck cells in a µIB-independent manner. Naked2 residues 1-173 redirect the apical adaptor protein NHERF1 to the basolateral plasma membrane of polarized Mkdck cells and deletion of residues 39-51 results in loss of polarized sorting. We propose that Naked2 acts as a monomeric adaptor to ensure the correct delivery and fusion of TGFα-containing vesicles to a distinct domain at the basolateral surface of polarized epithelial cells.
Involvement of the Small GTPase rab11 in Rhodopsin Trafficking

J. Mazelova, L. M. Astuto-Gribble, D. D. Deretic; Surgery/Ophthalmology, University of New Mexico, Albuquerque, NM

The rhodopsin-terminus sorting signal, VXPX-CAAX, regulates its incorporation into specialized transport carriers (RTC's), which are targeted to rod outer segments of retinal photoreceptors. Using a retinal cell-free system, we have previously established that the budding from the Golgi/TGN, and the incorporation of rhodopsin into RTCs, is regulated by direct binding of the VXPX-CAAX motif to the small GTP-binding protein ARF4. We find that the inhibition of RTC budding with an ARF4 blocking antibody is accompanied by the changes in subcellular distribution of ARF4 and the small GTPase rab11, previously localized to RTCs. Anti-ARF4 antibody resulted in the accumulation of ARF4 on the Golgi/TGN membranes and the release of rab11 into the cytosol, suggesting an involvement of ARF4 in the recruitment of rab11 to the RTC budding sites. Rab11 is an essential component of the budding machinery, as RTC budding in the cell-free assay was decreased by 30% with six-fold reduction of rab11 by cytosol immunodepletion. By confocal microscopy, rab11 was localized to punctate structures distributed along the Golgi/TGN, where it co-localized with a BAR domain protein involved in membrane bending and fission, ASAP1, an ARF-GAP with the preference for class II ARFs, such as ARF4. A regulatory ARF/rab11 binding protein Arfophilin/FIP3, was also present in these structures. Subcellular fractionation and co-immunoprecipitation experiments suggest that ASAP1, rab11 and FIP3 may be a part of a macromolecular complex whose assembly is regulated by the binding of ARF4 to rhodopsin. Sequential membrane dissociation of ARF4, ASAP1 and FIP3, and the retention of rab11 on RTCs, suggest that this complex may couple cargo sorting, formation of transport carriers, and concomitant recruitment of the molecular machinery necessary for RTC fusion. The presence of rab11 on RTCs may subsequently ensure the recruitment the Sec68 tethering complex involved in RTC fusion.

Dual Interaction of Arfophilin/Rab11-FIPs with ARFs and Rab11

H. Koga, H. W. Shinn, K. Nakayama; Department of Physiological Chemistry, Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan

In eukaryotic cells, intracellular membrane trafficking of proteins between organelles is mediated by vesicular carriers. Vesicles bud from a donor compartment, and dock and fuse with an acceptor one to deliver cargos. Small GTPases belonging to the ARF and Rab families regulate various steps of membrane trafficking. The ARF GTPases trigger budding of coated carrier vesicles by recruiting coat protein complexes onto donor membranes, whereas the Rab GTPases regulate targeting and docking/fusion of vesicles with acceptor membranes. These GTPase cycle on an inactive GDP-bound state and an active GTP-bound state, in which they are allowed to interact with various effector molecules. Both FIP3 and FIP4 (also referred to as arfophilin-1 and -2) have been recently identified as Rab11-interacting protein and bind not only to Rab11 but also to ARF5 and ARF6 through their C-terminal regions, suggesting their functional roles in vesicle trafficking. However, the regions responsible for Rab11- and ARF-binding have not been determined in detail, and their physiological roles are poorly understood. To elucidate the functions of FIP3 and FIP4 in vesicle trafficking and how they mediate a crosstalk between ARF5 and Rab11, we have investigated the interactions of FIP3 and FIP4 with ARF5, ARF6 and Rab11. Here we show that FIP3 and FIP4 have distinct C-terminal binding regions for Rab11 and ARF5; Rab11 binds the most C-terminal region of FIPs, whereas ARF5 binds a region immediately upstream of the Rab11-binding region. They bind ARF5 and Rab11 simultaneously but not competitively. Furthermore, when expressed in cells, FIPs recruit both ARF5 and Rab11 onto endosomal structures. These findings suggest that FIP3 and FIP4 regulate membrane traffic by coordinate binding to ARFs and Rab11.

Exo70p Mediates the Secretion of Specific Exocytic Vesicles at Early Stages of Cell Cycle for Polarized Cell Growth

B. He, F. Xi, J. Zhang, X. Zhang, W. Guo; Biology, University of Pennsylvania, Philadelphia, PA

In budding yeast, two classes of post-Golgi secretory vesicles carrying different sets of cargos typified by Bgp2p and invertase, respectively, are delivered to the plasma membrane for secretion. The exocyst complex is implicated in tethering these vesicles at the bud tip membrane prior to fusion. Here, we report that mutations in the exocyst component, Exo70p, specifically block the secretion of the Bgp2p vesicles. Further disruption of the invertase vesicles trafficking through endosomal compartments in the exo70 mutant is detrimental to cell growth. We also found that the secretion defect was most pronounced during the early budding stages, which delays the polarized growth of the daughter cells. The selective secretion block does not take place at the vesicle formation or sorting stage at the donor compartment, because the exocytic vesicles are properly generated and protein processing is normal in the exo70 mutant. Our study suggests that specificity of vesicle secretion can be regulated at the target membrane as well as the donor membrane. Exo70p, unlike other members of the exocyst complex, functions specifically at early stages of cell cycle in the exocytosis of the Bgp2p vesicles, which are critical for polarized cell growth.

CRMP-2 and Slp1 Mediate Anterograde Transport of TrkB through Kinesin-1

N. Arimura, K. Kaibuchi; Cell Pharmacology, Graduate School of Medicine, Nagaoka University, Nagaoka, Japan

Collapsin response mediator protein-2 (CRMP-2) is enriched at the distal part of growing axons in primary hippocampal neurons and plays a critical role in axon differentiation. We previously reported that CRMP-2 associates with tubulin heterodimer, and promotes microtubules assembly and axon elongation. CRMP-2 also binds to light chain of kinesin-1 (KLC), and serves as a cargo receptor of kinesin-1 to transport tubulin heterodimer or axonal proteins into the distal part of growing axon. This time, we report that CRMP-2 regulates the transport of TrkB, a receptor of neurotrophic factor. CRMP-2 forms a complex with synaptotagmin-like protein 1 (Slp1), Rab27 and KLC. These proteins locate along microtubules at growth cones. Slp1 is localized on a vesicular structure and is partially co-localized with Trks. Knockdown of Slp1, CRMP-2, or KLCs reduces the frequency of anterograde transport of TrkB-GFP. Thus, CRMP-2, Slp1, and Rab27 appear to mediate the anterograde transport of TrkB as a cargo receptor linking vesicles and kinesin-1. Since CRMP-2 is highly phosphorylated by GSK-3beta in the brains affected by Alzheimer's disease (AD), we would further discuss the potential role of CRMP-2 in AD.

Mechanisms of Activation and Golgi Targeting of Phosphatidylinositol 4-kinase II Alpha

B. Barylko, Y. Mao, P. Wlodarski, H. Sun, G. Jung, H. Yin, J. Albanesi; 1Department of Pharmacology, UT Southwestern Medical Center, Dallas, TX, 2Department of Physiology, UT Southwestern Medical Center, Dallas, TX

Phosphatidylinositol 4-kinase II (PI4KII) generates a pool of PI(4)P that regulates trafficking from the trans Golgi network (TGN) and endosomes. It is highly enriched in the TGN, and is also found on intracellular vesicles of the secretary/endoctic pathway. We previously reported that more than 90% of the PI4KIIa in cells is tightly associated with membranes by virtue of its palmitoylation in a cysteine-rich motif (C1C3C5C7) located within the catalytic domain. Here we present evidence that palmitoylation is essential for PI4KIIa's lipid kinase activity and for its enrichment at the TGN. When palmitoylated PI4KIIa partially redistributes to the plasma membrane, where it is otherwise nearly undetectable with normal palmitoylation. Enzymatic activity is rescued, and even enhanced, if all the cysteines in the palmitoylation motif are converted to the hydrophobic phenylalanine residues. This FFPFF mutant has a similar subcellular distribution as wild-type palmitoylated PI4KIIa, establishing the importance of a hydrophobic membrane-anchoring loop for PI4KIIa's kinase activity and TGN localization.

The Glut4 FQQI Motif Is Necessary for AS160-dependent Exit, but Not GGA-dependent Entry into the Insulin-Responsive Storage Compartment

E. Capilla, N. Suzuki, J. E. Pessin, J. C. Hou; Pharmacological Sciences, Stony Brook University, Stony Brook, NY

We have recently reported that newly synthesized GLUT4 enters into the insulin-responsive storage compartment without transiting to the plasma membrane in a process that is dependent upon the adaptin protein GGA (Golgi-localized γ-carboxyl-containing Arf-binding protein). Moreover, recent studies have shown that GLUT4 intracellular retention into the specialized compartment and insulin-stimulated translocation to the plasma membrane are regulated by AS160 (Akt Substrate of 160 kDa). Using a variety of loss-of-function and gain-of-function function GLUT4/GLUT1 chimeric proteins we have shown the structural domains involved in these GLUT4 initial sorting events and demonstrated that the amino terminus and the large cytoplasmic loop of GLUT4 are both necessary and sufficient to recapitulate normal GLUT4 trafficking in adipocytes. In the present study, taking advantage of these chimeras and several GLUT4 point mutations we have analyzed the specific motifs of GLUT4 that are important for GGA and AS160 regulation of the sorting of GLUT4 into and out of the insulin-responsive storage compartment. GLUT4 mutant at the Phylamines domain in position 5 to Alanine (GLUT4 AQQQ), when co-expressed with both wild type GGA and AS160, resulted in loss of GLUT4 intracellular retention in the basal state. Nevertheless, similarly to GLUT4 wild type, the plasma membrane localization of GLUT4 AQQQ was completely inhibited by co-expression of GGA dominant negative. In addition, co-expression of GLUT4 wild type with AS160 4P (which contains 4 of its 6 Akt phosphorylation sites mutated) abolished insulin-stimulated GLUT4 translocation. AS160 4P, however, was not able to inhibit insulin-stimulated GLUT4 AQQQ translocation. Together, these data demonstrate that the FQQI motif within the amino terminus of GLUT4 is necessary for the AS160 regulated intracellular retention but not for the GGA-dependent sorting of GLUT4 into the insulin-responsive compartment.
Membrane-mediated Interactions between Proteins Lead to Aggregation and Budding

B. Reynolds, M. Deserno, K. Kremer; MPI for Polymer Research, Mainz, Germany

Cellular control of membrane conformation is governed, in part, by proteins that individually induce a local curvature in the membrane. Such a protein could be a wedge-shaped trans-membrane protein that creates an asymmetry in the bilayer or it could be a peripheral membrane protein whose curved surface adsorbs to the membrane and distorts it. When two such proteins approach each other, but before the proteins themselves come into contact, the deformed regions of the membrane around them begin to overlap, causing a membrane-mediated interaction between the two proteins. We investigate the effect of such membrane-mediated interactions on curvature-inducing proteins using coarse-grained molecular dynamics simulations. We observe aggregation and budding of curvature-inducing proteins without any direct protein-protein interaction.

Computational Dissection of Adenovirus Motion on the Cell Surface

C. J. Burchhardt,1 J. F. Shalazarini,1 J. A. Helmuth,7 H. Ewers,5 P. Kroumoutsakos,2 U. F. Greber1; 1Institute of Zoology, University of Zürich, Zürich, Switzerland, 2Institute of Computational Science, ETH Zürich, Zürich, Switzerland, 7Institute of Biochemistry, ETH Zürich, Zürich, Switzerland

The earliest steps of virus host cell interactions are incompletely understood. In the case of human adenoviruses, they involve viral attachment to the coxsackie virus B adenovirus receptor CAR, and alpha v integrins followed by endocytic uptake and infection. How the virus finds the appropriate domain on the plasma membrane to engage in endocytosis or, alternatively, how the endocytic machinery is recruited to the receptors engaging the virus particle is unknown. Here we are using live fluorescence imaging at high temporal resolution to systematically map the motion of adenoviruses on the plasma membrane of human epithelial cells. Powerful single particle tracking algorithms allow us to generate large sets of virus trajectories. Surprisingly, the individual trajectories are of considerable heterogeneity. To unravel the information contained within these trajectories, we developed a novel segmentation approach based on neural networks. This approach revealed four distinct patterns, confined motions, slow drifting motions, fast drifting motion and nonclassified restricted motion. Adenovirus motion at the cell surface is mostly subdiffusive. Immediately upon attachment to the cells, viruses were mostly immobile, i.e. confined. Subsequently, short sequences of nonclassified restricted motion were observed, followed by confined motions and CAR and actin dependent slow drifting motions. Quantitative comparisons of the surface trajectories on alpha v integrin positive and negative cells indicate that integrins promoted the nonclassified restricted motions while reducing both slow drifts and confined motions. In addition, dual color fluorescence imaging experiments allow us to correlate virus surface motions with the recruitment of endocytic machinery, such as dynamin and clathrin light chain. Together, our data indicate that adenoviruses move in variable patterns of subdiffusive motions on the surface. These motions are determined in part by CAR and integrins, and are terminated by the recruitment of endocytic effector proteins leading to infectious virus entry.

Photobleaching Recovery Identifies Three Distinct Large-Scale Plasma Membrane Lipid Domains, Which Correlate with Differential Intracellular Trafficking

D. Tyteca, L. D'Auria, T. Medts, S. Carpenter, A. Platek, M. Mettlen, P. Van Der Smissen, P. de Diesbach, P. Courtoy; CELL Unit, Université Catholique de Louvain and Christian de Duve Institute of Cellular Pathology, Brussels, Belgium

Biological membranes show lateral heterogeneity in lipid composition and physical properties but the types, composition and size of lipid domains still remain poorly understood. Here, we provide evidence for large-scale lipid domains in the plasma membrane of CHO and J774 cells, based on long-range lateral diffusion upon photobleaching of various lipids substituted by BODIPY or NBD. Whereas BODIPY-glucosylceramide freely diffused in the plane of the membrane, BODIPY and NBD analogs of sphingomyelin and phosphatidylycercholine (16:0 and 18:1) displayed partial recovery (45% and 20%, respectively) after photobleaching of large fields (20 µm²), but full recovery in small fields (5 µm²). Diffusion of sphingomyelin and phosphatidylycercholine analogs (i) was not restricted by the actin cytoskeleton (insensitive to latrunculin B treatment); (ii) required enzymatic cholesterol (increased restriction upon methyl-beta-cycloextrin); and (iii) was restricted by endogenous glycosphingolipids (release of restriction upon inhibition of glycosphingolipid biosynthesis by D-PDMP). In addition, depletion by sphingomyelinase of endogenous sphingomyelin restricted diffusion of its two analogs, but facilitated that of BODIPY-phosphatidylycercholine. Taken together, our data indicate the coexistence of three distinct large-scale lipid domains, enriched respectively in glucosylceramide, sphingomyelin and phosphatidylycercholine, and showing increasing restriction in lateral mobility. Glucosylceramide, the most mobile lipid, also showed the highest intracellular accumulation and was targeted to the Golgi, whereas the poorly mobile phosphatidylycercholine was also poorly internalized but rapidly recycled to the cell surface. We suggest that membrane lipids endocytosis and their progression towards distal compartments of the endocytic apparatus are also controlled by their lateral diffusion. (Supported by Belgian FNRS and Region Wallonne)

Elasticity and Molecular Construct of Clathrin Coated Vesicles via Atomic Force Microscopy

A. J. Jin, K. Prasad, P. D. Smith, E. M. Lafer, R. J. Nossal; 1Dibeps/or/od, National Institutes of Health, Bethesda, MD, 2Biochemistry, University of Texas Health Science Center at San Antonio, San Antonio, TX, 3LIMB/NICHOD, National Institutes of Health, Bethesda, MD

Clathrin coated vesicles (CCVs) are multi-component protein and lipid complexes of 100 nm diameter. We consider each CCV to be composed of an outer clathrin lattice cage, inner clathrin vesicle, and an intermediate linkage layer with adaptor proteins and other cofactors, enclosing an aqueous core. To unravel the intricacy of their molecular constructs, we examine the mechanical properties of CCVs while developing new schemes of atomic force microscopy (AFM) and related analyses. By focusing on CCV deformation in quantitative response to varying AFM-substrate compression force, we have estimated that the bending rigidity of the CCV composite shell is 285±30 kT, which is about 20 times that of either the outer clathrin cage or inner vesicle membrane (Jin, et al., 2006. Biophys. J. 90:3333-44). This result indicates a flexible coupling between the clathrin core and the inner membrane that can be up or down regulated, potentially by orders of magnitude, during the vesicular trafficking in cells.

Functional Characterization of Stable Clathrin Patches at the Cell Surface

R. Zoncu, R. Perera, A. Raimondi, R. Sebastian, E. Diaz, D. K. Toomre, P. de Camilli; 1Cell Biology, Yale University/HHMI, New Haven, CT, 2Cell Biology, Yale University, New Haven, CT, 3Computer Science, University of Valencia, Valencia, Spain

The key components of endocytic clathrin coats and a variety of accessory factors have been identified and characterized. However, dynamics and regulation of clathrin coat assembly in living cells is only partially understood. Both electron microscopy and live microscopy studies have revealed a striking heterogeneity of clathrin structures. In particular, along with short-lived single clathrin-coated pits, stable large arrays of clathrin coats have been described and reported to be endocytically active. However, these structures remain poorly characterized. We have measured their average diameter and clathrin content. We have developed a novel live microscopy method, multi-angle Total Internal Reflection (MA-TIRFM), in order to investigate endocytosis from their average diameter and clathrin content. We have developed a novel live microscopy method, multi-angle Total Internal Reflection (MA-TIRFM), in order to investigate endocytosis from

Compartment Specific Function of Clathrin-light Chains

V. Poupart, V. Legendre-Guillermin, S. Thomas, M. Giraud, L. Bourbonniere, J. Philie, N. A. Bright, P. S. McPherson; 1Neurology and Neurosurgery, Montreal Neurological Institute, McGill University, Montreal, PQ, Canada, 2Clinical Biochemistry, Cambridge Institute for Medical Research, University of Cambridge, Cambridge, United Kingdom

Clathrin-coated vesicles (CCVs) are the major carriers for endocytic cargo at the plasma membrane and they also mediate intracellular trafficking between the trans-Golgi network (TGN) and the endosomal system. The structural unit of clathrin, the triskelion, is composed in brain of three copies of the clathrin-heavy chain (CHC) and three clathrin-light chains (CLCs) interacting in a non-covalent manner. While CHC provides the structural backbone of the clathrin lattice and helps to organize the machinery for membrane curvature and cargo selection, the roles of CLCs are not well defined. In order to examine the function of CLCs in cells, we have used small inhibitory RNA (siRNA) in COS-7 and HeLa cells to knock down the two CLC isoforms, CLCa and...
b. CLC knock down results in a slight decrease in CHC expression levels yet i) the remaining CHC associates in triskelia as does newly synthesized CHC, ii) clathrin-coated pits (CCPs) and CCVs containing clathrin adaptor protein 2 (AP-2) are observed at the plasma membrane by immunofluorescence and electron microscopy, iii) the endocytosis of multiple endocytic cargo is unchanged. In contrast, depletion of CLCs leads to a disruption in the distribution of the TGN/endosomal clathrin adaptor protein 1 (AP-1), whereas the related adaptors AP-2, AP-3, GGA2 and GGA3 are not affected. In addition, we observe a striking alteration in the distribution of the cation-independent mannose-6 phosphate receptor, which accumulates in a perinuclear area. These data reveal a surprising and previously unknown compartment and adaptor specific function for CLCs.

1104

NECAPs Contain a New Module in the PH Domain Superfamily That Serves as a Distinct Interface for FXDXF Motifs

B. Ritter,1 A. Y. Denisov,2 J. Phlice,3 V. Legendre-Guillen,4 K. Gehring,5 P. S. McPherson1; 1Montreal Neurological Institute, McGill University, Montreal, PQ, Canada, 2Department of Biochemistry and Montreal Joint Centre for Structural Biology, McGill University, Montreal, PQ, Canada

The NECAPs are a recently described family of AP-2-binding proteins associated with the coats of clathrin-coated vesicles. The N-terminal region of the NECAPs is highly conserved throughout species but shows no sequence homology to other proteins. Using NMR, we have now solved the structure of this region and homology searches reveal a fold in the PH domain superfamily. In a tandem MS-based screen for binding partners for the PH-like domain, we have identified amphiphysin I, confirm this domain as a general interface domain superfamily. In a tandem MS-based screen for binding partners for the PH-like domain, we identified amphiphysin I. Surprisingly, NMR analysis of the amphiphysin I/NECAP complex, coupled with mutational studies, revealed the FXDXF motif within the amphiphysin I insert domain as the binding site for the PH-like domain and accordingly, NECAP and AP-2 compete for binding to amphiphysin I. Moreover, the NECAP PH-like domain also mediates binding to other FXDXF motif-containing proteins, confirming this domain as a general interface for FXDXF motifs. So far, the FXDXF motif was thought to be exclusively an AP-2 alpha-ear-binding motif. Since FXDXF motifs are important for the organization and function of numerous endocytic accessory proteins, these data implicate the NECAP PH-like domain as an additional hub in the organization of the endocytic machinery.

1105

Solution Structure of Slal Homology Domain 1 and NPFX|D Binding Site Responsible for Endocytic Cargo Recognition

S. M. Di Michielo1, C. S. Krieger1, J. S. Pierson1,2; 1Biological Chemistry, UCLA, School of Medicine, Los Angeles, CA, 2CR-UK Institute for Cancer Studies, University of Birmingham, School of Medicine, Birmingham, United Kingdom

Adaptor proteins play important roles in endocytosis including recognition of internalisation signals in transmembrane cargo and linking the cargo to the endocytic machinery. Our laboratory has identified Slalp as an adaptor for the NPFX|1,2D endocytosis signal in Saccharomyces cerevisiae. A conserved Slalp domain, SHD1 (aa495-560), is responsible for NPFX|1,2D binding. We have now determined the solution structure of SHD1 bound to an NPFX|1,2D peptide by NMR spectroscopy and docking calculations. SHD1 is a novel domain variant based on the SH3-like topology which employs a distinct binding pocket to recognize the NPFX|D endocytosis internalisation signal. Signal-binding residues form a solvent exposed hydrophobic surface and were mutated to alanine and glutamate and purified mutant domains were characterized by far-UV CD spectroscopy to establish no major structural effects. Signal binding by purified wild-type and mutant domains was assessed by surface plasmon resonance with a peptide containing the NPF|SD signal. This assay wild-type SHD1 bound to the NPF|SD signal with a KD ~ 5 μmol and did not bind an endocytically inactive mutant form of the signal (NPASD). Results with the mutants defined an exposed hydrophobic pocket centered on 1531 as the key signal-binding determinant. Similar findings were obtained using yeast 2-hybrid and GST-fusion protein affinity binding to assay signal binding. NaCl concentration up to 0.75M had no effect on signal binding, as determined by GST-fusion protein affinity binding assay, supporting the importance of hydrophobic interactions. Our results reveal SHD1 as a novel endocytic signal-binding domain.

1106

The Clathrin Adaptor Eps15 Is a Golgi-associated Protein that Mediates TGN to Lysosome Vesicle Trafficking

S. Chi,1 H. Cao,2 M. A. McNiven1; 1Center for Basic Research in Digestive Diseases, Mayo Clinic College of Medicine, Rochester, MN, 2Department of Biochemistry and Molecular Biology and Center for Basic Research in Digestive Diseases, Mayo Clinic College of Medicine, Rochester, MN.

The trans-Golgi network (TGN) plays an important role in the sorting and packaging of newly synthesized proteins that are to be secreted or transported to the plasma membrane as well as nascent hydrolases that are trafficked to endosomes/lysosomes. However, the molecular mechanisms that support these essential processes at the TGN are still being defined. Eps15 (epidermal growth factor receptor pathway substrate clone 15) is a protein that is well known for its role in clathrin-coated vesicle formation and growth factor receptor internalization from the plasma membrane, where it binds to other endocytic proteins such as the clathrin adaptor protein AP-2. Interestingly, using purified polyclonal antibodies against Eps15 as well as expression of myc-tagged Eps15, we observed that Eps15 is not only present at the plasma membrane but also at the TGN and late endosomes. Therefore, we hypothesized that Eps15 might be a TGN-associated clathrin adaptor involved in the transport of Golgi cargo molecules such as lysosomal hydrolases. Indeed, we found that Eps15 localizes with a late endosomal, mannose-6-phosphate receptor-positive compartment and, further, that Eps15 and the TGN clathrin adaptor AP-1 co-immunoprecipitate from Golgi fractions of rat liver. Next, we tested whether Eps15 function is necessary for efficient TGN to endosome/lysosome trafficking by monitoring the maturation of the lysosomal hydrolase Cathepsin D that occurs upon transport to the lysosome. Using Western blot analysis to determine the amount of nascent versus mature, cleaved Cathepsin D, we observed a marked (20%) reduction in the cleaved form of Cathepsin D in mutant Eps15-expressing cells in comparison to wild-type Eps15-expressing cells. Together these findings support the exciting possibility that Eps15 plays important roles both in clathrin-based endocytic and Golgi vesicle formation and trafficking. The molecular mechanisms that differentiate these two roles of Eps15 and how they are regulated is currently under study.

1107

Synaptic Vesicle Recycling Requires Dynamin 1 Only during Sustained Stimulation

S. M. Ferguson,1 G. Brasnoj,2 M. Hayashi,3 M. Wolfel,4 C. Collesi,5 S. Giovedi,1 A. Raimondi,1 L. Gong,1 S. Paradise,1 E. O'Toole,1 R. Flavel,1 O. Cremona,4 G. Miesenbeck,2 P. V. De Camilli1; 1Department of Cell Biology/HIMI, Yale University School of Medicine, New Haven, CT, 2Department of Biochemistry, Weill Medical College of Cornell University, New York, NY, 3Department of Cell Biology, Yale University School of Medicine, New Haven, CT, 4JIRC Institute for Molecular Oncology Foundation, Universita Vita-Salute San Raffaele, Milano, Italy, 5Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, CO, 6Section of Immunology/HIMI, Yale University School of Medicine, New Haven, CT

Normal synaptic transmission is critically dependent on the continuous availability of neurotransmitter-containing synaptic vesicles. Much of this supply is accounted for by local endocytic recycling of vesicles that have recently undergone exocytosis. Numerous studies have demonstrated that the dynamin GT Pases play a general role in the fission of endocytic vesicles. Of the three dynams encoded by mammalian genomes, only dynamin 1 has a neuron-specific pattern of expression that increases in parallel with synaptogenesis and is therefore the isoform widely believed to be essential for synaptic vesicle endocytosis. We have now generated mice lacking the expression of dynamin 1. Surprisingly, dynamin 1 knockout mice are born apparently normal, with no major developmental defects at the nervous system. However, they fail to thrive, develop severe a neurological impairment and die by the second post-natal week. Functional and structural studies of neurons from these mice revealed the occurrence of synaptic transmission and the presence of synaptic vesicles. However, abnormal endocytic intermediates were also clearly present at a subset of synapses under both basal and stimulated conditions with an extreme abundance of clathrin-coated buds connected to each other and to the plasma membrane by extended tubules. Kinetic analyses of synaptic vesicle turnover in cultured dynamin 1 knockout neurons revealed a striking blockade of synaptic vesicle recycling only during intervals of strong stimulation. Collectively, these results confirm the importance of dynamin 1 in synaptic vesicle recycling, but show that it is not essential for this process. Thus, dynamin 1 may play a highly specialized role in mediating enhanced synaptic vesicle recycling during periods of elevated neuronal activity. Conversely, our data also supports a role for dynamin 1-independent mechanisms for synaptic vesicle endocytosis that are favored at lower levels of neuronal activity.

1108

Role of the Endocytic Protein Epsin in Cdc42 Regulation

B. G. Coon, D. F. Edwards, R. Aguilar; Biological Sciences, Purdue University, West Lafayette, IN

Epsin are endocytic proteins with a structured Epsin N-Terminal Homology (ENTH) domain and a carboxy-terminal region containing peptide motifs that interacts with ubiquitin and other endocytic proteins. We have recently demonstrated that the ENTH domain is both necessary and sufficient for viability of double epsin KO yeast cells. We have also shown that the ENTH domain binds guanine nucleotide triphosphate-activating proteins (GAPs) for Cdc42, a critical regulator of cell polarity and viability defects. Here we show evidence indicating conservation of this epsin-regulated Cdc42 pathway in mammalian cells (including human HeLa cells). We have found that mammalian epsin are capable of binding mammalian GAPs and that interference of the ENTH domain function in mammalian cells by using dominant negative approaches, leads to loss of...
cell viability and morphological abnormalities. Moreover, our results also suggest that the epsins contribute to the regulation of specific Cdc42 signaling pathways in yeast and mammalian cells. Taken together our data indicate that the epsin-regulated Cdc42 pathways are conserved and critical for normal cell physiology in metazoa.

1110

Functional Domain Analysis of Dictyostelium gpa4, a New Member of the Epsin Family

R. J. Brady, T. J. O'Halloran; Molecular, Cellular, and Developmental Biology, University of Texas at Austin, Austin, TX

As an integral component of clathrin-coated pits, epsin is thought to engage clathrin and key clathrin adaptors and tether these proteins to the membrane via an Epsin N-Terminal Homology (ENTH) domain. Functional analysis of epsin domains in different model systems has led to conflicting results about whether the lipid-binding function of the ENTH domain and the adaptor-binding motifs of the c-terminus can function independently. To address this issue, we generated an epsin-null mutant line in Dictyostelium discoideum. Although the ENTH domain of epsin was required for normal spore morphology, general phenotypes associated with clathrin-null cells were not observed in the epsin-null mutant. Conversely, we found epsin was unable to localize properly to membrane punctae in the absence of clathrin. Domain analysis revealed that, as with mammalian epsin, the ENTH domain was required to target epsin to the membrane, but the c-terminal binding motifs were critical for epsin to colocalize with clathrin on the membrane. To determine whether the ENTH domain contributed more than Pip2-binding function, we replaced the ENTH domain with the PH domain of PLC, a canonical Pip2-Binding domain. Remarkably, this chimera bound and sequestered clathrin into aberrant patches on the membrane, revealing that epsin is a potent recruiter of clathrin. However, the chimera did not localize into punctae and was not functional, indicating that the ENTH domain plays an important role in addition to lipid binding. While clearly targeting epsin to the membrane, the ENTH domain also may be required for epsin to appropriately facilitate the recruitment and organization of additional clathrin and adaptors to growing clathrin pits. Taken together, our results provide new insights into the contribution of the ENTH domain to epsin function.

1111

AAK1-mediated Numb Phosphorylation Redistributes Numb within the Cell

E. B. Sorensen, S. D. Conner; Genetics, Cell Biology and Development, University of Minnesota, Minneapolis, MN

The Drosophila Numb-associated kinase (Nak) was discovered as an interacting partner of Numb. Numb is a protein important for binary cell fate decisions by repressing Notch signaling transaction during neuronal development. Numb is highly conserved from flies to humans. In addition to its signaling role, mammalian Numb has been shown to function in clathrin-mediated endocytosis. The adaptor-associated kinase 1 (AAK1) is the human homolog of Nak. AAK1 is modeled to coordinate endocytosis by regulating AP2 affinity for receptors. AAK1 is stimutated by clathrin-mediated endocytosis and studies indicate that AAK1 may play a role in regulating Notch signaling through Numb phosphorylation. We have tested this idea using biochemical approaches to assess AAK1 kinase activity toward Numb. In vitro kinase assays demonstrate that AAK1 phosphorylates human Numb. We also find that Numb phosphorylation is stimulated by clathrin, consistent with previous reports for AP2. To investigate the impact of Numb phosphorylation in cells, we employed an adenovirus-mediated overexpression approach. We find that AAK1 overexpression leads to a redistribution of Numb to the perinuclear endosome. The observed redistribution is specific for AAK1 kinase activity as Numb distribution is unaffected in cells overexpressing AAK1 kinase dead mutants. We speculate that AAK1-dependent Numb redistribution prevents Numb from antagonizing Notch signaling.

1112

The Di-Leucine Motifs of LRP9 Bind to the Alpha/Sigma2 Hemicomplex of Adaptor Protein 2 (AP-2) and Mediate Its Endocytosis

B. Donay,1 J. Kinse1y,2 G. Bu,3 S. Konfeld1; Internal Medicine, Washington University School of Medicine, St. Louis, MO, 2Pediatrics, Washington University School of Medicine, St. Louis, MO

Low-density lipoprotein receptor related protein 9 (LRP9), a distant member of the LDLR family of proteins, is a type I integral membrane protein comprised of an extracellular or luminal domain of 427-aa, a transmembrane segment, and a 248-aa cytoplasmic tail. To date, there are no reports on the cellular distribution of LRP9 or the signals responsible for its localization. Indirect immunofluorescence microscopy of CHO cells stably expressing HA-tagged mouse LRP9 showed the protein to be predominantly localized to endosomes with a small amount on the plasma membrane. Analysis of the cytosolic tail revealed the presence of one YXX8 and 2 DEXXXLL consensus motifs that commonly mediate sorting to endosomes. Microscopy results indicated that either di-leucine motif is sufficient for proper endosomal targeting of the protein but mutation of both di-leucines resulted in retention at the cell surface. Mutation of the YYQQL motif, either alone or in combination with one of the two di-leucines, did not affect localization. Endocytosis assays with wild type or mutant receptor tails were in good agreement with the imaging data. In GST pull-down assays, each di-leucine motif bound the α2/δ hemicomplex of the clathrin adaptor AP-2 but not the individually expressed α2, β2, or δ2 subunits, or the β2/δ2 hemicomplex. The di-leucine motifs also bound the VHS domains of all three GGA proteins, providing the first documented example of di-leucine motifs with overlapping AP-1/AP-2 and GGA binding sequences. LRP9 also represents the first example of a transmembrane protein with an internal GGA-binding sequence in addition to the usual carboxyl terminal motif.

1113

A Role for the Acidic-Dileucine Motif of Vesicular Acetylcholine Transporter in Endocytosis

M. Kim, L. B. Hersh; Molecular, and Cellular Biochemistry, University of Kentucky, Lexington, KY

The carboxyl-cyttoplasmic tail of the vesicular acetylcholine transporter contains multiple sorting signals for its trafficking to synaptic vesicles. We recently showed that one of these signals, a dileucine motif, is involved in AP-1 binding and endocytosis. Dileucine motifs involve in addition to a dileucine pair, acidic residues four or five residue upstream. In VACHt this motif is S158EL. Serine 480 can be phosphorylated and it and the adjacent acidic residue are structurally important for endocytosis Deletion and site specific mutagenesis of this region in VACt showed that the dileucine motif is not the sole endocytosis motif. Although each leucine residue is important for endocytosis, leucine 485 plays a greater role than leucine 486. Mutation of serine 480 to alanine or glutamate 481 to alanine did not affect AP-1 binding, however these mutations increased cell surface VACt. Mutagenesis of the four residues SE/AA plus LL/AA inhibits endocytosis much more than the individual mutations LL/AA or SE/AA. The addition or deletion of single amino acids between the SE and LL residues increases cell surface expression of VACt. Taken together these results indicate that the entire SERDVELLL sequence represents the motif for the endocytosis of VACt.

1114

The Acidic Cluster Motif of the Cytoplasmic Tail of DC-SIGN Is Involved in Receptor Recycling and Intracellular Trafficking

H. Nassanian, S. Chantasrivast, B. Lee; Microbiology, Immunology, & Molecular Genetics, UCLA, Los Angeles, CA

Dendritic cell-specific ICAM-3-grabbing non-integrin (DC-SIGN) is a C-type lectin receptor expressed by DCs implicated in the binding of glycoproteins and pathogenic structures. The N-terminal cytoplasmic tail (CT) of DC-SIGN contains internalization and sorting signals that may play a role in receptor endocytosis and trafficking, including a dileucine motif (LL), a triadric cluster (EEE), and a tyrosine-based motif (YKSL). By generating endocytic motif mutants and performing biochemical and immunofluorescence experiments, we show the contribution of these motifs in the endocytosis, recycling, and intracellular trafficking of DC-SIGN. We have found that all three motifs play a role in endocytosis; whereas wild-type (wt) DC-SIGN internalized at a rate of 2%/minute, the CT mutants internalized at rates ranging from 0.5%-1.3%/minute. When studying DC-SIGN recycling after internalization, we found that the LL and EE
mutants recycled to the cell surface with faster kinetics compared to wt DC-SIGN, suggesting these motifs are involved in the intracellular trafficking of the receptor. Indeed, co-localization of wt DC-SIGN or the EE mutant with the intracellular markers Rab5 (early endosomes), Rab7 (late endosomes), LAMP-1 (lysosomes), and Rab11 (recycling endosomes) showed that the EE motif plays a role in receptor trafficking. While wt DC-SIGN colocalized with Rab5 and Rab11, the EE mutant did not. Both wt DC-SIGN and EE colocalized with LAMP-1 after 30 minutes of internalization, but only wt remained in LAMP-1 compartments after 45 minutes, indicating that the EE motif plays a role in retaining DC-SIGN in lysosomes. Both wt DC-SIGN and EE failed to colocalize with Rab7. We conclude that the dileucine motif, acidic cluster, and tyrosine-based motif present in the CT of DC-SIGN function to regulate receptor endocytosis, whereas receptor recycling is dependent on the dileucine and acidic cluster motifs. The acidic cluster is also highly responsible for the trafficking and targeting of DC-SIGN to intracellular compartments.

**1115**

NPFXD-mediated Endocytosis Is Required for Polarity and Function of a Yeast Cell Wall Stress Sensor

H. Piao, I. Machado, G. S. Payne; Biological Chemistry, UCLA School of Medicine, Los Angeles, CA

Previously we identified the actin-associated protein Slap1 as an adaptor for the NPFXD(1,2)D endocytic targeting signal in yeast. Here we report that Wsc1p, a cell wall stress sensor, depends on this signal-adaptor for endocytosis. Mutation of either NPFFD in Wsc1p or Slap1 NPFXD(1,2,D) binding domain blocked Wsc1p internalization. By live cell imaging, we demonstrate endocytotic requirement for Wsc1 localization, polarized distribution of Wsc1p to regions of cell growth was lost in the absence of endocytosis and endocytically-defective Wsc1p was not concentrated at sites of endocytosis. Mutations in genes necessary for endosome to the Golgi traffic caused redistribution of Wsc1p from the cell surface to internal compartments, indicative of recycling. In the absence of Wsc1p endocytosis, cells exhibited defects in polarized cell wall synthesis and increased sensitivity to cell wall perturbation. Our results reveal that the NPFXD(1,2,D)-Slap1 system is responsible for directing Wsc1p to intracellular recycling in an endocytosis pathway necessary to maintain yeast cell wall polarity. The dynamic localization of Wsc1p, a sensor of the extracellular wall in yeast, resembles polarized distribution of certain extracellular matrix-sensing integrins through endocytic recycling.

**1116**

Intracellular Autoactive Receptor Tyrosine Kinase Activity in the ER-Golgi Intermediate Compartment (ERGIC) Affects the Glycosylation and Trafficking of Other Cell Surface Proteins

L. C. Schecterson, M. P. Hudson, M. Ko, M. Bothwell; Physiology & Biophysics, University of Washington, Seattle, WA

Ligand-independent activation of receptor tyrosine kinases (RTKs) is a common theme in many diseases, including cancer. Transient transfection of 293 cells was used to determine the cellular localization of autocrine kinase activity of the Trk receptors TrkA, TrkB and TrkC, the FGF receptor 2 (FGFR2) and Crouzon craniosynostosis syndrome mutant FGFR2 C278F, and the PDGFR receptors A (PDGFRa) and B (PDGFRb). All autocrine forms were primarily retained intracellularly and were Endoglycosidase H (EndoH) sensitive, indicating they were in a high mannose (HM) state. Tunicamycin or Brefeldin A treatment did not affect the phospholipase-C(γ1) (PLC(γ1)) signaling pathway was activated by unglycosylated and HM forms of Trks, FGFR2s and the PDGFRs. The trafficking of other cell surface proteins, such as p75NTR and its homolog NR1I2, TNFR2, APP and Notch, was affected when co-expressed with the Trks and FGFR2 C278F. This study identified a novel ER-bound Bap29-related protein, termed Bap29varP, as an interacting protein. Bap29varP is 303 amino acid long and contains three transmembrane segments, and a large cytoplasmically located C-terminus. The C-terminal 33 amino acids interact with the N-terminus of Pgp. Protein analysis suggested that Bap29varP is nearly absent in Pgp +ve breast cancer cells due to rapid proteolysis, whereas, it is detectable in significant amounts in Pgp -ve cells. Forced expression of Bap29varP in Pgp +ve cells resulted in the accumulation of Pgp in calreticulin +ve vesicles that are also enriched with Bap29varP. Interestingly, expression of Bap29varP regulated the retention of Pgp at the cell surface. These data suggested that Bap29varP interacts with and retains Pgp in the ER, and protein synthesis of Bap29varP regulates the forward trafficking of Pgp to the cell surface. Possible implications of these results to the development of cancer drug resistance will be discussed.

**1117**

Bap29varP, a Novel Endoplasmic Reticular Protein Regulates the Cell Surface Expression of the Human P-Glycoprotein

U. S. Rao, P. S. Rao; Pharmaceutical Sciences, Texas Tech University Health Sciences Center, Amarillo, TX

Translocation of P-glycoprotein (Pgp) to the plasma membrane of cancer cells is the major obstacle for a successful chemotherapy. The mechanism by which Pgp is targeted to the plasma membrane is unknown. It is assumed that immediately after synthesis in the endoplasmic reticulum (ER), Pgp exits the ER to Golgi apparatus via COP II vesicles, from which it is trafficked to the cell surface through trans Golgi network. To identify novel proteins involved in Pgp trafficking to the cell surface, a yeast two-hybrid analysis was carried out using different regions in Pgp as bait. This study identified a novel ER-bound Bap29-related protein, termed Bap29varP, as an interacting protein. Bap29varP is 303 amino acid long and contains three transmembrane segments, and a large cytoplasmically located C-terminus. The trafficking of other cell surface proteins was also studied. ERGIC-53, the major protein in the ERGIC compartment, by sucrose gradient fractionation. These results demonstrate that there is a close relationship between RTK glycosylation and ligand-independent activation, and that the trafficking of other cell surface proteins may be affected by the activity of RTKs localized to ERGIC.

**1118**

Regulation of Glutamate Transporter EAAC1 Trafficking through an Interaction with RTN2B

Y. Liu, J. Rothstein; 2Neurology, Johns Hopkins University, Baltimore, MD, 2Neurology and Neuroscience, Johns Hopkins University, Baltimore, MD

Excitatory amino acid transporters (EAATs) are the primary regulators of extracellular glutamate concentrations in the central nervous system. Their dysfunction may contribute to several neurological diseases. To date, five distinct glutamate transporters have been cloned. In brain, EAAC1 (excitatory amino-acid carrier 1) is the primary neuronal glutamate transporter, localized on the presynaptic membranes that are close to release sites. Its contribution to synaptic actions, little is known concerning the regulation of EAAC1 trafficking from the ER to the cell surface. Previously, we identified an EAAC1 -associated protein, GTRAP3-18, an ER protein that prevents ER exit of EAAC1 when induced. Here we show that RTN2B, a member of reticulum protein family that mainly localizes to the ER, interacts with EAAC1 and GTRAP3-18 bind to different regions of RTN2B. Each protein can separately and independently form complexes with EAAC1. RTN2B interacts with EAAC1 in heterologous cells. Expression of short-interfering RNA (siRNA)-mediated knockdown of RTN2B decreases EAAC1 surface localization and overall expression in neurons. Overall, our results suggest that RTN2B functions as a positive regulator in the delivery of EAAC1 from the ER to the cell surface. In ER stress, up-regulated GTRAP3-18 retains EAAC1 in the ER by binding to EAAC1-RTN2B complex. These studies indicate that transporters exit from the ER controlled by the interaction with its ER binding partners represents a critical regulatory step in glutamate transporter trafficking to the cell surface.

**1119**

Characterization of Bn4, a Protein Phosphatase Type 1 Regulatory Subunit Involved in Chitin Synthase Targeting

J. R. Larson, J. P. Bharucha, K. Tatchell; Biochemistry and Molecular Biology, University of Washington, Health Sciences Center, Shreveport, LA

Chitin is an essential component of the cell wall of the budding yeast Saccharomyces cerevisiae. During vegetative growth, chitin is deposited first as a ring at the base of the bud, then later at cytokinesis as the primary septum. The majority of chitin is synthesized by chitin synthase III whose catalytic subunit (Chs3) resides in endosome-like vesicles termed chitosomes, and is targeted by an unknown mechanism to the bud neck where it interacts with its activator, Chs4. Bn4, a Protein Phosphatase Type 1 (PP1) targeting/regulatory subunit, is required for both Chs4 and Chs3 localization to the bud neck. We are investigating the role of Bn4 and Gc7, the yeast PP1 catalytic subunit, in the targeting of the chitin synthase machinery to the bud neck. We are using deletion analysis to determine which segments within Bn4 are required for its localization to the bud neck and for targeting the chitin synthase machinery. Previous studies showed that a Bn4 variant that cannot bind to Gc7 (Bn4VLGV7E53) has a null phenotype (Kozhoubets et al., Mol. Biol. Cell 14, p32). The reduced levels of Bn4VLGV7E53 at the bud neck led to the suggestion that Gc7 is required for Bn4 association with the septin ring. However, we have identified several Bn4 variants that accumulate at the septin ring at levels at or below that of Bn4VLGV7E53 but retain an intact Gc7-binding domain and target the chitin synthase machinery normally. These results suggest a more direct role of Gc7 activity in targeting components of the chitin synthase machinery to the bud neck.
Bchlp, Bud7p, and Chs5p Transport Fus1p to the Cell Surface in Saccharomyces cerevisiae

R. M. Barfield, R. W. Schekman; Molecular and Cell Biology, University of California, Berkeley, CA

The transmembrane protein Fus1p is required at the yeast cell surface for cell fusion to occur during mating. Fus1p fails to localize to the cell surface and is mislocalized to a Golgi/endosomal compartment in the absence of CHS5, a gene required to transport the chitin synthase Chs3p to the cell surface, suggesting Chs5p is important for transporting Fus1p to the cell surface. We have now evidence to suggest that two peripheral membrane proteins, Bud7p and Bchlp, act with Chs5p to transport Fus1p to the cell surface. In bud7 bchlp double mutants, Fus1p is mislocalized to a Golgi/endosomal compartment. In addition, bud7 bchlp double mutants have a cell fusion defect, a phenotype seen in fusl and chs5 mutants. Furthermore, Fus1p co-purifies with Chs5p, Bchlp, and Bud7p in vivo and interacts with Chs5p and Bchlp in a yeast-two-hybrid assay. We identified four acidic residues in the cytosolic tail of Fus1p that are important for these two-tandem interactions, suggesting these residues could be part of a sorting signal recognized by Chs5p, Bud7p, and Bchlp to direct transport of Fus1p to the cell surface. Bud7p, Bchlp, and two related proteins Chs5p and Bchlp are found in a high order complex with Chs5p (2,3). This complex can form a coat on synthetic membranes in the presence of the GTPase Arf1 and GTPγS reminiscent of the COPII coat important for ER to Golgi transport (3). We propose a model where Chs5p, Bud7p, Bchlp, and perhaps Chs6p and Bch2p, act as coat proteins to recruit Fus1p into vesicles formed at the Golgi destined for the cell surface. 1. Santos and Snyder. (2003). Eukaryotic Cell 4, 821-5. 2. Sanchatejat and Schekman. Mol Cell Biol, in press.

Ap-1 Interacts with the EGFR Juxtamembrane Domain to Influence Basolateral Sorting in Polarized Epithelial Cells

S. P. Ryan, C. R. Carlin; Dept of Physiology and Biophysics, Case Western Reserve University, Cleveland, OH

Sorting of the epidermal growth factor receptor (EGFR) in polarized epithelial cells is a highly regulated process, involving multiple sorting signals to influence the dynamic trafficking of the receptor. Among these signals is a dileucine motif 658-LL in the EGFR juxtamembrane domain, which has previously been shown to be involved in basolateral sorting of the receptor. We hypothesized that this 658-LL motif may recruit proteins in the endocytic compartment to regulate EGFR recycling to basolateral membranes. One such protein is AP-1, a clathrin adaptor that has been shown to be involved in recycling endosomes and is recruited to DxxxLL motifs. The EGFR contains a 654-TxxxLL motif that could potentially mimic a DxxxLL-type motif when threonine residue 654 (Thr-654) is phosphorylated by protein kinase C (PKC). Thus we tested the hypothesis that the EGFR juxtamembrane containing 654-TxxxLL creates a docking site for AP-1, and that Thr-654 plays an important role in the basolateral sorting of the EGFR in polarized epithelial cells. We have shown in vitro pull-down assays that the EGFR juxtamembrane co-immunoprecipitates AP-1. In addition, we have shown that EGFR is down-regulated upon EGF stimulation to a greater extent when Thr-654 is mutated to alanine (T654A), than both wildtype receptors or receptors with a T654D substitution. We therefore hypothesize that the 654 phosphorylation activates a latent AP-1 binding site at 658-LL, increasing the likelihood that ligand-stimulated receptors may recycle to the basolateral membrane in polarized epithelial cells. This may have important consequences for EGFR-dependent signaling in highly differentiated cells, which is tightly regulated because EGFRs are sequestered in adherens junctions.

Role of µ1B C-terminus in Lipid Binding and Exocyst Recruitment

S. M. King, I. C. Fields, E. Höfflin, F. Hölsc; Biochemistry, Molecular Biology, and Cell Biology, Northwestern University, Evanston, IL

Polarized epithelial cells co-express two closely related AP-1 complexes, AP-1A and AP-1B, involved in endosomal/luminal or basolateral sorting, respectively (Fölsch et al., 1999). Although these complexes differ only in the incorporation of their respective medium subunits µ1A or µ1B, they have largely non-overlapping functions and form distinct vesicle populations (Fölsch et al., 2001 & 2003). Furthermore, only AP-1B facilitates the recruitment of exocyst subunits to recycling endosomes for incorporation into AP-1B vesicles (Fölsch et al., 2003). Subsequently, the exocyst is believed to aid fusion of AP-1B vesicles with the basolateral membrane. In this study we have determined how µ1B specifies sorting to the basolateral membrane by examining the C-terminus of µ1B (C-µ1B). Upon membrane recruitment of AP-1B, C-µ1B becomes exposed to the cytosol and therefore available for association with protein and lipid partners (Heldwein et al., 2004). We have found that C-µ1B helps in establishing the identity of AP-1B vesicles by interacting with specific lipid domains on recycling endosomes. Additionally, C-µ1B is sufficient to trigger membrane recruitment of exocyst subunits. Consistent with these functions, over expression of C-µ1B causes mis-sorting of AP-1B-dependent cargos to the apical domain in a dominant negative manner. Therefore, by examining C-µ1B, we have begun to understand how AP-1B can fulfill its unique functions in basolateral targeting on a molecular level.

v-SNARE Cellubrevin Controls Basolateral Recycling of Cargo in Polarized Apical EPithelial Cells

I. Fields, M. Pyrnan, E. Höfflin, R. Kang, V. Proux-Gillardeaux, T. Galli, H. Fölsch; Biochemistry, Molecular Biology & Cell Biology, Northwestern University, Evanston, IL; 2Cell Biology, Yale University School of Medicine, New Haven, CT; INSERM Avenir Team, Institut Jacques Monod, CNRS UMR7592, Universities Paris 6 & Paris 7, Paris, France

In epithelial cells, polarized membrane trafficking to either the apical or basolateral plasma membrane is essential to maintain cellular polarity. Membrane fusion events are dependent on SNARE pairing between t-SNAREs localized in the target membrane and v-SNAREs localized on transport vesicles. MDCK cells express two major t-SNAREs, syntaxin 3 and syntaxin 4. Whereas syntaxin 3 plays a role in fusion events at the apical domain (Kreiter et al., 2003), syntaxin 4 is suspected to be necessary for basolateral trafficking pathways (Kreiter et al., 2003). However, the exact nature of basolateral vesicles dependent on syntaxin 4 for fusion has not been determined. Furthermore, the specific v-SNARE(s) involved in basolateral fusion events remained elusive. Here we show that the ubiquitously expressed cellubrevin v-SNARE localizes to the basolateral plasma membrane and where it co-localizes with the epithelial-specific clathrin adaptor complex AP-1B. Specific cleavage of cellubrevin with tetanus neurotoxin (TeNT) results in inhibition of cellubrevin function and leads to apical mis-sorting of cargos such as transferrin receptors and truncated LDL receptors, LDLR-T27, which rely on AP-1B for basolateral recycling from recycling endosomes. Moreover, we demonstrate that in fully polarized MDCK cells cellubrevin efficiently co-immunoprecipitates with syntaxins 3 and 4. We conclude that cellubrevin is needed for fusion of at least a subset of basolateral transport vesicles with their target membrane and that this fusion event most likely involves syntaxin 4.

Polarization-dependent, Selective Transport by KIF5B of an Apical Membrane Protein in MDCK Cells

X. Xue, F. Jaulin, E. Rodriguez-Boulan, G. Kreiter; Cell and Developmental Biology, Weill Medical College, Cornell University, New York, NY; 3Margaret M. Dyson Institute for Vision Research, Weill Medical College, Cornell University, New York, NY

For proper cell morphogenesis it is imperative to target membrane proteins correctly to distinct domains within the plasma membrane. Both before and after differentiation, long-range, vesicular transport is mediated by the kinesin family (KIFs) of microtubule-associated motors. However, the mechanism by which vesicular transport is redirected during differentiation to insure domain-selective membrane targeting is unclear. Using time-lapse fluorescence microscopy, we found that in polarized MDCK cells, expression of dominant-negative KIF5B inhibited the post-Golgi transport and targeted delivery of a GPPi-tagged apical marker, p75-GFP. By contrast, inhibition of KIF5B in non-polarized cells had no detectable effect on post-Golgi transport of p75-GFP. We confirmed that KIF5B interacts with p75-GFP in polarized MDCK cells by co-immunoprecipitation analysis. As expected from the functional studies, in lysates prepared from non-polarized epithelial cells KIF5B did not interact with p75-GFP. Inhibition of KIF5B did not impede apical trafficking in general, as we observed no change in the post-Golgi transport and targeting of either prionmin-GFP or GPI-GFP in polarized epithelial cells. Similarly, expression of dominant-negative KIF5B had no effect on post-Golgi trafficking of several GPPi-tagged basolateral membrane proteins, including E-cadherin and the low density lipoprotein receptor in either non-polarized or polarized MDCK cells. Finally, expression of dominant-negative KIF3C or KIF3D did not alter exit of p75-GFP from the Golgi or its targeting to the apical membrane in polarized MDCK cells, demonstrating that transport of p75-GFP is mediated selectively by KIF5B. Together, these results suggest that KIF5B and the different proteins are sorted to the apical membrane by distinct kinesin motors. More significantly, our results provide evidence that cell polarization is accompanied by a change in the machinery used to transport and target proteins to the plasma membrane in polarized epithelial cells. This polarization-dependent switch in kinesin utilization may be a direct cellular response to differentiative cues that lead to cell morphogenesis.

Phosphorylation of the Na,K-ATPase Alpha-1 Subunit at Ser943 Is Involved in the Control of the Na,K-ATPase Activity in MDCK Cells by Prostaglandins

M. L. Taub, T. Rajkhowa; Biochemistry, State University of New York at Buffalo, Buffalo, NY

Prostaglandins regulate renal sodium reabsorption by modulating the activity of the Na,K-ATPase in tubular epithelial cells. The major renal prostaglandin, PGE2, interacts with Gs coupled receptors, activating Protein Kinase A (PKA), as well as Gβ coupled receptors, activating Protein Kinase C (PKC). We have observed that activators of PKA and PKC increase Na,K-ATPase

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activity on the MDCK cell line, a model of distal tubule cells. Acute regulation of the Na,K-ATPase by PGE1 and 8-Bromo-cyclicAMP (8Br-cAMP) was studied by means of cell surface biotinylation studies, using MDCK cells cultured on transwells. A Western analysis of our biotinylated samples indicates an increase in the level of the Na,K-ATPase on the basolateral membrane of MDCK cells following a 30 min incubation with PGE1 and 8Br-cAMP. The Na,K-ATPase α subunit in MDCK cells contains a PKA phosphorylation site (Ser-943). In order to examine the possibility that the Na,K-ATPase α subunit is phosphorylated following PGE1 and 8Br-cAMP treatment, we immunoprecipitated the α subunit from MDCK cell lysates treated with PGE1, 8Br-cAMP or untreated. Immunoprecipitates were separated by SDS-PAGE, and transferred to nitrocellulose. Western blots were probed with an anti-phospho serine antibody, an antibody β antibody, as well as an antibody directed against the sequence in the phosphorylated α subunit, containing phospho Ser-943. Our results indicate an increase in serine phosphorylation of the α subunit in PGE1 and 8Br-cAMP treated MDCK cells, suggest that phospho Ser-943 is involved. Further studies are in progress to determine the role of PKA and/or PKC in mediating these observed effects of prostaglandin.

1126 RaLA Regulates the Delivery of Axonal and Dendritic Proteins to the Cell Surface in Cultured Hippocampal Neurons

B. Sampo, S. Kuech, G. Banker; Center for Research on Occupational and Environmental Toxicology, Oregon Health & Science University, Portland, OR. In epithelial cells, the small GTPase RaLA interacts with Sec5 and Exo84, two components of the exocyst complex, to regulate the delivery of proteins to the basolateral surface. Overexpression of active RaLA (RaLA72L) enhances the delivery of proteins to the basolateral but not the apical surface. We investigated the role of RaLA in neuronal membrane trafficking. When YFP-RaLA was expressed in mature hippocampal neurons, it labeled post-Golgi carriers that also contained NgCAM-CFP, an axonal protein. To determine whether RaLA regulates the delivery of proteins to the axonal surface, we expressed the RaA binding domain of Sec5 (RBD-Sec5) to sequester active endogenous RaA, and examine its effects on the surface expression of NgCAM. Overexpression of RBD-Sec5 did not affect the transport of NgCAM-containing post-Golgi carriers into the axon, but markedly reduced the expression of NgCAM on the cell surface. Expression of RBD-Sec5 also inhibited the delivery of the dendritic protein LDRL to the cell surface, causing the accumulation of LDRL-containing post-Golgi vesicles within the dendrites. Expression of constitutively active RaLA (but not a RaLA mutant that is unable to bind Sec5 and Exo84) led to an increase in the surface expression of both NgCAM and LDRL. Thus RaLA appears to regulate the delivery of both axonal and dendritic proteins to the plasma membrane. Although NgCAM and carriers are transported into both axons and dendrites, normally NgCAM is not efficiently delivered to the dendritic surface. Expression of constitutively active RaLA markedly enhanced the delivery of NgCAM to the dendritic surface, thereby greatly reducing the polarization of this protein. This suggests that the axonal polarization of NgCAM depends on the differential regulation of its exocytosis in axons and dendrites. Because LDRL carriers do not enter the axon, expression of active RaLA did not alter the polarization of this dendritic protein. Supported by NIH grant NS17112.

1127 Myristoylation and Membrane Association of Naked2 Is Required for Its Role as an Antagonist of Canonical Wnt Signaling

T. Hu, T. Van Raay, L. Solinka-Krezel, R. Coffey; 1 Medicine, Vanderbilt University, Nashville, TN; 2Biological Sciences, Vanderbilt University, Nashville, TN. The Wnt and epidermal growth factor receptor (EGFR) signaling pathways have been directly implicated in a broad range of processes including cancers. Recently, a major advancement was made in our understanding of how Wnt and EGFR signaling may cooperate; we found that the Wnt antagonist human Naked2, but not Naked1, escorts transforming growth factor α to the basolateral membrane of polarized epithelial cells. The role of Naked2 in Wnt signaling is poorly understood. Using zebrabith as an in vivo model, we demonstrate that Naked2 is both sufficient and required to antagonize canonical Wnt signaling. Further, we demonstrate that the N-terminal myristoylation sequence is required for this function. Mutating this site (G2A) dramatically reduces its ability to antagonize canonical Wnt signaling. Cell fractionation experiments both in vitro and in vivo demonstrate that while Naked2 is enriched in the membrane fraction, the G2A mutant is found mainly in the cytosol. This argues that Naked2 needs to be associated with the membrane in order for it to antagonize canonical Wnt signaling. Interestingly, we further show that this membrane localization reduces the half life of Naked2. In MDCK cells, we observe that wild type Naked2 is rapidly degraded (t1/2 ≥ 30 min) but the G2A mutant is more stable (t1/2 > 10 h). We demonstrate that association of Naked2 with the membrane is required for its degradation as the small membrane fraction of the G2A mutant (10%) contrast to MFI of 80 with a PGK promoter and MFI of 20 with the human CD40 promoter. Surprisingly, even though 100% of the B lymphocytes and CD34+ cells were infected with MOI of 100-2000, EYFP expression peaked at 80% for the CMV promoter and at 60% and 30% with the PGK promoter and CD40 promoter suggesting that subpopulations of B and CD34+ cells are able to silence these two promoters. In contrast, over 90% of M07e cells expressed EYFP following viral infection independently of the type of promoter. Viability was unperturbed in all cell types. Finally and in contrast to what was observed in cell lines, B lymphocytes and CD34+ cells were unable to initiate translation from a bicistronic expression cassette containing a PGK-IRES-GFP cassette. Thus our results demonstrate that an Ad5/F35 adenovector containing a CMV promoter is an efficient tool for the functional characterization of genes in activated B lymphocytes and in hematopoietic cells in general.
Objective: The objective of the current investigation was to identify the target genes for virus induced lymphoma by breast milk in our mouse model. Methods: Five BALB/c mice were used, one control and four experimental with lymphoma. Of 4 experimental mice 2 were offspring of mothers injected with temperature sensitive (ts) Moloney Marine Leukemia virus, (MoMuLV ts-1) and 1 was nursed by their own mothers; the other two were offspring of control mothers, (injected with media) but nursed by the surrogate infected mothers; the control was an offspring of a control mother raised by a surrogate control mother. Spleen tissue samples were used for DNA and total RNA isolation. Proviral DNA integration sites containing the gene of interest were determined by using inverse PCR (I-PCR). The expression of genes at/or near viral genome integration sites were determined by quantitative real time PCR (qRT-PCR). RNA expression levels were calculated for each gene using standard curves and normalized for GAPDH gene expression. Results: One hundred sixty-three proviral genomic insertion sites were identified, with 112 intergenic and 51 intragenic insertion. Maximum numbers of insertions (over 10) were found in chromosome 5, 7, 10, 11, 12 and 13. Five genes including Ncor 2, Elk 5, Ahil 1, Desert and Ifr 4, were identified, the expressions of which were up regulated from 1.29 fold to as high as 5 folds compared to control without lymphoma. Conclusion: We have identified 5 genes with known functions on nuclear receptor and cell signaling or regulation of lymphoid cell apoptosis. These genes were up regulated as high as 5 folds compared to control mouse. Viral genome integration might have contributed to this up regulation and lymphoma development in this model. This work was supported by American Cancer Society, Ohio Division and FM Douglas Foundation of St. Vincent’s Medical Center.

1131 Mapping Intracellular Protein:DNA Interactions: A More Robust and Efficient Alternative to ChIP (Chromatin Immunoprecipitation)

D. D. Hartzell, M. Urh, N. Karassina, G. Los, K. Wood; Promega Corporation, Madison, WI

Regulation of chromatin structure and gene expression is essential for normal development and cellular growth. Transcriptional events are tightly controlled both spatially and temporally by specific protein-DNA interactions. While significant advances in DNA tiling and microarrays have allowed for genome-wide screening of chromatin recognition sites, current methods to isolate intracellular protein-DNA complexes remain cumbersome and require co-immunoprecipitation, a process inherently sensitive to capture of non-specific DNA and proteins. To address these concerns a novel method has been devised for the covalent capture of protein-DNA complexes which does not require the use of antibodies. Proteins of interest are expressed in cells as HaloTag fusion proteins, crosslinked to DNA, and then captured on HaloLink resin, which forms a highly specific, cognate interaction with HaloTag. Due to the complete covalent linkage established between the resin and the crosslinked protein-DNA complexes, the resin can be stringently washed to remove non-specific DNA and protein much more efficiently than is possible by co-immunoprecipitation. The crosslinks are reversed to release purified DNA fragments from the resin. By improving specificity and reducing background interference during the isolation of protein-DNA complexes, this new methodology effectively increases the signal-to-noise ratio to permit detection of small changes in protein binding within a genome.

1132 Interaction of Intron Sequences of Apolipoprotein (a) Repeats with HepG2 Nuclear Extract

W. Tang, J. H. Wu; Microbiology and Immunology, Chang Gung University, Tao Yuan, Taiwan

Elevated blood lipoprotein (a) [Lp(a)] concentration is an independent risk factor for cardiovascular disease and stroke. Apolipoprotein (a) (Apo(a)) is a component of Lp(a) and contains 15-50 114 amino acid repeats. Each 342 nucleotide repeat is separated by an intron between repeat (designated as intron C) and an intron inside the repeat (designated as intron A/B). As blood Lp(a) level is inversely related to the repeat number of Apo(a), we hypothesize that the intron sequences of the repeat may play role in downregulating apo(a) promoter activity. Our previous studies revealed a down regulation of apo(a) promoter by intron A, B, C. We further investigated what cis element or trans factors were involved by gel shift assay and DNA affinity chromatography, respectively. The intron sequences were scanned for known transcription silencer sequences. These are GC factor (GCF) binding site with a consensus of GCGGCGG and silencing factor (SFA) binding sequence with a consensus of GGGCGGAGA. Several similar motifs are found in the apo(a) introns. The gel shift assays showed bindings of the DNA fragment containing the multiple GCF or SFA homolog with 1-2 base mismatch with the HepG2 nuclear extract (NE). GCF homolog was a better competitor than the SFA homolog. We further used the multimer of GCF homolog only as a probe and observed gel retardation and competition in the presence of HepG2 NE. Using streptavidin agarose to capture biotinylated GCF homolog plus HepG2 NE, we observed a major protein band in the eluate and the protein was identified as nuclear actin by MALDI-TOF experiment. Other minor bands were also observed but they were not identified.

In conclusion, the apo(a) introns in the repeats contain the known transcription downregulator GCF and SFA homolog and this complex that can interact with HepG2 NE and this may play role in downregulating apo(a) promoter activity.

1133 Revealing Combinatorial Regulation via an Integrated Quantification of Transcription Factor Binding

A. Beyer,1,2 C. Workman,3 J. Hollunder,4 T. Wilhelm,4 T. Ieke2; Bioengineering, UCSD, La Jolla, CA, T37b, FLI-Leibniz, Jena, Germany, T38b, Technical University of Denmark, Lyngby, Denmark, T38t, FLI-Leibniz, Jena, Germany

Systematic chromatin immunoprecipitation (ChIP-Chip) experiments have become a central technique for mapping transcriptional interactions in model organisms and humans. However, measurement of chromatin binding does not necessarily imply regulation, and binding may be difficult to detect if it is condition or co-factor dependent. To address these challenges, we present an approach for reliably assigning transcription factors (TFs) to target genes that integrates many lines of direct and indirect evidence into a single probabilistic model. Using this approach, we analyze publicly available ChIP-chip binding profiles measured for yeast TFs in standard conditions, showing that our model interprets these data with significantly higher accuracy than previous methods. We devised a new algorithm for identifying TF sets that cooperate to regulate a significant number of target genes. Pooling the high-confidence interactions reveals a large network containing 363 significant TF modules. We also analyzed multiple occurrences of binding sites. The results show that TFs exhibit distinct patterns of repeated binding sites that are TF specific. For instance we can show that these TFs preferentially have several binding sites in promoter regions (e.g. Skn7p), whereas others have at most one or two binding sites (e.g. Gat1p). In addition, the method predicts 980 novel binding interactions with high confidence that are likely to occur in so-far untested conditions. Indeed, using new ChIP-Chip experiments we were able to verify some of these predicted interactions. We outline the first approach for consistently integrating all available evidences for TF - target interactions and we comprehensively identify the resulting TF module hierarchy. Prioritizing experimental conditions for each factor will be especially important as increasing numbers of ChIP-Chip assays are performed in complex organisms such as humans, for which “standard conditions” are all-defined.

1134 Transcriptional Activation by the TRD1 Domain of Neuron-Restrictive Silencer Factor NR5F1/REST in COS-1 Cells

M. Hernandez-Barrales,2,3 J. Ayala-Lujan,5 J. Tapia-Ramirez,5; Ciencias Quimicas, Universidad Autónoma de Zacatecas, Guadalupe Zacatecas, Mexico, 2Department of Genetics and Molecular Biology, CINVESTAV-IPN, Mexico, D. F., Mexico

The transcription factor REST, a REI-silencing transcription factor, functions as a transcriptional repressor of multiple neuron-specific genes in non-neuronal cells and neuronal progenitors (Chong et al 1995). REST contains a DNA-binding domain and two repressor domains, TRD1 and TRD2, at the first one located at the N-terminal and the second at the C-terminal regions (Tapia-Ramirez et al 1997). Recent studies revealed that TRD1 repressed transcription by binding to mSin3 thereby recruiting HDAC, whereas TRD2 binds to another co-repressor, CoREST (Andrés et al 1999, Naruse et al 1999). The functionality of REST has concentrated on the repression of gene expression, however, several groups have suggested that REST and/or the RE-1, may have a dual function, as either repressor or activator, depending on the spatial and temporal context of its expression (Bessis et al 1997, Kallunki et al 1998). In this work, we investigated the dual function of the repressor domains of REST, therefore employed promoters containing TATA box: Thymidine kinase and Adenovirus promoter E1B and TATA-less promoters: Dehydrofolate reductase and the type II sodium channel. The promoters were cloned with five G4H4-bearing (UAS) sites and CAT reporter gene, transient transfection assays were performed in COS-1 cells. We found that TRD1 was able to activate almost all of the promoter tested over a wide range, meanwhile TRD2 maintained their repressor activity and the introduction of point mutations in the TRD1 domain reverted the transcriptional activation showed previously. This study suggests that TRD1 can be the responsible for dual function of REST. This work was supported in part by grant _30625-M_ from the National Council Science and Technology.

1135 PXET, Putative Xyloglucan Endotransglycosylase, Enhance Growth via BRs Signaling

I. Hwang, H. Chong, Chosun University, Gwangju, Republic of Korea

A specific inhibitor of the plant hormone is a very useful tool to study its function. Brassinazole(brz) is a specific inhibitor for brassinosteroids (BRs) biosynthesis. In order to expand our knowledge of the molecular mechanisms of plant steroid signaling, we performed a genetic screening in the medium containing brz in dark. Mutants insensitive to brz were shown to have long hypocotyls than the ones with the wild type plant. We recovered T-DNA flanking sequences from activation-tagged brassinazole insensitive (abr) mutant and named abr149. TAIL-PCR result indicated a T-DNA insertion at 2.4kb upstream of the putative xyloglucan endo-transglycosylase (PXET) gene in chromosome 2. Northern analysis and recapitulation results indicated that the
phenotypes of abz149 mutants were caused by the overexpression of the PXET gene. PXET-OX lines showed reduced fertility, small silique, early flowering, and taller than wild type. These results indicate that the PXET related with growth enhancement via BRs signaling.

1136 Distinctive Demethylating Effect of 5-aza-2'-deoxycytidine on Gene Coding Regions and Non-coding Repetitive Elements in NIH/3T3 Cells
H. W. Lim, M. Iwabuchi, N. Hattori, M. Suzuki, N. Hattori, S. Tanaka, S. Yagi, K. Shiozaka; Animal Resource Sciences, The University of Tokyo, Tokyo, Japan
5-aza-2'-deoxycytidine (5aza2C), a potent demethylating agent has been used to induce cell differentiation and global demethylation by inhibiting DNA methyltransferase (Dnmt). Previously, we reported that there are different preferences of DNA methyltransferases on specific genomic regions, in which Dnmt1 has preference on the repetitive elements whereas Dnmt3a and 3b primarily functions in gene regions in mouse embryonic stem cells. In present study, we investigated the effect of 5aza2C on repetitive elements, minor satellite repeats and endogenous C-type virus and several selected gene regions, Oct4, Per1 and Sad1 using mouse NIH/3T3 fibroblast cells by methylation sensitive southern blotting and bisulfite restriction mapping. Following a 3-day consecutive treatment with different concentrations of 5aza2C ranging from 0.001 μM and 5 μM, the methylation levels were decreased concentration-dependently at the repetitive elements. In contrast, the methylation levels at the gene regions fluctuated with concentration: unchanged at concentrations below 0.1 μM, decreased significantly at 0.1 μM, but higher concentrations (1 μM and 5 μM) of 5aza2C of did not cause demethylation in the gene regions. Therefore, the effect of 5aza2C was severe in repetitive elements compared to gene regions. Since there was no significant increase or decrease for the expression of Dnmt1, Dnmt3a and Dnmt3b between 5aza2C treated samples and the untreated control, this distinct demethylating effect was irrelevant to any expression changes of Dnmt after treatment. Furthermore, treatment with Trichostatin A (TSA), a histone deacetylase inhibitor, did not enhance the demethylating effect of 5aza2C in both repetitive elements and gene regions. The results obtained in this study may reflect preference of 5aza2C on Dnmt1. In conclusion, 5aza2C has distinctive demethylating effect on gene coding regions and non-coding repetitive elements in NIH/3T3 cells.

1137 FR901464, an Antitumor Microbial Metabolite That Causes Accumulation and Translation of Pre-mRNA, Binds to the SF3B Splicing Complex
D. Kaida,1,2 E. Tashiro,1,2 K. Ishigami,1 H. Watanabe,1 H. Nakajima,1 M. Imoto,1 M. Yoshida1,2 Chemical Genetics Lab., RIKEN, Wako, Saitama, Japan, 1Dept. Biosci. Infor., Fac. Sci. Tech., Keio Univ., Yokohama, Japan, 2Dept. of Applied Biol. Chem., Grad. School of Agricultural and Life Sciences, The Univ. of Tokyo, Tokyo, Japan, 3Exploratory Research Lab., Astellas Pharmaceutical Co., Ltd, Tsukuba, Japan
FR901464 (FR) is a novel antitumor compound that arrests the cell cycle at G1 and G2/M phases and induces apoptosis in a variety of tumor cell lines. We investigated the mechanism by which FR inhibits the cell cycle progression, and found that a C-terminal truncated form of p27Kip1 (p27*7) was accumulated in the FR-treated cells. Surprisingly, p27*7 was produced by translation of the p27 pre-mRNA with the intron 1 containing an in-frame termination codon. Further analyses indicated that FR accumulates pre-mRNAs of not only p27* but also other genes so far tested. In addition, localization of SC-35, a splicing factor that localizes in nuclear speckles, was changed and polyA-RNA accumulated in the nucleus after FR treatment. To identify the target molecule of FR, we synthesized a biotinylated FR probe that allowed isolation of FR-binding proteins, and identified the SF3B splicing complex as the FR-binding proteins in the HeLa cell extract by using CLASH. Interestingly, knockdown of these splicing factors using siRNA induced p27*7 production and change in the SC-35 localization. These results strongly suggest that FR binds and inhibits the splicing factors, thereby causing accumulation and translation of pre-mRNA.

1138 The Male Specific Gene from the Marine Red Alga Aglaothamnion oosumiense (Rhodophyta): Differential Expression of Gene from ESTs Analysis and Two Dimensional Electrophoresis
W. B. Kim, G. H. Kim; Biology, Kongju National University, Kongju, Republic of Korea
Sexual eukaryotes carry two classes of sex-related genes: sex determination genes act in individual organism to determine their gender or mating type and mate recognition genes encode traits that assure that mating occurs between the correct gender/mating type of correct species. Proteomic study using 2D gel electrophoresis showed that sex-specific proteins were 3.5% of total protein (59/1705). The number of sex-specific proteins was different in each sex (2% in female, 1.5% in male). About 13% (216/1705) of proteins expressed differentially according to sex. ESTs analysis showed that male and female plants have differentially categorized gene groups. The genes involved in transcription and protein destination were more abundant in the male than in the female, but the genes involved in metabolism and signal transduction were expressed more in the female. A full-length cDNA encoding male specific gene from database of ESTs analysis was isolated using the rapid amplification of cDNA ends-PCR method. Check up for male specific gene from real-time Polymerase Chain Reaction assays and got the result.

1139 Expression Analysis of the RPL9 Gene in Lung Tumours
Z. Dlumini, L. Mphalele; University of the Witwatersrand, Johannesburg, South Africa
The human ribosomal protein gene (RPL9) is approximately 5.5 kb in length and contains 8 exons. It is located in chromosome 4p13. This gene encodes a single domain ribosomal protein that is a component of the 60S subunit and it has been localized in the cytoplasm. The possible function of this gene is in cell growth and development as the highest expression levels of this gene has been observed in cells undergoing growth and differentiation and that the expression of expression declines as the tissue reaches developmental maturity. The aim of this study was to determine the expression pattern of RPL9 in various lung cancers. Was embedded tissue sections for the normal lung and lung cancer (small cell lung carcinoma, adenoarcinoma, large cell carcinoma and squamous cell carcinoma) were used to investigate the expression of RPL9. Antisense and sense RNA probes were synthesized by in vitro transcription and labeled with Digxygenase. Anti-sense RNA probe complimentary to the RPL9 mRNA was used to localize RPL9 mRNA transcript in tissue sections by in situ hybridization. Real time quantitative PCR was also done in lung cancer cell lines. RPL9 was found to be accumulating in the cytoplasm and was found to be up-regulated in lung cancer tissue sections as compared to normal lung tissue sections. The expression pattern for RPL9 in lung cancer was found to be significantly higher in small cell lung carcinoma, followed by Adenoarcinoma, Large cell carcinoma and least expressed in squamous cell lung carcinoma. Relative quantification showed that there was a 16 fold increase in the level of expression of the RPL9 mRNA in lung cancers. The up-regulation of RPL9 in lung cancer as compared to normal tissues suggests that RPL9 might be involved in the pathogenesis of these tumours.

1140 Regulation of ZBP1 Gene Expression in Metastasis
W. Gu, R. H. Singer; Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, NY
ZBP1 (zincode binding protein) was originally identified as an mRNA regulator, functioning in mRNA localization, stability and translational control. Recent studies revealed that ZBP1 is also a tumor related protein and plays a role in repression metastasis via controlling β-actin mRNA localization. To date, there is no information about how ZBP1 gene is regulated, which could facilitate our understanding of the relationship between ZBP1 regulation and metastasis. In this study, we used rat uterine adenoarcinoma lines as an experimental model to study the regulatory mechanism responsible for ZBP1 gene expression. These two cell lines were from the same tumor; the non-metastatic MTC cell line expresses high levels of ZBP1 and the metastatic MTLn3 cell line has suppressed ZBP1 expression. We demonstrated that repression of ZBP1 gene expression in MTLn3 cells is correlated with the hyper-methylation of its promoter. Transiently transfecting the cell lines with luciferase reporters driven by the ZBP1 promoter revealed that the reporters were actively expressed in MTC cells but were repressed in metastatic MTLn3 cells, implying that a regulatory mechanism exists to control ZBP1 expression in these cell lines. We have identified a 1.5 kb flanking region upstream of the ZBP1 transcriptional initiation site, which contained most of the activities for in vivo ZBP1 gene expression in MTC cells. How this promoter region positively or negatively controls the expression of ZBP1 gene in these two cell lines is under investigation. More interestingly, examining ZBP1 expression in samples of human breast tumors and metastatic tumors derived from the same origin, we detected a greatly reduced ZBP1 expression in the metastatic tissues. This suggests that down-regulation of ZBP1 expression could be a common phenomenon in metastasized breast tumors and it could become a valuable marker to predict metastasis.

1141 Engineering the PP7 Coat Protein for Fluorescent-tagging of mRNAs in Living Cells
J. A. Chao, S. C. Almo, R. H. Singer; Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, NY
The specific interaction between the MS2 coat protein (CP) and its cognate RNA stem-loop (SL) has been extensively exploited for molecular and cell biology including the labeling of mRNAs with fluorescent proteins. This technique allows for the fate of individual mRNAs to be followed in real-time in living cells. While this has opened the door for the interrogation of gene expression at the single cell and single molecule level, the reliance on a single RNA-protein complex limits the biological questions that can be addressed and prevents us from taking advantage of the myriad of fluorescent proteins that are now available. In order to expand our current ability to study mRNA in vivo, we have engineered the PP7 CP for use in tandem with the MS2 system. Like the MS2 CP, the PP7 CP binds tightly to its RNA SL and this interaction is orthogonal to any host RNA-protein complex. Importantly, biochemical experiments indicate that the PP7 CP binds its own RNA target 1000-fold tighter than the MS2 SL and we have confirmed this discrimination in a human U2OS cell line containing a transgene that contains 24 repeats of the MS2 SL. Construction of a similar transgene construct containing the PP7 SL is currently underway to validate the system. The MS2 CP-RNA protein interaction has been extensively characterized both biochemically and structurally. To better understand the molecular details of the PP7 CP-RNA interaction, we have crystallized the PP7 CP and determined its structure at 1.8Å resolution and are undertaking crystallization of the PP7 CP-RNA complex. Quantitative RNA-binding experiments will also be used identify which amino acids contribute significantly to the selectivity to the PP7 CP-RNA SL interaction. This work may allow us to design novel RNA-protein complexes that will further expand our ability to study mRNA in live cells.

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Generating Amino Acids Substitutions to Alter the Activity and Response Specificity of the Anti-Sigma Factor, ChrR
J. B. Rodríguez-Molina, 1, R. S. Greenwell, 1 T. J. Donohue; 1Industrial Biotechnology, University of Puerto Rico at Mayagüez, Mayagüez, PR, 2Bacteriology, University of Wisconsin-Madison, Madison, WI

The extracytoplasmic function (ECF) sigma factor, $\sigma^E$, of Rhodobacter sphaeroides stimulates transcription in response to singlet oxygen ($O_2$) stress. Its cognate anti-sigma factor, ChrR, is part of a family of zinc-binding anti-sigma factors (ZAS) that releases $\sigma^E$ in response to $O_2$ stress. ChrR is a two domain ZAS, where the N-terminal interacts with $\sigma^E$ and coordinates zinc, and the C-terminal is required for the response. $\sigma^E$ is coordinated by two cysteine residues (Cys$^E_1$ and Cys$^E_2$) and two histidines residues (His$^E_1$ and His$^E_2$) in ChrR. Another member of the ZAS family is RsrA in Streptomycetes coelicolor: RsrA inhibits the activity of $\sigma^E$ until disulfide stress occurs. Between ChrR and RsrA, three of the zinc ligands are conserved in a canonical zinc-binding motif, HXn-CXnH. The fourth zinc ligand differs between RsrA (Cys$^E_3$) and ChrR (His$^E_3$). A mutant ChrR protein containing a cysteine substitution at His$^E_3$ (ChrR-H6C) has been generated and remains functional to inhibit $\sigma^E$. To assess whether ChrR-H6C has altered specificity to stress responses, plate diffusion experiments have been conducted. Interestingly, cells containing ChrR-H6C can respond specifically to superoxide ($O_2^-$). The anti-sigma factor RseA of E. coli is a transmembrane protein that binds the activity of $\sigma^E$. RseA releases $\sigma^E$ in response to periplasmic stress. The structures of RseA and ChrR have been solved in complexes with their respective $\sigma$ factors, and both contain a three-helical structure that interacts with their cognate $\sigma$ factors. While ChrR requires zinc to form this three-helical structure, RseA does not. A zinc-free version of ChrR (ChrRAzA) is being generated by nested PCR mutagenesis reactions to substitute 9 amino acids of ChrR (with those from the RseA domain). This poster will also present on the ongoing experiment to generate ChrRAzA and assess its ability to inhibit $\sigma^E$ activity and respond to $O_2$.

1143

Transcriptional Regulation and Action at a Distance: Using Cells as Test Tubes to Probe DNA Mechanics In Vivo
H. G. García, 1 L. Bintu, 2 J. Kondes, 2 R. Phillips; 1Department of Physics, California Institute of Technology, Pasadena, CA, 2Department of Physics, California Institute of Technology, Pasadena, CA

DNA mechanics plays a central role in gene expression. Transcriptional control is realized through the interaction of the transcriptional machinery with DNA-bound transcription factors which can loop the DNA between two distant sites. DNA mechanics influences the gene expression readout of a wide variety of transcriptional networks. 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. Based on this quantitative understanding of DNA looping in the lac operon we propose to use an artificial lac system as a tool to probe DNA mechanics in vivo. In particular, by using a fluorescent reporter, we show that eukaryotic nucleosomal positioning sequences are indeed more flexible by characterizing their bending by Lac repressor in bacteria in the complete absence of nucleosomes. This work demonstrates that a careful interplay between quantitative data and quantitative models leads to a consistent picture of transcriptional regulation which yields falsifiable predictions.

1144

Expression of Retinoblastoma Binding Protein 6 (RbP6p) Gene in Human Immunodeficiency Virus Associated Nephropathy (HIVAN)
N. P. Numaite; Faculty of Health Sciences, School of Anatomical Sciences, University of the Witwatersrand, Parktown Johannesburg, South Africa

Background: Since Human Immunodeficiency Virus associated nephropathy (HIVAN) was discovered, HIVAN cases have increased drastically and now the leading cause of end stage renal disease (ESRD) deaths. HIVAN prevalence is constantly linked to people of African descent (90% blacks). Nephritic-range proteinuria, renal insufficiency, and enlarged kidneys are typical features in patients with HIVAN. This is especially the case in patients already with HIVAN. RbP6p isoform 3 a ubiquitinin-like, apoptotic gene when mutated or deleted in cells renders their resistance to apoptotic induction. The Human Immunodeficiency Virus (HIV) uses apoptosis as a means to survive immune attack. It encodes pro-apoptotic proteins which kill both infected and uninfected T lymphocytes through Tumour Necrosis Factor (TNF) or mitochondrial pathway. The aim of this study was to analyze the expression of the three human RbP6p isoforms at the protein level using immunocytochemistry Results: Preliminary results show that there are high levels of the RbP6p isoform 3 protein in the glomerulus and tubules. Higher levels of apoptosis were also demonstrated in glomerular and tubular cells. This was evidenced by brown stained spots in the cytoplasm and nucleus. Mononuclear interstitial infiltration also is a characteristic of HIVAN. In situ hybridization showed up-regulation of RbP6p isoform 3 mRNA. In normal kidney cells the RbP6p isoform 3 gene is mostly expressed in the cytoplasm, in the diseased kidney RbP6p isoform 3 translocates to the nucleus. Conclusion: Elevated levels of RbP6p isoform 3 and apoptosis in HIVAN suggest the involvement of RbP6p isoform 3 in the pathogenesis of this disease. It may be concluded that RbP6p isoform 3 gene is correlated with the levels of apoptosis in HIVAN.

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Early Response of Gene Expression to OAT3-mediated Cephalexin Toxicity Assessed by DNA Microarray Analysis
P. Jutabha, T. Muto, H. Endou, Y. Kanai; Pharmacology and Toxicology, Kyorin University School of Medicine, Tokyo, Japan

The primary site for renal elimination of drugs, toxins and their metabolites occurs in renal proximal tubule. It is the important tissue susceptible to the damage by the toxicants. Cephalexin, a first-generation of cephalosporin antibiotic, was known as nephrotoxicant transported into the renal proximal tubular cells by organic anion transporters (OATs). The mechanisms of nephrotoxicity induced by the compound have not yet completely understood. In this study, we examined the toxicity of cephalexin in OAT3-expressing renal proximal tubular cells (S2-OAT3) using cell viability assay. Transcriptionally-mediated cephalexin toxicity was assessed at the molecular level using microarray analysis. The low concentration of cephalexin induced the toxicity process in the cytoplasm, in the diseased kidney RbP6p isoform 3 localizes to the nucleus. Conclusion: Elevated levels of RbP6p isoform 3 and apoptosis in HIVAN suggest the involvement of RbP6p isoform 3 in the pathogenesis of this disease. It may be concluded that RbP6p isoform 3 gene is correlated with the levels of apoptosis in HIVAN.

1146

A Genomic Fossil Reveals Key Steps in Hemoglobin Loss by the Antarctic Icefishes
H. Dietrich, S. K. Parker, T. J. Near; 1Biology, Northeastern University, Boston, MA, 2Ecology and Evolutionary Biology, Yale University, New Haven, CT

Antarctic icefishes are the only vertebrates that do not have hemoglobin and erythrocytes in their blood. These startling phenotypes are associated in several icefish species with deletions of juvenile and adult globin loci, in which red-blooded teleosts are typically composed of tightly linked pairs of $\alpha$- and $\beta$-globin genes. It is unknown if the loss of hemoglobin expression in icefishes was the direct result of such deletions, or if other mutational events compromised globin chain synthesis prior to globin gene loss. In this study, we show that 15 of the 16 icefish species have lost the adult $\beta$-globin gene but retain a truncated $\alpha$ globin pseudogene. Surprisingly, a phylogenetically derived icefish species, Neopagetopsis ionah, possesses a complete, but non-functional, adult $\alpha$-globin complex. This cluster contains two distinct $\beta$-globin pseudogenes whose phylogenetic origins span the entire Antarctic notothenioid radiation, consistent with an origin via introgression. Maximum likelihood ancestral state reconstruction supports a scenario of icefish globin gene evolution that involves a single loss of the transcriptionally active adult $\alpha$-globin cluster prior to the diversification of the extant species in the clade. Through lineage sorting of ancestral polymorphism, two types of alleles became fixed in the clade, 1) the $\alpha$-globin...
pseudogene of the majority of species, and 2) the inactive αβ-globin complex of N. ionah. We conclude that the globin pseudogene complex of N. ionah is a "genomic fossil" that reveals key intermediate steps on the pathway to loss of hemoglobin expression by all icefish species. Supported by NSF grants OPP-9120311, 9402712, 9815381, 0809451, and 0336932 to H.W.D.

1147 Functional Interaction of Nucleolin, c-Jun and Sp1 in PMA-induced Gene Expression of Human PLA₂β
B. Chen, J. Tsou, W. Wang; Chang; Department of Pharmacology, National Cheng Kung University, Tainan, Taiwan

cPLA₂ is the major intracellular form of cPLA₂, which preferentially hydrolyzes membrane phospholipids at the sn-2 position to release arachidonic acid (1). Arachidonic acid metabolism through cyclooxygenase (COX) and lipoxygenase (LOX) pathways generates various biologically active lipids that play important roles in inflammation, thrombosis and tumor progression. It has been shown that PMA provides cPLA₂-activating signals. In this study we found that PMA induced the expression of cPLA₂β in human non-small-cell lung carcinoma (NSCLC) A549 cell. The two Sp1 sites of cPLA₂β promoter were essential for PMA-induced transcription and Sp1 was involved in the regulation of gene expression. PMA stimulation also facilitated access of c-Jun to the promoter resulting in the formation of c-Jun/Sp1 complex, and enhanced the complex binding to the promoter. Furthermore, we identified nucleolin binding to the Sp1 site of the cPLA₂β promoter. Nucleolin also cooperated with c-Jun/Sp1 to regulate the transcriptional activity of cPLA₂β gene. These results indicated that nucleolin/c-Jun/Sp1 complex played an important role in PMA-induced gene expression.

1148 SFHR-mediated Modification of Genomic HPRT without Random Insertion of the Modifying Oligonucleotide
B. Bedayat,1 H. Emamekhoo,1 A. Abdolmohammadi,2 L. Ye,3 J. Cheung,1 J. O’Neill,4 J. A. Nicklas,4 D. C. Gruenert1,2,5; 1Research Institute, California Pacific Medical Center, San Francisco, CA, 2Laboratory Medicine, University of California, San Francisco, San Francisco, CA, 3Cardiovascular Research Institute, University of California, San Francisco, CA, 4Pediatrics, University of Vermont, Burlington, VT, 5Medicine, University of Vermont, Burlington, VT

Gene therapy has opened the door to new therapeutic interventions for the treatment of genetic diseases. In addition to the standard cDNA-based approaches, alternative gene targeting strategies have been developed. In this study, an oligonucleotide gene targeting strategy, small fragment homologous replacement (SFHR), was used to correct a point mutation in exon 3 of the hypoxanthine guanine phosphoribosyltransferase (HPRT) gene that inactivates HPRT in a lymphoblastoid cell line (LT1-1B1) and allows the cells to grow in 6-thioguanine (6TG) containing medium. Because of their mutant status, LT1-1B1 cells will not grow in medium containing hypoxanthine/aminopterin/thymidine (HAT) unless the mutant HPRT is corrected. The LT1-1B1 cell line was transfected with a 579-bp small DNA fragment containing wild-type HPRT sequences at the site of the mutation using the AMAXA Nucleofector. Corrected LT1-1B1 clones were selected in HAT containing medium and further analyzed by PCR, reverse transcriptase PCR (RT-PCR), and Southern blot hybridization. Quantitative analysis showed a SFHR-mediated correction frequency of between 10⁻⁴ to 2 x 10⁻⁴. Southern blot analysis indicated a wild-type restriction fragment length polymorphism (RFLP) consistent with SFHR-mediated correction. No other sites of integration were observed. This work is supported by CPMC Research Foundation Funds

1149 The Transcriptional and Proteomic Response to Cold Shock and Recovery in Saccharomyces cerevisiae
K. D. Dahquist,1 W. T. Citi,2 M. Mejia,2 E. S. Eberhard;2 1Biology, Loyola Marymount University, Los Angeles, CA, 2Chemistry, Vassar College, Poughkeepsie, NY

Previous studies on the global transcriptional response of Saccharomyces cerevisiae to cold shock have revealed that the response can be divided into a set of early response genes (after 15 minutes to 2 hours of cold temperatures) and late response genes (after 12 to 60 hours of cold stress; Sahara et al., 2002; Kandor et al., 2004; Schade et al., 2004). The late response genes include the ESR genes induced by many environmental stresses (Schade et al., 2004). These findings need to be extended to fully characterize the pathways and processes to which the early response genes unique to cold shock, and characterize, and to predict the protein-level response. The global transcriptional response to cold shock and subsequent recovery was measured using DNA microarrays. Yeast cells were grown to early log phase at 30°C, then shifted to 13°C for 60 minutes, and then shifted back to 30°C for another 60 minutes. Samples were collected before cold shock, after 15, 30, and 60 minutes of cold shock, and after 30 minutes and 60 minutes of recovery at 30°C. The samples collected at each time point were split so that both total RNA and total protein lysate could be isolated from the same sample of yeast cells. The total RNA was isolated, amplified, and labeled with Cy3 and Cy5 dyes by the indirect method, and hybridized to DNA microarrays. Protein levels from the same yeast samples were determined by two-dimensional polyacrylamide gel electrophoresis, followed by spot intensity quantitation, and identification by MALDI-TOF mass spectroscopy. We have found that genes belonging to the glycolytic pathway were induced at both the transcriptional and translational level after 30 minutes of cold shock. In addition, genes that are induced or repressed by cold shock return to their pre-cold shock levels after 60 minutes of recovery at 30°C.

1150 Intergenic Transcripts Joining CXC Chemokine Genes CINC2 and Lungkine in Rat Lung
J. N. VanderHart,1 J. Schade,1 B. Bedayat,1 H. Emamekhoo,1 A. Abdolmohammadi,2 L. Ye,3 J. Cheung,1 J. O’Neill,4 J. A. Nicklas,4 D. C. Gruenert1,2,5; 1Research Institute, California Pacific Medical Center, San Francisco, CA, 2Laboratory Medicine, University of California, San Francisco, San Francisco, CA

Intergenic transcripts, containing exon sequences of one gene spliced to those of another, have been described in several systems but their significance is unclear. We present evidence that an intergenic transcript encodes CINC2β, a previously described rat CXC chemokine. The genes for CINC2 (Chemokine Induced Neutrophil Chemoattractant 2) and lungkine (CXXCL15) are 10 kb apart on rat chromosome 14. The CINC2 gene generates two chemokines, CINC2α and CINC2β, that differ only in 3′-terminal amino acids. The source of C-terminal variation is differential splicing of exons specific for CINC2α and CINC2β. In the rat DNA sequence, CINC2α-specific exon 4, encoding C-terminal amino acids, stop codon, 3′UTR and polyadenylation signals, predictably follows the third intron of the CINC2β gene. In contrast, CINC2β-specific exon 4, encoding C-terminal amino acids and the stop codon, shares sequence but not reading frame with exon 2 of the lungkine gene. The 3′UTR of CINC2β mRNA thus contains lungkine-coding-sequence exons 2, 3 and 4, and then terminates in a polyadenylation signal shared with lungkine. RT-PCR with upstream primers in exons 2 or 3 of the CINC2 gene and downstream lungkine primers generated the intergenic CINC2β transcript and several additional intergenic transcripts containing combinations of CINC2α and lungkine exon sequences. Only lung RNA contained these intergenic transcripts among several tissues examined including liver, brain, kidney and heart. CINC2-lungkine intergenic transcripts were observed in a rat distal lung epithelial cell line (X404) but not in a fibroblast line (Rat1). A similarly arranged pair of CXC chemokine genes located close to CINC2 and lungkine (MP2 and GRO) did not generate intergenic transcripts when tested by RT-PCR. Thus, the region of rat chromosome 14 encompassing the CINC2 and lungkine genes generates intergenic transcripts in pulmonary tissue. Supported by NIH grants HL24075 and HL57426.

1151 Characterization of trh924 in Thermosynechococcus elongatus BP-1, a Unique Prochlorophyte
S. D. Lane Njuguna; Biology, Mills College, Oakland, CA

Phycotoxins are photosensizers that enable plants to change light changes in light conditions and adapt via photomorphogenesis or phototaxis. Recently, phycotoxins were found in bacteria and fungi. The major proteins that bind to the GAF domains of these phycotoxins are categorized into four subgroups: the eukaryotic Ph domain, the cyanobacterial Cph domain, the bacterial BphP domain, and the putative photosensitizer Phr family, also referred to as the unorthodox Photos. The Phr family is the most divergent class in its members conserve the GAF domain but lack the PH domain. Thermosynechococcus elongatus BP-1, which was isolated from a hot spring in Beppu, Japan, is a unicellular, rod-shaped cyanobacterium. A computer prediction and similarity search revealed five Phr genes in Thermosynechococcus elongatus BP-1. In the phylogenetic tree of Phr proteins, Thfr924 is a "genomic fossil" that reveals key intermediate steps on the pathway to loss of hemoglobin expression by all icefish species. Supported by NSF grants OPP-9120311, 9402712, 9815381, 0809451, and 0336932 to H.W.D.

1152 Two Novel Splice Variants of Human Vascular Endothelial Growth Factor (VEGF) That Had Been Identified Using the Spliceriminer™ DNA Library Panel
L. Wu; Research and Development, Stratagene, La Jolla, CA
The SpliceMiner™ cDNA library Panel is designed to simplify the discovery of human alternative splice forms that would have been difficult or impossible to detect by other methods currently available. The SpliceMiner cDNA library panel is a comprehensive collection of the human transcriptome. It consists of ~4.6 million primary cDNA clones derived from 15 high quality cDNA libraries representing 28 cancer cell lines and 34 human tissues, including 14 sub-regions of the brain. The panel contains cDNA transcribed from full length and low abundance human mRNAs and is enriched for complete transcripts, while maintaining the diversity of message found in the original human source material. Recently, the SpliceMiner cDNA library panel was employed for screening alternate splice forms of the human vascular endothelial growth factor (VEGF) gene which was demonstrated to be expressed in a variety of tumor cells. PCR was employed on the SpliceMiner cDNA library panel using primers that are complimentary to the terminal exons of VEGF. Multiple PCR products of all size populations across the entire range of cell lines in the panel were sequenced and results were aligned against the published human genomic sequence. In one screen, five VEGF splice variants were identified. Isoform 2, 4 and 6 had been previously identified and published by other research groups. The other two novel splice variants originated from leukemia and colon cancer cell lines contain a putative exon between exon 3 and 4. The two newly-discovered splice variants are believed to be expressed at lower abundance. The separation of cDNA clones into a large number of pools, the SpliceMiner cDNA library panel increases the likelihood of obtaining new alternate splice forms that would have otherwise been masked by more abundant variants. The SpliceMiner cDNA library panel is a unique, efficient method for detecting human alternative splice forms.

Loc1p Is Required for Efficient Assembly and Nuclear Export of the 60S Ribosomal Subunit in Saccharomyces cerevisiae
C. R. Urbiniti, G. B. Gomisalver, J. P. Aris, R. M. Long
biology, Loyola Marymount University, Los Angeles, CA, Microbiology and Molecular Genetics, Medical College of Wisconsin, Milwaukee, WI, Anatomy and Cell Biology, University of Florida College of Medicine, Gainesville, FL

Loc1p was identified as an exclusively nuclear dsRNA-binding protein that affects the asymmetric sorting of ASH1 mRNA to daughter cells in Saccharomyces cerevisiae. In addition to its role in cytoplasmic mRNA localization, Loc1p was identified as a constituent of pre-60S ribosomes. Cells devoid of Loc1p display a defect in the synthesis of 60S ribosomal subunits, resulting in “halfform” polyribosomes. Previously, we reported that Loc1p is located throughout the entire nucleus; however, upon closer inspection we discovered that Loc1p is enriched in the nucleolus consistent with a role in 60S ribosome biogenesis. Given that Loc1p is an RNA-binding protein and presumably functions in the assembly of 60S ribosomal subunits, we investigated if Loc1p has a role in pre-RNA processing and nuclear export of 60S subunits. Analysis of pre-RNA processing revealed that loc1Δ cells exhibit gross defects in 25S RNA synthesis, specifically a delay in processing at sites A0, A1 and A2 in 35S pre-RNA. Furthermore, loc1Δ cells exhibit nuclear export defects for 60S ribosomal subunits, again, consistent with a role for Loc1p in the assembly of 60S ribosomal subunits. It is attractive to hypothesize that the two phenotypes associated with loc1Δ cells, namely altered ASH1 mRNA localization and ribosome biogenesis, are not mutually exclusive, but that ribosome biogenesis directly impacts mRNA localization.

The Major Vault Protein Is Related to the Prokaryotic Toxic Anion Resistance Protein Family, TelA
K. Suprenan, N. Bloom, B. Hendrick, K. Huffman, D. Corey, J. Fang, G. Lushington
Molecular Biosciences, University of Kansas, Lawrence, KS, Pharmacology and Biochemistry, University of Texas Southwestern Medical Center, Dallas, TX, Bioinformatics Core Facility, University of Kansas, Lawrence, KS, Molecular Graphics and Modeling Laboratory, University of Kansas, Lawrence, KS

Vaults are large, hollow ribonucleoprotein particles that are very abundant in certain tumors and multidrug resistant cancer cells. Recent studies have suggested that vaults may function in subcellular or nucleocytoplasmic transport and or as a scaffold in the epidermal growth factor signaling pathways important for cell survival. We initiated a sequence-based approach to understanding vault function because vault structure and composition are conserved amongst eukaryotes. We report that the major vault protein, MVP, is related to the tellurite-resistance protein, TelA, of Rhodobacter sphaeroides strain 2.4.1, given preliminary sequence analysis suggesting multiple conserved motifs between MVP and TelA, and a recently released NMR solution structure of MVP indicating 30% sequence identity conservation within structurally resolved regions and gaps present only in unstructured coil regions. Because the structural correspondence is not enough to guarantee a similar function, we examined whether mammalian cell vaults responded to tellurite treatment and importantly, whether vaults are sufficient for tellurite resistance. Knocking down the expression of vault mRNAs by specific siRNAs does not affect the cytotoxicity of tellurite in HeLa cells. However, in the presence of tellurite, large vault aggregates, or vautosomes, appear at the cell periphery in as little as 15 minutes. Vautosome formation is temperature-dependent, reversible, and occurs in normal human umbilical vein endothelial cells as well as transformed HeLa cervical cancer cells. Vautosomes are unique in that they do not colocalize with ubiquitinated proteins, metalllothionein, the stress granule protein, TIA-1, and other ribonucleoproteins such as PAPP-1 and HnRNP A2. We propose that vautosomes are an unresolved component of a stress response pathway that originated in bacteria. In this regard, vaults may act to integrate the normal cellular pathways of growth and survival with the stress responses evoked by toxic metalloids and chemotherapeutic agents.

Flex Appeal: Exploring the Structural Flexibility of the Functional Interaction between Ku and Telomerase
Department of Biology, Boston College, Chestnut Hill, MA, Department of Chemistry and Biochemistry, University of Colorado, Boulder, CO

Telomerase is a ribonucleoprotein enzyme that stabilizes chromosomes by adding telomeric DNA repeats to the 3’ ends of DNA. The RNA component of yeast telomerase, TELC1, binds a series of proteins, among them the catalytic subunits Est2p, Est1p, the Sm proteins, and the double stranded DNA end-binding protein Ku, which plays a role in recruiting telomerase to chromosome termini. Deletion of the 46-nucleotide Ku binding site on TELC1 (tel1Δ) results in reduced yet stable telomeres, as well as slower de novo telomere addition at broken DNA ends. Previous research has demonstrated that yeast maintain robust cell growth and nearly wild-type telomere length when the essential Est1p binding site is repositioned in the TELC1 molecule. These results showed that relative positioning of Est1p in telomerase is not rigidly constrained and that TELC1 may function as a flexible tether for proteins. Here, we test whether the relative positioning of Ku in telomerase is similarly flexible. We have created tel1 Δ alleles that reposition the Ku binding site within TELC1. We find, using an in vivo assay, that these mutant RNAs retain the ability to recruit telomerase. Strains expressing these tel1 Δ alleles are able to maintain native telomeres at a slightly longer length than tel1Δ strains. However, their ability to add a telomere de novo after induction of a double strand break shows little or no improvement over tel1Δ. These data suggest that there is some structural flexibility in the functional interaction between Ku and telomerase at telomeres, yet there is a limit to this flexibility when telomerase is recruited to broken DNA ends. We are currently exploring the molecular basis behind these differences.

Determinants of Rbp1p Localization in Specific Cytoplasmic mRNA-processing Foci, P-bodies
L. Jang, L. Buu, F. Lee
Institute of Molecular Medicine, National Taiwan University, Taipei, Taiwan

Rbp1p, a yeast RNA-binding protein, decreases the level of mitochondrial porin mRNA by enhancing its degradation, but the intracellular location of the Rbp1p-mediated degradation complex remains unknown. We show here that Rbp1p in xrn1Δ mutant yeast localizes in specific cytoplasmic foci that are known as P-bodies. The N-terminal and RRM1 domains of Rbp1p are
necessary but not sufficient for its localization in P bodies. Rbp1p forms oligomers through its C-terminal domain in vivo; N-terminally deleted, or RRMI mutated Rbp1p can be more efficiently recruited to P-bodies in an xrn1/3 strain expressing a full-length Rbp1p. Although POR1 mRNA is localized to P bodies in an xrn1/3 strain, this localization does not depend on Rbp1p. Decapping activator Dhh1p directly interacts with Rbp1p. In addition, the recruitment of Rbp1p to P-bodies does not require Dhh1p or Crep1. In wild-type cells, Rbp1p can localize to P-bodies under glucose deprivation or treatment with KCl. In addition, Rbp1p-mediated porin mRNA decay is elicited by Xrn1p, a 5' to 3' exonuclease. These results provide new insight into the mechanism of Rbp1p function.

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**Lacandonia Granules Are Present in Aloe vera and Tegetes erexcta Cell Nuclei**


Lacandonia granules are intranuclear ribonucleoprotein particles first described in the plant *Lacandonia schismatica* and further in other plants as *Tririx* and *Ginglyso biloba*. Here we used light, electron and atomic force microscopy to analyze the presence of these structures in other plants as *Aloe vera* and *Tegetes erexcta*, representative of monocots and eudicots. By using the specific method of ouimarin ammine we found that *A. vera* display ricinucatated chromatin while *T. erexcta* is chromocentric. Standard transmission electron microscopy and the preferential method for ribonucleoproteins indicate that Lacandonia granules are present in *A. vera* but are absent in *T. erexcta*. Results extend the observations on the existence of Lacandonia granules among plants, and suggest a wider distribution in nature (supported by DGAPA-UNAM IN215106-3).

1159

**The MicroRNA, miR-206, Concentrates in Ribosome-rich Regions of Both the Nucleus and the Cytoplasm**

J. C. R. Politz, F. Zhang, T. Pederson; Biochemistry/Molecular Pharmacology, University of Massachusetts Medical Center, Worcester, MA

MicroRNAs are small 22-24 nucleotide RNAs that have been found to regulate gene expression. miR-206 is a microRNA that is expressed at high levels in *Drosophila*, zebrafish and mouse skeletal muscle, and is thought to be involved in the control and/or maintenance of the differentiated state. We used oligo probes modified with locked nucleic acid nucleotides for in situ hybridization studies to characterize the intracellular localization of miR-206 during differentiation of rat myogenic cells. Like most microRNAs, which are presumed to suppress translation of target mRNAs in polyribosomas, we found that miR-206 occupies a cytoplasmic localization patterns in cultured myoblasts and differentiated myotubes, and that these levels increase in myotubes over the course of differentiation, consistent with previous findings in animal tissues. However, to our surprise, we also observed concentrated signal in the nucleus. A probe for miR-1, which differs from miR-206 by only three nucleotides, did not show nuclear localization. The results of both BLAST and Emboss searches showed that there is no significant complementarity between our probe for miR-206 and any non-coding RNAs (including rRNAs or snoRNAs) in the rat genome (other than miR-206 itself). We further characterized the intracellular localization of the miR-206 and found that a substantial fraction colocalizes with 28S RNA in both the cytoplasm and the nucleas. miR-206 is not concentrated in either the ribosomal centers of the nucleas nor in the dense fibrillar component, where polysorn RNA synthesis occurs and processing begins, but rather is localized in the granular component, the region of the nucleas where ribosomes assembly occurs. These results suggest that miR-206 may be associated with ribosomes not only in the cytoplasm, but also in the nucleas.

1160

**NMD Pathway is Dominant over the A2RE RNA Trafficking Pathway**

M. Ifrim, J. H. Carson2; 1Neuroscience, UConn Health Center, Farmington, CT, 2Molecular, Microbial & Structural Biology, UConn Health Center, Farmington, CT

Intracellular trafficking of normal RNA in eucaellular processes includes: processing in the nuclaus, export to the cytoplasm, transport to specific cytoplasmic localization sites, translation and degradation. RNAs containing premature termination codon (PTC) are processed in the nucleus but then diverge from the normal RNA trafficking pathway and are targeted for degradation by the Nonsense Mediated Decay (NMD) pathway. NMD is a surveillance mechanism that causes PTC-containing RNA to be degraded, preventing accumulation of truncated proteins. All RNA molecules are believed to undergo a pioneer round of translation soon after they are exported from nucleus, during which PTC containing RNAs are degraded by NMD. A2RE is an RNA localization sequence that functions in nuclear cells and is known to inhibit translation until the RNA is localized. We are trying to determine when and where in the RNA trafficking pathway nonsense-containing RNA is distinguished from normal RNA. We also want to determine if RNA containing both PTC and A2RE, is exported, localized and degraded or escapes NMD. Previous timelapse microscopy experiments suggested that PTC containing A2RE RNA is more stable than RNA without A2RE. In order to determine whether RNA containing both PTC and A2RE is degraded by NMD, we created a fluorescent reporter system for monitoring the level and localization of protein expression. Our results indicate that RNA containing both PTC and A2RE is subject to NMD. Also, fluorescent protein resulting from translation of PTC containing RNA is distributed mainly around the nucleus regardless of the presence or absence of A2RE, suggesting that the pioneer round of translation takes place soon after the RNA is exported from the nucleus, even for RNAs containing the A2RE. In conclusion our experiments suggest that NMD pathway is dominant over the A2RE pathway.

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**Efficient Gurken Secretion Requires Trailer Hitch, a Component of a Ribonucleoprotein Complex Localized to the ER in Drosophila**

J. E. Wilhelm, M. Busczak, S. Sayles; 1Biological Sciences, University of California, San Diego, San Diego, CA, 2Embryology, Carnegie Institution of Washington, Baltimore, MD

During *Drosophila* oogenesis, dorsomedial cells differentiate into dorsomedial-oocyte complexes that surround the egg chamber. The key signaling molecule in this process is gurken (grk), a TGF-alpha homolog that is a ligand for the epidermal growth factor receptor (EGFR). Gurken signaling is restricted to a subset of follicle cells bordering the oocyte by the gurken mRNA localized to the dorsal anterior region of the oocyte. Localized Gurken secretion activates EGFR, which is ubiquitously expressed in follicle cells, and only cells with activated EGFR adopt a dorsal fate. We have identified a new gene, *trailer hitch* (tral), that is required for dorsal-ventral patterning. Mutations in *tral* disrupt Gurken secretion leading to ventralized eggs. This phenotype is not secondary to a defect in *gurken* mRNA localization or oocyte polarity as both are normal in *trailer hitch* mutants. Surprisingly, biochemical purification of Tral revealed that it is part of a large RNA-protein complex that includes two components required for translational control of localized messages: the RNA helicase, Me31B, and the elf4E binding protein, Cup. Tral, Me31B, and Cup colocalize to subdomains of the endoplasmic reticulum (ER) that overlap and border ER exit sites. Consistent with a role of the complex in regulating ER exit, we found that the transcripts for two components of the ER exit site machinery, Sarl and Sec13, are also associated with the complex. Furthermore, mutations in *tral* alter the distribution of Sarl protein and disrupt normal ER exit site formation. We propose that the efficient secretion of Gurken requires the local translation of proteins at ER exit sites and that tral is required for this process. These findings raise exciting new possibilities for how the mRNA localization machinery could interface with the secretory pathway to promote efficient protein trafficking.

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**ZBP1 Mediates Dendritic mRNA Granule Localization through a Direct Interaction with a Novel Kinesin Light Chain (KLC)-like Protein in Neurons**

J. B. Dictenberg, K. J. Verhey, A. Friedland, J. Goldman, S. Huttelmeier; 1Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, NY, 4Cell Biology, Emory University, Atlanta, GA

Subcellular localization of mRNAs and local protein synthesis is necessary for proper outgrowth and plasticity in neurons. However the molecular details of how mRNAs granules are recognized by the microtubule motor transport machinery and how their interaction is regulated remains unknown. Here we show a new interaction between the conventional microtubule motor kinesin-I and an integral component of RNA granules, the zipcode-binding protein (ZBP1) which binds to the localization element (zipcode) within the 3-prime-UTR of beta-actin mRNA and targets it to neurites and growth cones in response to synaptic stimulation. ZBP1 associated with microtubules and kinesin-I (KHC and KLC), but not kinesin-II, in dendrites of hippocampal neurons. Immunoprecipitation of kinase-I subunits showed beta-actin mRNA, but not vinculin mRNA, associated in a zipcode-dependent manner. Yeast two-hybrid revealed a new, phylogenetically conserved kLC-like protein enriched in beta-actin RNA granules that localized to dendrites and growth cones of hippocampal neurons. Domain mapping revealed among plants, and suggest a wider distribution in nature (supported by DGAPA-UNAM IN215106-3).

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The Epigenome in Health and Disease Is Addressed by Modified Codes of “Non-Coding” Small RNA Bioaptamers: Not a State, but Patterns of Processes in Transition and Turnover

J. H. Wissler; ARC Cons-Institute for Applied Research & Didactics, Bad Nauheim, Germany

OBJECTIVE: Usually, the epigenome is considered as collection of biochemical chromatin/histone/DNA modifications indexing genetic information [review: Jenuwein, Science 297:2215-2218,2002; Lederberg, TheScientist 15:6,2001]. Hence, many epigenetic [non-Mendelian] phenotype alterations [EPA] basic to health and [e.g. conformational] disease may escape consideration. Aimed at understanding of control by RNA/RNP, epigenetic regulator proteins [ERP] were investigated. METHODS were developed for isolation of homologous domains with many other ERP [FEBS J. 217:291-306,2005; Spermatogenesis in the water fern Marsilea vestita is a rapid process that relies on mRNA and proteins stored in the dry microspore. The microspore consists of one cell, which when placed in water, undergoes nine division cycles to produce 7 somatic cells and 32 spermatids. After the divisions are completed, each spermatid undergoes a complex differentiation process, which includes nuclear elongation, cell coating, cytoskeletal formation and ciliogenesis. Mago nashi is a highly conserved protein and a component of the exon-on exon junction complex (EJC) for pre-mRNA splicing. It becomes associated with other EJC components and pre-mRNA to function in the nonsense-mediated mRNA decay pathway, enhancing translation and transport/localization of mRNAs for axis formation. In M. vestita, Mv-mago is important in early development establishing cytoplasmic domains that underlie cell fate specification and control of the planes of cell divisions. The morphology of RNAi-mago treated sperms combined with the localizations of proteins involved in spemogenesis (centrin, a-tubulin) and transcripts encoding proteins involved in RNA processing (Mv-elF4AIII, Mv-Prp19) show that cell fate specification is disrupted after knockdowns of Mv-mago. Even with mild knockdowns where division patterns are normal, anti-centrin antibody reveals anomalous basal body-like staining in sterile cells late in development. We suspect that Mv-mago affects the translation and distribution of cytoskeletal or motor proteins. Not surprisingly, knockdowns of the EJC components Mv-Y14 and Mv-elF4AIII closely resemble developmental anomalies observed with Mv- Mago dsRNA. Mv-mago protein becomes localized in dots in the cytoplasm of mainly spermogenetic cells at the end of the division cycles, up to the release of the gametes. Newly translated Mv-mago, Mv-Y14 and Mv-elF4AIII are essential for mago-dot formation. Mago-dots may be EJCs and appear to be essential for gamete development. [Supported by NSF grant MCB 0234423 to SMW].

Nuclear Pore Dynamics during Fungal Open Mitosis

U. Theisen,1 A. Straube,2 G. Stemberg1; Organismissche Interaktionen, Max-Planck-Institut für Mikrobiologie, Marburg, Germany, University of Edinburgh, Edinburgh, United Kingdom

OBJECTIVE: Nuclear pores are dynamic organelles required for nuclear import and export of proteins and mRNAs. In this study, we investigated the dynamics of nuclear pores using the yeast model system, S. cerevisiae. Our results showed that the assembly and disassembly of nuclear pores is regulated by the actin cytoskeleton. We also found that the nucleus is a dynamic organelle that can change its shape and position in response to changes in the cytoplasm. The nucleus is also a site of DNA repair and gene expression, and its dynamics play a role in the regulation of these processes.

LEMDomain Proteins and Nuclear Lamina Function: Loss of Drosophila MAN1 Causes Tissue-specific Defects

B. S. Pinto,1 S. R. Wilkinson,1 L. L. Wallra, P. K. Geyer, Biochemistry, University of Iowa, Iowa City, IA.

Distinct transcriptional territories are established in the nucleus through connections between chromatin and proteins in the nuclear lamina, a protein network that lines the inner nuclear membrane. One class of lamina proteins is the LEM domain family, which have a conserved domain that interacts with chromatin. LEM domain proteins are involved in the formation of the nuclear lamina, which plays a key role in gene expression and chromatin organization. The potential of the nuclear lamina to regulate gene expression is mediated by the LEM domain proteins, which form complexes with other proteins to regulate gene expression and chromatin organization. The LEM domain proteins are also involved in the formation of nuclear pore complexes, which are important for nuclear import and export of proteins and mRNAs.
increased the peripheral incidence of a Lacl-GFP visualized euclaxic LaclO locus. That neither well-characterized NETs such as emerin, LBR or LAP2 nor the other novel NETs tested had any influence on the distribution of these GFP-chromatin proteins serves as an internal control for the specificity of these NET functions. Thus it appears that some of these novel NETs can have a profound influence on chromatin architecture.

1168 Essential Role of Drosophila Lamin C in Nuclear Integrity and Chromatin Organization

B. V. Gurudatta, L. S. Shashidhara, V. K. Parmaik; Centre for Cellular and Molecular Biology, Hyderabad, India

Nuclear lamins are components of a filamentous network underlying the inner nuclear membrane termed the nuclear lamina. Lamins are important determinants of nuclear architecture and are also involved in vital functions of the nucleus such as replication, transcription and apoptosis. There are two kinds of lamins, B-type lamins that are expressed in all cells, and A-type lamins, which are differentially expressed during development. Mutations in the human lamin A gene cause a group of highly debilitating diseases called laminopathies. In order to understand the developmental role of A-type lamins, we have carried out a detailed genetic analysis of Drosophila lamin C (lamC) - a bona fide orthologue of vertebrate A-type lamins. We have generated lamC-specific alleles using P-element excisions and also made lamC RNAi transgenic lines. Mitotic clones with lamC excision allele or lamC RNAi were cell lethal, supporting the role of lamC in cell survival. The RNAi-mediated knock-down of lamin C in all cell types caused early lethality. The tissue-specific down-regulation of Lamin C resulted in a variety of phenotypes in wings, legs, thorax and eyes. The UAS-GAL4 mediated over-expression of Lamin C in various tissues resulted in the disruption of nuclear envelope organization and eventually manifested as developmental defects in muscles, wings and thorax. Perturbation in Lamin C expression affected lamina-chromatin interactions since the lamC excision alleles suppressed position effect variegation both at centromeric and telomeric sites, supporting a role for Lamin C in heterochromatic gene silencing. Heterochromatin protein 1 was either depleted (by lamC RNAi) or mis-localized (by over-expressed Lamin C) from its chromosomal sites. In summary, we provide evidence that Drosophila Lamin C is essential for nuclear integrity during development and is specifically required for chromatin organization.

1169 The Small Yeast Outer Nuclear Membrane Protein Apq12 Functions in Nuclear Pore Complex Biogenesis

J. J. Scarcelli,1 C. A. Hodge,1 C. N. Cole;1 Biochemistry, Dartmouth Medical School, Hanover, NH, 1Biochemistry and Genetics, Dartmouth Medical School, Hanover, NH

All transport of macromolecules between the nucleus and the cytoplasm occurs through nuclear pore complexes (NPCs), very large macromolecular assemblies embedded within the nuclear envelope and constructed using multiple copies of approximately 30 proteins, called nucleoporins. While the structure, organization, and function of various components of the NPC has been the focus of many studies, relatively little is known about NPC biogenesis. Here we report on Apq12p, a new player in this process. Apq12p was originally linked to mRNA processing and transport because apq12Δ cells accumulate mRNA with elongated poly(A) tails in their nuclei. Apq12p is a trans-membrane protein located in the outer nuclear envelope and endoplasmic reticulum. We find that cells lacking APQ12 display defects in the localization of a subset of nucleoporins, particularly those located asymmetrically at the cytoplasmic filament of the NPC. The cytoplasmic filament nucleoporins are the ones most important for mRNA export, so the defects observed in mRNA biogenesis and export are likely indirect consequences of aberrant NPCS. Electron microscopy reveals that deletion of APQ12 causes cessation of formation of membranous “studs” located around the nuclear envelope, as well as other defects in nuclear envelope morphology. In the absence of Apq12p, NPCs can be seen that are associated with the inner but not the outer nuclear envelope. Deletion of APQ12 leads to synthetic growth defects when combined with mutations in genes encoding several nucleoporins believed to provide structure to the NPC, as well as the integral membrane proteins POM152, POM34, and NDC1. We also show that the C-terminal truncation of Apq12p, which is predicted to be on the cytoplasmic side of the nuclear membrane, is required for function. The finding that inhibition of translation prevents mislocalization of GFP-tagged nucleoporins in apq12Δ cells strongly supports the idea that Apq12p plays a role in NPC biogenesis.

1170 Targeting Protein for Xklp2 (TPX2) Interacts with Both Lamins and Chromatin and Prevents Chromatin Decondensation in Xenopus Egg Extracts

L. O'Brien, C. Wiese; Biochemistry, UW-Madison, Madison, WI

The nuclear envelope, which separates a cell’s genetic material from the cytoplasm, disassembles at the onset of mitosis in most metazoans to allow assembly of the mitotic spindle. Reassembly of a functional nucleus after mitosis involves rebuilding the nuclear envelope around the decondensing chromosomes and continued nuclear growth. We recently described an essential role for the mitotic spindle assembly factor, TPX2, in nuclear reformation after mitosis (O'Brien and Wiese, 2006. J CB 173:685). The nuclear envelope, which separates a cell’s genetic material from the cytoplasm, disassembles at the onset of mitosis in most metazoans to allow assembly of the mitotic spindle.

1171 The Role of the Nuclear Envelope in Controlling the Migration and Anchorage of the Nucleus

D. A. Starr, M. D. Matthew, R. Rillo, M. Meyeron, D. M. Drainow; Center for Genetics and Development, University of California-Davis, Davis, CA

The position of the nucleus within the cell is important to a wide variety of eukaryotes. For example, budding yeast must move the nucleus to the bud neck, mammalian skeletal muscles must specialize nuclei at the neuromuscular junction, and in the developing human brain nuclei migrate towards the cortex. Defects in these processes have severe developmental defects and can lead to muscular dystrophy or lisencephaly. In order to position the nucleus, the cell must specify the outer nuclear membrane from the ER and transfer force across the nuclear envelope from the cytoskeleton to the nuclear matrix. Here we describe a mechanism where the C. elegans proteins UNC-83 and UNC-84 accomplish both of these important tasks. Digitonin extraction experiments suggest that UNC-84 is an inner nuclear membrane protein and UNC-83 is specific to the outer nuclear membrane. UNC-84 recruits UNC-83 to the outer nuclear membrane. We show by genetic methods in C. elegans and by a membrane bound yeast two hybrid assay that the SUN domain of UNC-84 directly interacts with the KASH domain of UNC-83 in the perinuclear space. Furthermore, both the SUN domain and the KASH domain are essential for nuclear migration in vivo. We have also undertaken a deletion analysis of the nucleoporin domain of UNC-83 and show that the C-terminal region is dispensable, and that the N-terminus is essential for nuclear migration. Finally, we have purified a His-tagged version of the SUN domain and are analyzing the KASH/SUN interaction in vitro. These data support the model that KASH and SUN proteins bridge the nuclear envelope, connecting the nuclear lamina to chromoskeletal components. This mechanism appears conserved across eukaryotes and is the first proposed mechanism to target proteins specifically to the outer nuclear membrane.

1173 C. elegans BAF and Its Kinase VRK Partake Directly in Postmitotic Nuclear Envelope Assembly

M. Gorjancic,2 E. Klerks,2 V. Galy,1 R. Santarella,1 C. Lopez-Iglesias,1 P. Askjaer,3,4 I. W. Mattaj3;1 European Molecular Biology Laboratory, Heidelberg, Germany, 3Institute for Research in Biomedicine, Barcelona, Spain, 2University of Barcelona, Barcelona, Spain

The nuclear envelope (NE) of eukaryotic cells provides an essential barrier that separates the genome from the cytoplasm. During mitotic prophase the NE disassembles to enable the chromosome segregation and in anaphase and telophase it reassemblies around the chromatin. We are studying Barrier-to-autointegration factor (BAF) that is an essential and highly conserved controller we found that BAF is directly involved in NE formation. With a series of temperature-shift experiments on Caenorhabditis elegans embryos either carrying a temperature sensitive point mutation in the chromosome segregation and in anaphase and telophase it reassemblies around the chromatin. We are studying Barrier-to-autointegration factor (BAF) that is an essential and highly conserved

2006 ASCB Annual Meeting Abstracts
Changes in Nup214 Domain Topology in Response to Chemical Effectors
S. M. Paullö, M. A. Powers, K. S. Ullman, B. Fahrenkrog1, M. E. Mueller Institute for Structural Biology, University of Basel, Basel, Switzerland, 2Department of Cell Biology, Emory University School of Medicine, Atlanta, GA, 3Department of Pharmacological Sciences, University of British Columbia, Vancouver, BC, Canada
Nuclear pore complexes (NPCs) span the nuclear envelope (NE) and provide major gateways for diffusion of small molecules, as well as signal-mediated transport of proteins, RNAs and RNP complexes across the nucleus. Approximately 30 different nucleoporins assemble the NPC, from which around one third are characterized by the presence of distinct polyglutamine motifs. Recent studies have identified critical amino acid residues within the RRM that are required for DNA binding. Data will also be presented on the potential role of the RRM in RNA binding and RNA metabolism.

Dissection of Vertebrate Nup53 Function through Domain Localization and Interaction Mapping
L. A. Hawryluk-Gara, R. W. Wozniak; Cell Biology, University of Alberta, Edmonton, AB, Canada
Nuclear pore complexes (NPCs) work in conjunction with soluble transport factors termed karyopherins to facilitate movement of macromolecules across the nuclear envelope (NE). The NPC is an evolutionarily conserved structure comprised of ~30 proteins termed nucleoporins (nups) that are present in multiple copies and organized into sub-complexes of interacting nups. In an effort to further understand how NPCs are formed and the mechanisms that control nuclear transport, we have examined the function of an essential vertebrate nucleoporin, Nup53. In these studies, we have investigated the role of various domains of Nup53 in mediating its assembly into the NPC and its interactions with neighboring nups. A series of N- and C-terminal truncations were examined by fluorescence microscopy to define domains necessary for NPC localization. In addition, in vitro binding studies were performed to map the domains of Nup53 necessary for its interactions with binding partners, namely the nucleoporins Nup93, Nup205, Nup155, and Ndc1. We showed that Nup53 interacts with these nups through a C-terminal domain where Nup155, Nup93, and Ndc1 interact with mutually exclusive domains. In line with our domain analysis of Nup53, we have initiated studies to examine the function of the RNA recognition motif (RRM) motif present within a central region of Nup53. We have observed a direct interaction between Nup53 and both single- and double-stranded DNA. Furthermore, mutational analysis identified critical amino acid residues within the RRM that are required for DNA binding. Data will also be presented on the potential role of the RRM in RNA binding and RNA metabolism.

Nuclear Size Control in Fission Yeast
F. R. Neumann, P. Nurse; The Rockefeller University, New York, NY
Many eukaryotic cells maintain a constant ratio between the volume of the nucleus and the cytoplasm. This requires a well orchestrated interplay between many processes which can influence nuclear and cell size, such as membrane biosynthesis, nucleo-cytoplasmic transport, and the cell cycle and growth machinery. Fission yeast is a good organism for studying nuclear envelope homeostasis due to the simple geometry of the cell and the nucleus. In metazoans, developmental changes, nuclear envelope breakdown during mitosis and the presence nuclear lamins add another layer of complexity to the control of nuclear envelope shape and size. We first describe nuclear growth during the cell cycle and further characterize how different cell size, growth conditions, DNA content, affect the nucleo-cytoplasmic ratio (N/C). Time-lapse studies reveal that the N/C volume ratio is kept at a constant throughout all stages of the cell cycle. This suggests that there is a rapid expansion of the nuclear envelope during mitosis, similar to budding yeast. Genetic and physical perturbations of this equilibrium show that nuclear growth is stopped at a high N/C ratio until cell size reaches a threshold upon which the nucleus and the cell will grow at the same rate. Taking advantage of the extensive repertoire of cell cycle and size mutants in fission yeast, we find that the N/C ratio is conserved over a 10-fold difference in cell sizes. In agreement with the cell cycle studies we find that cells arrested in G1 phase or G2 phase of the cell cycle have a conserved N/C ratio. Further increase of DNA content in diploid cells or in an endoreduplicating condition further undermines the strong conserved correlation between the size of the nucleus and the cell. Future experiments are aimed at elucidating the molecular mechanisms of this remarkable conservancy.

Mitic Remodeling of the Nuclear Envelope
D. R. Mackay, S. Elgort, A. J. Prunuske, K. S. Ullman; Huntsman Cancer Institute, University of Utah, Salt Lake City, UT
Remodeling and disassembly of the nuclear envelope during mitosis has been acknowledged as an important event in the metazoan cell cycle for over a century; however, the mechanisms by which this occurs and is coordinated with other mitotic events are still poorly understood. Recent studies in Xenopus egg extracts have demonstrated that the zinc-finger domains of two components of the nuclear pore complex, Nup214 and Nup358/RanBP2, interact with the COP1 customer complex and that this interaction is required for efficient nuclear disassembly, thus uncovering a previously unexplored function for the nuclear pore in this process. This mechanism appears to be conserved in mammalian cells, as overexpression of the Nup153 zinc-finger domain results in a two-fold increase in the number of mitotic cells in prophase concomitant with a decrease in the number of cells in metaphase, consistent with a delay in mitotic progression resulting from disrupted nuclear disassembly. To further understand the role of Nup153 in nuclear disassembly and how this process is coordinated with other mitotic events, we are taking a multifaceted approach using both RNAi and dominant negative strategies to disrupt Nup153 in somatic cells. Interestingly, we have found that mitotic arrest resulting from spindle stress is perturbed when Nup153 levels are decreased in HeLa cells, suggesting that these cells are either not progressing into mitosis or that the spindle stress checkpoint is not activated properly. A combination of FACs analysis and live imaging methods are currently being used to distinguish between these two possibilities. This work promises to provide new insight into the mechanism of nuclear remodeling and how it is coordinated with other cell cycle events.

The Role of Dyanin 3 in the Tests
K. S. Vaid1, J. A. Gutmann2, N. Babyk1, W. Deng2, M. A. McNiven1, M. Nohchi1, B. B. Finlay2, A. W. Vogel1, C. Lau1, S. Elgort1, 1Cellular and Physiological Sciences, University of British Columbia, Vancouver, BC, Canada, 2Michael Smith Laboratories, University of British Columbia, Vancouver, BC, Canada, 3Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN, 4Structural Analysis, National Cardiovascular Center Research Institute, Osaka, Japan
We report here that dyanmin 3 is the testis specific isoform of the vertebrate nuclear envelopeoporin Nup53. The nuclear pore complex is a major gatekeeper for diffusion of small molecules, as well as signal-mediated transport of proteins, RNAs and RNP complexes across the nuclear envelope. Approximately 30 different nucleoporins assemble the NPC, from which around one third are characterized by the presence of distinct polyglutamine motifs. Recent studies have identified critical amino acid residues within the RRM that are required for DNA binding. Data will also be presented on the potential role of the RRM in RNA binding and RNA metabolism.

Using a Chemical Genetic Approach to Protein Kinase A (PKA) as a Novel Tool to Investigate Male Fertility
D. J. Morgan, M. Weissenhaus, D. F. Babcock, C. Zhang, K. M. Slebk1, G. S. McKnight1, 1Pharmacology, University of Washington School of Medicine, Seattle, WA, 2Biophysics and Biophysics, University of Washington School of Medicine, Seattle, WA, 3Cellular and Molecular Biology, University of California, San Francisco, San Francisco, CA
We generated a novel strain of mice that expresses a point mutation in the catalytic-alpha subunit (Ca) of PKA. The Ca432A point mutation enhances the ATP binding pocket of PKA conferring sensitivity to pharmacological inhibition through the ATP analog, 1NM-PFI. Protein homogenates containing the mutant PKA were prepared from testes as well as other tissues from Ca432A

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The WD1 Scaffold RACK1 Is Essential throughout Development

J. L. Kadrams, M. A. Smith, S. M. Pronovost, M. C. Beckerle; Huntsman Cancer Institute and the Department of Oncological Sciences, University of Utah, Salt Lake City, UT

Primary regulator of spermatogenesis is an intrinsic genetic program involving male germ cell-specific genes. In this study, we analyzed the mouse spermatocyte UniGene library (Library 6787) containing 2155 gene-oriented transcript clusters, predicting that 230 of them are testis-specific genes, and identified 24 novel genes by computational bioinformatics tools, we found that a number of the gene products are potentially involved in various processes during spermatogenesis or fertilization. We performed transfection assay of the genes using GC-2 cells, providing information on the cellular characteristics of the gene products. Further, we generated antibodies against proteins encoded by a number of the novel genes and identified the proteins in spermatogenic cells and sperm. Taken together, we discovered the 24 authentic, novel genes with spermatogenic cell-specific expression by the integrative approach, and provide new and comprehensive information about the novel testis-specific genes by various in vitro and in silico analyses. Thus, this study establishes on a comprehensive scale a new basis for future investigation on functional characterization of the genes.

HeterotrimERIC G P R OTEIN(s) as an Src-activator in Xenopus Fertilization

T. Iwasaki, 1 K. Sato, 2, 3 Y. Kono, 3 Y. Koretomo, 4 G. Mammadova, 4 N. Yamaamoto, 5 H. A. K. M. Mahbub, 6 Y. Ueda, 6 Y. Fukami 1, 2, 7, 8 1 Research Center for Environmental Genomics, Kobe University, Kobe, Japan, 2 Department of Biology, Faculty of Science, Kobe University, Kobe, Japan, 3 The Graduate School of Science & Technology, Kobe University, Kobe, Japan, 4 A sperm induces a transient Ca2+ release, activation, and beginning of embryonic development in fertilized egg. We have reported that the Xenopus Src tyrosine kinase (xSrc) plays an important role in the egg activation signaling. xSrc, which localizes to the low-density detergent-insoluble fraction (LD-DIM or rafts) of egg plasma membrane, is activated and partially translocated to the cytosol within a few minutes after insemination. However, the mechanism of sperm-induced xSrc activation is still unrevealed. Previous studies demonstrated that microinjection of non-hydrolyzable GTP analog, GTPγS, an activator of GTP-binding proteins (G proteins), into Xenopus eggs resulted in egg activation. It was also shown that a pertussis toxin-insensitive G protein is involved in Xenopus egg activation (Kline et al. Dev Biol 1991). Another group showed that α subunits of G proteins, Gai and Gi, but not Gq, could directly interact with the kinase domain of e-Src and activate it (Ma et al. Cell 2000). We have reported that GTPγS promotes Src activation in the rafts isolated from Xenopus unfertilized eggs in vitro (Sato et al. Development 2002). These results suggest that G proteins act as an xSrc and egg activator. To assess this possibility, we tested whether GDPβS has an inhibitory effect on egg activation. GDPβS decreased the successful rate of egg activation in a dose-dependent manner. We have also shown that some signaling molecules including xSrc and Gi are present in the raft fraction prepared from unfertilized eggs. To analyze direct interaction between xSrc and G proteins, we prepared glutathione-S-transferase (GST)-fused α subunits of Xenopus G proteins. It is shown that GST-Gia, but not Gqβγ, can activate the xSrc in vitro. Our results support the hypothesis that G proteins such as Gai and Gi activate xSrc in the egg rafts at sperm-induced egg activation.

The WDI Scaffold RACK1 Is Essential throughout Drosophila Development

J. L. Kadrmas, M. A. Smith, S. M. Pronovost, M. C. Beckerle; Huntsman Cancer Institute and the Department of Oncological Sciences, University of Utah, Salt Lake City, UT

RACK1 shows that these proteins are highly conserved. Therefore, we have explored the function of RACK1 in Drosophila with 3 μM 1NM-PP1 blocked the increase in flagellar beat frequency that was caused by bicarbonate administration during capacitation. In contrast, the beat frequency of wildtype sperm increased from 2.6 Hz to 5.2 Hz in response to bicarbonate and was unaffected by 1NM-PP1. We conclude that the Drosophila mutant RACK1 is sensitive to inhibition by 1NM-PP1 in vitro and that tissue specific expression of this mutation will allow cell-type specific pharmacological inhibition of PKA in vivo to study the physiological roles of PKA. Supported by NIH grants: GM32875 (G.S.M.), HD12629 (D.F.B.), HL07312 (D.J.M.)
Within the mutant oocytes, egg chambers appear morphologically normal but mature eggs are not laid. Stage 14 eggs eventually accumulate within the ovarioles, with few early stage egg chambers present despite proper maintenance of germ-line stem cell populations. Our analysis highlights the critical importance of RACK1 in developing tissues.

1187 Highly Conserved Cysteines in the “VM Domain” of sV23 Are Essential for Eggshell Assembly in Drosophila

T. Wu, G. L. Waring; Biological Sciences, Marquette University, Milwaukee, WI

Extracellular matrices are complex molecular networks that not only provide mechanical support, but serve as reservoirs for bioactive molecules that control cell growth and differentiation. We are using the Drosophila eggshell, a specialized extracellular matrix amenable to biochemical, genetic, and morphological analyses, to study molecular mechanisms and motifs that are used to regulate its assembly in vivo. The oocyte proximal vitelline membrane (vm) layer consists of a small number of abundant proteins that possess a highly conserved 38 amino acid sequence termed the “vm domain”. Within the “vm domain” are 3 precisely spaced cysteines (CX3CX3CX3). To investigate their role in eggshell assembly we created a series of cysteine to serine substitution mutations in sV23, a major vitelline membrane protein. To assess the effects of the mutations we introduced each mutant transgene into an sV23 protein null female sterile mutant (fs(2)QJ42). Functional sV23 transgenes rescue the sterility and defective eggshells of fs(2)QJ42 females. A functional eggshell failed to assemble when the sV23 transgene had substitutions at either all three, or two of the three positions. Although sV23 accumulation was negligible in the triple cysteine mutant, sV23 accumulated at levels compatible with function in the double mutants. In wild type eggshells sV23 becomes incorporated into a large disulfide linked network during the terminal stages of oogenesis (12-14). In the double cysteine mutants sV23 formed small oligomeric disulfide linked complexes that failed to remain integrated in the eggshell correlated with the removal of its hydrophobic N-terminal segment via a normal, regulated cleavage event. While other proteins with “vm domains” were incorporated into large disulfide networks, their subsequent insolubilization via the formation of non-reducible cross-links was impaired, suggesting interdependence between the assembly of sV23 and other “vm domain” containing protein networks.

1188 Release of Calcium by Phosphatidic Acid in Xenopus Oocytes and Eggs

B. J. Stith,1 J. Juergens,1 J. Stafford,1 J. Snyder,1 M. Wood,2 D. Petcoff2; 1Biology, University of Colorado Denver, Denver, CO, 2Biology, Metropolitan State College, Denver, CO

The current model for fertilization in Xenopus laevis is that sperm activates Src tyrosine kinase which activates phospholipase C (PLC)γ (work by K-J Sato and others) to increase inositol 4,5-bisphosphate (IP2) and intracellular calcium. We have provided evidence that the missing step between sperm and Src activation is activation of phospholipase D1β2 which catalyzes the breakdown of phosphatidylcholine to phosphatic acid (PA): inhibiting the PA increase at fertilization inhibited IVB induction (but not sperm entry into the egg or the DAG mass increase), addition of 400 μM PA increased IP3 mass whereas herbimycin A, a tyrosine kinase inhibitor commonly used to inhibit Src, blocked PA elevation of IP3. PA also raised intracellular calcium whereas 2APB, an IP3 receptor blocker, inhibited calcium release. We now report that herbimycin A inhibited the PA- induced in vitro release of calcium from a preparation consisting of plasma membrane, cortex and cortical cytoplasm (PMC). Inhibited calcium release of PA action was obtained with 4 μM herbimycin, 50% inhibition with 2 μM herbimycin and 0% inhibition with 0.5 μM herbimycin (20 min preincubation). As an alternate method of intracellular calcium measurement in whole cells, we recorded the membrane potential of Xenopus oocytes as depolarization has been shown to be a measure of intracellular calcium. 400 μM PA-induced membrane depolarization (calcium release) that was inhibited by preincubation with herbimycin. PA addition shortly after a previous release of intracellular calcium by LPA (during the LPA refractory period) did not elicit a second release of calcium. This suggests that PA releases calcium specifically from intracellular stores used by LPA (e.g., IP3)- not through some nonspecific or alternate calcium release paths. As a control, ionomycin, which induces a nonspecific release of calcium, did induce a membrane depolarization when the ionophore was added at an equivalent time after LPA.

1189 Identification and Distribution of Carbohydrates on the Surface of Gametes in the Zebra Mussel Dreissena polymorpha and the Oyster Crassostrea virginica

L. C. Fallis,1 K. M. Kendro-McAnlis,1 J. W. Lynn,2 M. J. Misamore1; 1Biology, Texas Christian University, Fort Worth, TX, 2Biological Sciences, Louisiana State University, Baton Rouge, LA

Carbohydrates play a critical role in many cell-cell adhesions, including sperm-egg binding. The objective of this study is to identify carbohydrates associated with the surfaces of eggs and sperm and map their distribution during fertilization using two species of bivalve, the freshwater zebra mussel Dreissena polymorpha and the eastern oyster Crassostrea virginica as models. Both species are broadcast spawners with external fertilization. We used several lectins to characterize surface carbohydrates including: WGA, Con A, LCA, and GSII. Initial studies have found C. virginica and D. polymorpha have similar carbohydrate distributions such that the entire surface of eggs of both species labeled with WGA, Con A, and LCA. WGA also labeled the entire surface of sperm in both species. Con A, LCA, and GSII labeled only the lateral region of acrosome in acrosome-reacted sperm in both zebra mussels and oysters. GSII was the only lectin that specifically labeled the sperm acrosome but not the egg. During fertilization in D. polymorpha, GSII labels the acrosome in unbound, acrosome-reacted sperm and is localized to the binding site in sperm bound to the egg. Following incorporation of fertilizing sperm, the GSII labeling remains as a patch on the egg surface. Once an egg is fertilized, nonfertilizing bound sperm are detached from the egg surface at approximately 15 min postinsemination. The majority of the GSII labeling leaves with the detaching sperm. These studies suggest a role for N-acetylgalactosamine in initial sperm-egg interactions.

1190 The Role of Mos in Sea Urchin Fertilization and Early Mitotic Cell Cycles

M. M. Roux, K. R. Fotlz; MCDB and Marine Science Institute, University of California Santa Barbara, Santa Barbara, CA

The mitogen activated protein kinase (MAPK) cascade plays a crucial role in oocyte maturation, fertilization and early development. In eggs, this signaling module consists of MEKK (Mos), MEK and an ERK1/2 like MAP kinase, but its precise role in regulating the transition from arrested egg to the first mitotic cell cycle is unclear. The purple sea urchin Strongylocentrotus purpuratus is a unique model organism, spawning eggs that are fully mature and arrested at haploid pronuclear stage, making it possible to investigate regulation of MAPK signaling at fertilization specifically with respect to mitotic cell cycle progression and without the complication of meiotic completion. Of particular interest is the MEKK Mos. This kinase’s role in meiotic maturation has been extensively studied, but its role post fertilization and during mitosis remains controversial and elusive. We have identified the gene and cloned the cDNA encoding the S. purpuratus Mos ortholog (SpMos). The SpMos gene is a single exon containing a 1257bp open reading frame encoding a protein of ~48 kDa. SpMos mRNA and protein are expressed in sea urchin eggs and early embryos and expression of full length SpMos in Xenopus blastomeres causes cell cycle arrest, confirming that SpMos functions as a canonical Mos protein. Interestingly, SpMos has a unique N terminus extending 90 amino acids past the Xenopus Mos N-terminal domain and lacking the canonical phosphorylation site that regulates Mos protein degradation. SpMos lacking this extended N-terminus also causes Xenopus embryos arrest, suggesting that the N-terminus is not required for the arrest function. Experiments are under way to determine the potential functional role of the N-terminal domain (possibly in regulating degradation or activity) and to investigate the requirement of SpMos for normal cell cycle progression at fertilization in sea urchin zygotes.

1191 Milton Controls the Early Acquisition of Mitochondria by Drosophila Oocytes

R. T. Con, A. C. Spradling; Dept. of Embryology, Carnegie Institution/HHMI, Baltimore, MD

Mitochondria in many species enter the young oocyte en mass from interconnected germ cells to generate the large aggregate known as the Balbiani body, or mitochondrial cloud. Organelles and germ plasm components frequently associate with this structure. Balbiani body mitochondria are thought to populate the germ line, ensuring that their genomes will be inherited preferentially. We find that miton, a gene whose product was previously shown to associate with Kinesin and mediate axonal transport of mitochondria, is needed to form a normal Balbiani body. In addition, germ cells mutant for some mito or Kinesin heavy chain (Khc) alleles transport mitochondria to the oocyte prematurely and excessively, without disturbing Balbiani body- associated components. Our observations show that the majority of mitochondria by competitive bidirectional transport along microtubules and this is mediated by the Milton adaptor. These experiments provide a molecular explanation for Balbiani body formation and, surprisingly, show that viable, fertile offspring can be obtained from eggs in which the normal program of mitochondrial acquisition has been severely perturbed.
Cell shape changes, central to cellular function and development, require coordinated spatial regulation to redirect changes in cell form. Phosphoinositide phosphates (PIPs), phosphorylated derivatives of phosphaatylinositol, are highly spatially regulated lipids known to localize signaling events and cellular processes. The many lipid kinases and phosphatases that target specific PIPs therefore are crucial to cellular spatial-temporal regulation. Phosphoinositols are a large family of phosphoinositide 3-phosphate (PI(3)P) lipids implicated in human developmental myopathy and neuropathy diseases. However, little is known about the cellular roles and regulation of different Myotubularin phosphatases, or their specific functional PIP substrates, in mammalian myogenesis. Using RNAi in Drosophila cell culture, we identified myotubularin (mtm), one of six predicted in flies as important for cellular elongation. Characterization of the mtm RNAi defect in Kc167 blood cells suggests that regulation of PI(3)P-dependent membrane homeostasis is important to mediate cell shape change. We have generated loss and gain-of-function tmn mutant Drosophila to investigate roles for phosphoinositide regulation and endolysosomal function in developmental programs for cellular elongation. Using RNAi hairpins to knockdown mtm in vivo, we discovered that mtm is essential for proper development and survival past the pupal stage. We are currently characterizing defects leading to mtm RNAi induced lethality by assessing molecular markers of endolysosomal membrane compartments (Rab7-GFP and Rab5-GFP for endosomes; Lamp-GFP for lysosomes; FYVE-GFP and Aig1-GFP for PI(3)P and PI(3,5)P2, respectively) and cellular morphogenesis (actin-GFP and tubulin-GFP for cytoskeletal structure). To address specific roles for mtm and other encoded myotubularins we have determined ubiquitous mtm developmental expression by RT-PCR and in situ hybridization. We are currently localizing Mtm protein using antibodies and Mtm-GFP expression to assess spatial regulation of PI(3)P and PI(3,5)P2. A functional analysis of phosphoinositide regulators is an important first step towards understanding the dynamic subcellular pathways that orchestrate cell shape changes.
Ca++-dependent Phosphorylation of Ets-2 in Mechanotransduction Pathways in Cranial Suture Morphogenesis

J. Chen, J. Huang, C. Chew, K. H. Wenger, J. C. Yu, S. Huang, J. Huang, J. L. Borke; Biological Science & Technology, I-Shou University, Kaohsiung, Taiwan, 1Genesis Biotech Inc., Functional Discovery Unit, Taipei, Taiwan, 2Department of Biochemistry, Medical College of Georgia, Augusta, GA, 3Promega, Regensburg, Germany, 4Max-Planck-Institute for Molecular Medicine and Genetics, Medical College of Georgia, Augusta, GA, 5Orthopaedic Surgery, Medical College of Georgia, Augusta, GA, 6Surgery, Medical College of Georgia, Augusta, GA, 7College of Dental Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan, 8Oral Biology and Maxillofacial Pathology/Graduate Studies, Medical College of Georgia, Augusta, GA

Ets-1 is expressed in proliferating preosteoblastic cells, while Ets-2 is expressed in mature and differentiating osteoblasts. Ets proteins bind to DNA sequences in the promoter regions of target genes important for osteogenesis. We study the effects of mechanical strain on cells of the cranial sutures and associated bone. Our studies show that mechanical loading of sutures in organ culture produces a transient and reversible increase in cell Ca++. Our hypothesis is that mechanical loading of cranial sutures may be linked through Ets to changes in osteoblast gene expression. Loading may be derived from internal forces as the brain grows. The goal of our study is to determine if mechanical loading of cranial sutures is linked through the Ca++- dependent phosphorylation of Ets-2 in cranial bone gene expression. We use electrophoretic mobility shift assays to quantify Ca++-dependent phosphorylation of Ets-1 and Ets-2 from rat cranial sutures exposed to mechanical strain at a normal (high) extracellular Ca++ concentration of 10-3M and at a low extracellular Ca++ concentration of 10-6M Ca++. Based on existing literature and preliminary data, we suspect Ets-2 is phosphorylated by CaM Kinase II in vivo. The NetPhos 2.0 server was used to predict possible CaM kinase II phosphorylation sites on Ets-2. Based on query results, a nanoprobe array scanning through the entire sequence of Ets-2 was synthesized in situ on a stable membrane using Fmoc solid phase technology. In vitro kinase reactions were performed by incubation with CaM kinase II in the presence of radioactive ATP. Incorporated phosphate, indicating a positive reaction, was detected by autoradiography and phosphorimaging. Phosphopeptides were enriched by immobilized metal affinity chelation (IMAC) for MALDI-TOF/TOF mass spectrometry. Positive results from peptide array screening form the basis for further mutational analyses of Ets-2 in the mouse calvaria fibroblasts cell line MC3T3. (NSC(Taiwan) 95-2314-B-214 -007-MY2 Chen)

Nucleoredoxin, a Thioredoxin-related Redox-regulating Protein, Inhibits Wnt/β-catenin Signaling through Dishevelled

Y. Funato, H. Miki; Division of Cancer Genomics, Institute of Medical Science, University of Tokyo, Tokyo, Japan

Wnt signaling pathway is evolutionarily conserved from nematodes to mammals. It is important in organogenesis and stem cell maintenance, and its aberrant activation leads to tumorigenesis. Dishevelled (Dvl) is an essential intermediate in Wnt signaling pathway. Dvl transduces the signal from the Wnt receptor Frizzled to downstream components, leading to the stabilization of β-catenin and subsequent activation of the transcription factor TCF/LEF. However, the mechanism of Dvl action remains largely unknown. To elucidate the role of Dvl, we search for Dvl binding partners in vivo. Here, we report that nucleoredoxin (NRX), a member of the thioredoxin (TRX) family redox-regulating protein, is a novel Dvl-interacting protein. Overexpression of NRX selectively suppresses the Wnt/β-catenin pathway, and RNA-interference (RNAi) of NRX shows TCF/LEF activation, accelerated cell proliferation, and enhancement of oncogenicity via cooperation with Mek or Ras. Also, depletion of endogenous NRX in Xenopus embryos shows abnormal development via Wnt-β-catenin pathway activation. We find that cells respond to H2O2 stimulation by activating TCF/LEF. Redox-dependent phosphorylation of the cytoplasmic domain of NRX is impaired by NRX-RNAi. In addition, association between Dvl and NRX is inhibited by H2O2 treatment. These results implicate a hitherto unknown relation between the Wnt/β-catenin pathway and redox signaling through redox-sensitive association of NRX with Dvl.

Pinch Mediates Actin Organization and B-integrin Localization and Signaling during Drosophila Embryonic Dorsal Closure

A. M. Smith, J. L. Kadrmas, S. Pronovost, M. C. Elias, M. C. Beckerle; Huntsman Cancer Institute, University of Utah, Salt Lake City, UT

The coordination of integrin-mediated signaling and adhesion with cytoskeleton dynamics and cell shape change is essential for cell motility. An excellent model for the study of cell motility is Drosophila melanogaster embryonic dorsal closure. During dorsal closure, opposing lateral sheets of epithelia migrate, meet and suture at the dorsal midline. Dorsal closure is driven by a variety of forces, including cell shape changes in both the lateral epithelium and the underlying amnioserosa. Integrin mediated signaling and adhesion, the actin cytoskeleton, and the Jun kinase pathway form the basis for further mutational analyses of Ets-2 in the mouse calvaria fibroblasts cell line MC3T3. (NSC(Taiwan) 95-2314-B-214 -007-MY2 Chen)

Murine Palate Fusion Is Blocked by Down Regulation of Twist Expression

W. Yu, K. Svoboda; Biomedical Sciences, Baylor College of Dentistry, Dallas, TX

Epithelial-mesenchymal transformation (EMT) plays an important role in the disappearance of medial epithelial cells (MEE) during fusion of secondary palate. TGF beta3 regulates the disappearance of MEE during this process. Twist protein has a major role during EMT and our previous studies demonstrated that twist protein was upregulated just prior to palatal fusion. In this study, we demonstrated that Twist protein was downregulated by increasing the expression of Twist siRNA in vitro. The palatal shelves from E13.5 CD1 mice were dissected and placed on filter paper with the medial edges in contact. They were cultured in BGJb medium with 100, 200nm siRNA in 0.004% of transfection reagent, Lipofectamine. TGF beta3 was added (10ng/ml) in 221
Biochemical Evidence for the Role of Gαo in β-catenin/Wnt Signaling
K. E. Kalie, E. Lee; Cell and Developmental Biology, Vanderbilt University, Nashville, TN

Since the Wnt co-receptor Frizzled was identified to contain 7-transmembrane spanning domains, it has been speculated that G-proteins play a role in the beta-catenin/Wnt signaling pathway. Evidence for a key role of Gαo in the Wnt pathway has come from both cell culture and Drosophila genetic studies; however, this topic remains controversial. Using our biochemical system, we have shown that GTPγS and Gq/11 interact with β-catenin in Xenopus egg extracts and promote ubiquitin-mediated proteosome-dependent β-catenin turnover. "Hits" are currently being tested for their ability to perturb Xenopus embryogenesis as well as β-catenin/TCF-mediated gene transcription in cultured cells. Pharmacologic inhibitors of Wnt signaling would be useful in studying Wnt signaling during development and may be useful for perturbing Wnt signaling in certain disease states.

A Biochemical Screen for β-catenin/Axis Regulators
C. A. Thorne, E. Tahinci, K. Kalie, A. Salic, E. Lee; Cell and Developmental Biology, Vanderbilt University, Nashville, TN, Cell Biology, Harvard Medical School, Boston, MA

Wnt signaling is essential for proper development and is misregulated in many human cancers. β-catenin is a transcription factor that is stabilized at the protein level by activation of the Wnt pathway. In the absence of a Wnt ligand, a destruction complex, assembled by Axin, targets β-catenin for degradation via the proteasome. The destruction complex is inhibited in the presence of a Wnt signal, thereby leading to elevated levels of β-catenin that translocate to the nucleus and activate gene transcription. Recent studies in Drosophila as well as our unpublished biochemical data have shown that Axin degrades in response to Wnt signaling. Utilizing in vitro reconstitution of β-catenin degradation in Xenopus egg extracts, we have conducted a biochemical screen of the Wnt signaling pathway. Addition of β-catenin-nullified luciferase and Axin-nullified fusion proteins to Xenopus extracts in a 3×3-well format allowed us to simultaneously screen chemical libraries for regulators of β-catenin and Axin turnover. "Hits" are currently being tested for their ability to perturb embryonic development as well as β-catenin/TCF-mediated gene transcription in cultured cells. Pharmacologic inhibitors of Wnt signaling would be useful in studying Wnt signaling during development and may be useful for perturbing Wnt signaling in certain disease states.

Convergent Extension Movements in the Noncanonical PCP Signaling Pathway
G. Kim, J. Han; Division of Molecular and Life Sciences, Pohang University of Science and Technology, Pohang, Republic of Korea

Convergent extension (CE) movements in gastrulation are essential in establishing the body axis during early vertebrate development. Although the precise molecular mechanisms of CE movements are not clearly understood, the noncanonical Wnt/PCP pathway is known to be important in the control of CE movements. Here we present evidence that β-arrestin 2 (β-arrestin 2) is essential for the control of CE movements in Xenopus development. Overexpression Xenopus β-arrestin 2 (β-arrestin 2) inhibited CE movements and induced both activation of RhoA and JNK during gastrulation. Loss-of-function analysis using morpholino oligonucleotides likewise provided evidence that β-arrestin 2 is essential for CE movements and its defective phenotypes in Xenopus embryos were very similar to the morphogenetic defects caused by the inhibition of noncanonical Wnt signaling. Moreover, in the noncanonical Wnt signaling, β-arrestin 2 acted downstream of dishevelled in the planar cell polarity (PCP) signaling, but not Ca²⁺ signaling. We further demonstrated at molecular level that β-arrestin 2 interacted with phosphorylated dishevelled via amino and carboxyl regions containing DIX and DEP domain, was relocalized to the specific distribution of dishevelled, and was required for dishevelled activation of RhoA. Finally, we provided evidence that β-arrestin 2 is associated with N terminal quarter of hDaam1 and XRhOA, but not XRac1. Taken together, our results suggest that β-arrestin 2 plays a novel mediator to recognize the dishevelled activation (phosphorylation and membrane localization) in the noncanonical Wnt pathway and a functional association of dishevelled/β-arrestin 2-Daam1 specifically mediates the PCP/RhoA signaling to regulate Xenopus CE movements.

Biochemical Reconstitution of LRP6-mediated Axin Degradation in Xenopus Egg Extract
C. Cseleányi, K. Kalie, E. Tahinci, C. Thorne, E. Lee; Cell and Developmental Biology, Vanderbilt University, Nashville, TN

Axin is a scaffold that recruits β-catenin into a complex to mediate its degradation. Given limited Axin concentrations, models predicting that LRP6 promotes β-catenin stabilization via degradation of Axin are particularly appealing. We test the role of Axin as a target of LRP5/6 transduction. LRP6 intracellular domain (LRP6ICD) stabilizes β-catenin in Xenopus egg extracts and promotes ubiquitin-mediated, proteosome-dependent degradation of Axin independent of GSK3 inhibition and Dsh. We find, however, that Axin degradation is not required for LRP6-mediated β-catenin stabilization in egg extracts. An Axin mutant that can bind LRP6 but does not undergo LRP6-mediated degradation is still able to mediate Wnt signal transduction. We suggest that Axin degradation plays a role in controlling steady-state Axin levels to regulate canonical Wnt signaling following initial β-catenin stabilization.

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consequences of loss of APC function, both APC1 and APC2 must be mutant. We and others reported that APC2 APC1 double mutant clones in the adult wing blade exhibit cell fate transformations consistent with ectopic Wg signaling. We found that APC2 APC1 (double null) clones in wing marginal discs exhibit abnormal outpocketing and segregation from the surrounding epithelium. Clones with the segregation phenotype overproliferate and are largely restricted to areas of the disc not expressing Wg protein. Further, different alleles of APC, in combination with a null allele of APC1, exhibit a range in severity of the phenotype in wing discs and in adult wings. To determine whether the segregation phenotype is due to activation of Wg signaling, we have shown that blocking the Wg pathway in APC2 APC1 clones by expressing dominant negative TCF partially suppresses the segregation phenotype. Conversely, we have induced clones exhibiting the segregation phenotype by expressing stabilized Armadillo or mutant Axin to constitutively activate Wg target genes independent of APC function. To understand the basis for the segregation defect in APC2 APC1 clones, we are assessing polarity, adhesion, and cytoskeletal organization in the mutant tissue throughout wing development.

1209 Involvement of Myb Transcription Factors in the Function of Mesenchymal Stem Cells
A. Camelo, P. Garcia, J. Frampton; Infection and Immunity, Institute for Biomedical Research, Birmingham, United Kingdom
Two major populations of stem cells reside in the bone marrow of adults, that is, haemopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs). There is great interest in understanding the fundamental biology and potential for these two cellular populations, in part because of their importance for regenerative medicine. MSCs, like all adult stem cells, are capable of self-renewal while retaining the ability to differentiate into a number of stromal cell types, and possibly also neurons and cardiomyocytes. The regulation of stem cell self-renewal and commitment depends largely upon the activity of specific transcription factors. Myb family transcriptional regulators play a central part in the functioning of these HSCs; in particular c-Myb has critical roles in the maintenance of quiescence and commitment to differentiation, while B-Myb appears to exert an essential influence on proliferation. To date, no studies have investigated whether Myb family factors are similarly involved in MSCs. Here, we describe the isolation of a population of stromal cells from bone marrow that express the markers CD73, CD44 and CD90. These MSC-like cells are capable of self-renewal and are capable of differentiation into adipocytes and osteoblasts. Both B-Myb and c-Myb are expressed in these cells, but upon differentiation B-Myb expression is maintained or even increased whereas c-Myb expression decreases dramatically. In order to explore the significance of this pattern of Myb factor expression we are using Cre-loxP conditional gene deletion. We describe the distinct consequences of deletion of either gene in MSCs in terms of effects on both proliferation and differentiation.

1210 SVCT2 Expression during Brain Development
C. Silva-Alvarez, F. Nualart; Cell Biology, Universidad de Concepcion, Concepcion, Chile
Different cells transport vitamin C in its reduced form using the sodium-dependent ascorbic acid cotransporters (SVCT1 and SVCT2). SVCT2 is mainly expressed in the nervous system, however, there are no studies that describe the localization of SVCT2 during the central nervous system (CNS) development, which is important for the function of the CNS. We have carried out immunohistochemistry and in situ hybridization studies in embryonic preparations from different mouse brain developmental stages (E13 to E19). Our results indicate that SVCT2 is preferentially expressed in the melanoblast body of the radial glia, the ventricular glia body of the cortex during neurogenic stage (E12 to E17), while during the gliogenic stage SVCT2 expression downregulates and appears in cortical newborn neurons. A similar distribution pattern was observed in human brain samples from 9 weeks of gestation. These results suggest that SVCT2 is expressed in the radial glia during neurogenesis, decreasing it expression during gliogenic stage. For neurogenic development, the radial glia would have to keep its ventricular contact, uptaking vitamin C from cerebrospinal fluid (CSF). Grant FonDECYT 1050095, * CONICYT.

1211 Gene Expression Profiling of Melanocyte Stem Cells in the Mouse Hair Follicle
M. Osawa, R. Freter, G. Egawa, M. Mariko, S. Nishikawa; Laboratory for Stem Cell Biology, RIKEN Center for Developmental Biology, Kobe, Japan
Elucidation of molecular mechanisms underlying stem cell regulation is of great importance both for developmental biology and for clinical applications in regenerative medicine and cancer therapy. It is widely accepted that stem cells are regulated by their specialized microenvironment, termed stem cell niches. However, because of difficulties of identifying and manipulating the individual stem cells and their surrounding components in situ, the exact mechanisms underlying in the stem cell regulation by the niche have remained largely unknown. To overcome these difficulties, we are employing melanocyte stem cells (MSCs) that enable us to identify individual stem cells at the bulge region of the hair follicle. The relative quiescence of stem cells is maintained both in the animal and plant kingdoms and is thought to be of critical importance in maintaining the longevity of stem cells, although the exact molecular mechanisms involved in its regulation have remained unclear. To clarify molecular mechanisms implicating in fate-determination of MSCs, we compared transcriptional profiles of various subsets of immature melanoblast populations isolated from early stages of hair follicle morphogenesis. We obtained a cluster of genes that is transiently upregulated in melanoblasts at the onset when they get in a dormant status at the bulge region. These genes include molecules encoding transcription factors, adhesion proteins and stress-inducible factors, suggesting that a complex of signals is involved in the regulation of the stem cells by the niche. By establishing a vivo assay system to explore loss of function mutations, we are currently using this information to investigate how the cross-talks of these signals are implicated in the fate-determination of quiescent status of MSCs. We hope to provide new insights into our understanding of the nature of stem cell regulation at the molecular and cellular levels.

1212 Notch Signaling via Hes1 Transcription Factor Maintains Survival of Melanoblasts and Melanocyte Stem Cells
M. Moriyama, M. Osawa, S. Nishikawa; Laboratory for Stem Cell Biology, RIKEN Center for Developmental Biology, Kobe, Japan
Melanocytes exert an excellent model to understand molecular basis of various cell regulations, since genetic alterations in their regulations are easily identified by coat color defects. Melanoblasts (Mbs) including melanocyte stem cells (MSCs) are thought to be tightly regulated by cell-cell interactions with the surrounding epidermal keratinocytes, although the precise molecular mechanism has remained elusive. Notch signaling, whose activation is mediated by cell-cell interactions, is implicated in various aspects of cellular regulations such as cell fate determination, proliferation and survival. Here we investigated the role of Notch signaling in the maintenance of Mbs as well as MSCs. We found that Notch signaling is activated in both Mbs and MSCs. Conditional ablation of Notch signaling in the melanocyte lineage resulted in a severe defect in hair pigmentation, followed by intensive hair graying. The defect was caused by an apoptotic elimination of Mbs and MSCs. Furthermore, targeted overexpression of Hes1, a downstream molecule of Notch signaling, was sufficient to rescue Mbs from the apoptosis which was induced by the inhibition of Notch signaling. Thus, these data provide direct evidence that Notch signaling via Hes1 plays an indispensable role for survival of Mbs and MSCs in the epidermis. To define molecules that act at the downstream of Notch-Hes1 signaling pathway in Mbs, we performed gene expression analysis and found upregulation of several stress-responsive genes in the Mbs that were treated with a pharmacological inhibitor for Notch signaling. In fact, treatment of Mbs with an inhibitor for p38 MAPK, whose activation is induced by various stress responses, restored Mbs even in the absence of Notch signaling. Therefore, our findings demonstrate evidences for the implication of Notch signaling in the regulation of stress response.

1213 Aging-related Changes to Stem Cells and the Stem Cell Niche
L. Jones, C. Wong, M. Boyle, M. Rocha; Laboratory of Genetics, Salk Institute for Biological Studies, La Jolla, CA
Aging is characterized by compromised organ and tissue function. Adult stem cell populations maintain tissues such as blood, skin, and sperm throughout the lifetime of an individual; therefore, many consequences of aging may be due to loss of stem cell function. The transparency of the Drosophila testis, the spatiotemporal organization of germ cells, and the availability of markers for identifying various germ cell stages allow precise identification of germ line stem cells (GSCs) in vivo. GSCs and their associated somatic stem cells, known as cyst progenitor cells (CPCs), surround a cluster of post-mitotic somatic cells called the apical hub. Hub cells secrete a ligand Unpaired (Upd), which activates JAK-STAT signaling in the adjacent stem cells, thereby specifying stem cell fate. Thus, the hub is a critical component of the somatic niche that supports maintenance of the stem cell population. We are using the Drosophila male germline as a model system to analyze how the process of aging affects stem cell behavior. Testes from 50-day old males contain fewer differentiating germ cells and in aged testes. Our data suggest that aging-related changes within stem cell niches may be a significant contributing factor to reduced tissue homeostasis and regeneration in older individuals.
Dendritic Cells Expressing MHC-II, CD1a and Langerin Can Differentiate from CD133 Positive Human Cord Blood Cells

L. Giabban,1 L. Domenici,2 L. Pieri,2 S. Urbanil,2 G. Romano,1 M. Monici,3 R. Saccardi,4 V. Basile,1 A. Bosil,1 P. Romagnoli1; 1Department of Anatomy, Histology and Forensic Medicine, University of Florence, Firenze, Italy, 2Department of Hematology, University Hospital Careggi, University of Florence, Firenze, Italy, 3Department of Clinical Physiopathology, University of Florence, Firenze, Italy, 4Center for Laser Application in Medicine, Center of Excellence in Optronics, Firenze, Italy

Hematopoietic stem cells are classically selected by their expression of CD34, however CD133 has been shown to mark more primitive progenitors, i.e. hemangioblasts with a wider differentiation potential than CD34+. Since dendritic cells can differentiate in vitro from CD34+ cells, the object of this study was to determine if and under which conditions CD133+ cord blood cells can be expanded and differentiated into dendritic cells. Human cord blood was obtained from donations unsuitable for banking. Upon Foxc2 separation of mononuclear cells and immunomagnetic selection for CD133, the cells were expanded until day 7 with GM-CSF, TNF-α, IL-4, thrombopoietin, Flh-3L and SCF. They were then differentiated until day 18 in the same medium without thrombopoietin, Flh-3L and SCF. Some experiments were performed without TG-β, others with TG-β, 5 ng/mL since day 1 or since day 8, and 12.5 ng/mL since day 15.

CD133+ cells at the start of culture were 0.6-2.1 x 10⁶; expansion led to a 4-11 fold increase in the cell numbers; the cell proliferation slowed down during differentiation. Part of the cells adhered to the culture flask and had a dendritic shape. At the end of the experiments without TG-β, 36% cells expressed MHC-II and 12% expressed CD1a. With TG-β, there was a dose dependent inhibition; MHC of TG-β since the start of culture, many cells expressed langerin (49%). In conclusion: CD133+ cord blood cells can be expanded in vitro and induced to generate cells with features of dendritic cells; prolonged exposition to TG-β seems needed to induce the expression of the Langerhans cell specific marker langerin; preliminary expansion of the cells in the absence of TG-β may help to overcome the negative effect of TG-β on cell proliferation.

Activation of Transcription Factor FoxO3a in Mouse ES Cells Associated with Loss of Pluripotency

H. M. Romanska,1 Z. Gulzar,2 E. Lam,3 R. Howe,3 N. E. Lalani1; 1Pathology, University of Birmingham, Birmingham, United Kingdom, 2Department of Oncology and Cancer Research UK Labs, Imperial College London, London, United Kingdom

Background: PI3K/Akt signaling pathway plays a pivotal role in the regulation of pluripotency and self-renewal of mouse embryonic stem (MES) cells. Cellular responses to PI3K/Akt signaling can be antagonized by activation of the forkhead transcription factors. The aim of this study was to assess the temporal pattern of expression of Akt and FoxO3α, in relation to that of OCT-3/4, a marker of pluripotency, in differentiating cells. Method: MES cells (D3) were grown undifferentiated in MEM supplemented with LIF (1000 U/mL). Differentiation was induced by withdrawal of LIF from the medium and the formation of embryoid bodies (EBs). Expression of OCT-3/4, pAkt (phosphoThr308) and FoxO3α in a: undifferentiated (Day 0) and b) 14 day old EBs (EBd14) was evaluated by immunocytochemistry and Western blotting. Results and Conclusion: In undifferentiated cells (Day 0), of OCT-3/4 and phospho-Akt were highly expressed in the nuclei, whereas immunoreactivity for FoxO3α was restricted to the cytoplasm. By day 14 (EBd14) expression of both OCT-3/4 and phospho-Akt was markedly decreased with low level punctuate immunoreactivity noted in occasional cells. In contrast to the observable decline in OCT-3/4 and phospho-Akt proteins, FoxO3α was strongly expressed in the nuclei of early differentiating cells. This trend in FoxO3α expression (cytoplasmic to nuclear) was further confirmed by Western blotting. Our results suggest that negative regulation of PI3K signaling by activation of FoxO3α is associated with and might contribute to the loss of pluripotency and onset of differentiation of MES cells.

Transdifferentiation of Human and Rat Mesothelial Cells into Osteoblast-like Cells In Vitro

S. M. Lamkicy,1 R. G. Searles,1 S. E. Herrick,2 P. J. Thompson,1 C. M. Prele,1 S. E. Mutsaens; 1Asthma & Allergy Research Institute, Perth, Australia, 2University of Manchester, Manchester, United Kingdom

Telithrocyte formation was performed in vitro where cells were maintained in either osteogenic medium containing 10mM β-glycerophosphate, 50μg/ml ascorbic acid and 10mM dexamethasone or standard culture medium for 0-27 days. Rat mesothelial cells cultured in osteogenic medium expressed alkaline phosphatase, an early marker of the osteoblast phenotype, and formed mineralized bone-like nodules as demonstrated by von Kossa stain. Human and rat mesothelial cells expressed mRNA and protein of several key osteoblast markers, including bone specific core binding factor alpha 1 (Bcn1), osteoclast, osteopontin and bone sialoprotein by RT-PCR and Western blotting. Histological analysis of a human pleural MM biopsy demonstrated multiple foci of mineralized osteous metaplasia within the tumour. Bcfn1 immunostaining was also observed in several areas of its pleural MM tumour. In conclusion, we have strong evidence that mesothelial cells have the capacity to differentiate into cells of the osteoblast lineage, further illustrating their multipotent nature.

Difference in DNA Methylation Profile between Neurogenic and Glieogenic Progenitor Cells

K. Hirabayashi, N. Hattori, S. Yagi, S. Tanaka, K. Shiotia; Animal Resource Sciences/Veterinary Medical Sciences, The University of Tokyo, Tokyo, Japan

Neurons and glial cells are both derived from neural stem cells, and appear serially from mid-gestation to late-stage tissue of development. The isolated neural stem cells from each stage differentiate into neurons and glial cells, suggesting that the commitment of the neural stem cells to each cell lineage should be determined at this stage. DNA methylation is suggested to be involved in differentiation of the neural stem cells. To address how the commitment of the neural stem cells occurs during this period, we analyzed DNA methylation profiles of neurospheres, which were established from mouse embryos of day 11.5 (E11.5NSph) and 14.5 (E14.5NSph). When these NSphs were dispersed and cultured under the differentiation condition, 49.0% of cells from E11.5NSph were βIII-tubulin-positive and 5.7% of them were glial fibrillary acidic protein (GFAP)-positive, while 16.8% of cells from E14.5NSph were βIII-tubulin-positive and 21.6% of them were GFAP-positive, indicating that fates of cells in neurospheres were different between these stages. The DNA methylation profiles by restriction landmark genomic scanning (RLGS) represented the methylation status of approximately 2000 Nol sites among 6000 Nol sites scattered through genome-wide. In E14.5NSph, 32 hypo-methylated and 20 hyper-methylated loci were observed when compared to those in E11.5NSph. Based on the report that most of Notl sites are located in gene loci, the methylation status should be modulated at plurality of gene loci during the commitment period. In conclusion, neurospheres which were isolated from different developmental stage have distinct DNA methylation profiles, and the difference in their profiles suggested that establishment of DNA methylation profile should be involved in the commitment of neural stem cells.

Inhibition of Akt or NFκB Pathway Suppresses Development of CFU-GM from Murine Bone Marrow Cells

H. Choi,1 M. Kim,1 L. Han,1 Biological Science, University of Ulsan, Ulsan, Republic of Korea, 2IC, University of Ulsan, Ulsan, Republic of Korea

Hematopoietic stem cell (HSC) can undergo self-renewing or differentiate into different lineages. To investigate whether hematopoiesis from HSC involve NFκB and PI3-kinase/Akt-dependent pathway, we expressed dominant negative forms of IκB (dnIκB) and Akt (dnAkt) in HSCs obtained from murine bone marrow. These expression strongly suppressed colony formation of CFU-GM (colony forming unit-granulocyte/macrophage). Thus, both signal pathways seem to be associated with development of hematopoietic progenitor cells. GATA-1 expression was highly induced in cells transfected with adenovirus-dnIκB and -GFP control, its expression was much less increased in dnAkt-transfected cells, indicating that GATA-1 is involved in Akt-dependent but not NFκB-dependent pathway in suppressing colony formation of granulocyte/macrophage progenitor cells.

Investigation of Histone Variant Distribution in Human Embryonic Stem Cells

M. L. Shaw,1 E. J. Williams,2 J. Craig,3 K. H. A. Choo,2 R. Safery1; 1Epigenetics Research, Murdoch Childrens Research Institute, Parkville, Australia, 2Chromosome Research, Murdoch Childrens Research Institute, Parkville, Australia

Human embryonic stem cells (hESCs) have the ability to differentiate into all three germ layers. The differentiation process involves the coordinated regulation of three gene expression layers, most likely mediated by epigenetic remodelling of hESC chromatin. We are interested in studying the epigenetics of early stem cell differentiation, and in particular investigating the changes in
Muscle-derived Stem Cells Display Sexual Dimorphism under BMP4 Stimulation

K. A. Consi, J. B. Pellet, J. A. Jadlowiec, A. Usas, G. Li, J. Haard; Stem Cell Research Center, Children's Hospital of Pittsburgh and Department of Orthopedic Surgery, University of Pittsburgh, Pittsburgh, PA; Department of Bioengineering, University of Pittsburgh, Pittsburgh, PA; Carnegie Mellon University, Pittsburgh, PA; Department of Molecular Genetics and Biochemistry, University of Pittsburgh, Pittsburgh, PA.

Muscle-derived stem cells (MDSCs) are potential cell sources for orthopedic tissue engineering. This study investigated if the osteogenic differentiation of mouse MDSCs displays sexual dimorphism. Female and male MDSCs (F-MDSCs and M-MDSCs, respectively) were stimulated with bone morphogenetic protein (BMP)-4 (0-200 ng/ml) and assessed for osteogenic differentiation by gene expression and presence of alkaline phosphatase (ALP), an early osteogenic marker. Gene expression for Alp and Runx2 was upregulated within 12 hours of BMP4 stimulation, however no difference was observed between female and male populations. BMP4 stimulation led to an increase in ALP activity for all cells tested, regardless of sex. However, M-MDSCs displayed a significantly greater ALP activity than F-MDSCs at 2 and 3 days post-stimulation, but not at 4 days. To determine if M-MDSCs had more osteoprogenitors than F-MDSCs, the cells were single-cell sorted, the colonies stimulated with BMP4 for 3 days and stained for ALP. Since all colonies comprised a mixture of ALP negative and positive cells, they were scored as low ALP+ (<50% of the cells stained for ALP) or high ALP+ (>50% of the cells stained for ALP). M-MDSCs had significantly more colonies that were high ALP+ than did the F-MDSCs. MDSCs were also transduced to express human BMP4 and implanted into the intramuscular pocket of sex-matched mice. At 14 and 21 days post-implantation, a more consistent bone formation and of higher density was seen with M-MDSCs than F-MDSCs. This study indicates that although all MDSCs undergo osteogenic differentiation, M-MDSCs do so more readily than F-MDSCs. These findings warrant further investigation into sex-related differences of MDSCs and other stem cells, as well as identifying the mechanisms underlying these differences. This may then be used to further advance the use of stem cells in cell therapy and tissue engineering for bone healing.
distinguish between these possibilities using a variety of molecular genetic experiments. Mutants have additional defects in sense organ differentiation, and wing and eye development. We are currently exploring the common features of these phenotypes to identify the molecular functions of this cyclin.

1225 Quantitative Protein Measurements in Transitional Tumor Stem Cells Reveal Differences Not Seen with Nucleic Acid Techniques
D. W. Voehringer,1 S. Chan,2 A. Bhaimipati,1 P. Vander Horn,3 R. O'Neill,1 E. Weissman1; 1Inst. of Stem Cell Biology and Regenerative Medicine, Stanford School of Medicine, Palo Alto, CA
The discovery of tumor stem cells in acute myeloid leukemia a decade ago initiated a field of research that has seen accelerated growth in the past few years. Now researchers are describing tumor stem cells in a variety of hematopoietic and solid tumors. The impetus for much of this research has been the desire to identify targets for molecular medicine in these critical tumor populations. The molecular pathways that functionally define these cells are of particular interest as therapeutic targets. Techniques describing these pathways are different than in conventional biology as these cells are rare and often generate insufficient protein material to use standard protein methods. Quantitative measurements, where comparisons and contrasts are made between tumor stem cells and their surrounding progeny, are made using DNA microarray and qPCR approaches. Unfortunately, the rare nature of stem cells in these samples limits or prevents quantitative protein analysis. Here we describe a technique utilizing a nano-scale proteomic platform (Firefly) to measure a number of protein stem tumor cell proteins. To maximize sample efficiency, transitional tumor cells (TCC-) and transitional tumor stem cells (TCC+) were sorted directly into lysis buffer. 2,000 cells/well were sorted into 20 μl lysis buffer (HNTG - 50 mM HEPEs, 150 mM NaCl, 0.1% Triton X 100, 10% Glycerol). Following photolysis detection and quantification of signal was measured by HRP-labeled secondary chemiluminescence. Quantification of proteins that are not regulated at the transcriptional level, such as β-catenin, had similar RNA levels between TCC- and TCC+ cells. However, protein levels were strikingly different. A panel of RNA/proteins which define transitional tumor stem cells will be described along with examples that emphasize the importance of protein measurements in addition to nucleic acid measurements.

1226 Non-Radioactive ProLabel Detection of Protein-Protein Interactions in Co-IP Studies
Y. H. Nguyen, D. Li, Cell and Molecular Biology, Clontech Laboratories, Mountain View, CA
ProLabel technology is based on an enzyme fragment complementation (EFC) assay in which the enzyme, β-galactosidase, is genetically engineered into two fragments—the large enzyme acceptor and the small enzyme donor called ProLabel. Separately, the β-gal fragments are inactive, but in solution, they rapidly recombine to form an active β-galactosidase enzyme that hydrolyzes substrate to produce an easily detectable chemiluminescent or fluorescent signal. We have developed a sensitive, novel non-radioactive Co-IP assay for the confirmation of protein interactions typically isolated from a yeast two-hybrid screen, using AcGFP as a fluorescent tag for monitoring transfection efficiency as well as for the pull-down of the immunocomplex and ProLabel enzyme complementation assay as a readout of physical protein-protein interactions. Briefly, vector-specific primers are used in conjunction with Clontech’s In-Fusion technology to facilitate the efficient cloning and rapid transfer of bait and prey sequences amplified from yeast two-hybrid expression vectors into mammalian AcGFP and ProLabel expression vectors. HEK293 cells co-transfected with constructs expressing N-terminal fusions of AcGFP-bait and ProLabel-prey clearly show fluorescent expression and proper localization of the AcGFP-bait and ProLabel enzyme activity from the prey fusion. Co-immunoprecipitation studies using polyclonal antibodies demonstrated extremely low background and a greater than 300X increase in the co-immunoprecipitated ProLabel activity detected between interacting pairs (SV40 large T and p53) versus non-interacting pairs of proteins. The system eliminates the biobehaviors that are typically associated with co-construct co-immunoprecipitation and is an effective approach for the verification of protein-protein interactions.

1227 Applications of Artificial Microbial Mats
Z. O. Swankie,1 C. Blackford,1 A. Jamshed,1 T. Cote,1 B. M. Bebout,2 L. Prefurt-Bebout,2 R. Gonzalez-Plaza,2; 1Northwest Indian College, Bellingham, WA, 2NASA Ames Research Center, Moffett Field, CA
Microbial mats are complex networks of cyanobacteria and other microorganisms, which can grow in both fresh and salt water as well as in a variety of extreme environments. Microbial mats are among the first complex communities of organisms that grew in the young Earth, 3500 million years ago. Long-term research has focused on the role of microbial mats as the first detectable life forms on Earth and in the creation of the oxygenic atmosphere. In addition to continuing substantial roles in current Earth geochemical cycling processes, microbial mats offer potential commercial and research applications. We are investigating two of these possible uses. First, we are evaluating the possibility of using microbial mats as sources of hydrogen. Biologically produced hydrogen is more environmentally friendly than traditional hydrogen production in which fossil fuel is consumed during its production. Second, we are investigating the use of microbial mats as life support for the nematode C. elegans. C. elegans has been a valuable model organism for space studies. Current methods use E. coli for food to support the nematodes. Limited supplies of nutrients to support E. coli growth can be sent into space for a given mission. We suggest that the use of self-replicating microbial mats to support C. elegans could make possible longer periods of observation in order to better understand the long-term effects of space on biological systems. A critical requirement to address these and other questions could make possible longer periods of observation in order to better understand the long-term effects of space on biological systems. A critical requirement to address these and other questions.

1228 Identification of Chemical Inhibitors of the Rho GTPase Exchange Factor TRIO-GEFD1 Using the Yeast Exchange Assay
A. Blangy,1 N. Bouquier,1 C. Gauthier-Rouvière,1 S. Schmidt,1 A. Debant,1 J. Leonetti,2 P. Fort1; 1CRBM CNRS FRE2593, Montpellier, France, 2CNRS UMR 5160, Montpellier, France
Background information RhoGTPases are involved in many biological processes and participate in cancer development. Their activation is catalyzed by exchange factors (RhoGEFs) of the Dbl family. RhoGEFs display proto-oncogenic features, thus appearing as candidate targets for anticancer drugs. Dominant negative RhoGTPase mutants have been widely used to block RhoGEF signaling. However, these tools suffer from limitations, due to the high number of RhoGEFs and the complex mechanisms that control RhoGTPase activation. Results RhogT17N is a poor inhibitor of its exchange factor TRIO-GEFD1 in vivo: although it binds to TRIO-GEFD1, Rhog-T17N cannot bind to its effectors, which illustrates how negative mutants may produce misleading interpretations and emphasizes the need of new types of RhoGEF inhibitors. In that prospect, we adapted the Yeast Exchange Assay method to identify RhoGEF inhibitors. Using this novel approach, we screened a 3,500 chemical compound library and identified a potential inhibitor of TRIO-GEFD1 in vivo. This molecule inhibited TRIO-GEFD1 in vitro. Among the chemical analogs of this compound, we identified two molecules with better inhibitory activity. The three TRIO-GEFD1 inhibitors had no effect on ARHGEF17 and ARNO, two exchange factors for RhoA and Arf1 respectively. Conclusions The development of RhoGEF inhibitors appears as a necessary tool for the study of Rho GTPase signaling pathways. The Yeast Exchange Assay adaptation we present here is suitable to screen for chemical or peptide libraries and identify candidate inhibitors.

1229 A Novel In Vitro Assay for Measuring Protein Palmitoylation Dynamics
L. M. Borland, N. L. Allbritton, Dept. of Physiology and Biophysics, University of California, Irvine, Irvine, CA
Palmitoylation is the thioester linkage of fatty acid palmitate (C16:0) to cysteine residues on a protein. This dynamic and reversible post-translational modification increases the hydrophobicity of proteins, facilitating protein-membrane interactions, protein-protein interactions and intercellular trafficking of proteins to differing sites of action. Recently, fluorescence microscopy studies revealed that palmitoylation plays a significant role in transporting the MAPK signaling proteins H- and N-ras to sites of action near the plasma membrane (Rocks, et al., Science, 2005, 307: 1746-1757). Given that many cancers have a significant need to monitor and measure protein palmitoylation, unfortunately, few methods exist to quantitatively monitor protein palmitoylation, especially at the cellular level. In this study, we developed a capillary electrophoresis-based assay, using MEKC-LIF, to monitor and measure palmitoylation dynamics of a fluorescently-labeled peptide in vitro, with potential to extend such studies to the single-cell level. A fluorescently-labeled portion of the growth-associated protein (GAP, HiLyte Fluor488-MLCCMRRTL-NH2) was palmitoylated in vitro using a HEPEs-based acylation buffer and palmitoyl coenzyme A. Formation of a doubly-palmitoylated GAP peptide product was confirmed by MALDI-MS. The GAP peptide substrate was separated from the unreacted GAP and palmitoyl coenzyme A by liquid chromatography and then sequenced by nano-ESI-MS. The first 6 minutes from a total sample size of 1 nanoliter (10-9 L). The rate of in vitro palmitoylation with respect to time and increasing palmitoyl coenzyme A concentrations was examined. Mixture of GAP peptide substrate with cell lysate increased palmitoylated product production in a time-dependent manner. Conversely, palmitoylated GAP substrate was de-palmitoylated after addition of the

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cell lystate, demonstrating the reversibility of palmitoylation in vivo. Pharmacological manipulation of the GAP peptide substrate with 2-bromopalmitate, an inhibitor of palmitoylation, was also measured by MEKC-LIF. This novel CE-based assay for monitoring palmitoylation has applications for kinetic studies at the single cell level.

1230
Efficient siRNA Delivery Enhances the Potential of RNA Interference
A. Bolkato-Bellemin, M. Bonnet, P. Neuberg, P. Erbacher; Polyplus transfection, Illkirch, France
RNA interference (RNAi) is a potent technology for gene silencing based on an extremely selective interaction of short RNA duplexes (siRNA) with a single target in the mRNA, providing sequence-specific mRNA degradation and inhibition of protein synthesis. Currently, introduction of siRNA at 10-100 nM by transfection results in effective silencing of endogenous and exogenous genes in a variety of mammalian cells. Nevertheless, siRNA delivery remains a key step for efficient gene silencing. In order to fully exploit the promising potential of RNA interference, we have focussed our research on improving selectivity by using a delivery system efficient with at very low levels of siRNA. We present efficient gene silencing in mammalian cells using siRNA in the subnanomolar and picomolar range. We show complete and selective gene silencing at 1 nM siRNA and even down to 10-50 pM for several targeted genes (GAPDH, Lamina A/C, Vimentin, luciferase, GFP). Using subnanomolar and picomolar siRNA concentrations avoids off-target effects and also reduces toxicity drastically. Moreover, we demonstrate that efficient endogenous gene silencing with very low amount of siRNA occurs in many cell types (adherent cells, suspension cells and primary cells). Taken together our data show the great potential of RNA interference when delivering very small amounts of siRNA into mammalian cells.

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Protein Delivery to Live Cells
P. Erbacher, C. Weill, S. Biré, A. Adh; Polyplus transfection, Illkirch, France
The delivery of proteins to live cells offers a powerful alternative where other approaches have failed. Indeed, the conventional approach to studying protein function consisting in transfecting the corresponding cDNA sequence into cells is not always successful. The ability to introduce proteins to live cells opens new insights to a wide range of applications such as protein trafficking studies in live cells, live immunolabelling or even protein interference studies with antibodies. For efficient protein delivery, major hurdles such as crossing the plasma membrane and effective release of the protein in the cytoplasm still remain to be overcome. We present an optimized system for protein, peptide and antibody delivery to cells. This carrier forms complexes with the protein of interest, which are then internalized via anionic cell-adhesion receptors present on virtually all cells. The use of this carrier promotes efficient crossing of the plasma membrane, release from the endosome and allows protein diffusion within the cytoplasm as shown with fluorescent proteins, peptides and antibodies. The protein, in its native form is able to diffuse in the cytoplasm and resume cellular functions. Our data demonstrate the effective delivery of 1 µg or less of proteins, peptides or antibodies to a large variety of live eukaryotic cells, including primary and suspension cells, without any detectable side effects. The development of protein carriers represents novel applications for cell biologists in particular for the study of lethal proteins in living cells allowing a tight control of the time course and the amount of protein delivered.

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Characterisation and Heterologous Expression of Agaricus bisporus Lectins Which Inhibit Proliferation of Epithelial Tumour Cell Lines
J. Henderson,1 B. Herman,2 D. Eastwood,8 S. Sreenivasaprasad,1 K. Burton1; 1School of Science and Technology, University of Teesside, Middlesbrough, United Kingdom, 2WarwickHRI, Wellesbourne, United Kingdom
Colorectal cancer is the second most common cause of cancer-related deaths in the Western World with 28,000 cases per year in the United Kingdom. Agaricus bisporus is an economically important edible fungus. Despite its importance, the identification of two closely related lectin genes. The aims of this project are to clone these two lectin genes into vectors and transform them in unknown if these isoforms are formed by post-translational modification, if there are different genes for the four isoforms or if it is a combination of both possibilities. Recent work has demonstrated the effective delivery of 1 µg or less of proteins, peptides or antibodies to a variety of live eukaryotic cells, including primary and suspension cells, without any detectable side effects. The development of protein carriers represents novel applications for cell biologists in particular for the study of lethal proteins in living cells allowing a tight control of the time course and the amount of protein delivered.

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High-throughput Screening and Confirmation of Interacting Proteins in Living Cells
J. Lu; Surgery, McMaster University, Hamilton, ON, Canada
The major post-genome sequencing task is to determine the function of each of the genes in the human genome and thus the era of proteomics is now at the forefront. There is a growing demand for high throughputscreening technology platforms that will enable large scale screening of protein interactions. Unfortunately, the current methods used for the screening of protein interactions both in vitro (e.g. protein array) and in vivo (e.g. yeast two-hybrid) have many limitations. These include multiple costly and time-consuming complicated procedures, non-specific interactions, along with high rate of false positive/negative findings. We have developed a patented automatic approach, using fluorescence resonance energy transfer (FRET) principles to perform high throughput screening for novel interactions. This innovative method overcomes the aforementioned limitations and presents a unique route for the investigation of protein functions in their intact native state in vivo. Using this method we demonstrated increased transformation efficiency, higher stringency detection, decreased false positive results and high-throughput formats. Current applications of the technology in our lab include: 1. High throughput screening of potential drug candidates (small molecular chemicals, peptides etc.) on their effect on protein interactions and their effect on cells in vivo. 2. High-throughput identification of novel protein interactions in native living cells. 3. Confirmation of protein interaction in vivo in prokaryotic and eukaryotic cells. 4. Identification of non-protein molecular interactions. 5. Screening for potential candidate peptides or other chemicals that interact with special cellular target proteins towards the development of new molecular agents. For more information, contact: Sunita Asrani, Industrial Liaison Officer, Health Sciences, McMaster University, ON, Canada. Tel. (905) 525-9140 Ext. 28641 Fax: (905) 546-1372 Email: sunita@ip.mcmaster.ca

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Proteomic Analysis with IEF and On-Capillary Antibody Based Detection of Immobilized Proteins
The traditional western blot has been a mainstay of protein research for over 25 years. Although popular due to its relative simplicity, the method is slow, labor intensive, only semi-quantitative, and poorly suited to scarce samples. Additionally, quantitative results using the western blot can be difficult to produce. Similar challenges in nucleic acid analysis found a solution with capillary electrophoresis. Protein analysis using capillary electrophoresis has however proven more challenging due to the need to permanently affix the wide concentration range of separated proteins to a stationary phase for antibody-based detection. To overcome this problem, a novel capillary wall coating has been developed which allows rapid (60s) and permanent fixation of isoelectrically focused proteins to the inner wall of the capillary. Chemiluminescent or fluorescently tagged antibodies are then flowed through the open capillary allowing specific labeling and detection of captured proteins. Experiments show that the useful linear range of concentration of protein capture exceeds six orders of magnitude. This automated method has been demonstrated in the analysis of activation of a wide range of cell signaling proteins including AKT, ERK, BCL2, Myc and JNK.
Combination Therapy with Conditionally Replicating Adenovirus and Replication-incompetent Adenovirus Expressing P63γ Enhanced Adenovirus Transduction and Therapeutic Efficacy in Head and Neck Cancer Cell Lines

S. Shim, 1 S. Park, 1 M. Sung 1,2; 1Molecular Tumor Biology, Cancer Research Institute, Seoul National University, College of Medicine, Seoul, Republic of Korea, 2Department of Otorhinolaryngology – Head and Neck Surgery, Seoul National University, College of Medicine, Seoul, Republic of Korea

Low gene transfer rate is the most obstacles in a practical application of cancer gene therapy. Especially in case of head and neck cancer cell lines, gene delivery rate using adenoviral vector is not enough to show a meaningful therapeutic effect and high dose of adenovirus was required. Although the safety of adenovirus already has been proved, high dose of virus injection in human body is somewhat risky. To improve these points of issue, we used conditionally replicating adenovirus (CRAd) which is shown oncolytic action in cancer cells with abnormal Rb activity. Additionally, as a new therapeutic gene, we made recombinant adenovirus included p63γ gene which is one of the p53 family gene. As we hypothesized that conventional E1-deleted adenovirus can replicate competently when co-transfected with a CRAd to selectively supply E1 in tumors by combining with CRAd and adenovirus. As combining with CRAd and adenovirus carrying p63γ, we expected double effect that is the increase of killing gene expression in cancer cell and tumor killing effect. First, to identify increase of therapeutic gene expression, we combined with CRAd and adenovirus-lucase. The combination of CRAd and Adeno-lucase increased the transduction efficiency of lucase almost 20 times compared with transduction with Adeno-lucase alone. Next, we tried western blot to verify p63γ expression level. Combination of CRAd and Adeno-p63γ increased the expression of p63γ by 10-fold versus transduction with Adeno-p63γ alone. Although Adeno-lucase and Adeno-p63γ are E1-deleted replication-defective adenoviruses, adding small amount of CRAd could help excessive protein expression. Therefore, we did a MTT assay to measure lysis of tumor cell by combining CRAd and Adeno-p63γ. Treatment of head and neck cancer cell line with CRAd and Adeno-p63γ also enhanced anti-cancer effect. These results represent that this combination with CRAd and Adenovirus increased killing gene transfer rate and enhanced its anti-tumor effect.

Conventional ELISA vs. Micro-Immunoassays: The Advantages of Multiplexing

S. L. Call, J. D. Hoopes; Quansys Biosciences, Logan, UT

Multiplex protein assays provide researchers the ability to test many biomarkers at once. However, there are concerns about the uniformity of the data as it compares to conventional ELISA assays. The objective of this study is to compare results from conventional ELISA assays with results from higher density multiplexed micro-immunoassays. The conventional ELISA plates were prepared by coating 96-well plates with 50μl of 2μg/ml capture antibody. The micro-immunoassays were prepared by printing 50nl spots of each capture antibody into 96-well plates in a defined array. Each plate was treated the same, antigen incubation for one hour, washed three times with TBST, HRP-labeled detection for 15 minutes, and washed six times with TBST. The conventional ELISA plates were developed with TMB substrate, while the micro-immunoassays plates were visualized with chemiluminescent substrate captured by a CCD camera. Four cancer markers were tested in parallel for consistency: AFP, CA15-3, CA19-9, and CA125. Within the linear range of the assay, the R² residual for AFP was 0.94, 0.93 for CA15-3, 0.97 for CA125, and 0.95 for CA19-9. The results show that there is a strong correlation between conventional ELISA assays and micro-immunoassays. The greatest difference between the assays was that the micro-immunoassay was much more sensitive requiring significantly more reagents and time to perform the assay, though high values tended to saturate quicker. The other major observation is the ability of p53 to target to the cell membrane and cytoskeleton, and on the whole, micro-immunoassays, because of the use of chemiluminescence, which cannot be distinguished during a conventional ELISA. From this experiment we conclude that micro-immunoassays are a comparably if not superior alternative to conventional ELISA assays.

Reconstitution of Yeast Peroxisome Biogenesis from ER In Vitro

N. Yoda, 1 E. Naraba, 1 M. Robinson; 1GE Healthcare, Piscataway, NJ, 2London Bridge Fertility, Gynaecology and Genetics Centre, London, United Kingdom

For various genomic applications to cellular processes, such as those involved in meiosis and in embryogenesis, there is a need to develop protocols for the whole-genome amplification (WGA) from single cells. A robust WGA protocol will also be useful to cell biologists involved in other studies, such as molecular haplotyping, which require genetic analysis from single haploid cells. We have developed a simple two step, one tube protocol for representative whole-genome amplification from a single cell within 4 h using GenomiPhi™ V2 DNA amplification kit. We have also demonstrated the suitability of the amplified products for downstream SNP genotyping. For these experiments, tissue culture or isolated peripheral blood lymphocyte single cells were transferred using a mouth-controlled, fine hand-drawn micro-capillary pipette into 0.2 ml PC tubes containing 3 μl sample buffer from the GenomiPhi™ V2 DNA amplification kit. Single cells were lysed for 10 min by adding 1.5 μl cell lysis solution followed by the addition of 1.5 μl neutralizing buffer and 14 μl of amplification mixture containing reaction buffer, sample buffer and enzyme mix with Phu-29 DNA polymerase. The amplification was carried out in the same tube for 4 h at 30 °C. The amplified DNA was quantitated using PicoGreen™ dsDNA assay and was used for SNP-genotyping using a TaqMan™ assay without any purification. To demonstrate the robustness of our amplification protocol, >20 individual single-cell amplifications and >50 TaqMan™ genotyping assays using 2 cell types and at least three SNP genotyping assays were carried out by two operators. The results indicate the generation of 3-5 μig amplified DNA with > 95% call rate and > 95% concordance. In conclusion, we have demonstrated the single-cell WGA protocol to be simple and robust in providing a representative amplified product which could be used for further genotyping without any further purification.

Evaluation of the Role of the Endoplasmic Reticulum-Golgi Transit in the Biogenesis of Peroxisomal Membrane Proteins in Control and Peroxisome Biogenesis Mutant Mammalian Cells

A. A. Toro, 1 C. Arredondo, 1 J. G. Cordova, 1 C. C. Araya, 1 J. L. Palacios, 1 A. D. Venegas, 1 M. Morita, 1 M. J. Santos, 1 T. Imanaka; 1Biologia Celular y Molecular, Pontificia Universidad Católica de Chile, Santiago, Chile, 2Microbiology & Molecular Genetics, Pontificia Universidad Católica de Chile, Santiago, Chile, 3Dept. of Biological Chemistry, Faculty of Pharmaceutical Sciences, Toyoa Medical and Pharmaceutical University, Toyoa, Japan

Peroxisomes are thought to be formed by division of pre-existing peroxisomes after the import of newly synthesized proteins. In contrast to this model, it has been recently suggested that the peroxisomes are formed by division of pre-existing peroxisomes after the import of newly synthesized proteins. In contrast to this model, it has been recently suggested that the endoplasmic reticulum (ER) provide an alternative de novo mechanism for peroxisome biogenesis in some yeast and animal cells. To test a possible role of the ER in peroxisome biogenesis in mammalian cells, we evaluated the biogenesis of three peroxisomal membrane proteins (PMPs): ALDR (adrenoleukodystrophy related protein), PMP70 and Pex3 in mammalian cells. We constructed chimeric genes encoding the fusion protein of these 3 PMPs and green fluorescent protein (GFP) and transiently transfected these genes to CHO and human cells. We used wild control cells and peroxisomal biogenesis mutant CHO cells and human fibroblasts, in which normal peroxisomes were replaced by peroxisomal membrane ghosts. The expressed proteins were targeted to peroxisomes and peroxisomal ghosts correctly in the presence or absence of Bre6/kin A, a drug known to block the transit between the ER and Golgi apparatus. In addition, the number of normal peroxisomes, which the expressed proteins targeted, were essentially the same in both Bre6/kin A treated and untreated control cells. We also constructed a chimeric gene fusing the coding region of the ALDR gene, carrying an ER retention signal (DEKKMP) to the GFP gene. We transfected this gene to control cells and the fused protein was normally targeted to peroxisomes. We also tested the effect of different temperatures on the targeting of PMP-GFP proteins and found that these fused proteins are normally targeted at 10 and 15°C in control cells. These results suggest that ER and the classical ER-Golgi pathway does not play a major role in the biogenesis of mammalian PMPs.
Mechanism for the Coordination of Peroxisome Growth and Division
C. Gregg, T. Guo, T. Beukh-Viner, A. Goldberg, S. Bourque, P. Kryakov, F. Bama, K. Hung Yeung San, C. Sison, J. Solomon, V. Wong, V. I. Titorenko; Biology Department, Concordia University, Montreal, PQ, Canada
During the cell cycle, organelles must double in size and divide, in order to be accurately partitioned during cell division and inherited by the daughter cells. We have identified an unusual mechanism for the coordination of organelle growth and division in the yeast Yarrowia lipolytica. Yeast peroxisomes do not grow and divide at the same time. The growth of immature peroxisomal vesicles, which is accomplished by the stepwise import of matrix proteins, and their development into mature peroxisomes occurs before completely assembled mature peroxisomes undergo division. The division of immature peroxisomal vesicles is negatively regulated by Pex16p, a protein that binds lysophosphatidic acid (LPA) in the inner membrane leaflet of the vesicles. The binding of Pex16p to LPA prevents the formation of diacylglycerol (DAG), a particularly potent inducer of negative curvature and membrane bending, in a membrane-associated biosynthetic pathway. The stepwise import of distinct subsets of matrix proteins into different immature intermediates along the peroxisome assembly pathway causes the redistribution of a peroxisomal protein, acyl-CoA oxidase (Aox), from the matrix to the membrane. A significant redistribution of Aox occurs only in mature peroxisomes. Inside mature peroxisomes, Aox interacts with Pex16p. This interaction between Aox and Pex16p greatly decreases the affinity between Pex16p and LPA, thereby allowing LPA to enter the biosynthetic pathway leading to the formation of DAG in the inner membrane leaflet. The subsequent spontaneous movement of DAG into the outer leaflet promotes the reorganization of the bilayer configuration of the membrane and recruits the dynamin-like GTPase Vps1p to the cytosolic face of the peroxisomal surface. A peroxisome contains a distinct set of peroxisomal membrane proteins, Vps1p, and several actin cytoskeletal proteins is then assembled on the peroxisomal surface. This protein team promotes membrane scission and fission, executing the terminal steps of peroxisome division.

Global Analysis of Kinase and Phosphatase Function in Peroxisome Biogenesis in Yeast
B. Knoblach, F. D. Mast, C. Dobson, R. A. Rachubinski; Cell Biology, University of Alberta, Edmonton, AB, Canada
Compartimentalization of biochemical functions in membrane-bound organelles, an evolutionary hallmark of eukaryotic cells, concentrates enzymatic reactions within multiprotein complexes surrounded by or embedded in two-dimensional membranes. The spatial separation of biochemically distinct organelles provides a level of control unavailable to the prokaryotic cell. How the cell orchestrates the biogenesis of its organelles remains an open question of outstanding importance. Yeast peroxisomes constitute an ideal system to study organelle biogenesis, since their assembly is rapidly affected by changes in extracellular nutrients, and they are non-essential as long as cells are maintained under conditions not requiring their biochemical activity. They arise through the coordinated expression of ~26 PEX genes encoding peroxins, the organizers of peroxisomal membrane formation, matrix protein import, and organelle division. Here we present a genome-wide analysis of kinases and phosphatases affecting peroxisome biogenesis in baker’s yeast, Saccharomyces cerevisiae. Peroxisome formation was evaluated in a collection of 241 kinase and phosphatase gene deletion strains for functionality as measured by growth on myristate plates, the metabolism of which requires functional peroxisomes, and in the kinetics of its induction as measured by the appearance of peroxisomal thiolase chromosomally tagged with GFP in confocal fluorescence time-course microscopy. Based on over 3,600 quantitative descriptors, we categorized the strains into seven phenotypically distinct groups. Analysis of additional peroxisomal markers revealed that distinct regulatory mechanisms exist for different subsets of peroxisomal proteins. These mechanisms occur at both the transcriptional and post-transcriptional levels. Nutrient-dependent shuffling of several kinase and phosphatase effectors among the cytosol, nucleus, and peroxisome suggests underlying feedback mechanisms that control peroxisome biogenesis. We propose a model wherein the peroxisome itself temporally and spatially orchestrates the signaling molecules necessary for its formation, maintenance, and proliferation. This study represents, to our knowledge, the first global investigation of kinase and phosphatase activity in a complex biological process.

Species-specificity and Peroxisome Inducers Determine the Requirement of Sterol Glucoside for Pexophagy in Yeasts
T. Y. Nazarko,1,2 A. S. Polupanov,2 R. B. Manjithaya,1 A. A. Sibirny,2 S. Subramani1; 1Section of Molecular Biology, University of California, San Diego, La Jolla, CA, 2Department of Molecular Genetics and Biotechnology, Institute of Cell Biology, National Academy of Sciences of Ukraine, Lviv, Ukraine
Sterol glucosyltransferase, Ugt51/Atg26, is essential for degradation of methanol-induced peroxisomes of Pichia pastoris by both micro- and macropexophagic modes during glucose and ethanol adaptation, respectively. However, the role of the protein in pexophagy in other yeasts remains unclear. We show that glucose-induced degradation of either oleate- or amine-induced peroxisomes in Yarrowia lipolytica is a slow process that is completely independent of Atg26. As in Y. lipolytica, oleate- and amine-induced peroxisomes of P. pastoris were degraded by macropexophagy after the transfer of cells to glucose, but with the slow and high rates, respectively. PrAtg26 was not essential for these processes either. However, the rates of degradation of oleate- and amine-induced peroxisomes in P. pastoris were reduced in the absence of Atg26. Our comparative analysis of atg26 mutants in different yeasts suggests that P. pastoris specifically utilizes ergosterol glucoside to enhance the degradation of methanol-, oleate- and amine-induced peroxisomes. However, methanol-induced peroxisomes of P. pastoris apparently have lost the redundant ability to be degraded without the function of Atg26.

Mxr1p Up-regulates and Co-operates with Mpp1p to Transactivate Methanol-responsive Genes in Pichia pastoris
M. Yan, S. Subramani; Section of Molecular Biology, Division of Biological Sciences, University of California at San Diego, La Jolla, CA
P. pastoris Mxr1p is a transcription factor required for the induction of peroxisomal proteins necessary for the utilization of methanol and oleate as carbon sources (Lin-Cereghino GP et al, 2006). How the constitutively expressed Mxr1p activates carbon source-specific genes remains unknown. Here we show that in mex1Δ cells there is no expression of Mpp1p, a methanol-specific transcription factor first described in Hansenula polymorpha (Leao-Helder AN et al, 2003). In wild-type cells, Mpp1p is strongly induced by methanol and localized to nucleus, but its induction disappears in mex1Δ cells and the cells fail to grow on methanol. On the other hand, mpp1Δ cells have normal Mxr1p and grow normally in oleate. This indicates that MPP1 is one of the methanol-specific downstream genes of MXR1. Over-expression of one protein in the absence of the other restores alcohol oxidase (AOX1) expression partially, suggesting that cooperation between Mxr1p and Mpp1p exists at the level of the AOX1 promoter. We further found a 16 bp negative regulation site in the AOX1 5′ flanking region, which associates with (an) unknown repressor protein(s). The association of the repressor(s) with this site is reduced in methanol and is dependent on Mpp1p. The 16 bp consensus site is also found in other methanol-responsive genes such as dihydroxyacetone synthase (DMA5). We conclude that Mxr1p up-regulates Mpp1p and then co-operates with Mpp1p to transactivate methanol-inducible genes, partially by removing repressors.

Disturbed Regulation of SREBP/Insig Genes in Peroxisome-deficient PEX2 Zellweger Mice
W. J. Kovacs,1,2 K. N. Taps,3 J. E. Shackelford,2 M. J. Richards,3 S. J. Fissler,2 P. L. Faust,4 S. K. Krisans2; 1Anatomy & Cell Biology II, Justus Liebig University Giessen, Giessen, Germany, 2San Diego State University, San Diego, CA, 3Saint Louis University School of Medicine, St. Louis, MO, 4Columbia University, New York, NY
The marked deficiency of peroxisomal organelle assembly in the PEX2−/− mouse model for Zellweger syndrome and subsequent disturbed cholesterol homeostasis provides a unique opportunity to gain new insights into the regulation of cholesterol metabolism. Sterol regulatory element binding proteins (SREBPs) have been established as transcriptional regulators of lipid synthesis and Insig proteins are essential elements of the feedback regulation of cholesterol synthesis. Peroxisome-deficient PEX2 Zellweger mice exhibit decreased liver cholesterol levels (40%) in conjunction with increased hepatic and extrapituitary cholesterol biosynthesis. The treating PEXX− mice with oral bile acids prolonged postnatal survival and normalized hepatic and plasma total cholesterol levels. However, the mRNA levels for SREBP-2 and SREBP-2 target genes in lipogenic pathways were still significantly increased in the liver of bile acid-fed PEXX− mice despite normal hepatic cholesterol levels. The amount of HMGC-CoA reductase protein, the rate-limiting enzyme in the cholesterol biosynthetic pathway, was highly elevated in the liver of bile acid-fed PEXX− mice apparently owing to the increased expression and failure to accelerate repression of the enzyme. The current results also reveal a dysregulation of SREBP1c, Insig-2a and -2b expression and a previously unsuspected regulation of Insig-2b. Thus, peroxisome deficiency widely disturbs cholesterol homeostasis in mice by affecting the synthesis, transcriptional regulatory control, and cholesterol levels in plasma and tissues, and only some of these features are favorably altered by bile acid treatment.
The Role of Peroxoxins in the Development of Xenopus laevis

C. A. Cooper,1 P. A. Walton,2 S. Damjanovski;1 1Department of Biology, University of Western Ontario, London, ON, Canada, 2Department of Anatomy and Cell Biology, University of Western Ontario, London, ON, Canada

Peroxoxins are ubiquitous single membrane-bound organelles essential to eukaryotic cell function. They facilitate the chemical breakdown of fatty acids through the process of β-oxidation, and the elimination of hydrogen peroxide through the use of catalase. Of particular interest are the peroxoxins termed Pex proteins. These proteins have several functions including their involvement in the formation of peroxoxins and the recruitment of essential proteins to both the matrix and membrane of peroxoxins. Currently, research focuses on Pex proteins in human cell culture complementation studies to elucidate their function and protein interactions. The present study focuses on the role of peroxoxins in development, through the use of the model system Xenopus laevis. Full-length coding sequences for Xenopus peroxoxin proteins were identified, cloned and analyzed for sequence similarities with other species. Sequence analysis suggests that Xenopus peroxoxin function mimics that of mammalian function. In order to understand the function of peroxoxin proteins in development, the expression of key proteins was quantified during development through the use of RT-PCR. These data indicate that peroxoxin proteins are present in the egg prior to mid-blastula transition, which marks the initiation of the zygotic genome. Also, Pex proteins exhibit different expression patterns suggesting a dynamic need for certain peroxoxin events over the course of early Xenopus development. To test for the function of catalase in development, catalase was inhibited through microinjection of aminonitroazole, after which hydrogen peroxide levels were examined. Finally, the presence of peroxoxins was traced in early development by microinjecting GFP tagged with a peroxoxin targeting signal. Embryos were then fixed and sectioned to visualize peroxoxins at the sub-cellular level. These results suggest roles for peroxoxins in the cellular processes involved in the development of animals, as well as in normal cell function.

Biochemical and Morphological Characterization of Peroxoxins during Postnatal Development of the Mouse Brain

W. J. Kovacs, I. Neubert, B. Ahlemeyer, E. Baumgart-Vogt; Anatomy & Cell Biology II, Justus Liebig University Giessen, Giessen, Germany

The importance of peroxoxins in cellular metabolism is emphasized by the devastating consequences of peroxoxin deficiency in patients with peroxoxosomal biogenesis disorders (e.g. Zellweger syndrome). Zellweger syndrome patients exhibit neurological deficits and severe hypo- or demyelination of which completely unknown. Unfortunately, very little is known about the exact distribution and physiological role of peroxoxins in the brain. With the methods used in former publications, an increase in the number of peroxoxins in the rodent brain was observed in the first 3 weeks after birth, whereas hardly any peroxoxins were detected in the brain of adult rodents. In this study, enriched peroxoxin fractions were prepared from the cerebral cortex, hippocampus, and medulla oblongata of 2-, 15- and 49-day-old C57Bl/6J mice. The specific activity of the peroxoxosomal marker enzyme catalase was highest in brain regions from 2-day-old mice and significantly decreased in 15- and 49-day-old mice. Western blots of the enriched peroxoxin fractions were probed with antibodies against peroxoxin matrix (catalase, acyl-CoA oxidase 1, thiolase A) and membrane proteins (PMP70, Pex14p, Pex13p) as well as specific markers for distinct cell types of the CNS. Highest levels of peroxoxin proteins were detected in all brain regions of P2-mice. Double-immunofluorescence preparations of paraffin sections of distinct brain regions of P2-P15/P28 mice, probed with all antibodies, corroborated the biochemical data. All morphological data obtained with P2-brain sections were confirmed in primary cultures of neurons and astrocytes from newborn mice. Our data clearly show age-dependent differences in distribution and protein levels of catalase, peroxoxosomal β-oxidation enzymes, and peroxoxosomal membrane proteins in distinct brain regions. These results indicate the presence of distinct peroxoxosomal populations with different enzymatic compositions during postnatal development of the brain, suggestive for differences in peroxoxin function both in distinct cell types as well as during development.

Modifications of Peroxoxin Proteins Induced by Metal-catalyzed Oxidation

Y. Kwak, R. Donaldson; Biological Science, George Washington University, Washington, DC

Glyoxysomes are specialized peroxoxins present in oil-storing seeds like castor beans. Glyoxysomes contain glyoxylate cycle enzymes such as isocitrate lyase (ICL), malate synthase, and malate dehydrogenase. Hydrogen peroxide (H₂O₂), a reactive oxygen species (ROS), is produced in peroxoxins and can damage DNA, lipids, and protein. Peroxoxins in most eukaryotic cells, (yeast, mammals) produce ROS which could result in protein oxidation. Oxidized proteins have been implicated in the aging process, cancer, neurodegenerative diseases such as Alzheimer’s and Parkinson’s. Because ROS can induce molecular damage, cells have active antioxidant systems such as catalase (CAT) in peroxoxose to scavenge ROS. Glyoxysomes were isolated from castor beans and incubated with Ascorbate/Cu(II), or Fe(III)/H₂O₂ to induce metal-catalyzed oxidation (MCO). MCO results in the introduction of carbonyl groups into certain amino acids. The activity of ICL was decreased by exposure to these MCO conditions. Carbonyl moieties in protein side chains were derivatized with 2,4-dinitrophenylhydrazine (DNPH) or Biotin hydrazine, separated by SDS gel electrophoresis, and the protein bound hydrazones or Biotinhyrazones were detected in Western Blots using a DNP-specific antibody or Avidin which reacted to the expected carbonyl structure. The activity of ICL was increased by exposure to metal-free conditions. Carbonyl moieties in protein side chains were derivatized with 2,4-dinitrophenylhydrazine (DNPH) or Biotin hydrazine, separated by SDS gel electrophoresis, and the protein bound hydrazones or Biotinhyrazones were detected in Western Blots using a DNP-specific antibody or Avidin which revealed that ICL was oxidized. The proteins were separated by SDS-PAGE, trypsin digested and analyzed by MALDI-TOF-mass spectrometry revealing monoisotopic peaks at m/z 524, 529, 530, 535, 540, 544, and 0.806, which is consistent with the expected mass difference arising from biotinylation, suggest a site of oxidation. The locations of peptides are mapped to the 3D structure of a homologous ICL from Eminicella nidulans.

A Role for Endosomal Soling in the Adenosine A2 Receptor-mediated Suppression of Endotoxin (LPS)-induced TNFα Expression in Macrophages

J. A. Quipe,1 G. Hasko,2 S. J. Leibovich; 1Cell Biology and Molecular Medicine, Graduate School of Biomedical Sciences, UMDNJ, Newark, NJ, 2Surgery, New Jersey Medical School, UMDNJ, Newark, NJ

Macrophages are key cells involved in host defense. Toll-like receptors (TLRs) are innate immune receptors expressed by macrophages, and TLR ligation activates signaling pathways that result in expression of pro-inflammatory cytokines such as TNFα. Macrophages also express adenosine receptors (ARs). AR Ligation generally induces an anti-inflammatory response, down-regulating expression of inflammatory cytokines such as TNFα. We have previously reported that stimulation of macrophages with TLR 2,4,7 or 9 agonists with adenosine A2R agonists such as A230 expression, but strongly increased TNFα expression, and on the inhibition of TNFα expression by macrophages. The effects of various inhibitors of endosomal signaling (sucrose, ammonium chloride, chloroquine, bafilomycin-A1, concanamycin-A) on LPS-induced TNFα expression, and on the inhibition of TNFα expression by A2R agonists were examined. Macrophages were pre-incubated with endosomal inhibitors for 30 minutes, and were then treated overnight with LPS (100ng/ml) (TLR4 agonist), R837 (1μg/ml), LPS/NECA, or R837/NECA, in the presence of endosomal inhibitors. Conditioned media were then analyzed for TNFα by ELISA. Ammonium chloride, chloroquine and sucrose treatment had little effect on LPS-induced TNFα expression and did not affect the NECA-induced suppression of TNFα expression. Bafilomycin A1 (50nM-1μM) and concanamycin-A (200nM-2μM), however, had little effect on LPS-induced TNFα expression and significantly increased TNFα production in cells treated with LPS and NECA. Bafilomycin-A1 and concanamycin-A are inhibitors of v-ATPases that regulate vacuolar H+ content. These observations suggest an important role for endosomal signaling in the regulation of A2R-mediated suppression of LPS-induced TNFα expression.

Macrophages Express Granzyme B in Lesion Areas of Atherosclerosis and Rheumatoid Arthritis

W. J. Kim,1 H. Kim,1 Y. M. Kang,2 J. E. Park,3 W. H. Lee; 1Department of Genetic Engineering, School of Life Sciences and Biotechnology, Kyungpook National University, Daegu, Republic of Korea, 2Rheumatology Division, School of Medicine, Kyungpook National University, Daegu, Republic of Korea, 3Cardiology Division, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Republic of Korea

Granzyme B is a serine protease that is highly expressed in cytotoxic T cells (CTL) and natural killer (NK) cells. Granzyme B is a major mediator of the cytotoxic immune response by inducing target cell death when internalized in the presence of perforin. Recently, several studies have focused on another role of granzyme B, which is extracellular matrix (ECM) remodeling through degradation of ECM proteins. These observations suggest that granzyme B can be expressed by various cell types other than CTLs in tissues. In order to investigate the expression pattern of granzyme B in lesion areas of atherosclerosis and rheumatoid arthritis, we performed immunohistochemistry and in situ hybridization analyses of human atherosclerotic plaques and synovial tissues of rheumatoid arthritis- and osteoarthritis-joints. In atherosclerotic plaques, granzyme B was expressed in macrophages rich areas such as boundary between media and intima, areas around necrotic core, and shoulder regions. In synovial tissues of rheumatoid arthritis-joints, the expression of granzyme B was detected in T-cell rich follicular areas. Additionally, the expression of granzyme B was strongly observed in the lining layers where majority of the cells are macrophages and in perivascular areas where macrophages and small number of

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lymphocytes were mixed to form diffuse cellular aggregates. Relatively less granzyme B-positive cells were detected in the lining layers of osteoarthritic synovium. These observations indicate that macrophages would be added to the list of cells expressing granzyme B in human inflammatory diseases and that granzyme B may play roles in various inflammatory processes mediated by macrophages.

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A Role for Phosphatidylinositol-Specific Isomorphs of Phospholipase C (PLC) in the Switch of Macrophages from an Inflammatory to an Angiogenic Phenotype Induced by Adenosine A2A Receptor (A2AR) and Toll-like Receptor 4 (TLR4) Agonists

S. Grinberg, 1 S. J. Leitovich; 1 Cell Biology & Molecular Medicine, Graduate School of Biomedical Sciences, UMDNJ, Newark, NJ, 2Cell Biology & Molecular Medicine, New Jersey Medical School, UMDNJ, Newark, NJ

We have previously shown that co-stimulation of TLR2, 4, 7 or 9 and adenosine A2AR synergistically up-regulates VEGF expression in macrophages, while down-regulating TNFα expression. Treatment of murine macrophages with U73122, an inhibitor of phosphatidylinositol-specific PLC (PI-PLC), strongly promotes expression of VEGF in the presence of CGS21680 or NECA (A2AR agonists), while simultaneously down-regulating endotoxin (LPS)-induced TNFα expression. This suggests that the synergistic pathway induced by ligation of A2AR and TLR4 involves regulation of PLC. To determine whether LPS or NECA modulate PLCs, their effects on mRNA levels of the PLCs were determined by quantitative RT-PCR. Expression of PLCs β1, β2, β3, β4, γ1, γ2 and δ1 proteins were determined by Western analyses. LPS (100ng/ml) induced a rapid and selective decrease of PLCβ2 mRNA in murine macrophages. PLCβ2 protein levels were decreased within 1 hr of LPS treatment, and remained low for at least 26 hours. PLCβ2 protein levels were depleted by 8 hours, and remained low for at least 30 NECA alone had little effect on PLCβ2 levels, and did not affect the depletion of PLCβ2 by LPS. Since U73122 is a relatively specific PLCβ2 inhibitor, we used sRNA oligonucleotide constructs to specifically deplete PLCβ2 in RAW264.7 macrophage-like cells, and obtained ~70% depletion at both mRNA and protein levels. Treatment of these depleted cells with NECA resulted in the strong up-regulation of VEGF expression. Thus, both depletion of PLCβ2 by sRNA, and inhibition of PLCβ2 by U73122 sensitize macrophages to subsequent stimulation by A2AR agonists with promotion of VEGF expression. We propose that LPS down-regulates PLCβ2 expression in macrophages, thus playing a role in the regulation of VEGF expression by A2AR agonists.

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Vascular Endothelial Growth Factor (VEGF) Expression by Macrophages Is Synergistically Up-Regulated by Adenosine A2A Receptor Agonists and Endotoxin via the Hypoxia Response Element (HRE) in the VEGF Promoter

M. Ramasathan, G. Pinal-Enfied, I. Hae, S. J. Leitovich; Cell Biology & Molecular Medicine, New Jersey Medical School, UMDNJ, Newark, NJ

VEGF expression by macrophages is regulated by micro-environmental factors. Adenosine A2A receptor (A2AR) agonists together with agonists of Toll-like receptors (TLRs) 2, 4, 7 and 9 synergistically induce VEGF expression by macrophages. We now show that the TLR4 agonist Escherichia coli LPS and the A2AR agonists NECA and CGS21680 synergistically augment VEGF gene transcription. This transcriptional activation requires the hypoxia response element (HRE) in the VEGF promoter. Macrophages were treated with LPS and NECA or CGS21680, and steady-state levels of VEGF mRNA were measured. Strong induction (~ 10-fold) of VEGF mRNA was observed by 12 h. To determine if this induction was at the transcriptional level, RAW 264.7 cells (a macrophage-like cell line) were transiently transfected with pVEGF-Luc, a VEGF-promoter luciferase reporter construct. LPS/NECA treatment of the transfectants strongly induced VEGF promoter activity. Transfections with the VEGF promoter-luciferase constructs containing deletions of specific cis-elements showed that the HRE region of the VEGF promoter was essential for its activation by LPS/NECA. Deletion of the putative NFkB-binding region did not affect the LPS/NECA-induced activation of the VEGF promoter, suggesting that NFkB activation is not required for induction of VEGF expression. Since the HRE region is essential for hypoxia-induced VEGF gene expression via binding of the HIF transcription factor complex, we examined whether stimulation of macrophages with LPS/NECA induces HIF-1α expression, thus activating the VEGF promoter via the HRE. HIF-1α mRNA levels were significantly increased in LPS/NECA-treated macrophages, and this increase preceded the induction of VEGF mRNA. Further, LPS/NECA treatment of macrophages caused an increase in HIF-1α mRNA binding activity. An NFκB inhibitor, Bay 11-7085, blocked LPS-induced TNFα, but had little effect on LPS/NECA-induced VEGF expression. These data indicate that LPS/NECA-induced expression of the VEGF gene critically involves hypoxia-induced HIF transcriptional activity, while NFκB activation does not play a significant role in this induction.

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Low Intensity Pulsed Ultrasound Stimulates Macrophage Phagocytosis via Multiple Signaling Pathways

S. Zhou,1 A. Schmelz, Y. Li, T. Seufferlein,3 H. Gross,1 M. G. Bachem 1; 1Clinical Chemistry, University Hospital, Ulm, Germany, 2Trauma Surgery, University Hospital, Ulm, Germany, 3Internal Medicine, University Hospital, Ulm, Germany

We investigated whether low-intensity-pulsed-ultrasound (US) activates macrophages (Ma) and stimulates phagocytosis of E.coli. In addition we studied relevant signaling mechanism induced on the expression of several PLC isoforms were tested. Expression of mRNA for PLCs β1, β2, and β3 were determined by quantitative RT-PCR. Expression of PLCs β1, β2, β3, β4, γ1, γ2 and δ1 proteins were determined by Western analyses. LPS (100ng/ml) induced a rapid and selective decrease of PLCβ2 mRNA in murine macrophages. PLCC2 protein levels were decreased within 1 hr of LPS treatment, and remained low for at least 26 hours. PLCC2 protein levels were depleted by 8 hours, and remained low for at least 30 NECA alone had little effect on PLCC2 levels, and did not affect the depletion of PLCC2 by LPS. Since U73122 is a relatively specific PLCC2 inhibitor, we used sRNA oligonucleotide constructs to specifically deplete PLCC2 in RAW264.7 macrophage-like cells, and obtained ~70% depletion at both mRNA and protein levels. Treatment of these depleted cells with NECA resulted in the strong up-regulation of VEGF expression. Thus, both depletion of PLCC2 by sRNA, and inhibition of PLCC2 by U73122 sensitize macrophages to subsequent stimulation by A2AR agonists with promotion of VEGF expression. We propose that LPS down-regulates PLCC2 expression in macrophages, thus playing a role in the regulation of VEGF expression by A2AR agonists.

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Microtubule Dynamics in Fcgamma Receptor-mediated Phagocytosis

S. Pang,1 N. Qureshi, R. E. Harrison; 2 Laboratory of Medicine and Pathobiology, University of Toronto, Scarborough, ON, Canada, 2Department of Life Sciences, University of Toronto, Scarborough, ON, Canada

Phagocytosis is a cellular process by which particulate matter of >0.5μm is internalized. In animals, phagocytosis serves as a defence mechanism against foreign organisms. Macrophages are professional phagocytes and serve as "sentinels" in various tissues. IgG-coated pathogens are recognized by Fcgamma receptors (FcgammaRs) on patrolling macrophages. A signalling cascade ensues whereby a phagocytic cup form as pseudopods extend around the pathogen. The pathogen is internalized into a membrane-bound organelle termed the phagosome. A series of fusion events with endosomes and lysosomes occur as the phagosome travels towards the lysosome, ultimately acidifying the vesicle and destroying the pathogen. Microtubules (MTs) have been implicated in the retrograde transport of the phagosome and mediating lysosome fusion with the phagosome. A class of MT-associated proteins (MAPs), known as MT-plus (+)-end-tracking proteins (+TIPs), travel exclusively on MT (+) ends. We are currently using CLIP-170, the prototypical +TIP, to "track" (+) ends of MTs in mouse RAW 264.7 macrophages, CHO2A FcgammaR engineered phagocytes, and HeLa cells. Live imaging microscopy was done to visualize the dynamics of CLIP-170 and MTs in macrophages co-transfected with beta-tubulin-RFP and CLIP-170-GFP. We examined MT dynamics in resting macrophages and those undergoing phagocytosis. TIRF was used to visualize GFP-CLIP-170 at the plasma membrane during "frustrated phagocytosis" assays. Results indicate most MTs are dynamic in resting macrophages with CLIP-170 tracking the MT (+) ends. During phagocytosis, MTs and CLIP-170 were seen to co-localize to the forming phagocytic cup. In conclusion, MT (+) ends are targeted towards the phagocytic cup and CLIP-170 locates to MT (+) ends during phagocytosis.

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Extracellular ATP-induced Intracellular Ca2+ Dynamics without Following Normal Exocytosis

Y. Satoh, T. Nakamura, M. Osakada, T. Sano; Histology (Anatomy2), Iwate Medical University, Morioka, Japan

[Background/Aim] Adenosine trisphosphate (ATP) is a fundamental transmitter in various tissues, as well as intracellular energy source. Previous studies indicated that ATP induced histamine discharge from mast cells which possess many secretory granules, and the discharge was simply thought as exocytotic secretion. It is well known that intracellular Ca2+ ([Ca2+]i) increase as a key event in exocytosis in various cells including mast cells, but nobody observed whether ATP induces [Ca2+]i changes of mast cells, and whether ATP-induced histamine discharge is true exocytosis. [MATERIALS AND METHODS] Rat peritoneal mast cells were collected and loaded with Indo-1/AM or Fluo-4/AM for measuring of [Ca2+]i. The exocytosed granule matrices

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were stained by sulforhodamine B (SFRMB). The fluorescent images of the cells stimulated by ATP and/or compound 48/80 were consecutively acquired by confocal microscopy (Nikon RCM/Ab or Zeiss 510). [Results] ATP and ADP induced [Ca^{2+}], increase but not exocytosis. Adenosine and AMP has no effect, indicating that ATP-induced changes were mediated by P2 receptors, but not P1. UTP (P2Y2, 4, 6 agonist) did not induce [Ca^{2+}] changes, and removing of extracellular Ca^{2+} abolished ATP-induced [Ca^{2+}] changes. These suggested that most cells possess ion channel type receptors responding to extracellular ATP (i.e., P2X). DBzATP (P2X7 agonist) elicited prominent [Ca^{2+}], increase, but not β,γ-meATP (P2X1, 3 agonist) did not any responses. Intracellular non-exocytosed granule matrices were stained by SFRMB by high dose of DBzATP, indicating increasing plasma cell membrane permeability. Interestingly, after the ATP-induced [Ca^{2+}] changes, compound 48/80 induced exocytosis were inhibited. [Conclusions] ATP-induced histamine discharge is not physiological exocytosis, but pharmaco-pathological conditions in vitro. In tissue, ATP from nerve fibers or other damaged cells may inhibit secretory activity of mast cells.

Role of Reactive Oxygen Species in Leukocyte Transendothelial Migration
J. K. G. Gruenewald, A. J. Ridley; Ludwig Institute for Cancer Research, London, United Kingdom

Normal cellular redox homeostasis is a balance between the generation of free radicals or other reactive oxygen species (ROS) and the antioxidant response. Smoking, alcohol, poor diet as well as environmental pollution can lead to an imbalance and an increase of oxidative stress. To investigate the effect of ROS on leukocyte transendothelial migration, we set up an in vitro system as a model for transmigration using the human monocytic leukaemia cell line THP-1 and human umbilical vein endothelial cells (HUVECs). The effect of hydrogen peroxide (H_{2}O_{2}), applied extracellularly to THP-1 cells, on transendothelial migration was investigated. A significant decrease of transendothelial migration is induced by increasing H_{2}O_{2} levels and also by altering intracellular ROS levels using the NADPH oxidase stimulus phorbol 12-myristate 13-acetate (PMA) and the catalase inhibitor 3-amino-1,2,4-triazole (AT). This decrease was not observed in an endothelial cell-free transwell assay pointing to a transmigration defect specific for the interaction between leukocytes and endothelial cells. H_{2}O_{2}-treated THP-1 cells exhibit higher motility on HUVECs, whereas their adhesion on substrates such as VCAM-1, fibronectin and ICAM-1 is unaltered. We have tested the effects of H_{2}O_{2} on several signalling molecules implicated in cell migration, and have observed a change in the levels and activity of the small GTPase RhoA, which has previously been shown to be important for transendothelial migration. Altogether, our results suggest that ROS have multiple effects on THP-1 cell migration. We therefore propose that ROS play a regulatory role in leukocyte transendothelial migration.

Expression Profile of RhoGTPases and RhoGEFs during RANKL-stimulated Osteoclastogenesis: Identification of Essential Genes in Osteoclasts
H. Buraizer, S. Stephens, S. Ory, P. Forti, N. Morrison, A. Blangy, "GRDM, CNRS FRE2953, Montpellier, France; School of Medical Science, Griffith University, Queensland, Australia

Introduction. During the process of differentiation, adhesion to the bone matrix or osteolysis, the actin cytoskeleton of osteoclasts undergoes profound reorganization. RhoGTPases are key regulators of actin dynamics. They control cell adhesion, migration and morphology through their action on actin cytoskeleton. In mouse, there are 18 low molecular weight RhoGTPases. They regulate cell adhesion, migration, and have observed a change in the levels and activity of the small GTPase RhoA, which has previously been shown to be important for transendothelial migration. Altogether, our results suggest that ROS have multiple effects on THP-1 cell migration. We therefore propose that ROS play a regulatory role in leukocyte transendothelial migration.

IL-8 Induced Adhesion of Neutrophils Is Reduced by Fas Ligand (FasL) before Loss of CD47 and Phosphatidylserine Externalization Mark the Cells as Apoptotic
B. Hendey, P. Sagi, S. Lidder; Pharmacology, Rush University Medical Center, Chicago, IL

Previously work indicated that either Fas activation or rotterlin reduced treatment PMA stimulated neutrophil adhesion in a non-additive fashion. Fas activation and rotterlin also reduced the membrane localization of PKC-delta suggesting that Fas activation reduces adhesion by changing the localization of PKC-delta. Interestingly, the reduction in adhesion occurred before the cells could be recognized as apoptotic via Phosphatidylserine (PS) externalization. Since PMA is not a physiological agonist, adhesion in response to IL-8 and IMLP was examined to determine if adhesion and PKC-delta localization were sensitive to Fas activation. Treatment with either FasL or rotterlin, reduced IMLP-induced neutrophil adhesion but neither agent had an effect on IMLP mediated adhesion. Triton soluble membrane fractions were run using SDS-PAGE and Western blots were analyzed to determine if FasL affected membrane association of PKC-delta. FasL reduced the membrane localization of PKC-delta in response to either IL5 or IMLP. To determine if the reduction in IL-8 stimulated adhesion occurred at a time before the cells could be recognized as apoptotic, we also measured the effect of FasL on IMLP and IL8-stimulated neutrophils on subsequent PS externalization and CD47 expression. While 1 hr of Fas activation reduced adhesion, there was no significant effect of Fas activation on PS externalization. Likewise, at 2 hrs no effect of FasL on CD47 expression, a marker that has recently been shown to function as a “don’t eat me signal”. Only after 3 hrs of Fas activation would a fraction of the PMN be recognized as apoptotic via both loss of CD47 and externalization of PS. These results suggest that FasL exposure would decrease neutrophil adhesion in response to an inflammatory stimulus such as IL-8 before they would be recognized as apoptotic. However, the same FasL exposure would not reduce responsiveness to bacterial peptides.

Hematopoietic Cell Differentiation and Activation in Primary and Cultured Leukocytes
D. M. Fishwild, K. T. Tran; Applications Development, Guava Technologies, Hayward, CA

Cell differentiation and activation are complex processes generally believed to occur in a continuous fashion. However, techniques which monitor single cells rather than a population of cells often reveal these processes involve a series of discrete steps or switches. For example, as reported for promyelocytic precursor cells, HL60, stimulated with DMSO, differentiation to neutrophils occurs through a multi-step process in which cells are first primed and then differentiate by an all-or-none mechanism (Chang et al., BMC Cell Biology, 7:11; 2006). Using four-color flow cytometry, we extended these studies to examine the extra- and intracellular markers that correlate with these priming and differentiation steps. In addition, we examined the differences in expression of markers when HL60 cells were stimulated with PMA to induce differentiation to the monocytic lineage. Similarly, for primary leukocyte activation, the expression of such activation markers as CD25, CD69, CD38 and CD49a among others, can be tracked on individual cells and correlated with the cells’ proliferative capacity. Thus, four-color flow cytometry which can readily monitor differentiation and activation steps on a single cell level can better uncover the actual mechanisms involved in these complex processes than can analyses of populations of cells. This leads to a more complete understanding of the molecular mechanisms that control differentiation and maturation of hematopoietic cells crucial for the clinical manipulation of blood cell production.

Anti HLA Antibodies Treatment Precludes the Formation of Immunological Synapses and Rosettes between Macrophages and Lymphocytes
I. T. C. Novak, H. R. A. Cabral; 1 Institute of Cell Biology, National University of Cordoba-Argentina. Faculty of Medicine, Cordoba-Argentina, Argentina, 2Instituto de Biologia Celular, Cordoba, Argentina, 3Instituto de Biologia Celular, Univ Nac Cordoba, Argentina

An immunological synapse (IS) is formed when a T lymphocyte close adhered to an antigen-presenting cell (APC). We found that in cultures of autologous total human leukocytes, in which death of neutrophils occurs, macrophages and T lymphocytes form IS and rosettes (MLRs) -we considered a MLR-rosette when three or more T-lymphocytes are close adhered to the APC, in this case the macrophage (Am J Hematol 60: 285-8,1999). These phenomena are produced when the macrophages showed evidences of they phagocyting cell neutrophil materials. We included a step that consist in a centrifugation of the samples harvested from the cultures. The other known effects of Fas activation did not form IS or RMLRs. Macrophages present antigens to T helper lymphocytes trough their class II molecules . Here, we attempted to block the formation of IS and Rosettes RMLRs by means of anti HLA DR monoclonal antibodies. METHODOLOGY: Culture samples of human total leukocytes from 7 healthy donors were prepared at 48, 72 and 96 hours and submitted to anti HLA DR antibodies (Diatec, Norway) at a concentration of 15 micrograms/ml and incubated by 30 min. Then, we centrifuged the cells and prepared them as previously described. RESULTS: Nor IS neither rosettes were found in most of the samples

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Generation of Suppressor CD8+ T Cells by Triggering 4-1BB

Y. Kim, B. Choi, W. Kim, W. Kang, B. Kwon

Immunology, Immunomodulation Research Center, Ulsan, Republic of Korea, 2LSU Eye Center, New Orleans, LA

A superantigen, staphylococcal enterotoxin A (SEA) is known to induce a strong proliferative response of both CD4+ and CD8+ T lymphocytes through a specific interaction with Vβ3 TCR. It has been reported that 4-1BB stimulation in vivo results in the suppression of SEA-specific CD4+ T cells, which is partially mediated by the increase of TGF-β-expressing CD8+ T cells. Since we previously found that the 4-1BB-mediated CD4+ T suppression was mediated by the indoleamine 2,3-dioxygenase (IDO) in collagen-induced arthritis, we investigated whether the anti-4-1BB-mediated CD4+ T suppression in vivo was dependent on the IDO. 4-1BB stimulation in vivo led to the induction of IDO in dendritic cells (DCs) and monocytes/macrophages stimulated by the disease, whose NADPH oxidase is inactive, suffer recurrent bacterial infections. Here, we test the hypothesis that the NADPH oxidase is present in NETs by immunofluorescence and confocal microscopy analysis. We also evaluate the production of reactive oxygen species (ROS) in NETs by chemiluminescence. We first show that NETs, detected with DAPI staining by immunofluorescence, are formed in neutrophils treated with LPS but not in unstimulated neutrophils. The use of poly-L-lysine at high concentrations was also effective for the development of NETs. NETs were effectively disassembled when neutrophils were treated with deoxyribonuclease (DNase) before or after stimulation. DNase treatment did not affect cell viability in neutrophils. The NADPH oxidase components p47-phox and p22-phox were clearly detected in NETs by immunofluorescence analysis. Co-localization of p47-phox and p22-phox in punctate structures was evident, suggesting that the NADPH oxidase could be assembled in the NETs. The kinetics of ROS production in response to heat-killed Gram positive and Gram negative bacteria was attenuated by treatment with DNase. This suggests that ROS production is decreased when NET assembly is prevented. Deficiency in Rab27a, an enzyme that regulates the Golgi organization, subcellular localization of paxillin, and actin cytoskeletal remodeling; all of which may need to be coordinately regulated during cell migration. To understand the physiological roles of GIT2, we here generate mice in which GIT2 gene is disrupted. We found that loss of GIT2 leads to an immunodeficient state, which is primarily due to the dysfunction of neutrophils. Loss of GIT2 causes impaired chemotactic directional sensing and hyperproduction of superoxide in GPCR-stimulated bone marrow neutrophils. We found that GIT2 is an essential component of the Gβγ-mediated directional sensing machinery, and is also necessary for suppressive control of the superoxide production. Moreover, GIT2 was found to be necessary for the proper orientation of superoxide production. Therefore, GIT2 links chemotactic directionality and proper production of superoxide, in quantity and in direction, in GPCR-stimulated neutrophils, and is hence important for innate immunity.

Neutrophil Directional Sensing and Superoxide Production Linked by GIT2


Department of Medical Sciences, National Defense Medical College, Saitama, Japan, 2Department of Molecular Pathology, RIKEN Kobe, Kobe, Japan, 3Graduate School of Biostudies, Kyoto University, Kyoto, Japan

Neutrophils play important roles in innate immunity and in initiation of an acute response to infection. During such responses, neutrophils are activated, move towards the site of inflammation and actively produce antimicrobial agents, including a number of reactive oxygen species (ROS). These ROS are first produced as superoxide anions by NADPH oxidase and are essential for the killing of invaders upon neutrophil phagocytosis. Neutrophil production of superoxide anions is tightly coupled with the activation state of neutrophils. Strict suppression of superoxide production in resting neutrophils is essential to prevent non-specific injury of blood vessel endothelial cells, tissues and organs. We have previously identified GIT family members of ADP-ribosylation factor (Arf) GTPase-activating proteins (GAPs), as binding proteins to an integrin-signaling protein, paxillin. By regulating Arf1 activity, we have shown that GIT2 has a potential role in regulating the Golgi organization, subcellular localization of paxillin, and actin cytoskeletal remodeling; all of which may need to be coordinately regulated during cell migration. To understand the physiological roles of GIT2, we here generate mice in which GIT2 gene is disrupted. We found that loss of GIT2 leads to an immunodeficient state, which is primarily due to the dysfunction of neutrophils. Loss of GIT2 causes impaired chemotactic directional sensing and hyperproduction of superoxide in GPCR-stimulated bone marrow neutrophils. We found that GIT2 is an essential component of the Gβγ-mediated directional sensing machinery, and is also necessary for suppressive control of the superoxide production. Moreover, GIT2 was found to be necessary for the proper orientation of superoxide production. Therefore, GIT2 links chemotactic directionality and proper production of superoxide, in quantity and in direction, in GPCR-stimulated neutrophils, and is hence important for innate immunity.

Role of Interferon Response Factor 8 (IRF8) in Development of Follicular Dendritic Cells of IRF8 Knock Out Mice

C. Qi, Laboratory of Immunopathology, NAID/NHLI Rockville, MD

Activated B cells rapidly expand and differentiate in germinal centers (GC). This activity is dependent on follicular dendritic cells (FDC) that trap immune complexes, present them to B cells and signal B cell resistance to apoptosis. FDC are dependent on CD40L+ T cells and LTα+ B cells for their development from non-hematopoietic cells in bone marrow. The transcription factor, interferon regulatory factor 8 (IRF8), regulates the differentiation and function of macrophages, granulocytes and other dendritic cells through activation or repression of target genes. IRF8 also affects B cells by activating transcriptional regulation of CDC20 and BCL6. To determine if IRF8 is involved in development and regulation of FDC, we used immunohistochemistry to study expression of IRF8 and other genes in spleens of wild type (IRF8+/+) and IRF8 knockout mice (IRF8−/−), comparing them for: GC and follicular structure; marginal zone (MZ) cells including MZ and macrophage-rich lymphocytes and sinus endothelial cells; extrafollicular myeloid cells; FDC; B-cells; and T-cells. Primary follicles of IRF8−/− mice exhibited markedly larger lymphocytes with increased total GC in IRF8−/− relative to wild type variable in size with a reduced density of lymphocytes and irregular distributions of FDC1+ FDC. In normal spleens, FDC of wild type mice were distributed by point counting at higher levels than FDC from knockout mice. The present findings suggest that IRF8 modulates certain features of GC FDC with the highest rate of apoptosis in follicles of IRF8 knockout mice implying that the ability of FDC to protect B cells from death is reduced. These results may have implications for understanding autoimmunity and B cell lymphomagenesis.

Protective Effect of Curcumin and Silibinin on Inflammatory Cell-mediated Hepatotoxicity

D. A. O. Clarke, E. Niam, D. Hill, Biology, Morgan State University, Baltimore, MD

The hepatotoxicity induced by Alpha-naphthylisothiocyanate (ANIT) in rodents is mediated by inflammatory cell infiltration and activation. This hepatotoxicity models that of drug-induced cholestasis in humans. Curcumin, a polyphenolic plant pigments characteristic to the tropical root Tumeric, has demonstrated anti-inflammatory and antioxidant activity. Silibinin, a component of polyphenolic flavinoid extracts from milk thistle, has also demonstrated anti-inflammatory and anti-oxidant activity. Because of the inflammatory cell-mediated hepatotoxicity of ANIT and the anti-inflammatory activity of both curcumin and silibinin the current study address the hypothesis that co-pretreatment with silibinin/curcumin affords protection against ANIT-induced hepatotoxicity. To substantiate this hypothesis, male Sprague Dawley rats were treated orally with silibinin (200-250 mg/kg) and curcumin (200-250 mg/kg) prior to treatment with ANIT (150-250 mg/kg). 24 hours after ANIT treatment, samples of rat livers and plasma were processed for analysis of hepatic injury. The histopathological analysis of ANIT-induced liver injury indicated a pronounced infiltration of inflammatory cells and overt hepatocellular necrosis. ANIT-induced liver injury was also manifested as significant elevations in plasma levels of liver enzymes including alanine aminotransferase (ALT, SGPT) and aspartate aminotransferase (AST, SGOT). Co-pretreatment with silibinin and curcumin was protective against the plasma injury indicated a pronounced infiltration of neutrophils and overt hepatocellular necrosis.
elevations of hepatic enzymes including ALT/SGPT and AST/SGOT. In addition, histological and immunohistochemical analysis indicated that co-pretreatment with silybinin and curcumin effected hepatic infiltration of inflammatory cells and the degree of hepatic necrosis. These data suggest that co-pretreatment with silybinin/curcumin affords protection against ANIT-induced hepatotoxicity. (Supported by NIEHS Grant ES 00338-02)

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Characterization of an Interacting Protein with TRPV1

S. Lee1, Y. Oh2, K. Um1, I. Baybarz3; 1Hankyong National University, Anung, Kyonggi-do, Republic of Korea, 2Graduate School of Bio-Information Technology, Hankyong National University, Anung, Kyonggi-do, Republic of Korea

TRPV1, previously known as VR1, is a capacitative, proton- and heat-sensitive non-selective cation channel. Although its channel activity is reportedly modulated through protein-protein interactions, to date very few VR1 interacting proteins have been identified. In order to find proteins that interact and regulate the channel activity of TRPV1, we performed yeast two hybrid screening technique. We found a protein, called Eferin, which interact with TRPV1 in a specific manner. Interaction of Eferin with TRPV1 is confirmed by GST-pull down assay and co-immunoprecipitation. Also TRPV1 and Eferin were shown colocalized by immunohistochemistry. However, the overexpression of eferin did not increase the channel activity significantly in response to capsaicin. The reason for the slight effect of Eferin on channel activity might be the presence of endogenous Eferin in HEK293 cells. Therefore, we knocked down the endogenous Eferin in TRPV1-stably transfected HEK293 cells by siRNA technology. We found that TRPV1 expression in membrane is much decreased to show decreased channel activity to capsaicin by knocking down the Eferin. This work was supported by Brain Korea 21 Project, Ministry of Education of Korea.

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Effects of Nicotine on the Change of Dopamine and G Protein-coupled Receptor Kinase 3 (GRK3) Levels in PC12 Cells

M. Yeom, D. Han, H. Hwang, J. Han, S. Kim, H. Lee, D. Hahm; Kyung Hee University, Yongin-si, Republic of Korea

G protein-coupled receptor kinases (GRKs) are a family of serine/threonine kinases involved in the agonist-induced homologous desensitization of G-protein coupled receptors (GPCRs). It is known that G-protein receptor kinase 3 (GRK3) seems to be involved in the development of tolerance and dependence, processes closely related to drug addiction such as opioid and cocaine. But, the involvement of GRK3 in the mechanism of nicotine addiction is poorly understood. In this study we aim to elucidate the cellular mechanisms of GRK3 in the release of dopamine and GRK3 expression in dopaminergic PC12 cells. dopamine levels were determined by high-performance liquid chromatography (HPLC)-electrochemical detection (ECD) assay and those of GRK3 mRNA and protein by the real-time PCR and immunoblot assay, respectively. Nicotine induced the secretion of dopamine in PC12 cells. Also, nicotine dose-dependently decreased the levels of GRK3 mRNA and protein, both. The GRK3 levels reduced by nicotine were gradually increased as time went by, but were maintained at lower levels than in control, non-transfected PC12 cells, during the experiment. These results suggest that the expression levels of GRK3 in dopaminergic neural cells may affect the secretion of dopamine after nicotine treatment.

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Intracellular Retention of GluR6a Kainate Receptors by a Point Mutation Outside the Ligand-binding Domain

P. Vivithanaporn1, W. Marszalcz2, G. T. Swanson3; 1Department of Pharmacology and Toxicology, University of Texas Medical Branch, Galveston, TX, 2Department of Molecular Pharmacology and Biological Chemistry, Northwestern University, Chicago, IL

Biosynthesis of ionotropic glutamate receptors (iGluRs), including the kainate receptor (KAR) family, is controlled by various trafficking motifs within the subunit proteins. Previous results have suggested that domains intrinsically linked to receptor function, such as ligand-binding and gating domains, can play an important role in subcellular trafficking and egress from the endoplasmic reticulum (ER). The mechanism of this quality-control process is not understood. In these studies we aim to elucidate the cellular mechanisms of KARs, the process of desensitization, and changes in protein trafficking and assembly. Desensitization is a well-characterized biophysical phenomenon in iGluRs with an associated physical model formulated from structure-function and crystallographic studies. We determined how changes in desensitization rates correlated with altered subcellular trafficking of the GluR6a KAR biosynthesis by mutating two amino acids located outside the ligand-binding domain; these residues are known to be determinants of desensitization for KARs. We introduced conservative and non-conservative mutations to change our target amino acids, E662 and R663, and measured plasma membrane expression in biochemical assays and functional properties in patch-clamp physiological recordings. Plasma membrane expression of myc-GluR6a receptors relative to total receptor protein was measured by cell ELISA assays in transfected COS-7 cells. Substitution of R663 with a glutamate [GluR6(R663E)], which slows receptor desensitization by 2-fold, greatly diminished the plasma membrane expression of myc-GluR6a receptors by 24-fold (n = 3).

In contrast, a conservative substitution [GluR6(R663K)] did not alter plasma membrane expression of the receptors. These results were reproduced in transfected primary hippocampal neurons. These results demonstrate that a linker region of the GluR6a subunit, which previously was associated specifically with desensitization, also can influence receptor trafficking.

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Neurotrophic Regeneration during the Corneal Wound Healing after Refractive Surgery

T. Blanco-Mezquita1, J. Merayo-Lloves1, C. Martinez-Garcia2, S. Bonini1, A. Lambiase1, R. Pronec4; 1OIBA, Universidad de Valladolid, Valladolid, Spain, 2Cell Biology, Universidad de Valladolid, Valladolid, Spain, 3Laboratory of Ophthalmology, University of Rome, Rome, Italy, 4IIBIL, Universidad de Coimbra, Coimbra, Portugal

OBJECTIVES The cornea is one of the most densely innervated tissues of the body, which is highly abounded by sensory nerves and autonomic nerve fibers. Corneal nerves fibers exert important trophic influences on the corneal epithelium and contributes to maintain the cornea and promote wound healing after external injuries. The aim of this work is to evaluate the neurotrophic role of effrons axons in the corneal wound healing process after refractive surgery in hens. METHODS Lohmann Brown hens underwent Photorefractive Keratotomy (PRK) were divided into different groups treated with topical administration of 0.2% marine Nerve Growth Factor (NGF) group A, Balanced Salt Solution (BSS) group B and group C received no treatment. Clinical monitoring was made during two months. Eyes were examined at sequential time points and fixed in 1% buffered formalin. Sections were stained with H-E and Masson trichrome. Occludin, Claudin-5, Hsp-70, Alpha-SMA, Trk-A and Gap-43 were detected by immunohistochemistry. RESULTS Statistically significant differences in the grade of haze and pachymetry measurements were found. There were not differences in the expression of Occludin and Claudin-5 in the corneal epithelium, among groups A significant increase in Hsp-70, Alpha-SMA and Trk-A was observed in the NGF group. Statistically significant differences were observed in the expression of GAP-43 between groups. Gap-43 expression was minor in NGF group. CONCLUSIONS Topical NGF modulates tropanic axon regeneration, which probably influence in the actual corneal wound healing process.

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Protection of PC12 Cells from Polyglutamine-rich Fragments of Huntingtin

Y. Zhang, A. M. Tartakoff; Pathology, Case Western Reserve University, Cleveland, OH

The neurotoxicity of polyglutamine-containing proteins is central to the pathogenesis of Huntington’s Disease and several other autosomal dominant disorders. In each case, otherwise innocuous proteins acquire enlarged polyglutamine tracts due to increased numbers of trinucleotide repeats in the corresponding open reading frames. For unknown reasons, in each disease a distinct region of the brain is affected. Although several strategies have been identified to apply proteins or drugs which protect against polyglutamine toxicity using model organisms, few protective factors have been identified for higher eukaryotic cells. We therefore have made use of a marine PC-12 cell culture model in which an ecdysoneregulated promoter induces synthesis of a GFP-tagged form of the N-terminus of huntingtin which includes a large polyglutamine tract (103 repeats) (Aiken et al. Neurobiol. Dis., 16, 546 (2004)). Upon induction, the GFP-tagged product become visible with one day and apoptosis is quickly apparent, as judged by cell detachment and binding of annexin V. To test the impact of selected proteins, we have transfected these cells with plasmids encoding human Hsp70, a mutant form of Hsp70, or the accessory protein, Hdj1. To identify transfected cells, a plasmid encoding dsRed was always included. Induction begins one day after transfection and cultures are followed over the three following days. We observe that toxicity is rapid in cells which receive only ds-Red, or ds-Red in conjunction with the Hsp70 mutant, or Hdj1 plasmids. By contrast, Hsp70 is distinctly protective, resulting in essentially complete survival for at least two days, even though the transfecinants still have very large polyglutamine tracts. It is unlikely that widespread overexpression of Hsp70 can be of therapeutic value; however, these observations illustrate the utility of this culture model and motivate the identification of additional corrective cDNA's. Supported by the HigfiQ Foundation.
1271 Cariporide, a Specific Na'/H' Exchanger (NHE) Inhibitor, Attenuates Apoptotic Cell Death Caused by Mitochondrial Ca2+ Increase in Neuronal Cortical Cells
S. Hyun,1,2 B. Lee,1,2 D. Lee,1,2 S. Kim,1 S. Lee,1 K. Yi,1 S. Yoo,1 S. Lee,1,2 E. Baik,1,2 C. Moon,1,2 Y. Jung,1,2 1Department of Physiology, School of Medicine, Ajou University, Suwon, Republic of Korea, 2Brain Korea 21 for Medical Science, School of Medicine, Suwon, Republic of Korea, 3Brain Korea 21 for Molecular Science and Technology, Suwon, Republic of Korea, 4Brain Disease Research Center, School of Medicine, Ajou University, Suwon, Republic of Korea, 5Medical Science Division, Korea Research Institute of Chemical Technology, Daejeon, Republic of Korea. Ischemia, which often occurs during seizure and stroke, gives rise to glutamate release. Glutamate induces activation of Na'/H' exchanger (NHE) and subsequently accelerates the process of neuronal cell death via intracellular Ca2+ overload. Glutamate-induced neuronal cell death also requires mitochondrial Ca2+ uptake. In the present study, we examined effect of cariporide, well known as NHE inhibitor, on mitochondrial Ca2+-induced neuronal cell death in cultured neuronal cells and in vivo. Treatment with cariporide reduced glutamate-induced apoptotic cell death (Caspase-3 activity, TUNEL-positivity) in neuronal cortical cells. Dual peaks of [Ca2+]i rise were observed after glutamate exposure. Cariporide suppressed only second [Ca2+]i increase and reduced mitochondrial Ca2+ uptake. In addition, cariporide significantly recovered the large reduction in mitochondrial membrane potential and cytochrome c release to cytosol induced by glutamate exposure. In vivo brain ischemia model, cariporide produced protective effects, decreasing the infarct size. Taken together, Cariporide attenuates mitochondrial Ca2+-induced apoptotic neuronal cell death through inhibition of NHE in neuronal cortical cells in vivo and in vitro. This work was supported by a grant (CIBM2-A300-001-1-0-2) from the center for Biological Modulators of the 21st Century Frontier R&D Program, the Ministry of Science and Technology, and by a grant from the Korea Science and Engineering Foundation (KOSEF) through the BDRC at Ajou University.

Keywords: Na'/H' exchanger (NHE), neuronal cortical cells, Ca2+, mitochondria, apoptosis, glutamate

1272 Presenilin-dependent erbB4 Nuclear Signaling Regulates the Timing of Astrogenesis in the Developing Cerebral Cortex
S. P. Sardi,1,2 J. Murtie,1,2 S. Koirala,1,2 B. A. Patten,1,2 G. Corfis1,2 1Neurobiology Program, Children's Hospital Boston, Boston, MA, 2Department of Neurology, Harvard Medical School, Boston, MA
Receptor tyrosine kinases (RTKs) are known to regulate gene transcription through activation of complex multilayered kinase cascades. However, recent studies suggested that erbB4, an EGFR receptor family member, might signal in a different way. Activation of this RTK results in erbB4 cleavage by TACE and presenilin, generating a soluble intracellular domain (E4ICD). These studies raised the possibility that E4ICD could translocate to the nucleus and directly impact transcription. We hypothesized that identification of E4ICD binding proteins would provide insights into the mechanisms and biological roles of this novel signaling pathway. For that we first activation by neuregulin-1, erbB4 associates with the adaptor protein TAB2 and the nuclear corepressor N-CoR in neural stem cells. Once erbB4 is cleaved by the pro tease, the E4ICD/TAB2/N-CoR complex translocates to the nucleus, where it binds to promoters of astrocytic genes, repressing their expression and thus preventing astrogenesis. Consistent with these observations, erbB4 knockout mice show precocious astrogenesis. Furthermore, this phenotype is rescued by reintroduction of a cleavable isoform of erbB4 but not by a cleavage-resistant isoform. Our studies: (1) define a novel mechanism by which a RTK signals directly to the nucleus to regulate transcription and influence cell fate choices; (2) reveal a new role for presenilin in mammalian brain development (the inhibition of astrogenesis via E4ICD release); and (3) provide new insights into the mechanisms that control the timing of astrogenesis in the developing brain. Importantly, our findings might be relevant to other RTKs since other receptors undergo ligand-induced shedding of their extracellular domain, which can trigger presenilin cleavage. Furthermore, since presenilin plays a critical role in Alzheimer’s disease and erbB4, which is increased in neurodegenerative disease, regulates key aspects of neuronal function, it is possible that this signaling mechanism might be at play in this devastating neurodegenerative disease.

1273 Ontogeny of Muscarinic Receptor Expression in Zebrafish
R. J. Nuckels, J. A. Smith, D. M. García; Biology, Texas State University, San Marcos, TX
Muscarinic acetylcholine receptor (mACHR) activation initiates pigment granule movement in the RPE (retinal pigment epithelium) of fish. We are interested in discovering the pathways that link receptor activation to pigment granule movement. Since there are different downstream signaling pathways associated with the different subtypes of mACHR, we aim to identify which subtypes of receptors are present in the eyes of the zebrafish, Danio rerio. Using the publicly available zebrafish genome, we have identified putative sequences for the five known subtypes of muscarinic receptors (m1-m5) plus possible variants of the m3 and m5 receptors. Based on these sequences we have designed primers for the m-odd receptor subtypes (m1, m3, and m5) and cloned two of these (m3 and m5). We isolated tissue from larval and adult zebrafish and determined which m-odd muscarinic receptors were expressed. We removed the eyes from larval and adult zebrafish and we isolated spleen from the adult fish. Using RT-PCR, we found that the putative muscarinic receptor subtypes, m1, m3, and m5 and a variant of m5 were expressed in the larval eyes. We also have found that m3 and the variant of m5 are expressed in whole larval fish as early as 3 days post fertilization. Additionally, after isolating adult zebrafish spleen and eyes, we show that the m5 variant is expressed. These results support previous studies that show that pigment granule movement in the RPE of fish eyes use a muscarinic acetylcholine receptor mediated signaling pathway.

1274 The Importance of Tetraspan Vesicle Membrane Proteins for GABAergic Neurotransmission in Caenorhabditis elegans
C. Abraham, L. Bai, R. E. Leube; Department of Anatomy and Cell Biology, Johannes Gutenberg University, Mainz, Germany
Regulation of the intracellular trafficking of Glutamate receptors is likely to play an important role in synaptic plasticity. We have used the role of the TVPs we have chosen the model organism Caenorhabditis elegans that features only one member of the physisins (SPH-1), gynins (SGN-1) and secretory carrier-associated membrane proteins (SCAMPs, SCM-1), respectively. Previous analyses demonstrated that single, double and triple mutants of the encoding genes developed normally and presented an inconspicuous neuronal architecture with ordered synaptic contacts. These animals also exhibited normal thermotactic, chemotactic, electrophysiological and aldicarb responses (Abraham et al., 2006). We now present evidence, however, that TVP mutants show increased sensitivity to the GABA-receptor antagonist pentylenetetrazole (PTZ) reacting with pronounced muscle spasms. This paper describes two genes, unc-101 and UNC-108/Rab-2, whose mutations alter the distribution of GLR-1. Here I will describe two of these genes. The unc-101 gene encodes an ortholog of mammalian Rab-2. Loss of function alleles of both genes were identified in mutations causing increased abundance of GLR-1 in the ventral nerve cord. AP-1 has been implicated in TGN-Plasma membrane, and Early Endosome-TGN transport pathways. Rab-2 has been implicated in retrograde trafficking of proteins from the golgi to the ER. Here I will discuss our efforts to determine the mechanism by which UNC-101/1 and UNC-108/Rab-2 regulate GLR-1 trafficking.
Anti-Inflammatory Cytokines as Mediators in the Pathogenesis of Endothelial Shock

M. E. Cresencio, R. López Marure; Fisiología, Instituto Nacional de Cardiología, México D. F., Mexico

Introduction:
M. Roomi, V. Ivanov, A. Niedzwiecki, M. Rath; Dr. Rath Research Institute, Santa Clara, CA

RESULTS

Taken together these results suggest that the major pathway of ATP-induced [Ca^{2+}]i increase in prostatic smooth muscle cells is a Ca^{2+} influx via P2X receptors.

Effect of ATP on Calcium Dynamics in Prostatic Smooth Muscle Cell of Golden Hamsters

M. Matsuura, T. Kuroda, Y. Sato; Histology, Iwate Medical University, Morioka, Japan

OBJECTIVE: We have reported the heterogeneous responses of vascular smooth muscle cells to neurotransmitters. All contractile cells but striated smooth muscle cells are designated as “smooth muscle cells”, therefore it is conceivable that non-vascular “smooth muscle cells” show different nature to transmitters. It is well known that ATP is released from adrenergic nerve terminals. Here, we study the effect of ATP in prostate smooth muscle cell of golden hamsters using an imaging technique. [METHODS] We used male Golden hamsters (120-140 g). Prostatic gland endpieces which were surrounded by smooth muscle cells were isolated and soaked in Hepes-buffered Ringer’s Solution (HR) (pH 7.4). Connective tissues around the endpieces were digested by purified collagenase (100 units/ml) for 2 hours at room temperature. Then the glandular endpieces, kept structural integrities, were loaded by Indo-1/AM (5 µM) for 2 hours at room temperature. They were placed on cover slides in chambers which were coated with Cell-Tak® 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 [Ca^{2+}]i in the smooth muscle cells was observed. The response was abolished when extracellular Ca^{2+} was removed from perfused HR. The calcium wave was observed and it was moved to next cells one after another. It looks like having a polarization.

Conclusions: In present study, we therefore suggest that the major pathway of ATP-induced [Ca^{2+}]i increase in prosthetic smooth muscle cells is a Ca^{2+} influx via P2X receptors.

The expression of these receptors is a key event in determining the pro-invasive activity in melanocytes. In studies involving melanoma cell lines, we have found that ATP and its analogs modulate the expression of adhesion molecules, leading to changes in cellular morphology and migration. These findings suggest a potential role for ATP and its receptors in the regulation of melanoma aggressiveness.

Comparison of Effects of Serotonin (5-HT) on Intracellular Calcium Dynamics between Rat Testicular and Cerebral Arterioles

M. Matsuura, T. Kuroda, T. Saino, Y. Satoh; Histology (Anatomy2), Iwate Medical University, Morioka, Japan

Comparison of Effects of Serotonin (5-HT) on Intracellular Calcium Dynamics between Rat Testicular and Cerebral Arterioles


Different Statins Inhibit the Proliferation and Induce Cell Death Like Necrosis of Cervical Cancer Cell Lines

T. M. H. involved in taste, smell and temperature sensing. Either
The proteins predicted from the expressed sequences have similarities to chemoreceptors in other organisms such as Dictyostelium, Caenorhabditis and specialized vertebrate sensory cells involved in taste, smell and temperature sensing. Either Tetrahymena have primitive receptors that can recognize these and other agonists or some of these compounds can act independently of specific receptors. We have cloned many of these genes and we have begun generating macromolecular knockout mutations to identify the specific function of each. Supported by NSF grant MCB-0445362 to T.M.H.
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IGF-1 and IGF-2 Mediated Altered Regulation of Ornithine Decarboxylase and Spermidine Spermine N\(^2\)-acetyltransferase Expression in H-ras Transformed Cells Involves Multiple Signaling Pathways
R. A. R. Hurts, M. D. Marchett; Biology, University of Prince Edward Island, Charlottetown, PE, Canada

The polyamines (putrescine, spermidine, spermine) are ubiquitous low-molecular weight aliphatic amines that play roles in both cell proliferation and differentiation. Polyamine biosynthesis is regulated by the activity of ornithine decarboxylase (ODC), a key rate limiting enzyme in the polyamine biosynthetic pathway. Conversely, spermidine/spermine \(N^2\)-acetyltransferase (SSAT) is a key rate limiting enzyme in the catabolism of polyamines. The insulin-like growth factor (IGF) system plays an important role in the growth and development of many tissues and regulates overall growth and has been implicated in tumorigenesis. The aim of this study was to investigate the possible link between IGF-mediated cellular signaling during H-ras mediated cellular transformation and the expression of ODC and SSAT. Using untransformed, parental 10T1/2 cells and H-ras transformed NR3 cells (capable of benign tumor formation) ODC and SSAT expression was evaluated in response to exposure to IGF-1 and IGF-2 in vitro. Results from Western blot analyses suggest that ODC and SSAT protein levels are differentially induced in NR3 cells in response to IGF-1 and IGF-2 following exposure for 1 to 24 hours, while no apparent alterations in either ODC or SSAT protein levels in response to IGF-1 and IGF-2 were noted in 10T1/2 cells. Further studies determined that involvement of protein kinase-C and mitogen activated protein kinase mediated signaling pathways are necessary for the IGF-mediated induction of ODC and SSAT in NR3 cells. The results suggest that a part of the altered growth regulatory program which results following H-ras mediated cellular transformation involves a heightened responsiveness of both ODC and SSAT to IGF-1 and IGF-2. (N.S.E.R.C. funded)

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Regulation of p53 Protein Synthesis Following DNA Damage by Phosphorylation of eIF4E
Y. Zhang, D. Yang; Department of Basic Biomedical Science, Sanford School of Medicine, University of South Dakota, Vermillion, SD

The eukaryotic translation initiation factor 4E (eIF4E) is essential for efficient cap-dependent translation. eIF4E is known to be regulated by its inhibitory binding proteins (4E-BPs) and by its phosphorylation. However, the role of eIF4E phosphorylation in translational control still remains controversial. The tumor suppressor protein p53 plays a critical role in suppressing cell transformation and maintaining genetic integrity. This activity is achieved by the accumulation of p53 protein after DNA damage and by p53-induced activation of a number of genes that mediate either cell growth arrest or cell death. Although it is known that p53 is stabilized after DNA damage, there is also clear evidence indicating that p53 induction is regulated by protein synthesis in response to DNA damage. The mechanism for the translational regulation of p53, however, is poorly understood. In this study, the function of eIF4E phosphorylation in translational control of p53 induction after DNA damage was investigated. Our results showed that etoposide treatment caused a rapid increase in eIF4E phosphorylation as well as increased p53 synthesis. The addition of a specific inhibitor of eIF4E kinase, 4Ei, not only inhibited eIF4E phosphorylation but also resulted in reduced synthesis of the p53 protein. RT-PCR analysis further demonstrated that the inhibition of eIF4E phosphorylation was accompanied by a decrease in the abundance of p53 mRNA in the polyribosomal fraction following etoposide treatment. In addition, the attenuation of p53 translation by the suppression of eIF4E phosphorylation was found to promote cell-cycle progression from G1 to S phase. This study provides the first evidence indicating that phosphorylation of eIF4E by 4Ei is critical for increased p53 protein synthesis in response to DNA damage.

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Anti-angiogenic Activity of Processed Rhus verniciflua STOKES
W. Choi, J. Lee, Y. Chang, E. Lee, C. Lee, K. Ahn, S. Kim; 1Department of Oncology, Graduate School of East-West Medical Science, Kyung Hee University, Yongin, Republic of Korea, 2Lab of Angiogenesis and Chemoprevention, College of Oriental Medicine, Kyung Hee University, Seoul, Republic of Korea

Rhus verniciflua STOKES (R. verniciflua) is a traditional medicinal plant that is used for the treatment of inflammatory disease and uterine cancer in Oriental folk medicine. However, anti-cancer mechanisms of R. verniciflua STOKES still remain unclear. Thus, in the present study, the anti-cancer effects of R. verniciflua STOKES with focus on angiogenesis were evaluated in vitro and in vivo. R. verniciflua STOKES inhibited vascular endothelial growth factor (VEGF)-induced proliferation and migration of human umbilical vein endothelial cells (HUVECs) in a concentration-dependent manner. Also, R. verniciflua STOKES inhibited tumor growth and tumor weight in LLC-bearing C57BL6 mice. These results demonstrated that R. verniciflua STOKES showed anti-cancer effect through inhibition of angiogenesis and suggest that it may be valuable to develop anti-cancer drugs.

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Rutin Isolated from the Actinometa lobatum Maxim Exerts Antithrombotic Activity
K. Kim, J. Lee, Y. Chang, H. Lee, E. Lee, B. Shim; 1Department of Oncology, Graduate School of East-West Medical Science, Kyung Hee University, Yongin, Republic of Korea, 2Lab of Angiogenesis and Chemoprevention, College of Oriental Medicine, Kyung Hee University, Seoul, Republic of Korea

Actinometa lobatum Maxim has been utilized for the treatment of thrombosis and ulcerative skin disease. However, its antithrombotic effect remains unclear. Thus, in the present study, the antithrombotic mechanism was investigated in human platelets by rutin isolated from the Actinometa lobatum Maxim. In platelet aggregation assay, rutin significantly inhibited collagen-induced human platelet aggregation at 400 µM. In addition, rutin inhibited the expression of glycoprotein (GP) IIb/IIIa and (thromboxane A2)TXA2 formation in collagen-induced human platelet aggregation. In coagulation assay, rutin significantly prolonged activated PT and aPTT compared with untreated control in a concentration dependent manner. Furthermore, oral administration of rutin resulted in an inhibition of experimental pulmonary embolism in ICR mice. Taken together, these results suggest that rutin may exert its antithrombotic activity via inhibition of platelet aggregation, coagulation activity, GP IIb/IIIa complex expression and TXA2 activities.

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DMNQ S64 Induces Apoptosis via ROS Activation and NF\(\beta\)B Inhibition in A549, Human Non Small Lung Cancer Cells
Y. Rhee, M. Park, H. Lee, E. Lee, K. Lee, B. Shim; 1Department of Oncology, Graduate School of East-West Medical Science, Kyung Hee University, Yongin, Republic of Korea, 2Lab of Angiogenesis and Chemoprevention, College of Oriental Medicine, Kyung Hee University, Seoul, Republic of Korea

6-ppim (1-propoxyiminoalkyl)-5,8-dimethoxyoxy 1,4-naphtoquinone S-64 (DMNQ S64) was synthesized as a shikonin derivative. In the present study, the underlying apoptotic mechanism of DMNQ S64 was examined. DMNQ S64 exerted cytotoxicity against A549 lung carcinoma cells with IC50 of 25 µM. Apoptotic bodies were observed in DMNQ S64 treated A549 cells by DAPI staining assay and caspase-3 activity was observed by immunofluorescence staining. DMNQ S64 also increased ROS portion in a time dependent manner by flow cytometric analysis. Western blotting revealed that DMNQ S64 effectively reduced the expression of mitogen activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK), cleaved poly(ADP-ribose) polymerase (PARP) and activated caspase-8. Furthermore, Luciferase assay has shown DMNQ S64 significantly inhibits NF\(\beta\)B and the phosphorylation of SRE. Taken together, these results suggest that DMNQ S64 may induce apoptosis via ROS activation and inhibition of NF\(\beta\)B and phopho-p38.

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Anti-angiogenic Activity of Sojuktang in Vascular Endothelial Growth Factor Induced Human Umbilical Vein Endothelial Cells
Y. Oh, Y. Rhee, J. Lee, K. Kim, H. Lee, E. Lee, K. Ahn, S. Kim; 1Department of Oncology, Graduate School of East-West Medical Science, Kyung Hee University, Yongin, Republic of Korea, 2Lab of Angiogenesis and Chemoprevention, College of Oriental Medicine, Kyung Hee University, Seoul, Republic of Korea

Angiogenesis, a formation of new blood vessels from pre-existing ones, has been shown to play an important role both in animal development and pathologic conditions like tumor growth and metastasis or cardiovascular disease. In present study, the anti-angiogenic activity of ethanol extract of Sojuktang (EST), an oriental herbal formula, was evaluated in vascular endothelial growth factor (VEGF)-stimulated human umbilical vein endothelial cells (HUEVC). EST significantly inhibited VEGF induced proliferation of HUEVC at non-toxic concentration of 5 µg/ml. EST also has shown inhibitory effects on the migration and tube formation of HUEVCs stimulated by VEGF in a concentration dependent manner. Similarly, VEGF induced neovascularization was disrupted by EST in chick chorioallantoic membrane (CAM) assay. In addition, EST significantly reduced the level of prostaglandin E2 in VEGF treated HUEVCs by ELISA assay. These results suggest that EST can be applied to angiogenesis dependent diseases including cancer.
3,3'-dihydroxybenzene (DIM) Induces Apoptosis in Prostate Cancer Cells

S. Park,1 E. Kim,2 H. Shin,3 D. Kwon,1 Y. Surh,1 J. Park2,1 Food Science and Nutrition, Hallym University, Chuncheon, Republic of Korea, 2Center for Efficacy Assessment and Development of Functional Foods and Drugs, Hallym University, Chuncheon, Republic of Korea, 3Korea Food Research Institute, Sungnam, Republic of Korea, 4College of Pharmacy, Seoul National University, Seoul, Republic of Korea

DIM is a major in vivo product of acid-catalyzed oligomerization of indole-3-carbinol, which is present in cruciferous vegetables and has been reported to have anti-carcinogenic properties. The present study examined the mechanisms by which DIM inhibits human prostate cancer cells. To examine the effects of DIM on the growth of LNCaP (wild-type p53) and DU145 (mutant p53) human prostate cancer cells, the cells were cultured with various concentrations of (0 - 30 μM) DIM. DIM substantially decreased viable cell numbers and induced apoptosis of LNCaP and DU145 cells in dose-dependent manners. Western blot analysis of total cell lysates revealed that DIM increased cleavage of caspase-8, -9, -7, -3, and poly (ADP-ribose) polymerase in both LNCaP and DU145 cells. In addition, DIM decreased the translocation of cytochrome c and Smac/Diablo from the mitochondria to the cytosol. In LNCaP cells, DIM did not affect the protein levels of p53, Bcl-2, or Bax, whereas DIM increased Bax and truncated Bid levels in DU145 cells. It has been demonstrated that DIM inhibits cell proliferation and induces apoptosis in prostate cancer cells. The induction of apoptosis may be mediated through changes in mitochondrial membrane permeability and the activation of the caspase pathways.

The Proteome of the Endoplasmic Reticulum in Cancer

E. Abdou,1 M. Taheri,1 L. Roy,2 G. Thiubault,1 D. Boismenu,3 J. Hayes,4 F. Servant,5 R. Kearney,5 J. Paiement; 1Department of Pathology and Cell Biology, University of Montreal, Montreal, PQ, Canada, 2Joint Research Center, National Sun Yat-Sen University-Kaohsiung Medical University, Kaohsiung, Taiwan, 3Biomedical Engineering, McGill University, Montreal, PQ, Canada, 4Biomedical Research Centre, University of Dundee, Dundee, United Kingdom, 5Biomedical Engineering, McGill University, Montreal, PQ, Canada

The protein composition of endoplasmic reticulum (ER) isolated from dissected liver tumor nodules of fatoxacin Bt-treated rats was compared with that of ER from control liver. Tandem mass spectrometry (MS) peptide counts and immunoblot validation were used to identify and determine the relative expression level of the proteins in highly enriched ER fractions. LC-MS/MS tandem MS was carried out on protein samples obtained after separation by 2D gel electrophoresis, peptides were quantified and a variety of antibodies have been used against specific proteins of relevance to the ER proteome and cancer. Many proteins relevant to cancer were up-regulated in ER from dissected liver tumor nodules. These include inhibitors of apoptosis (i.e. paxidoxin 1, Bax inhibitor 1, RNAse I, Inhibitor and Rack1), proteins involved in transcription (i.e. Y box protein 1, BTF3), in mRNA metabolism (i.e. HnrNP K, HnrNP D, PABP1, Hua) in translation (i.e. eIF-1A, eIF-2, eEF-2A, eEF-3), in ribosome biogenesis (i.e. EB1 and ribosomal proteins including S3a, S11, S14, L6, L23a) and in membrane traffic (COP12 customer complex). Several proteins of the cytochrome p450 family were down-regulated whereas a number of alkido-ketoreductases were up-regulated in ER from tumors. In contrast proteins of the protein folding machinery (i.e. Bip, calnexin, calreticulin, endoplasmin) were expressed in equal amounts in ER from dissected tumor nodules compared to those in control ER. Many novel proteins of relevance to the ER proteome and cancer were uncovered, several of these were observed in higher concentration in ER membranes from tumors. Finally immunoblot analysis using anti-phosphotyrosine antibodies revealed higher amounts of a number of phosphoproteins in ER from dissected liver tumor nodules. Thus the molecular profile of the ER in hepatocellular carcinoma is different from ER in control liver. This difference may be relevant to liver tumor biology.
Effect of GSK3 Phosphorylation on HIF-1α Stability and Transcriptional Activity

S. A. Hale, K. Lounsbury; Pharmacology, University of Vermont, Burlington, VT

Cancerous tumors adjust their signaling pathways to facilitate survival in a hypoxic environment. The pro-angiogenic transcription factor hypoxia inducible factor α (HIF-1α) is stabilized in hypoxia and promotes tumor cell survival, proliferation, and metastasis by inducing transcription of many targets including vascular endothelial growth factor (VEGF). Previous work in our lab has shown increases in both HIF-1α and VEGF in advanced stages of ovarian cancer, indicating a strong correlation between HIF-1α, VEGF, and cancerous states. Despite current research describing HIF-1α signaling and regulation, there remains a dearth of information investigating HIF-1α phosphorylation status and regulation of phosphorylation. Current work in our laboratory involves connecting the role of phosphorylation states of HIF-1α to the regulation of HIF-1α transcriptional activity and proteasomal degradation. GSK3 (glycogen synthase kinase) has been implicated in the phosphorylation of HIF-1α although the downstream effect of GSK3 on HIF-1α has yet to be clarified. Considering the canonical role of GSK3 in proteasomal degradation of other oncoproteins such as c-myc, β-catenin, and cyclin D1, and the presence of GSK3 consensus sequences within HIF-1α, we hypothesized that phosphorylation by GSK3 would target HIF-1α for proteasomal degradation as well. In support of this hypothesis, we show here that concurrent treatment of ovarian cancer cells with BIO (a GSK3 inhibitor) and hypoxia leads to an increase in stabilization of HIF-1α that is echoed by an increase in VEGF transcription, suggesting an augmentation of HIF-1α transcriptional activity. Further, in vitro studies show that after phosphatase treatment the HIF-1α protein exhibits an increase in electrophoretic mobility that is partially reversed by treatment with GSK3. From these results, we propose a model whereby GSK3 phosphorylates and subsequently destabilizes HIF-1α thus halting HIF-1α-dependent VEGF transcription.

Determination of the Anti-Cancer Activity of Fluorescent Cyanine Dye Constructs in MCF-7 Cell Lines

M. W. Baker, 1 D. A. Hill, 1 C. Ntam, 1 N. Flemming, 2 N. Smith, 2 A. Winstead, 2 D. Curry, 2 R. Williams, 2 L. Jones 2; 1Biology, Morgan State University, Baltimore, MD, 2Chemistry, Morgan State University, Baltimore, MD

Breast cancer is a persistent and invasive disease. In 2004-2005, the CDC reported that approximately 225,990 women received their first positive breast cancer diagnosis, and approximately 42,850 women died from the disease. Breast cancer exists in various forms including ductal carcinoma in situ (DCIS, non-invasive), invasive ductal carcinoma (IDC, metastatic), or lobular carcinoma (invasive or non-invasive). Several forms exhibited resistance to conventional chemotherapeutics. This resistance can lead to increased breast cancer-related mortality. Thus, oncologists and biomedical researchers are interested in newly developed applications or technologies that can indicate and treat this insidious disease. Collaborative studies between our laboratory and the Chemistry Department at Morgan State University have resulted in the manipulation of fluorescent technology to develop several classes of fluorescent cyanine dye-based microsensor constructs (CDBMC). These constructs are designed to indicate intracellular alterations using fluorescent intensity. The “Hydroxyl” class of CDBMC demonstrated the ability to undergo intracellular uptake, correlate fluorescent intensity with cytotoxicity and induce cellular lethality. Thus, the current study was designed to determine if the “Hydroxyl” class of CDBMC could undergo intracellular uptake, correlate fluorescent intensity with cytotoxicity, and induce cytotoxicity within the MCF7 breast cancer cell line. MCF7 cells were incubated with CDBMC (20 μM) for 6 hours. After this incubation, MCF7 cells were collected and analyzed for cytotoxicity. Cell growth and proliferation, enzyme release, viability and cell attachment were used as markers of cytotoxicity. The results demonstrated that CDBMC caused increased enzyme release and decreased cell viability, attachment, growth and proliferation (Supported by DOE ER63580, NSF 0236753).

Von Hippel-Lindau Protein Induced Variation of Microtubule Dynamic Instability Probed by Automated GFP-labeled EB3 Spot Detection and Tracking

A. V. Matov, 1 C. Thoma, 2 W. Krek, 2 G. Danuser 1; 1Cell Biology, The Scripps Research Institute, La Jolla, CA, 2Cell Biology, Swiss Federal Institute of Technology, Zurich, Switzerland

Von Hippel-Lindau (VHL) disease is an autosomal-dominant inherited cancer syndrome. Affected individuals develop tumors in the kidney, pancreas, adrenal gland, retina and brain. Most effects have been associated with pVHL function in an E3-ligase complex targeting hypoxia-inducible factor α (HIFα) for degradation under normoxic conditions and thereby blocking the HIFα-mediated hypoxic transcriptional program. Hergovich et al., 2003, reported that pVHL may also have a stabilizing effect on microtubule (MT) dynamic instability and this may be implicated in deregulating cell migration during invasion and metastasis. To study pVHL functions in MT dynamics regulation we analyzed the movement of GFP-labeled EB3 plus-end binding protein using multi-particle tracking of EB3 spots. EB3 spot tracking measures MT assembly, revealing statistics of growth velocity and catastrophe frequency. However, we found that many of the EB3 spot trajectories are collinear, suggesting that they may belong to the same MTs sampled at different time points. These MTs lose EB3 decoration during pause and shrinkage, rendering them temporarily invisible. To test this hypothesis, we are investigating the possibility of computationally grouping collinear tracks to estimate MT shrinkage velocity and rescue frequency. Tracking of MT plus ends reveals a bimodal distribution of growth speeds in VHL-deficient cancer cell lines (786-O). Specifically, a large population of MTs grows at a slow speed with a narrow distribution, but many MTs exhibit growth at higher rates with a broader variation. Upon reconstitution of pVHL, MT growth becomes restricted to only the slower of the two modes. This initial data supports the notion that the presence of pVHL stabilizes MTs by oppressing a secondary hyperactive dynamic mode. Currently we are developing a new cell model to confirm these results. We will knock down VHL protein expression by miR30-based short-hairpin RNA (shRNA/mir) in primary cell lines and quantify the respective change in MT dynamics.

Cortactin Regulates Invadopodia Activity and Tumor Invasion by Head and Neck Squamous Cell Carcinomas

E. S. Clark, 1 A. Whigham, 2 W. Yarbrough, 3 A. Weaver 4; 1Pathology, Vanderbilt University, Nashville, TN, 2Otolaryngology, Vanderbilt University, Nashville, TN, 3Otolaryngology and Cancer Biology, Vanderbilt University, Nashville, TN, 4Otolaryngology and Cancer Biology, Vanderbilt University, Nashville, TN

Invadopodia are actin-rich processes that regulate extracellular matrix degradation and thought to be involved in cancer cell invasion and metastasis. Cortactin is a critical component of invadopodia that links many potential processes in invadopodia, including actin assembly, signaling, and membrane trafficking. Cortactin is also gene-amplified in 30% of head and neck squamous carcinomas, which has been reported to confer poor prognosis. We examined the role of cortactin in both invadopodia formation and tumor invasion by HNSCC cells in a novel xenograft rat tracheal model. We found that overexpression of cortactin increased invasion through Matrigel transwells and extracellular matrix degradation mediated by invadopodia. Interestingly, cortactin expression correlated more highly with ECM degradation than with the formation of actin-rich invadopodia puncta, suggesting that cortactin is not required for Arp2/3 complex activation at these structures but instead acts at a subsequent step. In vivo, cortactin overexpression increased invasiveness of tumors derived from these cell lines. This study links the function of cortactin in invadopodia to invasion at the tumor scale, providing a potential mechanism for aggressiveness of cortactin-overexpressing HNSCC cancers.

Proteomic Analysis and Identification of Early Stage Liver Cancer Markers

A. Menthner, 1 L. Siconolfi-Bacis, 2 J. Lim, 1 R. H. Angellett, 1 P. M. Nowakoff 1; 1Department of Developmental and Molecular Biology, Albert Einstein College of Medicine, Bronx, NY, 2Department of Pathology, Albert Einstein College of Medicine, Bronx, NY

Liver cancer in humans is diagnosed at late stages. Proteomics was used to identify early stage liver cancer markers in a rat liver cancer model. The rat model (Solt et al., Am J Path, 1977) exhibits stage-specific cancer progression analogous to human liver. Unique protein expression patterns in liver tissue and serum at early stages of cancer were analyzed and compared to normal liver and serum. Serum was analyzed to provide markers for early cancer detection in a comparatively non-invasive procedure. Serum was depleted and labeled with isotopic tags (iTRAQ), followed by chromatographic separation to permit detection of low abundance proteins. Identification and quantification of proteins was performed by tandem mass spectrometry. Tissue sample markers provide clues about the mechanism of progression of cancer. Proteins were isolated from frozen sections of liver tissue. Cancerous tissue contains both precancerous/cancerous nodules and surrounding normal liver. Tissue sample protein quantitation was determined using a micro-BCA assay (Bicinchoninic acid), developed in house, which consumes 1 to 2 μl of the sample. Quantitative differences in protein expression were assessed using the difference-in-gel electrophoresis (DIGE) technology (GE Amersham). Potential proteins markers are being confirmed by fluorescent immunohistochemistry. Proteomic analysis using 2-dimensional gels and MS (mass spectrometric) data revealed differences in liver tissue and serum between early stage cancer versus control. Proteomics is a powerful tool with the potential to identify unique protein patterns associated with early stages of liver cancer in a rat model analogous to human liver cancer. Supported in part by NIH Grant CA101150.

Investigation of Nuclear Import and Export Signals within Parafibromin, the Tumor Suppressor Associated with Parathyroid Tumorigenesis

M. A. Hahn, D. J. Marsh; Cancer Genetics, Kolling Institute of Medical Research, Royal North Shore Hospital and Department of Molecular Medicine, University of Sydney, NSW, Australia
Parafibromin is a putative tumor suppressor encoded by HRPT2, mutations in which have been implicated in familial Hyperparathyroidism-Jaw Tumor Syndrome and sporadic parathyroid carcinoma. The precise function of parafibromin and how its aberration leads to parathyroid tumorigenesis is poorly understood. In an initial study in which we investigated the cellular localization of parafibromin, wild type or various mutants of this protein were fused to an enhanced green fluorescent protein (EGFP) tag and used to identify a functional-bipartite, evolutionarily conserved nuclear localization signal (NLS) at residues 125-139. The aim of the studies presented here is to extend this work by assessing the nuclear import or export activity of three other putative NLS and three nuclear export signals (NES) that we have identified within parafibromin. Localization data obtained from HEK293 cells transiently transfected with each NLS fused to EGFP, or full length paraffin in which each NLS had been mutated fused to EGFP, revealed that all four NLS possessed nuclear import activity. However the NLS at residues 125-139 was clearly the primary determinant of parafibromin nuclear localization. Each NES sequence when fused to EGFP and transiently transfected into HEK293 cells localized EGFP primarily to the cytoplasm suggesting that each of these signals possessed nuclear export activity. However when these signals were mutated in fragments of parafibromin fused to EGFP they increased cytoplasmic localization of EGFP without decreasing the NLS signal that was expected if these signals were in fact nuclear export. In summary, despite the presence within parafibromin of four NLS and three NES sequences that are capable of nuclear import or export when each is fused to EGFP, the nuclear localization of parafibromin is mediated primarily by one NLS. Studies designed to examine potential roles for the other NLSs and the functional significance of abrogation of these signals are currently underway.

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Integrin-linked Kinase Controls Notch1 Signaling by Down-regulation of Protein Stability
S. Han, J. Kim, J. Mun, M. Kim, J. So, S. Mi-Sun, M. Kim, S. Lee, J. Park, E. Choi, J. Seong, C. Cho, R. Fassler, H. Park; SBST, Chonnam National University, Gwangju, Republic of Korea, 2Department of Dermatology, Chonnam National University, Gwangju, Republic of Korea, 3Department of Biochemistry, Kyungpook National University, Taegu, Republic of Korea, 4National Creative Research Initiative Center for Cell Death, School of Life Science and Biotechnology, Konkuk University, Seoul, Republic of Korea, 5Laboratory of G Protein Coupled Receptors, Graduate School of Medicine, Konkuk University, Seoul, Republic of Korea, 6Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Taejon, Republic of Korea, 7Department of Molecular Medicine, Max Planck Institute of Biochemistry, Martinsried, Germany

Integrin-linked kinase (ILK) is a scaffold and protein kinase that acts as a pivotal effector in integrin signaling for various cellular functions. GSK-3β, a downstream kinase of ILK, has already been reported as a positive regulator of Notch1 signaling. Accordingly, we hypothesized the existence of signal crosstalk between integrin and Notch1 signaling, and evaluated this crosstalk by means of ILK. ILK markedly reduced the protein stability of Notch1 and suppressed Notch1 binding to RBP-JK. The kinase activity of ILK was essential for the inhibition of Notch1 signaling, but Akt and GSK-3β, the downstream effectors of ILK, were not involved in the negative regulation of Notch1 by ILK. Notably, the protein level and transcriptional activity of endogenous Notch1 was higher in the ILK null cells than in ILK wild-type cells, and the endogenous Notch1 was increased by the blocking of the proteasome representing the enhancement of the proteasomal degradation of Notch1 by ILK. Notch1 was ubiquitinated prior to proteasomal degradation, and we investigated the involvement of Fbw7, an E3 ligase for the ubiquitination of Notch1, and the ubiquitination of Fbw7 was stimulated in the presence of ILK. Furthermore, we found that down-regulation of Notch1C and up-regulation of ILK in basal cell carcinoma (BCC) and melanoma patients, but not in squamous cell carcinoma (SCC). These results suggest that ILK down-regulates the protein stability of Notch1 through the ubiquitin-proteasome pathway by means of Fbw7.

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Tip60 Histone Acetyltransferase Acts as a Negative Regulator of Notch1 Signaling by Means of Acetylation
M. Kim, S. Han, J. Kim, S. Kim, J. Mo, J. So, H. Park; SBST, Chonnam National University, Gwangju, Republic of Korea

The Notch signaling pathway appears to perform an important function in the determination of cell fate, as well as differentiation, in a wide variety of organisms and cell types. In our present study, we provide evidence that UV irradiation-induced Tip60 histone acetyltransferase (HAT) proteins reduced Notch1 activity to a marked degree. Accumulated UV irradiation-induced Tip60 suppresses Notch1 transcriptional activity via the dissociation of the Notch1-IC-RBP-JK complex. In vivo binding between endogenous Tip60 and Notch1-IC in HEK293 cells was verified in this study by coimmunoprecipitation. Interestingly, the physical interaction of Tip60 with Notch1-IC occurs to a more profound degree in the presence of RBP-JK, but does not exist in a trimeric complex. Using Notch1-IC and Tip60 deletion mutants, we also determined that the N-term, which harbors six asparagine repeats of Notch1-IC, interacts with the zinc-finger and Acetyl-CoA domain of Tip60. Furthermore, here we report that Notch1-IC is a direct target of the acetyltransferase activity of Tip60. Collectively, our data suggest that Tip60 is an inhibitor of the Notch1 signaling pathway, and Tip60-dependent acetylation of Notch1-IC may be relevant to the mechanism by which Tip60 suppresses Notch1 signaling.

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Identification of a Novel Oncogene, Cancer-upregulated Gene 2 (CUG2)
S. Lee, B. Cho, B. Lee, J. Jung, S. Koh; 1Department of Microbiology, Chungnam National University, Daejeon, Republic of Korea, 2LG Life Sciences, Ltd./R&D Park, Daejeon, Republic of Korea

We examined genome-wide differences in gene expression between tumor tissues and normal tissues in order to identify differentially regulated genes in tumors. Cancer-upregulated gene 2 (CUG2) was identified as an expressed sequence tag (EST) that exhibits significant differential expression in multiple human cancer types. CUG2 showed weak sequence homology with the down-regulator of transcription 1 (DRT1) gene, a human transcription repressor. We found that EGFP-CUG2 fusion proteins were predominantly localized in the nucleus, suggesting their putative role in gene regulation. In addition, CUG2-overexpressing mouse fibroblast cells exhibited distinct cancer-specific phenotypes in vitro. Taken together, these findings suggest that CUG2 is a novel tumor-associated gene that is commonly activated in various human cancers and exhibits high oncogenic activities.

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Oncogenic Property of PTK6 Depending on Subcellular Localization
H. Kim, L. Soo-Taeck; Dept. of Biochemistry, Yonsei University, Seoul, Republic of Korea

Protein-tyrosine kinase 6 (PTK6; also known as Brk) is an intracellular tyrosine kinase and is expressed in and around 60% of breast tumors. Altered localization of protein tyrosine kinases has been shown to play an important role in cancer progression. Here we have analyzed oncogenic property of PTK6 depending on its subcellular localization. To generate membrane-targeting and nuclear forms of PTK6, we made HEK 293 cell lines expressing either Myr-PTK6 containing a Src myristoylation signal or NES-PTK6 containing SV40 T-antigen nuclear localization sequence. The Myr-PTK6 was mainly localized in plasma membrane and showed enhanced abilities of PTK6 to promote proliferation and prevent apoptosis of HEK 293 cells, as well as to promote anchorage-independent colony formation. The NLS-PTK6 was localized in nucleus but lost these oncogenic abilities. In addition, a pattern of tyrosine-phosphorylated proteins in Myr-PTK6-expressing cells was quite different from that in NLS-PTK6-expressing cells, suggesting that PTK6 in each subcellular compartment associates with and phosphorylates a distinct set of proteins. Targeting of PTK6 to plasma membrane would be a valuable prognostic indicator for various tumor progressions and identification of substrates phosphorylated by the membrane-targeted PTK6 may provide potential targets for therapeutic intervention.

1303
Oxidative Modification of Caspase 9 Mediates Its Interaction with Apaf-1, Auto-cleavage, and Activation
J. Yang, Y. Zuo, J. Yi; Cell Biology, Shanghai Jiao Tong University School of Medicine, Shanghai, China

Caspase 9 must undergo proteolytic activation through binding with Apaf-1 in the cytochrome c/ATP-dependent pathway to exert apoptosis. Intracellular reactive oxygen species (ROS) and redox state were increasingly reported to regulate apoptosis and is closely associated with Caspase 9 activation, however the direct relationship of ROS and Caspase 9 activation has been unclear. The present study aimed to elucidate the role of ROS and possible mechanisms in Caspase 9 activation. The results firstly showed that in reductive cellular environment Caspase 9 activation initiated by various apoptotic stimulus staurosporin or TNFα was weakened, while in oxidative environment, Caspase 9 activation was facilitated. Therefore, in general, redox state regulated Caspase 9 activation. We then applied hydrogen peroxide (H2O2) to directly mimic redox alteration, finding that it allowed the auto-cleavage and activation of Caspase 9, and in the process the thiol of caspase 9 were oxidized. H2O2 also led to formation of the complex of Caspase 9 and Apaf-1, while DTT could separate the complex, suggesting that H2O2 resulted in oxidation of caspase 9 and formation of intermolecular disulfide bond between procaspase 9 and Apaf-1. To exclude possibility that H2O2 indirectly activated Caspase 9 through increased cysteine c, we reconstituted an in vitro mitochondria deplention system. The result showed that thiol-specific oxidant dimide could induce Caspase 9 activation, demonstrating that the oxidized procaspase 9 processing and activation maybe independent of cysteine c increase induced by H2O2. Finally in an in vitro Caspase 9 and Apaf-1 recombinant system, diamide caused disulfide-mediated interaction with Apaf-1, auto-cleavage and activation of Caspase 9. Taken together, caspase 9 could be oxidized by certain amount of ROS, and thus oxidative modification of procaspase 9 mediated interaction with Apaf-1, auto-cleavage and activation of procaspase 9. This finding explicates the mechanism by which oxidative stress initiates cell apoptosis.
Mitochondrial Manganese Superoxide Dismutase Protects Ovarian Cancer Cells from Oxidative Stress-induced Apoptosis

K. Wong, B. Yeung, A. Wong; Zoology, The University of Hong Kong, Hong Kong

The manganese superoxide dismutase (MnSOD), located in the mitochondria, is a major antioxidant enzyme that plays an important role in protecting cells from oxidative damage. MnSOD has been suggested to have tumor suppressor function in many cancer types. Surprisingly, the levels of MnSOD in ovarian carcinomas were found elevated compared with normal ovarian epithelium. In this study, we aimed to investigate the levels of MnSOD protein in ovarian cancer cell lines and ovarian surface epithelial (OSE) cells and a possible link between MnSOD expression and resistance to apoptosis. We showed that MnSOD protein was abundant in most ovarian cancer cell lines but was at very low levels in OSE. MnSOD overexpression in ovarian cancer cells caused a ~50% decrease of cell proliferation and an increase of apoptosis, whereas targeted inhibition of endogenous MnSOD using small interfering RNA promoted growth of these cells, confirming the effect was MnSOD specific. Furthermore, stimulation of mitochondrial superoxide (O₂⁻) production induced an increase of MnSOD expression, suggesting that MnSOD may alleviate the reactive oxygen species stress in these cells. Our data also showed that MnSOD overexpression protected ovarian cancer cells from apoptosis induced by treatment with rotenone, hydrogen peroxide, or hypoxia-mimicking agents cobalt chloride and deferoxamine compared with the parental andneo control cell lines. Together, these data suggest upregulation of MnSOD in ovarian cancer cells is one of the mechanisms which may increase resistance to oxidative stress and apoptosis in cancer cells.

Gapex-5, a Rab31 Guanine Nucleotide Exchange Factor That Regulates Glut4 Trafficking in Adipocytes

I. J. Lodhi, S. H. Chiang, L. Chang, D. Vollenweider, T. T. Watson, M. Inoue, J. E. Pessin, A. R. Saltiel; Life Sciences Institute, University of Michigan, Ann Arbor, MI, Department of Pharmacological Sciences, Stony Brook University, Stony Brook, NY

Insulin stimulates glucose uptake into muscle and adipocytes by promoting translocation of the facilitative glucose transporter Glut4 from intracellular storage compartments to the plasma membrane. In the absence of insulin, Glut4 is retained intracellularly, although the mechanism underlying this process remains uncertain. Using the T1CD1-transducing protein CIPM as bait in a yeast two-hybrid screen, we cloned a RasGAP and VPS9 domain-containing protein, Gapex-5BME-6. The VPS9 domain is a guanine nucleotide exchange factor for Rab31, a Rab5 subfamily GTase implicated in trans-Golgi Network (TGN)-to-endosome trafficking. Overexpression of Rab31 blocks insulin-stimulated Glut4 translocation by retaining Glut4 vesicles inside the cell, whereas knockdown of Rab31 potentiates insulin-stimulated Glut4 translocation and glucose uptake. Gapex-5 is predominantly cytosolic in untreated cells; its overexpression promotes intracellular retention of Glut4 in adipocytes by maintaining Rab31 in an active state. Insulin recruits the CIP4-Gapex-5 complex to the plasma membrane, thus reducing Rab31 activity and permitting Glut4 vesicles to translocate to the cell surface. These data suggest that in the basal state, Glut4 is retained in cells by cycling between the TGN and endosomes due to the activity of Rab31, and escapes this futile cycle after insulin stimulates the translocation of Gapex-5 to the plasma membrane.

RNA Interference-mediated Allele-specific Silencing Rescues Sialic Acid Levels in the Dominant Disorder Sialuria

R. Klotowijk, P. Savelkoul, C. Ciccone, D. Krasnewich, W. Gahl, M. Huizing; NIHgRI, NIH, Bethesda, MD

Sialuria is a rare autosomal dominant disorder of the sialic acid biosynthesis. UDP-GlcNAc 2-epimerase/MannNAc kinase, encoded by the GNE gene. This results in loss of feedback inhibition of GNE by CMP-sialic acid, and overproduction of sialic acid. Sialuria patients manifest variable signs and symptoms, including mild hepatomegaly and developmental delay. Since dominantly inherited disease alleles are attractive therapeutic targets for allele-specific silencing mediated by RNA interference (RNAi), we employed this method in fibroblasts of sialuria patients. Smaller interfering RNA (siRNA) was designed to specifically target a sialuria GNE nonsense mutation (c.787G>T, R263L). This siRNA was transfected into patients’ fibroblasts and the extent of silencing was assessed after 48 hours. After silencing, allele-specific real-time PCR analysis demonstrated that expression of the mutant GNE transcript was decreased by 66 ± 3 (SD) % (n=3). Furthermore, HPLC analysis of fibroblast extracts showed that sialic acid levels decreased 59 ± 15 % (n=3) after silencing. Finally, GNE enzymatic activity measurements showed a 41 ± 6 % (n=3) recovery of feedback inhibition by CMP-sialic acid. These results demonstrate that RNAi can correct the underlying metabolic defect in vitro in a human inborn error of metabolism. Allele-specific RNAi therapeutics in sialuria provides an example for correcting dominant-negative mechanisms through elimination of specific transcripts.

Characterizing the Molecular Mechanism of the Antitherogenic Effect of Lycopene

C. J. Wu, C. Lia; Biological Science and Technology, China Medical University, Taichung, Taiwan

Lycopene has been shown to be associated with a decreased risk of chronic diseases such as atherosclerosis. However, the role of lycopene in preventing atherogenesis have not been clearly elucidated. The aim of this study was to investigate the effect of lycopene-containing diet on the lipid metabolism of hamster fed with a high cholesterol diet. The molecular mechanisms of hypolipidemic and antioxidant effects of lycopene were also elucidated in vitro. For an 8-week feeding period, 50 male Golden Syrian hamsters (7 week old) were randomly assigned to 5 diet groups, including control, 0.2% cholesterol, lupid, low (3%) and high (9%) dose of lycopene. Upon sacrifice, the serum was subjected to analysis of total cholesterol, LDL, HDL, and triglyceride. Our results indicated that high dose of lycopene significantly reduced the serum level of total cholesterol and LDL for 15% and 11%, respectively. HDL was increased for 22% as compared to the control. However, triglyceride level was not affected by lycopene. Regarding the antioxidative activity, the effect on LDL oxidation of lycopene was determined by relative thiorbarbituric acid-relative substances(TBARS) assay and diene conjugation. Our results showed that lycopene were able to decrease MDA production in TBARS assay and delayed the diene conjugation in the Cu²⁺-mediated oxidation of LDL. The protein levels of several antioxidant enzymes in liver, including catalase,superoxide dismutases and glutathione peroxidase were also determined. All three enzymes were found to be up-regulated significantly by lycopene as compared to the cholesterol group. Real-time PCR analysis showing the reduced level of LDL receptor mRNA in hepatocytes further supported the lipid-lowering effects of lycopene. Two dimension protein gel analysis with LC-MS/MS analysis indicated the close association of Lycopene and its cognate ATP10C protein in the insulin mediated clearance of glucose by the peripheral tissues.

Insulin Signaling Pathway in a Novel Mouse Model of Type 2 Diabetes

M. S. Dhur, H. E. Reaves, C. S. Sommardahl, D. K. Haines, J. S. Yuan; 1Large Animal Clinical Sciences, University of Tennessee, Knoxville, TN, 2Department of Pharmacological Sciences, Stony Brook University, Stony Brook, NY

RNA interference (RNAi) can correct the underlying metabolic defect in vitro in a human inborn error of metabolism. Allele-specific RNAi therapeutics in sialuria provides an example for correcting dominant-negative mechanisms through elimination of specific transcripts.

Insulin Signaling Pathway in a Novel Mouse Model of Type 2 Diabetes

M. S. Dhur, H. E. Reaves, C. S. Sommardahl, D. K. Haines, J. S. Yuan; 1Life Sciences Institute, University of Michigan, Ann Arbor, MI, 2Department of Pharmacological Sciences, Stony Brook University, Stony Brook, NY

The etiology of human obesity and insulin resistance is complex with both genetic and environmental factors playing a role. These polygenic diseases can be more easily studied in murine models and then translated into human homologs and phenotypes. Two independent lines of mice, heterozygous for the pink-eyed dilution (p) locus deletions represent a novel polygenic mouse model of type 2 diabetes. Apo10c, a putative aminophospholipid transporter, mapping to the p locus on mouse chromosome 7 is a strong candidate for the phenotype observed in these mice. This phenotype is diet-induced. Metabolic profiling to investigate obesity-related phenotypes showed that mutants heterozygous for Apo10c and inheriting the deletion maternally, exhibit a significantly higher body weight, adiposity index, plasma triglyceride and insulin concentrations. These mice are glucose intolerant and insulin resistant. Insulin-stimulated glucose transport is significantly (P<0.05) decreased in the peripheral tissues. There is a clear difference in the severity and onset of insulin resistance compared with the parental and neo control cell lines. Together, these data suggest upregulation of MnSOD in ovarian cancer cells is one of the mechanisms which may increase resistance to oxidative stress and apoptosis in cancer cells.
A Mutation in the DXXE ER-Exit Code of Kir6.2 Causes Congenital Hyperinsulinism by Impairing Sar1 Dependent ER Export

T. K. Tanja,1 J. Mankouri,2 S. Kamoun,1 A. Smith,3 S. Sjöblad,4 A. Carlson,2 S. Iverson,2 H. Christiansen,2 A. Sivapuradasanab,1 Institute of Membrane and Systems Biology, Faculty of Biological Sciences, University of Leeds, Leeds, West Yorkshire, United Kingdom, 2Department of Paediatrics, University Hospital of Lund, Lund, Sweden, 3Department of Paediatrics, Malmö University Hospital, Malmö, Sweden, 4Department of Paediatrics, Odense University Hospital, Odense, Denmark

The ATP-sensing potassium (KATP) channel of the pancreatic β-cell is an octameric complex composed of four subunits each of Kir6.2 (K inward rectifier) and sulfonylurea receptor 1 (SUR1). Kir6.2 is responsible for the K+ ion conductance and SUR1 for regulation of Kir6.2 function. KATP channels couple glucose metabolism to membrane potential and thus play a central role in glucose stimulated insulin secretion. Genetic mutations in the genes encoding the channel subunits can cause congenital hyperinsulinism (CHI). CHI mutations impair channel function or trafficking thereby resulting in persistent depolarisation of the β-cell membrane and unregulated insulin secretion and severe hypoglycaemia. Here we report the discovery of a new mutation, E282K, in Kir6.2 subunit in a patient diagnosed with CHI. We have examined the consequence of this mutation on channel trafficking using immunocytocchemistry and function using electrophysiology. In heterologous expression systems, this mutation prevented maturation of SUR1 and surface expression by retaining the channel in the ER. However, the mutant subunit was able to reach the cell surface and form functional channels when co-assembled with the wild-type subunit; this suggests that ER retention is due to mis-folding of the mutant subunit. Further mutagenesis studies suggested that E282K could be part of a di-acidic DXXE ER exit code, raising the possibility that KATP channels may assemble with Sec23/Sec24 proteins and exit the ER as COPII vesicles in a process that requires hydrolysis of GTP by Sarl-GTPase. Consistent with this possibility, a dominant negative form of Sarl-GTPase (one which is unable to hydrolyse GTP) prevented the ER exit of wild-type channels. We conclude that KATP channels use the DXXE motif as a code to exit the ER. Functional importance of this motif is highlighted by the severity of the disease (CHI) caused by a genetic mutation disrupting this code. Funded by the MRC-UK.

1310 Ubiquitinated-Protein Aggregates Form in Pancratic β-cells during Diabetes-induced Oxidative Stress and Are Regulated by Autophagy

N. A. Kami,1 M. Kiraly,1 A. Volchuk,3 M. Vranic,3 J. H. Brumell1; 1Cell Biology, Hospital for Sick Children, Toronto, ON, Canada, 2Department of Physiology, University of Toronto, Toronto, ON, Canada, 3Division of Cell and Molecular Biology, University Health Network, Toronto, ON, Canada

Previous studies indicate that diabetes-induced oxidative stress can lead to protein misfolding and degradation by the ubiquitin-proteasome system. In this study, we examined protein ubiquitination events in pancreatic sections from Zucker Diabetic Fatty rats. We observed large aggregates of ubiquitinated proteins (Ub-proteins) in insulin expressing β-cells and surrounding acinar cells. The formation of these aggregates was also observed in INS1 β-cells after exposure to high glucose, allowing us to further characterize this phenotype. Aggregate formation was maximal 24 h after high glucose exposure and was not affected by protein synthesis inhibitors. Oxidative stress was sufficient to induce Ub-protein aggregate formation and the addition of antioxidants resulted in a significant decrease in their formation during high glucose treatment. Clearance of the Ub-protein aggregates from the cytosol was observed during recovery in normal glucose media. Despite the fact that the 20S proteasome was localized to Ub-protein aggregates, epoxomicin treatment did not affect their clearance, indicating the proteasome does not degrade proteins localized to these structures. Surprisingly, the autophagy inhibitor 3MA blocked aggregate clearance during recovery and was sufficient to induce their formation in low glucose media. Together, these findings demonstrate that diabetes-induced oxidative stress induces ubiquitination and storage of long-lived proteins into cytoplasmic aggregates, Autophagy, not the proteasome, plays a key role in regulating their formation and degradation. To our knowledge, this is the first demonstration that autophagy acts as a defense to cellular damage incurred during diabetes.

1311 ABCA1-mediated Cholesterol Eflux Is Modulated by Ion-Channels

J. M. Karwatsky, S. Nandi, Y. Feng, X. Zha; Ottawa Health Research Institute, Ottawa, ON, Canada

ATP binding cassette (ABC) proteins are primarily membrane-associated proteins that transport a diverse array of substrates across membranes. ABCA1 is the molecular basis for Tangier disease due to functional mutations. The physiological consequence of this disease is extremely low concentrations of high density lipoprotein (HDL) in plasma. Normally, ABCA1 promotes the efflux of cellular cholesterol to extracellular acceptor, such as lipid-poor apolipoprotein A-I (apoA-I, A1), to form HDL. Cells with non-functional ABCA1 cannot efflux cholesterol to HDL, and consequently large quantities of cholesterol are stored within cells. Clinically, atherosclerosis is associated with macrophages that are overloaded with cholesterol, a condition that can lead to heart disease. The mechanism by which ABCA1 mediates cholesterol efflux is still poorly understood. Particularly, the precise molecular function of ABCA1 remains unknown. Given the fact that many ABC transporters function as or influence ion-channels, we hypothesized that ion channels may be active participants of cholesterol efflux. We therefore tested the effect of channel blockers in cholesterol efflux. We demonstrate that replacement of extracellular Ca2+ with channel blocking anions effectively inhibit cholesterol efflux. The relative degree of efflux inhibition was: SCN− > gluconate− > aspartate−. Moreover, efflux was inhibited by the ion-channel modulator DIDS. Finally, our results indicate that altering of intra- and extra-cellular Ca2+ also had a pronounced effect on cholesterol efflux. These findings demonstrate for the first time that ion movement and concentration directly modulate ABCA1-mediated cholesterol efflux.

1312 Cigarette Smoke Induces Steatosis (Fatty Liver Disease) through Stimulation of TNF Alpha

Y. Huan, M. Martins-Green; Cell Biology and Neurosciences, University of California, Riverside, Riverside, CA

The most common cause of chronic liver disease in many countries, nonalcoholic fatty liver disease (NAFLD), is closely related with the increasing prevalence of obesity, diabetes mellitus, and metabolic syndrome. The first hepatic pathological stage of NAFLD is steatosis, the accumulation of lipid in the liver tissue. Steatosis can further progress to liver fibrosis and cirrhosis. However, the mechanisms involved in these stages are not clear. Cigarette smoking is a major risk factor for cardiovascular disease as well as hypertension and hyperlipidemia, and has recently been linked to NAFLD. We hypothesized that cigarette smoke stimulates liver cells to produce proinflammatory cytokines that increase lipid production.

The pro-inflammatory cytokine tumor necrosis factor alpha (TNFα) stimulates inflammatory, apoptotic, and fibrogenic responses that contribute to the progression of NAFLD and levels of TNFα are elevated in patients with nonalcoholic steatohepatitis (NASH), a NAFLD stage between steatosis and cirrhosis. To test our hypothesis, we used cultured hepatocytes, exposed them to cigarette smoke and that the activation of SREBPs can be blocked by an antibody to TNFα, we are currently investigating the regulation of this process in hepatocytes exposed to “second-hand” cigarette smoke. Understanding the regulatory mechanisms of this process will help understanding of NAFLD, and may provide insight into diabetic conditions that are related to NAFLD.

1313 Reverse Signaling Initiated from GITRL Induces NF-kappaB Activation through P42/p44 MAPK in Macrophages

E. M. Bae,1 W. J. Kim,1 H. U. Bae,2 Y. M. Kang,1 J. E. Park,1 B. S. Kwon,3 H. W. Lee; 1Department of Genetic Engineering, School of Life Sciences and Biotechnology, Kyungpook National University, Daegu, Republic of Korea, 2Rheumatology Division, School of Medicine, Kyungpook National University, Daegu, Republic of Korea, 3Cardiology Division, Samsung Seoul Hospital, Sungkyunkwan University, Seoul, Republic of Korea

Glucocorticoid-induced TNFR family-related protein ligand (GITRL) is the eighteenth member of the tumor necrosis factor superfamily (TNFSF18) and is known to interact with its cognate receptor GITR (TNFRSF18). In order to investigate the potential role of GITRL in the pro-inflammatory activation of macrophages and the signaling pathway induced by GITRL, we stimulated THP-1 macrophage cell line and human primary macrophages with anti-GITRL monoclonal antibody or GITR:Fc fusion protein and analyzed cellular responses. Stimulation of GITRL induced expression of pro-inflammatoric cytokines and matrix metalloproteinase (MMP)-9 and up-regulated intracellular adhesion molecule-1 (ICAM-1) expression levels. Activation of these pro-inflammatory mediators required activation of NF-kappaB, which was demonstrated by complete blockade of MMP-9 activation by NF-kappaB inhibitors such as etyyl pyruvate, sulfasalazine, and N-tosyl-L-phenylalanylchloromethyl ketone. Immunofluorescence analysis of subcellular location of NF-kappaB revealed nuclear translocation of p50 subunit within 40 min after the activation. GITRL-induced macrophage activation also required transient activation of ERK, since ERK inhibitors, PD98059 and U0126, blocked MMP-9 activation and Western blot analysis revealed transient phosphorylation of ERK within 5 min after the stimulation. Presence of ERK inhibitors, nuclear translocation of NF-kappaB p50 subunit was blocked indicating that GITRL stimulation induces ERK activation which subsequently induced NF-kappaB activation. Furthermore, expression of GITRL and GITR was detected in macrophages in inflammatory disease specimens such as atherosclerotic plaques and synovial tissues of rheumatoid arthritis. These observations raise the possibility that GITRL mediated inflammatory activation of macrophages is involved in the pathogenesis of inflammatory diseases.

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Cardioprotective Effect of Onion (Allium cepa) through Inhibition of Oxidative Stress and Mitochondrial Death Pathway

D. Lee, J. C. P. Roos, S. Park, M. Kim, S. Lee, E. Bahk, C. Moon, E. Ko, S. Park, J. Jung; Department of Physiology, School of Medicine, Ajou University, Suwon, Republic of Korea.  

Our results suggest that psychosines dysregulate membrane concentrations (1-5 micromolar), but not ceramides, disrupt endocytosis: mis-trafficked membrane and ingested materials accumulate in a large vesicular compartment. This multivesicular body bears most lysosomal markers but has a neutral pH, is negative for acid phosphatase and positive for lysobisphosphatidic acid - consistent instead with late endosomes. The ultrastructure of γ finger two of PPAR γ, although both are expressed in a similar quantity. Compared to the WT, the mutant form has reduced capability to bind DNA. Mutation of a zinc-binding amino acid in zinc-γ resistance, dyslipidemia, and partial lipodystrophy (FPLD). In three subjects with FPLD, diabetes mellitus and hypertriglyceridemia, PPARG was screened for mutations by direct sequencing, adipocytes and other cellular regulatory processes. Mutations in the PPARG as well as in the LMNA gene, encoding the nuclear proteins lamin A/C, have been reported to cause insulin resistance, dyslipidemia, and partial piodoprosthesia (FPD). In three subjects with FPD, diabetess mellitus and hyperglycemia, PPAR γ was screened for mutations through direct sequencing after mutations in LMNA were excluded. 293T cells were transfected with a reporter gene plasmid and WT or mutant PPAR γ. Reporter gene activity was measured to determine if the PPAR γ mutant has decreased transcriptional activity. To assess whether WT and mutant protein are expressed in similar quantity, the protein was fixed with a FLAG-tag and detected by immunoblotting. Electrophoretic mobility shift assays were performed to investigate the capability of the mutant PPAR γ to bind DNA. We identified a PPAR γ mutation, C190S that causes PFPD and metabolic alterations. The mutation was not present in the unaffected family members or unaffected controls. The mutation is in the DNA binding domain, located within zinc finger two at the one of the zinc-binding amino acids. The C190S mutant had a significantly lower ability to activate a PPAR γ reporter gene than WT PPAR γ in absence and presence of rosiglitazone, although both are expressed in a similar quantity. Compared to the WT, the mutant form has reduced capability to bind DNA. Mutation of a zinc-binding amino acid in zinc-finger two of PPAR γ leads to an altered protein-DNA binding pattern, resulting in a partial loss of function, which in turn is associated with FPD. As distinct mutations in the LMNA gene also lead to PFPD, an interaction or common pathway between PPAR γ and lamin A/C has to be discussed.

Psychosines Mis-trafficked Endocytosed Cargo to a Pre-lysosomal Compartment: Relevance to the Pathogenesis of Gaucher Disease

J. C. P. Roos, S. Z. Wang, T. M. Cox; Department of Medicine, University of Cambridge, Cambridge, United Kingdom

In Gaucher’s disease, glucocerebrosidase deficiency leads to the accumulation of glucosylphosphoinositol (psychosine) and glucosylceramide. Multinucleate “Gaucher” cells form from macrophages and begin to store other lipids unrelated to the enzymatic defect. Psychosines have been reported to inhibit cytokinesis in cells expressing G-protein linked receptor 65 (GPR65). Signalling via GPR65 is also known to affect phosphorylation, calcium release and result in the formation of actin clots. Could psychosine-mediated signalling shed light on the formation of these multinucleated cells and account for the accumulation of lipids that are not substrates for glucocerebrosidase in Gaucher disease? Methods: We investigated the effects of psychosines and other lysophospholipids on human macrophages and monocyte-related cell lines expressing GPR65. Trafficking was evaluated by pulse-chase experiments using fluorescent lipids and markers of pH. Changes induced by lysophospholipids were visualized by electron microscopy and compared with Gaucher cells from affected spleen. Confocal fluorescence and electron microscopy were used to identify sub-cellular compartments. GPR65 expression in Gaucher spleen and other cells was confirmed by RT-PCR. Results: Psychosines stimulate sub-pathophysiological concentrations (1-5 micromolar), but not ceramides, disrupt endocytosis: mis-trafficked membrane and ingested materials accumulate in a large vesicular compartment. This multivesicular body bears most lysosomal markers but has a neutral pH, is negative for acid phosphatase and positive for lysobisphosphatidic acid - consistent instead with late endosomes. The ultrastructure of γ finger two of PPAR γ, although both are expressed in a similar quantity. Compared to the WT, the mutant form has reduced capability to bind DNA. Mutation of a zinc-binding amino acid in zinc-finger two of PPAR γ leads to an altered protein-DNA binding pattern, resulting in a partial loss of function, which in turn is associated with FPD. As distinct mutations in the LMNA gene also lead to PFPD, an interaction or common pathway between PPAR γ and lamin A/C has to be discussed.
There is a growing body of evidence suggesting that the declines seen with aging could be due to a decreased capacity of the ER to catalyze the posttranslational processing of newly synthesized proteins. In particular, we found in an autopsy series that low levels of ERp57 in the cerebral spinal fluid were invariably associated with brain atrophy. Remarkably, secretory and membrane protein co-aggregation with pre-existing PrP or Htt aggregates could be reduced by improving the efficiency of translocation into the ER. These findings suggest that the normally undetectable and minor population of mis-segregated secretory and membrane proteins can substantially modify the dynamics of cytosolic protein aggregates to affect traffic of constitutively cycling proteins like the transferrin receptor. These observations are consistent with the hypothesis that synapsin IIb regulates the formation and/or maintenance of synapses.

### Objectives

A. Ashok, R. S. Hegde; Cell Biology and Metabolism Branch, National Institutes of Health, Bethesda, MD

BACKGROUND: Aging is an unique characteristic of all multicellular eukaryotes. Numerous hypotheses have been proposed as the basis for these declines: such as accumulation of lethal mutations, oxidant injury, the "Hayflick" phenomenon and loss of telomerase activity. Two major problems with these models are that they are not all addressing the specific causes of aging, and the process of aging is not random. Our hypothesis is that aging is a result of the accumulation of cellular dysfunction, which in turn can be reduced by targeting specific components.

### Results

**O. Chakrabarti, N. S. Rane, R. S. Hegde; NICHD/CBMB, National Institutes of Health, Bethesda, MD**

Numerous neurodegenerative diseases are characterized by chronic accumulation of cytosolic protein aggregates initiated primarily by a single specific protein. This progressive aggregation of one protein is thought to dominate the functioning of various biochemical pathways and factors to ultimately cause cellular dysfunction and death. Conversely, attenuation of the cellular aggregate burden is often correlated with alleviation of cellular dysfunction. However, the parameters that influence the initial formation, persistence, or clearance of cytosolic aggregates remain largely unknown. To address this question, we have used a combination of multi-color live-cell imaging and biochemistry to simultaneously follow the fate of various cellular proteins in the presence and absence of cytosolic aggregates composed of either the mammalian Prion protein (PrP) or poly-glutamine containing Huntingtin (Htt). Surprisingly, pre-existing aggregates of PrP or Htt caused the progressive accumulation of cytosolic protein and membrane aggregates over the course of 4 days. This intracellular accumulation was not due to an inhibition of protein trafficking through the secretory pathway. Instead, pre-existing aggregates altered the fate of the normally small proportion of secretory and membrane proteins that failed to be segregated into the endoplasmic reticulum (ER). Rather than being degraded rapidly, non-translocated polypeptides co-associated with cytosolic aggregates to facilitate their growth and persistence. Remarkably, secretory and membrane protein co-aggregation with pre-existing PrP or Htt aggregates could be reduced by improving the efficiency of translocation into the ER. These findings suggest that the normally undetectable and minor population of mis-segregated secretory and membrane proteins can substantially modify the dynamics of cytosolic protein aggregates to potentially influence the pathologic course of neurodegeneration.

### Conclusion

From Birth to Death: Systematic Comparative Analysis of the Fate of Disease-causing PrP Mutants

A. Ashok, R. S. Hegde; Cell Biology and Metabolism Branch, National Institutes of Health, Bethesda, MD

Prion diseases are neurodegenerative disorders characterized by the aberrant metabolism of the mammalian prion protein (PrP), a widely expressed GPI-linked cell surface protein of unknown function. Although most studies are aimed at understanding the transmission of prion diseases, roughly one-third of cases involve dominantly inherited mutations in PrP. The phenotypic similarities between transmissible and heritable prion diseases have suggested shared mechanisms of neuronal damage that in neither case is well understood. To address this central issue, we are combining our insights from in vitro, biochemical, cell biological, and imaging methodologies to trace the biosynthesis, trafficking, and metabolism of PrP. Systematic comparative analyses of disease-causing mutations in two disease-modifying polymorphic backgrounds are being used to identify defining features underlying their common neurodegenerative outcomes. Our ongoing analysis has allowed the classification of PrP mutants into two qualitatively distinct groups. The first group, represented by five mutants, influences the initial translocation of PrP across the ER membrane to alter the proportion of PrP molecules that are generated as a transmembrane protein. The effects are modest (affecting only -5-10% of total PrP molecules) and appear to have little or no effect on the steady state localization, biochemical behavior, or modification of PrP in cultured cells. The second group, represented by six mutants, shows no effect on ER translocation, core modifications, or topology. However, -5-10% of these mutant PrP molecules change their biochemical properties and trafficking itineraries at a post-ER compartment. The long-lived nature of this altered form results in its increased accumulation at steady state, being detected as an incompletely modified insoluble population of PrP. Thus, two key points in PrP metabolism, one at the ER and another at a post-ER compartment, have emerged as especially sensitive to mutational deviation and represent candidates for key events in prion disease pathogenesis.

### A Method to Obtain Schwann Cell Enriched Cultures from Adult Rabbit Sciatic Nerve

I. De la Fuente Ayuso, 1.2 I. Alcalde, 1.4 O. L. Gamboa, 1,3 M. J. Gayoso, 2,3 1,2Cell Biology, University of Valladolid, Valladolid, Spain, 1Incy, University of Valladolid, Valladolid, Spain

Objectives: Obtaining a large number of viable Schwann cells (SCs) is one of the most promising approaches to repair nervous system injuries. We present here a fast protocol, which is easily reproducible and scalable, to obtain highly enriched cultures of adult rabbit SCs. This method of nervous system diseases. Methods: 10-15 fragments from a rabbit sciatic segment of 1.5 cm, were preincubated in vitro for a week with DMEM, 10% fetal bovine serum (FBS) and neuregulin (NRG1). After dissociation with trypsin and collagenase, cells were plated and incubated for six days in the same type of medium. Results: At 13 days in vitro, this method yields a total of around 2.85 x 10^6 cells, with an average of 88.76% p75 positives and 94.04% s100 positives. Conclusions: Nerve in vitro predegeneration with DMEM with FBS and NRG1 together with subsequent culture in the same conditions are sufficient and suitable to obtain adult SC enriched cultures in under two weeks. (Supported by a grant FIS PI 03/1533)
The Ubiquitin Protease Ataxin-3 Auto-Regulates Its Protein Levels and Post-translational Modification

S. V. Todi, M. N. Laco, H. M. Wen, H. L. Paulson; Neurology, The University of Iowa, Iowa City, IA

Spinocerebellar Ataxia 3 (SCA3) is one of nine inherited neurodegenerative disorders caused by expanded polyglutamine regions in the respective proteins. SCA results from an expansion in the MJJD1 gene, which encodes the protein ataxin-3 (AT3). AT3 was recently shown to be a deubiquitinating enzyme and poly-ubiquitin binding protein that functions in protein quality control pathways. Here we present data that AT3 auto-regulates its cellular protein levels in a catalytic site-dependent fashion. An inactivating mutation of the AT3 catalytic site leads to a marked increase in steady state levels of AT3 protein, which can be reduced by coexpression of active AT3. Consistent with this, in-vitro and in-vivo experiments show that catalytically inactive AT3 is more resistant to proteasomal degradation than its wild type counterpart. These results led us to investigate whether inactive AT3 is more stable as a result of altered ubiquitination of the protein. Studies in transfected cells show that AT3 is in fact ubiquitinated and that AT3 ubiquitination pattern is dependent upon its DUB activity; inactive AT3 is more heavily ubiquitinated than wild type AT3. We next investigated the hypothesis that wild type AT3 can lead to lower levels of coexpressed inactive AT3 by directly deubiquitinating it. However, our research indicates that wild type AT3 does not alter the ubiquitination pattern of inactive AT3 in trans. Instead, our data suggest that AT3 forms a homomeric complex with other AT3 proteins and that, through this interaction, AT3 affects their fate, possibly by recruiting inactive AT3 more readily into a proteasomal-targeted complex. We believe that AT3 auto-regulates its cellular levels, possibly via a catalytic site-dependent interaction with the proteasome. This is important because it suggests that AT3 may not only alter substrate protein ubiquitination patterns, but conceivably also by associating with them and affecting their fate.

Activation of p38 MAPK Negatively Modulates Induction of Matrix Metalloproteinase-9 Expression in Rat Astrocytes


As an extension of our previous study showing Erk1/2 pathway mediates lipopolysaccharide (LPS) induced MPP-9 over-expression in rat primary astrocytes (Glia 41:15-24, 2003), we investigated the role of p38 pathway on the regulation of MPP-9 expression in LPS-stimulated rat primary astrocytes. LPS treatment activated both p38 and JNK in rat primary astrocytes. Treatment of a specific p38 MAPK inhibitor SB203580, but not the JNK inhibitor SP600125, increased the LPS-stimulated MPP-9 expression, which was dependent on the concentration of SB203580. Anti-inflammatory cytokines, such as IFN-γ and IL-4, activated p38 MAPK and decreased the LPS-induced MPP-9 production. When the activation of p38 MAPK was blocked by SB203580, the inhibitory effects of these cytokines on MPP-9 induction were abolished. Finally, direct injection of SB203580 into the lateral ventricle of the rat brain increased the LPS-induced MPP-9 activity from the cerebral cortex. Altogether, these results suggest that p38 activation modulates the inflammatory stimulation-induced over-expression of MPP-9, both in primary astrocytes and in the rat cerebral cortex, which enables fine-tuning of the MPP-9 expression in neuroinflammatory condition.

Carboxyfullerenes Attenuate NMDA-induced Excitotoxic Injury in Cortical Neuronal Cultures: A Structure-Activity Study

S. S. Ali, M. Behrens, L. Dugan; Medicine, University of California San Diego, La Jolla, CA

Carboxyfullerenes are small-size molecules with unique chemical and biomedicinal properties. Motivated by the extraordinary free radical scavenging ability of the parent fullerene C60 molecule, we synthesized and explored the applications of its water-soluble malonic acid derivatives as potential neuroprotective agents. We discovered that these compounds are capable of eliminating superoxide radical, which is the main source of cellular oxidative stress, through a catalytic mechanism mimicking superoxide dismutase action (Ali et al. Free Radical Biology and Medicine, 37 (2004) 1191). The qualitative structure-activity relationship of 7 different malonic acid C60 derivatives against NMDA-induced excitotoxic injury in cortical neuronal cultures was studied. Attempts to correlate neuroprotective efficacy of carboxyfullerenes with their reactivity towards superoxide radical were also carried out. Moreover, quantum mechanical semi-empirical calculations were performed and the obtained parameters were employed to rationalize the observed reactivity trend. Distribution of the malonic acid substituents around the C60 surface, rather than their population, was found to determine the reactivity of drugs towards superoxide, and hence, their neuroprotective efficacy. Computer-generated modeling studies suggested that the enhanced potency of C3 compound (relatively large dipole moment) and the inefficacy of its D3 isomer (zero dipole moment) may be due to the potential electrostatic interaction between the drug and the excitotoxic superoxide radical. These results confirm that the neuroprotective action of carboxyfullerenes is due to their antioxidative properties rather than to a hypothetical ionic-channel regulatory effect.
significant decrease in phallolidin fluorescence intensity occurred in the filopodia and the peripheral lamellipodial region compared to control neurons, suggesting that thimerosal may affect either assembly dynamics or localization of F-actin in growth cones. The immediate effects of thimerosal on growth cone behavior suggest concentrations as low as 500 nM can inhibit axon growth to target cells during development.

#### HSV-mediated Transfer of Erythropoietin Reduces Injury and Improves Functional Recovery in Spinal Cord Trauma

M. Mata1; 1Neurology, University of Michigan, Ann Arbor, MI, 2VAMC, Ann Arbor, MI

Erythropoietin (EPO) is a pleiotropic cytokine which has been shown to have significant neuroprotective effects in several models of injury to the CNS. We determine whether vector-mediated expression of EPO in spinal cord would reduce damage and improve outcome after blunt trauma. A non-replicating herpes simplex virus (HSV)-based vector containing the human EPO gene under the control of the HCMV IEp (vector DHEPO) was constructed. Two hours after a 150 kDblunt trauma to the T10 thoracic spinal cord (Infinite Horizons Impactor), 1 μL containing 1.8x10^6 plaque forming units of DHEPO or control vector DHZ was injected on each side of the midline at the level of the injury. Motor behavior assessed by open field walking, grid-walk, and an automated Tread-scan and sensory behaviour assessed by thermal latency and mechanical threshold over 8 weeks after injury. Transgene expression, histological and BBB assessments were determined in addition to activation of MAPK and PI3K after injury. Increased expression of EPO was detected in spinal cord of SCI animals treated with DHEPO by RT-PCR and WB. SCI rats treated with DHEPO showed marked improvement in motor behavior compared to the DHZ treated animals but there was no improvement in sensory function. DHEPO-treated animals had less disruption of the blood brain barrier, decreased hemorrhage and lower RSA extravasation that injured DHZ animals after injury. While p-AKT was increased at 3, 10 and 28 days after injury, p-ERK was increased only at 28 days in the DHEPO-inoculated SCI. Expression of NeuN was greater in the DHEPO-SCI animals compared to the DHZ group and NeuN banding pattern by WB in DHEPO treated group was similar to that of sham animals. Gene therapy with HSV-mediated expression of EPO after SCI may be used to protect blood brain barrier function, reduce tissue injury and improve function.

#### Cytoskeleton Alterations Induced by Herpes Simplex Virus Type 1 Infection in Neuronal Cultures

A. Zambrano,1 L. Solis,2 N. Salvadores,12 M. Cortés,1,2 C. Mendez,1,2 C. Orih1, Instituto de Microbiología, Facultad de Ciencias, Universidad Austral de Chile, Valdivia, Chile

Herpes simplex virus type 1 (HSV-1) belongs to the family Herpesviridae, genus Simplexvirus, it is ubiquitous, neurotropic and the most common pathogenic cause of sporadic acute encephalitis in humans. Herpes simplex encephalitis (HSE) is associated with neurological and neurophysiological disorders, which afflict patients all life long. However, at present, there is not evidence about the role of HSV-1 triggering neurodegenerative process. In cell lines, HSV-1 induce cytoskeleton alterations, these alterations involve the overall disruption of one or more elements of the cytoskeleton. In our laboratory, primary cortical neurons were infected with HSV-1 to study change in microtubule dynamics and alterations of actin filaments of cortical neurons. Our results show a clear neuronal death, axonal injury and neurites disruption in neuronal cultures infected by HSV-1. Immunofluorescence and western blot analysis showed that HSV-1 induced a significant change in microtubule dynamics and also induced actin filaments alterations. These results suggest an important role of HSV-1 infections on neuronal cytoskeleton.

#### Receptors for Microtubule Motors in the Inner Tegmentum of Herpes Simplex Virus

K. Radtke, A. Wolfstein, B. Sodeik; Department of Virology, Hannover Medical School, Hannover, Germany

After entry into cells, capsids of Herpes Simplex Virus Type 1 (HSV1) are transported along microtubules towards the nucleus for viral genome uncoating, transcription and replication. Newly synthesized capsids leave the nucleus through the cytosol to host membranes for envelopment and further viral spread. Cytosplasmic dynein and its cofactor dynactin transport cytosolic capsids towards the microtubule minus-end during virus entry. However, the motor proteins conveying and directed transport during virus egress from cells as well as the viral interaction partners are not known. In order to identify HSV1 receptors for microtubule motors, we generated capsids with different tegument protein composition: (i) capsids without tegument proteins, (ii) capsids with only inner tegument proteins such as VP1-3 and UL37, and (iii) capsids with inner and outer tegument proteins. To analyze motor protein binding to these capsids, they were incubated with cytosol and either collected by sedimentation and analysed by immunoblot, or examined via immuno-gold labelling, negative-contrasting and electron microscopy. Capsids which had only inner tegument proteins attached showed an increase in dynein and dynactin binding compared to capsids with inner and outer tegument proteins. Tegumented capsids also bound the plus-end directed motors kinesin-1 and kinesin-2. In contrast, capsids without tegument showed little interaction with microtubule motors. This is consistent with Wolfstein et al. (Traffic 2006; 7:227-37), who demonstrated that capsids exposing inner tegument proteins showed an increased transport along fluorescently labelled microtubules in vitro.
GMF-induced Production of Proinflammatory Mediators in Glial Cells Mediates Neuronal Cell Death

A. Zaheer, 1 S. Knight, 2 S. Zaheer, 2 S. K. Sahu 2; 1Neurology, VA Medical Center & University of Iowa, Iowa City, IA, 2Neurology, University of Iowa, Iowa City, IA, 3Neurology and Neurosurgery, University of Iowa, Iowa City, IA

The glia maturation factor (GMF), a highly conserved brain-specific protein, isolated, sequenced and cloned in our laboratory, has proinflammatory and immunomodulatory functions in the central nervous system. GMF is localized mainly in astrocytes but is also present in some neuronal populations. Recently, we established that overexpression of GMF in astrocytes, leads to immune activation of microglia in vitro, through secretion of granulocyte-macrophage-colony stimulating factor. In the present study, we evaluated the effects of GMF-induced expression and release of proinflammatory mediators by glial cells in the neuronal cells in culture. The overexpression of GMF in mixed culture of primary glial cells with a replication-defective adenovirus carrying GMF cDNA caused a significantly increased production of GM-CSF, TNF-α, IL-1β, and IL-12 p40. Additionally, the GMF-overexpressing glial cell conditioned-medium was cytotoxic to primary neuronal cells and N18 mouse neuroblastoma cells in culture. The increased production of proinflammatory cytokines as well as the neuronal cell cytotoxicity was significantly blocked (greater than 80%) by down-regulating the GMF expression using a GMF-specific siRNA. Control scrambled siRNA had no such effects. This GMF led death of neuronal cells may have important implications in neurological disorders such as multiple sclerosis and Alzheimer’s disease. Supported by VA Merit Review award and NIH grant NS47145 (to AZ)

The Juvenile Myoclonic Epilepsy-related Protein EFHC1 Interacts with the Redox-sensitive TRPM2 Channel Linked to Cell Death

M. Katano, 1 Y. Mori, 2 Y. Hara 2; Kyoto University, Kyoto, Japan, 3Iowa University, Iowa City, IA

The transient receptor potential M2 channel (TRPM2) is the Ca2+ permeable cation channel controlled by cellular redox status via beta-NAD+ and ADP-ribose (ADPPr). TRPM2 activity has been reported to underlie susceptibility to cell death. However, little is known about the intracellular mechanisms that regulate oxidative stress-induced cell death via TRPM2. We report here a molecular and functional interaction between the TRPM2 channel and EFHC1. Immunoprecipitation analysis demonstrated physical interaction of the N- and C-terminal cytoplasmic regions of TRPM2 with the EFHC1 protein. Co-expression of EFHC1 significantly potentiated hydrogen peroxide (H2O2)- and ADPPr-induced TRPM2 channel activation in intracellular Ca2+ measurements and electrophysiological ionic current recordings in HEK293 cells. Furthermore, EFHC1 enhanced TRPM2-mediated susceptibility of HEK293 cells to H2O2-induced cell death. These effects of EFHC1 were reversed by JME mutations. The results reveal a possible regulatory action of EFHC1 on TRPM2 activity which enhances cell death, suggesting that TRPM2 contributes to the expression of JME phenotypes by mediating disruptive effects of JME mutations on EFHC1-induced neuronal death.

Pathways of Microglia Activation and Neuron Killing in a Model of the Ischemic Stroke Penumbra

V. Kasahara, 1 K. Takahashi, 2 R. Prusa, 3 D. Ethell, 4; 1Toronto Western Research Institute, Toronto, ON, Canada, 2Department of Physiology, University of Toronto, Toronto, ON, Canada

Microglia, the resident immune cells of the CNS, are important for orchestrating acute brain inflammation following stroke and trauma. During a stroke, glutamate is released by damaged neurons and is thought to act both through ionotropic glutamate receptors (mGluR) and metabotrophic receptors (mGluR). To study the underlying processes, we developed an in vitro model of microglia activation that is more relevant to the stroke penumbra, where microglia are exposed to stressed neurons. Microglia were grown on porous Transwell™ inserts, and then exposed to oxygen glucose deprivation (OGD). Cortical biopsies were stored in glutaraldehyde for investigation by electron microscope (Phillips CM100) and in formaldehyde for immunohistochemical investigation with antibodies against caspase 3, Bcl-2, NSE, and CD68. Total RNA was extracted from brain tissue. cDNAs were synthesized from RNA using promoter primer, fragment transcripts were hybridized on nylon membrane. Results. A spectrofluorometer-based assay showed that OGD-treated neurons activated microglial NF-kB, a transcription factor well known to promote pro-inflammatory functions of innate immune cells. In this in vitro model, the trigger for microglia activation appears to be glutamate acting on metabotropic glutamate receptors, since their neurotoxic properties were decreased by treating microglia with an mGluR1 antagonist (EGLU), and increased by an mGluR1 agonist (DGC IV). Consistent with the role of TNF-α in microglia-mediated killing, mGluR1 stimulation with DGC IV evoked TNF-α, but not NO, production. Importantly, we found that blocking Kv1.1 channels in the microglia inhibited NF-kB activation and reduced their neurotoxicity in this stroke penumbra model, as it did in a model using LPS-activated microglia. This novel in vitro model of the ischemic penumbra should prove useful for delineating processes more relevant to neuron-inflammation following stroke, and for assessing potential treatments. Supported by grants to LCS from the Heart & Stroke Foundation.

Gene Expression Profile and Serum Levels of NSE and S100b Protein in Patients with Trauma Brain Injury during 10 Days of Hospitalization

R. Prusa, 1 D. Vajtr, 1 J. Kukacka, 1 L. Houstava, 2 F. Samal, 2 A. Kracmarova, 3 M. Merkerova, 3 O. Benada 4; 1Clinical Biochemistry and Pathobiology, Charles University, 2nd Medical Faculty, Prague, Czech Republic, 3Neuropathology, Charles University, 3rd Medical Faculty, Prague, Czech Republic, 4Institute of Hematology, Charles University, 1st Medical Faculty, Prague, Czech Republic, 1Electronic Microscopy, Academy of Science, Prague, Czech Republic

Objective. The aim of this study was to evaluate cell damage by means of serum markers, electron microscopy, immunohistochemistry, and gene expression profile in patients with trauma brain injury according Glasgow coma score during 10 days of hospitalization. Methods. 12 patients with trauma focal brain injury were investigated and divided into group (n=6) with improvement of GCS during 10 days of hospitalisation to 15 points, and group of patients (n=6) without improvement of GCS. Serum NSE and S100B concentrations were measured on Elecsys2010 (Roche). Cortical biopsies were stored in glutaraldehyde for investigation by electron microscope (Phillips CM100) and in formaldehyde for immunohistochemical investigation with antibodies against caspase 3, Bcl-2, NSE, and CD68. Total RNA was extracted from brain tissue. cDNAs were synthesized from RNA using promoter primer, fragment transcripts were hybridized on nylon membrane. Results. A spectrofluorometer-based assay showed that OGD-treated neurons activated microglial NF-kB, a transcription factor well known to promote pro-inflammatory functions of innate immune cells. In this in vitro model, the trigger for microglia activation appears to be glutamate acting on metabotropic glutamate receptors, since their neurotoxic properties were decreased by treating microglia with an mGluR1 antagonist (EGLU), and increased by an mGluR1 agonist (DGC IV). Consistent with the role of TNF-α in microglia-mediated killing, mGluR1 stimulation with DGC IV evoked TNF-α, but not NO, production. Importantly, we found that blocking Kv1.1 channels in the microglia inhibited NF-kB activation and reduced their neurotoxicity in this stroke penumbra model, as it did in a model using LPS-activated microglia. This novel in vitro model of the ischemic penumbra should prove useful for delineating processes more relevant to neuron-inflammation following stroke, and for assessing potential treatments. Supported by grants to LCS from the Heart & Stroke Foundation.

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Resorption. Moreover, our findings strongly implicate PLEKHM1 as a component of Rab7 signaling pathways and late endosomal trafficking in osteoclasts. However, recent studies have shown that VLDLR is also involved in neuronal migration during brain development. Although VLDLR knockout mice display normal plasma lipid levels, they develop extensive retinal neovascularization (RNV), subretinal hemorrhages and choroidal anastomoses. Here we explore the function of VLDLR in the retina and the mechanism of RNV caused by deficiency of VLDLR. VLDLR expression in adult and developing mouse retinas was examined by RT-PCR. Northern blot, in situ hybridization and Western blot. Cell type specificity was studied by immunocytochemistry using antibodies against vldlr, and a panel of antibodies specific to different retinal cells including rod and cone photoreceptors, bipolar, amacrine, horizontal, Muller and ganglion cells. Visual function was assessed by both rod-derived and cone-derived electrotetrogramnic recordings. Our results show that VLDLR is expressed in the retina, although at levels lower than in the heart, skeletal muscle and brain. Expression is associated with the retinal vasculature and initiates at the onset of vascular development in the neonatal mouse retina. VLDLR KO mice display a normal distribution and density of retinal neurons and Muller glial cells, and exhibit normal visual function comparing to age-matched controls. We conclude that the abnormal neovascularization seen in VLDLR KO mice is unlikely due to its role in retin signal transduction of neuronal patterning since these mice have normal visual function and normal density and distribution of retinal neurons. It is also unlikely that the RNV is caused by deficiency in tricyclic VLDR metabolism since these mice display a normal lipid profile. We therefore suggest that the RNV in KO mice may be due to VLDLR-mediated specific interaction with novel ligands either directly or indirectly involved in angiogenesis.

DNase II-deficiency Disrupts Immune Function and Eye Development in Drosophila

C. Seong, A. Varela-Ramirez, R. J. Aguila; Biology, University of Texas at El Paso, El Paso, TX

DNase II belongs to a unique family of proteins that are required for degradation of ingested DNA. In eukaryotes, phagocytic cells utilize this enzyme to degrade the DNA of engulfed apoptotic cells or invading organisms. Since phagocytosis is an essential component of innate immunity, we hypothesized that the loss in this enzyme would result in a phagocyte-deficiency and possibly other defects. Using RNA interference and available DNase II-deficient lines, we have recently demonstrated that DNase II-deficiency results in severe immunodeficiency against bacterial infection due to the disruption of hemocyte (plasmatocyte) function. Microarray and real-time PCR analyses also indicate that DNase II-deficient flies exhibit an abnormal regulation of various genes including anti-microbial peptide genes. Expression of the ahse II-RNAi construct in other tissues did not lead to detectable defects with the exception of defects in eye development. Similar eye defects were also detected upon the specific expression of the ahse II-RNAi construct in the eye. However, no defects were detected in control animals or when expression was restricted to other tissues. Although no human patients have been linked to a deficiency in this enzyme, DNase II polymorphisms have been associated with increased risk of renal disorder among Systemic Lupus Erythematosus (SLE) patients. Our ongoing studies should greatly enhance our understanding of the molecular and biochemical roles of an enzyme that is essential for engulfment-mediated DNA degradation, normal immune function and eye development.

Xanthine Oxidase in Conjunctival Epithelial Cells of Humans Suffering from Dry Eye

J. Čejková, T. Ardan, C. Čejka, G. Sechova, K. Jirsova; Eye Histochemistry and Pharmacology, Institute of Experimental Medicine, Academy of Sciences of the Czech Republic, Prague, Czech Republic

Conjunctival cells from patients with dry eye produce excessive xanthine oxidase and express xanthine dehydrogenase, enzymes which catalyze the conversion of hypoxanthine and xanthine to uric acid. Since these reactions generate superoxide anion and hydrogen peroxide, xanthine oxidase may be involved in oxidative damage of the ocular surface.

Inhibitory Effects of Stewartia Koreana Extract on Osteoclast Differentiation and Bone Resorption

C. Park, H. Kim, D. Yang, D. Jung, N. Baek, J. Kim, Z. Lee, H. Kim; Seoul National University, Seoul, Republic of Korea, Kyung Hee University, Seoul, Republic of Korea

Several plants and herbs have long been used to treat osteoporosis as folk medicine in oriental countries. In this study, we aimed to screen extracts and natural products from edible plants for potential application to osteoporosis prevention and therapeutic effects. The in vitro screening was performed with culture of osteoclasts, differentiated with receptor activator of nuclear kappa B ligand (RANKL), macrophage colony stimulating factor (M-CSF), and in the presence or absence of extracts and natural products for 6 days. Osteoclast formation was tested by tartrate-resistant acid phosphatase staining. TRAP-positive multinucleated cells containing more than three nuclei were counted. Of the several plants-derived materials, Stewartia koreana extract (SKE) showed inhibitory effects on osteoclast differentiation and bone resorption. We examined the expression of SKE on osteoclast signaling pathways, SKE reduced levels of NFATc1 and c-Fos, crucial transcriptional regulators for osteoclastogenesis. SKE also downregulated mRNA levels of c-Fos and NFATc1 in RANKL-stimulated BMMs. Finally, SKE showed a great inhibitory effect on LPS-induced bone resorption in mouse model. Taken together, we demonstrated that SKE strongly inhibited bone resorption in vitro and in vivo by blocking osteoclast differentiation signal.

Plekhi1 Is Involved in Vesicular Transport in Osteoclasts and Causes Osteopetrosis in the Incisors Absent Rat and Human

L. Van Wesenbeek, P. R. Ogden, F. P. Coxon, A. Frattini, P. Moens, B. Van Hul, J. Timmermans; Laboratory of Cell Biology and Histology, University of Antwerp, Antwerp, Belgium, Dept of Experimental Medicine, University of L'Aquila, L'Aquila, Italy

The osteopetroses are a heterogeneous group of bone disorders characterized by a reduction in bone resorption and a generalized net accumulation of skeletal mass. We have now identified the plekhi1 gene as the gene responsible for the osteopetrotic phenotype of the incisors absent rat and have identified a splice site mutation in the human PLEKHI1 gene in a family diagnosed with an intermediate form of osteopetrosis. Electron and confocal microscopy analysis demonstrated that osteoclasts (derived from M-CSF-dependent peripheral blood monocytes) from the patient differentiated normally. However, unlike osteoclasts from sib-brother lacking the mutation, osteoclasts from the patient showed hardly any evidence of resorptive activity when cultured on dentine discs, and had a more flattened morphology. This is the first evidence for a role of the plekhi1 protein in bone metabolism. The presence of RUN and PH domains suggests that the plekhi1 protein may be involved in small GTPase signaling. Overexpression studies in HEK293 cells demonstrated that the plekhi1 protein is partially associated with intracellular vesicles and colocalizes with Rab7 and Rab9, but not with Rab5 or Rab6, suggesting that these vesicles are late endosomes/lysosomes. Moreover, in cells overexpressing Rab7, plekhi1 becomes completely localized to Rab7-positive vesicles, an effect that is not seen with overexpression of Rab9. Inhibiting the prenylation of Rab proteins with the specific Rab-GTPase inhibitor 3-PiHPC disrupted the endosomal localization of both Rab7 and plekhi1, while coexpression of a dominant negative Rab7 mutant and plekhi1 also resulted in a mainly cytosolic distribution of both proteins, suggesting that plekhi1 interacts with Rab7. In conclusion, the identification of PLEKHI1 as a new osteoporosis gene indicates its crucial role in bone resorption. Moreover, our findings strongly implicate plekhi1 as a component of Rab7 signaling pathways and late endosomal trafficking in osteoclasts.

Inhibition of RANKL-induced Osteoclastogenesis, by DMS, a Sphingosine Kinase Inhibitor through Down Regulation of NFATc1

H. Kim, E. Chang, C. Park, H. Kim; College of Dentistry, Seoul National University, Seoul, Republic of Korea

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Therefore, CpG-ODNs may inhibit osteoclast differentiation through by down-regulating TREM-2 expression in osteoclast precursor s. This fact suggests a novel mechanism of TLR9 derived macrophages and pre-osteoclasts in response to RANKL but not in osteoclasts. In line, CpG-ODNs inhibited RANKL-induced NFAT activity but not NF-κB. In osteoclasts included filamin A and gelsolin, suggesting a role of actin remodeling during early stage of differentiation. To identify differentially expressed proteins, lysates from surface-biotinylated cells were subjected to avidin column and those biotinylated proteins were separated on SDS PAGE. After gel staining, proteins in 12 bands showing significant increase or decrease during differentiation were in-gel trypsin digested and eluted. Finally, peptides were subjected to reverse-phase liquid chromatography connected to tandem mass spectrometer and identified by SEQUEST search. As a result, we have identified 160 proteins differentially expressed between macrophages and pre-osteoclasts. Proteins increased in pre-osteoclasts included filamin A and gelsolin, suggesting a role of actin remodeling during early stage of differentiation. Expression of proteases specific for macrophages such as c-fms, CD14, and F4/80 decreased, confirming that pre-osteoclast lost phenotype of macrophages. Interestingly, expressions of integrin subtypes either increased or decreased in pre-osteoclasts compared with those in macrophages. Collectively, these results suggest that actin remodeling and regulation of signaling upon cell-cell/cell-extracellular matrix contact may be controlled at a protein level during early osteoclast differentiation.

Matrix Metalloproteases (MMPs) which are a family of zinc-dependent endoproteases cleave extracellular matrix components and other physiologically functional proteins. Using our degradomic approach using proteomic techniques, we have identified apolipoprotein E (apoE) as a putative MMP-14 substrate from human plasma. ApoE which is synthesized in the liver and several extracellular tissues is involved in lipid metabolism and protection of atherosclerosis. Digestion of the purified ApoE with various MMPs demonstrated that apoE is the most susceptible for MMP-14 among the tested MMPs. The 34-kDa apoE was initially cleaved into 28-kDa, 23-kDa, 21-kDa, 20-kDa, and 11-kDa fragments by MMP-14. By N-terminal sequencing and MALDI-TOF mass determination of the MMP fragments, cleavage sites of apoE by MMP-14 were determined to be between A176-I177, P183-L184, P202-L203, and Q249-I250. The cleavage sites were confirmed by identification of the C-terminus of each MMP fragment using isotope labeling (O18/D18) of the C-termini of MMP fragments, protease digestion, and MALDI-TOF mass spectrometry of the peptides. The cleavage pattern of the lipid-bound apoE by MMP-14 was the same as that of the lipid-free apoE. The vesicle size of the lipid-bound apoE was decreased upon digestion with MMP-14. Considering the important role of apoE for lipid metabolism and atherosclerosis protection, our findings suggest that MMP-14 may play an important role for development of hyperlipidemia and atherosclerosis by degradation of apoE.

CpG oligodeoxynucleotides (Cpg-ODNs) mimicking bacterial DNA, stimulate osteoclastogenesis via Toll-like receptor 9 (TLR9) in receptor activator of NF-kappa B ligand (RANKL)-primed osteoclast precursors. In contrast, activation of TLR9 in early osteoclast precursors results in inhibition of RANKL-induced osteoclast differentiation. However, it is not yet defined well how TLR9 activation differently modulates it. In this study, we demonstrated that TLR9 activation modulates the expression of triggering receptors expressed by myeloid cells (TREM-2), known to co-stimulatory signals required for RANKL. During osteoclastogenesis, the surface expression of TREM-2 was increased in response to RANKL and which was inhibited by CpG-ODNs treatment through the flow cytometry analysis. However, mRNA level of TREM-2 did not regulated by TLR9 co-stimulation. Interestingly, TREM-2 level was up-regulated in bone marrow-derived macrophages and pre-osteoclasts in response to RANKL but not in osteoclasts. In line, CpG-ODNs inhibited RANKL-induced NFAT activity but not NF-kB and AP-1 activity. These data indicated the role of PKH2 in osteoclastogenesis. Furthermore, CpG-ODNs differentially modulate the expression of co-stimulatory molecules for RANKL-induced osteoclast differentiation.
Disease Research CBHR, Brain Research Institute, University of Niigata, Niigata, Japan, 2Mental Retardation and Birth Defect Research, National Institute of Neuroscience, NCNP, Tokyo, Japan, 3Department of Physiology and Biophysics and Department of Neurology, Howard Hughes Medical Institute, University of Iowa, Iowa City, IA

Reduced level in glycosylation of α-dystroglycan causes a group of muscular dystrophies, which have been called α-dystroglycanopathies. Six causative genes have been implicated: three responsible proteins, namely protein O-mannosyltransferase 1 (POMGnT1), and protein O-mannosyltransferases 1 and 2 have been characterized as glycosyltransferases; the functional roles of the other three which include fukutin, LARGE, and fukutin-related protein remain largely unknown. Here we demonstrate a novel function of fukutin and LARGE. The cytochemical staining of the proteins, immunoprecipitation analysis, in vitro measurement of POMGnT1 activity, mutagenesis of fukutin and metabolic labeling of α-dystroglycan were used in this study. These two proteins co-localized to cis-Golgi compartment with POMGnT1 by forming a complex in cultured myoblasts. Enzymatic assay revealed that only POMGnT1 has N-acetylglucosaminyltransferase activity, whereas fukutin and LARGE have no activity by themselves, but strongly activate POMGnT1. Mutated fukutins were not expressed in cis-Golgi and were unable to activate POMGnT1.Brains from patients with Fukuyama-type muscular dystrophy and N2 mouse also showed decreased levels in protein O-mannosyl N-acetylglucosaminyltransferase activities. These findings suggest that the three α-dystroglycanopathies whose responsible proteins are fukutin, LARGE and POMGnT1, are most likely to be caused by a common pathomechanism, that is a defect in the second step in O-mannosylglycan synthesis of α-dystroglycan.

1352 Ankyrins-B and -G Cooperate in a Stepwise Pathway for Sarcomere Targeting and Costamer Localization of the Dystrophin Glycoprotein Complex

G. Ayala, J. Q. Davis, V. G. Bennett; Cell Biology and IHMI, Duke University Medical Center, Durham, NC

Costameres are specialized sarcomere domains, connecting striated muscle contractile machinery to the extracellular matrix (ECM). Costameres contain the Dystrophin Glycoprotein Complex (DGC), which forms physical bridges connecting the actin cytoskeleton and the ECM. The DGC preserves sarcomere integrity during contraction-relaxation cycles. Mutated genes encoding DGC proteins cause muscular dystrophy due to weakening of the sarcolemma and its disruption when subjected to physical stress. Cellular pathways governing DGC sarcomeral targeting and costamer localization are poorly understood. Ankyrins are cytoplasmic adaptor proteins known to localize integral membrane proteins to specialized membrane domains. We show for the first time that ankyrin-B is required for targeting the DGC to the sarcolemma, while ankyrin-G is required for localization of sarcomeral DGC to costameres. In neonatal ankyrin-B null mice, multiple DGC proteins are missing from the sarcolemma. siRNA knockdown of muscle ankyrin-B in adult mice in vivo recapitulates this phenotype. Ankyrin-B-depleted cells exhibit Evans Blue dye uptake following exercise. Following ankyrin-G knockdown in vivo, the DGC is sarcolemmal but mislocalized. Strikingly, these fibers also are penetrated by the dye showing sarcolemmal fragility. This implies that sarcolemmal presence of the DGC is not sufficient. Specific localization of the DGC to costameres is physiologically critical and is ankyrin-G dependent. Ankyrin-B binds directly the DGC proteins dystrophin (Dp71) and γ-sarcoglycan but not β-dystroglycan. Ankyrin-G binds dystrophin and γ-sarcoglycan, and with high affinity to β-dystroglycan, the main DGC transmembrane axis. We propose a model of DGC transient targeting interaction with ankyrin-B and a stable anchoring to costameres by ankyrin-G. We describe here a new principle: a two-step cellular pathway where ankyrin-B mediates targeting of proteins to the plasma membrane and ankyrin-G subsequently restricts them to specialized membrane domains that are critical for their physiological roles.

1353 Paxillin Upregulation in Dystrophin Deficiency Increases Contractility and Mapk Signaling but is Pro-survival

S. Sen, 1 Michael DeBakey Institute for Comparative Cardiovascular Science, Small Animal Clinic, College of Veterinary Medicine, College Station, TX, 2Dept. of Biomedical Engineering, Texas A&M University, College Station, TX, 3Dept. of Systems Biology and Translational Medicine, Texas A&M Health Science Center, College Station, TX, 4Michael DeBakey Institute for Comparative Cardiovascular Science, Small Animal Clinic, College of Veterinary Medicine, College Station, TX, 5Dept. of Biomedical Engineering, Texas A&M University, College Station, TX

Development and normal functioning of myotubes depend on an optimal balance between adhesion and cellular protrast. Normal muscle cells posses both integrin-based adhesions and dystroglycan-complex based adhesions. However, the latter are mostly missing in dystrophin deficiency and is known to cause integrin upregulation. Here we show that adhesion signaling protein paxillin is also upregulated, allowing study of its functional effects in muscle cells. We demonstrate that paxillin, a multifunctional protein found to localize both in focal adhesions and cytoskeletal structures, is also an essential signaling molecule of the contractile loop. Extended studies in cell cultures show that overexpression of GFP-paxillin in C2C12 myotubes promotes contractility and contributes to enhanced sarcomeric organization with no significant changes in adhesion. Finally, we confirm that the activation MAPK signaling is a common pathway of dystrophin deficiency, and paxillin promotes the de-differentiation associated phenotypic changes by modulating pro-survival signals.

1354 TGF-beta and BMP Signaling in Hypertensive Vascular Remodeling

N. Poppovic, 1 J. D. Neiger, 2 M. Miller, 3 T. Fossain, 2 J. D. Humphrey, 1 E. Wilson, 1 Dept. of Systems Biology and Translational Medicine, Texas A&M Health Science Center, College Station, TX, 2Michael DeBakey Institute for Comparative Cardiovascular Science, Small Animal Clinic, College of Veterinary Medicine, College Station, TX, 3Dept. of Biomedical Engineering, Texas A&M University, College Station, TX

Hypertensive vascular remodeling is characterized by phenotypic switching of muscle cells (SMC) to less differentiated phenotype. Bone morphogenetic proteins (BMP) and transforming growth factor beta (TGFβ) proteins in SMC act through the same TGFβ control element (TCE) and have opposing effects on SMC differentiation. The goal of our study was to determine the role of BMP and TGFβ signaling in hypertensive vascular remodeling. Aortic coarctation in miniswine was used to cause 2 weeks of sustained hypertension (mean arterial pressure>150 mmHg). Ponce 10k oligoarray chips were used to compare gene expression in aortic tissue from 3 animals proximal (high pressure) and distal (low pressure) to coarctation.

1355 Hypoxia-inducible Factor-1α Is Stabilized by Angiotensin II in Vascular Smooth Muscle Cells

E. L. Page, 1 M. Levine, 2 D. E. Richard; 1Centre de Recherche de l'Hôtel-Dieu de Québec, Québec, PQ, Canada, 2Molecular and Clinical Nutrition Section, Digestive Diseases Branch, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD

Hypoxia-inducible factor-1α (HIF-1α) is a decisive element in the transcriptional regulation of many genes induced under low oxygen conditions. Under normoxia, HIF-1α, the active subunit of HIF-1, is hydroxylated on proline residues by specific iron and 2-oxoglutarate dependant oxygenases named prolyl-hydroxylases (PHDs), enzymes using also ascorbate and oxygen as cofactors. This hydroxylation permits the binding of pVHL, the recognition component of an E3 ligase complex, leading to the ubiquitination-degradation of HIF-1α by the proteasome. In hypoxic conditions, hydroxylation and ubiquitination are blocked and HIF-1α accumulates in cells. Our studies have shown that non-hypoxic stimuli like vasoactive hormones may induce HIF-1α in vascular smooth muscle cells (VSMC). We have determined that angiotensin II (Ang II) increases HIF-1α levels through transcriptional and translational mechanisms. Our current studies suggest that Ang II may also modulate HIF-1α induction through its stabilization. Utilization of cycloheximide, a protein synthesis inhibitor, shows that the half-life of HIF-1α under Ang II stimulation is comparable to the half-life in hypoxic conditions. The use of a fusion protein between HIF-1α and luciferase shows that the stabilization of this protein is increased in VSMC treated with Ang II. Our data also show that the ubiquitination level of HIF-1α is decreased in Ang II-treated VSMC as compared to non-stimulated cells. Our studies demonstrate that the proteasome binding of HIF-1α protein is diminished in VSMC treated with Ang II. Ang II also decreases HIF-1α hydroxylation since pVHL binding is diminished in Ang II-treated cells. Additionally, an ascorbate supplementation of cells, a PHD cofactor, completely inhibits HIF-1α induction by Ang II. These results identify protein stabilization as a third mechanism responsible for HIF-1 induction by Ang II in VSMC. These mechanisms could modulate the expression of a number of genes controlled by the HIF-1 transcription factor under Ang II stimulation of VSMC.

1356 Apium graveolens Modulates Sodium Valproate-induced Reproductive Toxicity in Rats

A. Amin, A. Hamza; Biology, UAE University, Al-Ain, United Arab Emirates
Sodium valproate, a common treatment of epilepsy and other psychiatric disorders, is known to have severe toxic effects on tests both in experimental animals and in humans. The present study was designed to investigate the protective effect of Apium graveolens against the sodium valproate-induced tests injury. Testicular toxicity was induced by the administration of sodium valproate (500 mg/kg/day) once for 7 consecutive days. Protective group received daily doses (200 mg/kg/day) of AG crude extract for 23 days prior to sodium valproate administration. Sodium valproate-induced reproductive toxicity was assessed based on the weight of testes, sperm analysis and serum concentration of sexual hormones. The relative weights of testes and epididymes and the sperm numbers viability were all decreased following the valproate administration. Testosterone levels dropped while FSH level increased following the drug administration. Severe histopathological changes in tests were observed such as degeneration of seminiferous tubules and degeneration of germ cells. These biochemical and histological changes were also associated with alterations of oxidative stress markers. Levels of malondialdehyde have increased, while superoxide dismutase activity has decreased. Pretreatment with Apium graveolens extract has effectively attenuated all the sodium valproate-induced effects suggesting a protective role of Apium graveolens extract against experimental sodium valproate-induced toxicity. Apigenin content was estimated and was shown as a major fraction of the Apium graveolens extract.

1357 Epidemiology of Vascular Risk Factors and Heat Shock Proteins (Hsps)  
M. Guassioua, J. Ioli, G. Daciones, J. E. Rivas, J. C. Salgado, A. B. D. Padua, G. A. Barreiro, I. Vancurova; Biology, St. John’s University, Queens, NY

1358 Compound Heterozygosity for Mutations in LMAA Causes a Progeria Syndrome without Prenatal A Accumulation  
V. L. E. Verstraeten, B. C. J. L. V. M. B. L. R. M. V. M. S. Terra, C. C. Ghosh, H. Vu, S. Robinson, I. Vancurova; Experimental Medicine & Surgery Unit, Hospital General Universitario Gregorio Maranon, Madrid, Spain, 2Department of Biochemistry, Hospital General Universitario Gregorio Maranon, Madrid, Spain, 3Preventive Medicine, Hospital General Universitario Gregorio Maranon, Madrid, Spain

1359 Modulation of Glucocorticoid Sensitivity by Thioredoxin Reductase 1  
K. Sohn, C. Kim, S. Jang, D. Choi, S. Back, Y. Seo, J. Park, J. Lee; Dermatology, Chungnam National University, Daejeon, Republic of Korea

1360 The RAS/MEK/HIF-1 Pathway Upregulates pro-apoptotic BNIP3 in Nitric Oxide-induced Cell Death  
H. An, J. Lee, Y. Kim, H. Lee, S. Paik; 1Biology, Chungnam National University, Daejeon, Republic of Korea, 2Chemistry, Korea Advanced Institute of Science and Technology, Daejeon, Republic of Korea

1361 Inhibitor of CRM1-Dependent Nuclear Export Inhibits TNFα Release from Stimulated Human Monocellular U-937 Cells  
C. C. Ghosh, H. Vu, S. Robinson, I. Vancurova; Biology, St. John’s University, Queens, NY
cells. In contrast, release of another pro-inflammatory cytokine, interleukin-8 (IL-8), is not inhibited by LMB. In addition, we show that the mechanism of LMB inhibition of TNFα release consists of a specific inhibition of mRNA TNFα levels. Our data indicate that when there is excessive, persistent inflammation that results in tissue injury, as observed in sepsis, acute respiratory distress syndrome, or arthritis, the inhibition of TNFα release from activated mononuclear cells by the CR1M-dependent nuclear export mechanism could be used as a new therapeutic anti-inflammatory target.

1362 Hepatitis Delta Virus Biogenesis Requires Eososomal Tumor Susceptibility Gene 101

M. Mato, 1 J. S. Glenn, 2 B. Aroeti 1; 1Department of Cell and Animal Biology, Hebrew University of Jerusalem, Jerusalem, Israel, 2Division of Gastroenterology and Hepatology, Stanford University School of Medicine, Stanford, CA

Hepatitis delta virus (HDV) is an important cause of acute and chronic liver disease for which there is no current effective medical therapy. HDV is only found in patients that are also infected with hepatitis B virus (HBV). The HDV virion is composed of a RNA genome, small and large hepatitis delta antigens (HDAg-S and HDAg-L, respectively) and the surface protein of the HBV (HBsAg) embedded within a lipid envelope. The HDAg-S is expressed early during infection and is required for HDV RNA replication. The HDAg-L is produced in a later stage by editing processes, which add nineteen amino acids to the C-terminus of the small protein. Prenylation of the cysteine in the CXXX box positioned at the C-terminus of the HDAg-L is required for protein association with the endoplasmic reticulum and viral particle assembly. The overall goal of this project is to better understand the mechanisms of HDV assembly and budding. We hypothesize that a proline-rich region preceding the CXX box, functions as a late domain that is essential for viral particle budding. We reasoned that the late domain is required for interactions with Tsg101 (tumor susceptibility gene 101), a protein associated with the ESCRT complex and the biogenesis of exosomes budding in the lumen of multivesicular bodies. We hypothesized that a similar machinery is recruited by HDAg-L to mediate HDV budding into the lumen of the ER, or other organelles. We found that both, over-expression of wt Tsg101, or a dominant negative mutant form of Tsg101, diminished HDV budding in COS7 cells. Furthermore, depletion of Tsg101 by siRNA also decreased viral particle release. Tsg101 co-immunoprecipitates with anti-HDV antibodies. Our data signif for the first time the importance of eososomal sorting mechanisms in the biogenesis of hepatocellular viruses, whose budding is thought to take place in the endoplasmic reticulum.

1363 Prevotella intermedia LPS Stimulates Release of TNF-α through MAPK Signaling Pathways in Monocyte-derived Macrophages

S. Kim, 1 E. Choi, 1 J. Jin, 1 C. Kim, 1 I. Choi 1; 1Department of Periodontology, College of Dentistry, Pusan National University, Busan, Republic of Korea, 2Department of Life Science, College of Medical and Life Science, Busan, Republic of Korea

The purpose of this study was to investigate the effects of lipopolysaccharide from Prevotella intermedia, a major cause of inflammatory periodontal disease, on the production of TNF-α and the expression of TNF-α mRNA in differentiated THP-1 cells, a human monocytic cell line. We also investigated the potential involvement of the three main MAPKs signaling pathways in the induction of TNF-α production. LPS from P. intermedia ATCC 25611 was prepared by the standard hot phenol-water method. THP-1 cells were incubated in the medium supplemented with phorbol myristate acetate (PMA) to induce differentiation into macrophage-like cells. The amount of TNF-α was determined by ELISA using a commercially available kit. The effect of P. intermedia LPS on TNF-α transcription and accumulation of TNF-α mRNA was confirmed by real-time PCR. The levels of total and phosphorylated MAPKs (ERK1/2, p38 MAPK and JNK1/2) in differentiated THP-1 cells were determined by immunoblotting. We found that P. intermedia LPS can induce TNF-α mRNA expression and stimulate the release of TNF-α in differentiated THP-1 cells in the absence of other stimuli. Treatment of the cells with P. intermedia LPS resulted in a time-dependent increase in phosphorylated forms of MAPKs. Furthermore, all three MAPK inhibitors reduced the phosphorylation of their specific MAPKs by P. intermedia LPS. The MAPKs signaling pathways appeared to play a dominant role in the induction of TNF-α production by P. intermedia LPS as documented by a very significant inhibition of TNF-α mRNA and protein production after blocking with three MAPK inhibitors.

1364 Increased Oxidative Stress in Myoblasts from Inclusion Body Myopathy Patients

S. Sabedi, 1 S. Zanotti, 1 C. Di Blasi, 1 A. Ruggeri 2, S. Romaggi 3, F. Colleoni 3, H. Lochmuller 4, P. Bernasconi 5, M. Mora 5; 1Neuromuscular Diseases and Neuroimmunology, National Neurological Institute C. Besta, Milano, Italy, 2Neurologische Klinik und Poliklinik, Ludwig-Maximilians-Universitat, Muenchen, Germany

Sporadic inclusion body myositis (sIBM) is the most common disorder of skeletal muscle in aged humans, is of unknown etiology and pathogenesis and lacks definitive treatment. It shares biochemical features with Alzheimer's disease (AD), including coprophilic deposits, which are immunoreactive for beta-amyloid peptide (Abeta) and AbetaPP. Traditionally considered as an inflammatory myopathy, inclusion body myositis is now rather thought to be a disease of muscle aging, just like AD is considered a disease of brain aging. It is characterized by progressive vacuolar degeneration with accumulation of insoluble misfolded proteins such as AbetaPP; beta-amyloid, phosphorylated tau protein, prion protein (PrP), presenilin-1, alpha-synuclein, and others. There are numerous other familial forms of inclusion body myopathy, either autosomal dominant or recessive, similar to SIBM for the presence of vacuolar degeneration and toxic proteins in muscle fibers, but without inflammation, and therefore called hereditary inclusion body myopathies (hIBM). There is increasing evidence that oxidative stress, is involved in neurodegenerative disorders as well as in IBM. In order to investigate whether oxidative stress is increased in primary muscle cultures, we evaluated nitric oxide basal content in myoblasts from s-IBM and h-IBM patients and in control myoblasts. We found a 20-30 % increase in nitric oxide content in IBM myoblasts compared to control cells, and a 30-80% increase with greater variability in sIBM cells probably in relation with the inflammatory changes observed in this patient group. Toxic protein deposition in muscle fibers is likely to have a multifactorial origin in sIBM and has genetic causes in IBM forms. The most important factors contributing to accumulation of toxic proteins are those of aging; among these, increased oxidative stress and, possibly, inadequate denaturation systems, are likely to play a role.

1365 Induction of Inflammatory Cytokines and Their Repression by Small Molecules in a Mouse for EBS

S. Löffèl, 1 H. Lu, 2 P. Zigrino, 2 T. Magini 2; 1University of Bonn, Institute of Physiological Chemistry, Bonn, Germany, 2University of Cologne, Department of Dermatology and Center of Molecular Medicin, Cologne, Germany

Keratins K5 and K14 form the major intermediate filaments of basal epidermis. Their primary function is to impart mechanical strength to cells, as highlighted by dominant mutations causing inherited skin disorders including epidermolysis bullosa simplex (EBS). Moreover, keratins mediate the response to apoptosis and cause pigmented changes in keratinocytes. Until recently, EBS was widely regarded as a mecanobullous disease, resulting from a weakened cytoskeleton, followed by extensive cytolysis. It was postulated by others that certain K14 mutations induce TAF-α, increase apoptosis and mediate an unfolded protein response, thereby adding to EBS pathomechanisms. Here, we compared K5- mice to controls by Affymetrix analysis to examine a potential induction of inflammatory pathways which might contribute to EBS. We found an increase in interleukin (IL)-6, CXCL1 and IL-1β in K5- mice versus controls. These results were confirmed by real-time PCR. In contrast, we found no alteration in TAF-α, neither in K5- nor in a mutant K14 cell culture model, employing Affymetrix and ELISA assays. We conclude that keratin mutations induce certain inflammatory cytokines but not TAF-α. Following a clinical report using tetracyclines to treat EBS patients, we extended our studies on K5- mice to investigate whether systemic tetracycline application leads to a reduction of skin blistering and less fragile epidermis. Here, we demonstrate that systemic treatment of pregnant K5- females with doxycycline prolonged the survival of K5- pups from 1 to 8 hours. Microwave and TaqMan real-time PCR showed a down-regulation of MMP-13, serine protease and IL-1 beta, indicating an affect of doxycycline on transcription. Our findings lead to the hypothesis that mutations in keratins induce a stress response which can lead to inflammation, changes in gene expression and translational arrest. Collectively, our data offer novel small molecule-based therapy approaches for EBS.

1366 Structure and Molecular Basis of the Interactions of Dipeptidyl Peptidase IV with Adenosine Deaminase and HIV-1 Transactivator

H. Fan, 1 W. Weihofen, 1 K. Ludwig, 2 C. Boettcher, 1 J. Lin, 1 F. Tani, 1 M. Ledermann, 3 S. Stehling, 1 W. Reutter, 1 W. Sanger, 2 Institut fuer Molekularbiologie und Biochemie, Charite-Universitaetsmedizin, Berlin, Germany, 3Institut fuer Kristallographie, Freie Universitaet, Berlin, Germany

INTRODUCTION: Dipeptidyl peptidase IV (DPPIV, CD26; EC. 3.4.14.5) is a widely distributed multifunctional membrane glycoprotein. This protein is engaged in immune functions by co-stimulatory effects on activation and proliferation of T-lymphocytes, binding to adenosine deaminase (ADA) and regulation of various chemokines and cytokines. It's peptidase activity is inhibited by HIV-1 Transactivator (Tat) suggesting that DPPIV mediates immunosuppressive effects of Tat protein (1). METHODS: The activity of DPPIV was measured by cleavage of the substrate Gly-Pro-pNA. The ADA activity was assayed by spectrophotometric monitoring the conversion of adenosine to inosine. The 3D structure was obtained by cryo-TEM and single particle analysis. X-ray data were collected at BESSY II, Berlin, Germany and at beamline ID14-2, ESRF, Grenoble. These data were processed with DENZO and SCALEPACK. RESULTS: |
The page contains scientific text discussing mitochondrial disorders, including the molecular diagnosis of mitochondrial disorders, molecular diagnosis of 22 tRNA genes, and the mutations observed in Korean patients with mitochondrial cytopathies. The text also mentions the molecular design of mitochondria and the resolution video-microscopy techniques used to study endosomes. The page further discusses the role of Rab GTPases in vesicle formation, tethering, and fusion, and the cellular roles of mitochondrial fission and fusion in inherited human diseases.
Student Mobility Profile: An Instrument for Measuring the Effectiveness of Efforts to Improve Teaching and Learning
W. S. Bradshaw,1 J. Nelson,2 J. D. Bell;3 Microbiology and Molecular Biology, Brigham Young University, Provo, UT, 1Physiology and Developmental Biology, Brigham Young University, Provo, UT

The process of educational renewal, including efforts to improve the learning of cell biology, should incorporate assessment - a rigorous evaluation of whether or not changes made to a course actually achieved their intended goals. The Student Mobility Profile (SMP) is a novel statistical instrument designed to facilitate such an assessment. The SMP will process data obtained from the beginning and end of a scholastic interval, scores from a first and final exam, or from a pre and post semester problem-solving exercise, for example. An important advantage is that it can be applied post hoc allowing one to utilize existing data from past versions of a course for comparison to recent offerings in which innovations have been attempted. The results of the analysis indicate the presence or absence of systematic factors leading to either improvement or decline in performance of the student population over the time period being assessed. Consequently, instead of indicating whether students have simply acquired the factual information from the course, the analysis reveals the extent to which they have improved in their ability to learn. For example, the teacher might discover whether students have developed additional expertise in practicing an important scientific skill, such as drawing valid conclusions from experimental data. The theory upon which the SMP is based will be provided, along with examples of its application in university science courses. The results of computer simulations to determine the range and limitations of this instrument will also be reported.

Yeast as a Model System for Complex Neurological Problems and Drug Discovery
S. Lindquist,1 A. A. Cooper,2 A. D. Gritle,3 A. Cashikar,4 M. L. Daenwally,1 L. J. Sui,1 T. F. Oteirola,1 C. M. Haynes,2 J. K. Hill,1 B. Bhullar,1 K. Liu,1 K. Xu,1 K. E. Straathorn,4 F. Liu,1 S. Cao,1 K. A. Caldwell,1 G. A. Caldwell,1 G. Marischky,2 R. D. Kolodner,3 J. LaBaer,4 J. Roche,1 N. M. Bonini;2 Whitehead Institute for Biomedical Research, Cambridge, MA, 1School of Biological Sciences, University of Missouri-Kansas City, Kansas City, MO, 2Department of Biology, University of Pennsylvania, Philadelphia, PA, 3Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University, West Lafayette, IN, 4Department of Biological Sciences, University of Alabama, Tuscaloosa, AL, 1Institute of Proteomics, Harvard, Cambridge, MA, 5Institute for Cancer Research, UCSF, La Jolla, CA

Protein conformational changes govern most processes in cell biology. We have developed yeast models of protein folding diseases and used them to investigate mechanisms of cytotoxicity and to screen for novel therapeutic targets. Strong evidence links the misfolding of alpha-synuclein (αSyn) to Parkinson’s Disease (PD). Cellular toxicity depends on the level of αSyn expression and is associated with cytoplasmic inclusions. Our yeast αSyn overexpression model recapitulates many features of αSyn-related pathology, including the inhibition of phosphatase D, the production of reactive oxygen species, and problems in vesicle trafficking. The earliest defect following αSyn induction in yeast was a block in vesicle trafficking from the endoplasmic reticulum (ER) to the Golgi. To identify relevant biochemical pathways and targets we screened an expression library for genetic enhancers and suppressors of αSyn toxicity. The largest class of toxicity modifiers includes proteins functioning at this same trafficking step, including the Rab GTPase Ypt1p, which is also associated with cytoplasmic αSyn inclusions. Elevated expression of Rab1, the mammalian YPT1 homolog, protects against αSyn-induced dopaminergic neuron loss in whole-animal models of PD, and in cultures of rat midbrain, demonstrating the relevance of these results obtained in yeast to mammalian neurons. We also screened >150,000 small molecules for toxicity suppressors. Strikingly, the two most effective compounds in our screen also selectively rescued cultured rat dopaminergic neurons from rotenone and from A35T αSyn expression. We have expanded our efforts to screen for toxicity enhancers and suppressors in a yeast Huntington’s Disease model expressing human huntingtin exon. Our work presents a paradigm for how toxicity is the result of the interaction of particular disease-associated proteins with eukaryotic cell biology and not merely a non-specific response to protein misfolding. The specific pathways elucidated by our yeast models provide new targets for therapeutic intervention.

Genetic Mutations in Cardiac Hypertrophy: Rare Events or Common Etiologies
C. Seidman, Department of Genetics, Harvard Medical School, Boston, MA
Unexplained cardiac hypertrophy, which affects 4% of the population, is a recognized risk factor for adverse cardiovascular events. Human molecular genetic studies have been productive for delineating triggers of hypertrophic remodeling. Studies of hypertrophic cardiac myopathy (HCM) revealed sarcomere protein gene mutations and to date over 400 different mutations in 11 genes have been identified. In part, genetic diversity explains the variable clinical manifestations of HCM. Genetic analyses of unexplained cardiac hypertrophy genetic in subjects from the Framingham Heart Study revealed sarcomere gene mutations in approximately 14%, indicating that allelic variation in sarcomere genes cause both monogenic disorders and common forms of hypertrophy. Some patients clinically diagnosed with HCM do not have sarcomere protein mutations, but defects that alter glycogen storage and metabolism to cause LVH. Gene mutations in the AMP-dependent kinase g2 subunit (PRKAG2) mutations produce a cardiomyopathy characterized by glycogen accumulation in myocyte vacuoles, ventricular hypertrophy and electrophysiological abnormalities (Wolf-Parkinson-White Syndrome and progressive degenerative conduction system disease). Human mutations in the lysosome-associated membrane protein-2 (LAMP2) cause X-linked lysosomal glycogen storage disease that presents with cardiac hypertrophy and pre-excitation, with variable degrees of skeletal myopathy and cognitive impairment, and evolves to heart failure. To delineate cell and molecular responses triggered by these different genetic causes of LVH, we engineered human mutations into mice. Using these models we determined the relationship between histopathology and clinically important events such as arrhythmia vulnerability. Biochemical analyses and transcriptional profiling reveal the cell and molecular signals triggered by gene mutations that result in hypertrophic growth. This discovery system defines potential therapeutic targets to abrogate remodeling and improve prognosis in cardiac hypertrophy.

Development of Novel Cancer Therapeutic Agents Based on Human Apoptotic Pathways
W. Xiang, H19), Department of Biochemistry, UT Southwestern Medical Center at Dallas, Dallas, TX
Apoptosis is a form of cell death that is executed by chains of controlled biochemical reactions. Apoptosis plays important roles during animal development, immune response, elimination of damaged cells, and maintenance of tissue homeostasis. However, apoptosis defects are also associated with tumorigenesis, tumor metastasis, and resistant to therapy. We have been working to understand specific and general biochemical mechanism of apoptosis defects in human cancer cells and trying to design chemical strategies to counter these defects so that we can induce apoptosis specifically and efficiently in a cancer cell specific manner. Progress toward this goal has been made when we designed a small molecule that takes on the function of an apoptosis promoting protein Smac. Smac promotes apoptosis by binding and neutralizing a group of inhibitor of apoptosis protein (IAP) in human cells. Smac normally localizes in mitochondria. During apoptosis, Smac is released from mitochondria to cytosol where IAPs are located. However, in many cancer cells, this release is blocked by the elevation of Bcl-2 and related proteins. Moreover, IAP proteins are also often elevated in many human cancer cells as well. Our Smac-mimicking small molecule can pass through cell membrane and neutralize cytosolic IAP proteins directly, thereby circumventing both Bcl-2 and IAP-mediated apoptosis block that often used by cancer cells. This molecule sensitizes over 80% cancer cell lines from a variety of tissue origins to apoptosis while does no harm for normal fibroblasts and endothelial cells. In animal tumor xerographic studies, the molecules demonstrated excellent efficacy as well. Interestingly, there are about 20% human cancer cell lines shown sensitivity to Smac mimetic treatment alone, indicating that an apoptotic pathway was already turned on in these cells that was blocked by IAPs. Using combination of Smac mimetic treat this might, we will use the development of Smac mimic molecules for cancer therapy as an example to discuss ways to develop novel anti-cancer agents based on our studies on the biochemical pathway of apoptosis in human cancer cells.
Our studies show that caspase-11 interacts physically and functionally with Apal. Inhibition of Apal or caspase-11 expression reduces cell motility. Actin dynamics is altered in caspase-11 knockout cells. These results reveal a novel function of caspase-11 in regulating actin dynamics and cell migration and suggest caspase-11 might be able to regulate inflammatory response at multiple levels.

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Structural Knockout of an ASC Speck Rescues Cells from Apoptosis; the Speck Is a Platform for Apoptosis

K. Ke, A. L. Waite, N. Richards, A. J. Hunt, D. L. Garmy; University of Michigan, Ann Arbor, MI

ASC is a 22-kDa adapter protein composed of a PYD and a CARD domain that is capable of interacting with proteins upstream of IL-1β and NF-κB. Through its interacting partners, ASC affects a wide spectrum of pathways critical to apoptotic and inflammatory events. The activation of the apoptotic pathway in ASC expressing cells often coincides with the formation of an ASC aggregate widely known as a "speck". Although cells with specks progress to apoptosis, it is not clear if the speck is a byproduct or is required for the mediation of the apoptotic process. We studied the functional role of the speck through a technique called structural knockout. An ultrafast laser was used to physically remove the speck from transfected HeLa cells in a surgical procedure we call speckectomy. In contrast to gene knockout, RNAi, and antibody inhibition, this procedure allows spatial and temporal control over the removal of particular sub-cellular structures in cells. Ablation using tightly focused ultrafast laser pulses virtually eliminates the collateral damage that typically associated with laser microsurgery. Our real time tracking of the life cycle of cells expressing ASC and forming a speck revealed that over 48 hours, 60 percent of such cells die, 30 percent undergo mitosis, and 10 percent undergo unsuccessful mitosis. In contrast, cells in which the speck is removed by speckectomy, 75 percent undergo apoptosis; 25 percent undergo unsuccessful mitosis. In the speckectomized cells that undergo mitosis, mitotic duration and morphology is normal. The survival of cells that undergo speckectomy is nearly identical to control cells that express ASC but do not form specks: 75 percent mitosis and 25 percent cell death. Our results strongly support the notion that the physical aggregate known as the speck participates actively in the apoptotic process, perhaps as an agglutinating platform for the apoptotic machinery.

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A Morphologically Conserved Non-Apoptotic Program Promotes Linker Cell Death in C. elegans

M. C. Abraham, Y. Lu, S. Shaham; Developmental Genetics, The Rockefeller University, New York, NY

Apoptosis, a form of programmed cell death characterized by stereotypical morphological features including chromatin compaction and cytoplasmic shrinkage, requires the activity of caspase proteases. Conserved gene pathways controlling caspase activity have been described. Although non-apoptotic, caspase-independent programmed cell death pathways have been postulated, little is known about their molecular constituents or their possible in vivo functions. We have shown that the death of the Caenorhabditis elegans linker cell during development is independent of the casp-3 caspase and all other known C. elegans cell death genes. Linker cell death proceeds efficiently even in animals carrying multiple mutations that block the death of other C. elegans cells destined to die. We demonstrated that the linker cell still dies when we ablate neighboring cells or when the linker cell is abnormally positioned, suggesting the linker cell employs a cell-autonomous program to promote its demise. Furthermore, although the linker cell can be engulfed by the canonical apoptotic cell engulfment program when the cell is at an abnormal position, a previously unidentified engulfment program is required for its clearance at its normal location. Electron microscopy of dying linker cells reveals non-apoptotic features including nuclear crenellation in the absence of chromatin condensation, swelling of mitochondria and endoplasmic reticulum, and accumulation of single- and multi-layered cytoplasmic membrane-bound structures. Remarkably, similar morphological changes occur during the normal developmental death of neurons in the vertebrate spinal cord and ciliary ganglia. Linker cell death is controlled by the microRNA let-7 and by the Zn-finger transcription factor LIN-29, both components of the main developmental timing pathway in the animal. LIN-29 functions within the linker cell to promote its demise, consistent with the idea that the linker cell employs a cell-autonomous death program. We propose that the program executing linker cell death is conserved and may play important roles during vertebrate development.

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Autophagy Functions as a Tumor Suppression Mechanism by Limiting Metabolic Stress

E. White; Rutgers University, Piscataway, NJ

Defective autophagy renders immortalized epithelial cells highly tumorigenic, but how tumor cells survive metabolic stress in vivo during tumorigenesis is not known. In apoptosis-deficient cells, inhibition of autophagy by Akt activation or by anisomycin (which is a specific inhibitor of the mammalian target of rapamycin (mTOR)) results from its ability to mitigate these bioenergetic stresses during ECM detachment.

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Autophagy Restricts Apoptosis during ECM Detachment

J. Debnath, C. Fung; Department of Pathology, University of California San Francisico, San Francisco, CA

The individual units (acini) comprising glandular epithelium possess a hollow lumen that is filled during early oncogenesis. While investigating the generation of luminal space using an in vitro 3D cell culture model, we found that lumen formation involves the selective apoptosis of centrally-located cells within developing acini. However, a hollow lumen still forms when apoptosis is blocked, indicating additional processes regulate luminal clearance. During lumen formation, the central cells of acini contain numerous autophagic vacuoles, suggesting autophagy, a lysosomal process through which a cell digests its own contents, may influence cell fate in the lumen. To clarify the role of autophagy in epithelial cells, we have examined how autophagy regulators (called ATGs) influence 3D morphogenesis. Remarkably, RNAi-mediated downregulation of Beclin1/ATG6 enhances proliferation in developing acini. Although the resulting acini grow larger in size, they exhibit enhanced luminal apoptosis and remain hollow. Remarkably, Beclin1/ATG6 knockdown does not cooperate with Bcl-2-mediated apoptotic inhibition to fill the lumen, despite the decreased autophagy and increased proliferation in these structures. Because epithelial cells die in the lumen due to extracellular matrix (ECM) deprivation, we have further investigated how ECM detachment (anoikis) regulates autophagy. ECM detachment strongly induces autophagy in epithelial cells, confirmed by electron microscopy and LC3/ATG8 relocation to autophagosomes. Importantly, matrix-detached cells still exhibit autophagy when apoptosis is blocked by Bcl-2 expression. In contrast, siRNA-mediated knockdown of ATG5, ATG6/Beclin and ATG7, inhibits autophagy in ECM-detached cells and enhances apoptosis during anoikis. Hence, autophagy protects ECM-detached cells from apoptosis. Recently, we have found that AMP-activated protein kinase (AMPK), is activated during anoikis, indicating that ECM-detached cells exhibit evidence of energy (ATP) depletion. We are currently investigating if the pro-survival effects of autophagy during anoikis result from its ability to mitigate these bioenergetic stresses during ECM detachment.

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Regulation of Mammary Epithelial Anoikis and Morphogenesis by the BH3-Only Protein Bmf

T. Schmelzle,1 A. A. Maileux,1 M. Overholtzer,1 J. S. Carroll,2 N. L. Solimini,1 E. S. Lightcap,3 O. P. Veiby,3 J. S. Brugge,11 Cell Biology, Harvard Medical School, Boston, MA; 2Medical Oncology, Dana-Farber Cancer Institute, Boston, MA; 3Millennium Pharmaceuticals, Inc., Cambridge, MA

Mammary epithelial cells cultured in a three-dimensional basal membrane substrate model system assemble into polarized, proliferation-arrested structures with a hollow lumen that resemble acini found in the mammary gland. Using such a 3D model with human MCF-10A cells our objective is to identify and characterize the signaling pathways and mechanisms that are required to form and maintain the normal acinar architecture in vitro. Microarray experiments revealed a pronounced proliferation arrest signature starting around culture day 5 as the principal transcriptional program during morphogenesis whose hallmark was the analysis of several different proliferation markers. We have also exploited potential similarities between anoikis (epithelial cell death in response to loss of adhesion) and the selective death of inner cells of 3D structures during lumen formation. Our comparative microarray studies have identified the proapoptotic BH3-only protein Bmf as a candidate regulator involved in MCF-10A anoikis and 3D lummn formation. Bmf RNA levels are upregulated in suspension and morphogenesis samples, and knockdown of Bmf by RNAi results in significant protection from cell death in both culture conditions. Knockdown of BH3-only proteins other than Bmf or Bid, however does not affect apoptosis in MCF-10A anoikis or morphogenesis, suggesting specific processing of death signals rather than a BH3-level ‘threshold’. Transcriptional regulation of Bmf is specifically observed in response to detachment from matrix and significantly suppressed upon oncogenic activation of the MEK/Erk and PI3K pathways. Moreover, downregulation of Bmf, when combined with a proliferative oncogene, confers anchorage-independent growth. Taken together, our findings contribute new insights into the mechanisms whereby oncogenes and signaling aberrations disrupt the regulation of normal mammary epithelial morphogenesis and serve as a model for events associated with the initiation and progression of epithelial tumors.
Innovations in Fluorescence Imaging of Cellular Functions Using Fluorescent Proteins
A. Miyawaki; Lab for Cell Function Dynamics, RIKEN Brain Science Institute, Wako City, Japan

Fluorescence imaging has enabled us to decipher spatio-temporal information coded in complex tissues. Genetically encoded probes that enable fluorescence imaging of enzymatic activity have been constructed by fusing fluorescent proteins to functional proteins that are involved in physiological signaling. The probes are introduced into an intact organism and targeted to specific tissues, cell types, or subcellular compartments, thereby allowing specific signals to be extracted more efficiently than was previously possible. I will describe how this approach has met cell-biologists' demands and desires.

Site-specific Protein Labeling with Biophysical Probes in Living Cells Using Enzyme Ligases
A. Ting; Department of Chemistry, Massachusetts Institute of Technology, Cambridge, MA

Our lab develops new methodology for imaging protein trafficking and function in living cells. We will describe site-specific protein labeling techniques based on enzyme ligases, quantum dot targeting strategies, protein-protein interaction detection methods, and single molecule imaging of neuronal receptors.

Demonstration of p130Cas as a Direct Mechano-sensor by In Vitro Stretching of Single Molecules
Y. Sawada,1 M. Tamada,2 B. J. Dubin-Thaler,2 R. Sakai,3 S. Tanaka1 M. P. Sheetz2,1 Department of Biological Sciences, Columbia University, New York, NY, 2Graduate School of Medicine, Kobe University, Kobe, Japan, 3Growth Factor Division, National Cancer Center Research Institute, Tokyo, Japan, 4Department of Orthopaedic Surgery, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan

Cellular responses to mechanical force underlie many critical functions from normal morphogenesis to carcinogenesis, cardiac hypertrophy, wound healing and bone homeostasis. Recent studies indicate that various signaling pathways are involved in force transduction, including tyrosine phosphorylation. However, because suitable in vitro experimental systems have not been available, direct sensors of forces, i.e. force receptors, have not been identified with the exception of ion channels. We previously showed that cytoskeletal complexes (Triton cytoskeletons) can transduce stretching forces into focal contact protein binding and Rap1 activation. Because we found that tyrosine phosphorylation of p130Cas (By Src family kinases (SFKs)) was involved in stretch-dependent Rap1 activation in intact cells, we explored whether activation of the kinase was primarily responsible for stretch-increased phosphorylation of Cas and found that cell stretching caused no apparent change in c-Src activity while Cas phosphorylation increased. To test if modification of Cas could enhance its phosphorylation, we developed an experimental system for in vitro stretching of single molecules (ISS). Using the ISS system, we mechanically stretched bacterially expressed Cas substrate domain (CasSD) and found stretching of CasSD remarkably enhanced its phosphorylation by c-Src, FynT, or Abi1 but not Nck or Zap-70 kinases, independent of kinase activation. By YFP amino-terminal swapping assay based on the interaction between amino- and carboxy-terminal domains of YFP, which was expected in bimolecular fluorescence complementation, we confirmed that CasSD molecules are stretched in the ISS system. In intact cells, an antibody that bound to unfolded CasSD preferentially recognized Cas molecules in the peripheral regions of spreading cells where higher forces were expected and phosphorylated Cas was found. Thus, Cas converts force into a biochemical signal through mechanical unfolding of its substrate domain, causing priming to phosphorylation. We suggest that Cas substrate priming is the first example of a more general mechanism for signal transduction.

Computationally Multiplexed Imaging of Rho GT-Pase Activities
M. Machacek,1 L. Hodgson,2 P. Nabant,3 O. Pertz,4 F. Shen,5 K. M. Hahn,6 G. Danuser5; 1Department of Cell Biology, TSRI, La Jolla, CA, 2Department of Pharmacology, University of North Carolina at Chapel Hill, Chapel Hill, NC, 3Department of Immunology, TSRI, La Jolla, CA, 4Department of Pathology and Moores Cancer Center, University of California at San Diego, La Jolla, CA

We present a computational approach to reconstruct the coordinated dynamics of a theoretically unlimited number of protein activities visualized by biosensors. Biosensors are emerging as a powerful tool for measuring the activities of functionally related proteins and by doing so systematically uncovering the spatio-temporal characteristics of entire signaling networks. In practice, this approach is limited by the number of spectrally separable biosensors and by the formidable technical complexity of multiplex biosensor measurements. Here, we demonstrate that by statistical analysis of the random temporal fluctuations of signaling network activity, functional relationships between network components can be established. The hierarchy of signaling events in the entire network can be inferred indirectly, by compiling activity fluctuations from a series of separate experiments, each using a distinct set of biosensor probes. Registration of activity fluctuations on a common time scale across the experiments is achieved either by measuring two activity probes per experiment, with one serving as the time fiduciary linking different experiments, or by observing a morphological event sequence that can serve as a fiduciary. We use this approach to reconstruct the timing of the small GTPase family members Rac1, RhoA and Cdc42 during cell protrusion. We found that the GT-Pases were activated in a strictly conserved sequence relative to protrusion. Activation of RhoA was synchronous with protrusion, Cdc42 was delayed by 20 sec, and Rac1 by 70 sec, respectively. As a proof of principle we confirmed this relative timing by monitoring Cdc42 and RhoA activities simultaneously in the same cell. We found an exact quantitative match between the indirectly and the directly reconstructed event sequence.

High-Resolution, Doxycycline-dependent Phototoxic Gene Expression in Eukaryotic Systems
S. B. Cambride,1 F. Calegari,2 K. Anastassidou,3 D. Geisser,1 T. Bonhoeffer1; 1Max-Planck-Institute of Neurobiology, Munich-Marienried, Germany, 2Max-Planck-Institute of Cell Biology and Genetics, Dresden, Germany, 3Biotec Centre, Dresden, Germany

To improve our understanding of gene function, tools are needed which allow the manipulation of gene function in a specific cell at a specific time. Unfortunately, none of the current conditional gene expression paradigms provides the required spatial and temporal resolution. To overcome this, we synthesized a reversibly inhibited, photosensitive (‘caged’) doxycycline analogue for precise light-controlled activation of transgenes based on the Tet-on system. With this method, gene expression can simply be induced with DAPI excitation light after adding the membrane-permeant caged doxycycline directly to the medium. Phototoxic gene expression was achieved in diverse biological systems including mice, plants, and Xenopus. We demonstrate that this method allows induction of transgenes with very high spatial resolution, including single cells. Moreover, levels of UV light needed for induction were harmless as no signs of toxicity could be detected. Thus, phototoxicated gene expression will be a very powerful tool for biomedical research.

Optical Tweezers as a Sensor for Intracellular Mechanical Properties of Endothelial Cells
M. Mengistu,1 D. H. Ou-Yang,2 L. J. Lowe-Krentz2; 1Biological Sciences, Lehigh University, Bethlehem, PA, 2Physics, Lehigh University, Bethlehem, PA

Characterizing the mechanical properties of cells is becoming increasingly important for understanding processes such as cell movement, shape, growth, and even the development of disease. In this study, we explore the mechanical properties of endothelial cells, whose dysfunction leads to the development of atherosclerosis. We employ the optical tweezers technique as a sensor, a technique that allows us to probe very local mechanical properties to account for cytoskeletal inhomogeneity, as well as measure cell dynamics. We extracted the viscoelastic moduli, G’ and G”, which are measures of elasticity and viscosity respectively, in endothelial cells using endocytosed polystyrene beads and intrinsic granular structures as probes. Our results show that endothelial cells have a dominant elastic modulus, with an average G’ value varying between 3.44±102 - 7.13±102 dyn/cm2. These results were consistent with intracellular cytoskeletal distributions, where the higher G’ values correspond to probed regions of the cell that are nearest to the nucleus, suggesting that cell elasticity increases in cytoskeleton-rich regions near the nucleus relative to cell peripheries. We also treated the cells with cytochalasin B and nocodazole, to depolymerize actin filaments and microtubules respectively, then measured the viscoelastic moduli to obtain their contribution in the mechanical properties of endothelial cells. The disruption of actin filaments affected cell dynamics drastically, but did not significantly change cell elasticity, with average G’ values around 300 dyn/cm2. Microtubule depolymerization, on the other hand, lead to an increase in cell elasticity, G’=103 dyn/cm2, possibly due to compensating stress fibers made by endothelial cells. Our results establish optical tweezers as effective and non-invasive probes of viscoelastic moduli in endothelial cells, where they can be applied to study the effect of flow on their mechanical properties, as well as mechanosensing and mechanotransduction events carried out by these cells.
Polarized Targeting of E-cadherin to Sites of Cell-Cell Contact in Early Embryos and Epithelial Cells Requires Ankyrin-G and Beta-2-spectrin

K. Kizhatil,1 J. Q. Davis,1 W. Yoon,1 B. L. M. Hogan,1 V. Bennett1; 1Cell Biology and HHMI, Duke University, Durham, NC, 2Cell Biology, Duke University, Durham, NC

Cadherins define specialized membrane domains at sites of cell-cell contact. In epithelial cells, E-Cadherin function requires an interaction with the membrane skeleton. However, the mechanism of E-cadherin-membrane skeleton interaction is unknown. We report here that E-cadherin targeting to sites of cell-cell contact requires the membrane skeleton proteins ankyrin-G and beta-2-spectrin. Moreover, we also present the first evidence that ankyrin-G binds the cytoplasmic domain of E-cadherin directly with high affinity. Ankyrin-G and beta-2-spectrin colocalize with E-cadherin at the plasma membrane of mouse early embryo blastomerses and relocate to sites of cell-cell contact during compaction. Depletion of ankyrin-G or beta-2-spectrin in one cell of a 2-cell embryo by siRNA injection reduces E-cadherin at sites of cell contact between the daughter cells of the injected cell. These daughter cells fail to compact and fail to undergo cytokinesis. In polarized human bronchial epithelial (HBE) cells the levels of ankyrin-G and beta-2-spectrin increase in parallel with the height of the newly formed lateral membrane. Depletion of ankyrin-G or beta-2-spectrin by siRNA results in inability to form the HBE lateral membrane and also disrupts the de novo biogenesis of the lateral membrane during cytokinesis. Several experiments in HBE cells demonstrate that ankyrin-G and beta-2-spectrin collaborate in a lateral membrane assembly pathway. Depletion of ankyrin-G or beta-2-spectrin in HBE cells results in the accumulation of E-cadherin in the trans-Golgi network (TGN) but not the cis/Golgi, ER or endosomes. Accumulation of E-cadherin in the TGN also resulted from microtubule disruption using nocodazole in HBE cells. Nocodazole washout allowed visualization of trafficking intermediates of E-cadherin which contained ankyrin-G and beta-2-spectrin. These results suggest a model where E-cadherin binds ankyrin-G which is then coupled to microtubule motors proteins by beta-2-spectrin for post-TGN transport along microtubules to sites of cell-cell contact in epithelial cells and early embryos.

Tyrosine-phosphorylated Cortactin Regulates Rosettes II and MLCK to Maintain the Integrity of Cell-Cell Contacts

F. M. Helwani,1 S. Verna,1 S. A. Weed,4 A. S. Yap3; 1Institute of Molecular Biosciences, University of Queensland, Brisbane, Australia, 4Mary Babb Randolph Cancer Center, West Virginia University, Morgantown, WV

Epithelial architecture is critically influenced by the ability of E-cadherin adhesion receptors to support the integrity and cohesion of cell-cell interactions. It has long been recognised that E-cadherin functions in close cooperation with the actin cytoskeleton; indeed, E-cadherin can actively regulate actin during cell-cell contact formation and reorganisation. More recently, we demonstrated that E-cadherin could influence at least two modes of actin regulation: 1) Arp2/3 mediated actin assembly; and 2) Myosin II-dependent contractility. The molecular intermediates involved in linking E-cadherin adhesion to these different actin regulatory processes are a current area of focus in our laboratory. One such intermediate is cortacin, a multifunctional actin binding protein implicated in a wide variety of cellular processes and signalling pathways within the cell. Cortacin function is tightly regulated by tyrosine phosphorylation in a number of cellular contexts. Previously we and other groups identified a role for cortacin in regulating Arp2/3-based actin assembly at cadherin contacts. Here we report an additional role for cortacin at cadherin contacts that is distinct from its role in Arp2/3 regulation. We found that cortacin controls Myosin II activity at cadherin contacts in a Src-dependent manner. Cortacin was necessary for the recruitment and activation of Myosin II at cadherin contacts, a process that may reflect the ability of cortacin to bind and recruit MLCK to contacts. Importantly, tyrosine phosphorylated cortacin was essential for its role in regulating Myosin II. Therefore we propose that cortacin might provide a link between these two functionally distinct modes of cadherin-regulated actin organization, by integrating signalling pathways that are activated downstream of E-cadherin adhesion.
Microtubule Plus-end Tracking Proteins Target Gap Junction Proteins Directly from the Cell Interior to Adhesions Junctions
Gap junctions are intercellular channels that connect the cytoplasms of adjacent cells. For gap junctions to properly control organ function and electrical synchronization in the heart and brain, connexin 43-based hemichannels must be correctly targeted to cell-cell borders in enriched regions called plaques. Mislocalization of plaques in cardiomyocytes is a major cause of ischemia related sudden cardiac death. While it is generally accepted that plaques form via lateral diffusion of hemichannels following microtubule-mediated delivery to the plasma membrane, we provide evidence for direct targeting of hemichannels to plaques at cell-cell junctions through a pathway dependent on microtubule dynamics, the adherens junction proteins N-cadherin and beta-catenin, the microtubule plus-end tracking protein (+TIP) EB1, and its interacting protein p150(Glucl). Live cell microscopy reveals cortical capture of microtubules at cell-cell borders as well as an intimate association between microtubules and gap junction plaques. Dynamic studies using both fluorescence recovery after photobleaching (FRAP) and total internal reflection fluorescence (TIRF) demonstrate rapid, preferential delivery of cytoplasmic hemichannels to adherens junctions in a microtubule dependent fashion. siRNA knockdown and cadherin blocking peptides confirm involvement of EB1, p150(Glucl), beta-catenin, and cadherin on gap junction plaque formation. In conclusion, we have found that preferential tethering of microtubule plus-ends at the adherens junction promotes delivery of connexin hemichannels directly into plaques at the cell-cell border. Moreover, hemichannel delivery to the cortical membrane is facilitated by interaction between +TIP proteins and the adherens junction. Together, these data support a novel mechanism for gap junction delivery to points of cell-cell contact.

Cellular Responses to Pore-forming Toxins
G. van der Goot, L. Garidel; Global Health Institute, Ecole Polytechnique Federale de Lausanne, Lausanne, Switzerland
Many pathogenic organisms produce pore-forming toxins as virulence factors. Target cells however mount a response to such membrane damage. We have found that upon infection with pore-forming toxin producing bacteria, cells respond by switching on lipid metabolic pathways that promote survival. More specifically, toxin induced membrane permeabilization leads to a decrease in cytoplasmic potassium, which triggers activation of the NALP3 and IPAF inflammasomes, which in turn activate caspase-1. We demonstrate a novel role for caspase-1, which is to trigger the activation of the central regulators of membrane biogenesis, the Sterol Regulatory Element Binding Proteins (SREBPs). Upregulation of lipid metabolic pathways in turn promotes cell survival.

Coxsackievirus Entry through Epithelial Tight Junctions Requires Occludin and the Small GT-Pases Rab34 and Rab5
C. B. Coyle, J. M. Bergelson; Infectious Diseases, The Children’s Hospital of Philadelphia, Philadelphia, PA
Group B coxsackieviruses (CVB) infect by crossing the intestinal mucosa, which is lined by polarized epithelium. We previously found that CVB infection of polarized epithelial cells in vitro requires virus movement to the tight junction (TJ), and that interaction with the coxsackievirus and adenovirus receptor (CAR), a TJ component, is essential for subsequent internalization and infection. Virus entry from the TJ depends on functional cavelin 1, as well as on phosphorylation of cavelin, unlike many endocytic processes it is independent of dynamin 1. Because virus entry is accompanied by a transient loss of junctional integrity, we have used fluorescence microscopy to examine the localization of TJ proteins during the early stages of infection. In cells exposed to virus, a single TJ protein, occludin, moved from the junction and was observed both with virus in cavelin-containing vesicles, and in typical macroinosomes. Depletion of occludin with siRNA prevented virus entry and infection, trapping virus at the TJ, as did inhibitors of macropinocytosis (amiloride and rottlerin). Rab34, a GTPase that functions in macropinocytosis, was concentrated at the TJ even in infected cells, and both dominant-negative Rab34 and Rab34 siRNA prevented internalization of virus and occludin. Furthermore, dominant-negative Rab5 prevented infection and entry, as did siRNA depletion of Rabankyrin-5, a Rab5 effector also implicated in macropinocytosis. These results indicate that virus entry is coupled to the internalization of occludin by a macropinocytic process, and that in addition to functional cavelin) Rab34, Rab5, and Rabankyrin-5 are needed for virus entry from the TJ. The results suggest that, in polarized epithelial cells, traffic from the TJ may involve cellular machinery associated both with cavelar endocytosis and macropinocytosis.

Differential Activation and Function of Rho GT-Pases during Salmonella-Host Cell Interactions
J. C. Patel, J. E. Galan; Section of Microbial Pathogenesis, Yale University New Haven, CT
Salmonella enterica, the cause of food-poisoning and typhoid fever, has evolved sophisticated mechanisms to modulate Rho-family GT-Pases to mediate specific cellular responses such as actin remodeling, macropinocytosis, and nuclear responses. These responses are largely the result of the activity of a set of bacterial proteins, SopE, SopE2 and SopB, which upon delivery into host cells via a type III secretion system, activate specific Rho-family GT-Pases either directly (SopE and SopE2) or indirectly (SopB). We show here that different Rho-family GT-Pases play a distinct role in Salmonella induced cellular responses. In particular, we find that upon infection Salmonella elicits GTP loading on Rac and Cdc42 independently. Activation of Rac leads to actin cytoskeleton rearrangements whilst Cdc42 activation mediates nuclear responses, such as IL8 induction. Moreover, we report that SopB stimulates cellular responses by activating SGEF, a host cell encoded guanine nucleotide exchange factor (GEF) for RhoG, which we found also plays a central role in the actin cytoskeleton remodeling stimulated by Cdc42 activation mediates nuclear responses, such as IL8 induction. We demonstrate a novel role for caspase-1, which is to induce the activation of the central regulators of membrane biogenesis, the Sterol Regulatory Element Binding Proteins (SREBPs). Upregulation of lipid metabolic pathways in turn promotes cell survival.

Membrane Nanotubes Connect Human T-cells and Could Facilitate Intercellular Transmission of HIV-1
S. Sowinski,1 C. Jolly,2 Q. Sattentau,2 D. Davis1; 1Cell and Molecular Biology, Imperial College London, London, United Kingdom, 2Pathology, William Dunn School of Pathology, Oxford, United Kingdom
We report that T-cells could be connected by long intercellular membrane tethers, a third of such connections having derived from cytokinesis. Connections derived from cytokinesis contained both filamentous actin and microtubules, and were accessible to the small cytoplasmic dye calcein. In contrast, T-cell membrane nanotubes formed independently of cell division contained filamentous actin but not microtubules, and excluded the cytoplasmic dye calcein. Membrane tethers from differentially labeled cells did not freely mix within membrane nanotubes, and instead met at junctions, revealing synaptic connections within T-cell membrane nanotubes. Furthermore, we observed that the HIV-1 Gag and Env proteins were present in membrane nanotubes connecting HIV-1-infected CD4+ T-cells to uninfected CD4+ T-cells. HIV-1 Gag and Env proteins were detected within uninfected T-cells linked via membrane nanotubes to chronically infected T-cells suggesting that membrane nanotubes may provide a novel mechanism for direct cell-to-cell dissemination of HIV-1.

Differential Mechanisms of Chlamydia Egress from Non-phagocytic Cells
K. Hybiske, R. S. Stephens; Department of Infectious Diseases, School of Public Health, University of California Berkeley, Berkeley, CA
The egress of infectious Chlamydia from its host cell is a critical stage in its pathogenic cycle. Although it has been proposed that release may occur by a cytokidal or exocytic mechanism, this process remains poorly understood at the cellular and molecular level. To this end, we employed time-lapse confocal fluorescence videomicroscopy to determine what processes mediate Chlamydia egress. HeLa cells with stable expression of cytosolic GFP were generated by retroviral transduction. Upon infection with Chlamydia, the bacteria were readily internalized and sequestered into a parastropliphic vacuole, or inclusion, that increased in size within the cytoplasm of the host cell. Due to the inherent ability of the Chlamydia inclusion to exclude cytosolic GFP, our technique enabled unambiguous visualization of inclusions and their dynamic processes in live cells. Analysis of infected cells in the throes of Chlamydia egress revealed two distinct mechanisms of lysis: a lytic mechanism involving rapid rupture of the inclusion and host cell; (i) a novel process we have named “extrusion”, in which the inclusion is pushed outward from the cell body into a tethered, membrane- and cytoplasm-bound compartment. Extrusion-based egress had a 53% occurrence rate, while lysis occurred for 47% of the cells that underwent egress. Additionally, we characterized essential cellular mediators of each egress pathway. Lysis was comprised of a stepwise sequence of intracellular permeabilizations - first the Chlamydia inclusion, subsequently followed by nuclear permeabilization and finally plasma membrane rupture. Treatment with cycloheximide, yielded no effects on either egress mechanism. Thus, we have identified and characterized two pathways for Chlamydia release from infected cells; extrusion represents a novel release mechanism for obligate intracellular bacteria.
Mechanism of Monomeric Actin sequestration by Toxofilin from Toxoplasma gondii: A Structural-Functional Study

S. Lee, Y. Li, G. Rebowsky, I. Tardieux, R. Dominguez; 1Physiology, University of Pennsylvania, Philadelphia, PA, 2Département des Maladies Infectieuses, Institut Cochin, Paris, France

Toxoplasma gondii is a parasite belonging to the phylum Apicomplexa, which includes human pathogens of major medical importance, such as Plasmodium the agent of malaria. In contrast to other pathogens such as Listeria, which utilize the host-cell actin cytoskeleton to move within and between cells, T. gondii has developed its own actin cytoskeletal system, necessary for host invasion. T. gondii is unique in that it presents a strikingly low amount of F-actin, suggesting that G-actin-binding proteins play a key role in the regulation of actin dynamics and pathogenicity. Toxofilin is an abundantly expressed G-actin-binding protein of T. gondii. The mechanism of G-actin sequestration by toxofilin is unknown. We determined the crystal structure of toxofilin complexed with actin. Toxofilin interacts extensively with actin, displaying multiple, apparently independent, actin-binding sites. The central region of toxofilin binds in the cleft between actin subdomain 1 and 3, a common binding site for many actin-binding proteins. Despite the lack of sequence identity with known eukaryotic actin-binding proteins, this interaction is strikingly similar to that of the WASP-homology domain 2 with actin. However, toxofilin presents two additional, and so far unique, actin-binding sites. This result suggested that a single molecule of toxofilin could interact with multiple actin molecules simultaneously. Analytical ultracentrifugation studies of complexes of toxofilin fragments with actin confirmed this observation, and indicated that toxofilin could bind up to three actin monomers. Toxofilin is also the only actin-binding protein shown so far to interact directly with the nucleotide in actin, resulting in a dramatic increase in G-actin polymerization. This ability to bind multiple actin monomers and bind G-actin could be used as a tool to delve into toxofilin's role in T. gondii, emphasizing its importance for pathogen invasion. This structure provides a molecular target for the development of drugs against this deadly group of parasites.

1400 Determinants of the Interaction between the CENP-A Nucleosome and Its Associated Complexes

D. R. Foltz,1 D. W. Black,1,2 L. E. T. Jansen,1,2 A. O. Bailey,3 J. R. Yates,4 D. W. Cleveland; 1Cell Biology, Ludwig Institute for Cancer Research, La Jolla, CA, 2Department of Cellular and Molecular Medicine, UCSD, La Jolla, CA, 3Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, PA, 4Department of Cell Biology, The Scripps Research Institute, La Jolla, CA

Proper chromosome segregation requires the establishment of a specialized chromatin domain known as the centromere that is characterized by its incorporation of a specialized nucleosome containing the histone H3 variant CENP-A. This centromeric histone is thought to be important for the epigenetic inheritance of the centromere. The CENP-A nucleosome is responsible for recruiting a set of six protein variant CENP-A proteins comprised of CENP-C, H, M, N, U(50) and T. The CENP-A complex is in turn responsible for recruiting a set of CENP-A distal (CAD) components (CENP’s K, L, P, Q, R and S). CENP-T and CENP-S contain a region of similarity to a subdomain of the histone fold present in all histone H3 variants. Here we show that knockdown of CENP-T results in the disruption of the CENP-A complex and dramatically alters mitotic progression. Deletion of histone-like domain of CENP-T eliminates it centromeric localization. Tandem affinity purification of CENP-T co-purifies components of the CENP-A complex. Use of deuterium exchange mass spectrometry demonstrates that the 22 amino acid CENP-A Targeting Domain (CADT) that is responsible for localizing it to the centromere confers a unique structural rigidity to both the pre-nucleosomal CENP-A/H4 heterotetramer and to nucleosomes assembled from it, independent of the DNA template. To assess whether this unique nucleosome structure is recognized by CENP-A nucleosome recruiting partners, histone H3-CADT containing nucleosomes have been purified by tandem affinity purification and their composition compared with that of histone H3.1 or CENP-A nucleosomes. The FACT complex, as well as CENP-D, -E, and -F, originally isolated with CENP-A nucleosomes, are also found associated with the centromere targeted histone H3 nucleosomes containing the FACT complex, demonstrating that at least a subset of the CENP-A nucleosome interaction partners are able to recognize the unique, CENP-A dependent CENP-A nucleosome structure.

1401 Xenopus Cenp-V Is Required to Anchor Microtubules to Kinetochores and Centrosomes in Mitosis

M. J. Emanuele, T. Stukenberg; Biochemistry and Molecular Genetics, University of Virginia, Charlottesville, VA

The anchorage of microtubules within the mitotic spindle apparatus is critical for generating the forces required for chromosome movement. We report here the characterization of the Xenopus Cenp-V (Centromere associated protein V) and demonstrate its role in microtubule attachment at centrosomes and kinetochores. Cenp-V was identified using sequence analysis searches for the a vertebrate homolog of yeast Dam1. We show that Cenp-V localizes to the kinetochore, centrosome and mitotic spindle in Xenopus extracts. Cenp-V depletion results in an elongated mitotic spindle with reduced tubulin incorporation and unaligned chromosomes. Cenp-V interacts by immunoprecipitation with the kinetochore proteins Zwint, Mis12 and CLIP-170. Inhibition of Cenp-V reduces chromosome-microtubule binding in vitro, and the effect is equivalent to that observed for inhibition of the outer plate protein Ndc80. Since stably attached spindle microtubules are resistant to depolymerization we challenged bipolar spindles assembled in cyclized extracts with a low dose of nocodazole. We found that Cenp-V inhibition generated spindles lacking stabilized microtubules. Cenp-V also interacted with the centromere protein gamma-tubulin. After Cenp-V depletion, centrosome mediated astras nucleated with normal kinetics but rapidly lost their ability to anchor microtubules. Real time imaging revealed microtubule bundles floating away from centrosomes in Cenp-V depleted extracts. We conclude that Cenp-V has a role in microtubule binding at both the centrosome and kinetochore in Xenopus extracts.

1402 Molecular Dissection of Kinetochore-based Regulation of Microtubule Dynamics in Drosophila

D. W. Basu, J. Tupper, J. Sharpe; 1Physiology and 2Pharmacology, College of Medicine, Bronx, NY, 3Biology, University of North Carolina, Chapel Hill, NC

The centrosome is a multiprotein complex that serves as the primary interface between chromosomes and microtubule spindle. As such, kinetochores perform a variety of regulatory and mechanical functions central to chromosome segregation during mitosis. A particularly interesting facet of kinetochore function is its ability to control the assembly state of associated microtubule plus-ends: Kinetochore-induced microtubule depolymerization stimulates poleward chromatid motility, while polymerization drives the movement of chromatids towards the spindle equator. To dissect the molecular machinery responsible for this, we have utilized RNAi to deplete all of the known effectors of microtubule assembly/dissassembly and severing in Drosophila S2 cells and assayed the effects on both chromosome motility and microtubule assembly state using live cell microscopy and photobleaching/FRAP analyses. The proteins examined in this study include I) the microtubule assembly promoters CLIP199, EB1, Minispinules and Mast II) the disassembly promoters KLP67A, KLP59C and KLP10A and III) the severing proteins Katamin, Spastin, and Fidgetin. Our findings reveal a remarkable integration of the activities of these three classes of proteins. We have observed functional antagonism between multiple assembly and disassembly factors throughout mitosis indicating that the net assembly state of kinetochore-associated microtubule plus-ends is determined by the cumulative activities of these proteins. Alternatively, severing proteins appear to enable kinetochore microtubule dynamics but do not directly influence the their assembly state. Prior to anaphase, severing proteins are required for normal microtubule polymerization while subsequently, after anaphase onset, severing is required for normal microtubule depolymerization. In light of these findings, a new model of kinetochore-driven chromosome motility will be presented.

1403 Defining Discrete Steps in Centriole Assembly Using an In Vivo Imaging Approach

A. Dammann, A. Desai, K. Oegema; Department of Cellular and Molecular Medicine, University of California–San Diego, Ludwig Institute for Cancer Research, La Jolla, CA

Centrioles are small cylindrical structures consisting of a 9-fold symmetric array of stable microtubules. Centrioles have two critical functions in cells: (1) they recruit pericentriolar material to form centrosomes that contribute to cell division, and (2) they template the formation of cilia, which perform a variety of motile and sensory functions. The first post-fertilization division of the C. elegans embryo has recently emerged as a powerful system to study centriole assembly. A set of 4 C. elegans proteins (SAS-4, SAS-5, SAS-6 and ZYG-1) have been identified that localize to centrioles and are specifically required for their duplication. Due to their small size, centriole duplication has typically been studied by electron microscopy, which has limited the repertoire of experiments that can be used to study them. To circumvent this problem, we are developing light microscopy-based methods to visualize centriole assembly in living embryos. We began by analyzing the kinetics of recruitment of two centriole components, SAS-4 and SAS-6, that are incorporated into daughter centrioles during assembly and do not subsequently exchange with the cytoplasm. Ultrastructural analysis has shown that centriole assembly proceeds in two discrete steps: 1) the formation of a structural intermediate called central tube, followed by 2) assembly of the microtubule containing outer centriole wall. Our analysis of the recruitment of SAS-6 and SAS-4 indicates that they are incorporated during the assembly of the central tube and outer centriole wall, respectively. As the first constituents of these centriolar substructures to be identified, this work represents an important step towards a molecular understanding of centriolar development. The methods described here have demonstrated should also serve as a useful tool for defining the relationship between the steps in pathway of centriole assembly, and in analyzing the role of other components implicated in duplication.
HsSAS-6 Is a Cell Cycle Regulated Centriolar Protein Required for Daughter Centriole Formation

We have characterized hPOC5, required for procentriole assembly but for that of the distal end of centrioles, revealing that centriole duplication and centriole differentiation correspond to structurally distinct assembly processes (1). Interestingly, hPOC5 family revealed an evolutionary divergence between Drosophila or C. elegans, which have lost hPOC5 homologues, and the other animals. The divergence of centriole structure in those species could result from the loss of the distal part of centrioles, concomitantly with the loss of genes from the POC5 family, or from centrin sub-families (2). Candidates for hPOC5-interacting proteins were identified by a two-hybrid approach. We selected a p83 protein whose interaction was confirmed biochemically. p83 defines a conserved family in eukaryotes, and the Chlamydomonas homologues is present in centriole fractions (3). In agreement, a GFP-tagged version of p83 labeled centrioles in human cells. When overexpressed, GFP-p83 labeled microtubules. Its binding stabilized microtubules and progressively induced the formation of long bundles along the plasma membrane or of a thick and continuous bundle apposed to the nucleus periphery, in which microtubules were closely linked by regularly spaced bridges. Endogenous p83, expressed at a low level, is likely restricted to centrioles. We speculate that p83 would interact with the intraluminal centrin- and hPOC5-containing structures and bind centriole blades to stabilize them or participate in their assembly. Interestingly, the p83 family is conserved in Drosophila and C. elegans. Characterizing the mechanisms in which p83 participates could help deciphering the evolution of centriole structure and function among divergent animal species. 1- Azimzadeh, et al., Submitted. 2- Azimzadeh J, Bornens, M (2004) In Centrosomes In Development and Disease Ed EA Nigg, Wiley-Vch 3- Keller et al., Curr Biol. 2005 Jun 21;15(12):1090-8.

Dissecting the Centriole/Basal Body Structure in Animal Cells

J. Azimzadeh,1 A. Delouve,1 U. Euteneuer,1 L. Daviet,1 M. Bornens1; 1Cnrs/umr144, Institute Curie, Paris, France, 2Zellebiologie, Adolf-Butenandt-Institut, University of Munich, Germany, 3Hybrigenics, Paris, France.

We have characterized hPOC5, a conserved centrin-binding protein containing SH3-like centrin-binding repeats, concentrated in the distal lumen of centrioles from human cells. hPOC5 is not required for procentriole assembly but for that of the distal end of centrioles, revealing that centriole duplication and centriole differentiation correspond to structurally distinct processes (1). Interestingly, hPOC5 family revealed an evolutionary divergence between Drosophila or C. elegans, which have lost hPOC5 homologues, and the other animals. The divergence of centriole structure in those species could result from the loss of the distal part of centrioles, concomitantly with the loss of genes from the POC5 family, or from centrin sub-families (2). Candidates for hPOC5-interacting proteins were identified by a two-hybrid approach. We selected a p83 protein whose interaction was confirmed biochemically. p83 defines a conserved family in eukaryotes, and the Chlamydomonas homologues is present in centriole fractions (3). In agreement, a GFP-tagged version of p83 labeled centrioles in human cells. When overexpressed, GFP-p83 labeled microtubules. Its binding stabilized microtubules and progressively induced the formation of long bundles along the plasma membrane or of a thick and continuous bundle apposed to the nucleus periphery, in which microtubules were closely linked by regularly spaced bridges. Endogenous p83, expressed at a low level, is likely restricted to centrioles. We speculate that p83 would interact with the intraluminal centrin- and hPOC5-containing structures and bind centriole blades to stabilize them or participate in their assembly. Interestingly, the p83 family is conserved in Drosophila and C. elegans. Characterizing the mechanisms in which p83 participates could help deciphering the evolution of centriole structure and function among divergent animal species. 1- Azimzadeh, et al., Submitted. 2- Azimzadeh J, Bornens, M (2004) In Centrosomes In Development and Disease Ed EA Nigg, Wiley-Vch 3- Keller et al., Curr Biol. 2005 Jun 21;15(12):1090-8.

Activities and Regulation of Full-length Formin Proteins

S. Maiti,1 J. B. Moseley,1 A. DuPage,1 O. Sokolova,2 N. Grigorieff,2 B. L. Goode1; 1Biology Department, Brandeis University, Waltham, MA, 2Biochemistry Department, Brandeis University, Waltham, MA.

Formins nucleate actin assembly and processively cap the fast-growing ends of actin filaments, but the molecular mechanisms regulating their activities remain poorly understood, in part because the activities of full-length formins have not been characterized. Here, we isolated five different full-length formin proteins (human Daam1, mouse mDia1 and mDia2, and budding yeast Bni1 and Bnr1), all of which have a conserved diaphanos auto-inhibitory domain (DAD). We find that Daam1, mDia1, mDia2, and Bnr1 are strongly auto-inhibited, whereas Bni1 is constitutively active. Thus, the presence of a DAD is not a reliable predictor of formin autoinhibition. Truncation of DAD from mDia1 disrupts autoinhibition and induces a significant increase in Stokes radius consistent with release from a compact form. Structural analysis by electron microscopy and single particle averaging supports this view. RhoA is sufficient to only partially activate N-terminally truncated mDia1 molecules or trans-inhibited mDia1 fragments, and minimally activates intact, full-length mDia1. This suggests that additional cellular factors besides RhoA are required to release mDia1 from an autoinhibited state. Finally, our analyses reveal an unexpected role for the DAD in promoting formin-mediated actin assembly that involves direct interactions between the DAD and actin. This function likely explains why DAD is conserved in both autoinhibited and constitutively active formins.

Dynamic Formation of Actin Bundles by Formins

A. Michelou,1 J. Berro,2 E. Derivery,1 R. Pateras-Boujelma,1 C. Guerin,1 H. N. Higgs,2 J. C. Stauger,1 J. L. Martel2,1 L. Blanchon1; 1DRDC, Laboratoire de Physiologie Cellulaire Végétale, CEA Grenoble, Grenoble, France, 2Laboratoire TIMC-TIMB, UMR CNRS 5525, Grenoble, France.

We characterize the dynamic formation of actin bundles by full-length formins. We show that AFH1 does not remain attached at the growing barbed end of elongating filaments, as would be expected for a processive assembly factor, but rather it moves from the end to the side of an actin filament following nucleation. This behavior is crucial for the mechanism of actin bundle initiation by AFH1. However, processive formin as mDia2 is able to generate also actin bundles with a different polarity compare to AFH1. We propose a model for bundle formation that requires tight coordination between the nucleation events, the rate of elongation of actin filaments, and the thermal fluctuation of flexible polymers induced by Brownian motion.

Assembly of the Contractile Ring in S. pombe

D. Vasyukonis,1 J. Q. Wu,2 S. Hao,2 T. Kamine,2 B. O'Slaughnessy,1 T. D. Pollard2; 1Chemical Engineering, Columbia University, New York, NY, 2Cellular, Molecular and Developmental Biology, Yale University, New Haven, CT.

We used high resolution quantitative confocal microscopy and mathematical modeling to study the assembly of the actomyosin contractile ring during cytokinesis of S. pombe. The contractile ring in fission yeast is formed from the condensation of a broad band of ~70 membrane-bound “nodes” containing myosin-II (Myo2p), formin (Cdc12p), and other proteins (Wu et al. J. Cell Biol. 2006). We observed nodes in cells expressing myosin regulatory chain Rlc1p triply labeled with GFP. Nodes condense into rings through movements and coalescence events. A typical movement episode lasts ~ 20 s during which a node moves with a speed ~ 40 nm/s. A large fraction of node motions can be attributed to pair-wise node motions. Before the onset of condensation the nodes appear to execute Brownian motion with a diffusion coefficient ~dT = 4n m2/s. We thus estimate that the force exerted on a moving node is F = v kT / D > 4 pN, in the same range as the force exerted by a few molecular motors. These observations support a picture where myosin pulls nodes together by pulling on an actin filament, which is nucleated in a node by Cdc12p and grows to establish a transient connection with a neighboring node. To test if such a mechanism is robust enough to account for the uniform contraction of the broad band of nodes into a ring, we performed numerical simulations of simple models, which involve establishment of node connections, node movement, connection breakage, and our measured experimental parameter values. If we assume that the connection probability decreases with increasing distance between nodes, we observe a high probability of formation of disconnected aggregates, instead of a continuous ring. We speculate on possible mechanisms that may have evolved to bypass such instabilities.
Dynamic actin networks drive cell locomotion, phagocytosis, and intracellular motility of lipid vesicles, organelles, and invasive pathogens. Arp2/3 complex is the key component of these networks by virtue of its ability to form actin filament branches. Here, I will describe how we applied a hybrid approach that involves a combination of electron tomography, image analysis, computational docking, and modeling to obtain a detailed model of the branch junction at near-atomic resolution.

1410
A Structural Look into the Mechanism of Actin Polymerization-based Motility
F. Ferron, D. Chereau, M. Boczkowska, G. Rebowski, R. Dominguez; Department of Physiology, University of Pennsylvania School of Medicine, Philadelphia, PA. Elan Pharmaceuticals, Inc, San Francisco, CA.

Actin-polymerization-based proteins drive multiple cellular processes, including cell locomotion, cell division, endocytosis, and the movement of various infectious pathogens. Despite dramatic progress during the last ten years in our understanding of the proteins and regulatory pathways that lead to such motile processes, the molecular bases of actin-polymerization-based movement remain a mystery. Actin, a 375 amino acid-long ATPase and the most abundant protein in eukaryotic cells, constitutes the "fuel" for such cellular motilities. This is in part due to actin’s ability to exist in two states, a monomer (G-actin) and a filamentous polymer (F-actin). However, actin polymerization is harnessed into directed movement by the interactions of actin with hundreds of proteins, generally known as actin-binding proteins (ABPs). These proteins determine the location, rate and timing for actin assembly into functional networks. Two families of ABPs, Ena/VASP and WASP, have emerged as key regulators of actin cytoskeleton remodeling. Ena/VASP is linked to actin filament elongation, whereas WASP plays a role in filament nucleation and branching mediated by Arp2/3 complex. Although Ena/VASP and WASP are considered two fundamentally different regulators of cytoskeleton dynamics, our structural studies suggest a striking resemblance in the way these two proteins function during nucleation and elongation. In particular, their roles in these processes are mediated by similar G- and F-actin-binding domains belonging to the WASP-homology (WH2) family, and by long poly-Pro regions, which mediate the binding of profilin-actin complexes. Based on various crystal structures (including those of the transitional state in elongation by VASP and WH2-assembled actin minifilaments) and a throughout biophysical characterization of some of the transitional complexes involved in nucleation and elongation, we will discuss emerging principles underlying the mechanism of actin-polymerization based motility.

1411
PAK1-regulated Cofilin Activity Spatially Organizes Lamellipodium and Lamella Networks and Controls Leading Edge Protrusion
V. D. Delorme, M. Machack, T. Wittmann, C. Waterman-Storer, G. Danuser; Immunoology, The Scripps Research Institute, La Jolla, CA. Cell Biology, The Scripps Research Institute, La Jolla, CA, CA. UCSF, San Francisco, CA.

Actin filaments at the leading edge of migrating epithelial cells are organized in two distinct but overlapping modules: the lamellipodium and the lamella. Factors exist to regulate the intersection and dynamics of the two networks differentially and specifically to allow efficient cell edge protrusion. Cofilin constitutes an excellent candidate regulator: by virtue of its F-actin severing and depolymerizing activity, cofilin is known to promote actin remodeling. Paradoxically, cofilin severing activity may also decrease the mechanical stability of the lamellipodium and thus reduce cell protrusivity. To test this, we used quantitative Fluorescent Speckle Microscopy and measured F-actin dynamics in vivo in the two actin networks upon perturbation of the full signaling pathway of cofilin. We show that cofilin activity is regulated by PAK1, but not ROCK, at the cell leading edge. Increase of active cofilin downstream of active Rac, achieved using either PAK1 inhibitory domain, kinase-defective LIMK, or non-phosphorylatable active cofilin mutants, induces the formation of a unique region in which F-actin undergoes a fast retrograde flow. We provide evidence that this region displays kinetics and molecular characteristics of the lamellipodium. In addition, active cofilin increases the rate of F-actin treadmill in vivo and contributes to the formation of a wide region of F-actin polymerization at the cell edge. This effect is due to an enhancement of the density of polymerization-competent free barbed-ends but happens in an Arp2/3-independent fashion. Finally, classification of lamellipodium/lamella speciﬁcs in cells expressing active cofilin suggests a decoupling of the lamellipodium and the lamella at the leading edge. Tracking of the leading edge and measure of protrusion rates indicate that enhanced cofilin activity leads to a loss of spatio-temporal leading edge dynamics and to a decrease in net protrusion. We propose a model by which cofilin regulates lamellipodium/lamella spatial organization that is required for cell protrusion.

1412
Defective ER-to-Golgi Transport Caused by a Mutation in the COPII Protein SEC23A Is Responsible for the Human Disease Cranio-Lenticulo-Sutural Displasia

Intra-Golgi Vesicular Transport and Glycosylation Disorders
D. Ungar, M. F. Hughson; Molecular Biology, Princeton University, Princeton, NJ.

Eukaryotic glycoproteins contain a variety of glycan structures ensuring their specific functionality. Diversification of the glycans occurs in the Golgi by a large number of glycosyltransferases and glycosidases. To ensure correct synthesis of the glycans, different sets of enzymes are stationed to different Golgi cisternae. Therefore the Golgi constantly needs to sort the enzymes from the moving secretory cargo. This is done by a combination of cisternal retention and vesicular transport, during which resident enzymes are packaged into retrograde vesicles for recycling within the Golgi. The conserved oligomeric Golgi (COG) complex is a hetero-oligomeric peripheral membrane protein functioning in intra-Golgi retrograde vesicle transport. Several mutations in COG subunits have been found to cause congenital glycosylation disorders, suggesting that COG is necessary for the correct sorting of glycosyltransferases, which create a variety of developmentally important glycans. To understand its molecular function, we investigated the structure of mammalian COG. The complex consists of two lobes, each containing a different heterotrimeric subunit assembly, that are connected by the remaining two subunits. We further find that three Golgi localized Rabs (Rab1, 2 and 6) in the TGN generate functional COG complexes.

1413
Intra-Golgi Vesicular Transport and Glycosylation Disorders
D. Ungar, M. F. Hughson; Molecular Biology, Princeton University, Princeton, NJ.

Cranio-lenticulo-sutural dysplasia (CLSD) is an autosomal recessive syndrome featuring cranial and skeletal defects and cataracts that segregates with a missense mutation in the coding region of the SEC23A gene. SEC23A and its homolog SEC23B are two of the human genes encoding the SEC23 protein, an essential component of the COPII protein coat responsible for formation of vesicles budding from the endoplasmic reticulum (ER) destined for the Golgi complex. Electron microscopy and immunofluorescence studies of SEC23A-/- primary fibroblasts show obvious disorganization of the ER. Additionally, SEC31, another essential component of the COPII protein coat, is observed to be mis-localized to the cytoplasm in these cells by immunofluorescence. Purified mutant SEC23A is able to bind membranes and activate GTP hydrolysis by the COPII protein SAR1A at a level similar to wild-type. ER-derived vesicle budding assays performed in vitro with purified mutant SEC23A reveal that the mutation results in loss of functional budding activity. Surprisingly, the loss of budding activity is observed when SAR1B is used in the assay, but activity is restored if the highly homologous SAR1A is used instead. The loss of function can also be suppressed in vitro by addition of excess SEC13/SEC31A protein complex, suggesting that SEC13/SEC31A can interact with COG. This activity was confirmed through binding assays demonstrating the mutant SEC23A protein is impaired in its ability to recruit SEC13/SEC31A to both synthetic liposomes and microsomal membranes. Our observations suggest CLSD is caused by disrupted ER export of secretory proteins required for normal morphogenesis, and our in vitro data begin to explain the molecular basis for the disease.
The Dicytostele LvsB Is Localized on Post-Lysosomes and Controls the Fusion of Endocytic Vesicles

E. Kypri, A. De Lozanne; University of Texas at Austin, Austin, TX

The Chediak-Higashi Syndrome (CHS) is a rare autoso-mal recessive immunodeficiency disorder characterized by albinism and recurrent infections. At the cellular level, CHS is characterized by the presence of enlarged lysosomes and secretory defects. The gene affected in humans with CHS encodes a 430KDa protein named LYST (lysosomal trafficking regulator). To date, it is not clear how LYST contributes to lysosome biogenesis. Dicytostele discoidium contains a gene homologous to CHS termed lvsB that encodes the LvsB protein. LvsB mutants model the lysosomal defects associated with the Chediak-Higashi Syndrome. The aim of our studies is to determine the molecular function of LvsB and to provide insights into the regulation of lysosome biogenesis and the Chediak-Higashi syndrome. To better understand the role of LvsB, we used a knock-in approach to introduce the coding sequence of GFP at the 5’end of the lvsB gene. Imaging of cells expressing GFP-LvsB revealed that it is localized in the cytosol and on vesicular compartments of various sizes. Staining with antibodies against different endolysosomal markers revealed that GFP-LvsB colocalized with vacuolin on post-lysosomes. This result indicated that LvsB must exert its function primarily on the post-lysosomal compartment. Interestingly, LvsB mutants showed abnormally enlarged post-lysosomes that frequently exhibited altered acidity. Utilizing a fluid phase marker in a fusion assay we showed that, in the absence of LvsB, the terminal post-lysosomal compartments fuse inappropriately with earlier lysosomes. In agreement with this fusion assay, LvsB mutants exhibited frequent intermixing of lysosomal and post-lysosomal markers. Based on our data we propose that LvsB helps define the identity of late endosomal compartments by inhibiting their incorrect fusion with earlier compartments.

A Defect in the Metabolism of the Signaling Lipid, PI3,5P2 Causes Neurodegeneration in Mice

Y. Zhang,1 C. Y. Chow,2 S. C. Richardson,1 R. C. Piper,1 R. J. Westrick,1 M. H. Meisler,1 L. I. Weissman1; 1Department of Cell and Developmental Biology, Life Sciences Inst., University of Michigan, Ann Arbor, MI, 2Department of Human Genetics, University of Michigan, Ann Arbor, MI

The low abundance signaling lipid, phosphatidylinositol 3,5-biphosphate (PI3,5P2) is produced in the endocytic pathway. Its physiological functions are not known. PI3,5P2 is unique in that its levels change dramatically in response to specific stimuli. For example, in the yeast, S. cerevisiae, exposure to hyperosmotic shock, causes PI3,5P2 levels to transiently rise more than 20 fold within 5 minutes. The return to basal levels, even in the continued presence of esmyle, is achieved by 30 minutes. As an approach to determine the physiological roles of PI3,5P2, in mammals, we tested and found that proteins required for synthesis and turnover of PI3,5P2, are present in all tissues examined. This wide distribution suggests that PI3,5P2 plays multiple roles in signaling events that occur on internal membranes. Here we report that mice hypomorphic for the PI3,5P2, regulator, Vac14, die soon after birth. While development appears to be normal, there is massive neurodegeneration in both the central and peripheral nervous system. Cell bodies of multiple neurons are vacuolated, and large holes are present in areas where neurons should be present. Vacuolation appears specifically in neurons, however, other cell-types have the potential to form large vacuoles. That vacuoles appear first in neurons suggests that either PI3,5P2 functions in a neuronal-specific compartment and/or that neurons by virtue of their long processes are more sensitive to general defects in membrane transport. These findings strongly suggest that the general signaling lipid, PI3,5P2, is specifically essential for neuronal survival, and raise the possibility that selected human neuropathies are caused by mutations in PI3,5P2, signaling pathways.

Autophagy, Cell Death, and Development

S. Pattingre,1 Y. Wei,1 Q. Xu,1 N. Mizushima2,3 B. Levine1,4; 1Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, TX, 2Department of Birerregulation and Metabolism, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan, 3Precursory Research for Embryonic Science and Technology, Japan Science and Technology Agency, Kawaguchi, Japan, 4Department of Microbiology, University of Texas Southwestern Medical Center, Dallas, TX

Apoptosis and autophagy are both tightly regulated biological processes that play a central role in tissue homeostasis, development, and disease. Previously, we have shown that the anti-apoptotic protein, Bcl-2, interacts with the evolutionarily conserved autophagy protein, Beclin 1. Bcl-2 binding inhibits Beclin 1-dependent autophagy and autophagy gene-dependent cell death. Moreover, the binding and dissociation of the Bcl-2/Beclin 1 complex is regulated by nutritional status. In this meeting, new data will be presented regarding the biochemical regulation of Bcl-2/Beclin 1 binding. We will also describe studies using autophagy gene null embryonic stem cells to assess the role of autophagy in programmed cell death during mammalian development. Together, our data demonstrate important interrelationships between the autophagy and apoptosis pathway in mammalian physiology and development.

Rules for Nuclear Localization Sequence Recognition by Karyopherin beta2/transportin

Y. Chook; Pharmacology, UT Southwestern Medical Center, Dallas, TX

Karyopherin beta2/transportin bind nuclear localization and export signals (NLSs and NESs) to mediates nuclear trafficking. Diversity and complexity of signals recognized by Kap2 have been previously predicted for new Kap substrates. We have solved the crystal structure of Kap2/transportin bound to the NLS of hnRNP A1, and further analyses reveal three rules for NLS recognition by Kap2: NLSs are structured disorderly in free substrates, have overall basic character, and possess a central hydrophobic or basic motif followed by a C-terminal R/H/KX(2), S/PY consensus sequence. We demonstrate the predictive nature of these rules by identifying NLSs in seven previously known Kap2 substrates and uncovering 81 new candidate Kap2 substrates in an initial bioinformatics endeavor. Of the members of this predicted group with annotated subcellular localization, ~90% are reported to be nuclear localized. We have experimentally validated all seven new NLSs of known Kap2 substrates and five new bioinformatics-predicted substrates for Kap2 recognition as well as Ran-mediated dissociation. These studies define and validate a new NLS that could not be predicted by primary sequence analysis alone. We refer to this new class of prevalent NLS as PY-NLSs. This class of signals can be further subdivided according to their central motifs into hydrophobic or hPI-NLSs and basic or bPY-NLSs. We have recently solved crystal structures of Kap2 in complex with two additional hPI-NLSs found in Kap2 substrate TAP and SAM68, as well as a bPY-NLS found in hnRNP M. The C-terminal R/H/KX(2), S/PY consensus sequence in all three additional structures are structurally conserved, explaining the importance of this key motif and its conservation through evolution for Kap2 recognition. The four Kap2-substrate structures now shows how significant diversity within PY-NLSs are accommodated by the karyopherin, and the structures also allow refinement of the NLS consensus sequences for more extensive bioinformatics prediction of new substrates.

Nanomechanical Selective Gating in the Nuclear Pore Complex

R. Y. H. Lim,1 B. Fahrenkrog,1 J. Deng,2 K. Schwarz-Herion,1 J. Köser,1 U. Aebi1; 1M.E. Müller Institute for Structural Biology, Biozentrum, University of Basel, Basel, Switzerland, 2Department of Materials Research and Engineering, Singapore, Singapore

The intrinsic surrounding the nuclear pore complex (NPC) lies in its ability to restrict or promote cargo translocation between the cytoplasm and nucleus i.e. selective gating. To come to understand the modus operandi of the selective gating mechanism necessitates a detailed knowledge of both the biochemical identities and the corresponding physical responses of the NPC machinery. In this regard, biological approaches are key in identifying the protein-protein interactions that govern cargo translocation, but provide only marginal assessments of its physical foundations. To obtain a rational understanding of the mechanical nature of the selective gate, it is pertinent to study its principal components with respect to different environmental conditions and related transport factors, at the relevant length scale i.e. nanoscale. In our laboratory, we have developed a single-hairpin, inter-implanar approach to unravel such effects. Specifically, phenylalanine-glycine (FG)-molecules are tethered to gold nanostructures designed to mimic the NPC geometry. Atomic force microscopy (AFM) measurements reveal that the thermal fluctuations of FG-molecules serve as an entropic barrier to would-be entrants to the NPC. Privileged access to the NPC is provided by transport receptors (e.g. importin-β), which negotiate the entropic barrier by causing a collapse of the FG-molecules via receptor-FG binding interactions. This striking effect is only reversed by RanGTP, which sequesters importin-β and inhibits FG-binding. Immunogold-labeling electron microscopy complement these findings by showing that the FG-domains of Kap153 are reversibly collapsed in vivo by importin-β and RanGTP. We anticipate that the receptor-driven collapse of the FG-domains defines the physical aspects of selective gating, and propose that the flux of collapsing and distending FG-domains serves to promote the translocation of receptor-cargo complexes while simultaneously maintaining the entropic barrier. In closing, we will demonstrate how the aforementioned principles of nanomechanical selective gating can be applied to the construction of a de novo designed “nanomill” NPC.
Baculovirus Infection Reveals a New Role for an Importin Alpha Isoform in Directing Integral Membrane Proteins to the Inner Nuclear Membrane

S. C. Brauneagl, S. Saksena, M. Summers 1,2,3, Texas Agricultural Experimental Station, Texas A&M University, College Station, TX, Department of Biochemistry and Biophysics, Texas A&M University, College Station, TX, Department of Entomology, Texas A&M University, College Station, TX.

Baculovirus infection produces an amplified pulse of viral integral membrane proteins directed specifically toward the inner nuclear membrane (INM) for viral envelope assembly in the nucleus. A viral INM-sorting sequence has been identified from studies of these proteins and features of this sequence are retained in mammalian INM-directed integral membrane proteins. Using the viral sequence to identify interacting proteins specific to the pathway of viral protein transit from the ER to the INM has identified a cellular, truncated, membrane-bound importin (importin α-16). Importin α-16 binds the INM-sorting sequence during protein synthesis at the junction of the translocon-associated proteins, importin α-16 remains adjacent to the INM-sorting sequence after it integrates into the ER membrane and is released from the translocon. Thus importin α-16 is the first known sorting factor that can discriminate INM-directed integral membrane proteins during translation and insertion into the ER membrane, and facilitate their transit from the ER to the INM. The identification of importin α-16 provides evidence for a new model of INM-directed protein trafficking that may include functional activity for transit across the lateral channels of the nuclear pore complex. Analyses of the six human importin α genes reveal that humans encode five unique isoforms of importin α. When tested in an in vitro translation assay followed by treatment with a chemical crosslinking reagent, one human isoform covalently crosslinks to the baculovirus INM-sorting signal sequence. These data suggest that the observations originally made with the insect importin α-16 also apply to similar isoforms generated in humans. The model of importin α-16 mediated trafficking of integral membrane proteins to the INM, and new observations made with human isoforms of importin α will be discussed.

The Nuclear Export Factor Xpo1p Regulates the Turnover of Mad1p at Kinetochores

R. J. Scott, R. W. Wozniak; Department of Cell Biology, University of Alberta, Edmonton, AB, Canada

Nuclear pore complexes (NPCs) are strategically positioned along the surface of the nuclear envelope where they control the exchange of macromolecules between the cytoplasm and the nucleoplasm. The majority of the component parts of the NPC function in transport by either directly interacting with soluble cargo proteins or establishing the structural framework of the NPC. However, there are several proteins that interact with the NPC but play no obvious role in transport suggesting that the NPC is involved in other cellular processes. Two such NPC residents are the proteins Mad1p and Mad2p, which function in the spindle assembly checkpoint (SAC). These proteins are bound to the NPC during interphase and are recruited onto mitotic kinetochores during SAC activation. We have previously shown that S. cerevisiae Mad1p dynamically associates with both NPCs and kinetochores during SAC activation and that its association with kinetochores is energy dependent. Here we will present new data showing that the cycling of Mad1p on and off kinetochores and the NPC is dependent on the GTPase Ran and the soluble nuclear export factor Xpo1p. In mutants of the yeast RanGEF, FPR20, Mad1p turnover at the kinetochores is inhibited during SAC activation. Similarly, overexpression of a dominant negative mutant of Ran incapable of GTP hydrolysis allows association of Mad1p with kinetochores but prevents its turnover. Kinetochore turnover of Mad1p is also inhibited by mutations in Xpo1p. Consistent with the hypothesis that Xpo1p is present in the nucleoplasm and at spindle pole bodies throughout the cell cycle, but is recruited kinetochores during SAC activation. Finally, the results of experiments examining the direct interactions of Mad1p with Xpo1p and the role of this export factor in regulating the SAC will be discussed.

Export of mRNA through the Nuclear Pore Complex

K. Weis, C. S. Weichr, J. P. Erzberger, J. S. Flick, J. M. Berger, J. Thorrer; Department of Molecular & Cell Biology, University of California, Berkeley, Berkeley, CA

Export of mRNAs from the nucleus is essential for eukaryotic gene expression. Mature mRNAs are packaged into export-competent mRNA-protein particles (mRNPs) in the nucleus and subsequently transported across the nuclear pore complex (NPC) to the cytoplasm. mRNA export requires the essential DExD/H-box ATPase Dbp5, however, the precise role of Dbp5 in mRNA export is unknown and how mRNAs are directionally translocated across the NPC remains poorly understood. We have identified the nuclear pore protein Gle1 as an essential cellular activator of Dbp5. While Dbp5 alone is unable to bind RNA or effectively hydrolyze ATP under physiological conditions, addition of Gle1 dramatically stimulates these activities of Dbp5. A gle1p mutation that is deficient for Dbp5 stimulation in vitro does not affect Dbp5 expression in vivo, indicating that activation of Dbp5 is an essential function of Gle1. Functional analysis of the crystal structure of Dbp5’s C-terminal domain also reveals commonalities between Dbp5 activation by Gle1 and eIF4A activation by eIF4G, suggesting that these interactions may define a general model for the regulation of cellular DExD/H-box proteins by trans-acting factors. Interestingly, Gle1 binds directly to inositol hexakisphosphate (IP6) and IP6 potentiates the Gle1-mediated stimulation of the ATPase activity of Dbp5 in an RNA-dependent manner. We have isolated novel dominant alleles of Gle1 and DBP5 that rescue mRNA export phenotypes associated with the lack of IP6 production. Importantly, the dominant Dbp5 protein variant mimics the effect of IP6 on Dbp5 activation by Gle1 in vitro. These results define specific roles for Gle1 and IP6 in mRNA export and suggest that local activation of Dbp5 at the NPC by Gle1 and IP6 is critical for mRNA export.

A Role for the Nuclear Pore Protein, Nup60p in the Localization of ASH1 mRNA

E. A. Powrie, S. Shenoy, R. Singer; Cell Biology, Albert Einstein College of Medicine, Bronx, NY

Localization of ASH1 in the budding yeast Saccharomyces cerevisiae is one of the paradigms of mRNA localization. ASH1 is very highly expressed in late anaphase and actively localized to the bud-tip where it is locally translated and selected into the daughter cell nucleus where it functions as a GATA-like transcriptional repressor of the HO endonuclease. In addition to the She proteins that were initially identified as affecting ASH1 mRNA localization, other proteins that have more subtle effects on ASH1 localization (e.g. Loc1p, Khd1p, Puf6p) have been discovered. High resolution imaging of a localizing ASH1 reporter construct under wild type and mutant conditions demonstrates that the mRNA co-localizes with the nuclear pores, suggesting a potential role for nuclear pores in localization. Viable deletion strains (of nucleoporins and nuclear periphery-associated proteins) were selectively screened for abnormal ASH1 localization by in situ hybridization. The nuclear basket protein Nup60p displayed a defect in the efficacy of endogenous ASH1 transcript localization. Characterization of the nup60p deletion defect showed that a significant proportion of the ASH1 mRNA was retained in the mother cell. Preliminary analysis using in situ hybridization techniques showed that in nup60p deletion strains, the transcription site is internalized relative to its usual position at the nuclear periphery. Whether there is a cause and effect relationship between the aberrant localization phenotype and the delocalization of the transcription site is being investigated. As the identification and understanding of how trans-acting protein factors affect ASH1 localization expands, events that occur at every part of the transcript life cycle, from transcription to translation are ultimately critical to maintaining the wild type phenotype. Supported by GM557071.

Cell Signaling by Receptor Tyrosine Kinases: From Bench to Bedside

J. Schlessinger; Department of Pharmacology, Yale University School of Medicine, New Haven, CT

Receptor tyrosine kinases (RTKs) comprise a large family of membrane receptors that control many critical cellular processes. It is now well established that various human diseases and pathologies are caused by dysfunction in RTKs or in the intracellular signaling pathways that they activate. We have used mass spectrometry and X-ray crystallography to demonstrate that tyrosine autophosphorylation of the catalytic tyrosine kinase domain of FGF-receptor-1 (FGFR1) is mediated by a sequential and precisely ordered reaction. We also demonstrate that the rate of catalysis of two FGFR substrates is enhanced by 50 to 100 fold following autophosphorylation of the first site in the activation loop while autophosphorylation of the second site in the activation loop results in 500 to 1000 fold increase in the rate of substrate phosphorylation. We propose that FGFR1 is activated by a two-step mechanism mediated by strictly ordered and regulated autophosphorylation suggesting that distinct phosphorylation states may provide both temporal and spatial resolution to receptor signaling. Genetic models in mice provide new opportunities for exploring and developing new treatments for diseases caused by dysfunctions in RTKs and in their intracellular signaling pathways. Inhibitors of tyrosine kinases have been successfully applied for the treatment of cancers driven by activated tyrosine kinases. Support of U011126 is a new grant biacis actions of several tyrrosine kinases including e-kai, PDGFR and VEGFR. Sentox has been approved by the FDA for the treatment of advanced kidney cancers, gastrointestinal stromal tumors (GIST) and Gleevec resistant GIST. Finally, a novel scaffold-based drug discovery approach will be described that enables the development of many new families of inhibitors for protein kinases and other enzymes that play a role in cell signaling.
Transactivation of Epidermal Growth Factor Receptor in Human Mammary Epithelial Cells Involves Multiple Receptor Types Operating via Distinct Signaling Modules

N. Bollinger, D. Ippolito, H. Wiley, K. D. Roldand; Biological Sciences, Pacific Northwest National Laboratory, Richland, WA

It has been established that oncogenes can induce complex, interconnected communication networks that allow cells to integrate various extracellular signals. The transactivation of epidermal growth factor receptor (EGFR) is involved in many cellular processes such as proliferation, differentiation, and migration. The overall objective of this study was to determine which receptor types and signaling pathways were involved in transactivation of the EGFR in human mammary epithelial cells (HMEC). We found that G-protein-coupled receptors (LPA receptors, purinergic receptors, and Calcium-sensing receptors), cytokine receptors (VEGF and GH receptors), IGF receptor and TNF-α receptors are all capable of initiating cross-talk measured as increased ERK phosphorylation that could be blocked by inhibiting EGFR kinase activity. In contrast, the IGF receptor appears to activate the ERK pathway independent of EGFR. We found that transactivation of the EGFR generally involves activation of membrane associated metallopeptases of either the ADAM (A Disintegrin And Metalloproteinase-) or matrix metallopeptase (MMP) families, resulting in the proteolytic cleavage of membrane-bound EGFR ligands. Specifically, our results suggest that GPCR-mediated transactivation of the EGFR in HMEC cells required activation of ADAM 17/TACE when the IGF-1 receptor used alternate metallopeptases. Analysis of the ligands responsible for transactivation in HMEC cells indicated that although amphiuregulin is the dominant ligand released by IGF receptor activation, GPCR transactivation involves multiple ligands, including TGFα and epiregulin. These data suggest that multiple pathways are responsible for the selective release of different ligands that activate the EGFR in HMEC. Understanding the various receptors and the complex mechanisms involved in EGFR transactivation may lead to identification of therapeutic targets in diverse physiological disorders, such as cancer.

Membrane Mucin Muc4 Induces Density Dependent Changes in Erk Activation in Mammary Epithelial and Tumor Cells

C. A. Carothers Carraway, V. Pino, K. Carraway; University of Miami School of Medicine, Miami, FL

The membrane mucin molecule has been shown to alter cell behavior through both anti-adhesive effects on cell-cell and cell-extracellular matrix interactions and its ability to act as an intramembrane ligand for the receptor tyrosine kinase ErbB2. The extracellular-regulated kinase (Erk) pathway is regulated by both cell-matrix and cell-cell adhesion. An analysis of the effects of Muc4 expression on Erk phosphorylation in mammary and epithelial cells which exhibit both adhesion dependent growth and contact inhibition of growth showed that the effects are density dependent, with opposing effects on proliferating cells and on contact-inhibited cells. In these cells, cell-matrix interactions through integrins are required for activation of the Erk mitogenesis pathway. However, cell-cell interactions via cadherins inhibit the Erk pathway. Expression of Muc4 reverses both of these effects. In contact-inhibited cells, Muc4 appears to activate the Erk pathway at the level of Raf-1; this activation does not depend on Ras activation. The increase in Erk activity correlates with an increase in cyclin D1 expression in these cells. This abrogation of contact inhibition is dependent on the number of mucin repeats in the mucin subunit of Muc4, indicative of an anti-adhesive effect. The mechanism by which Muc4 disrupts contact inhibition involves a Muc4-induced relocalization of E-cadherin from adherens junctions at the lateral membrane of the cells to the apical membrane. Muc4-induced abrogation of contact inhibition may be an important mechanism by which tumors progress from an early more benign state to invasiveness.

SUZ12 Mediated NF-IL6 Gene Silencing Plays a Functional Role in Tumorgenesis

C. Ko; The Institute of Basic Medical Sciences, College of Medicine, National Cheng Kung University, Tainan, Taiwan

Polycomb group (PcG) proteins function as epigenetic chromatin modifiers and involve in cancer development and also in the maintenance of embryonic and adult stem cells. Recent reports have demonstrated that PcG protein EZH2 is highly expressed in metastatic prostate cancer and lymphomas. SUZ12 is a recently identified PcG protein which binds with EZH2, a histone H3 methyltransferase (HMT), and EED to form Polycomb repressive complexes (PRC). SUZ12 has been identified as an E2F target gene and its mRNA is up-regulated in a number of different human tumors, such as tumors of the colon, breast, and liver. On the other hand, previous reports showed that NF-IL6 (human C/EBPα) induced growth arrest in breast cancer cells and was a crucial regulator of pro-apoptotic gene expression during mammary gland involution. These data suggested that NF-IL6β was a potential tumor suppressor. Kirmizis et al. has shown that knock-down of SUZ12 induced C/EBPs gene expression. From my result, it showed that SUZ12 could bind to NF-IL6β promoter region and inhibited its transcriptional activity which resulted in decreasing NF-IL6β gene expression. Moreover, NF-IL6β gene expression could be induced by treatment with DNA methyltransferase inhibitor 5-AzaC in different cancer cell lines. Taken together, these results suggested that SUZ12 might play a functional role in NF-IL6 gene silencing. In the future, I would like to identify and clarify the connection between SUZ12 and NF-IL6 gene silencing in cancer cells and whether overexpression of NF-IL6β can block the SUZ12-induced tumorigenic phenotype.

Signaling Pathway Regulating Heat Shock Responses by Hsp27 and Peroxiredoxin 6

Y. Kim, Y. Kim, J. Jeong; College of Pharmacy, Ewha Womans University, Seoul, Republic of Korea

Heat shock stress induces a wide variety of biological processes, including ROS generation, inhibition of protein synthesis, elevated expression of heat shock proteins (Hsps), and induction of thermotolerance and apoptotic cell death in a dose dependent manner. However, the exact mechanisms of these processes were not well understood. Recently, proteosome inhibitor can induce Hps and thermotolerance as well as heat shock responses. To determine whether heat shock response is occurred similar way to proteosome inhibitor, we examined the differential protein expressions with systematic way in a radiation-induced fibrosarcoma cell line (RIF) and found that Hsp27 and peroxiredoxin6 (PRX6) were highly overexpressed and heavily modified in various sites identified using nano-LC-ESI-q-TOF MS/MS/MS. Based on the results, the heat shock signaling pathways in Hsp27 and PRX6 overexpressed cells were examined in ROS generation, poly-Ub accumulation, MAPK activation, Hsp synthesis, and thermotolerance. The results demonstrate that heat-induced signaling pathways were regulated by various key proteins including Hsp27 and PRX6 in protein-specific manner. [Supported by KOSEF grant FPR05A2-480, CCS, CCS & DDR and Brain Korea 21]

Lithium Induces Mitochondrial Biogenesis Independently of Glycogen Synthase Kinase-3-Beta and Inositol Monophosphatase Inhibition

I. T. Strouweeg, C. D. Maas; GCNS, University of Kentucky, Lexington, KY

Lithium treatment of these cells led to an increase in cell size, similar to the Wnt hypertrophic effect. Along with these changes there was an increase of mitochondrial mass as evidenced by an increase in mitochondrial genomic DNA compared to nuclear DNA. This increase in mitochondrial mass was accompanied by a proportional increase in ATP production and mitochondria membrane potential indicating that there was no change in mitochondrial efficiency. This increase of mitochondrial biogenesis was due to an increase in the mRNA and protein levels of oxidative phosphorylation components, which was triggered by an increased expression of the transcription factors NRF1 and 2 and the mitochondrial transcription factor TFAM. Though lithium also increased the levels of the inactive Phospho-S9-GSK3β, these changes were not a result of GSK3β inhibition as these effects were not reproduced with other GSK3β inhibitors, valproate and indirubin, or by expression of the inactive K85R-GSK3β. Although lithium also affects the inositol pathway by inhibiting inositol monophosphatase (IMPase), these effects could not be reversed with the addition of myo-inositol to restore the inositol intracellular pool. Together our results suggest a novel effect of lithium on mitochondrial biogenesis that is independent of GSK3β and IMPase inhibition and reveal a new target pathway for lithium that might be relevant to Wnt signaling.
1431 Hypoxia Inducible Factor-1 Is a Determinant of Hepatic Oxygen Consumption In Vivo

K. Tsukada, 1 T. Tajima, 1 T. Kawamura, 1 N. Goda, 1 R. S. Johnson, 2 M. Suematsu 2; 1Department of Biochemistry & Integrative Medical Biology, Keio University, Tokyo, Japan, 2Molecular Biology Section, Division of Biological Sciences, College of Veterinary Medicine, The Ohio State University, Columbus, OH, USA

Hypoxia-inducible factor (HIF)-1α is an oxygen-sensing transcriptional factor that dictates expression of erythropoietin, TGF-β and gas-generating enzymes such as NO synthase-2 and heme oxygenase-1. Since the gene disruption causes embryonic lethality, the roles of HIF-1α in adult mice remain largely unknown. To determine the role of HIF-1α in liver, we have herein generated mice harboring a floxed HIF-1α allele, and employed the albumin-Cre transgenic line to inactivate the gene site-specifically in hepatocytes. Intravital observation of the hepatic microcirculation combined with phosphorescence-assisted local oxygen measurements revealed that no difference in the local oxygen tension was evident in portal and central veins between HIF-KO and control mice. However, because of a higher erythrocyte velocity in microvasculature, the net oxygen consumption in the lobules appeared to be greater in HIF-KO mice than in the controls. The greater oxygen consumption in the HIF-KO liver appeared to result from an increase in the consumption at the hepatocellular level, so far as judged from measurements of the oxygen consumption in primary cultured hepatocytes. Although the whole mechanism remains unknown, the current results suggest a crucial role of HIF-1α in modulation of cellular oxygen consumption.

1432 Endosomal NADPH Oxidase Regulates c-Src Activation Following Hypoxia/Reperfusion Injury

Q. Li, 1 Y. Zheng, 1 W. Zhou, 1 J. Engelhardt 2; 1Anatomy and Cell Biology, The University of Iowa, Iowa City, IA, 2The Center for Gene Therapy, The University of Iowa, Iowa City, IA

As a tyrosine kinase, c-src has been shown to activate NFkappaB following hypoxia/reoxygenation (H/R) and ischemia/reperfusion (I/R) by directly phosphorylating IkappakBAlpaha. Our previous studies (J Clin Invest, 2004, 113(5): 746-55) have suggested that reactive oxygen species (ROS) are required for c-Src activation following H/R and I/R injuries in hepatocytes. In the present study, we investigated potential sources of ROS required for c-Src activation following H/R with the hypothesis that NADPH oxidases were involved. Our results demonstrate that following H/R injury, c-Src movement from the plasma membrane to intracellular vesicles was associated with enhanced NADPH-dependent superoxide production in isolated endosomes. Blocking endocytosis, by expressing a dominant negative mutant of dynamin, inhibited activation of NADPH oxidase, c-Src activation, and the ability of c-Src to tyrosine-phosphorylate IkappakBAlpaha in endomembranes following H/R. Quenching intra-endosomal ROS by loading newly formed endosomes with SOD1 and catalase, inhibited c-Src activation and its ability to tyrosine-phosphorylate of IkappakBAlpaha, without affecting c-Src recruitment into endosomes. These findings suggest that NADPH oxidase derived ROS are important for c-Src activation but not subcellular relocalization following I/R injury. Using reductant and adenoviral mediated expression of GPx-1, we demonstrate that hydrogen peroxide appears the key ROS required for c-Src activation following H/R. This finding suggests that NADPH oxidase derived superoxides are the source of endogenous hydrogen peroxide required for H/R activation of c-Src. Using siRNA mediated knockdown of Rac1 (a small GTPase and required co-activator of NADPH oxidases), we also demonstrate that Rac1 is required for both the endocytosis and reoxidation-mediated activation of c-Src. Inhibition of Rac1 also prevented H/R-induced ROS production by the endosomal compartment. In summary, our data suggest that Rac1-dependent NADPH oxidases play a critical role in activating c-Src following H/R injury. This may be a useful therapeutic target for I/R related disease.

1433 C/EBPβ mRNA Is Stabilized by UV Radiation Via Activation of the p38 MAP Kinase Pathway and HuR

B. Li, J. DeWille; Department of Veterinary Biosciences, College of Veterinary Medicine, The Ohio State University, Columbus, OH, USA

Inhibition of c-Src by treatment of A549 adenocarcinoma cells with the tyrosine kinase inhibitor, PP1, or treatment with Schisandrin B (SchB), both known to disrupt TGF-β signaling, led to a decrease in C/EBPβ mRNA levels. Treatment of cells with UV radiation induced an increase in C/EBPβ mRNA levels which was also blocked by the participation of the transcription factor, HuR. These findings suggest that UV radiation-induced stabilization of C/EBPβ mRNA is regulated by cellular proteins that bind to the C/EBPβ mRNA 3' UTR AU rich elements (ARE). In this study we treated HC11 mouse mammary epithelial cells with UVB or Anisomycin (AN) and demonstrated increased activation (phosphorylation) of p38 MAP kinase and increased C/EBPβ mRNA stability. Treatment with SB203580, a p38 MAP kinase inhibitor resulted in a decrease in C/EBPβ mRNA stability. UV radiation induces the nuclear -> cytoplasm translocation of HuR, an ARE-binding protein that functions in mRNA stability. We show that UV radiation-induced HuR translocation is also blocked by SB203580. Furthermore, repression of HuR by HuR siRNA treatment led to inhibition of C/EBPβ mRNA stability by UV radiation. HuR immunoprecipitation analysis demonstrated that HuR can bind to C/EBPβ mRNA in vivo after UV treatment. These results suggest that UV radiation activates p38 MAP kinase leading to nuclear/cytoplasmic shuttling of HuR and subsequent stabilization of C/EBPβ mRNA. Finally, previous studies from our lab have demonstrated that increased C/EBPβ expression dramatically reduces the growth of human breast cancer cell lines. The results from the present study suggest that pharmacological interventions aimed at activation of the p38 MAP kinase pathway may increase C/EBPβ mRNA stability and promote growth arrest and/or programmed cell death of breast cancer cells.

1434 Inhibition of ATR Protein Kinase Activity by Schisandrin B during DNA Damage Response

H. Nishida, 1 N. Tatewaki, 1 T. Magara, 1 Y. Nakajima, 1 K. M. Ko, 1 Y. Hamamori, 1 T. Konishi, 1 Applied Life Sciences, Niigata University of Pharmacy and Applied Life Sciences, Niigata, Japan; 2Biochemistry, Hong Kong University of Science and Technology, Hong Kong, China, 3Cardiology, Baylor College of Medicine, Houston, TX

Inhibition of DNA damage checkpoint genes, such as ATR, reduces cell growth and interrupts cell cycle progression (J Biol Chem, 2003, 278: 35515-22). In addition, knockdown of ATR, caused growth arrest (Cancer Lettre, 2003, 202: 281-90). We previously reported that treatment of Schisandrin B (SchB) significantly reduced the A549 adenocarcinoma cell viability after UV exposure. Of importance, SchB treatment also disrupts G2/M checkpoint in the UV-exposed cells. In vitro immunoprecipitation protein kinase activity assay of ATR was clearly decreased by increased concentration of SchB. Consistent with potential inhibition of ATR by SchB, UV-induced phosphorylations of ATR effectors such as p53 and Chk1 are remarkably reduced by SchB in ATM-deficient cells (AT patient derived AT2KY cells and siATM treated cells) but not in siATR-treated cells. SchB does not affect an activity of another protein kinase, ERK (p44/p42). Taken together these findings suggest that SchB specifically inhibits ATR protein kinase in cells following DNA damage.

1435 Arsenic-induced p53-DNA Binding and Activity in Epithelial Cells

M. Sandrowal, 1 M. Morales, 1 A. OrtegA, 1 P. Ostrosky-Wegman, 1 E. López-Bayghen 1; 1Genética y Biología Molecular, Cinvestav, Mexico DF, Mexico, 2Genética y Toxicología Ambiental, Instituto de Biología, UNAM, Mexico DF, Mexico

Exposure to arsenic is associated with an increased incidence of skin pathologies as hyperkeratosis and cancer. Arsenic effects include genotoxicity, cell-proliferation changes, alterations in DNA repair and methylation patterns. Proliferation and differentiation coordinated expression of structural and regulatory proteins, is probably lost in arsenic-associated-skin pathologies. We evaluated whether the changes in epithelial cells including human keratinocytes after arsenic exposure, are mediated through modifications in the activity of the tumor suppressor p53, a potent mediator of responses against genotoxic damage, and a pivotal proliferator-control. We tested if arsenic modulates the abilities of p53 as a transcription factor in epithelial cells treated with increasing concentrations of sodium arsenite for up to 24h, using a p53-consensus sequence in EMSAs. A time and dose-dependent response in p53-DNA binding occurs under arsenic exposure. We test if changes in the proliferation and differentiation process during arsenic exposure can be mediated by modifications in the expression levels of p53 and human involucrin, an important differentiation marker in cornified envelope of terminally differentiated keratinocytes. Significantly, using concentrations higher than 0.5μM, we noticed a decrease in proliferation and increased levels of involucrin in keratinocytes, supporting the idea that arsenic activates p53 (C33-A). An increase in proliferation, in cells under treatment is accompanied by an increase in cyclin D1 promoter activity. Signaling events include PI3 kinase and Protein Kinase B activation for an activated p53 pathway, favouring the notion that p53-controlled transcriptional mechanisms are important to understand modifications in the proliferation-differentiation balance severely impaired during skin carcinogenesis.
1436 Tobacco Carcinogens Stimulate Different Signal Pathways in Breast Cancer M. W. Hance,1 M. S. Dhar,1 H. K. Plummer; 1Pathobiology, University of Tennessee, Knoxville, TN, 2Pathobiology and Large Animal, University of Tennessee, Knoxville, TN

Breast cancer is a leading cause of new cancers in women, and smoking is a controversial risk factor. However, the tobacco-specific metabolite (3-aminomethyl-3-pyridyl)-1-butanone (NNK) has been identified as a high affinity agonist for β-adrenergic receptors. Our previous data suggests a joint role of G-protein inwardly rectifying potassium channel 1 channels and β-adrenergic signaling in the growth regulation of some breast cancers. In addition, polyacrylic polyacrylic hydrocarbons (PAH) from cigarette smoke and other sources induce breast cancer. In the present studies, we investigated the effects of the PAH 7,12-dimethylbenz[a]anthracene (DMBA) and NNK on β-adrenergic signaling using the estrogen receptor (ER) [ER+] cell line MDA-MB-453 and the ER (-) cell line MDA-MB-361. DMBA decreased β-adrenergic protein levels, but neither 10 nM nor 100 nM NNK increased β-adrenergic levels in MDA-MB-453 cells. However, in the MDA-MB-361 cells, DMBA increased β-adrenergic protein expression, and neither 10 nM nor 100 nM NNK had effects on β-adrenergic expression. We then studied the cAMP-PKA-CREB signaling pathway since binding of agonists to β-adrenergic receptors may activate this pathway. Treatment of the MDA-MB-361 cell line with 100 nM or 10 nM NNK or 5 μM DMBA did not lead to PKA phosphorylation at times ranging from 5 minutes to 2 hours. In addition, DMBA decreased cAMP levels while the β-adrenergic agonist formoterol stimulated cAMP production indicating that DMBA affects stimulation of cAMP differently than β-adrenergic stimulation. However in the MDA-MB-453 cell line, both 100 nM and 10 μM NNK treatment led to PKA phosphorylation at all time periods. DMBA had no effect on PKA phosphorylation in MDA-MB-453 cells. This collective data indicates that DMBA and NNK have different effects on the β-adrenergic signaling pathway, including different effects based on ER expression. Supported by Philip Morris USA Inc./Philip Morris International and University of Tennessee Center of Excellence.

1437 The Effects of Insulin on Serum Deprivation-induced Cell Death of SK-N-BE(2) Cells That Cannot Produce Insulin-induced ROS C. Yeo, J. Lim, J. Kim, K. Hur; Department of Life Sciences, Ewha Womans University, Seoul, Republic of Korea

Insulin can suppress serum deprivation-induced cell death in most cell lines. However, upon insulin stimulation, serum deprivation-induced cell death is accelerated in SK-N-BE(2) neuroblastoma cells while it is suppressed in SK-N SH neuroblastoma cells. Moreover, SK-N-BE(2) cells are more resistant to serum deprivation compared to SK-N-SH cells. SK-N-BE(2) cells cannot produce reactive oxygen species (ROS) in response to insulin while SK-N-SH cells can. We examined the time course of Akt activation, in order to address the molecular mechanisms for opposing oxygen species in these cells. In SK-N-SH cells, Akt activation occurred up to 48 hrs only in the presence of insulin. In SK-N-BE(2) cells, Akt activation was gradually increased up to 48 hrs upon serum deprivation even in the absence of insulin. Moreover, the levels of active Akt was lower in the presence of insulin than in the absence of insulin in SK-N-BE(2) cells. Similarly, the production of ROS was gradually increased upon serum deprivation and the increase of ROS production was suppressed by insulin. The resistance of SK-N-BE(2) cells to serum deprivation-induced cell death was abolished when cells were treated with N-acetylcysteine. These results suggest that the increase of ROS levels in SK-N-BE(2) cells in serum-free condition is a major reason for the abnormal response to insulin.

1438 Microarray Analysis of Diabetic Rat Wounds Treated with Either V.A.C.® Therapy or Moist Wound Healing K. Derrick, A. McNulty, K. Norbury, K. Kieswetter; KCI, Inc., San Antonio, TX

Vacuum Assisted Closure® Therapy (V.A.C.® Therapy) is extensively used to treat both acute and chronic wounds. Use of this therapy is widely known to help promote wound healing. Whole-genome studies were designed to gain insight into the biological processes which V.A.C.® Therapy may affect. Three cm wounds were made dorsally on 8 diabetic rats (one wound per animal). Wounds were treated with either V.A.C.® Therapy (125mm Hg, continuous negative pressure) using V.A.C.® Gammagraf® Dressing or with Tegaderm™. After 2 days of treatment, wound tissue was harvested. The Applied Biosystems microarray platform was used to assess gene expression for all 16 pieces of tissue (8 normal from wound creation, 8 wound from day 2). A total of 8,445 unique genes (p<0.05) experienced fold changes in gene expression between day 0 and day 2 post wounding in the 2 treatment groups. This was divided into 7,359 genes changed in V.A.C.® Therapy treated tissue vs. 4,563 genes changed in Tegaderm™ treated tissue. After differential analyses, data were entered into the PANTHER classification system (www.pantherdb.org) to assess biological processes which were differentially affected by V.A.C.® Therapy. For V.A.C.® Therapy treated tissue, significantly more signal transduction related genes were expressed than expected (p=1.85x10^-10). Significantly more genes related to intracellular signaling were also differentially expressed during the two days of therapy (p=2.74x10^-7). Cell motility related genes were also overexpressed in V.A.C.® Therapy treated animals (p=1.77x10^-14). These data show that, in studied rats, V.A.C.® Therapy influenced specific biological processes during wound healing, which may account for some of the benefits of this therapy.

1439 Helicobacter pylori VacA Enhances PGE2 Production through the Induction of COX-2 Expression via p38 MAP Kinase/ATF-2 Cascade in AZ-521 Cells T. Hirayama,1 J. Hisatsune,1 E. Yamasaki,1 A. Wada,1 K. Yahiro,2 J. Moss2; 1Dept. of Bacteriology, Nagasaki University, Nagasaki, Japan, 2Pccm, nhlbi, National Institutes of Health, Bethesda, MD

Helicobacter pylori produces a vacuolating exotoxin, VacA, which causes vacuolation and gastric injury. We investigated whether VacA affected cyclooxygenase-2 (COX-2) expression in AZ-521 cultured gastric epithelial cells, resulting in the production of prostanooids from arachidonic acid. Treatment of AZ-521 cells with VacA increased amounts of COX-2 mRNA in time- and dose-dependent process. Inhibitor of p38 MAP kinase, SB203580, almost completely blocked the elevation of COX-2 mRNA levels, whereas an MKK inhibitor, PD98059, which is known to block the Erk1/2 signal cascade partially suppressed it. VacA-induced accumulation of COX-2 mRNA was reduced in AZ-521 cells overexpressing a dominant-negative p38 MAP kinase construct, suggesting that VacA increased COX-2 expression in AZ-521 cells through a signaling pathway that included p38 MAP kinase. In agreement, PI-PLC, which inhibited VacA-induced p38 MAP kinase activation, blocked VacA-induced COX-2 expression, whereas two inhibitors (NPPB and bafilomycin A1) of VacA-induced vacuolation, had no effect on p38 MAP kinase activation and COX-2 expression in AZ-521 cells, suggesting that P3MAP kinase activation was independent of the vacuolating activity of VacA. In parallel with COX-2 expression, VacA increased PGE2 production by AZ-521 cells, which was remarkably inhibited by SB203580 as well as NS-398, a COX-2 inhibitor. In addition, VacA-induced PGE2 production was markedly attenuated in AZ-521 cells stably expressing dominant-negative p38 MAP kinase. VacA increased COX-2 transcription by more than 2.5-fold in AZ-521 cells transfected with WTCOX-2(-327/+59) promoter, whereas VacA had no activation effect in AZ-521 cells transfected with a mutated CRE site, but not with mutated NFκB or NF-IL6 sites, suggesting direct involvement of the ATF-2/CREB-binding region in VacA-induced COX-2 promoter activation. These results show that VacA enhances PGE2 production in AZ-521 cells through the induction of COX-2 expression via the p38 MAP kinase/ATF-2 cascade.

1440 Photomechanical and Biochemical Characterization of LOV2 Mutant Domains of Arabidopsis thaliana H. Kim,1 E. Baik,1 K. Im,1 S. Choi,1 Y. Yoo,1 I. Han; 3; 1Korea Science Academy, Busan, Republic of Korea, 2IRC, University of Ulsan, Ulsan, Republic of Korea, 3Biological Science, University of Ulsan, Ulsan, Republic of Korea

The LOV domains are the photoreceptive modules of the phototropins and related chroomoproteins. LOV domain fusion proteins purified from E. coli are highly fluorescent and undergo a formation of a helical adduct between the FMN chromophore and a conserved active-site Cys residue. However, the molecular and structural basis of LOV domains regulate the activity of phototropin is not well understood. In order to investigate photomechanical activity of LOV domain, we have used three mutant oat LOV2 domains (C39A/K77E, C39A/V67A, and C39A/T53P) obtained from LOVC39A, of which fluorescence and absorption properties are very similar to those of wild type LOV domain, by using PCR-driven random mutagenesis method. Amino acid substitution of Trp 53 with proline had deleterious effect on fluorescent activity of LOV domain. Compared to C39A, C39A/V67A exhibited only two-thirds of fluorescent activity, whereas two inhibitors (NPPB and bafilomycin A1) of VacA-induced vacuolation, had no effect on p38 MAP kinase activation and COX-2 expression in AZ-521 cells, suggesting that P3MAP kinase activation was independent of the vacuolating activity of VacA. In parallel with COX-2 expression, VacA increased PGE2 production by AZ-521 cells, which was remarkably inhibited by SB203580 as well as NS-398, a COX-2 inhibitor. In addition, VacA-induced PGE2 production was markedly attenuated in AZ-521 cells stably expressing dominant-negative p38 MAP kinase. VacA increased COX-2 transcription by more than 2.5-fold in AZ-521 cells transfected with WT COX-2(-327/+59) promoter, whereas VacA had no activation effect in AZ-521 cells transfected with a mutated CRE site, but not with mutated NFκB or NF-IL6 sites, suggesting direct involvement of the ATF-2/CREB-binding region in VacA-induced COX-2 promoter activation. These results show that VacA enhances PGE2 production in AZ-521 cells through the induction of COX-2 expression via the p38 MAP kinase/ATF-2 cascade.

1441 Regulation of Arabidopsis thaliana Ku Gene Expression by Abscisic Acid P. Liu, W. Chang, Y. Wang, R. Pan; Life Science, National Tsing Hua University, Hsinchu, Taiwan

Ku antigen (Ku70/Ku80) is a regulatory subunit of DNA-dependent protein kinase (DNA-PK), which participates in the regulation of DNA replication, cell cycle and telomere maintenance. The plant hormone abscisic acid (ABA) plays a central role in stress responses and is described as a plant growth inhibitor. In this study, we aimed to find out whether Arabidopsis thaliana Ku
genes (At.Ku) were regulated during ABA inducing slow growth in 3-week-old seedlings. Firstly, some ABA responsive elements of At.Ku promoters are predicted from PlantCARE (http://www.plantcare.com/) and PLACE (http://www.dna.nic.go.in/PLACE/) websites and At.Ku promoter-GUS (β-Glucuronidase) analysis in transgenic Arabidopsis indicated that activity of At.Ku promoter was inhibited by ABA. In addition, real time quantitative PCR analysis showed their repression in a time-and concentration-dependent manner. Moreover, ABA-mediated inhibition of At.Ku gene expression was confirmed by inhibitor treatment and mutant analysis of ABA biosynthesis. Furthermore, At.Ku gene repression under ABA responses may be regulated through the pathway of Ca²⁺/phospholipase D alpha (PLDα) mitogen-activated protein kinase (MAPK) and some transcription factors, abf1 and abf2. Finally, our results revealed an interaction between ABA, auxins and GA for modulating At.Ku gene expression. Taken together, these results demonstrated that At.Ku gene is presumably regulated by ABA.

Identification of a New Gene Required for Cell Wall Separation in Corynebacterium glutamicum

Y. Tagne, M. Imi, H. Yakuwa; Research Institute of Innovative Technology for the Earth, Kyoto, Japan, Nara Institute of Science and Technology, Nara, Japan

Corynebacterium glutamicum is a gram-positive bacterium extensively used in industrial amino acid production. This bacterium exhibits a unique cell division system called snapping division, completely different from model microorganism like Escherichia coli or Bacillus subtilis. However, the detailed mechanism of this division system is mostly unknown. Elucidation of the cell division mechanism of this bacterium may contribute to higher amino acids productivity, given that C. glutamicum secretes bulk of its amino acids when cell division is arrested. Of 2990 genes of C. glutamicum, we focused on cws1. This gene consists of deduced 610 amino acid residues with a 50-residue signal peptide. Insertional inactivation of cws1 resulted in a 3-fold elongation of the cell compared to the wild type. Multiple lines were observed on the cell under light microscopy. Complementation of the G2/M checkpoint proteins cyclin B and cdk1 was not decreased if nuclear Ca²⁺ was buffered, but the mitotic index was increased by 47% in these cells, and virtually all mitotic cells were in early prophase and failed to undergo centrosome duplication. Knockdown of the Ca²⁺-binding protein centrin-2 had a similar effect; BrdU uptake was decreased by 75% in these cells, there was a 48% increase in mitotic index, and nearly all mitotic cells were in early prophase and failed to undergo centrosome duplication. These findings show that cell growth is regulated by nuclear rather than cytosolic Ca²⁺ signals and suggest that the specific role of nuclear Ca²⁺ is to permit progression through early prophase, by centriole-mediated centrosome duplication.

Mechanisms Underlying the P35-mediated Prevention of Adriamycin-induced Apoptosis and Polyploidy/Multinucleation

Y. Lin, F. Wang; The Institute of Biochemistry and Molecular Biology, National Yang-Ming University, Taipei, Taiwan

The p53 tumor suppressor executes its function either by maintaining the cell survival through arresting cell cycle progression for DNA damage repair or eliciting apoptosis, and has long been regarded as a key therapeutic target for cancer treatment. Adriamycin is a topoisomerase II inhibitor widely used in the treatment of a variety of cancers. To explore the cytoprotective function of p35 on adriamycin treated cells, we established clones of H1299 cells stably expressing the temperature-sensitive p53 mutant, tsp53V143A, previously shown to activate the genes related to the p53 tumor-suppressor pathway. Adriamycin leads to the down-regulation of p21 Waf1/Cip1 after p53 activation, while the role of p21 Waf1/Cip1 in mediating the cytoprotective function of p53 was not known. We found that p21 Waf1/Cip1 is required for suppression of the adriamycin-induced polyploidy/multinucleation. Together our findings suggest that the cytoprotective action of p53 against adriamycin induced cell death requires the expression of p21 Waf1/Cip1 in vitro, indicating the importance of this gene in p53-mediated cancer chemotherapy.

Investigation of the Role of Conserved H59 and H157 for the Functionality of Pin1

M. L. Bailey, C. J. Brandl, D. W. Litchfield, B. H. Shilton; Biochemistry, University of Western Ontario, London, ON, Canada

Pin1 catalyzes the cis to trans isomerization of peptide-polyglycine bonds preceded by a phosphorylated serine or threonine residue, and has emerged as an important regulator of cell division. Pin1 consists of two domains: a WW domain that binds phosphorylated proteins and an isomerase (PPIase) domain; however, the precise catalytic mechanism and how isomerase activity relates to the catalytic mechanism is unknown. is a conserved histidine residues (H59 and H157) found in the Pin1 active site that were initially proposed to play critical roles in the catalytic mechanism. Interestingly, mutation of H59GQG still yielded a functional protein in yeast complementation assay. Similarly, substitution of the nearby H157GQG produced a functional protein. However, it was most surprising to find that a non-functional H595L mutant of Pin1 could be rescued by substitution of H157 with L. To examine this finding further, both H59 and H157 were mutated to multiple amino acids to determine their role in the mechanism of Pin1. This work provides evidence that H59 and H157 are not as critical to Pin1 catalysis as originally suggested and that the current catalytic mechanism for this protein may need to be refined.

Down-regulation of γ-tubulin Inhibits Cell Proliferation and Induces Apoptosis in Human Breast and Prostate Cancer Cells

A. Banerjee, R. F. Ludueña; Department of Biochemistry, The University of Texas Health Science Center, San Antonio, TX

γ-tubulin is a member of the tubulin superfamily and plays a very important role in the mechanism of mitotic spindles. Although the major function of γ-tubulin is to nucleate mitotic spindles, recent studies have implicated its role in the regulation of cell cycle. In human system, γ-tubulin isoforms, namely γ1 and γ2 tubulin, are expressed. We have studied the expression of these two isoforms in MCF-7 breast cancer cells by RT-PCR. We find that γ1 is the major isoform while γ2 is expressed in trace amounts in MCF-7 cells. In an effort to study whether targeted depletion of γ-tubulin may affect cell growth, antisense phosphorothioate oligodeoxycytidine nucleotides (ODNs) targeted to human γ1 and γ2 tubulin mRNA were prepared. Transfection of human breast and prostate cancer cells showed that both antisense ODNs inhibited cell growth in a dose-dependent manner. Western analysis of the cell extracts from antisense-transfected cells showed significant reduction in the level of γ-tubulin, while the same treatment with scrambled ODN had no effect. TUNEL staining of cells indicated that antisense-transfected cells underwent significant apoptosis as compared to the untreated cells. Supported by a DOD BCRP grant DAMD17-98-1-8244 to AB, the Welch Foundation grant AQ-0726 and a DOD BCRP grant W81XWH-05-1-0238 to RFL.
Tetraploidy and Cell Cycle Progression Revised
A. M. Kryzwicz-Racka, G. Sluder; Cell Biology, University of Massachusetts Medical School, Worcester, MA
Cleavage failure is believed to be dangerous to the organism because the meiotic and mitotic divisions are coupled. Normal human cells do not have a tetraploidy checkpoint (Uetake and Sluder, J. Cell Biol. 2004;165(5):609–15), which characterizes the long term practical consequences of cytokinesis D induced cleavage failure in RPE1 cells expressing GFP centrin 1 to tag the centrosomes. Lineage analysis from time lapse records reveals that 60% of binucleate cells progress through the first interphase to mitosis, and approximately 70% assemble bipolar spindles due to centrosome bundling. Even though diploid control cells in the same preparations continue proliferating, more and more of the progeny of tetraploid cells arrest in interphase at each cell cycle. None divide a fourth time even when all mitoses were bipolar. To gain insight into this phenomenon, we induce cleavage failure and 60-180 min. after exit from mitosis, we microscopically cut each tetraploid cell between the nuclei to produce two mononucleate cells. Since the centrosomes often cluster between the nuclei, we produce cells that have 0, 1, or two centrosomes at G1. We find more than 50% such mononucleate cells continue to proliferate for at least 4-7 cell cycles (depending on movie duration and confluence). Also, tetraploid cells generated by the fusion between a G1 cell from which the centrosome was removed and a control G1 cell never divided more than twice. These results indicate that the eventual arrest of tetraploid cells is due to the extra genomic content, not to cell size or inappropriate centrosome number. Thus, cleavage failure per se is not sufficient to produce a proliferating population of genomically unstable cells at risk for transformation.

Plk Interacts with Cdc14A and Is Essential for Orchestrating Faithful Chromosome Segregation
K. Yuan,1 H. Hu,2 R. Hsu,2 G. Fu,2 B. Liu,1 Z. Zhu,1 X. Yao,2 Hefei National Laboratory, Hefei, China, 2Fudan University School of Medicine, Shanghai, China, 3Shanghai Second Medical University, Shanghai, China
Chromosome segregation in mitosis is orchestrated by protein kinase and phosphatase signaling cascades. Previous studies showed that overexpression of human phosphatase Cdc14A, an antagonist of CDK1, affects several aspects of cell division. However, the molecular mechanism underlying this regulation has remained elusive. To identify the proteins that participate in Cdc14A signaling in mitosis, we carried out proteomic search for mitotic proteins associated with Cdc14A. To this end, mitotic extracts from HeLa cells stably expressing FLAG-Cdc14A were purified using a FLAG antibody-affinity chromatography. The proteins selectively bound to Cdc14A were removed from the gel and analyzed by LC-MS/MS. Of several known proteins selectively bound to Cdc14A, the presence of a polo-like protein (PLK) was novel and particularly exciting. Polo-like kinases are key regulators of mitosis, regulating many aspects of the cell cycle, including mitotic entry, chromosome segregation, mitotic exit, and cytokinesis. Interestingly, this PLK interacts with and phosphorylates Cdc14A. Our mass spectrometric analysis of Cdc14A revealed that Cdc14A is phosphorylated by the PLK in vitro and in vivo. To examine the functional relevance of such phospho-regulation of Cdc14A, phospho-mimicking mutant of Cdc14A was expressed in HeLa cells. Importantly, overexpression of the phospho-mimicking and non-phosphorylatable mutants caused aberrant chromosome alignment with a prometaphase delay and aberrant chromosome segregation into daughter cells, suggesting the temporal regulation of Cdc14A phosphorylation and activity is critical for orchestrating mitotic events. Currently, we are employing biosensor to monitor the spatio-temporal profile of Cdc14A activation and illustrate how the kinase-phosphatase interplay orchestrates mitotic exit.

Microcephalin (MCPH1) Is Required for Cell-Cycle Progression in the Early Drosophila Embryo
J. L. Rickmyre, A. Y. Frist, L. A. Lee; Cell and 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. To date, no animal models for primary microcephaly due to mutations in MCPH1 have been reported. We have identified maternal-effect lethal mutations in the Drosophila homolog of human MCPH1. Early Drosophila embryogenesis consists of rapid S-M cell cycles driven by stockpiles of maternal RNA that occur without gaps or cytokinesis. These streamlined cycles as well as the genetic tractability of Drosophila offer ideal conditions in which to study cell-cycle regulation during development. Embryos from mutant MCPH1 females undergo mitotic arrest in the early syncytial divisions and contain short, rounded spindles that lack centrosomes. We have genetically determined that this is a secondary effect 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. Current efforts are directed towards elucidation of the mechanism by which mutation of MCPH1 leads to Chk2 activation as well as placement of MCPH1 within a molecular framework.

An RNAi Screen for Genes That Are Synthetically Lethal with san-l(ok1580)
T. Yamamoto, R. Kitagawa; Molecular Pharmacology, St. Jude Children’s Research Hospital, Memphis, TN
Chromosome segregation in mitosis is orchestrated by protein kinase and phosphatase signaling cascades. Previous studies showed that overexpression of human phosphatase Cdc14A, an antagonist of CDK1, affects several aspects of cell division. However, the molecular mechanism underlying this regulation has remained elusive. To identify the proteins that participate in Cdc14A signaling in mitosis, we carried out proteomic search for mitotic proteins associated with Cdc14A. To this end, mitotic extracts from HeLa cells stably expressing FLAG-Cdc14A were purified using a FLAG antibody-affinity chromatography. The proteins selectively bound to Cdc14A were removed from the gel and analyzed by LC-MS/MS. Of several known proteins selectively bound to Cdc14A, the presence of a polo-like protein (PLK) was novel and particularly exciting. Polo-like kinases are key regulators of mitosis, regulating many aspects of the cell cycle, including mitotic entry, chromosome segregation, mitotic exit, and cytokinesis. Interestingly, this PLK interacts with and phosphorylates Cdc14A. Our mass spectrometric analysis of Cdc14A revealed that Cdc14A is phosphorylated by the PLK in vitro and in vivo. To examine the functional relevance of such phospho-regulation of Cdc14A, phospho-mimicking mutant of Cdc14A was expressed in HeLa cells. Importantly, overexpression of the phospho-mimicking and non-phosphorylatable mutants caused aberrant chromosome alignment with a prometaphase delay and aberrant chromosome segregation into daughter cells, suggesting the temporal regulation of Cdc14A phosphorylation and activity is critical for orchestrating mitotic events. Currently, we are employing biosensor to monitor the spatio-temporal profile of Cdc14A activation and illustrate how the kinase-phosphatase interplay orchestrates mitotic exit.

Cell Cycle Dependent Changes in Mitochondrial Morphology
K. Mitra, J. Lippincott-Schwartz; NICHD, NIH, Bethesda, MD
Mitochondria are highly dynamic in morphology, but little is known about the significance of this dynamic nature. Here, we investigate whether mitochondrial dynamics is linked to changes in energy production during the cell cycle. Using live cell imaging approaches employing GFP-tagged mitochondrial marker, we found that mitochondrial morphology is highly regulated during the cell cycle. Cells arrested at the G1/S boundary had very long mitochondria that formed a network with a single continuous matrix based on Fluorescence Recovery After Photobleaching (FRAP) results. Time-lapse imaging of mitochondria and an S-phase-specific marker, PCNA, revealed that the network formed specifically at late G1 and persisted through early S phase, gradually breaking apart as the cell progressed through later stages of S phase. Interestingly, this PLK interacts with and phosphorylates Cdc14A. Our mass spectrometric analysis of Cdc14A revealed that Cdc14A is phosphorylated by the PLK in vitro and in vivo. To examine the functional relevance of such phospho-regulation of Cdc14A, phospho-mimicking mutant of Cdc14A was expressed in HeLa cells. Importantly, overexpression of the phospho-mimicking and non-phosphorylatable mutants caused aberrant chromosome alignment with a prometaphase delay and aberrant chromosome segregation into daughter cells, suggesting the temporal regulation of Cdc14A phosphorylation and activity is critical for orchestrating mitotic events. Currently, we are employing biosensor to monitor the spatio-temporal profile of Cdc14A activation and illustrate how the kinase-phosphatase interplay orchestrates mitotic exit.

The Role of Yeast NDR Kinase Chkl in Polarized Morphogenesis
C. Kuriscik,1 P. Nazarov,1 C. Zhang,2 K. M. Shokat,2 F. C. Luca; 1Department of Animal Biology, University of Pennsylvania, Philadelphia, PA, 2Department of Cellular and Molecular Pharmacology, University of California, San Francisco, San Francisco, CA
Mitochondria are highly dynamic in morphology, but little is known about the significance of this dynamic nature. Here, we investigate whether mitochondrial dynamics is linked to changes in energy production during the cell cycle. Using live cell imaging approaches employing GFP-tagged mitochondrial marker, we found that mitochondrial morphology is highly regulated during the cell cycle. Cells arrested at the G1/S boundary had very long mitochondria that formed a network with a single continuous matrix based on Fluorescence Recovery After Photobleaching (FRAP) results. Time-lapse imaging of mitochondria and an S-phase-specific marker, PCNA, revealed that the network formed specifically at late G1 and persisted through early S phase, gradually breaking apart as the cell progressed through later stages of S phase. Interestingly, this PLK interacts with and phosphorylates Cdc14A. Our mass spectrometric analysis of Cdc14A revealed that Cdc14A is phosphorylated by the PLK in vitro and in vivo. To examine the functional relevance of such phospho-regulation of Cdc14A, phospho-mimicking mutant of Cdc14A was expressed in HeLa cells. Importantly, overexpression of the phospho-mimicking and non-phosphorylatable mutants caused aberrant chromosome alignment with a prometaphase delay and aberrant chromosome segregation into daughter cells, suggesting the temporal regulation of Cdc14A phosphorylation and activity is critical for orchestrating mitotic events. Currently, we are employing biosensor to monitor the spatio-temporal profile of Cdc14A activation and illustrate how the kinase-phosphatase interplay orchestrates mitotic exit.
During cell, tissue and organ development, conserved signaling networks coordinate multiple processes associated with cell growth and proliferation. The NDR protein kinase family was implicated in regulating cellular morphogenesis, maintenance of cell integrity and cell cycle progression in a variety of cell types, however the molecular functions of NDR kinases are unknown. We previously established that the Saccharomyces cerevisiae NDR kinase Cbk1 functions in a conserved signaling network that is essential for maintaining cell integrity and controlling daughter specific gene expression (Weiss et al., 2002; Neill et al., 2002; Kurischko et al., 2005). To elucidate the essential role of Cbk1, we investigated the consequence of Cbk1 inhibition in synchronized cells. We found that Cbk1 kinase inhibition delayed bud emergence and growth. Cytological analyses revealed Cbk1 inhibition disrupted endocytosis and exocytosis but did not interfere with actin polarity establishment. Moreover, Cbk1 kinase inhibition triggered the Swi1-dependent cell cycle checkpoint, indicating that Cbk1 is important for coordinating polarized growth with cell cycle progression. Collectively, these data suggest that NDR kinases control cellular morphogenesis and development via regulating polarized secretion.

1453
Rfp1 and Rfp2, RING Finger Proteins with a SUMO-Interacting Motif, Regulate Chromosome Segregation in Fission Yeast
H. Sun, Salk Institute for Biological Studies, La Jolla, CA
Small ubiquitin-like modifier (SUMO) is conjugated to target proteins through a pathway similar to ubiquitylation. Most SUMOylation targets function in nucleus. The mechanism through which SUMOylation regulates cellular processes may lie in the interaction between SUMO-binding proteins and the SUMOylated targets. We have identified two proteins in Schizosaccharomyces pombe, named Rfp1 and Rfp2, each with an N-terminal SUMO-interacting motif (SIM) and a C-terminal RING domain. Consistent with their similarity in amino acid sequence, rfp1 and rfp2 compensate for each other genetically. Together they are essential for cell growth. Spores with both rfp1 and rfp2 genes missing survive for only a limited number of cell divisions and fail to grow into visible colonies. Viable strains of double null mutant cells show slow growth, elongated cell bodies, and aberrant nuclear segregation. The growth defect of double mutant cells can be rescued by expression of either wild-type Rfp1 or Rfp2, but not their SIM or RING domain mutants. Rnf4, a mammalian protein with significant sequence similarity to Rfp1 and Rfp2, appears to be their functional homologue, as it also rescues the rfp1/rfp2 double null phenotype. Nevertheless, while Rnf4 can act as a ubiquitin E3 ligase, presumably through its RING domain, we did not detect any ubiquitin or SUMO ligase activity with Rfp1 or Rfp2. When fluorescent protein-tagged forms are expressed under a mid-strength nmt promoter, both Rfp1 and Rfp2 appear as multiple nuclear spots. However, an Rfp2 mutant without SUMO-interacting motif, but not the comparable Rfp1 mutant, appears as only a single nuclear spot that is positioned next to a nucleus marker. We conclude that Rfp1 and Rfp2 as SUMO-interacting proteins may be involved in regulating chromosome segregation and progression through mitosis.

1454
Role of MicroRNAs in Drosophila Germ Line Stem Cells That Can Induce Their Own Niche
H. T. Ruohola-Baker, E. Ward, H. Scherberbata, S. Reynolds, S. Hatfield, J. Yu, K. Fischer; Biochemistry, University of Washington, Seattle, WA
A critical question in stem cell biology is how stem cell division is controlled; too little disturbs tissue homeostasis, too much can result in cancer. We have shown the necessity of microRNA pathway for germ line stem cell (GSC) function. Analysis for GSCs mutant for dicer-1 (dcr-1), the dRNAseII essential for miRNA biogenesis, revealed a dramatic reduction in the rate of germ line cyt production. Based on cell cycle markers and genetic interactions, we conclude that dcr-1 GSCs are delayed in the CDK-inhibitor p21/p27/Dacapo-dependent G1 to S transition. We show that GFP from Dacapo3’UTR-sensor construct is up-regulated in dcr-1 mutant GSCs that lack functional microRNAs, revealing that Dacapo 3’UTR is responsive to functional miRNAs. Work is in progress to identify the microRNAs critical for stem cell division. Stem cells are maintained and retain their capacity to continue dividing due to the influence of a microenvironment, termed a niche. Niche is important to maintain ‘stemness’ in a wide variety of tissues, the mechanism by which niches are maintained is unknown. We show that Notch activation can induce the expression of niche cell markers even in an adult fly. Delta over-expression in the germ line, or expression of cNotch in the somatic cells, results in extra somatic cells expressing niche cell markers. In turn, these ectopic niche cells induce ectopic GSCs, a sign of active niche. Conversely, when GSCs do not produce functional Delta and Serrate ligands, the TGF-beta pathway is not activated in the GSCs, the GSCs differentiate and the niche is lost. Importantly, Notch activity is not required in GSCs but in the somatic cells. These data show that a feedback loop exists between the stem cells and niche cells and may provide insight into how cancer can spread throughout an organism via populations of cancer stem cells.

1455
Cytokines Maintain Lymphocyte Homeostasis through the Activity of Cdc25A
C. Kittaparin, 1 W. Li, 1 S. Durum, 1 A. R. Khaleed, 1 Biomolecular Science Center, University of Central Florida, Orlando, FL, 2National Cancer Institute, NIH, Frederick, MD
Homeostasis of the peripheral immune system is maintained through signals transduced by cytokines like interleukin-7 (IL-7). In the absence of IL-7 cells undergo apoptosis, while excess IL-7 leads to proliferation. However, the mechanisms by which IL-7 regulates these life and death processes are poorly understood. We observed that expressing an anti-apoptotic protein, such as BCL-2 or inhibiting a pro-apoptotic protein, such as BAX, protected cells from death but did not support growth, suggesting the existence of previously unrecognized IL-7 dependent proliferative factors. To identify these factors, we examined cell cycling in cytokine-dependent lymphocytes and found that the G1-to-S phase progression was not solely dependent on the synthesis of cyclin-dependent kinases (cdks) and cyclins or the inactivation of inhibitors. Rather it was the removal of inhibitory phosphorylations on cdks by the phosphatase, Cdc25A, that primarily transduced cytokine-mediated growth signals. We determined that cytokine deprivation led to cell cycle arrest by inducing the activity of the stress kinase, p38 MAP kinase (MAPK), which in turn phosphorylated and destabilized Cdc25A. As a result, the substrates of Cdc25A, phosphorylated cdks, remained inactive, retinoblastoma (Rb) protein was hypophosphorylated, and cells arrested prior to induction of apoptosis. Inhibiting p38 MAPK or expressing a mutant Cdc25A, in which two p38 MAPK target sites, Ser75 and Ser123, were substituted for alanines, rendered cells resistant to cytokine withdrawal, driving the cell cycle independent of a cytokine growth stimulus. Cells expressing the Cdc25A mutant, in the absence of cytokines, actively synthesized DNA, expressed hyperphosphorylated Rb protein, maintained glucose uptake and did not undergo apoptosis, even in the presence of elevated levels of the inhibitor, p27kip. Cdc25A is thus a novel transducer of cytokine-mediated growth signals and regulation of its activity through phosphorylation by p38 MAPK is a means by which cytokines, like IL-7, can control lymphocyte proliferation.

1456
Linking Epidermal Proliferation and Differentiation: A Novel Phenomenon in Skin Homeostasis
L. Barnes, 1 2 J. Zanet, 1 M. Dumas, 1 A. Gandarillas, 2 LVMH Recherche, Saint Jean de Braye, France, 3Laboratoire Dermatologie Moleculaire, Université Montpellier 1, Montpellier, France
Human epidermis is a frequent target of hyperproliferative alterations and abnormal differentiation processes. However, the mechanisms that link cell growth and differentiation and maintain epidermal homeostasis are not understood. We have previously shown that primary differentiating keratinocytes continue DNA synthesis and become polyplloid in the absence of cell division (endoreplication). Cell growth, cell cycle, differentiation and genomic stability are thus dynamically linked. This phenomenon might have a physiological importance in keeping correct stem cell function and differentiation. We have now unravelled and studied this phenomenon in normal human epidermis in situ. To this aim, we have applied to human skin up-to-date techniques for the study of cell cycle regulation, DNA replication and genome amplification. All these investigations converge on the importance of endoreplication in normal human epidermis. This novel epidermal phenomenon may have a central role in co-ordinating proliferation with differentiation, thus maintaining skin homeostasis.

1457
Establishment of Radiosensitive Non Small Cell Lung Cancer Cell Line and Expression Profiles of the Radiodifference-related Genes
Y. Lee, 1 M. Mollah, 1 B. Kim, 1 C. Kim, 1 Y. Seo, 1 J. Park, 1 M. Cho, 1 J. Lee, 1 Department of Dermatology, College of Medicine, Chungnam National University, Daejeon, Republic of Korea, 2Department of Radiation Oncology, College of Medicine, Chungnam National University, Daejeon, Republic of Korea
Non-small cell lung cancers commonly develop resistance to radiation and chemotherapy. To gain an insight into the molecular mechanism underlying radiosensitivity, we established radiosensitive cell line using a human non small cell lung cancer cell line H460. H460 cells were irradiated with X-rays by an accumulated dose of 40 Gy over 10 weeks. After this treatment, we got radiosensitive cells and named H460R. To assess the susceptibility to radiation, we compared the several characteristics of H460 and H460R cells. These include morphological features, clonogenic ability, radiosensitivity, and cell growth kinetics. We then performed DNA microarray to examine the differences in gene expression profiles between mother and radiosensitive cells. In radiosensitivity test, H460R cells showed marked increase in radiosensitivity as compared with H460 cells. In addition, H460R cells also showed resistance to UVB and camptothecin (CPT), suggesting that these cells acquired resistance against broad range of external death stimuli. The radiosensitivity of H460R cells were repeatedly confirmed by clonogenic assay and FACS analysis. Primary cell culture, cell cycle, apoptosis, DNA repair, cell communication, cell death regulation and development. Our results suggest that many of biological characteristics were affected during the process of establishing radiosensitivity, and provide important clues for the future investigations on molecular events underlying radiation response.
1458 Blm-s, a Novel Proapoptotic Molecule, Induces Apoptosis in a Bax-dependent Manner

W. Liu, H. Huang* (equal contribution), S. Chen, S. Hou, P. Huang; Graduate Institute of Pathology, College of Medicine, National Taiwan University, Taipei, Taiwan. Department of Pathology, School of Medicine, National Taiwan University Hospital, Taipei, Taiwan. Molecules mediating neuronal apoptosis during embryonic development have long been research focus. In this study, we report a novel Bcl-2 family member enriched in mouse embryonic nervous system, Blm-s (Bcl-2-like molecule-gmnl transcript). Northern blotting reveals Blm-s expression is limited to a later developmental stage in mice, when CNS morphogenesis and physiological neuronal death occur. Blm-s contains three Bcl-2 homology (BH) domains and an ER membrane retention signal. In transfected cells, we demonstrated overexpression of Blm-s kills by apoptosis via a common mitochondrial pathway. Additionally, BH domains are required for Blm-s-mediated apoptosis. Blm-s interacts selectively with Bcl-2 and counteracts Bcl-2 antiapoptotic activity. Although not directly interacting with Bax, Blm-s induces apoptosis in a Bax-dependent manner. Furthermore, RNA in situ hybridization on γ-irradiation-treated E15.5 mice reveals such double strand break stress activates transcription of Blm-s. In summary, Blm-s is a novel Bax-dependent proapoptotic molecule and can be up-regulated after DNA damage.

1459 Rotkitamycin Induces Mitochondria Defect and Caspase-dependent Apoptosis

T. Shinomiya, M. Fukui, Y. Nagahara; 1Radioisotopes, National Research Institute of Child Health and Development, Tokyo, Japan, 2Biotecnology, Tokyo Denki University, Tokyo, Japan [Objective] Macrolides are a well-known family of antibiotics. We examined whether various macrolides induce any cytotoxic effects to human lymphoma cell lines (Jurkat and HL-60), and studied the effect of thirteen macrolides on the cell viabilities. After 40 hr incubation with 10μM or 40μM of macrolides, respectively, Rotkitamycin (RKM) exhibited strong cytotoxic effect by a MTT colorimetry assay. So we investigated whether the observed lymphoma cell death involved any particular apoptotic pathways, in further detail. [Methods and Results] RKM-treated Jurkat cells showed DNA ladders on agarose gel electrophoresis and sub-G1 DNA content cells on flow cytometric analysis. RKM-treated cells were detected in the activations of caspase 3 and 9 by the western blot analysis using anti-caspase 3 or anti-caspase 9 antibodies. Pre-treated with caspase inhibitor z-VAD-fmk, the DNA degradation and the caspases activation were blocked. DNA fragmentation and caspases activation estimated the progression of apoptosis. We went on to investigate mitochondrial membrane potentials in RKM-treated cells by the DiOC6(3) staining and flow cytometric analysis. RKM reduced the mitochondria membrane potential in the cells. Furthermore, mitochondria were prepared from Jurkat cells and incubated with none or 10μM RKM for 30 min at 37°C. Mixtures were incubated with 50 nM DiOC6(3) for 15 min then the membrane potential was analyzed by flow cytometry. Isolated mitochondria incubated with RKM resulted decreased mitochondrial membrane potentials. By the western blot analysis using anti-cytochrome c antibodies, the RKM-treatment revealed the concentrations of cytochrome c, decreased in mitochondria particles and increased in mitochondrial supernatants, respectively. [Conclusions] We concluded that RKM directly affected the mitochondria, decreased mitochondrial membrane potential, released cytochrome c from mitochondria into cytosol, activated caspase 9 and 3 and induced apoptosis in Jurkat and HL-60 cells.

1460 Scaffold Protein Intersectin-1s (ITSN-1s) Regulates Mitochondrial-dependent Apoptosis

S. A. Predescu, D. N. Predescu, I. Knezevic, A. B. Malik; Pharmacology, University of Illinois at Chicago, Chicago, IL. Intersectins (ITSNs) are multidomain adaptor proteins that have been implicated in endocytosis, regulation of actin polymerization, and Ras/ MAP kinase signaling. We have previously shown by in vitro biochemistry and protein overexpression studies that ITSN-1s expression is required for caveolea fission and internalization in cultured endothelial cells (ECs). In this study, using the RNAi approach to knock down ITSN-1s protein expression followed by morphological and biochemical analysis of ECs deficient in ITSN-1s, we demonstrate a novel role of ITSN-1s as an anti-apoptotic protein. Down-regulation of ITSN-1s expression in ECs by siRNA triggered the mitochondrial pathway of apoptosis as determined by specific morphological and biochemical changes such as genomic DNA fragmentation, extensive mitochondrial fission, blebbing of the mitochondrial membrane, widening of the narrow tubular cristae, swollen and unstacked Golgi apparatus and activation of the pro-apoptotic proteins BAK and BAX, resulting in cytochrome c efflux from mitochondria. Moreover, Bcl-X1 expression prevented BAX activation and inhibited the apoptotic ECs death caused by down regulation of ITSN-1s expression, consistent with a novel role of ITSN-1s in regulation of signaling pathways required for cell survival.

1461 Activation of Dual Apoptotic Pathways by E2F4 in Human Intestinal Crypt Epithelial Cells

H. Gameau, L. Alvarez, M. C. Paquin, C. Lussier, N. Rivard; Anatomy and Cell Biology, University of Sherbrooke, Sherbrooke, PQ, Canada. The E2F transcription factors control cell cycle progression. The localization of E2F4 in intestinal epithelial cells is cell cycle-dependent, being cytoplasmic in quiescent differentiated cells but nuclear in proliferative cells. Objective. To investigate the impact of constitutive nuclear expression of E2F4 in normal intestinal epithelial cells. Methods. Adenoviruses expressing fusion proteins between GFP and either E2F4 wild-type (E2F4wt) or E2F4-NLS containing a strong NLS from SV40LgT were used to infect non immortalized human intestinal epithelial cells (HIEC). Survival regulatory proteins were analyzed by Western blot. Caspase activities were analyzed with fluorogenic substrates. Cell death was analyzed by trypan blue exclusion assay and electron microscopy. Results. 1- Examination of living cells revealed that HIEC-expressing E2F4-NLS acquired a fibroblastic morphology few hours after infection, in contrast to non-infected epithelial HIEC or HIEC-expressing E2F4wt. 2- Two days after infection, HIEC-expressing E2F4-NLS died and detached; no exploded cells or flocculent densities in organelles or cytosol that would signal necrosis were observed. 3- An induction of p35 (5.5-fold), PUMA (3.5-fold), FAS (2-fold), BAX (2-fold) and RIP (4-fold) was also observed in HIEC-expressing E2F4-NLS comparatively to cells-expressing E2F4wt, by contrast, E2F1 protein expression remained unaltered. 4- The lentiviral infection of a sRNA which specifically knocked-down p53 expression significantly attenuated cell death induced by E2F4-NLS expression. 5- Caspase-3, 8 and 9 activities were enhanced by respectively 12, 3 and 2-fold in HIEC-expressing E2F4-NLS. 6- The caspase inhibitor z-VAD-fmk (100 μM) abolished caspase activities but only 60% reduced death induced by E2F4-NLS. 7- Inhibition of calcines (E6-d, 50 μM) and caspases prevented death of HIEC-expressing E2F4-NLS. 8- Chromatin margination, but not nuclear fragmentation, is noted in cells expressing E2F4-NLS. Conclusion. Persistent nuclear localization of E2F4 induces apoptosis of normal intestinal epithelial cell by at least two mechanisms: a p53/caspase-dependent pathway and a caspase-independent pathway involving calcines.

1462 Aquaporins 8 and 9 Translocate to the Plasma Membrane during Apoptosis and in Response to Trypsin or Collagenase and Likely Involve Integrin Signaling and Myosin IIs

W. K. Braddy, B. L. Gragg, J. S. Coley, R. L. Ross, C. M. Yengo, N. A. McConnell, G. E. White, F. M. Hughes; 1Biological, University of North Carolina at Charlotte, Charlotte, NC, 2Oncology, University of Wisconsin at Madison, Madison, WI, 3Biological and Environmental Sciences, Longwood University, Farmville, VA. Early during apoptosis cells detach from the substratum and round up. Subsequent efflux of K+ then drives the Apoptotic Volume Decrease (AVD). We have shown that during the AVD the water leaves through aquaporins (AQPs) and that the expression level of AQPs directly affects the rate of cell death. In the present study we investigated the cellular localization of AQP8 and AQP9 during apoptosis. Surprisingly, immunoreactivity in nondegrading rat ovarian granulosa cells and the uterine cancer cell line RUCA-1 was intracellular but translocated to the plasma membrane following induction of apoptosis, logically to increase the water permeability and allow the cell to die as quickly as possible. Using RUCA-1 cells we further demonstrated translocation in response to multiple apoptotic signals suggesting it is a signal-independent phenomenon. Interestingly, cell detachment by trypsin also stimulated translocation, as demonstrated by coimmunolocalization with TNF-R1 and Western blotting of isolated plasma membrane and vesicles, suggesting that cleavage of cell adhesion proteins will stimulate this event. Plating cells on collagen, we observed translocation of an AQP9-GFP fusion protein (but not a GFP-only control) in response to collagenase, implicating the disturbance of integrin signaling as a trigger for translocation. In addition, cytochalasin D prevented translocation while colchicine had no effect, suggesting that translocation takes place along microfilaments. Finally, immunolaosilated AQP9-containing vesicles contained the actin motor proteins myosin IIA, IIB and IIC by Western blot, although myosin IIC was highly enriched. Together, the results demonstrate translocation of AQP9 and 9 to the plasma membrane during apoptosis, logically to increase the rate of death, and implicating cleavage of integrins and a cessation of their signaling in stimulating this translocation. Furthermore, we identified three non-muscle myosins (IIA, IIB and IIC) associated with AQP9 vesicles which may be important in vesicular movement to the plasma membrane and/or within the cell.

1463 Mathematical and Experimental Analysis of Effector Caspase Amplification in the Death Receptor Network in Single-Cells

A proposed mechanism of ligand-induced single-cell rapid-all-or-none death is positive feedback in the apoptotic network. However, we experimentally show in HeLa cells that this hypothesis is not supported. We derive an experimentally verified mass-action ODE model of apoptosis. Model simulations indicate that in single HeLa cells, the mitochondrial death pathway is responsible for rapid, all-or-none apoptosis. We use singular perturbation techniques for model reduction, enabling us to find a theoretical threshold parameter that, when crossed, initiates fast, all-or-none mitochondrial cell death. Moreover, our model theoretically predicts, and we experimentally verify, that Bcl-2 over-expressing and XIAP knock-down cells produces "undead" single cells, that is, partially cleaved PARP in single cells that do not apoptose. Finally, we experimentally show that these undead cells proliferate, thus theoretically suggesting a mechanism of creating and/or perpetuating DNA-damaged cells.

1464 Histone Deacetylase Inhibitors Induce Apoptosis in Both Type I and Type II Endometrial Cancer Cells J. Li; S. Jiang; S. Dowdy; X. Meng; K. Podrata; S. Jiang; Department of Obstetrics and Gynecology, Mayo Clinic, Rochester, MN; 2Shandong Provincial Hospital, Jinan, China; 3Department of Pharmacology, Mayo Clinic, Rochester, MN

Objective. To investigate the inhibitory effects of histone deacetylase inhibitors on Type I and Type II apoptotic endometrial cancer cell proliferation. To characterize the molecular pathway involved in cell apoptosis induced histone deacetylase inhibitors. Methods. ARK2, Ishikawa, and AN3 cells cell lines representing both Type I and Type II endometrial cancers were treated with various concentrations of oxamflatin and HDAC inhibitor-1. Cell apoptosis was determined by flow cytometry, nuclear staining, Western blotting, and mitochondrial membrane potential assays. Results. Compared to controls, there was a 95% reduction in the growth of ARK2 cells following administration of histone deacetylase inhibitors and this response was dose-dependent. These agents also caused profound morphologic changes and loss of mitochondrial membrane potentials consistent with the induction of apoptosis. Cleavage of PARP, caspase-9, and caspase-8 was detected, indicating the activation of apoptotic cascades in endometrial carcinoma cells. This effect was present in both serous and endometrioid cell types. Conclusion. These results suggested that oxamflatin and HDAC inhibitor-1 has potent cytotoxicity in endometrial cancer cells by inducing cell apoptosis. Histone deacetylase inhibitors may be potentially useful chemotherapeutic drugs for women diagnosed with different types of endometrial carcinomas.

1465 Ultrasensitive Immunooassays for Cleaved PARP as an Indicator of Apoptosis W. Zheng, C. Foutain, J. DeSimone, J. Wang, K. J. Reagan; Signal Transduction Assay R&D, BioSource, Invitrogen Corporation, Camarillo, CA

Poly (ADP-Ribose) Polymerase (PARP) is a 116 kDa nuclear protein and a classic indicator of apoptosis. PARP is activated by cleavage by ICE family members, such as caspase-3 and -7, between amino acids Asp214 and Gly215 to yield a 85 kDa fragment. We report sensitive and quantitative cell-based methods (both an ELISA and Luminex bead based immunoassay) to analyze the amount of PARP p85 fragment as an indicator of the extent of apoptosis in cell lysates and tissue homogenates. In this study, Jurkat cells were grown in 96-well plate and treated with staurosporine at different doses and time intervals to induce apoptosis. In comparison to a caspase-3 protease assay, the PARP p85 specific immunooassay can quantitate apoptosis with a least 10 times the sensitivity. The assay can detect apoptosis in as few as 20-50 cells. The Luminex assay can be combined with other signaling markers of pro or anti-apoptotic activity.

1466 Mcl-1 Protein Binds to an Immediate Early Gene, IEX-1, and Modulates Apoptosis Signaling S. Yoon; S. M. Ryu; K. Lee; Y. Kang; M. Won; D. C. Lee; S. J. Kim; S. Y. Lee; J. J. Ko; J. Bae; 2Graduate School of Life Science and Biotechnology, CHA Research Institute, Seongnam, Republic of Korea; 3Comprehensive Gynecologic Cancer Center, Bundang CHA General Hospital, Pochon CHA University, Seongnam, Republic of Korea; 4Department of Life Science, College of Natural Science, Chung-Ang University, Seoul, Republic of Korea

Mcl-1 (myeloid cell leukemia 1) is a distinguished member of anti-apoptotic Bcl-2 family protein, and studies have been demonstrated pivotal roles of Mcl-1 protein in controlling cell death and survival under various conditions. To further understand the signaling pathway of Mcl-1 protein, we have screened 3 x 10^6 independent clones of human ovarian cDNA library using yeast two-hybrid system. We have identified IEX-1 (immediate early response gene X-1), also known as IER3, Dif-2, gly96 or p22/PRG-1, as a Mcl-1-interacting protein and investigated the two-hybrid system. We have identified IEX-1 (immediate early response gene X-1), also known as IER3, Dif-2, gly96 or p22/PRG-1, as a Mcl-1-interacting protein and investigated the possible roles of IEX-1 and Mcl-1 in their apoptosis signaling pathways. IEX-1 is a stress- and growth-induced gene up-regulated under various conditions, but the functional role of IEX-1 in the circumstances aforementioned has not been clearly understood yet. In the current study we confirmed the association of Mcl-1 with IEX-1 in the ovarian cell line SK-OV-3 as assessed by immunoprecipitation. Further, IEX-1 induced apoptosis of different cell lines as determined by cell viability assay and FACS analysis. Overexpression of Mcl-1 effectively attenuated IEX-1-induced cell killing effect, and IEX-1-mediated cell death effect was further augmented in a Mcl-1 knock downed stable cell line. In addition, IEX-1, itself, was able to block Mcl-1-mediated cell survival effect. Therefore, the current study reports that Mcl-1 and IEX-1 inhibit each other’s function by the direct association and suggests them as regulators in apoptosis signaling pathways.

1467 Ceramide Induces p38 MAPK and JNK Activation through a Mechanism Involving Thioredoxin-interacting Protein-regulating Pathway C. Chen, C. Lin, Y. Lin; Microbiology and Immunology, National Cheng Kung University Medical College, Tainan, Taiwan

Ceramide has been demonstrated as a second messenger, which is generated from sphingolipid metabolism by sphingomyelin hydrolysis or de novo synthesis pathway in various apoptotic stimuli. However the transcriptional regulation of ceramide on apoptotic signaling remains unclear. Here, using microarray analysis, we showed that ceramide induced the transcription of thioredoxin-interacting protein (Txnip), an endogenous inhibitor of thioredoxin. The increased Txnip mRNA and protein expression was further confirmed by RT-PCR, Western blotting, and confocal microscopy. Ceramide-upregulated Txnip co-localized with thioredoxin and reduced thioredoxin activity. Because thioredoxin might inhibit apoptosis-regulating signal kinase 1 (ASK1) and downstream p38 MAPK and JNK, we therefore investigate the effect of ceramide on ASK1, p38 MAPK, and JNK activation. Ceramide first activated ASK1 by inhibiting PKB-mediated inhibitory residue Ser-83 phosphorylation and increasing the activating residue Thr-845 phosphorylation. Ceramide further caused p38 MAPK and JNK activation time-dependently. Treatment with p38 MAPK inhibitor, SB203580, or JNK inhibitor, SP600125, partially reduced ceramide-induced cell apoptosis. Interestingly, ceramide-induced p38 MAPK and JNK phosphorylation was significantly inhibited by actinomycin D and Txnip siRNA treatment, which indicated that ceramide may act through Txnip-mediated transcriptional mechanism to regulate p38 MAPK and JNK phosphorylation. Taken together, our results show that ceramide exhibits a transcriptional regulation via Txnip-thioredoxin signaling to activate ASK1, which then trigger p38 MAPK and JNK activation leading to apoptosis.

1468 The Expression of Retinoblastoma Binding Protein 6 in Correlation to P53 in Lung Cancer L. R. Motadi, Z. Dlamini, K. D. Bhoola; 1MCB, Wits University, Johannesburg, South Africa; 2Centre for Allergy, Asthma and Respiratory Disease, University of the Western Australia, Perth, Australia

Tumor of the lung is the main cause of cancer related deaths and the incidence of such tumors shows a continuous. Apoptosis also known as programmed cell death (PCD), which is a genetically controlled mechanism essential for the maintenance of tissue homeostasis, proper development, and the elimination of unwanted cells. Defect in apoptosis signaling have been reported to contribute to resistance to tumour cells including lung cancer. Retinoblastoma binding protein 6 (RbBP6) gene, located on chromosome 16p21 encodes for RbBP6 protein 13kDa which consist of only the domain (DOMAIN) and RbBP6 protein which contains several other domains including Zinc finger and p53 binding domain which is believe to be involved in p53 dependent pathway. The aim of this study was to establish the relationship between apoptosis levels and RbBP6 expression in A549 cell lines, MRC5 cell lines and lung cancer tissues. A total of 98 histologically confirmed cases of various lung cancers were analyzed for protein expression of RbBP6 and p53 regulators of apoptosis. RT-PCR revealed that the levels of the transcripts of RbBP6, p21, Bax and Bcl2 were variable and that there was no direct correlation between them. The ratio of Bcl2/Bax was around 1.5 which indicated increased levels of antiapoptotic molecules at the transcript level. The protein expression levels of Bcl2, Bax and RbBP6 ranged from negative to positivity. Immunostaining showed cytoplasmic expression with Bcl2 showing basal positivity whereas RbBP6 showed some nuclei expression. From this small analysis of various lung cancers, it appears that the balance is in favour of anti-apoptosis which is predominantly determined by the Bcl2/Bax ratio.

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Alpha-Crystallins Modulate p53 Phosphorylation Status to Attenuate UVA-induced Apoptosis

J. Liu, J. Qin, Q. Yan, D. W. Li; Biochemistry & Molecular Biology, University of Nebraska Medical Center, Omaha, NE, College of Life Sciences, Hunan Normal University, Changsha, China

UV-induced apoptosis is linked to cataractogenesis of the ocular lens. The ocular lens normally guards apoptosis of the lens epithelial cells through the function of the lens structure proteins, alpha-crystallins. In our previous studies, we have demonstrated that alpha-crystallins can negatively regulate UVA-induced apoptosis through suppression of the ERK-mediated pathway and activation of the AKT signaling pathway. In the present study using UVA irradiation, Western blot analysis and apoptosis assays, we present evidence to show that alpha-crystallins can modulate the phosphorylation status of the tumor suppressor, p53 to antagonize UVA-induced apoptosis. Thus, our results demonstrate that alpha-crystallins can regulate UVA-induced apoptosis through different mechanisms. Supported by NIH/NEI IR01 EY115765 and the Lotus Professorship Funds from Hunan Province Government.

A Zinc Finger Containing Protein, P99 Induces Cell Death

D. Kang, J. Kim, J. Lim, J. Yu; Korea Basic Science Institute, Chunchon Center, Chunchon, Republic of Korea

We show here functional study of p99, a new anaphase promoting complex/cyclosome (APC/C) substrate using imaging tools such as three dimensional colocalization technique, long-term live imaging and FRET (fluorescence resonance energy transfer) method. APC/C is known to have important roles during cell cycle. The evidence that these complexes are related with a variety of cellular events such as neural cell differentiation and genetic stability beyond cell cycle is rising. We adopted small pool expression screening to find new substrates of APC/C with some modifications. We isolated many interesting candidates as APC/C substrates. Among them, a zinc finger containing protein, p99 was selected to investigate the functional consequences of its degradations. We show a full length p99 is ubiquitinated in vitro. Interestingly, p99 is expressed in a nucleus as speckles and co-localized with SC-35, a marker of IG (interchromatin granule cluster). Over-expression of p99 disturbed normal cell cycle progression. Live fluorescence imaging show p99 induces cell death in dose dependent manners. The physiological function of p99 and meaning of its degradation is under investigation.

Molecular and Celluar Characteristics of Apoptosis and Cell Growth in Cervical Cancer Cells Following RNAi Mediated Knockdown of Survivin, Cdc2 and Eg5


Resistance to apoptosis is a hallmark of cancer cells. The ability to regulate apoptosis, holocytochrome c is released from mitochondria and associates with Apaf1 and procaspase 9, forming a complex called the apoptosome. Apoptosome formation results in activation of caspase 9 initiating a proteolytic caspase cascade and the ultimate demise of the cell. The analysis of cytochrome c release is important in determining the commitment to apoptosis in model signaling systems. Widely employed cellular fractionation strategies for determination of cytochrome c release generally involve mechanical disruption of cells resulting in incomplete or excessive rupturing of cells and mitochondria. Subsequent analysis of subcellular fractions by Western blot can only be regarded as qualitative, reflecting the quality of the fractionation procedure. We have developed a fluorescent assay for determination of mitochondrial cytochrome c localization on a per cell basis. The assay relies on the selective permeabilization of the cellular membrane for release of cytosolic components while leaving the mitochondrial membrane intact. Cytochrome c is detected with a monoclonal antibody by flow cytometry or fluorescent microscopy. To determine the usefulness of the assay in measuring apoptotic cell death, Jurkat cells were treated with Actinomycin D and cytochrome c release was determined by flow cytometry. In parallel, samples were processed for annexin V binding, DNA fragmentation (TUNEL), and propidium iodide staining. Cytochrome c release was detected at earlier time points of the cell death response when compared to the TUNEL based assay and propidium iodide staining. Furthermore, unlike annexin V staining, the cytochrome c stained cells are fixed making the assay amenable to mult-paramater analysis of apoptotic cells as well as convenient storage of cells for future analysis. Unlike traditional cellular fractionation protocols, the assay may be useful for high throughput screening as well as the analysis of scarce samples such as primary cells.

Soft Substrate Induces Apoptosis by the Disturbance of Ca2+ Homeostasis in Renal Epithelial LLC-PKI Cells

W. Chiu, Y. Wang, M. Tang, M. Shen; Institute of Basic Medical Sciences, National Cheng-Kung University, Tainan, Taiwan, Department of Pharmacology, National Cheng-Kung University, Tainan, Taiwan

Different rigidities of adhesive substrate affect cellular functions in different ways. Here, we cultured a renal epithelial cell line (LLC-PKI) and a tumor cell line (HeLa) on substrates of different rigidities and compared the cell type-specific responses. The culture dish was coated with a very thin layer of collagen gel (control group) or overlaid with collagen gel (soft substrate). LLC-PKI cells contracted as they grew on collagen gel and the apoptotic bodies obviously appeared with time. The protein levels of procaspase-12 and its downstream target procaspase-3 were decreased when LLC-PKI cells cultured on collagen gel. µ-calpain was activated on collagen gel. In contrast, there was no significant change in cytochrome c reduction, mitochondrial membrane potential, and the protein levels of procaspase-8 and procaspase-9. Moreover, soft substrate caused elevated cytosolic Ca2+. Ca2+ overload in ER and upregulation of capacitative calcium entry. Ca2+ chelator or channel blocker partially rescued the collagen-gel induced apoptosis by inhibiting µ-calpain activation. In contrast, for HeLa cells cultured either on...
Molecular Determinants of Mob1 Localization at the Spindle Poles, Kinetochores, and Central Spindle of Dividing Cells

L. J. Wilneth, J. Rashe, S. Shrestha, C. B. Shuster; Biology, New Mexico State University, Las Cruces, NM

Maintenance of chromosomal ploidy requires chromosome segregation and cytokinesis be precisely coordinated in space and time. We have focused on the localization dynamics of Mob1 family members, which are required for activation and localization of the NDR family kinases, and are essential for mitotic progression in yeast. There are 4 human Mob1 isoforms, and to discriminate between these highly similar proteins, Mob1-A-D were fused to EGFP. Transient expression of all four isoforms in HeLa cells revealed partially redundant localization patterns through mitosis and cytokinesis. Mob1 proteins were found on interphase centrosomes, with enrichment late in G2 and remaining through cytokinesis. Spindle pole localization was microtubule-independent, suggesting that Mob1 is a true centrosomal protein. Additionally, Mob1 recruitment to kinetochores could be detected in control cells from prophase until anaphase onset, as well as in cells arrested with nocodazole or monastrol. Upon anaphase onset, Mob1 was found at the spindle poles and central spindle, with equatorial localization maintained throughout cytokinesis. Mob1 localization at metaphase and cytokinesis is suggested to be microtubule-dependent, because Mob1A localization was examined in cells depleted of INCENP and PLK1 by siRNA. INCENP depletion resulted in lagging chromosomes during anaphase and cytokinesis failure, and while Mob1A localization at the centrosomes was unaffected, kinetochore- and central spindle localization was lost. Conversely, PI3 depletion, did not affect kinetochore localization, although spindle pole localization was reduced. Together, these results suggest that there may be multiple regulatory inputs determining Mob1 localization, including the possibility that Mob1 functions downstream of the chromosomal passenger complex. Supported by P200R016480 and S06GM008136.

NEK2A Regulates the Interaction of Hecl with CENP-H and the Attachment of Spindle Microtubules to the Kinetochore

A. Jimenez-Dalmaroni, M. Théry, V. Racine, F. Julicher, M. Bornens; 1Max Planck Institute for the Physics of Complex Systems, Dresden, Germany, 2UMR 144, Institut Curie, Paris, France

Chromosome segregation in mitosis is orchestrated by the interaction of the kinetochore with spindle microtubules. Our recent study shows that NEK2A interacts with MAD1 at the kinetochore and possibly functions as a novel integrator of the spindle checkpoint signaling. However, it is unclear how NEK2A regulates kinetochore-microtubule attachment in mitosis. Here, we report that NEK2A modulates the interaction of Hecl with CENP-H and the attachment of spindle microtubules to the kinetochore during mitosis. Hecl1 interacts with CENP-H in vitro and in vivo via extended coiled-coil domains located in the middle of the two molecules, respectively. Our immunofluorescence study revealed that Hecl1 is localized at the metaphase kinetochore exterior to CENP-H with the globular domain oriented toward microtubule plus end. NEK2A phosphorylates Hecl1 at Ser165 during mitosis while such phosphorylation regulates Hecl1-CENP-H interaction in vitro and in vivo. Interestingly, phosphorylation of Ser165 is not essential for assembly of Hecl to kinetochore as non-phosphorylatable mutant Hecl1S165A is localized to the kinetochore. Moreover, Hecl1 interacts with kinetochore microtubules. However, there was a significant increase in attachment errors, including syntelic attachment and monotelic attachment. In addition, these Hecl1S165A-overexpressing cells display a chromosome-bridge phenotype with sister chromatids inter-connected. These findings reveal a key role for the NEK2A-mediated phosphorylation of Hecl1 in governing proper kinetochore-microtubule attachment and support a notion by which NEK2A integrates kinetochore-microtubule attachment into spindle checkpoint signaling. Using fluorescence resonance energy transfer measurements in living cells, we are evaluating how NEK2A orchestrates the Hecl1-CENP-H interactions in kinetochore assembly and chromosome segregation.

B-Raf Is Required for Spindle Formation and Chromosome Congregation in Human Somatic Cells

M. E. B. Brooks, T. Gandaglio, J. V. D. M. Molecular Oncology, Moffitt Cancer Center and Research Institute, Tampa, FL

B-Raf is a member of the Raf family of serine/threonine protein kinases that serves a prominent role in regulating cell proliferation. The cellular mechanisms through which B-Raf mediates cell proliferation remain poorly understood. Here we report a novel role for B-Raf in regulating spindle formation in somatic cells. We show by epifluorescence and confocal microscopy that B-Raf localizes at the mitotic spindle and spindle-associated structures during the stages of mitosis. A detergent-insensitive pool of B-Raf is tightly associated with the centrospheres throughout the cell cycle. Furthermore, phospho-state specific antibodies detect phospho-B-Raf (Thr599/Ser602) at the centrioles, kinetochores, and outer regions of chromosomes during prometaphase, metaphase, and anaphase, and at the midbody during cytokinesis. To determine the functional significance of B-Raf localization at the spindle apparatus, RNA interference was used to down-regulate B-Raf in human cells. Reduction of B-Raf resulted in pleiotropic effects on the mitotic spindle and chromosome alignment in over 80% of mitotic cells. Similar results were observed using a second siRNA that targeted a different exon in B-Raf. In contrast, reduction of C-Raf (Raf-1) had no effects on spindle formation or chromosome alignment suggesting that B-Raf, and not Raf-1, is critical for regulating the mitotic spindle. Additionally, we showed that depletion of B-Raf disrupts spindle pole focusing in Xenopus egg extracts, a system in which B-Raf is activated exclusively during mitosis. In summary, our results demonstrate that B-Raf is required for proper mitotic spindle assembly and chromosome alignment in human somatic cells.

Theoretical and Experimental Description of Mitotic Spindle Orientation

A. Jimenez-Dalmaroni, M. Théry, V. Racine, F. Julicher, M. Bornens; 1Max Planck Institute for the Physics of Complex Systems, Dresden, Germany, 2UMR 144, Institut Curie, Paris, France

The architecture and adhesiveness of cell microenvironment is a critical factor regulating spindle orientation in vitro. It has been shown that the orientation of the spindle during HeLa cell division in vitro depends on the spatial distribution of adhesive cell contacts. It has also been demonstrated, that minus-end directed cortical force generators exert forces on astral microtubules. Here we show that the observed spindle orientation in HeLa cells can be understood as the result of the action of cortical force generators interacting with astral microtubules. We assume that these force generators are activated by cortical signals which are associated with retraction fibers connecting the mitotic cell body to its adhesive microenvironment. The force generators exert a torque on the spindle which depends on its relative orientation with respect to the microenvironment geometry and leads to spindle rotation. We developed a simple physical description of this spindle mechanics which allows us to calculate angular profiles of the torque acting on the spindle and the angular distribution of spindle orientations. Our model could account for the spindle orientations observed in a large variety of different adhesive patterns. Remarkably it also describes the shape transition from symmetrical to asymmetrical orientation observed for pattern of similar type. Our results suggest that a slight modification of cell microenvironment is sufficient to provide either main polarizing signals and stimulate symmetrical orientation or a single polarizing signal and stimulate asymmetrical orientation of the spindle. It supports the hypothesis that, in vivo, niche architecture is established so as to embed stem cells in a geometrical configuration close to this transition. 1. Fuchs, E. et al. Cell (2004). 2. Thery, M. et al. Nat Cell Biot (2005). 3. Grill, S. W. et al. Science (2003).

Bod1, a Novel Kinetochore Protein Required for Chromosome Biorientation

I. M. Porter, G. A. Khoudels, J. S. Anderson, J. W. Bow, J. R. Swedlow; 1Division of Gene Regulation and Expression, University of Dundee, Dundee, United Kingdom, 2Division of Biochemistry and Molecular Biology, University of Southern Denmark, Odense, Denmark

Through proteomic analysis of Xenopus chromosomes and a secondary RNAi live cell imaging screen we have identified a novel protein required for proper chromosome alignment during mitosis that we have named Bi-orientation defect 1 (Bod1). Bod1 is a small 22 kDa protein that is a member of a family of three closely related proteins that appear to have arisen due to gene duplication. GFP tagged Bod1 localised to centrosomes throughout the cell cycle and also localised to kinetochores from prometaphase through to anaphase. We performed time-lapse microscopy of HeLa cells expressing GFP tagged Bod1 and observed that Bod1 was not recruited to the centromeres of chromosomes until prometaphase. Bod1 recruitment to centromeres was not mitosis-dependent, suggesting that Bod1 is a microtubule associated protein. Co-localisation studies indicate that the majority of these attachments are stable suggesting that the kinetochores are intact. Together, these results suggest that Bod1 is a novel protein required for the maintenance of chromosome biorientation at metaphase.
A Conserved Role of Kinesin-14 Family Proteins HSET/XCTK2 in Controlling Spindle Morphology

S. Cai, S. C. Ems-McClung, C. E. Walczak, C. Biochemistry Program, Indiana University, Bloomington, IN; Medical Sciences Program, Indiana University, Bloomington, IN

Kinesin-14 family proteins are minus-end directed motors, which drive microtubule movements and play key roles during mitosis. We showed previously that the Xenopus Kinesin-14 XCTK2 is regulated by Ran via the association with importin alpha/beta to a bipartite NLS in the tail of XCTK2. Binding of importin alpha/beta to XCTK2 inhibits its association with microtubules and blocks its ability to cross-link microtubules during spindle formation. Whether Ran-regulation of the Kinesin-14 family is conserved in vertebrate cells is not known. We found that knockdown of the human Kinesin-14 HSET by RNAs resulted in shorter spindles, and overexpression of HSET increased spindle length. These results are consistent with the role of HSET in microtubule cross-linking and sliding in the spindle. To ask whether HSET is regulated by Ran, we identified the NLS in the tail of HSET and generated NLS mutants. Transfection of NLS-mutated-HSET caused an accumulation of HSET in the cytoplasm, which resulted in strong microtubule bundling, suggesting that one role of the NLS is to sequester HSET from cytoplasmic microtubules. In mitosis, overexpression of the NLS-HSET mutants also caused spindle defects, consistent with its role in microtubule cross-linking. To compare HSET with XCTK2, we generated full-length XCTK2 with a mutated NLS. Addition of a 5-10 fold molar excess of NLS mutant XCTK2 over endogenous levels to extracts disrupted spindle assembly by causing excessive microtubule sliding. This effect is additive with expression of wild-type XCTK2, which resulted in enhanced spindle formation. Our results are consistent with a model in which Ran regulates the association of XCTK2/HSET with importin alpha/beta to prevent aberrant cross-linking and bundling of microtubules and to sequester XCTK2/HSET in the nucleus. Upon NLS, XCTK2/HSET is released from importin alpha/beta in the vicinity of chromosomes to facilitate microtubule cross-linking and organization of the spindle.

Organization and Dynamics of the Budding Yeast Metaphase Spindle

H. Qi, Y. Ding, L. Zhu, U. Rath, J. Girtton, K. M. Johansen; Biochemistry, Biophysics & Molecular Biology, Iowa State University, Ames, IA

The concept of a spindle matrix has long been proposed based on theoretical considerations of the requirements for force production to help organize and stabilize the microtubule spindle during mitosis. However, molecular evidence corroborating its existence and function has been elusive. In *Drosophila* we have recently identified four nuclear proteins, Skeleton, Chromator, Megator, and EAST that interact with each other and that redistribute during prophase forming a fusiform spindle structure that persists in the absence of polymerized tubulin. Two of these proteins, Skeleton and Chromator, are localized to chromosomes during interphase whereas the other two, Megator and EAST, occupy the intranuclear space surrounding the chromosomes. Megator is a 260 kD protein with a large NH-terminal coiled-coil domain that is capable of self-assembly when expressed in S2 cells indicating that Megator has the necessary molecular properties to serve as the structural basis for the spindle matrix complex. To further analyze Megator's role in spindle function we used EMS mutagenesis to generate 43 new alleles that reduced adult survival rates by more than 50% when heterozygous with the null Megator allele. The new Megator alleles fall into three distinct complementation groups possibly reflecting an essential role of Megator in three different cellular contexts. Currently, we have identified one allele from complementation group I, 22-6, with clear mitotic spindle defects. In 22-6 the ipMTs are misaligned, incompletely formed, and with abnormal congression and segregation of chromosomes. In mutant brains (n=9) 317 out of 346 neuroblasts examined had such phenotypes versus only 13 out of 265 neuroblasts in control brains (n=12) (χ² test). These results strongly suggest that Megator mutations can result in severe chromosome segregation defects as well as in spindle abnormalities and support the hypothesis that Megator plays a functional role in mitosis. (Supported by NSF grant MCB0445182).

Role of the Bipolar Kinesin-5, KLP61F, in Poleward Flux and Spindle Elongation in *Drosophila* Embryos

I. Brust-Mascher, D. Cheemanthatham, P. Shroff, J. M. Scholky; Molecular and Cell Biology, University of California, Davis, Davis, CA

Mitosis involves a highly choreographed sequence of motility events that depend upon the action of microtubule (MT)-based force generators, namely MT polymer ratchets, kinesins and dyneins. Here we focus on the role of KLP61F during the metaphase-anaphase A steady state and during anaphase B spindle elongation. In previous studies we proposed that the bipolar kinesin KLP61F (Sharp et al, 2000, MBC 11:241) persistently slides dynamically unstable ipMTs outward, while the depolymerase KLP10A (Rogers et al, 2004 Nature 427:364) acts at the poles to convert ipMT sliding to flux before anaphase B. The inhibition of this depolymerization at anaphase B onset suppresses flux and allows ipMT sliding to drive spindle elongation (Brust-Mascher and Scholky, 2002, MBC, 13:3967, Brust-Mascher et al, 2004, PNAS 101:15493). The proposal that KLP61F slides ipMTs during both the steady state and during spindle elongation predicts that this motor contributes to poleward flux within ipMTs. To test this we are using Fluorescence Speckle Microscopy and time lapse confocal microscopy to measure flux and spindle pole movement in the syncytial embryo. Injection of an antiKLP61F function blocking antibody produces a gradient of defects. The most severe defect occurs during prometaphase and spindle pole movement in the syncytial embryo. Tracking kinetochore-proximal chromosome tags further allows us to measure and compare the dynamics of individual kinetochore microtubules in wild type and mutant cells, which has revealed that kinetochore proteins are key regulators of kinetochore microtubule dynamics.
The Endocytic Coat Protein ARH Associates with Motor and Centrosomal Proteins and Is Involved in Mitosis

S. Lehtonen, R. Nielsen, N. Iino, J. J. Ryan, H. Zhou, M. G. Farquhar; Department of Cellular and Molecular Medicine, University of California San Diego, La Jolla, CA; Ludwig Institute for Cancer Research, University of California, La Jolla, CA; Department of Cellular and Molecular Medicine and Ludwig Institute for Cancer Research, University of California San Diego, La Jolla, CA

ARH is an endocytic adaptor protein that is essential for LDL receptor mediated uptake of LDL in hepatocytes and which we found is required for uptake of ligands by megalin (Nagai, MBC 14: 4984-4996, 2003). This binds clathrin and AP2, and the NPXY sequence present in the cytoplasmic tail of LDLR superfamily members. Here we report novel and unexpected findings showing that ARH is a core centrosomal protein. Moreover, we found localization of ARH to be regulated in a cell-cycle dependent manner: When cells entered mitosis, ARH was recruited to kinetochores and spindle poles where it co-localizes with the motor protein cytoplasmic dynein and dynactin. Furthermore, during cytokinesis ARH was detected in the midbody of HeLa cells. By co-IP followed by mass spec analysis ARH was found to form a complex with y-tubulin complex proteins and dynine. Centrosomes are involved in several cell cycle regulatory events in addition to serving as microtubule nucleation sites and dynein, a microtubule-based molecular motor, participates in cell division and vesicular trafficking. Depletion of ARH with siRNA in Rat-1 cells caused a G1 delay, and immuno/fluorescence showed that microtubules seem to be organized more randomly in ARH depleted cells compared to control cells. These data suggest that ARH has structural and/or functional roles that are needed for normal progression of mitosis.

The ENDO Network Couples Cyclin Stability to the Assembly of the Mitotic Spindle and Checkpoint Activation

K. H. Ban, J. Z. Torres, J. J. Miller, A. Mikhailov, M. V. Nachury, J. J. Tung, C. L. Rieder, P. K. Jackson; Program in Cancer Biology, Stanford University, Stanford, CA; Department of Pathology, Stanford University, Stanford, CA; Genentech, Inc., South San Francisco, CA; Program in Molecular Pharmacology, Stanford University, Stanford, CA; Division of Molecular Medicine, Wadsworth Center, Albany, NY

The activation of cyclin-dependent kinase 1 (CdK1) drives chromosomal and microtubule organization, and at a later time downregulates its own activity by initiating the ubiquitination and destruction of cyclin B by the Anaphase Promoting Complex/Cyclosome (APCC) E3 ligase. Studies to date have emphasized the role of checkpoint signaling at the kinetochore to provide the critical delay for APC/C activation. However, recent work has demonstrated an additional role for checkpoint-independent mechanisms in restraining APC/C activation in early mitosis when kinetochores are not yet competent to generate the necessary APC/C inhibitory signals. Here, we show that a novel regulatory pathway, called the ENDO (Emi1/NuMA/Dynein-dynactin) network, forms a critical APC/C inhibitory mechanism on spindle poles in human tissue culture cells. We demonstrate that a specialized pool of the APC/C inhibitor Emi1 (~15%) persists in early mitosis and binds to the APCC on the mitotic spindle poles from prophase to anaphase. This organization is driven by the minus-end directed motor dynein-dynactin and the spindle protein NuMA. We find that Emi1 binds directly to the microtubule binding domain of NuMA and this interaction anchors the Emi1-APCC complex to the spindle pole. Inactivation of Emi1 in mitosis leads to the loss of pole-associated cyclin B due to the premature activation of APC/C at the poles. Because NuMA function requires Cdk1 activity, this premature destruction of cyclin B disrupts NuMA-dependent anchorage of microtubules at the poles thus resulting in pole fragmentation and aberrant spindle formation. Surprisingly, we find that perturbing the END network abolishes the recruitment of checkpoint proteins to the kinetochores, suggesting that cyclin B/Cdk1-dependent organization of the spindle pole is linked to the activation of the kinetochore-directed spindle checkpoint. We propose that the END network functions to couple localized cyclin stabilization to both spindle assembly and checkpoint activation following prophase.

Identifying the Kinetochore-derived “Wait Anaphase” Signal of the Mitotic Checkpoint

A. Kulukian, D. W. Cleveland; Biology, University of California, San Diego, La Jolla, CA; Ludwig Institute for Cancer Research, University of California, San Diego, La Jolla, CA

The mitotic checkpoint is the major cell cycle control mechanism for maintaining diploid chromosome content. Prevention of premature anaphase onset requires the production by unattached kinetochores of a diffusible “wait anaphase” inhibitor that is thought to function by inhibiting the ability of Cdc20 to activate the APC/C, the E3 ubiquitin ligase that targets mitotic regulators such as cyclin B and securn for degradation. The exact composition of this inhibitor(s) has not been established, although the mitotic checkpoint components BubR1 and Mad2 can bind Cdc20 directly and have, along with Bub3, been implicated as potential components of this inhibitor. In addition, Mad1 and Bub1, stably bound components of unattached kinetochores, have also been shown to play a role in the generation of the checkpoint signal, potentially through the recruitment of diffusible components (including Mad2 and BubR1) to the mitotic spindle and the possible modification of those components upon recruitment. To identify the kinetochore-derived anaphase inhibitor, we have attempted to reconstitute mitotic checkpoint signaling in vitro from purified components. Using cyclin B ubiquitination as an assay for APC/C activity, purified recombinant checkpoint proteins Mad2, Bub3, and BubR1 are demonstrated to act synergistically to inhibit APC/C/Cdc20, with the combination at least four times more potent than each alone. Moreover, purified chromosomes (with unattached kinetochores) amplify, by a further 10 fold, the overall generation of a Cdc20-specific APC/C inhibitor. Fluorescently labeled Mad2 is shown to localize to kinetochores as further proof of its role as part of a kinetochore-generated inhibitory signal. This provides an assay for identifying the kinetochore-derived mitotic checkpoint inhibitor and determining how unattached kinetochores catalyze the production of this inhibitor.

Mechanisms of Poleward Chromosome Movement

E. L. Grishchuk, M. L. Molotskov, J. S. Spirodnov, A. K. Yetefromov, F. I. Atullakhakovov, J. R. McIntosh; MCB Biology, University of Colorado, Boulder, CO; National Research Center for Hematology, Moscow, Russian Federation

Poleward chromosome movement during mitosis is dependent upon the activities of minus-end directed, microtubule-dependent motors, and it requires the depolymerization of microtubules. To learn the relative roles of these factors in the process, we have undertaken two experimental approaches. Microtubule depolymerization is studied in a novel experimental system, which allows the direct measurement of the depolymerization force. By conjugating glass microbeads to microtubules through strong inert linkages, and by using laser tweezers we have shown that depolymerization of microtubules can exert a brief tug on an attached bead (Grishchuk et al, Nature 2005). Analysis of these interactions with a molecular-mechanical model of microtubule (Molodtsov et al, PNAS 2003) suggests that a single disassembling microtubule can generate about ten times the force that is developed by a motor enzyme. Thus, this mechanism might be the primary force for chromosome motion. This experimental system is being applied to analyze force production by depolymerizing microtubules that are linked to cargo via processive couplers. We are also studying the roles of minus-end directed motors in poleward chromosome motions in vivo using fusion yeast (Grishchuk and McIntosh, submitted). This organism has three such motors: dynein and two kinesin-14s, Pkl1p and Klp2p. The maximum rate of poleward chromosome movement was unaffected by the deletion of any of these motors, suggesting that microtubule depolymerization can cause such movements in vivo. However, Klp2p, which localizes to kinetochores, contributed to the effectiveness of poleward movement by promoting the shortening of kinetochore fibers. Pkl1p and dynein were required for efficient chromosome bi-orientation. In pkl1a, whose product normally localizes to the spindle poles, the checkpoint that monitors chromosome bi-orientation was defective, leading to premature anaphase. Electron microscopy suggests that Pkl1p contributes to error-free bi-orientation by promoting the normal organization of spindle poles, while dynein helps to anchor microtubule minus ends.

Cytoplasmic Dynein Drives Fast Poleward Motion during Kinetochore Attachment in Vertebrates

Z. Yang, C. Rieder; Laboratory of Cell Regulation, Division of Molecular Medicine, Wadsworth Center, New York State Department of Health, Albany, NY; Biomedical Sciences, SUNY School of Public Health, Albany, NY

During mitosis in vertebrates kinetochores become attached to the forming spindle as they interact with dynamic microtubules growing from the separating centrosomes. Due to the random nature of this process it is not unusual for one kinetochore to become attached to the spindle before its sister does. Under this condition the chromosome “monoorient” and rapidly moves towards the pole it is attaching to (C.L. Rieder and S.P. Alexander. J. Cell Biol., 110:81-95, 1990). Although this rapid motion is through to be mediated by kinetochore-associated cytoplasmic dynein, there is no direct evidence that this is so. To examine this in detail we tracked kinetochore motion during nuclear envelope breakdown in live human U2OS CENP-B-GFP cells after knocking down zw10 by siRNA. This approach allowed us to selectively deplete kinetochores of cytoplasmic dynein, without inhibiting normal bipolar spindle assembly. As expected, we found that shortening of numerous kinetochores exhibited a rapid poleward motion up to 5 minutes after nuclear envelope breakdown, some with rates > 20 μm/min. However, after zw10 siRNA treatment the number of rapid poleward kinetochore motions was greatly reduced, the normal dynamics of chromosome congression was perturbed, and the duration of prometaphase was considerably prolonged. From our data we conclude that cytoplasmic dynein is involved in the initial rapid poleward motion of attaching chromosomes, and also in normal congression movements. We are currently exploring the role of dynein in anaphase poleward motion.

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A Change in the Distribution of Microtubule Plus Ends Contributes to the Anaphase B Switch in Drosophila Mitotic Spindles

D. K. Cheeresarabathur, G. Civelękoglu-Scholey, J. Braut-Mascher, P. Sommi, A. Mogilner, J. Scholey; MCB, University of California, Davis, Davis, CA

Spindle elongation during anaphase B in Drosophila embryos is initiated by the inhibition of minus end depolymerization within microtubules (MTs), which allows the sliding apart of interolar (ip) MTs to exert forces that drive spindle pole separation. Here we monitored the dynamics of the plus ends of MTs within the spindle during mitosis by using time lapse microscopy of the GFP-labeled plus end tip tracker protein EB1. Analysis of EB1 revealed a change in the distribution of growing MT plus ends, which are localized throughout the spindle prior to anaphase B, but concentrate in the overlap region of ipMTs at the equator at anaphase B onset. This change in plus end distribution during the transition is reflected in MT polymer dynamics measured using fluorescence recovery after photobleaching (FRAP) of GFP-tubulin. Our studies reveal that spindle MTs display very high dynamicity (recovery half time, 5-10 sec) at all stages of mitosis on all subsets of MTs. However, the fractional recovery of spindle MTs becomes spatially non-uniform at the onset of anaphase B, with MTs proximal to the poles recovering to a significantly lower extent (~ 45%) than elsewhere (80-90%), indicating the emergence of a stable subset of MTs near the poles. Quantitative modeling suggests that this depends on the establishment of a spatial gradient in MT dynamic instability parameters, with MT plus end catastrophe frequencies being highest at the poles and decreasing toward the equator. The resulting redistribution of growing MT plus ends to the ipMT overlap zone, together with the suppression of ipMT depolymerization at the poles, constitutes a mechanical switch that initiates spindle elongation.

Kinesin-14 and the Dam1 Complex Ensure Microtubule-dependent Kinetochore Transport Towards Spindle Poles by Distinct Mechanisms

K. Tanaka, E. Kitamura, Y. T. U. Tanaka; School of Life Sciences, University of Dundee, Dundee, United Kingdom

To ensure high-fidelity chromosome segregation, kinetochores must be properly captured by spindle microtubules. Kinetochore are initially captured by the lateral sides of single microtubules and subsequently transported towards spindle poles. Poleward kinetochore transport is especially crucial when kinetochores are located at a distance from the mitotic spindle. We found that at least two mechanisms are involved in microtubule-dependent poleward kinetochore transport in Saccharomyces cerevisiae. First, Kar3, a kinesin-14 family member and a microtubule minus-end directed motor, localizes at kinetochores and drives poleward kinetochore sliding along the lateral surface of microtubules. Second, following capture by microtubule lateral sides, kinetochores are tethered at the distal ends of microtubules and pulled poleward as microtubules shrink. The first mode of kinetochore transport is often converted to the second one, but the opposite conversion rarely happens. The second mode leads to more processive kinetochore transport and crucially depends on the Dam1 complex. The Dam1 complexes accumulate at the microtubule plus ends as microtubules shrink, providing evidence that the complex forms rings encircling microtubules in vivo, as reported in vitro. We suggest that the Dam1 complex plays an essential role in converting microtubule depolymerization into the poleward kinetochore-pulling force. Thus Kar3 and the Dam1 complex ensure microtubule-dependent kinetochore transport by distinct mechanisms.

The Kinesin-13 Family Member, KLP59D, Contributes to Both Poleward Flux and Pacman-based Chromosome Motility in Drosophila S2 Cells

U. Rath, G. C. Rogers, D. J. Sharp; 1Department of Physiology and Biophysics, Albert Einstein College of Medicine, Bronx, NY, 2Department of Biology, University of North Carolina, Chapel Hill, NC

Kinesin-13 family members destabilize microtubules and perform important and conserved roles during mitosis. Previously, we reported that Drosophila contain three distinct Kinesin-13s, termed KLP10A, KLP59C, and KLP59D. In embryos, two of these, KLP10A and KLP59C depolymerize microtubule minus- and plus-ends, respectively, to drive Anaphase A by a “Pacman-Flux” mechanism. Here, we assess the mitotic functions of KLP59D. Our data reveal that KLP59D targets to outer kinetochores and centrosomes. Following KLP59D knockdown in S2 cells using RNAi, the rate of anaphase A decreases by ~50%. Similar results were obtained following KLP59D antibody injections into embryos. Photobleaching experiments performed in S2 cells expressing fluorescently labeled microtubules and chromosomes reveal that KLP59D exerts its impact on anaphase by inducing the depolymerization of both ends of kinetochore associated microtubules. Specifically, knockdown of KLP59D results in a ~80% reduction in plus-end depolymerization along with a 20% reduction in microtubule minus-end depolymerization during anaphase. Thus, KLP59D is uniquely important for chromosome segregation in Drosophila cells.

Production of a Human Artificial Chromosome with a Conditional Centromere


Kinesin-13 family members destabilize microtubules and perform important and conserved roles during mitosis. Specifically knockdown of KLP59D results in a ~80% reduction in plus-end depolymerization along with a 20% reduction in microtubule minus-end depolymerization during anaphase. Thus, KLP59D is uniquely important for chromosome segregation in Drosophila cells.

Identification of Genes Involved in Centromeric Chromatin Formation

C. M. Betts, S. Erhardt, B. Mellone, M. H. Karpen, A. F. Straight; 1Biochemistry, Stanford University, Stanford, CA, 2Genome Biology, Lawrence Berkeley National Lab, Berkeley, CA

Accurate chromosome segregation is essential for maintenance of genomic stability. Attachment of the chromosomes to the spindle apparatus is mediated by kinetochores assembled on the chromosome's centromere. All eukaryotic centromeres require the replacement of histone H3 in the nucleosomes of centromeric chromatin with the histone variant CENP-A. Thus, to understand how the site of centromere formation is determined it is necessary to understand how CENP-A is specifically incorporated into centromeric chromatin. Although many centromere proteins have been biochemically identified, the mechanisms of centromeric chromatin formation have yet to be elucidated. To identify factors involved in centromere determination, we used an RNAi based approach to screen the entire Drosophila genome for defects in centromeric chromatin formation. Cultured Drosophila cells were treated with double stranded RNAs corresponding to each gene in the Drosophila genome to deplete the messenger RNA encoded by each gene. Cells were fixed and immunofluorescence was performed to localize cdc (the Drosophila CENP-A homologue), HP1 (a marker for heterochromatin), and the DNA intercalating dye DAPI. We acquired fluorescent images of each depletion using an automated microscope and we developed conditions to screen the entire Drosophila genome for defects in centromeric chromatin formation. Cultured Drosophila cells were treated with double stranded RNAs corresponding to each gene in the Drosophila genome to deplete the messenger RNA encoded by each gene. Cells were fixed and immunofluorescence was performed to localize cdc (the Drosophila CENP-A homologue), HP1 (a marker for heterochromatin), and the DNA intercalating dye DAPI. We acquired fluorescent images of each depletion using an automated microscope and we developed...
computational methods to rapidly analyze the image data and identify genes that disrupted Cenp-A localization. We have identified 17 genes that were not previously implicated in centromere formation but that cause a loss in Cenp-A incorporation into centromeres.

1498

Centromere Specification Requires a Conserved Myb Domain-containing Protein Family

P. S. Maddox, J. Menon, F. Hyndman, K. Oegema, A. Desai; LICR, UCSD, La Jolla, CA

Nucleosomes containing the histone H3 variant CENP-A create the structural foundation for kinetochores - the primary chromosomal attachment sites for spindle microtubules which mediate chromosome segregation during cell division. The mechanisms that selectively load CENP-A nucleosomes and maintain their restricted distribution, while largely unknown, are critical for genomic stability. To identify factors required for CENP-A loading, we utilized a functional genomics approach in the nematode C. elegans. Secondary analysis of a gene set defined by a genome-wide RNA interference (RNAi) screen identified 4 proteins whose depletion recapitulates the "kinetochore-null" (KNL) phenotype observed following depletion of CENP-A. Of these, only one protein, KNL-2, is required to load CENP-A nucleosomes onto chromatids. This function is specific, as KNL-2 is not required to load histone H3-containing nucleosomes. Consistent with a close functional interaction on chromosomes, KNL-2 and CENP-A co-localize at centromeres, and are coordinately required for kinetochore assembly, mitotic chromosome structure, and chromosome segregation. Biochemical isolation of KNL-2 associated chromatin specifically enriched CENP-A relative to histone H3 indicating close physical proximity of these two proteins on DNA. KNL-2 is a member of a conserved and previously uncharacterized sub-family of the Myb domain-containing protein superfamily. Analysis of human KNL-2 (Hsk2NL-2) revealed a conserved function in centromere assembly. Hsk2NL-2 localizes to centromeres and is required for localization of CENP-A to centromeres and kinetochore assembly. These results implicate a novel protein class, known to interact with DNA as well as histones, in centromeric chromatin assembly, a critical event in segregation of the genome during cell division.

1499

Assembly of Centromeric Chromatin Requires DNA Repair by Ung2

S. G. Zeitlin,1 C. Tai,2 B. R. Chapados,1 G. Shuphang,1 B. Kavl1,1 J. J. Y. Wang3; 1Cellular and Molecular Medicine, UCSD, La Jolla, CA, 2Biology, UCSD, La Jolla, CA, 3Molecular Biology, TSRI, La Jolla, CA, 4Faculty of Medicine, NTNU, Trondheim, Norway, 5Moores Cancer Center, UCSD, La Jolla, CA

CENP-A is an essential histone H3 variant found in all eukaryotes examined to date. Previously, we showed that the incorporation and removal of uracil from DNA by the nuclear uracil DNA glycosylase, Ung2, are required for CENP-A assembly into sperm chromatin in cell-free Xenopus egg extracts. Here we examine whether such DNA modifications are also important for CENP-A assembly in mammalian cells. To distinguish new CENP-A assembly from maintenance of existing CENP-A in chromatin, we generated stable inducible cell lines that express CENP-A-GFP upon addition of tetracycline. Both siRNA against Ung2 and expression of Vpr, which induces Ung2 degradation, produced a consistent loss of detectable Ung2 and CENP-A-GFP protein, without detectably affecting pre-existing untagged CENP-A. These results suggest that Ung2 is required for assembly of newly synthesized CENP-A, but is not required for maintenance of CENP-A that is already assembled at centromeres.

1500

Structural Alterations and Conformational Rigidity Conferred to Centromeric Nucleosomes by the CENP-A Targeting Domain (CATD)

B. E. Black,1,2 L. E. T. Jansen,3 D. R. Foltz,7 P. S. Maddox,1 D. W. Cleveland2; 1Biochemistry and Biophysics, University of Pennsylvania School of Medicine, Philadelphia, PA, 2Ludwig Institute for Cancer Research, UCSD, La Jolla, CA

CENP-A targets the chromosomal region of its own propagation is not understood. Here we show, using fluorescent pulse-chase experiments that CENP-A remains stably associated with centromeres through multiple cell divisions and is equally partitioned among sister centromeres generated during S phase. Strikingly, loading of new CENP-A on replicated centromere DNA does not take place until after cells have entered the next G1 phase. We further show that passage through mitosis, proceeding normally or by artificially bypassing the mitotic checkpoint, is required for CENP-A assembly into centromeric chromatin. These findings demonstrate a direct coupling between cell cycle progression through mitosis and the assembly of the next generation of centromeres.”

1501

Propagation of Centromeric Chromatin Requires Exit from Mitosis

L. E. T. Jansen, B. E. Black, D. R. Foltz, D. W. Cleveland, Ludwig Institute for Cancer Research, La Jolla, CA

Centromeres direct chromosomal inheritance by providing the foundation for the assembly of the kinetochore, a large multi-protein complex required for spindle microtubule attachment at cell division. CENP-A, a histone H3 variant, assembles with histone H3, independent of DNA template. Substitution of the CATD into histone H3 generates the same more rigid nucleosome as does CENP-A. In human cells, while depletion of CENP-A is lethal, recruitment of normal levels of kinetochore proteins, centromere-generated microtubule attachment, and viability can be rescued by this chimeric H3 containing the CATD. The CATD is confined to the structured “core” of the nucleosome, comprised of the loop 1 and alpha-2 helix of the CENP-A histone fold domain, demonstrating that much (if not all) of the essential features of CENP-A are within the rigid core of the nucleosome. Together, these data support a model of centromere identity maintained by a unique nucleosome structure that serves to distinguish the centromere from the rest of the chromosome.

1502

Mapping the Assembly Pathways That Specify Formation of the Trilaminar Kinetochore Plates in Human Cells

S. Liu,2 J. Rittere,7 S. Jablonski,1 J. Famulski,1 G. Chan,7 T. Yen1; 1Cellular and Molecular Medicine, UCSD, La Jolla, CA, 2Biology, UCSD, La Jolla, CA, 3Molecular Biology, TSRI, La Jolla, CA, 4Faculty of Medicine, NTNU, Trondheim, Norway, 5Moores Cancer Center, UCSD, La Jolla, CA

While the physical nature of the epigenetic mark defining the location of the centromere on the chromosome is not known, nucleosomes in which CENP-A replaces histone H3 are at the foundation of the centromeric chromatin. Hydrogen/deuterium exchange mass spectrometry is now used to show that assembly into nucleosomes imposes stringent conformational constraints, reducing solvent accessibility in almost all histone regions by >3 orders of magnitude. Nucleosomes assembled with CENP-A are substantially more conformationally rigid than those assembled with histone H3, independent of DNA template. Substitution of the CATD into histone H3 generates the same more rigid nucleosome as does CENP-A. In human cells, while depletion of CENP-A is lethal, recruitment of normal levels of kinetochore proteins, centromere-generated microtubule attachment, and viability can be rescued by this chimeric H3 containing the CATD. The CATD is confined to the structured “core” of the nucleosome, comprised of the loop 1 and alpha-2 helix of the CENP-A histone fold domain, demonstrating that much (if not all) of the essential features of CENP-A are within the rigid core of the nucleosome. Together, these data support a model of centromere identity maintained by a unique nucleosome structure that serves to distinguish the centromere from the rest of the chromosome.

1503

Understanding the Arrangement of Structural Kinetochore Proteins within a Kinetochore-Microtubule Attachment Site in Budding Yeast

A. Joglekar, D. Bouck, J. Molk, K. Bloom, E. Salmon; University of North Carolina, Chapel Hill, NC

The eukaryotic kinetochore is a complex protein structure comprised from more than 60 different proteins. Understanding the mechanism by which this protein assembly regulates microtubule polymerization dynamics and generates force requires knowledge of the three dimensional architecture of kinetochore proteins within the kinetochore-microtubule attachment. Budding yeast with only one microtubule attachment per kinetochore provides an ideal model system for studying the kinetochore-microtubule attachment. Using quantitative fluorescence microscopy of representative proteins from each essential kinetochore protein complex in budding yeast, we have counted the copy number of each protein complex within a kinetochore-microtubule attachment. We find that the attachment site is composed of a standardized number of structural proteins, which ranges from one or two for inner kinetochore proteins such as Mtr2p (hCENP-C) to more than 16 for the outer kinetochore complex DAM/DASH. To determine the localization of these proteins within the attachment, we are currently adapting the Simultaneous High Resolution Colocalization (SHREC) technique for use in vivo. SHREC is used to measure nanometer scale distances between two fluorophores emitting different wavelengths by simultaneous two-color imaging and subsequent localization of each fluorophore with high resolution. Yeast cells expressing two kinetochore proteins tagged with GFP and mCherry can be arrested in mid-anaphase for two-color imaging of single kinetochores. We will use SHREC imaging and analysis to localize the centroid of each of the two kinetochore protein clusters with nanometer scale accuracy. This will allow us to measure the relative axial localization of kinetochore proteins within a kinetochore-microtubule attachment. Combined
with biochemical and structural data, the copy numbers and localization data for structural kinetochore protein complexes will allow us to compile a high resolution structural description of the protein assembly interfacing centromeric DNA and a microtubule plus-end.

1504

The CENP-H/I Complex Proteins Are Required for Faithful Chromosome Segregation in Vertebrate Cultured-Cells and Are Essential for Early Development in Mice

T. Fukagawa,1 M. Okada,1 I. M. Cheeseman, 2 T. Hori1; 1Department of Molecular Genetics, National Institute of Genetics, Mishima, Shizuoka, Japan, 2Ludwig Institute for Cancer Research, La Jolla, CA

Chromosome segregation in eukaryotes requires a multi-protein structure termed the kinetochore, which assembles on centromeric DNA to mediate the binding of spindle microtubules to chromosomes and chromosome movement. Several components of the kinetochrome, including CENP-A, CENP-C, CENP-H, CENP-I, and the Mis12 complex proteins, have been identified. However, comparison to budding yeast kinetochores, where more than 60 kinetochore proteins have been identified, suggests that other components of the vertebrate kinetochore remain to be isolated. To identify additional vertebrate kinetochore components, we utilized two parallel biochemical strategies to isolate a multi-subunit complex, which includes the established kinetochore components CENP-H and CENP-I and nine other proteins (CENP-K, -L, -M, -N, -O, -P, -Q, -R, -50), from both human and chicken cells (Okada et al., Nature Cell Biol., 2006). Here, we present data for these proteins using chicken and human DT40 knockout and RNA interference analysis in human cells. Our results demonstrate that the CENP-H1 complex can be divided into three functional sub-complexes, each of which is required for chromosome alignment and faithful chromosome segregation. Interestingly, DT40 knockout of one sub-complex (CENP-O class) were not lethal: however, our live cell observations found that the time for these knockout cells to complete mitosis is longer than that for wild-type cells. We also observed severe mitotic defects in these knockouts with apparent premature sister chromatid separation when the mitotic checkpoint was activated, indicating that CENP-O class proteins are required for recovery from spindle damage. To examine the function of CENP-O class proteins in the organismal context, we generated knockout mice of CENP-50, a member of CENP-O class proteins. Surprisingly, CENP-50 gene-disrupted mice could not survive beyond day 7.5 postcoitus due to accumulation of mitotic errors. Thus, although CENP-50-deficient DT40 cells are viable, CENP-50 is essential for centromere function during early embryogenesis.

1505

Biological and Functional Analysis of Human CENP-H/CENP-I and CENP-H Kinetochore Complexes

S. E. McClelland1, S. Borusu2, P. Meraldi2, A. D. McAins2; 1Chromosome Segregation, Marie Curie Research Institute, Oxted, United Kingdom, 2Institute for Biochemistry ETH Zurich, Zurich, Switzerland

A fundamental challenge in cell biology is to determine by which mechanisms kinetochores assemble on centromeric DNA, capture microtubules and ultimately regulate subsequent chromosome movement during cell division. Human kinetochores are complex molecular machines that are 80-100-nm thick, 0.5-1.0-µm in diameter and mediate the coordinated attachment to ~30 microtubules. The sequence of events including centromere attachment and spindle checkpoint signaling. As a first step, we have determined the biophysical properties of multiple kinetochore proteins using gel-based gradient centrifugation and size-exclusion chromatography. From these experiments we have determined that kinetochores are comprised of diverse sub-complexes, including the previously characterized NDC80 and MIND/Mis12 complexes, which are required for microtubule attachment and force generation respectively. We have also determined that the recently identified nucleosome-associated complex (NAC) is assembled from a set of smaller sub-complexes. These include a 100KDa CENP-H containing complex, an 89KDa Sim4R-Fta1R complex and a Chl4R dimer. Consistent with these findings, inactivation of Chl4R or CENP-H by RNAi-mediated protein depletion leads to distinct mitotic defects. Thus, the Chl4R and CENP-H sub-complexes represent distinct functional units within the kinetochore which implies that the NAC can modulate multiple kinetochore functions during chromosome segregation.

1506

Analysis of Cenp-h Function in Kinetochore Assembly

B. Moree, A. Straight; Biochemistry, Stanford University, Stanford, CA

During mitosis, kinetochores function as a molecular scaffold physically linking chromosomes to the dynamic microtubules of the mitotic spindle apparatus. Assembly of functional kinetochores requires localization of more than 60 proteins to the centromere, yet the coordination of the events necessary for kinetochore assembly is poorly understood. The centromere, a specialized chromatin domain on each chromosome, serves as the assembly substrate for the loading of diverse kinetochore protein complexes essential for mitotic checkpoint activity, microtubule binding, and chromosome segregation. Central to building functional kinetochores is the loading of the essential centromere protein CENP-H at centromeres. CENP-H constitutively associates with centromeres in somatic cells and functions as a molecular bridge between centromeric chromatin and the microtubule binding proteins of the kinetochore. To determine the role of CENP-H in centromeric chromatin formation and mitotic kinetochore assembly, we are using Xenopus laevis egg extracts to follow kinetochore assembly in vitro. Xenopus egg extracts are arrested at metaphase of meiosis II and assemble functional meiotic spindles when sperm chromatin is added to the extract. Cenp-h is present in Xenopus egg extract by western blot but does not localize to centromeres of meiosis II chromosomes suggesting that it may be dispensable for kinetochore function in meiosis II. When sperm chromatin is added to extracts made from mitotically activated Xenopus eggs, Cenp-h is assembled at centromeres. These results suggest that Cenp-h is assembled only on mitotic centromeres and may reflect fundamental differences in the composition of meiotic and mitotic kinetochores.

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Structural and Functional Analysis of the Human Zw10 Interacting Protein, Hzwint-1

L. J. Vos, G. K. T. Chan; Oncology, University of Alberta, Edmonton, AB, Canada

The human hzw10 interacting protein, hzwint-1, is a novel kinetochore protein originally identified in a yeast 2-hybrid screen for interactors of Zw10 (Starr et al. 2000). Hzw10 and hrod form a complex which recruits dynein/dynactin to the kinetochore and is essential components of the mitotic checkpoint (Chan et al. 2000). Hzwint-1 localizes to the kinetochore in prophase, prior to hzw10 localization, and remains at the kinetochore until anaphase, after hzw10 has dissociated. In addition to hzw10, we have found that hzwint-1 interacts with hHec1, a component of the conserved Ndc80 complex, and hMis12, a conserved inner kinetochore protein, in yeast 2-hybrid assays. As well as interacting with hHec1 and hMis12, hzwint-1 has similar dynamics as examined by fluorescence recovery after photobleaching. As such we propose that hzwint-1 is a structural protein to which hzw10 binds. To test this hypothesis, we are performing mutagenesis based domain mapping to determine which regions of hzwint-1 are necessary for kinetochore localization and which are required for the interaction with hzw10, hHec1 and hMis12. We have produced 16 truncation mutants, 23 random-insertion mutants and 25 site-directed mutants. By transferring these mutants into a green fluorescent protein fusion vector and transiently transfecting HeLa cells, we have determined that the N-terminal of hzwint-1 is crucial for kinetochore localization. By employing the mutants in a yeast 2-hybrid assay we are mapping the hzw10, hHec1 and hMis12 interaction domains. Yeast 2-hybrid results are confirmed with GST pull-down assays. We are analyzing the effect of these hzwint-1 mutants have on kinetochore assembly, chromosome segregation and the mitotic checkpoint in cells that have reduced level of endogenous hzwint-1 proteins by siRNA knockdown.

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HZW10 Response to the Loss of Tension Is Dependant on Aurora B Kinase Activity

J. K. Famulski, G. K. T. Chan; Oncology, University of Alberta, Edmonton, AB, Canada

Faithful chromosome segregation depends on the bi-polar attachment of microtubules (MTs) at kinetochores leading to chromosome alignment at the metaphase plate. The mitotic checkpoint is thought to monitor both kinetochore-MT attachments as well as tension across sister kinetochores by accumulating checkpoint proteins at kinetochores. Here we report the finding that human Zeste White 10 (hzw10), a known mitotic checkpoint protein, accumulates on kinetochores in response to the loss of tension. Using immunofluorescence, we established that once chromosomes align at the metaphase plate, and are therefore under equal tension, hzw10 signal is diminished at kinetochores. However, when HeLa cells were treated with low dose Taxol which is known to inhibit MT dynamics and therefore prevent tension across sister kinetochores (Lampson et al. 2005), we observed an accumulation of hzw10 at metaphase kinetochores. We have verified this finding through time-lapse microscopy using a stable HeLa cell line expressing EGFP-hzw10 treated with low dose taxol. Furthermore, we have observed that EGFP-hzw10 kinetochore dynamics are retarded in cells treated with low dose taxol. Hzw10R1 is an Aurora B checkpoint protein that is also known to accumulate at kinetochores lacking tension and its accumulation has been shown to be dependent on Aurora B kinase activity (Hauf et al. 2003; Ditchfield et al. 2003). We have tested how the Aurora B kinase activity, whether Aurora A kinase activity or Aurora B kinase activity, plays a role in the response of hzw10 to the loss of tension. Interestingly, we find that inhibition of Aurora B, by treatment with ZM447439, prevents the accumulation of hzw10 at kinetochores in the presence of low dose taxol. This suggests that Aurora B function is required for Hzw10R1 and hzw10 accumulation at kinetochores in response to the loss of tension. We therefore conclude that hzw10 accumulates at kinetochores lacking tension in an Aurora B kinase dependent manner.
Structural and Functional Analysis of the Kinetochore Protein, hZwilch

S. Shoba, 1 S. Cheng, D. McDonald, 2 H. Cho, 3 G. Chan, 3 Department of Oncology, Faculty of Medicine & Dentistry, Edmonton, AB, Canada; 3Department of Biochemistry & Chronic Inflammatory Disease for Research Center, Ajou University School of Medicine, Suwon, Republic of Korea.

During mitosis, the kinetochore interacts with the microtubules and mediates chromosome movement to ensure accurate chromosome segregation. The kinetochore is also important in mitotic regulation as a docking site for mitotic checkpoint components. hZwilch localizes to kinetochores during metaphase as expected. Using transient transfection and fluorescence microscopy to analyze the Zwilch mutants, we found that both the N and C-terminal regions are important for kinetochore localization.

Molecular Dissection of Mammalian Kinetochore Proteins Identifies a Novel CENP-E-interacting Protein, Cenp-V, Essential for Mitotic Checkpoint

J. Du, 1 S. Hua, 1 X. Cai, 1 D. Liu, 1 Z. Guo, 1 J. Li, 1 X. Zhou, 2 A. Shaw, 2 M. Zhu, 1 S. Liang, 3 Z. Cheng, 1 D. Cleveland, 1 X. Yao, 2 1Cellular Dynamics, Heifei National Laboratory, Heifei, China; 2Physiology, Morehouse School of Medicine, Atlanta, GA; 3Proteomics, Human Normal University, Changsha, China; 4Cell Biology, Ludwig Institute for Cancer Research, La Jolla, CA.

Our previous studies show that mitotic kinesin CENP-E represents a link between attachment of spindle microtubules and the mitotic checkpoint signaling cascade (NCB 2: 484-491). However, the molecular mechanism underlying CENP-E-mediated spindle checkpoint signaling remains elusive. To identify the proteins that participate in the CENP-E-mediated spindle checkpoint signaling, we carried out proteomic search for kinetochore proteins associated with CENP-E. To this end, mitotic extracts from HeLa cells stably expressing FLAG-CENP-E were purified using a FLAG antibody-affinity chromatography. The proteins selectively bound to CENP-E were removed from the gel and analyzed by LC-MS/MS. Of several known proteins selectively bound to CENP-E, the presence of a 59 kDa previously uncharacterized protein was novel and particularly exciting. Our immunofluorescence microscopic analysis shows that this protein co-distributes with CENP-E to the kinetochore of HeLa cells during early prometaphase and departs from kinetochores upon the metaphase alignment. We therefore designated it as CENP-V (centromere-associated protein V). Using immunoelectron microscopy, CENP-V is shown to target to the outermost region of the developing kinetochores upon the nuclear envelope breakdown. Throughout chromosome congression, CENP-V is a constituent of the corona fibers, extending up to 90 nm away from the kinetochore outer plate. Our yeast two hybrid assay revealed that the C-terminal tail of CENP-E binds to CENP-V, which was confirmed by a pull-down assay using bacterially recombinant proteins. Significantly, depletion of CENP-V abrogates the localization of CENP-E to the kinetochore and results in chromosome mis-segregation. Real-time analyses show that elimination of CENP-V caused a substantial mitotic arrest. In addition, the tension across the sister kinetochore is reduced in the absence of CENP-V, suggesting that CENP-V links spindle microtubules to kinetochores. Taken together, our studies strongly support a model in which CENP-V functions together with CENP-E in spindle checkpoint signaling cascades at the kinetochore.

Tripin Is a New Inner Centromere Protein That Recruits MCAK and Prevents Aberrant Kinetochore Attachments

H. Huang, 1 J. Feng, 1 J. Famulski, 1 J. Rattner, 2 S. Liu, 1 G. Kao, 1 R. Muschel, 1 G. Chan, 1 T. Yen, 1 Fox Chase Cancer Center, Philadelphia, PA; 1Dep. of Experimental Oncology, Cross Cancer Institute, University of Alberta, Edmonton, AB, Canada; 3Dep. of Cell Biology & Anatomy, Faculty of Medicine, University of Calgary, Calgary, AB, Canada.

In eukaryotes, nuclear pore complexes (NPCs) are evolutionarily conserved structures, which allow bidirectional transport of macromolecules between the cytoplasm and the nucleus. NPCs are composed of proteins termed nucleoporins, often organized in complexes. Among them, the Nup107-160 complex, which is composed of 9 nucleoporins in metazoans, plays an important role in NPCs reassembly at the end of mitosis. We previously demonstrated that a fraction of the Nup107-160 complex is targeted to kinetochores at the onset of mitosis. However, the molecular mechanisms involved in this targeting as well as the biological relevance of this mitotic-specific localization remained uninvestigated. Here, we show that one of the Nup107-160 complex subunits, Nup133, interacts with CENP-F and that CENP-F partially contributes to the targeting of the Nup107-160 complex to kinetochores. In addition, siRNA experiments revealed that the Ndc80 complex plays a key function in the recruitment of the Nup107-160 complex to kinetochores. We further demonstrate that specific mislocalization of the Nup107-160 complex from kinetochores induces a checkpoint-dependent mitotic delay. These cells showed impaired chromosome congression, reduced tension and less stable kinetochore-microtubule attachment. Current studies are underway to identify the downstream effectors of the Nup107-160 complex that may mediate its function at kinetochores.

Targeting and Function of the Nup107-160 Complex at Kinetochoreksites

M. Zuccolo, 1 A. Alves, 1 S. Bolhy, 1 J. Sibarita, 1 T. Fukagawa, 1 T. Yen, 1 X. Yao, 2 1Umr144-CNRS, Institut Curie, Paris, France; 2National Institute of Genetics and SOKENDAI, Mishima, Shizuoka, Japan.

Our previous studies show that mitotic kinesin CENP-E represents a link between attachment of spindle microtubules and the mitotic checkpoint signaling cascade (NCB 2: 484-491). However, the molecular mechanism underlying CENP-E-mediated spindle checkpoint signaling remains elusive. To identify the proteins that participate in the CENP-E-mediated spindle checkpoint signaling, we carried out proteomic search for kinetochore proteins associated with CENP-E. To this end, mitotic extracts from HeLa cells stably expressing FLAG-CENP-E were purified using a FLAG antibody-affinity chromatography. The proteins selectively bound to CENP-E were removed from the gel and analyzed by LC-MS/MS. Of several known proteins selectively bound to CENP-E, the presence of a 59 kDa previously uncharacterized protein was novel and particularly exciting. Our immunofluorescence microscopic analysis shows that this protein co-distributes with CENP-E to the kinetochore of HeLa cells during early prometaphase and departs from kinetochores upon the metaphase alignment. We therefore designated it as CENP-V (centromere-associated protein V). Using immunoelectron microscopy, CENP-V is shown to target to the outermost region of the developing kinetochores upon the nuclear envelope breakdown. Throughout chromosome congression, CENP-V is a constituent of the corona fibers, extending up to 90 nm away from the kinetochore outer plate. Our yeast two hybrid assay revealed that the C-terminal tail of CENP-E binds to CENP-V, which was confirmed by a pull-down assay using bacterially recombinant proteins. Significantly, depletion of CENP-V abrogates the localization of CENP-E to the kinetochore and results in chromosome mis-segregation. Real-time analyses show that elimination of CENP-V caused a substantial mitotic arrest. In addition, the tension across the sister kinetochore is reduced in the absence of CENP-V, suggesting that CENP-V links spindle microtubules to kinetochores. Taken together, our studies strongly support a model in which CENP-V functions together with CENP-E in spindle checkpoint signaling cascades at the kinetochore.
algorithm to find the centroids of kinetochore fluorescent speckle images to determine how the Ndc80 complex is oriented within the kinetochore at dimensions beneath the 200 nm Abbe limit of resolution in the light microscope. Metaphase HeLa cells were fixed and labeled with 9G3 to Hecl and a polyclonal antibody to Spc24. Green and red fluorescent images of secondary antibodies were collected using spinning-disk confocal microscopy and 200nm optical sections. For in-focus sister kinetochore pairs, the centroid of the Hecl fluorescent label was always outside the centroid of the Spc24 label by an average distance delta = 43±4 nm (N=120). At metaphase, sister kinetochores become pulled apart to 1-2 μm from a separation of about 0.6 μm in prophase. At metaphase, delta was independent of this two-fold variation in centromere separation indicating that under tension, all Ndc80 complexes in the kinetochore are stiff and oriented with their long axes parallel with the kMT-centromere axis. Preliminary analysis of the Hecl marker relative to GFP-CENP-A at centromeric DNA shows an average separation at metaphase sister kinetochores of 85nm, being smaller at lower and higher at lower values of centromere stretch (range: 50-110nm). This suggests that the protein linkage between CENP-A and the Ndc80 complex is compliant and tension-sensitive. Supported by NIH GM24364 and NIH GM60678.

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NudC Is Required for Plk1 Targeting to the Kinetochore and Chromosome Congression
M. Nishino, Y. Kurawa, B. R. Brinkley, S. Lin, L. Yu-Lei; Department of Cell Biology, Baylor College of Medicine, Houston, TX. Program in Cell and Molecular Biology, Baylor College of Medicine, Houston, TX. Department of Cell Biology, Baylor College of Medicine, Houston, TX. Department of Molecular Pathology, The University of Texas M.D. Anderson Cancer Center, Houston, TX
The equa distribution of chromosomes during mitosis is crucial for maintaining the integrity of the genome. Essential to this process are the capture of spindle microtubules by kinetochores and the congression of chromosomes to the metaphase plate. Polo-like kinase 1 (Plk1) is a mitotic kinase that has been implicated in microtubule-kinetochore attachment, tension generation at kinetochores, tension-responsive signal transduction, and chromosome congression. The tension-sensitive substrates of Plk1 at the kinetochore are unknown. Here, we demonstrate that human nuclear distribution protein C (NudC), a 42 kDa protein initially identified in Aspergillus nidulans and shown to be phosphorylated by Plk1, plays a significant role in regulating kinetochore function. Plk1-phosphorylated NudC co-localizes with Plk1 at the outer plate of the kinetochore. Depletion of NudC reduced end-on microtubule attachments at kinetochores and resulted in defects in chromosome congression at the metaphase plate. Importantly, NudC-deficient cells exhibited mislocalization of Plk1 and the Kinesin-7 motor CENP-E from prometaphase kinetochores. Ectopic expression of wild-type NudC, but not NudC containing mutations in the Plk1 phosphorylation sites, recovered Plk1 localization at the kinetochore and rescued chromosome congression. Thus, NudC functions as both a substrate and a spatial regulator of Plk1 at the kinetochore to promote chromosome congression.

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BubR1 and APC/EB1 Cooperate to Maintain Metaphase Chromosome Alignment
J. Zhang, Y. Mao; Cell Biology and Pathology, Columbia University College of Physicians and Surgeons, New York, NY
Accurate segregation of generic material in mitosis requires dynamic attachment of chromosomes to spindle microtubules, which is mediated largely by kinetochores. A major question has been how kinetochores capture and maintain interactions with dynamic microtubules. Using immunodepletion from and antibody addition to Xenopus egg extracts, we show that the kinetochore protein Arc21 and the APC/EB1 tumor suppressor BubR1 forms a complex with two microtubule associated proteins (MAPs) APC (adenomatous polyposis coli) and EB1, which localizes to the ends of microtubules embedded in kinetochores. We further demonstrate that both APC and EB1 are essential for metaphase chromosome congression. These findings support a model in which BubR1 kinase may directly regulate APC/EB1 functions involved in stable kinetochore microtubule attachment.

The Role of Baculovirus WASP-like Protein P78/83 in Viral Replication
T. Ohkawa, E. D. Goley, J. Mancuso, W. E. Cande, J. A. D'Alessio, J. B. Woodruff, L. E. Volkman, M. D. Welch; Molecular and Cellular Biology, University of California, Berkeley, CA, Plant and Microbial Biology, University of California, Berkeley, CA
Of the many pathogens that exploit the actin cytoskeleton during infection, baculoviruses are remarkable for the unique actin rearrangements they require for replication. Early in infection, baculovirus nucleocapsids induce actin cable formation in the cytoplasm of the host cell, a process hypothesized to mediate efficient translocation to the nucleus, which is the site of viral replication. Later, baculoviruses induce the polymerization of actin within the nuclei of infected cells, an apparently novel phenomenon that is essential for nucleocapsid assembly. We report that actin filaments in the nuclei of cells infected with Autographa californica Multiple Nucleopolyhedrovirus (AcMNPV) are highly dynamic, turning over with a half-life similar to filaments at the leading edge of motile cells. We demonstrate that dynamic nuclear actin polymerization by AcMNPV depends on its p78/83 protein, which functions as a WASP-like activator of Arp2/3-mediated actin polymerization. Both p78/83 and Arp2/3 complex translocate into the nucleus during infection, coincident with the presence of nuclear actin filaments. Mutations in p78/83 that abolish its in vitro nuclear-polymerizing activity prevent both nuclear actin polymerization and virus progeny production. Furthermore, silencing the expression of an Arp2/3 subunit by RNA interference results in a significant drop in viral titer. Using electron microscopy, we show that defects in p78/83 and Arp2/3-mediated actin polymerization result in aberrant nucleocapsid morphogenesis and envelopment and packaging of virions. Actin nucleation by Arp2/3 complex and p78/83 in the nucleus is therefore essential for AcMNPV virion assembly and replication. We anticipate that further study of this novel mode of pathogenesis will reveal elusive regulation and function of nuclear actin and actin regulatory factors in both pathogenic and normal cellular conditions.

Regulation of the Proteasome by Yin6/int6 and an Actin Regulatory Protein, Arc21
R. Cabrera, J. Suo, E. C. Chang; Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX
The “int” genes were originally identified as loci where mouse mammary tumor virus (MMTV) integration induces breast tumor formation. Nearly all “int” genes have well characterized molecular functions that are critical for oncogenesis; in contrast, the role of int6 in tumorigenesis remains largely unknown. In order to better understand how int5 can mediate cancer formation, we have recently uncovered a highly conserved function of int6 by characterizing the int6 ortholog, named yin6, in the genetic model system fission yeast. Our data suggest that yin6 regulates localization and assembly of the proteasome. Inactivation of int6 weakens the proteasome leading to chromosome instability and abnormal mitosis. To further decipher how int6 regulates proteasome localization and assembly, our lab performed a screen to isolate S. pombe CDNAs that, when overexpressed, rescue the growth defect of yin6 null (yin6Δ) cells. This study focuses on arc21, encoding a potential subunit of the Arp2/3 complex, which has been implicated in the regulation of actin polymerization. Our data suggest that arc21 is an essential gene and its inactivation is associated with loss of cell polarity. Furthermore, our data show that an Arc21-GFP fusion protein colocalizes with F-actin, a pattern that can be disrupted by the actin polymerization inhibitor Latrunculin A. These results support the hypothesis that Arc21 binds actin and regulates actin function. Overexpression of Arc21 can rescue the proteasome defects observed in yin6Δ cells. Additionally, yin6Δ cells show an aberrant localization of Arc21-GFP, and, like many mutants defective in F-actin, are hypersensitive to actin polymerization inhibitors. These data suggest a model in which yin6 mediates proteasome localization and assembly by acting via arc21 and the actin cytoskeleton.

Actin Bundling Protein Fascin Promotes Cancer Metastasis
D. Vignjevic, M. Schoumacher, K. Janssen, F. elMarjou, D. Louvard, S. Robine; Institute Curie, Paris, France, Technische Universität, München, Germany
Cancer cells become metastatic by acquiring a motile and invasive phenotype. This step requires remodelling of the actin cytoskeleton and the expression of exploratory, sensory organelles known as filopodia. Efficient bundling of actin filaments within filopodia, is critical for filopodia function. Fascin was the only actin bundling protein found to be localized along the whole length of all filopodia and its targeted depletion by siRNA lead to the substantially reduced number of filopodia. Using qRT-PCR and immunohistochemistry, we found that fascin expression was significantly increased in human and mouse intestinal tumors compared with normal epithelium, in tumor stage depended manner. Expression was unchanged in benign lesions and early tumor stages, but significantly upregulated in invasive and metastatic lesions. A possible role of fascin on the cell growth of human adenocarcinoma cells HT29 were tested: 1) ex vivo, in cell culture assays and 2) in vivo, by subcutaneous injection of cells into SCID mice. In both cases cell proliferation was not altered by fascin expression. Next, we tested whether fascin can promote invasion and migration: 1) in vitro, in matrigel coated trans-filter assays and 2) in vivo, by tail vein injection of cells into SCID mice. Fascin expressing cells had higher migrational potential in vitro; and in vivo led to more frequent distant metastasis in the lungs. Moreover, mice injected with fascin positive cells developed severe paralysis, which was caused by invading tumors along the spine. To be able to follow all the steps of metastasis in vivo, we designed transgenic mouse metastasis model that express active form of K-ras, mutated form of APC and fascin in the intestine. In conclusion, we propose that up-regulation of fascin, by promoting the formation of filopodia, could be a significant component in the acquisition of invasive phenotype in carcinomas.
Aip/ Deletion in Mutant Actin Yeast Strains

M. McKane, K. Wen, P. Rubenstein; Biochemistry, University of Iowa College of Medicine, Iowa City, IA

Proper eukaryotic cellular function requires careful regulation of actin cytoskeletal assembly and disassembly. This regulation requires a fine balance between filament stabilization and fragmentation or severing. A major F-actin severing protein is cofilin. Yeast cofilin is a relatively poor severing protein whose activity is somehow activated by Aip1. We constructed yeast/muscle hybrid actins in which muscle actin-specific residues were substituted for their yeast counterparts in subdomains 1 and 2. These substitutions cause yeast phenotypes that seem to arise from enhanced filament fragmentation. If true, decreasing severing protein activity may rescue the phenotypes caused by the mutant actins. To test this hypothesis, we introduced into an aip1 strain a mutant yeast actin 3A/casubl1 which carries all 18 muscle-specific subdomain 1 residues except with 3 instead of 4 acidic N-terminal residues normally found in muscle actin. This F-actin fragments much more readily than WT actin. With this actin, Aip1 deletion partially rescues polarized actin structures and the endocytosis defect associated with the actin, and makes the cells sensitive to growth at 37°C instead of 37°C-lethal. Repetition of the experiment in an aip1/Δ background with the additional insertion of the three subdomain 2 substitutions into 3Ac/casubl1 actin caused cell death. Thus, increased F-actin instability can be at least partially overcome by decreased filament severing. We repeated the experiment with H372R actin. The original H372R strain is characterized by thickened actin cables, loss of mitochondrial function and mitochondrial DNA, and hypervacuolated vacuoles. We thought deletion of Aip1 in the H372R strain might be lethal because of what appeared to be the enhanced stability of the actin. Interestingly, with this actin, aip1/Δ prevents loss of mitochondrial function. There are aggregated actin patches, and abnormal vacuole morphology persists. This result implies a role for Aip1 other than as a facilitator of yeast cofilin function.

Overexpression of Nebulin Fragments in Spreading Embryonic Cardiomyocytes

Z. Panavici, X. Deng, M. Esham, C. L. Moncman; Molecular & Cell Biochemistry, University of Kentucky, Lexington, KY

Nebulin is a large actin binding protein that in concert with tropomodulin plays an important role in regulating the lengths of thin filaments in vertebrate skeletal muscle. Since its identification in the 1970s, nebulin was thought to be expressed only in skeletal muscle; however, in recent years there is a growing body of evidence that indicate the expression of nebulin isoforms in vertebrate cardiac tissue. To investigate the role of nebulin during myofibrilligenesis in spreading cardiomyocytes, we have expressed fragments of the nebulin cDNA tagged with the jellyfish green fluorescent protein (GFP) in chicken embryonic cardiomyocytes. Nebulin fragments from both the superrepeats and single repeats were expressed minus and plus the nebulin linker region. Expression and incorporation of the nebulin fragments was monitored by confocal microscopy over a three day timecourse post transfection and compared with the distribution of cardiac sarcomeric protein by immunolabeling. We find that expression of nebulin modules derived from the superrepeats displayed a diffuse to punctate distribution. Occasional cells exhibited alignment of the recombinant protein along the myofilaments at early timepoints. By 3 days postdaylighting, the majority of the expressing cells contained the nebulin superrepeat along the thin filaments, but not within the Z-line. Nebulin modules from the single repeats incorporate into the cardiac myofilaments at the level of the Z-line. Expression of the nebulin superrepeat conjoined with the nebulin linker did not alter the punctate distribution of the modules. However expression of the single repeats abutted to the nebulin linker resulted in both a B-band and Z-line distribution. The expression of the superrepeats and the single repeats resulted in disruption of the myofilament network. These data indicate that nebulin fragments, like nebulin, alter myofilament organization.

Functional Analysis of Cyclase-associated Proteins in Caenorhabditis elegans

K. Ono, S. Ono; Pathology, Emory University, Atlanta, GA

Cyclase-associated protein (CAP) is a conserved actin monomer-binding protein that is also implicated in mediating several signal transduction pathways to the actin cytoskeleton. CAP promotes ADF/cofilin-dependent actin filament turnover by enhancing dissociation of actin monomers from ADF/cofilin and recycling actin monomers for polymerization. Unicellular organisms have only one CAP gene. However in mammals, two CAP isoforms, CAP1 and CAP2, are present, but their functional difference is not understood. The nematode Caenorhabditis elegans has two CAP genes, cas-1 and cas-2, on separate chromosomes. CAS-1 and CAS-2 proteins are 42 % identical in their amino acid sequences and show 25-38 % identity with CAP proteins from other species. In particular, CAS-1 and CAS-2 exhibit high degrees of similarity to other CAP proteins in their C-terminal halves, which contain a proline-rich sequence and an actin-binding site. cas-1 knockout animals are homozygous lethal, while cas-2 knockouts are homozygous viable. Further phenotypic characterization revealed that the cas-2 mutant has disorganized rachis, which is the actin-rich structure in the core of hermaphroditic gonad. They have relatively normal rachis as young adults. However, as they become gravid adults, the actin-rich lining of the rachis was progressively diminished, and large actin rods were formed inside the rachis. UNC-60A, a non-muscle ADF/cofilin isoform, was expressed in the rachis and localized to small aggregates in wild-type, while in the cas-2 mutant, UNC-60A was associated with the large actin rods. These results suggest that CAS-2 promotes dissociation of actin from UNC-60A and regulates actin dynamics in the gonad, and that CAS-1 and CAS-2 may have distinct in vivo functions.

Interactions of APC with the Actin Cytoskeleton and Direct Regulation by the Microtubule Plus End Tracking Protein EB1

J. B. Mosley, F. Bartoloni, K. Okada, Y. Wen, G. G. Gundersen, B. L. Goode; Anatomy and Cell Biology, Columbia University, New York, NY

Adenomatous polyposis coli (APC) is a 310 kDa multi-domain protein that is truncated in most colorectal cancers, indicating that functions associated with its C-terminals are required for tumor suppression. The C-terminals mediate binding to microtubules and the microtubule plus-end tracking protein EB1, and APC localizes to microtubule plus ends in living cells. In addition, APC has been localized to actin-rich regions of cells, but the mechanism and functional significance of this localization has remained unclear. Here, we show that human APC binds directly to filamentous F-actin and characterize the dynamic exchange of APC between the actin and microtubule cytoskeletons. In biochemical assays, a C-terminal fragment of human APC (APC-basic) directly associates with and bundles F-actin, while CAP1 and CAP2, are present, but their functional difference is not understood. The nematode Caenorhabditis elegans has two CAP genes, cas-1 and cas-2, on separate chromosomes. CAS-1 and CAS-2 proteins are 42 % identical in their amino acid sequences and show 25-38 % identity with CAP proteins from other species. In particular, CAS-1 and CAS-2 exhibit high degrees of similarity to other CAP proteins in their C-terminal halves, which contain a proline-rich sequence and an actin-binding site. cas-1 knockout animals are homozygous lethal, while cas-2 knockouts are homozygous viable. Further phenotypic characterization revealed that the cas-2 mutant has disorganized rachis, which is the actin-rich structure in the core of hermaphroditic gonad. They have relatively normal rachis as young adults. However, as they become gravid adults, the actin-rich lining of the rachis was progressively diminished, and large actin rods were formed inside the rachis. UNC-60A, a non-muscle ADF/cofilin isoform, was expressed in the rachis and localized to small aggregates in wild-type, while in the cas-2 mutant, UNC-60A was associated with the large actin rods. These results suggest that CAS-2 promotes dissociation of actin from UNC-60A and regulates actin dynamics in the gonad, and that CAS-1 and CAS-2 may have distinct in vivo functions.

A Role for Tropomodulin 3 in Polarization and Junction Formation in Epithelial Cells

K. L. Weber, R. B. Nowak, R. S. Fischer, V. M. Fowler; The Scripps Research Institute, La Jolla, CA

The function of an epithelial monolayer is dependent on the transformation of individual cells into a polarized epithelial sheet, within which each cell has a tall, cuboidal morphology with clearly defined apical and basal surfaces, and lateral membranes which form junctions with neighboring cells. Cell junctions ultimately integrate the cytoskeletons of adjacent cells via the thin filaments, but not within the Z-line. Nebulin modules from the single repeats abutted to the nebulin linker resulted in both a B-band and Z-line distribution. The expression of the superrepeats and the single repeats resulted in disruption of the myofilament network. These data indicate that nebulin fragments, like nebulin, alter myofilament organization.

2006 ASCB Annual Meeting Abstracts
Bcr-Abl Alters Cellular Adhesion and Actin Dynamics during Development in Drosophila melanogaster

T. L. Stevens, 1 K. D. Miller, 1 N. B. Artabazon, 1 S. E. Tittermary, 1 M. Peifer 1; 1Biology, Randolph-Macon College, Ashland, VA; 2Biology, University of North Carolina at Chapel Hill, Chapel Hill, NC

Bcr-Abl is an activated fusion protein that has been linked to leukemia in humans. Bcr-Abl results from a reciprocal translocation between chromosomes 9 and 22 that fuses sequences of the bcr gene to the abl gene. Relative to its normal, cellular counterpart c-Abl, Bcr-Abl has increased tyrosine kinase activity. The exact cellular events that lead to cancer are unclear, but studies in culture suggest that altered actin dynamics and aberrant cell movement in cells expressing Bcr-Abl may be key events in promoting leukemia. In order to examine the effects of Bcr-Abl in the context of an organism, we used transgenic flies carrying the bcr-abl oncogene. We found that Drosophila embryos expressing Bcr-Abl in the epithelium die with defects in processes that require cell shape changes, migration, and regulated cellular adhesion. Time-lapse studies revealed that expression of Bcr-Abl drastically altered actin-based projections in migrating epithelial cells. Fewer filopodia were observed in cells that express Bcr-Abl; they were replaced with lamellipodia, and these lamellipodia persisted for longer periods of time. We hypothesize that some of the effects of Bcr-Abl may be through the phosphorylation of targets that regulate actin dynamics and cellular adhesion and that phosphorylation regulates their function. In support of this, Bcr-Abl localized primarily to cell junctions, where overall levels of tyrosine phosphorylation were increased. One substrate of Ab1 tyrosine kinase activity that is altered by the presence of Bcr-Abl is the actin regulator, Enabled (Ena). In embryos expressing Bcr-Abl, we observed increased tyrosine phosphorylation of Ena and other proteins that associate with Ena. Furthermore, Ena was mis-localized from cell junctions and from the leading edge of migrating cells in embryos expressing Bcr-Abl. These results suggest that at least some of the effects of Bcr-Abl on cell migration may be through the actin regulator Ena.

Sulfur mustard (SM, bis-2-chloroethyl sulfide) produces large, debilitating blisters at the epidermal-dermal junction of human skin. It also produces corneal keratopathy and disruption of airway epithelium, typically after a clinical latent phase of 8-24 hours. Almost 90 years after SM was first used as a chemical weapon in WW I, we are beginning to detect and understand the earliest structural lesions and mechanisms that precede this latent expression of clinical pathology. Using cultures of human epidermal keratinocytes (HEK), corneal epithelial cells (HCEC-3), and techniques of multiphoton microscopy, immunofluorescent imaging, and gene-array analysis, we have shown that exposure to SM (400 μM x 5 min) induces early (≤ 1 h) progressive disruption in the organization of cytoskeletal F-actin. Results from 3 HEK postexposure profiles confirm that the early effects on F-actin and on related changes in specific mRNA expression are remarkably and statistically reproducible. Multidimensional scaling of Affymetrix gene-chip results showed that SM induces a disparate and progressive increase in abnormal gene expression that begins gradually in the first hour then accelerates dramatically in the second hour. Image-based searches showed that abnormal F-actin polymerization precedes significant 2-h decreases in the mRNA signals for Cdc42, a small GTP-binding protein that impacts F-actin polymerization. Early changes in F-actin were, however, coincident with significant changes in mRNA signals for some “upstream activators” (guanine nucleotide exchange factors) and some “downstream targets” of Cdc42 like PEX-β (ARIGEF-7), and an occasional stress related gene, e.g., PAK-2. Our results indicate that early effects of SM disrupt structural and functional feedback loops in an F-actin/Cdc42/PIX-β signaling pathway.

F-actin Column Chromatography as a Tool to Analyze Modification of Actin-binding Proteins

A. G. Terasaki, 1 S. Sakamoto, 1 J. Suzuki, 1 H. Suzuki, 1 H. Hashimoto, 1 K. Ohashi; 1Graduate School of Science and Technology, Chiba University, Chiba, Japan, 2Faculty of Science, Chiba University, Chiba, Japan

F-actin affinity chromatography has been used to analyze proteins associated with the actin cytoskeleton. We found previously identified actin-binding proteins such as Arp2/3 complex and novel actin binding proteins (e.g., lasp-2) were enriched in eluates column eluents of chicken brain (Terasaki et al., 2002 and Terasaki et al., 2004). Lasp-2 has putative phosphorylation sites and some components of Arp2/3 complex also have been suggested to exist as phosphorylated form. The modification would affect their activities and complex formation. In this approach, we analyzed isoellectric points of lasp-1, lasp-2, p34-Arc, and p21-Arc in F-actin column eluates because proteins in any modification or specific isoforms might have high affinity to actin and bind to the column specifically. 2D electrophoresis and immunoblotting analysis revealed that all of the proteins showed multiple isoellectric points in chicken brain and specific spots of each protein increased in F-actin column eluates. These findings suggest that the F-actin binding column would be a tool to analyze relationship between modification and activity of actin-binding proteins.

Anthrax Lethal Toxin Paralyses Actin-based Motility by Blocking Phosphorylation of Hsp 27

R. During; Medicine/Infectious Disease, University of Florida, Gainesville, FL

The Bacillus anthracis lethal toxin (LT) blocks the host’s immune response permitting fatal bacteremia. We have discovered that LT paralyzes human neutrophils, preventing them from reaching the site of anthrax infection. LT accomplishes this task by blocking the formation of actin filaments required for the shape changes associated with amoeboid movement. Similarly, when HeLa cells are exposed to LT the ability of the intracellular pathogen Listeria monocytogenes to induce actin filament branching through the Arp2/3 complex is markedly impaired. To identify the involvement SPIN90 in the regulation of dendritic actin networks. Activation of SPIN90 by aluminium fluoride promotes the formation of lamellipodia and its association with cytoskeleton components. SPIN90 can directly bind to F-actin which is mediated via end of C terminus, stimulate actin polymerization, and induce actin filament branching through the Arp2/3 complex. Furthermore, deletion of F-actin binding region on failed to localize at the membrane edge and impairs its capacity to promote the formation of lamellipodia, suggesting that F-actin binding region is essential for the formation of the actin cytoskeleton and regulation of actin cytoskeleton at the leading edge of cells.

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WH2 domain located at the C-terminal region of CAP1 plays an important role in ATP-G-actin binding. Our data demonstrates that mammalian CAP1 also interacts with profilin. We are currently mapping the actin and profilin binding sites of mammalian CAP1 and examining the biological roles of these interactions in cultured mammalian cells.

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**Drebrin-A, a Spine Specific Actin-binding Protein in Neuron, Inhibits the ADF/cofilin-dependent Actin Dynamics and Actin-Mysyn- V Interaction**

R. Ishikawa, 1 A. Takahashi, 1 K. Oseki, 1 M. Watanabe, 2 M. Igarashi, 1 A. Nakamura, 1 K. Kohama 1 1Department of Molecular and Cellular Pharmacology, Gunma University School of Medicine, Maebashi, Japan, 2Division of Molecular and Cellular Biology, Graduate School of Medical and Dental Sciences, Niigata University, Niigata, Japan

Drebrin-A is an actin-binding protein localized in dendritic spine of mature neuron, and suggested to affect spine morphology. In last year’s ASCB meeting, we reported the purification of drebrin-A, and showed the inhibition by drebrin-A of the activities of caldesmon. In this report, we examined the effects of drebrin-A on the activities of other actin-binding proteins localizing in dendritic spines, and found that drebrin-A inhibited the activity of ADF/cofilin. Enhancement of actin polymerization by ADF/cofilin was canceled in the presence of drebrin-A, which were confirmed by the measurements of (i) the turbidity of the solutions and (ii) the fluorescence intensity of pyrene-lebelled G-actin. F-actin cosedimentation assay revealed that drebrin-A inhibited the actin-binding activity of drebrin-A. The ADF/cofilin-induced decrease in precipitated actin was also canceled in the presence of drebrin-A. We also found that drebrin-A inhibited the Mg-ATPase activity of myosin V. In viro motility assay revealed that the attachment of F-actin to glass surface coated with myosin-V was inhibited by drebrin-A, but once F-actin attached to the surface, sliding speed of F-actin was unaffected in the presence of drebrin A. These results suggest that drebrin-A may affect spine dynamics through regulating the activities of ADF/cofilin and myosin-V.

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**UNC-87, a *C. elegans* Calponin-repeat Protein, Inhibits Actomyosin Contractility**

S. Yamashiro, K. Ono, S. Ono; Pathology, Emory University, Atlanta, GA

Calponin is an actin-binding protein that is distributed in smooth muscle and non-muscle cells and has been suggested as a regulator of actomyosin contractility. However, its precise in vivo function is still unclear. In the nematode *C. elegans*, UNC-87 has seven calponin repeats that are found in the C-terminal region of calponin, but does not have a calponin-homology (CH) domain, which is found in a variety of actin-binding proteins. Previous studies showed that UNC-87 binds and cross-links F-actin in vitro, is associated with thin filaments in body wall muscle, and is important for maintenance of the myoﬁbrils. We have shown that UNC-87 stabilizes actin ﬁlaments by inhibiting ADF/cofilin-dependent actin dynamics in vitro and in vivo. In this study, we report evidence that UNC-87 is a negative regulator of actomyosin contractility. We found that UNC-87 inhibited actin-activated Mg2+-ATPase activity of rabbit skeletal muscle heavy meromyosin. UNC-87 also inhibited slug formation of actin ﬁlaments on a glass surface coated with skeletal muscle heavy meromyosin. Immunostaining of UNC-87 showed that both striated body wall muscle and non-striated ovarian cells UNC-87 localized to a restricted region of the thin filaments near the dense bodies where actin ﬁlaments are anchored. On the other hand, tropomyosin localized to a region of the thin ﬁlament more distal to the dense bodies. These results suggest that UNC-87 has a distinct function from tropomyosin and acts as a negative regulator of actomyosin contractility.

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**Biochemical Basis of Deafness-causing Actin Mutations**

K. E. Bryan, P. A. Rubenstein; Biochemistry, University of Iowa, Iowa City, IA

The actin cytoskeletal apparatus in the auditory hair cell plays a central role in sound detection. Recently it was discovered that six point mutations in non-muscle γ-actin cause early onset autosomal dominant nonsyndromic hearing loss. The molecular basis for the deafness is unknown. We have engineered each mutation into yeast actin to investigate the effects of these mutations on actin function in vivo and in vitro. Yeast expressing each of the mutant actins as its sole active actin is viable. Four strains exhibit significant growth deficiencies in complete media and an inability to grow on glycerol as a sole carbon source implying a mitochondrial defect. These four strains exhibit abnormal mitochondrial morphology. All of the mutations result in abnormal actin cytoskeletal patterns. Five of the six mutants display strain-specific vacuole morphology abnormalities. Two of the purified mutant actins exhibit decreased monomer thermal stability and increased rates of nucleotide exchange, suggestive of increased protein flexibility. Only the V370A actin displays abnormal polymerization kinetics. V370A filaments were one-third as long as WT filaments, and mixtures of WT and V370A actins display kinetic properties and filament lengths proportional to the mole fraction of each actin used in the mixture, indicating that the mutant actin had no dominant effect on filament stability. The lack of polymerization effects in most of the mutants implies that hearing loss arises because of inability of the mutant filaments to be properly regulated. Therefore, we have begun to assess the effects each mutation has on the interaction of actin with actin-binding proteins. Three mutants, T89I, K118M, and V370A exhibit allele-specific abnormalities in their interactions with yeast coﬁlin. These three mutants also show differential effects on their interaction with yeast Arp2/3. Our results implicate misregulation of actin filament behavior as the molecular basis of the hearing loss associated with these mutations.

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**Conserved Residues of the ILWQE Module Mediate the F-actin Binding and Dimerization of Talin1**

S. J. Smith, R. O. McCann; Biochemistry, University of Kentucky, Lexington, KY

The ILWQE module is an ancient, conserved C-terminal actin-binding element found in animal talins, H1p/12, and fungal Sla2. The module has a consensus length of 186 amino acids arranged in four conserved blocks. Dysregulation of the expression of ILWQE module proteins has several negative consequences. Talin1 mutants lacking the ILWQE module do not form a linkage between the extracellular matrix and actin cytoskeleton. Overexpression of H1p/12 mutants without the ILWQE module displays a similar phenotype to H1p/12 mutants, while cation-coated vesicles accumulated due to delayed or blocked internalization. Yeast Sla2 lacking the ILWQE module results in abnormal endocytosis. These results indicate that the ILWQE module forms a link between the actin cytoskeleton and other cellular compartments, such as the plasma membrane and endocytic vesicles. We have previously shown that the ILWQE module contains a dimerization motif and that the C-terminal Block 4 is essential for both dimerization and F-actin binding. We have used site-directed mutagenesis of Block 4 of mouse Talin1 to determine whether dimerization and actin binding are coupled in the ILWQE module. The mutant ILWQE modules displayed a significant decrease in F-actin binding affinity. We used gel filtration chromatography, cross-linking, and analytical ultracentrifugation to correlate actin binding with dimerization. Our results show that the regulation of dimerization and actin binding of the ILWQE module are coupled. Future research will examine the mechanisms of F-actin binding and the role of the dimerization motif in ILWQE module-containing proteins.

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**Prelude to Invasion: The Shigella Protein, IpaA, Targets Integrins and Rho Activity to Reorganize the Host Cell Cytoskeleton**

K. DeMali,2, A. Jue,2 K. Burridge2; 1Biochemistry, University of Iowa, Iowa City, IA, 2Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC

Shigella, the causative agent of dysentery, invades epithelial cells by reorganizing the host cell actin cytoskeleton. This process requires the release of a set of Ipa proteins through a Shigella type-III secretion apparatus. IpaA is one of the proteins secreted by *Shigella*, and it has been shown to bind to vinculin. The idea that this interaction with vinculin mediates IpaA’s effects on the cytoskeleton has been rapidly accepted. Here we have examined how IpaA reorganizes the actin cytoskeleton. We show that IpaA does not require vinculin expression or an intact vinculin binding site to exert its effects. Rather, we find that IpaA acts via increasing Rho activity, resulting in the subsequent phosphorylation of the myosin light chain. IpaA also decreases integrin affinity for extracellular matrix ligands by interfering with talin recruitment to integrin cytoplasmic domains. The mechanism for IpaA’s effects will be addressed. The combination of these two effects, namely weakened adhesion and increased contractility, account for IpaA’s effect on the actin cytoskeleton. Based on these findings, we propose a new role for IpaA at the site of bacterial entry whereby IpaA regulates integrin affinity for ligands and stimulates contractility.

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**How Do Cells Shape Actin-based Protrusions?**

S. A. Dilks, S. DiNardo; Cell and Molecular Biology, University of Pennsylvania, Philadelphia, PA

The ability of cells to organize actin filaments into stable structures is vital to many cellular functions such as nutrient absorption, hearing, and sensory input. Although the actin-based protrusions thatunderlie these processes have distinct, elaborate shapes, it is unknown how cells form shaped protrusions. To better understand this process, we are using a well studied and genetically manipulatable model system, the *Drosophila* embryo. In this tissue, certain cells of the ventral epidermis produce an actin-based protrusion (called a dendrite), which exhibits a characteristic, reproducible looking shape. This shape is coordinated along a cell row, and each cell row produces a distinctly shaped protrusion. Although it is unknown what proteins are
in involved in this process, certain properties of actin crosslinking proteins may provide insight into the mechanism of shaping protrusions. Drosophila have homologues of the vertebrate actin crosslinkers villin (quail), fascin (singed), and small espin (forked), and loss-of-function alleles of singed and forked cause defects in denticle shaping (Dickinson & Thatcher, 1997). We now show that singed and forked localize to denticles differentially, consistent with our hypothesis that different actin crosslinkers play distinct roles in shape determination. We further hypothesize that the coordination between crosslinkers and other cytoskeletal elements with cellular signaling cascades is required for shape production. In the Drosophila embryonic epidermis, development of the denticle result in embryonic, role of the different inhibitors of PI 3-kinase and MLCK. Although svb is both necessary and sufficient for denticle formation (Payre et al, 1999), we show that ectopic expression of svb alone does not result in properly shaped protrusions. Furthermore, we now show that singed is positively regulated by svb. In total, our data support the hypothesis that actin crosslinking proteins are crucial in creating the characteristic hooking shape of denticles.

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Phagocytosis: Effects of Several Inhibitors of PI 3-kinase and Myosin Light Chain Kinase

D. V. Zuckroll1, L. Octavio1, L. A. Hufnagel1, School of Arts and Sciences, Massachusetts College of Pharmacy and Health Sciences, Boston, MA. 1Cell and Molecular Biology, University of Rhode Island, Kingston, RI

Phagocytosis in the ciliated protist, Tetrahymena thermophila, shares many properties in common with phagocytosis in mammalian cells. We previously reported that certain inhibitory effects of actin inhibitors latrunculin and cytochalasin on Tetrahymena thermophila on phagocytosis are mimicked by effects of an inhibitor of PI-3 kinase and myosin light chain kinase (MLCK), wortmannin. Like actin inhibitors, wortmannin blocked nascent phagosome closure, evidenced by the formation of oversized nascent phagosomes that failed to detach from the oral aperture. To determine which of wortmannin's enzyme inhibitory activities might be required for the inhibition of nascent phagosome release, we examined the effects of LY 294002 (a structurally unrelated inhibitor reported to inhibit PI 3-kinase, but not MLCK) and ML-7 (an inhibitor specific for MLCK), in comparison with wortmannin effects. Wortmannin inhibited the rate of phagosome formation by ~ten times lower than "natural" pregnancy rates (Goldstein et al., 2000). This study investigates how a single dose of 1.25mgCC given to ovariectomized rats before the implantation priming hormon, consisting of a single dose of progesterone for three days and a dose of estradiol-17β on day three (P-P-PE) (Psychylos, 1963), alters the expression and distribution of the actin binding proteins, α-actin, gelsolin and vinculin. Actin binding proteins show a specific distribution in uterine epithelium during implantation, linking the actin cytoskeleton to integrin expression on the uterine surface. Integrins may aid "adhesiveness" for apposition of blastocysts to the uterine epithelium, the first step of implantation. The results of the immunocytochemistry on frozen uterine sections using mouse monoclonal antibodies against α-actin, gelsolin and vinculin and peroxidase-conjugated secondary antibodies, indicate that CC, when administered before the P-P-PE regimen, down regulates the expression of vinculin, does not significantly alter the expression of gelsolin and up regulates α-actin, on the apical surface of the uterine luminal epithelium, when compared to P-P-PE treated animals. All three proteins are down regulated on the apical surface of the luminal epithelium and glands in all groups when compared to pregnant controls. Vinculin alone was localized in the basolateral compartment of the uterine epithelial cells in the CC treated groups. By down regulating these proteins on the uterine surface and up-regulating vinculin on the basolateral membrane of the epithelium, CC may therefore impede adhesion of blastocysts at implantation. An understanding of how CC affects the uterine epithelial cells during implantation will allow for the possibility of exogenously manipulating uterine receptivity to control fertility and improving assisted reproductive technique out-comes.

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Cdc42 Interaction with Its Effector Proteins Observed by FRET and FCCS

S. Thanhka1, L. Ping1, W. Bu1, K. Lim1, T. Wehland2, S. Ahmed1, Regenerative Medicine, Centre for Molecular Medicine, Singapore. 2Department of Chemistry, National University of Singapore, Singapore

Rho GTPases (e.g. Rac1, Cdc42 and RhoA) are members of the Ras super family of GTP binding proteins. Rho GTPases play a crucial role in many biological functions including; cell polarity, movement, adhesion and cytoskeletal organization. We are investigating the role of Cdc42 pathways in filopodia and neurite formation and have identified IRS53, N-WASP and Toca-1 as important effector proteins in these pathways. IRS53 is an adaptor protein with a partial CRIB motif, an F-actin binding domain and a SH3 domain. N-WASP is an activator of the Arp2/3 complex, has a CRIB motif and acts as a scaffolding protein. Toca-1 can bind both N-WASP and Cdc42 and plays a role in regulating N-WASP. We are developing fluorescence microscopy techniques to follow Cdc42-effector complex formation and regulation of F-actin dynamics in vivo. Here, we present data using FRET ( Förster resonance energy transfer) and FCCS (Fluorescence Cross Correlation Spectroscopy) to monitor the spatial and temporal interaction of Cdc42 with IRS53, N-WASP and Toca-1. This study was carried out by using GFP and mRFP tagged proteins. FRET was observed by acceptor bleaching on single cells. FCCS was done by simultaneous excitation of GFP and mRFP by 514 laser on a customised Zeiss microscope. Our data show that FCCS is a sensitive methodology to measure protein-protein interaction that can be used to quantitate interactions in vivo. This work illustrates that the combination of FRET and FCCS represents a powerful approach to investigate cell signaling pathways in vivo.

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The Role of Actin-related kinases in Tetrahymena Phagocytosis: Effects of Several Inhibitors of PI 3-kinase and Myosin Light Chain Kinase

B. V. Zackroll1, L. Octavio1, L. A. Hufnagel1, School of Arts and Sciences, Massachusetts College of Pharmacy and Health Sciences, Boston, MA. 1Cell and Molecular Biology, University of Rhode Island, Kingston, RI

Phagocytosis is the ciliated protist, Tetrahymena thermophila, shares many properties in common with phagocytosis in mammalian cells. We previously reported that certain inhibitory effects of actin inhibitors latrunculin and cytochalasin on Tetrahymena thermophila on phagocytosis are mimicked by effects of an inhibitor of PI-3 kinase and myosin light chain kinase (MLCK), wortmannin. Like actin inhibitors, wortmannin blocked nascent phagosome closure, evidenced by the formation of oversized nascent phagosomes that failed to detach from the oral aperture. To determine which of wortmannin’s enzyme inhibitory activities might be required for the inhibition of nascent phagosome release, we examined the effects of LY 294002 (a structurally unrelated inhibitor reported to inhibit PI 3-kinease, but not MLCK) and ML-7 (an inhibitor specific for MLCK), in comparison with wortmannin effects. Wortmannin inhibited the rate of phagosome formation by ~50% at a concentration of 0.1 μM, and induced oversized nascent phagosomes in most cells in the 0.2-2 μM concentration range. However, while LY 294002 significantly reduced the rate of phagosome formation over the 4-40 μM concentration range, it did not induce formation of oversized nascent phagosomes. Moreover, ML-7, up to 10 μM, did not significantly affect the rate of phagocytosis or induce giant phagosomes, even though at similar concentrations it inhibited another myosin-dependent process, cell division. These results demonstrate that while two different inhibitors of PI-3 kinase interfere with Tetrahymena phagocytosis, a specific MLCK inhibitor does not, and furthermore that the effect of wortmannin on phagosome closure cannot be explained by its activity in blocking either PI-3 kinase or MLCK. We conclude that PI-3 kinase, but not MLCK, is required for Tetrahymena phagocytosis, and hypothesize that the induction of giant phagosomes by wortmannin may be due to effects on still another enzyme, such as a polo-like kinase, recently reported to influence actin dynamics in other cells.

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Palladin Interacts with SH3 Domains of Src and SPIN90 and Is Required for Src-induced Cytoskeletal Remodelling

M. Ronty1, A. Taivainen1, C. A. Otey2, E. Ehler3, W. K. Song4, O. Carpen5, 1Department of Pathology, University of Helsinki, Helsinki, Finland. 2Department of Cell and Molecular Physiology, University of North Carolina at Chapel Hill, Chapel Hill, NC. 3King's College London, The Randall Division of Cell and Molecular Biophysics, London, United Kingdom. 4Department of Life Science and Molecular Disease Research Center, Gwangju Institute of Science and Technology, Gwangju, Republic of Korea. 5Department of Pathology, University of Turku, Turku, Finland

Palladin and SPIN90 are widely expressed phosphoproteins, which play important roles in organization of actin cytoskeleton. Palladin is expressed as multiple isoforms that bind to several actin-associated proteins such as ezrin, VASP, alpha-actinin, profilin, ArgBP2 and LASP1. SPIN90 on the other hand binds to Nck, WASP, beta-pix and dynamin. Both proteins localize to cardiomyocyte Z-discs and probably play a role in the maintenance of sarcomere integrity. In this study we demonstrate an interaction between palladin and SPIN90 and characterize the role of these proteins in PDGF and Src-induced cytoskeletal remodelling. We show that SPIN90 SH3 domain directly binds palladin's amino-terminal poly-proline sequence. In non-muscle cells the proteins co-localize to PDGF-induced membrane ruffles and lamellipodia. The effect of PDGF on the cytoskeleton is at least partly mediated by the Src kinase, since PP2, a specific Src kinase family inhibitor, could block PDGF-induced changes. We therefore studied the behavior of palladin and SPIN90 in cells transfected with an active Src kinase construct. Again, the proteins co-localized in membrane protrusions implying that both proteins are involved in the cytoskeleton remodelling induced by Src. Knock-down of endogenous SPIN90 did not inhibit the cytoskeletal rearrangement. Instead, knock-down of palladin with a specific siRNA resulted in cytoskeletal disorganization and inhibition of Src-induced remodelling. Further studies indicated that palladin is tyrosine phosphorylated in cells expressing active Src. Since the SH3 domain of SPIN90 shows high homology to Src SH3, we also tested if palladin directly binds to Src and could show that this is indeed the case. Taken together we show that palladin's poly-proline motif binds to SPIN90 and Src SH3 domains, that palladin and SPIN90 are rearranged coordinately during PDGF-induced cytoskeletal remodelling, and that palladin directly links to Src mediated signaling.

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ICAM1-mediated RhoG Activation Is Required for Leukocyte Transmigration Across the Endothelium

J. D. Van Buul,1,2 M. Allingham,1 T. Samson,1 R. Garcia-Mata,1 K. Burridge1; 1Cell & Developmental Biology, UNC, Chapel Hill, NC. 2Molecular Cell Biology, University of Amsterdam Sanquin, Amsterdam, The Netherlands.
During transendothelial migration (TEM), leukocytes induce endothelial cells to form transmigratory cups, dynamic membrane protrusions that partially surround adherent leukocytes. However, little is known about the signaling pathways that regulate these structures. Here we show that Rhôh is activated downstream from ICAM1 engagement and that this requires the intracellular tail of ICAM1. Moreover, ICAM1 co-localizes with the Rhô-specific guanine-nucleotide exchange factor SGEF and with RhôG, but not with Rac1. In addition, we show that SGEF interacts with the intracellular tail of ICAM1 through its SH3 domain. Depletion of Rhôh by siRNA decreased the formation of transmigratory cups and attenuated leukocyte TEM, but not initial adhesion. Silencing SGEF resulted in a partial reduction in cup formation and TEM. Together these results define a new signaling pathway involving the exchange factor SGEF and RhôG downstream from ICAM1 that is critical for leukocyte TEM.

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Role of Dynin Light Chain 1 in the Movement and Dynamics of the Investment Cones of Drosophila melanogaster
B. S. Desai, A. Sarkar, R. Choudhary, D. Kar, A. Ghosh-Roy, K. Ray; Department of Biological Sciences, Tata Institute of Fundamental Research, Mumbai, India
During late stages of spermiogenesis, F-actin rich conical structures called Investment Cones (IC) form around the compacted sperm nuclei, which then move caudally to extrude cytoplasmic contents and tightly invest spermatids with plasma membrane. Although F-actin dynamics and the pro-apoptotic proteases are shown to regulate the movement, the exact mechanism remained unclear. Earlier, we have shown that the Dynin Light Chain 1 (Dlc1) is enriched in ICs and its partial loss in certain ddlc1 mutants proportionally reduces the F-actin levels at the cone (Ghosh-Roy et al., MBC, 2005, 16:3107). We have now found that levels of Dlc1 play a crucial role in regulating the IC movements as well. In dlcl1 hemizygous mutants, average rates of IC displacements were reduced causing an increased accumulation of the ICs at the basal ends of the testes and an overall reduction in the fraction progressed values. The IC distribution defect, however, was not altered by additional loss of Dsc64C, dalcl1(CI74), or Gld3 (P150) genes products. Furthermore, conditional loss of dynamin function significantly altered the progressed IC fractions within eleven hours. Surprisingly though, such defect was not observed in the WASp, eps15, syndapin and several other related mutants. Altogether, these suggested a novel mechanism of F-actin dependent propulsion of the ICs involving Dlc1 and dynamin.

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Tyr53-phosphorylation of Dictyostelium Actin Inhibits Filament Nucleation, Elongation, and Stability
X. Liu, S. Shu,1 M. Hong,2 R. L. Levine,1 E. D. Korn2; 1Laboratory of Cell Biology, NHLBI, NIH, Bethesda, MD, 2Laboratory of Biochemistry, NHLBI, NIH, Bethesda, MD
Dictyostelium actin was previously shown to become phosphorylated, uniquely on Tyr53 adjacent to the DNase I-binding loop, when cells in the amoeboid stage are subjected to stress, including heat shock, inhibition of oxidative phosphorylation, and exposure to heavy metal salts, and also late in the developmental cycle. As much as 50% of the actin in spores or heat-shocked vegetative amoebae is phosphorylated on Tyr53. The properties of Tyr53-phosphorylated actin had not previously been studied. We have now purified Dictyostelium actin, separated phosphorylated and unphosphorylated actin by FPLC chromatography on a Mono P column, and compared the biochemical and biochemical properties of Tyr53-phosphorylated actin and unphosphorylated actin. Tyr53-phosphorylation substantially reduces actin's ability to inactivate pancreatic DNAse I activity, increases actin's critical concentration for polymerization from 0.5 to 1.25 μM, and greatly reduces the rate of actin polymerization; inhibits substantially, and possibly completely, the ability of elongation from the pointed-end of actin filaments; reduces the rate of elongation from the barbed-end by 75-80%; and delays the hydrolysis of ATP that accompanies polymerization. Tyr53-phosphorylation also destabilizes actin filaments. In contrast to the typical long (>2 μm) filaments of unphosphorylated actin, polymerized Tyr53-phosphorylated actin forms either short, 15-16-μm bars, or a variable mixture of short bars and filaments with obvious breaks. The mixture of small oligomers and broken filaments is converted to more typical, long filaments upon addition of myosin subfragment 1. Tyr53-phosphorylation does not affect the ability of filamentous actin to activate myosin ATPase. Tyr53-phosphorylated and unphosphorylated actin co-polymerize in vitro, and, as we have shown by immunofluorescence staining, co-localize in pseudopods and filopods of vegetative amoebae. The effects of Tyr53-phosphorylation on actin polymerization may explain, at least in part, why cells subjected to stress round up and lose their adherence to the substratum.

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Analysis of Actin Organization and Dynamics in Migrating Epithelial Cells
J. I. Lim, L. Ji, C. M. Waterman-Storer, G. Danuser; Cell Biology, The Scripps Research Institute, La Jolla, CA
Cellular contraction is a crucial component of cell motility. The cell is thought protrude through actin polymerization at its leading edge, and utilizes its actin-myosin cytoskeletal network to generate contraction forces to "pull" itself forward against resistance to the extracellular matrix. How contraction and contraction events are coordinated is unclear. To analyze this process PtK1 cells were microinjected with fluorescently labeled actin and myosin regulatory light chain and imaged via multi-spectral spinning disk confocal microscopy. We have identified three dynamic organization states of actin-myosin based contractions in migrating epithelial cells (PtK1): 1. "Contraction-Depolymerization" in which the interface between converging actin networks is correlated with local actin disassembly; 2. "Shear" in which adjacent actin networks move anti-parallel to one another; 3. "Compression-Bundling" in which compression of the lamella network induces formation of transverse actin bundles. To further characterize these contraction states, the degree of local correlated motion between actin and myosin were investigated using correlational qFSM (quantitative Fluorescent Speckle Microscopy). Actin and myosin (speckles) were analyzed in contracting (converging) cytoskeletal networks. In all three contraction states, actin and myosin primarily displayed identical kinematics (direction and speed). However, some cells exhibiting the "Contraction-Depolymerization" phenotype showed differential speeds between actin and myosin motion, indicating motion of one system relative to the other. Often, cells exhibiting the contraction-depolymerization phenotype exhibited enhanced protrusive activity at their leading edge. We will confirm this observation with time correlation analysis to determine if differential speeds between actin and myosin (decoupling) are linked to cell protrusion events.

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Actin-mediated Rocketing of Phagosomes Induced by Mechanical Pressure
M. Clarke, A. Müller-Taubenberger, G. Gerisch; Molecular, Cell, and Developmental Biology, Oklahoma Medical Research Foundation, Oklahoma City, OK, 3Max-Planck-Institut für Biochemie, Martinsried, Germany
We have found that mechanical pressure from an agar overlay can trigger actin-mediated rocketing of phagosomes in Dictyostelium cells. Living cells expressing fluorescent fusion proteins that label actin filaments and other cytoskeletal components were fed S. cerevisiae, then covered with a thin layer of agar, which was blotted to remove excess moisture. Actin assembled wherever the plasma membrane pressed against a phagosome, and asymmetric deposition of actin led to phagosome rocketing. The class I myosin MyoII and the Arp2/3 complex became enriched at sites of phagosome-plasma membrane contact. GFP-ARP3 persisted briefly at the plasma membrane when the phagosome moved away, indicating that Arp2/3 was more concentrated at the plasma membrane than at the phagosome membrane. The actin filaments trailing the moving phagosome were labeled with two probes, mRF-LimE, which binds to newly formed actins, and coronin-GFP, which binds to slightly older filaments. These probes showed that new actin is always added close to the moving phagosome. Our data suggest that the force generated by a rocketing phagosome is sufficient to displace the nucleus and the entire microtubule apparatus. This mechanism may keep the cell cortex free of trafficking phagosomes until exocytosis commences, and may also prevent rigid particles from hindering a cell's movement through confined spaces.

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In Vivo Dynamics of Type II Plasmid Segregation Systems
C. S. Campbell, J. S. van Zon, M. Zuccolotto, D. Mullins; 1University of California San Francisco, San Francisco, CA, 2Centre for Integrative Systems Biology, Imperial College London, London, United Kingdom, 3Umr144-CNRS, Institut Curie, Paris, France
The R1 Par operon is a self-contained plasmid partitioning system composed of three parts: parC, ParR and ParM. parC is a stretch of DNA consisting of 10 sequential repeats, each of which binds ParR. The ParR-parC complex in turn binds the actin homolog ParM. Previous studies have shown that ParM forms filaments nearly identical to those of eukaryotic actin filaments and that ParM filament bundles appear to position plasmids at each end of a rod-shaped cell. We recently demonstrated that ParM filaments are dynamically unstable and can elongate bidirectionally in vitro. These observations led to a model in which ParM filaments continually search the cytoplasm and eventually capture a ParR bound parC region on a plasmid. Insertional polymerization at the ParM-parR interface will then push the plasmids to opposite ends of the cell and hold them in place until cell division, ensuring that each daughter cell receives a copy. To test this model directly in live cells, we used GFP labeled lacI to visualize lacI sites present on a plasmid containing a functional R1 Par operon. We observe that the plasmids make both

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diffusive and directed movements wherein two plasmids move away from each other very rapidly. These directed movements occur many times during a single cell division, indicating that segregation is a very dynamic process. In addition, we have visualized GFP labeled ParM filaments and observe very similar dynamics were filaments polymerize, undergo catastrophe, and rapidly depolymerize in less than a minute. We also observe the formation and dissolution of plasmid clusters. These clusters have reduced dynamics and are more frequently localized towards the center of the cell. Our results indicate that plasmid partitioning by ParM is a very dynamic process and suggest a new model for polymer-based plasmid segregation.

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Does the Phosphorylation of Tropomodulin Control Actin Filament Dynamics?

R. A. Lewis, K. L. Weber, D. J. Moyer, V. M. Fowler; Cell Biology, The Scripps Research Institute, La Jolla, CA

Tropomodulins (Tmdos) are a family of actin-binding proteins that regulate actin dynamics by capping the slow-growing (pointed) ends of thin filaments. Tmdos interact with both tropomyosin and actin filaments to prevent depolymerization and elongation at the pointed end, thereby stabilizing the filament. Tmd1 caps stable actin structures such as sarcomeres in cardiacmyocytes and short membrane-bound filaments in erythrocytes but is also found in actin filaments undergoing turnover in lens fiber cells and neurons. It is currently unknown how actin pointed end capping is controlled; thus, understanding the regulation of Tmdos in both dynamic and static structures will provide insight into the regulation of cell shape changes during migration and development. Phosphorylation may provide a mechanism to regulate the function of Tmd1l by changing the sub-cellular localization of Tmd1 or by altering interactions with other proteins which could influence the dynamics of actin filaments. We demonstrate that Tmd1 is phosphorylated on serine2 (S2) in red blood cells. This serine residue is conserved in both Tmd1 and Tmd4 from several species suggesting that phosphorylation of this residue may play a role in regulating Tmd1 function. Phosphorylation of Tmd1l is enhanced by phorbol ester treatment of erythrocytes indicating that this event may occur by the protein kinase C pathway. To determine the role of phosphorylation in regulating Tmd1 function, we created FLAG-tagged versions of the wildtype protein, an S2A (unphosphorymorphic) mutant, and an S2E (phosphomimetic) mutant. Using these constructs we assay for altered localization using immunofluorescence staining. In addition, interaction studies by anti-FLAG immunoprecipitation will test how Tmd1 is regulated by phosphorylation on S2 and identify the kinase responsible for this regulation. The phosphorylation of Tmdos may function to modulate capping and actin pointed end dynamics, allowing for the formation of both stable and static actin structures throughout development.

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Changes in Cytoskeletal Tension Rapidly Phosphorylate Vasodilator-stimulated Phosphoprotein (VASP) in Calcium and ROCK-dependent Manner

K. Yasuda, M. Sbia, J. Yost, M. Yoshigi; Pediatrics, Toyohashi Municipal Hospital, Aichi, Japan, 3Huntsman Cancer Institute, University of Utah, Salt Lake City, UT

BACKGROUND AND OBJECTIVE: Vasodilator-stimulated phosphoprotein (VASP) is highly expressed in smooth muscle-rich organs, where cells experience various mechanical stresses (stretch, shear, and compression). We previously identified VASP and its binding partner zyxin as mechano-sensitive focal adhesion constituents and regulators for mechanically induced actin cytoskeletal remodeling (J Cell Biol 171:209:2005). VASP regulates the actin cytoskeletal remodeling via its phosphorylation kinetics. However, how mechanical stress affects on VASP phosphorylation remains to be determined. METHODS: We applied uniaxial cyclic stretch or fluid shear stress on human aortic smooth muscle cells in vitro and determined VASP phosphorylation at serine 157 and serine 239 residues via immunoblots and densitometric quantification. We also developed a new device that allowed us to rapidly change basal length of scaffold and lyse cells while scaffold length is held (unphasic uniaxial stretch/relax stimulation). RESULTS: Within two minutes upon cyclic stretch (10%, 0.5Hz) or unphasic stretch/relax (10%), VASP was robustly (>80%) phosphorylated both at serine 157 and 239 residues. Fluid shear stress (15 dynes/cm2) weakly (<30%) phosphorylated VASP. Interestingly, VASP phosphorylation by cyclic stretch and shear stress had an initial robust peak at 5-15 minutes which decayed after one hour, and the second peak about 4-6 hours, which appeared to be transcriptionally regulated. Using pharmacological inhibitors for PKA and PKC, we demonstrated that fluid shear stress (15 dynes/cm2) weakly (<30%) phosphorylated VASP. These data suggest that mechano-induced VASP phosphorylation is regulated by unique signaling pathways distinct from commonly recognized pathways. Functional aspects regarding mechano-induced VASP phosphorylation and stress fiber formation remain to be determined in the future.

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Actin Assembly Processes Required for c-Met-mediated Membrane Ruffling and Listeria Invasion

T. Bosse, J. Ehinger, A. Czuchra, S. Benesch, A. Steffen, K. Schloen, H. H. Niemann, G. Scita, T. E. B. Stradal, C. Brakebusch, K. Rottner; 1Helmholtz Centre for Infection Research, Braunschweig, Germany, 3Max Planck Institute of Biochemistry, Martinsried, Germany, 5Institute of Molecular Biotechnology, Vienna, Austria, 3Institut Curie, Paris, France, 1IEO/FSM, Milan, Italy, 2University of Copenhagen, Copenhagen, Denmark

Reorganizations of the actin cytoskeleton drive various essential cellular processes such as protrusion of the front edge during migration, the engulfment of particles during phagocytosis as well as diverse types of specific host-pathogen interactions. These actin reorganizations are frequently initiated by binding of extracellular ligands to their receptors eliciting signal transduction pathways, which can be exploited by certain bacterial pathogens. A prominent example for such a pathway is the gram-positive bacterium Listeria monocytogenes, which by interaction of bacterial Internalin B (InlB) with the hepatocyte growth factor / scatter factor (HGF/SF) receptor, also called c-Met, induces its invasion into non-epithelial cells. In recent years, regulators of bacterial Internalin B (InlB) with the hepatocyte growth factor / scatter factor (HGF/SF) receptor, also called c-Met, induces its invasion into non-epithelial cells. In recent years, regulators of actin assembly processes required for c-Met-mediated membrane ruffling and Listeria Invasion have been identified. In this context, the Rho GTPases, which promote actin filament polymerization, play a central role. However, the precise mechanisms by which these Rho GTPases regulate actin dynamics during Listeria invasion and c-Met-mediated membrane ruffling remain to be determined. In this study, we addressed the question whether actin dynamics during Listeria Invasion and c-Met-mediated membrane ruffling are regulated by the Rho GTPases. To address this question, we performed Fluorescence Recovery After Photobleaching (FRAP) analyses. Full recovery of GFP-actin in individual microspikes occurred in 26 seconds. Increasing F-actin stability with cytochalas-D and jasplakinolide significantly slowed GFP-actin recovery, indicating that recovery was dependent on dynamic actin. In addition, the regulation of actin dynamics in different actin-rich regions was explored by examining GFP-actin recovery in dendrite spines and in microspikes. Recovery of GFP-actin in dendrite spines occurred at a slower rate than did GFP-actin in microspikes, suggesting different factors contribute to actin turnover in different actin-rich regions. Interestingly, the recovery kinetics of F-actin in microspikes was the same in mature and immature neurons. This result suggests that actin turnover in microspikes is independent of neuronal maturity. Taken together these data suggest that mechano-induced VASP phosphorylation and stress fiber formation remain to be determined in the future.

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Dynamics of Actin in Bundled Filaments in Cortical Neurons

Y. Lin, L. Redmond; Pharmacology & Toxicology, Medical College of Georgia, Augusta, GA

The actin cytoskeleton is essential for a multitude of cellular functions including determining cell shape, polarity, and motility. Actin performs these functions when it is assembled into branched networks or bundled into filaments yet remains dynamic and responsive to a variety of cellular signals. In an effort to understand mechanisms of neuronal differentiation, the actin cytoskeleton was examined in primary cortical neurons in vitro. Phalloidin staining revealed F-actin rich protrusions extending from the soma in some, but not all, neurons. We call these structures microspikes. Immunocytochemical and SEM analysis indicated these microspike cytoskeletal structures were composed of bundles of actin filaments. Further, confocal time-lapse imaging of GFP-actin containing microspikes indicated that the microspikes did not change in number or location over time. To begin to investigate actin dynamics in the microspikes, we expressed GFP-actin in neurons and performed Fluorescence Recovery After Photobleaching (FRAP) analyses. Full recovery of GFP-actin in individual microspikes occurred in 26 seconds. Increasing F-actin stability with cytochalasin-D and jasplakinolide significantly slowed GFP-actin recovery, indicating that recovery was dependent on dynamic actin. In addition, the regulation of actin dynamics in different actin-rich regions was explored by examining GFP-actin recovery in dendrite spines and in microspikes. Recovery of GFP-actin in dendrite spines occurred at a slower rate than did GFP-actin in microspikes, suggesting different factors contribute to actin turnover in different actin-rich regions. Interestingly, the recovery kinetics of F-actin in microspikes was the same in mature and immature neurons. This result suggests that actin turnover in microspikes is independent of neuronal maturity. Taken together these data suggest that microspikes are composed of dynamic actin, which forms stable F-actin rich cytoskeletal structures that persist during neuronal differentiation.

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Coordinated Control of Actin Assembly through IQGAPI and Rho GTPases

Y. Cai, H. Kan, G. S. Bloom; Department of Biology, University of Virginia, Charlottesville, VA

The assembly of branched actin networks provides the force for plasma membrane protrusion during cell motility and morphogenesis. The multi-subunit Arp2/3 complex is essential for network formation by simultaneously binding to the sides of pre-existing filaments, and nucleating new filaments from their minus ends. This property of Arp2/3 requires activation by other factors, for example N-WASP, which must in turn be activated by directly binding proteins, like IQGAPI (Benseker, et al. submitted) or activated (GTP-bound) Cdc42 (Rohatgi, et al. 2006 ASCB Annual Meeting Abstracts)
Consistent with a role of Rac1 in PIPKIα smooth to saltatory or erratic motility. These myriad interactions define a multi-scale state diagram of signaling. PIPKIα increases comet tail actin density, which results in decreased β-actin speed. Changing the rate of branch formation, and thus the degree of branching within the tail, affects the transition from dependence on ActA distribution (ultrapolar, unipolar, and bipolar). An unknown molecular detail we must specify is the nature of the mechanical interaction, which we call a tether, between an actin filament and the bacterium. Simulations tuned to satisfy our principal constraints produce realistic bond energies for this tether on the order of a few kT. Furthermore, these simulations produce actin densities within the comet tail within a 5-fold range of experimental estimates. For these tuned simulations we find, for example, that decreasing the F-actin depolymerization rate increases comet tail actin density, which results in decreased bacterial speed. Changing the rate of branch formation, and thus the degree of branching within the tail, affects the transition from smooth to salatory or erratic motility. These myriad interactions define a multi-scale state diagram of Listeria motility.

Differential Roles of Type I Phosphatidylinositol 4-Phosphate 5-Kinases in Cell Motility and Cell Spreading

W. T. Chao, A. C. Daquinag, J. Kunz; Molecular Physiology and Biophysics, Baylor College of Medicine, Houston, TX

Cell motility is dependent on the dynamic reorganization of the actin cytoskeleton at membrane protrusions and sites of cell-matrix contact. The lipid phosphatidylinositol-4,5-bisphosphate (PIP2) is a key second messenger that regulates actin cytoskeletal reorganization. PIP2 has been previously shown to modulate cell motility and cell-matrix adhesion and its synthesis at the plasma membrane appears upregulated during these processes. PIP2 is synthesized by Type I phosphatidylinositol-4-phosphate 5-kinases (PIPKis). There are three isoforms of PIPKI (Iα, Iβ, and Iγ) in mammals and all three can induce actin reorganization upon overexpression. However, it is not clear whether they normally have redundant roles in the regulation of actin dynamics and cell motility. Here we demonstrate that siRNA-mediated knock-down of either PIPKIα or PIPKIβ inhibits cell migration and cell spreading. In contrast, PIPKIγ knock-down enhances migration speed, and there was a proportional increase in cell spreading kinetics. Thus, silencing of each PIPKI isoform affects the migration machinery suggesting that these isoforms play non-redundant roles during cell motility. Here we report on further studies on the role of PIPKIγ. We demonstrate that PIPKIγ is required for actin assembly induced by local activation of integrin signaling. PIPKIγ, but not PIPKIβ or PIPKIα, is essential for membrane translocation of two key regulators of actin dynamics: the small GTPase Rac1 and the actin binding protein cortactin. Consistent with a role of Rac1 in PIPKIγ-mediated signaling, constitutive membrane targeting of Rac1 partially suppresses the motility defects of PIPKIγ knock-down cells. Together our data suggest that specific PIPKI isoforms are involved in different aspects of motility and control the recruitment of specific yet distinct signaling and scaffolding proteins to discrete membrane microdomains.

Stress Fibers Are Generated by Two Distinct Actin Assembly Mechanisms in Motile Cells

P. Hotulainen, P. Lappalainen; Institute of Biotechnology, Helsinki, Finland

Stress fibers play a central role in adhesion, motility, and morphogenesis of eukaryotic cells, but the mechanism of how these and other contractile actomyosin structures are generated is not known. By analyzing stress fiber assembly pathways using live cell microscopy, we revealed that these structures are generated by two distinct mechanisms. Dorsal stress fibers, which are connected to the substrate via a focal adhesion at one end, are assembled through formin (mDia1/DRF1)-driven actin polymerization at focal adhesions. In contrast, transverse arcs, which are not directly anchored to substrate, are generated by endwise annealing of myosin bundles and Arp2/3-nucleated actin bundles at the lamella. Remarkably, dorsal stress fibers and transverse arcs can be converted to ventral stress fibers anchored to focal adhesions at both ends. Fluorescence recovery after photobleaching analysis revealed that specific actin cross-linking in stress fibers is highly dynamic, suggesting that the rapid association-dissociation kinetics of cross-linkers may be essential for the formation and contractility of stress fibers. Based on these data, we propose a general model for assembly and maintenance of contractile actin structures in cells.
Regulation of Actin Dynamics-driven Processes during Drosophila Development
R. Stephan, R. Fricke, A. Mertens, C. Klämbt, S. Bogdan; Institute of Neurobiology, University of Muenster, Muenster, Germany
Actin polymerization is a key process for many cellular events during development. To a large extent, the formation of filamentous actin is controlled by the WASP and WAVE proteins that activate the Arp2/3 complex in different developmental processes. Wave function is regulated through a protein complex containing Scar, Kette and Abi. We have recently shown that the Abi protein also plays a central role in activating Wasp mediated processes. Abi directly binds to Wasp and is able to activate Wasp dependent F-actin formation in vitro and in vivo. Here, we report that the Drosophila Abi-Wasp function is generally required to promote sensory organ development by modulating EGFR signaling. Expression of a membrane-tethered activated Abi protein leads to tyrosine hyperphosphorylation and an increase in MAPK activity. Additionally, co-expression of activated Abi and EGFR dramatically enhances the induction of extra-sensory organs whereas inhibition of EGFR signaling leads to a suppression of the dominant phenotype. In agreement with this observation Abi is able to associate with the EGFR in a common complex at the membrane. Thus, in vivo different Abi complexes may set the balance between Wasp and Wave in different actin based developmental processes. In order to identify further proteins that regulate WASP and WAVE, we have conducted different genetic and biochemical approaches and selected different new candidates. A biochemical and functional analysis of one novel Abi interacting candidate will be presented.

Leiomodin: A Novel Actin Filament-nucleating Factor
D. Chereau,1 M. Bozcowska,1 I. Fujigawa,2 G. Rebowski,1 D. B. Hayes,1 P. Erhardt,1 T. D. Pollard,1 R. Dominguez2; 1Elan Pharmaceuticals, South San Francisco, CA, 2Physiology, University of Pennsylvania School of Medicine, Philadelphia, PA
The actin cytoskeleton undergoes constant remodeling during a variety of cellular processes, including cell morphogenesis and motility. The de novo assembly of actin filaments is highly regulated in vivo and requires the participation of actin filament-nucleating factors. To date, three types of filament nucleating factors have been identified: Arp2/3 complex, formin and spire. These nucleating factors appear to have specialized functions and work under the control of dedicated signaling pathways and activator proteins. Here we report the discovery of a novel nucleating factor of the actin cytoskeleton in muscle cells, leiomodin (Lmod). Like most cytoskeletal proteins, Lmod is a modular protein. The N-terminal portion of Lmod shares sequence similarity with tropomodulin, a filament pointed-end capping protein. Like tropomodulin, this portion of Lmod contains a flexible region involved in tropomyosin binding, followed by a globular domain that binds actin. However, compared to tropomodulin, Lmod presents a large C-terminal extension, which contains a poly-Pro region and additional actin-binding sites of the WASP-homology domain-2 (WH2) type. We show here that full-length Lmod increases dramatically the rate of actin filament nucleation. Functional domain mapping further demonstrates that this activity requires the presence of the tropomodulin-like globular domain as well as the C-terminal poly-Pro-WH2 extension, but not the flexible N-terminal tropomyosin-binding region. Yet, Lmod nucleates actin filaments efficiently in the presence of tropomyosin, suggesting that its function is to nucleate tropomyosin-decorated actin filaments in muscle cells.

The Susceptibility of Actin Filament Populations to Latrunculin A and Cytochalasin D Is Regulated by Tropomyosin Composition
S. J. Creed,1 N. Bryce,1 J. T. Stelm,1 P. Gunning2; 1Oncology Research Unit, The Children's Hospital Westmead, Westmead, NSW, Australia, 2Discipline of Paediatrics and Child Health, University of Sydney, Sydney, NSW, Australia
Actin altering drugs such as Latrunculin A and Cytochalasin D have been used to study the cytoskeleton for a number of years. While it is known that effects of these drugs are cell type dependent, it has been widely accepted that all actin filament populations are equally susceptible. However, the discovery that spatial and temporal regulation of tropomyosin isoforms results in dramatically different filament populations leads to the hypothesis that these drugs may not affect all filament populations in the same manner. Therefore, several conclusions on biological functions of actin filaments drawn from studies using these drugs may not always be accurate. The aim of this study was to determine if molecularly distinct filament populations have altered susceptibility to actin destabilizing drugs. The Tm5NM1 and Tm3 tropomyosin isoforms were over-expressed in neuro-epithelial cells. We examined the organization of microfilaments and susceptibility to Latrunculin A and Cytochalasin D using immunofluorescence and western blot analysis of detergent soluble and insoluble fractions. Over-expression of Tm5NM1 resulted in Tm5NM1-containing stress fibers with decreased detergent solubility. Tm3 over-expression caused an abundance of filopodia containing shorter Tm3-associated filaments with increased susceptibility. Filament populations containing Tm5NM1 demonstrated greater resistance to drug treatment, with filament organization maintained at higher concentrations than observed for control cells. Conversely, Tm3-containing filaments showed increased susceptibility. Supporting this, a Latrunculin A time course demonstrated faster filament breakdown in Tm3 cells. Combined the data suggest that filament populations containing Tm3 are shorter and more dynamic making them more susceptible to Latrunculin and Cytochalasin. Preliminary data examining ADF, an actin severing protein, suggests ADF is associated with Tm3 containing filaments at the filopodia, providing a possible mechanism for this phenomenon. We conclude that tropomyosins direct organization and dynamics of actin filaments, altering the sensitivity of the filaments to actin destabilizing drugs.

Actin Cross-linking with Cholera RTX Toxin
D. S. Kurdyanov,2 A. Durer,1 C. L. Cordero,1 S. A. Benchas,1 R. E. Reisler,2 K. J. Fullmer-Satchell1; 1Department of Chemistry and Biochemistry, UCLA, Los Angeles, CA, 2Department of Microbiology, University of Western Ontario, London, Canada
Vibrio cholerae RTX toxin causes rounding of mammalian epithelial cells in culture by dismantling actin filaments via a unique mechanism. The 47.8 kDa actin-crosslinking domain of RTX (ACD-RTX) covalently cross-links actin subunits in vitro and in vivo forming a ladder of oligomers similar to the ladder of longitudinal oligomers obtained by cross-linking of F-actin by azidonitrophenyl putreiscine (ANP). In contrast to ANP induced cross-linking, ACD-RTX cross-linking is blocked in F-actin; yet it requires high MgCl2 concentrations. CaCl2 and KCl (at concentrations high enough to polymerize actin) do not support the cross-linking in the absence of MgCl2. Actin polymerization and cross-linking reactions compete with each other when ACD and MgCl2 are added to G-actin simultaneously. The purified dimers of ACD-cross-linked actin do not accelerate the polymerization of intact actin, indicating that they do not nucleate new filaments growth. The toxin cross-linked oligomers, however, do not polymerize under physiological salt conditions, but the polymerization is rescued with either phalloidin or cofilin, as confirmed by light scattering, pelleting assays, and electron microscopy. We locate the cross-linking sites on actin, we used fluorescently labeled yeast actin mutant in limited enzymatic and chemical proteolysis experiments. Our preliminary data suggest that ACD-RTX covalently attaches SD2 of one actin protomer to the SD3 of another protomer in longitudinal manner. This type of interaction between the subunits is consistent with the Holmes model of actin filament. Yet, given the impaired polymerization of the cross-linked oligomers, their conformation must be different from that in F-actin. Additionally, a number of factors that destabilize F-actin - latrunculin, gelsolin S1, Kabiramide C, and Cys374 labeling with TMR - do not interfere with the ACD-RTX induced cross-linking. Thus, our data suggest that RTX toxin destabilizes actin filaments in an unusual way - by covalently capturing one of the unstable conformations of actin oligomers.

An APC2-Diaphanous Complex Organizes Actin in the Drosophila Syncytial Embryo
R. L. Webb,1 J. S. Weinberg, M. Zhou, S. E. Clark,2 B. M. McCartney1; 1Biological Sciences, Carnegie Mellon University, Pittsburgh, PA, 2Biological Sciences, Colby College, Waterville, ME
Organization and rearrangement of the cytoskeleton is essential for many cellular processes. Adenomatous polyposis coli (APC) family proteins associate with actin and microtubules and influence their behavior, however the precise molecular mechanisms involved are poorly 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 actin and microtubules is necessary for cytoskeletal rearrangements involved in synchronous nuclear divisions. We examined embryos null for Drosophila APC2 and observed incomplete actin rings and lack of actin furrow extension during syncytial mitoses. To determine the mechanism by which APC2 functions to organize actin, we evaluated the role of the formin Diaphanous (Dia), which functions to organize actin during Drosophila cellularization and is in a complex with APC and EB1 in migrating mammalian cultured cells. Similar to APC2 mutant embryos, dia mutants have defects in actin rings and furrow extension. We found that APC2 and Dia form a complex in syncytial embryos, and that localization of APC2 to actin rings and furrows is dependent on Dia. Further, we found that reduction of dia in APC2 mutant embryos enhances the severity of APC2 actin defects. We asked whether an APC2-Dia complex is downstream of Rho1, as predicted from other studies, by examining RhoGEF2 and Rho1 mutant syncytial embryos. The actin defects in Rho1 and RhoGEF2 mutants are overlapping but distinct from those of APC2 and dia, suggesting that they are functioning in parallel pathways. Based on their functions in other systems, APC2 and Dia may influence actin directly, or indirectly through microtubules. To distinguish between these possibilities we are using Total Internal Reflection Fluorescence microscopy to assess cortical microtubules in live wild type and mutant embryos. Furthermore, we are probing the relationship between APC2, Dia and EB1 in this system.

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The PH Domain Proteins Slm1 and Slm2 Are Novel Effectors of Sphingolipid Signaling Pathways during the Response to Heat Stress

A. Daquinag, M. M. Madri, J. Kunz; Molecular Physiology and Biophysics, Baylor College of Medicine, Houston, TX

The redundant and essential PH domain containing proteins Slm1 and Slm2 were previously identified as targets of the PI4,5P2 and TORC2 signaling pathways. Here, we demonstrate that Slm1 and Slm2 are also targets of sphingolipid signaling pathways during environmental stress conditions. We show that upon depletion of cellular sphingolipid levels Slm1 function becomes essential for survival under heat stress. We further demonstrate that in response to heat stress Slm proteins are regulated by a phosphorylation/dephosphorylation cycle involving the sphingolipid-activated protein kinases Ptk1/Pkh2 and the calcium/calmodulin-dependent protein phosphatase calcineurin. Mass spectrometry analysis identifies Ser659 in Slm1 as one of the residues phosphorylated during heat shock. Characterization of mutant Slm1 variants that mimic dephosphorylated and phosphorylated states, respectively, demonstrate that phosphorylation at Ser659 is vital for survival under heat stress and may play a role in the sphingolipid- and Pkh-dependent repolarization of the actin cytoskeleton. Finally, we present evidence that Slm proteins are also required for the trafficking of the raft-associated arginine permease Cari to the plasma membrane, a process that requires sphingolipid and phospholipid synthesis. Thus, Slm proteins are required for sphingolipid-dependent functions including actin polarization and aspects of vesicle trafficking in response to environmental stress conditions.

Myristoylated Alanine-rich Protein Kinase C Substrate PSD: Specific Functions and Possibilities for Regulation

E. G. Yarmola, J. Kress, T. V. Yarmola, M. R. Bubb; Medicine, University of Florida, Gainesville, FL

Myristoylated alanine-rich protein kinase C substrate (MARCKS) is a prominent phosphorylation substrate in the brain that binds calmodulin and acidic phospholipids, and has been implicated in cellular processes associated with cytoskeletal remodeling. The effect of the MARCKS active site, phosphorylation site domain (PSD) on actin polymerization is complex and requires detailed characterization. Our data characterize specific effects of PSD on actin filament dynamics, and suggest how these effects could incorporate into the overall PSD activity, depending on conditions. We demonstrate that MARCKS PSD nucleates actin filaments but does not change the effective nucleus size. PSD inhibits barbed end elongation at low ratios to actin consistent with capping effect, and promotes elongation at higher ratios, converging with the elongation rate for gelsolin-capped filaments. PSD crosslinks actin filaments and inhibits depolymerization in the cooperative manner consistent with crosslinking. PSD decreases actin critical concentration in presence and absence of gelsolin in a manner quantitatively consistent with its effect on the rates of elongation and depolymerization. Phospholipid PI(2) differentially modulates the effect of MARCKS PSD on actin polymerization. It eliminates inhibitory effect of PSD on elongation and depolymerization, and increases its nucleation effect. We suggest that the effect of phosphorylation on the PSD activity, we created several pseudophosphorylated peptides in which different serine residues in PSD were replaced with glutamic acid. These peptides crosslink actin filaments and inhibit actin depolymerization with lower activity depending on the specific site(s) of phosphorylation. Our data suggest that MARCKS PSD function is a combination of several effects that could be tightly regulated in cells. MARCKS could be used as a switch that determines whether quiescent actin bundles are stabilizing the membrane-cytoskeletal interface or whether a dynamic population of actin filaments is actively altering morphological plasticity by remodeling the cytoskeleton.

Mechanosensing and Mechanotransduction by the Na-H exchanger NHE1 for Actin Filament Assembly

J. R. Mackley,* C. Frantz,* L. LeClaire,* E. Almeida; Cell and Tissue Biology, University of California, San Francisco, San Francisco, CA; *NASA Ames Research Center, Moffett Field, CA

Mechanical forces regulate tissue organization and remodeling, and cell architecture and function. Our understanding of how cells sense and transduce mechanical force, however, is limited compared with what is known about sensing and transducing chemical cues. In specialized sensory cells mechanotransduction units include a plasma membrane ion transport protein anchored to the actin cytoskeleton. We previously reported that in fibroblasts the plasma membrane Na-H exchanger NHE1, which is anchored to actin filaments by binding ERM (ezrin, radixin, moesin) proteins, is mechanosensitive. Although integrins are also mechanosensitive and their activation stimulates NHE1 activity, we now report that mechanosensitivity of NHE1 is independent of integrin-matrix adhesion. Cells plated on a polystyrene strip mounted in a uniaxial loading system were mechanically stimulated by applying a 5 min 1% deformation and release. NHE1 activation in response to mechanical load was similar in fibroblasts plated for four hours on strips coated with either fibronectin or poly-L-lysine, and in 48 h cultures of wild-type or beta-1-null mouse embryonic tetarocarcinoma cells. We also found that mechanotransduction by NHE1 is necessary for dynamic actin filament assembly. Although the abundance of F-actin in quiescent, unstimulated fibroblasts is NHE1-independent, total F-actin increased 2-fold in fibroblasts expressing NHE1 but did not change in NHE1-deficient fibroblasts with mechanical load. Mechanical load also increased the number of new actin free barbed ends in cells expressing NHE1, but this was markedly attenuated in NHE1-deficient cells. The Arp2/3 complex is a primary nucleator of new actin filaments. Phosphorylation of the Arp2 subunit, which is necessary for Arp2/3 nucleating activity, increased with mechanical load and the increase was dependent on NHE1 activity. These data suggest that NHE1 functions within a mechanotransduction unit because it is activated by mechanical force independent of integrin-matrix adhesion and is necessary for mechanosensitive actin filament assembly. Supported by NASA grant NNA04CC53A.

Hydrogen/Deuterium Exchange Studies of Twinfilin-capping Protein Interactions and Dynamics

M. C. Merckel, M. R. Emmett, V. O. Paavilainen, P. Lappalainen, A. G. Marshall, R. Tuma; Institute of Biotechnology, Helsinki University, Helsinki, Finland, National High Magnetic Field Laboratory, University of Florida, Tallahassee, FL

Twinfilin (Twf) is an evolutionarily conserved actin-binding protein that regulates cytoskeletal dynamics by sequestering actin monomers and by binding to filament barbed ends [1]. Twf is composed of two actin-depolymerization factor homology (ADF-H) domains followed by a ~30 residue long C-terminal tail. CP binds to the barbed ends of the filaments and works as a 'cap' by preventing the addition and loss of actin monomers. Twf is composed of two actin-depolymerization factor homology (ADF-H) domains followed by a ~30 residue long C-terminal tail. CP binds to the barbed ends of the filaments and works as a 'cap' by preventing the addition and loss of actin monomers. CP binds to the barbed ends of the filaments and works as a 'cap' by preventing the addition and loss of actin monomers. CP binds to the barbed ends of the filaments and works as a 'cap' by preventing the addition and loss of actin monomers. CP binds to the barbed ends of the filaments and works as a 'cap' by preventing the addition and loss of actin monomers. CP binds to the barbed ends of the filaments and works as a 'cap' by preventing the addition and loss of actin monomers. CP binds to the barbed ends of the filaments and works as a 'cap' by preventing the addition and loss of actin monomers. CP binds to the barbed ends of the filaments and works as a 'cap' by preventing the addition and loss of actin monomers. CP binds to the barbed ends of the filaments and works as a 'cap' by preventing the addition and loss of actin monomers. CP binds to the barbed ends of the filaments and works as a 'cap' by preventing the addition and loss of actin monomers. CP binds to the barbed ends of the filaments and works as a 'cap' by preventing the addition and loss of actin monomers. CP binds to the barbed ends of the filaments and works as a 'cap' by preventing the addition and loss of actin monomers. CP binds to the barbed ends of the filaments and works as a 'cap' by preventing the addition and loss of actin monomers. CP binds to the barbed ends of the filaments and works as a 'cap' by preventing the addition and loss of actin monomers. CP binds to the barbed ends of the filaments and works as a 'cap' by preventing the addition and loss of actin monomers. CP binds to the barbed ends of the filaments and works as a 'cap' by preventing the addition and loss of actin monomers. CP binds to the barbed ends of the filaments and works as a 'cap' by preventing the addition and loss of actin monomers. CP binds to the barbed ends of the filaments and works as a 'cap' by preventing the addition and loss of actin monomers. CP binds to the barbed ends of the filaments and works as a 'cap' by preventing the addition and loss of actin monomers. CP binds to the barbed ends of the filaments and works as a 'cap' by preventing the addition and loss of actin monomers. CP binds to the barbed ends of the filaments and works as a 'cap' by preventing the addition and loss of actin monomers. CP binds to the barbed ends of the filaments and works as a 'cap' by preventing the addition and loss of actin monomers. CP binds to the barbed ends of the fi...
vertebrate, the existence of two types of fibers is important since muscle should play a multiple of works depending on its location in the body. But the differentiation mechanism of these muscle fiber subtypes is unclear. We analyzed whether the differentiation of myoblasts into myofibers had variability between fast and slow types. Myoblasts from 7-day-old chick embryo were cultured for 7 days and formed into muscle fibers. When cells were stained immunohistochemically, more than 60% of fibers produced only MyHC, and remaining fibers produced both MyHC and MyHC in the control group. However, when the extract of slow muscle was supplemented to the culture medium, about 35% of muscle fibers expressed only MyHC and 65% of that expressed both MyHC and MyHC. The same result was obtained when the extract of slow muscle was added into the culture of cloned mouse myoblast, C2C12 cells. The factor, which induces the differentiation of slow muscle, is considered to be secreted protein, since it denatured by heating, but recovered by the dialysis after the urea denaturation. We performed DNA screening from adult chicken slow muscle cDNA library, and we discovered one gene, which induced the differentiation into slow muscle fiber when the product was supplemented into the culture medium. The results presented here show that an environment of myoblasts controls the subtype differentiation of muscle fibers.

1568 S100B Activates Quiescent Myoblasts and Satellite Cells
F. Riuszi, G. Sorci, R. Donato; Department of Experimental Medicine & Biochemical Sciences, University of Perugia, Perugia, Italy
We reported that low-density and high-density myoblasts, the effects of S100B on myoblasts, the effects of S100B on differentiation and the duration of exposure condition the sensitivity of myoblasts to S100B, suggesting that S100B might play a dual regulatory role during embryonic myogenesis and muscle regeneration.

1569 S100B in Myoblasts Interferes with MyoD Expression
R. Donato, C. Tubaro, C. Arcuri; Department of Experimental Medicine & Biochemical Sciences, University of Perugia, Perugia, Italy
The Ca2+-modulated protein of the EF-hand type, S100B, which participates in the regulation of several intracellular processes including cell cycle progression and differentiation, is expressed in related to the process of muscle development, the effects of S100B on myoblasts and inhibition of S100B expression by RNA interference. L6 myoblasts stably overexpressing S100B (clone L6C8) showed inability to express the muscle-specific transcription factor, myogenin, and the late differentiation marker, myosin heavy chain (MHC) and to fuse into myotubes, suggesting that S100B might interfere with myoblast differentiation. However, these effects were not consequent to increased proliferation of L6C8 myoblasts. Interestingly, no obvious decrease in the extent of activation of p38 MAPK, which is crucial for myoblast terminal differentiation, were detected in L6C8 myoblasts under differentiation conditions. Also, transfection with MKK4E, an active mutant of the p38 MAPK upstream kinase MKK3, while stimulating myogenin and MHC expression in mock-transfected myoblasts (clone L6C11) as expected, failed to counteract the inhibitory effect of S100B overexpression on myogenin and MHC expression in L6C8 myoblasts. Thus, S100B might interfere with myoblast differentiation and myotube formation by acting downstream of p38 MAPK. Inability of L6C8 myoblast to form myotubes was accompanied by reduced p21 induction and no MyoD expression while Myf-5 expression was unchanged, compared with L6C11 myoblasts. Also, transient transfection with the muscle-specific transcription factor, MyoD, but not Myf-5, restored L6C8 myoblast differentiation and fusion, while silencing S100B expression in wild-type myoblasts by RNA interference resulted in stimulation of MyoD, myogenin and MHC expression and myotube formation. Finally, the transcriptional activity of NF-kB, a negative regulator of MyoD expression, was greatly enhanced in L6C8 myoblasts. Thus, intracellular S100B might participate in the modulation of myoblast differentiation by interfering with MyoD expression in an NF-kB-dependent manner.

1570 Glucocorticoid Induces Accelerated MyoD Protein Degradation via Activation of the N-terminal Ubiquitination Pathway in Muscle Cells
L. Sun, J. S. Trausch-Azar, A. Ciechanover, A. L. Schwartz; 1Department of Pediatrics and Molecular Biology and Pharmacology, Washington University School of Medicine, St. Louis, MO, 2Vascular and Tumor Biology Research Center, Technion-Israel Institute for Technology, Haifa, Israel
MyoD is a tissue-specific transcriptional activator that acts as the master switch for skeletal muscle differentiation and development. Previous studies in surrogate, non-muscle cells have shown that MyoD protein degradation is mediated by the ubiquitin-proteasome system via two distinct pathways characterized by the site of ubiquitination of the protein, namely an N-terminal dependent pathway and a lysine-dependent pathway. However, the biological roles of the two ubiquitination pathways in MyoD protein degradation during muscle differentiation remain unclear. Therefore, we investigated the protein stabilities of two MyoD mutants, which are deficient either in N-terminal ubiquitination or internal lysine ubiquitination respectively, in muscle cells with or without glucocorticoid administration. Prolonged glucocorticoid administration at pharmacological doses is known to inhibit muscle differentiation and induce muscle wasting, a catabolic condition associated with stimulated proteolysis via the ubiquitin-proteasome system. In both C2C12 myoblasts and myotubes, we show that N-terminal blocked MyoD is rapidly degraded with a t1/2 ~ 1 hour, which is comparable to that of wild type MyoD, while lysine-less MyoD is more stable, with a t1/2 ~ 7 hours. Glucocorticoid administration induced accelerated protein degradation of both endogenous and exogenous wild type MyoD in C2C12 myotubes (t1/2 from ~1 hour to ~0.4 hour). However, the two ubiquitination pathways appear to be affected differently by glucocorticoid. In C2C12 myotubes, exposure to dexamethasone appears to stabilize N-terminal blocked MyoD (t1/2 ~ 3.4 hours), whereas lysine-less MyoD is destabilized (t1/2 ~ 1.2 hours). Taken together, our results suggest that glucocorticoid induces accelerated MyoD protein degradation, perhaps via activation of the N-terminal ubiquitination pathway. Thus, our findings support different roles for the two ubiquitination pathways in MyoD protein degradation in muscle cells, and suggest an important role for the novel N-terminal ubiquitination pathway in muscle cells under catabolic conditions.

1571 The Role of Phospholipase D and Phosphatidic Acid in the Mechanical Activation of mTOR Signaling in Skeletal Muscle
T. A. Hornberger, K. B. Sakihisa, S. A. Huang, S. Chien; Bioengineering, University of California San Diego, La Jolla, CA
Signaling by the mammalian target of rapamycin (mTOR) has been reported to be necessary for mechanical load-induced growth of skeletal muscle. The mechanisms involved in the mechanical activation of mTOR signaling are not known, but several studies indicate that a unique (PI3K- and nutrient-independent) mechanism is involved. In this study, we have demonstrated that a novel regulatory pathway for mTOR signaling, which involves phospholipase D (PLD) and the lipid second messenger phosphatidic acid (PA), plays a critical role in the mechanical activation of mTOR signaling. First, an elevation in PA concentration was sufficient for the activation of mTOR signaling. Second, the isomers of PLD (PLD1 and PLD2) are localized to the z-band in skeletal muscle (a critical site of mechanical force transmission). Third, mechanical stimulation of skeletal muscle with intermittent passive stretch or vivo-induced PLD activation, PA accumulation and mTOR signaling. Finally, pharmacological inhibition of PLD blocked the mechanically-induced increase in PA and the activation of mTOR signaling. Combined, these results indicate that mechanical stimuli activate mTOR signaling through a PLD-dependent increase in PA. Furthermore, we showed that mTOR signaling was partially resistant to rapamycin in muscles subjected to mechanical stimulation. Since rapamycin and PA compete for binding to the FRB domain on mTOR, these results suggest that mechanical stimuli activate mTOR signaling through an enhanced binding of PA to the FRB domain on mTOR.

1572 Functional Variation of Troponin between Smooth and Striated Muscles in Photicodrphates
T. Obinata, Y. Tandon, N. Sato, M. Ogawara, K. Kubokawa; 1Department of Biology, Chiba University, Chiba-shi, Japan, 2Ocean Research Institute, The University of Tokyo, Tokyo, Japan
Troponin is a protein characteristic of striated muscles in vertebrates. It is known to be an inhibitor for muscle contraction or actin-myosin interaction and Ca2+ removes the inhibition. We previously detected troponin in the smooth muscle of ascidian, one of protochordate animals, and found that its function is distinct from that of vertebrate striated muscle troponin, namely the ascidian troponin functioned as a Ca2+-dependent accelerator of muscle contraction (Endo & Obinata, 1981). This unique property of ascidian troponin was attributed to the characteristics of
troponin 1 (TnT) and T (TnT). In this study, we were interested in whether troponin in proteochondrate striated muscles functions as an inhibitor for muscle contraction or as a Ca++-dependent accelerator. We used amphioxus, Branchiostoma belcheri, as a model animal of proteochondrates to obtain striated muscle, because obtaining sufficient amount of striated muscle from ascidian is difficult. Troponin was extracted from frozen amphioxus tissue and partially purified. This troponin activated myosin in interaction in the absence of Ca++. To further clarify the properties of each troponin component, we prepared recombinant TnT and TnI of amphioxus and compared them with chicken troponin C. The complex markedly inhibited actomyosin ATPase in the presence of Ca++, the but the inhibition was eliminated by Ca++. Just as in the case of chicken or rabbit skeletal muscle troponin, Amphioxus TnI inhibited actomyosin ATPase strongly in a Ca++- independent manner. Amphioxus TnT shared common properties with chicken troponin. These results strongly suggest that troponin in proteochondrate striated muscles functionally resembles troponin in vertebrate striated muscle but is distinct from the smooth muscle troponin.

1573 Mutagenesis of Phosphorylation Sites in Drosophila Flightin Impair Flight Muscle Structure and Function

B. Barton,¹ G. Ayer,¹ D. W. Maughan,¹ J. O. Vigoreaux²,³; ¹Biology, University of Vermont, Burlington, VT, ²Molecular Physiology & Biophysics, University of Vermont, Burlington, VT

Flightin is a myosin rod binding protein that in Drosophila is expressed exclusively in the asynchronous, indirect flight muscles (IFM). Hyperphosphorylation of flightin precedes the emergence of flight competency in newly eclosed adults and plays an essential role in IFM structural stability and mechanics. Flightin null (fln) mutants display disorganization of muscle A-bands. Mutations that affect flightin expression or phosphorylation result in muscle hypercontraction, loss of power output, and flightlessness. Despite its essential role in IFM, flightin is not found in vertebrate striated muscle. The goal of this study is to reconstruct the evolutionary history of flightin to determine its origin as an IFM-specific protein. Flightin was identified in 32 insects (Orders Thysanura, Orthoptera, Hemiptera, Coleoptera, Hymenoptera, Lepidoptera, Diptera) and six crustaceans (Orders Crustacea, Isopoda, Decapoda). Analysis of flightin sequence revealed a highly variable (fast evolving) amino terminal region (first ~82 residues) and a ~52 amino acid central region within which residues that remain conserved or neutralized are abundant. The central region is shared by C. elegans and D. melanogaster (i.e., Pancrustacea) but absent in all other metazoans. Within the conserved region, 9 amino acids are invariant including an N-terminal tryptophan (W), 4 tyrosines (Y), and a C-terminal arginine (R). We refer to this conserved region as the WYR-W domain. The presence of flightin in the ancestrally wingless bristlefoot suggests this gene is present in all insects. The identification of flightin in C. elegans indicates the origin of more than 400 million years ago. Phylogenetic analysis of amino acid sequences suggests functional historical variation in selective forces have affected the evolution of flightin in higher insects, probably as a result of the newly evolved IFM-specific function. One possibility is that flightin is under dual evolutionary constraints (purifying and positive selection) to fulfill a conserved muscle physiological function and a rapidly evolving behavior-associated function. We propose that if WYR functions as a myosin rod binding domain.

1574 Evolutionary Analysis of Flightin Reveals a Putative Myosin Rod Binding Domain

F. Soto-Adames, J. Vigoreaux; Biology, University of Vermont, Burlington, VT

Flightin is a hyperphosphorylated, myosin rod binding protein that is necessary for the structural stability of thick filaments. In Drosophila melanogaster, flightin is expressed exclusively in the asynchronous indirect flight muscles (IFM), renowned for their enhanced stretch activation response. Mutations that affect flightin expression or phosphorylation result in muscle hypercontraction, loss of power output, and flightlessness. Despite its essential role in IFM, flightin is not found in vertebrate striated muscle. The goal of this study is to reconstruct the evolutionary history of flightin to determine its origin as an IFM-specific protein. Flightin was identified in 32 insects (Orders Thysanura, Orthoptera, Hemiptera, Coleoptera, Hymenoptera, Lepidoptera, Diptera) and six crustaceans (Orders Crustacea, Isopoda, Decapoda). Analysis of flightin sequence revealed a highly variable (fast evolving) amino terminal region (first ~82 residues) and a ~52 amino acid central region within which residues that remain conserved or neutralized are abundant. The central region is shared by C. elegans and D. melanogaster (i.e., Pancrustacea) but absent in all other metazoans. Within the conserved region, 9 amino acids are invariant including an N-terminal tryptophan (W), 4 tyrosines (Y), and a C-terminal arginine (R). We refer to this conserved region as the WYR-W domain.

1575 Characterization and Functional Analysis of the Drosophila UNC-45 Chaperone In Vitro and In Vivo

C. F. Lee, Biology, San Diego State University, San Diego, CA

UNC-45 is a chaperone important for muscle function. It may be responsible for folding the myosin head into the correct conformation, which is essential for muscle function. In addition, UNC-45 may also prevent myosin from denaturing at elevated temperatures, e.g., during exercise. Here, we explore UNC-45 localization using immunofluorescence confocal microscopy in Drosophila melanogaster and use electron microscopy to demonstrate that UNC-45 protects myosin heads from denaturing and fusing at elevated temperature. We find that UNC-45 is localized to the blastoderm in two-hour-old embryos and co-localizes with non-muscle myosin. In 14-hour-old embryos, UNC-45 is expressed strongly in the body-wall muscles, pterygophore muscle, and the heart tube. Lighter staining areas could indicate UNC-45 function in other tissues. In third instar larval body wall muscle, UNC-45 preliminarily shows an alternating staining pattern with phalloidin labeled actin in a subset of muscles, which implies its localization in the A-bands of sarcomeres. Further, UNC-45 expression is up-regulated by heat-shock in vivo, muscle, and the heart tube. Expression was observed in the same set of muscles (pharyngeal, vulval, body wall) in which UNC-45 is capable of maintaining the two-headed structure of myosin at elevated temperatures, as shown by electron microscopy of chicken skeletal muscle myosin at 25ºC and 42ºC. In contrast, chicken myosin at 42ºC in the absence of UNC-45 showed fused heads. Our studies indicate that phosphorylation of Thr158 and/or Ser162 is necessary for subsequent phosphorylation of other sites. All three transgenic strains express all nine phosphovariants. These results suggest that phosphorylation of Thr158 and/or Ser162 is necessary for subsequent phosphorylation of other sites. All three transgenic strains show normal, albeit long, IFM sarcomeres in newly eclosed adults. In contrast, sarcomeres in fully mature flies show extensive breakdown while those in fln0 are not as disordered. The fiber hypercontraction phenotype that characterizes fln is evident in fln0 and fln0 flies but partially rescued in fln0. Skinner fiber mechanics of newly eclosed flies show alterations in viscous and elastic moduli for fln0 and fln0 resulting in a significant reduction in oscillatory power output. Flightin sequence analysis revealed that Thr158 and Ser162, but not Ser139, Ser141, and Ser145, are conserved among 15 Drosophila species (~40-60 million years divergence time). Our studies indicate that phosphorylation of Thr158 and/or Ser162 fulfills an essential role in IFM structural stability and mechanics while phosphorylation in vivo may enhance secondary, species-specific functions.

1576 A Protein Phosphatase Is a Binding Partner for Protein Kinase 2 (PK2) of UNC-89 (obscurein) in C. elegans

H. Qadota, T. Ferrara, K. Mercer, G. Benian; Pathology, Emory University, Atlanta, GA

In C. elegans, unc-89 mutants display disorganization of muscle A-bands. unc-89 encodes 6 major polypeptides, as large as 800 kDa, composed of immunoglobulin (Ig), fibronectin type III (Fn3), SH3, DH, PH, and protein kinase domains (Benian et al., 1996; Small et al., 2004). Several UNC-89 isoforms contain two protein kinase domains, PK1 and PK2. Molecular modeling indicates that PK1 is inactive, whereas PK2 is catalytically active. Antibodies localize the UNC-89 proteins to the M-line. To gain further insight into the function of UNC-89, we screened a yeast 2-hybrid library, using portions of UNC-89 containing PK2, as baits. The screen resulted in 42 positive clones after re- transformation. These clones identified the same gene, B0379.4. Both isoforms, B0379.4a (38 kDa) and b (55 kDa), were represented. The only recognizable domain in B0379.4 is a serine protein phosphatase. When expressed in E. coli, B0379.4 has phosphatase activity towards p-nitrophenylphosphate in vitro. For the PK2 kinase domain, interaction with B0379.4 required both the autoinhibitory sequence lying C-terminal of the kinase domain, and the Ig and Fn3 domains lying N-terminal of the kinase domain. Moreover, when the analogous regions from the two other giant kinases of C. elegans, twitchin and TTN-1 (Ce titin), were tested, they failed to interact with B0379.4. Transgenic animals were generated carrying a plasmid in which 7.5 kb of sequence upstream of the 5'end of B0379.4 was fused to GFP. Expression was observed in the same set of muscles (pharyngeal, vulval, body wall) in which unc-89 is expressed. Rabbit antibodies were generated that recognize B0379.4 from worm extracts of wild type, and in truncated form from two knockout mutants. By immunofluorescence microscopy anti-B0379.4 localizes to the M-line and to a portion of the I-band. One hypothesis being tested is that PK2 phosphorylates B0379.4 to influence its phosphatase activity.

1577 unc-94 Encodes a Tropomodulin in C. elegans

T. Stevenson¹, K. Mercer,¹ C. Conley,¹ N. Szewczyk,² G. Benian;¹ Pathology, Emory University, Atlanta, GA, ²Aames Research Center, Moffett Field, CA

unc-94 is one of about 40 genes in C. elegans, that when mutant, show reduced motility and disorganization of myofilaments. The original allele, unc-94(x177) was recovered by Zengel and Epstein (1980) by a motility-requiring selection. We have recovered a new allele, n20, by an F1 non-complementation screen. Both alleles show reduced motility and brood size, and disorganization of muscle structure. By polarized light microscopy, there is alteration between normal and increased width of individual A-bands. The localization of a number of known sarcomeric proteins was examined in unc-94 mutants. Each of these proteins shows some degree of mis-localization, but the most dramatic effect is in the localization of F-actin, with some, abnormally many filaments appearing below the level of the cell cortex to a level between 500-600 nm below the cell surface. Among unc-94 mutants, include: (1) obtaining the Unc-94 polarized light phenotype using RNAi for C06A5.7, and (2) achieving transgenic
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rescue of the Unc-94 mutant phenotype by using cosmid C06A5. After creating transgenic animals that carry putative promoter sequences for tmd-1.a and mid-1.b fused to GFP, we determined that isoform-a is primarily expressed in body wall muscle, while isoform-b is found mostly in the pharyngeal and anal depressor muscles, and the spermatheca.

1578

Proper Localization of Paramyosin Within Thick Filaments Depends upon UNC-98 and UNC-96, Two C. elegans M-line Proteins
R. Miller, K. Mercer, H. Qudota, T. Stark, G. Benian; Pathology, Emory University, Atlanta, GA

Mutations in unc-96 or unc-98 cause reduced motility and a characteristic defect in muscle structure: M-lines and dense bodies are disrupted, and by polarized light microscopy birefringent needles are present. UNC-96 is a 48 kD protein with little similarity to known proteins (Mercer et al. 2006). UNC-98 is a 37 kD protein consisting of four C2H2 Zn finger domains (Mercer et al. 2003). Antibodies localize both UNC-96 and UNC-98 to M-lines in wild-type animals. Non-null mutations in unc-15, the structural gene for paramyosin, also show similar needle-like birefringent needles in mutant animals. Additionally, triple null unc-15+/+ unc-96+/+ and unc-15+/+ unc-98+/+ transheterozygotes, with only one wild-type copy of each gene, have more severe polarized light phenotypes than unc-96, unc-98, or unc-15 heterozygotes alone. ELISAs demonstrate that UNC-98 and UNC-96 interact with paramyosin in vitro. By yeast 2-hybrid, the N-terminus of UNC-96 and C-terminus of UNC-98 interact with a similar region of the coiled-coil rod of paramyosin. Additionally, genetic, cellular, and biochemical data indicate that UNC-98 and UNC-96 interact (Mercer et al. 2006). Our working hypothesis is that UNC-96 and UNC-98 work together to promote thick filament maintenance, either by promoting incorporation of paramyosin into thick filaments or by promoting degradation of paramyosin that has dissociated from thick filaments.

1579

New UNC-97 / PINCH Interactors Localized at Muscle Focal Adhesions in C. elegans
H. Qudota, K. Mercer, K. Kaibuchi, G. Benian; Pathology, Emory University, Atlanta, GA; Cell Pharmacology, Nagoya University, Nagoya, Japan

Myofilaments within C. elegans body wall muscle cells are organized and anchored to the muscle cell membrane by dense bodies and M-lines. These structures are both analogous and homologous to vertebrate focal adhesion plaques. To clarify the molecular mechanisms regulating these intergins-associated attachment structures, we are investigating protein-protein interactions between integrin-associated proteins using a systematic two hybrid approach. Previously, we found a protein cluster associated with integrin including PAT-4 (ILK), PAT-6 (actopaxin), UNC-97 (PINCH), UNC-112 (Mig-2). From a collection of yeast two hybrid constructs consisting of candidate proteins localized at dense bodies and M-lines, we found novel UNC-97/PINCH interactors: UNC-95, LIM-8 and LIM-9. All three proteins contain a LIM domain, which is essential for interaction with UNC-97/PINCH. UNC-97/PINCH is composed of 5 LIM domains and we found that the first LIM domain of UNC-97 is responsible for binding to UNC-95, LIM-8, and LIM-9. LIM-8 and LIM-9 can bind to UNC-96, a novel M-line protein (Mercer et al. 2006). These interactions were confirmed with in vitro binding assays using purified fusion proteins expressed in bacterial cells. UNC-97 and UNC-95 have been reported to be localized at M-lines by using GFP fusions. We investigated expression profiles of two novel gene products, LIM-8 and LIM-9, by promoter-GFP fusions and found that these two genes are expressed in body wall muscle cells. We also prepared antibodies against UNC-97, UNC-95, UNC-96, and UNC-98. We confirmed the localization of UNC-97 and UNC-95 at dense bodies and M-lines, but not in nuclei. With immunostaining of worm adult muscle, LIM-8 and LIM-9 are localized both at dense bodies and M-lines. Taking together the biochemical and cell biological data, we propose a model that the UNC-97/PINCH protein functions for the maintenance of cell-substratum adhesion through the cooperation of three LIM domain proteins.

1580

MAPping the Eukaryotic Tree of Life: The Evolution of the MAP215/Dis1 Family of Microtubule-associated Proteins
D. L. Gard; Biology, University of Utah, Salt Lake City, UT

The MAP215/Dis1 family of proteins is an evolutionarily ancient family of microtubule-associated proteins, with characterized members in all major kingdoms of eukaryotes. An extensive BLAST search of public databases (including both EST and genome sequences) identified partial or complete sequences predicted to encode more than 160 of the MAP215/Dis1 family members from diverse eukaryotic species, including 11 Protists, 3 Stemnophelidae, 32 algae and plants, 44 fungi (39 Ascomyces, 5 Basidiomycetes, and 1 Zygomyces), and more than 70 species in 7 phyla of animals. MAP215/Dis1 proteins in all plants, most animals (except nematodes), and fungi (except ascomyces) are characterized by the presence of four TGT domains, each ~240 amino acids in length and consisting of 1-5 HEAT repeats, suggesting that the gene duplication events from which they are derived occurred prior to the divergence of the major kingdoms of eukaryotes. The two TGT proteins of nematodes and ascomycete fungi are postulated to be derived from ancestral four TGT proteins through deletions and gene rearrangement. MAP215/Dis1 proteins in plants, most animals (except nematodes), at least one fungal (U. maydis), and several protists, also contain a single copy of a moderately conserved ~18 amino acid sequence similar (~45% identical) to the Tau repeat of vertebrate brains. The widespread occurrence of this Tau repeat in vertebrates suggests that it arose early in the evolution of the MAP215/Dis1 family.

1581

Evolution of Alternative Splicing in the MAP215/Dis1 Proteins of Vertebrates
S. K. Evans, E. D. Sanzenbacher, D. L. Gard; Biology, University of Utah, Salt Lake City, UT; Jordan Applied Technology Ctr, West Jordan, UT

We previously identified developmentally-regulated transcripts encoding two isoforms of MAP215 (MAP215M and MAP215S), a member of the MAP215/Dis1 family of microtubule-associated proteins. These isoforms differ in the presence (XMAP215M) or absence (XMAP215S) of a 36 amino acid insert (ins2) encoded by a single exon. XMAP215M transcripts are expressed during oogenesis and present in gastrula embryos and adults. BLAST searches revealed that genes encoding MAP215/Dis1 proteins of teleost fish and mammals lack sequences encoding ins2. However, a related sequence (55% identical; 77% similar) is encoded by the MAP215/Dis1 gene of chickens. We used nested primer pairs and sequential RT-PCR and PCR to clone and sequence cDNAs spanning the ins2 exon of MAP215/Dis1 transcripts from a variety of vertebrates. Transcripts encoding ins2 were identified in oocytes or eggs from several species of amphibians and reptiles, but were absent from somatic transcripts in those species. Nine phosphorylatable amino acids (S/T), including a predicted target for the cell cycle kinase CDK1, are conserved in ins2 sequences from four amphibian species obtained to date (X. laevis, X. tropicalis, R. pipiens, and Bufo sp.), suggesting that phosphorylation of ins2 plays an important role in regulating MAP215M activity during amphibian development. Six of the conserved S/T, including the CDK1 target, have been lost to deletion or mutation in ins2 of the garter snake (Thamnophis sp.). These results support the hypothesis that (1) the ins2 exon arose in a common ancestor of terrestrial vertebrates subsequent to their divergence from fish; (2) ins2 may play an important role in XM215 function in amphibians; and (3) that ins2 function has been lost, through divergence or deletion of ins2 sequences, from other terrestrial lineages. Supported by grant #MCB-0212000 from the N.S.F. and the University of Utah.
To identify putative targets and other components of the Aurora complex in plants, we have performed yeast two-hybrid screening using AtAurora1. Candidate Aurora interactors localize in a centrosome-dependent and centrosome-independent processes during microtubule spindle assembly in Xenopus. In sperm asters with fewer microtubules, Interestingly, targeting of the microtubule nucleator, γ-tubulin, to sperm centrosomes is not affected in these samples. Consistent with a centrosome-independent role for AtXEN1 in aster assembly, XEN1 depleted extracts assemble unoriented microtubules in response to RanGTP, suggesting a role for XEN1 in microtubule organization. Addition of recombinant XEN1-C-terminus to XEN1 egg extracts, or to microtubules assembled in vitro, causes the microtubules to become highly bundled. Recombinant XEN1-C-terminus can recruit γ-tubulin from egg extracts and is able to interact with microtubules directly in vitro. Based on these observations, we hypothesize that XEN1 functions in both centrosome-dependent and centrosome-independent processes during microtubule spindle assembly in Xenopus.

Transcripts Encoding Multiple TACC Proteins Are Expressed During Development of the African Frog Xenopus laevis
J. F. Plant, D. L. Gard, Biology, University of Utah, Salt Lake City, UT

Transforming acidic-coiled-coil (TACC) proteins are a conserved family of proteins that have been implicated in the localization and anchoring of the MAP215/Dis1 proteins to the centrosome. Characterized by a highly conserved TACC domain predicted to form an acidic coiled-coil, TACC proteins have been identified in a diverse range of animals, including both invertebrate and vertebrate species. The invertebrates D. melanogaster and C. elegans each express a single TACC protein. In contrast, mammals, including humans and mice, express three TACC proteins characterized by divergent TACC domains. Previous studies identified a single member of the TACC family (Maskin) in the African frog, Xenopus laevis. Sequence comparison suggests that Maskin is most closely related to TACC3 of mammals. A BLAST search of the incomplete genome of the related frog, X. tropicalis, identified three independent sequences encoding putative TACC proteins, one of which corresponds to the Maskin gene. A BLAST search of Xenopus laevis cDNAs identified partial sequences related to TACC1 and TACC2. Using primers designed from these sequences, we used RT-PCR and PCR-RACE to clone transcripts encoding two putative TACC1 and TACC2 proteins from X. laevis. RT-PCR and in-situ hybridization demonstrated distinct temporal and spatial patterns of expression of TACCs 1-3 during Xenopus development. Supported by grant #MCB-0212900 from the National Science Foundation and by the University of Utah.

Identification of Arctica, a Novel Kinetochore-binding Protein That Interacts with Aurora Kinase in Plants
S. Couture,1 D. Van Damme,1 D. Inze,2 D. Geeler3
1Department of Plant Systems Biology, Flanders Interuniversity Institute for Biotechnology (VIB), Ghent University, Ghent, Belgium
2Faculty of Bioscience Engineering, Department of Plant Production, Ghent University, Ghent, Belgium

Introduction: From budding yeast to human, Aurora serine/threonine kinases are essential for the successful execution of cell division. Aurora kinases form chromosomal passenger complexes (CPCs) that are responsible for the establishment of a bipolar mitotic spindle, accurate segregation of chromosomes and the completion of cytokinesis. Although Aurora has been well-studied in animal and yeast, plant Aurora kinases have only recently been identified (Demidov et al, 2005). The Aurora kinase family in Arabidopsis has 3 Aurora homologues. Structure and expression of AtAurora1 and AtAurora2 suggest that these genes arose by a recent gene duplication, whereas the diversification of plant alpha and beta Aurora kinases predates the origin of land plants. Intracellular localization of GFP-tagged AtAurora1 and AtAurora2 shows labelling of microtubule spindle and the growing cell plate of dividing tobacco BY-2 cells while GFP-AtAurora3 localizes to the kinetochores.

Objective and methods: To identify putative target partners and other components of the Aurora complex in plants, we have performed yeast two-hybrid screening using AtAurora1 as a bait. To validate the interaction data, 37 Aurora interactors were GFP-tagged and localized in dividing tobacco BY-2 cells. Results and conclusions: 7 putative interactors localize in a dynamic cell-cycle-specific manner. The candidate Arctica shows the classical double-dot kinetochore pattern and translocates to the growing cell plate during cytokinesis. This unknown Aurora-interactor has no homology with yeast or animal Aurora kinases, suggesting that plants have developed divergent Aurora kinase signalling pathways involved in chromosome segregation and cytokinesis. The interaction between Arctica and AtAurora1 is investigated using co-localization experiments with GFP and RFP and radioactive kinase assays. Knock-out, knock-down and overexpression studies will be performed to analyze the role of the new Aurora interactor in plant growth and development. This approach will enable us to provide insights into the role of the Arctica-Aurora complex in plants.

Cytoskeletal Components of an Invasion Machine: The Apical Complex and Conoid of Toxoplasma gondii
E. Nagayasu,1 F. Zhang,2 K. Hu,3 J. M. Murray1; 1Cell & Developmental Biology, University of Pennsylvania, Philadelphia, PA, 2Biology, Indiana University, Bloomington, IN

Toxoplasma gondii is an obligate intracellular human parasite in the phylum Apicomplexa, which includes many important human and veterinary pathogens. Central to both invasion and proliferation of Toxoplasma is the apical complex, a group of cytoskeletal and membrane-bound organelles. The complex includes the "conoid", an extraordinary array of 14 spirally arranged tubulin-containing fibers (not microtubules) that is actively motile during invasion (Hu et al., 2002). We are studying the structure and formation of the conoid, particularly the basis for assembly of the non-microtubular tubulin polymer. The primary sequence of the T. gondii tubulins seems unlikely to account for the novel polypeptide form, as these same tubulins assemble into canonical 13-pf microtubules elsewhere in the cell. Furthermore, human tubulin expressed in T. gondii is readily incorporated into the conoid. Candidate accessory proteins that might account for the unusual polymer structure were identified in a proteomics screen (Hu et al. 2005), and we have now characterized several. TgDCXP (55.08188) is one such protein. Its C-terminal region contains a DCX domain, which was originally found in human doublecortin, a neuronal microtubule-associated protein, which has been shown to interact with microtubules. In spindle positioning, the Kar9p and dynein pathways are required to accurately position the mitotic spindle. We are currently testing the hypothesis that dimerization of Bik1p is important for its binding to microtubules. In spindle positioning, the Kar9p and dynein pathways are required to accurately position the mitotic spindle. We are currently testing the hypothesis that dimerization of Bik1p is important for its binding to microtubules. The conoid is a highly dynamic structure that is comprised of a CAP-Gly head domain believed to bind microtubules, a serine rich region, a central coiled-coil, and a C-terminal cargo-binding domain. A structure-function analysis using both purified full length Bik1p and truncated proteins showed that in contrast to full length Bik1p, the CAP-Gly domain together with the serine rich region is not sufficient to bind microtubules. We are currently testing the hypothesis that dimerization of Bik1p is important for its binding to microtubules. In spindle positioning, the Kar9p and dynein pathways are required to accurately position the mitotic spindle. Bik1p has recently been shown to physically interact with proteins of both pathways. To begin to dissect its role in each of these pathways, a screen was carried out to obtain bik1 mutants lacking subsets of these interactions. One mutant was identified that interacted poorly with both Kar9p and Kip2p. Sequencing identified a mutation in bik1 that results in a premature termination at position 61 (Y61H). Phenotypic characterization of Y61H revealed that its cytoplasmic microtubules are short. Bik1p-Y61H tagged with GFP is localized as discrete punctae throughout the cell. Biochemical analyzes showed that the Bik1-Y61H protein was about 8 kD smaller than wild-type, suggesting that this mutation affects the stability of Bik1p. Further analyses revealed that the bipartite Y61H is intact, suggesting that it is clipped at its N-terminus, the region believed to bind microtubules. A possible candidate to carry out this clipping is Lap4p, a leucine aminopeptidase found in the cytoplasm and vacuole. Lap4p has been previously reported to interact with Bik1p by affinity capture methods. We are currently testing the novel hypothesis that limited proteolysis of wild-type Bik1p may regulate its function.

Cytoskeletal Components of an Invasion Machine: The Apical Complex and Conoid of Toxoplasma gondii
E. Nagayasu, F. Zhang, K. Hu, J. M. Murray1; 1Cell & Developmental Biology, University of Pennsylvania, Philadelphia, PA, 2Biology, Indiana University, Bloomington, IN

Mutational Analysis of Bik1p the Yeast Ortholog of CLIP-170
S. D’Souza,1 H. Hoops,2 H. V. Goodson,3 R. K. Miller2; 1Dept. of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, IN, 2Dept. of Biology, University of Rochester, Rochester, NY, 3Dept. of Biology, State University of New York-Geneseo, Geneseo, NY

Bik1p, the yeast homolog of the mammalian microtubule binding protein CLIP-170, plays an important role in spindle positioning by stabilizing cytoplasmic microtubules. Like CLIP-170, the structure of Bik1p is comprised of a CAP-Gly head domain believed to bind microtubules, a serine rich region, a central coiled-coil, and a C-terminal cargo-binding domain. A structure-function analysis using both purified full length Bik1p and truncated proteins showed that in contrast to full length Bik1p, the CAP-Gly domain together with the serine rich region is not sufficient to bind microtubules. We are currently testing the hypothesis that dimerization of Bik1p is important for its binding to microtubules. In spindle positioning, the Kar9p and dynein pathways are required to accurately position the mitotic spindle. Bik1p has recently been shown to physically interact with proteins of both pathways. To begin to dissect its role in each of these pathways, a screen was carried out to obtain bik1 mutants lacking subsets of these interactions. One mutant was identified that interacted poorly with both Kar9p and Kip2p. Sequencing identified a mutation in bik1 that results in a premature termination at position 61 (Y61H). Phenotypic characterization of Y61H revealed that its cytoplasmic microtubules are short. Bik1p-Y61H tagged with GFP is localized as discrete punctae throughout the cell. Biochemical analyzes showed that the Bik1-Y61H protein was about 8 kD smaller than wild-type, suggesting that this mutation affects the stability of Bik1p. Further analyses revealed that the bipartite Y61H is intact, suggesting that it is clipped at its N-terminus, the region believed to bind microtubules. A possible candidate to carry out this clipping is Lap4p, a leucine aminopeptidase found in the cytoplasm and vacuole. Lap4p has been previously reported to interact with Bik1p by affinity capture methods. We are currently testing the novel hypothesis that limited proteolysis of wild-type Bik1p may regulate its function.
A. Adelbola, J. Farel, D. Goryunov, R. Liem; Pathology, Columbia University, New York, NY

Plakins are a family of cytoskeletal proteins that interact with cytoskeletal microfilaments to each other and to cell junctional complexes. In epithelia, plakins form junctions between intermediate filaments and desmosomes, as well as hemidesmosomes. The epithelial plakins are generally composed of a so-called plakin domain that interacts with cell junctional complexes, a coiled-coil rod that allows for protein dimerization and a plakin repeat region that interacts with intermediate filaments. Bullous pemphigoid antigen 1 (BPAG1) and microtubule-actin crosslinking factor 1 (MACF1) are members of the plakin family that exhibit tissue-specific alternative splicing. Like the epithelial isoform, BPAG1 and MACF1 isoforms expressed predominantly in the brain and muscle also contain the plakin domain. However, they do not contain the coiled-coil rod domain and plakin repeat region, rather these isoforms are composed of N-terminal calponin-homology domains that can bind to actin filaments, an internal plakin repeat region (BPAG1 muscle isoform), 23 spectrin repeats, and a C-terminal microtubule binding domain (MTBD). Recent reports reveal that the MTBD of human BPAG1 interacts with the microtubule plus-end binding protein, EB1 (Slep et al., 2005). We report the alternative splicing of the MTBD of BPAG1 and MACF1. This alternative splicing uses different 3′ exons to yield four additional variants of the neural and muscle isoforms of BPAG1 and MACF1. We have examined the expression profile and microtubule binding properties of each newly identified isoform. We also determine if the alternative splicing alters BPAG1 and MACF1 interaction with the EB1 family members.

Bim1p also interact with proteins involved in the sumoylation pathway—

sumoylation regulates spindle positioning in yeast. Here, we show that Kar9p and Bim1p are likely to be modified by SUMO/Smt3p. Both interact with SMT3 by two-hybrid analysis. However, neither interact with a mutant form of SMT3 in which the terminal glycine required for conjugation has been mutated to alanine. Both Kar9p and Bim1p also interact with proteins involved in the sumoylation pathway—UBC9 an E2 enzyme required for SUMO conjugation; NIP1 an E3 enzyme conferring specificity for SUMO targets; and PSS1 a weak suppressor of SUMO. Because Kar9p and Bim1p physically interact with each other, the two simplest bridging models were tested by deleting either KAR9 or BIM1 from the two-hybrid reporter strain and testing for the reciprocal interaction. In both cases, Kar9p and Bim1p retained their interactions with the sumoylation machinery. The Kar9p-Ube9p interaction was confirmed in vivo by affinity chromatography. Experiments to characterize the cellular and molecular requirements for this interaction of Kar9p with the sumoylation system are in progress. Our data support the hypothesis that sumoylation regulates spindle positioning in yeast.

A central question in cell biology is how cells determine and maintain the size of their organelles. Mitotic chromosome segregation is orchestrated by dynamic interaction between spindle microtubules and the kinetochores. In a proteomics discovery of previously uncharacterized microtubule-associated proteins, we identified a unique human microtubule-associated protein named CAP140. CAP140 localizes to microtubules in interphase, associates with the mitotic spindle during mitosis, and redistributes to the central body during cytokinesis. Our biochemical characterization reveals that CAP140 binds to microtubules via its central coiled-coil domain. While endogenous CAP140 is located along cytoplasmic microtubules in interphase and on the spindle in mitosis, overexpression of CAP140 resulted in exclusive interphase microtubule bundles without affecting early mitotic spindle organization. However, overexpression of CAP140 central domain induces profound bundling of cytoplasmic microtubules in interphase cells and aberrant spindles in mitosis suggesting the integrity of CAP140 is essential for spindle stability. Indeed, depletion of CAP140 by RNA interference results in severe mitotic delays and defective cytokinesis. The mitotic abnormalities seen in CAP140-depleted cells include multiple spindle poles and stimulated spindle assembly. Truncation mutants demonstrate that the central region of CAP140 is important for localization to the mitotic spindle, and to the center of the midbody, whereas the C-terminal tails, with the high sequence homology to tau, bearing microtubule depolymerase activity. These results suggest a crucial role CAP140 in the organization of the bipolar mitotic spindle, and mitotic progression, and define CAP140 as a novel key factor for proper spindle assembly. Currently, we are studying the real-time chromosome movements in living cells depleted CAP140 and elucidating the phospho-regulation of CAP140 in mitosis.

One role of Kar9p in spindle positioning is to link the actin and microtubule cytoskeletons. The microtubule binding protein and EB1 homologue Bim1p links Kar9p to the microtubule. The type V myosin links Kar9p to the actin cytoskeleton. However, little is known about the molecular mechanisms that regulate Kar9p function. SUMO/Smt3p is a conserved small ubiquitin-related modifier that is covalently attached to other proteins as a post-translational modification. SUMO is analogous to ubiquitin in several ways, but functionally different. Unlike ubiquitin, sumoylation does not target proteins for degradation. Although targets of sumoylation are associated with a diverse set of biological processes, it was not previously known whether sumoylation regulates the microtubule-dependent process of spindle positioning in yeast. Here, we show that Kar9p and Bim1p are likely to be modified by SUMO/Smt3p. Both interact with SMT3 by two-hybrid analysis. However, neither interact with a mutant form of SMT3 in which the terminal glycine required for conjugation has been mutated to alanine. Both Kar9p and Bim1p also interact with proteins involved in the sumoylation pathway—UBC9 an E2 enzyme required for SUMO conjugation; NIP1 an E3 enzyme conferring specificity for SUMO targets; and PSS1 a weak suppressor of SUMO. Because Kar9p and Bim1p physically interact with each other, the two simplest bridging models were tested by deleting either KAR9 or BIM1 from the two-hybrid reporter strain and testing for the reciprocal interaction. In both cases, Kar9p and Bim1p retained their interactions with the sumoylation machinery. The Kar9p-Ube9p interaction was confirmed in vivo by affinity chromatography. Experiments to characterize the cellular and molecular requirements for this interaction of Kar9p with the sumoylation system are in progress. Our data support the hypothesis that sumoylation regulates spindle positioning in yeast.

E. S. Kannegaard, W. F. Marshall; Biochemistry, University of California San Francisco, San Francisco, CA

Cilia are known to be crucial for many developmental and physiological processes, yet cilia biogenesis and maintenance are not well understood. In particular, apart from intraflagellar transport proteins, molecular players in the process of ciliogenesis are largely unknown. Two recent studies utilizing the unicellular green alga Chlamydomonas reinhardtii, which has two flagella that are homologous to human cilia, have determined the proteome of the flagellum and identified genes that are upregulated upon deflagellation. By analyzing these datasets we have identified a set of genes that are upregulated during flagellar regeneration for which the gene products are not components of the flagellum itself, suggesting that there is a class of proteins related to those in the Chlamydomonas flagellum that are homologous to human cilia, have determined the proteome of the flagellum and identified genes that are upregulated upon deflagellation. By analyzing these datasets we have identified a transcriptional circuit that may help increase system stability during flagellar regeneration.

Dynamics, Feedback, and Bifurcations in an Organelle Size Control System

W. Marshall, W. Ludington, I. Zamora; Biochemistry, UCSF, San Francisco, CA

A fundamental question in cell biology is how cells determine the size of organelles. Flagella are a convenient system to address the question of organelle abundance since their size can be described by a single number—length. An interesting feature of flagellar length control is length equalization—when one flagellum is severed from a cell with two flagella, it will grow back, but as it does so, the other unperturbed flagellum will shorten until the two flagella reach the same length. This has been taken as evidence that the cell "knows" how long its flagella are, and "instructs" the long one to shorten. Based on studies of flagellar microtubules, we have proposed a simple model in which length is determined by the balance of turnover and intraflagellar transport. We will show that simulations based on this model can predict the length equalization of flagella. Numerical explorations with the model predict that as the level of intraflagellar transport machinery is increased, the system should undergo a supercritical Hopf bifurcation, leading to sustained limit cycle oscillations. To test this prediction, we searched a panel of mutants and identified one with increased intraflagellar transport, and as predicted the flagella no longer can attain a stable steady-state length but instead undergo continual oscillations. Our model can be viewed as an integral-only control system, and such systems are prone to instability due to the phenomenon of "integrator wind-up". Model control systems are never integral-only, and always employ additional control loops of a derivative or proportional nature to counteract the instability caused by the integrator. We have searched for such additional loops within the flagellar length control system and have identified a transcriptional circuit that may help increase system stability during flagellar regeneration.

K. A. Wenner, J. L. Feldman, W. F. Marshall; Biochemistry, UC San Francisco, San Francisco, CA

A central question in cell biology is how cells determine and maintain the size of their organelles. Chlamydomonas reinhardtii flagella provide an excellent model system for studying this problem, as Chlamydomonas is tightly and precisely controlled. It is known that the distal tips of flagella are unstable and undergoing continuous turnover, therefore the precise flagellar length must be maintained by balancing assembly and disassembly at the tip of each flagellum. Several models have been proposed to explain the mechanism responsible for this balance. We would like to investigate these models by examining flagellar length mutants using a combination of genetics and microscopy to test predictions derived from these models of length control.
1595 Regulation of the Flagellar Length Control Kinase LF4 by Phosphorylation

M. M. McLaren, B. Raber, N. F. Wilson; Anatomy & Cell Biology, Oklahoma State University Center for Health Sciences, Tulsa, OK

Regulation of flagellar length in Chlamydomonas oda7 (L1, L2, LF3, and LF4) have been identified that when mutated generate cells with abnormally long flagella. LF4 was recently shown to encode a MAP kinase. Immunoblot analysis revealed that in cell bodies, LF4p migrates as a doublet at 73/72 kDa whereas in flagella, LF4p migrates as a single band at 65 kDa. Both isoforms in cell bodies are recognized by an anti-phosphoTEY antibody that recognizes the activated form of MAP kinases. Interestingly, the flagellar form of LF4p was not recognized by the anti-active MAP kinase antibody. Consistent with the observation that the cell body isoforms of LF4p are enzymatically active, LF4p from cell bodies but not flagella was able to phosphorylate myelin basic protein in an in-gel kinase assay. These observations suggest that LF4p exists as an active kinase in cell bodies while the flagellar form is maintained in a largely inactive state. In vitro mixing experiments suggest that the flagellar form of LF4p is inactivated by the activity of one or more flagellar phosphatase(s). We have utilized the genome sequence of Chlamydomonas to identify phosphatases(s) that regulate the kinase activity of LF4p in flagella. We have identified 8 putative MAP Kinase Phosphatases (MKPs) in the genome of Chlamydomonas that could potentially regulate LF4p. We are using Northern analysis to determine which of these 8 MKPs are upregulated during flagellar regeneration. To date, 1 (MKP1) of the 4 phosphatases screened in such a manner is upregulated during flagellar regeneration. Currently, we are completing our screening of the remaining phosphatases by Northern analysis. In addition, we are generating RNAi constructs for knock-down of gene expression for MKP1, as well as substrate-trapping mutants for this phosphatase.

1596 Importance of Golgi for the Regulation of Flagellar Length in Chlamydomonas

W. Dettler; Molecular Biosciences, University of Kansas, Lawrence, KS

The importance of IFT for flagellar microtubule assembly and maintenance is established but the role of the membrane for flagellar growth or maintenance is not understood. Haller & Fabraz (1998, BBRC 24:597) reported that brefeldin A (BFA), which disrupts Golgi, arrested flagellar growth in Gonium, suggesting that membranes may partly regulate flagellar growth. To study the role of membranes in flagellar maintenance, Chlamydomonas fla10 and pf18 cells were treated with BFA to answer the following questions: (a) Does BFA block flagellar growth or induce flagellar shortening? Cells were delflagellated by pH shock and measured during regeneration. On cells treated with 0-4.0 µg/ml BFA, flagella grew to 70-80% the length of control flagella but then shortened to 51% of control lengths within 120 min; greater inhibition was observed with 10-15 µg/ml BFA. After BFA removal, flagella rapidly grew to control lengths. On nonflagellated cells, BFA induced flagella to shorten to 50% of control length within 3 hrs and to 10% of control length with longer treatment. This occurred on fla10 cells and on pf3B23 cells, which shorten during the day. (b) Are new processes (delivered from Golgi) required to maintain flagellar length? Flagella slightly shortened on cells treated with 30 µg/ml cycloheximide for up to 6 hrs, but shortening was significantly less than that produced by BFA. (c) Does BFA affect IFT? Examination of pf18 flagella with DIC (Dettler, 2005; ICB 170:649) revealed normal IFT. (d) Does BFA affect Chlamydomonas Golgi? By TEM, Golgi were present in control cells but absent in cells treated with 10µg/ml BFA. IFT particles were present in both control and BFA-treated flagella. Taken together, these data indicate that flagellar length depends, in part, on the regulated delivery of membrane to flagella via the Golgi that may be independent of the IFT-mediated delivery of newly synthesized flagellar proteins.

1597 The Ciliary Transcriptome

W. Marshall; L. Zamora; Biochemistry, UCSF, San Francisco, CA

The assembly of a cilium requires production of hundreds of proteins. We have analyzed the transcriptional program of ciliogenesis, both as a way to identify genes involved in ciliary assembly or function, and also in order to explore the role of transcriptional regulatory circuits in coordinating the assembly process. The system we employ is flagellar regeneration in Chlamydomonas - upon flagellar severing induced by pH shock, hundreds of genes become transcriptionally upregulated, including virtually all known flagellar proteins. How does the nucleus know to turn these genes on? We have investigated the cues that drive activation of the ciliogenesis program, and will present evidence that transcriptional activation is NOT triggered by the pH shock itself, nor by the loss of the flagellum, but rather by the active process of flagellar assembly. This suggests a feedback system for the assembly machinery into the nucleus. By analyzing flagellar gene transcription in a collection of pre-existing flagellar assembly mutants, we have found a mutant that regenerates flagella but no longer activates flagellar genes during regeneration. This gene, which encodes a component of the intraflagellar transport machinery, may therefore act to couple the two processes of assembly and transcription. In terms of ciliary gene-discovery, a major drawback of the transcriptional profiling approach has been the possibility that some of the genes identified may simply represent a stress response to the pH shock or severing. By using mutants which block transcription of known flagellar genes following delflagellation, we have developed a strategy to classify transcriptome elements as bona fide ciliogenesis genes versus generalized stress response genes.

1598 Ca2+-dependent Interaction of the Heavy Chain-associated LC4 Light Chain and Intermediate Chain 1 within Chlamydomonas Outer Arm Dynein

M. Sakato, S. M. King; MMSB, University of Kansas Health Center, Lawrence, KS

Chlamydomonas flagella exhibit waveform conversion upon an increase in intraflagellar [Ca2+] from pCa 6 to 4. Mutational studies suggest that the outer row of axonemal dynein arms is involved in this Ca2+-mediated regulatory pathway. Outer arm dynein consists of three heavy chains (α, β, and γ), two intermediate chains, multiple light chains and the docking complex. We have previously shown that α- LC4 directly activates ATP-sensitive microtubule binding by a dynein subparticle containing the β and γ heavy chains. The γ heavy chain-associated LC4 light chain is a member of the calmodulin family and binds 1-2 Ca2+ with Kd ~ 3 x 10^{-5} M in vitro, suggesting it may act as a Ca2+-sensor for outer arm dynein. We further investigated interactions between L4C and the γ heavy chain in vivo. Two I2Q consensus motifs for binding calmodulin-like protein are located within the stem domain of the γ heavy chain. In vitro experiments indicate that LC4 is permanently tethered C-terminal of this region, and that it undergoes a Ca2+-dependent interaction with the I2Q motifs. In addition, chemical crosslinking revealed that LC4 moves into close proximity of the intermediate chain IC1 in the presence of Ca2+. Interestingly, the sedimentation profiles of the γ heavy chain subunit changes subtly following Ca2+ addition suggesting that the entire complex alters conformation in response to Ca2+. We propose that a Ca2+-dependent conformational change within LC4 has a direct effect on the stem domain of the γ heavy chain and eventually leads to alterations in mechanical interactions between microtubules and the motor domain(s) of the outer arm dynein.

1599 The Chlamydomonas Oda7 Protein Links Outer Row and Inner Row Dyneins

J. Freshour, R. Yokoyama, D. R. Mitchell; Cell and Developmental Biology, SUNY Upstate Medical University, Syracuse, NY

Most Chlamydomonas mutations that disrupt the assembly of outer row dyneins encode either dynein motor complex subunits, or subunits of doublet microtubule attachment sites for dynein motors. We identified the gene products of two outer row dynein assembly loci on LG1, ODA47 and ODA48, through positional cloning and characterization of mutations that disrupt each gene. Both loci were cloned by walking from near-by molecular markers; their identities were confirmed by phenotypic rescue with genomic clones and by comparing wild type and mutant copies of each gene. The oda7 strain contains a deletion of exon 1 of gene C_410113 (GI Chlamydomonas genome database V2.0); the oda6 strain contains a frame shift in the first coding exon of gene C_1330506. Both genes encode leucine-rich repeat (LRR) proteins in the SDS22 superfamily, which includes outer row dynein light chain 1. ClustalW analysis suggests that most flagellated organisms contain single homologs of LC1, Oda7 and Oda6. Among flagellates, four genes a 60 kD protein on western blots of both whole cells and flagella. Oda7 is present in flagella of all single mutants examined except oda7, thus we could not genetically identify an axonemal structure essential for its assembly. After extracted by 0.6 M NaCl from axonemes of wild type or oda7 mutants such as p28, Oda7 preferentially co-sediments on sucrose gradients with Inner row dynein. In extracts from 11 mutants it co-sediments with outer row dynein. Sedimentation rate shifts to the top of the gradient in extracts of mutants that lack both outer row and Inner row dynein. We hypothesize that Oda7 resides in a doublet-associated complex that interacts with both outer row and Inner row dyneins. Supported by a grant from the National Institutes of Health to DRM.

1600 Regulation of Flagellar Inner Arm Dynein 11: The β Heavy Chain Motor Domain Is Necessary for Control of Microtubule Sliding by Phosphorylation

L. A. Fox, D. Tritschler, M. E. Porter, W. S. Sale; Cell Biology, Emory University School of Medicine, Atlanta, GA, Genetics, Cell and Development, University of Minnesota, Minneapolis, MN
The inner arm dynein I1, also known as the f-dynein, is a two-headed axonemal dynein required for regulation of microtubule sliding and control of flagellar bending. The mechanism of regulation involves the radial spokes, axonemal kinetics (PKA; CK1) and control of phosphorylation of the intermediate chain IC138 (Hendrickson et al., 2004). To test the role of the two different dynein heavy chain (DHC) motor domains in regulation, we measured microtubule sliding in axonemes from pf17 mutant cells that also lack either the 1α or 1β motor domains of I1 (Myster et al., 1999; Perrone et al., 2000 and see Toba et al., these Proceedings). The motor domain mutants were generated by crossing pf17 to transformants expressing the N-terminal region of the appropriate DHC in a null mutant background. These N-terminal DHC constructs selectively delete the motor domains but permit the assembly of the remainder of the I1 dynein complex. Genotypes of paralyzed “triple” mutants were confirmed by PCR and Western blotting. In 1 mM MgATP, microtubule sliding velocity was greatly reduced in axonemes from both triple mutants (~90 µm/sec - similar to microtubule sliding in pf17) compared to wild type (~18.0 µm/sec). Addition of the kinase inhibitors PKI or DRB rescued microtubule sliding in pf17 axonemes. Similarly, the kinase inhibitors rescued sliding in pf17 axonemes lacking the 1α DHC motor domain. However, the kinase inhibitors had no effect on microtubule sliding in pf17 axonemes lacking the 1β DHC motor domain. Based on these, and other data indicating I1 and IC138 assembly is required for regulation (e.g. Yang and Sale, 2006; Bower et al., these Proceedings), we conclude that the I1 DHC, but possibly not the 1α DHC, motor domain is necessary for regulation of microtubule sliding by the IC138 phosphorylation pathway.

1602 The Role of the IC97 in I1-Dynein Assembly and Axonemal Anchoring
Cell Biology, Emory University School of Medicine, Atlanta, GA; Genetics, Cell Biology & Development, University of Minnesota, Minneapolis, MN; Molecular, Cellular, and Developmental Biology, University of Colorado, Boulder, CO; Cell Biology, Emory University, Atlanta, GA

Our goal is to understand how the BOP5 gene product, the IC138 phosphophotoprotein, regulates the activity of the I1 inner arm dynein (Hendrickson et al., 2004). Here we focus on an unusual motility mutant, 6F5, obtained by insertional mutagenesis. We previously found that ~90% of the IC138 gene is deleted in 6F5, and that the motility defect co-segregates with the loss of IC138 (Bower et al., ASCB 2005). Contrary to expectation, we also found that most I1 subunits are present in 6F5 axonemes, but both IC138 and IC97 are missing (see also Wirschel et al., these proceedings). Fractionation of dynein extracts by sucrose density gradient centrifugation revealed that the remaining I1 subunits dissociate into two distinct peaks. Thus IC138 and IC97 appear to stabilize the I1 dynein, but they are not required for its assembly in the axoneme. Analysis of 6F5 axonemes by electron microscopy and computer image averaging revealed a defect at the base of the I1 dynein, in close proximity to radial spoke I1. Microtubule sliding velocities are also reduced. Transformation with wild-type IC138 restores assembly of both IC138 and IC97 and increases sliding velocities to wild-type levels. These observations suggest that the IC138 is required for activation of the I1 dynein motor domains by the radial spokes. To test this hypothesis, we have now analyzed sliding velocities in a double mutant lacking both the radial spoke head and IC138. As expected, treatment with protein kinase inhibitors increased sliding velocities in pf17 axonemes, but not in pf17 6F5 double mutants. Together with similar observations on I1 motor domain mutants (Fox et al., these proceedings), our results indicate that IC138 (and IC97) play a key role in mediating signals between the radial spokes and the I1 dynein motor domains (supported by NIH).

1603 Characterization of the Protein Kinase CK1 That Regulates Dynein-driven Microtubule Sliding and Is Anchored in Chlamydomonas Flagellar Axonemes
A. Gokhale, M. Wirschel, W. S. Sale; Cell Biology, Emory University, Atlanta, GA

Biochemical, functional and pharmacological evidence reveal that caspase kinase 1 (CK1), is anchored in the flagellar axoneme and regulates microtubule sliding. The mechanism of regulation appears to require inner arm dynein I1 and involves control of phosphorylation of IC138 (Yang and Sale, 2000, Hendrickson et al., 2004). To test the hypothesis that CK1 is anchored to the outer doublet, near I1 dynein, we have cloned and characterized CK1 identified in the flagellar proteome (Pazour et al., 2005). MS/MS analysis of axonemal fractions revealed multiple unique peptide corresponding to one gene located on scaffold 20 (GI version 3). The same gene model was identified in a eyespot proteome, indicating that CK1 is targeted to multiple cell functions (Schmidt et.al, 2006). Analysis of mapped BAC clones containing the CK1 gene indicates that it maps to linkage group XII/XIII. The predicted 38 kDa protein is highly conserved in the N terminal and catalytic domains. Polyclonal antibodies were made to a unique 34 amino acid sequence at the C-terminus. Western blots show a single band in axonemes that migrates at about 36 kDa. The protein is protease resistant and associated with axonemal structure. In summary, we have characterized a putative CK1 in axonemes that is conserved in both cell and tissue specific functions. Our current objective is to test the hypothesis that the axoneme contains a CK1 specific binding protein. EDC treatment of axonemes results in a robust CK1 cross-linked product of ~90 kDa. We are in process of identifying this protein ~60 kDa axonemal protein.

1604 IDA6 Encodes a Conserved Coiled Coil Polypeptide Associated with the Dynein Regulatory Complex
D. Tritschler, E. O'Toole, C. Perrone, M. E. Porter; Genetics, Cell Biology & Development, University of Minnesota, Minneapolis, MN; Molecular, Cellular, and Developmental Biology, University of Colorado, Boulder, CO

The dynein regulatory complex (DRC) is a group of tightly bound axonemal polypeptides thought to mediate signals between the radial spokes, nexin linkages, and dynein motor domains (Mastronarde et al., 1992; Gardner et al., 1994; Piperno et al., 1994; Nicastro et al., 2006). Several motor mutants lacking DRC components are also associated with defects in the assembly of a crescent shaped structure in the DRC and an associated dynein head domain. To identify the IDA6 gene product, we screened the flagellar and basal body proteomes (Li et al., 2004, Pazour et al., 2005) for candidate genes that are linked to the dynein regulatory complex. Genotypes of paralyzed “triple” mutants were confirmed by PCR and Western blotting. In 1 mM MgATP, microtubule sliding velocity was greatly reduced in axonemes from both triple mutants (~90 µm/sec - similar to microtubule sliding in pf17) compared to wild type (~18.0 µm/sec). Addition of the kinase inhibitors PKI or DRB rescued microtubule sliding in pf17 axonemes. Similarly, the kinase inhibitors rescued sliding in pf17 axonemes lacking the 1α DHC motor domain. However, the kinase inhibitors had no effect on microtubule sliding in pf17 axonemes lacking the 1β DHC motor domain. Based on these, and other data indicating I1 and IC138 assembly is required for regulation (e.g. Yang and Sale, 2006; Bower et al., these Proceedings), we conclude that the I1 DHC, but possibly not the 1α DHC, motor domain is necessary for regulation of microtubule sliding by the IC138 phosphorylation pathway.

1605 Identification of a DF2/P22/GASS Required for Assembly of the Dynein Regulatory Complex
R. Bower, D. Tritschler, J. Mueller, K. Vander Waal, C. Perrone, M. E. Porter; Genetics, Cell Biology & Development, University of Minnesota, Minneapolis, MN

The Chlamydomonas pf2 gene encodes a highly conserved coiled coil protein that is the orthologue of the growth arrest specific gene GAS8 (Rupp and Porter, 2003). Closely related sequences are found in other organisms that assemble motile cilia, consistent with the hypothesis that PF2 is a conserved component of the dynein regulatory complex (DRC). The DRC is a group of tightly bound axonemal polypeptides thought to mediate signals between the radial spokes, nexin linkages, and dynein arms (Mastronarde et al., 1992; Gardner et al., 1994; Piperno et al., 1994; Nicastro et al., 2006). To further characterize the DRC in Chlamydomonas, we generated a series of antibodies against PF2/GASS. Western blot analysis of DRC mutant axonemes

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confirmed that the antibodies are specific for PF2, but also showed that PF2 is modified in sup-pf-3 axonesmes. Cloning and sequence analysis subsequently showed that the sup-pf-3 mutation was the result of transposon insertion into the PF2 locus. RT-PCR revealed the presence of cryptic splice sites, and the resulting transcripts contain in-frame deletions within the PF2 coding sequence. The truncated PF2 subunits still assemble into the axoneme, but other DRC subunits are missing (see Huang et al., 1982). Transformation of pf2 and sup-pf-3 with epitope-tagged PF2 rescues the motility defects. High salt extraction and sucrose gradient centrifugation demonstrated that PF2 is part of a large complex in wild-type and rescued strains, but sediments as a smaller complex in sup-pf-3 extracts. These results suggest that the missing PF2 domains in sup-pf-3 are critical for the assembly of the DRC. Dikarya rescue experiments with a PF2-GFP strain also indicated that the time course of DRC assembly is much slower than might be predicted simply on the basis of intrflagellar transport. (Supported by NIH).

1606 Microtubule Binding and Assembly of a Calmodulin and Radial Spoke Associated Complex (CRC) E. E. Dynek, F. Smith; Biological Sciences, Dartmouth College, Hanover, NH
As a first step towards defining the role of calmodulin in modulating ciliary motility, we used an immunoprecipitation approach to identify calmodulin interactors in the axoneme. In an accompanying abstract (authors Dynek and Smith) we present data supporting the discovery of a calmodulin associated complex that includes three polypeptides, one of which (CaM-IP2) is an AKAP binding protein. This complex is associated with the radial spoke protein RSP3 (an AKAP) locus. The band at the AKAP localization in the CRC complex, which lacks RSP3, is confirmed that the antibodies are specific for PF2, but also showed that PF2 is modified in sup-pf-3 axonesmes. Cloning and sequence analysis subsequently showed that the sup-pf-3 mutation was the result of transposon insertion into the PF2 locus. RT-PCR revealed the presence of cryptic splice sites, and the resulting transcripts contain in-frame deletions within the PF2 coding sequence. The truncated PF2 subunits still assemble into the axoneme, but other DRC subunits are missing (see Huang et al., 1982). Transformation of pf2 and sup-pf-3 with epitope-tagged PF2 rescues the motility defects. High salt extraction and sucrose gradient centrifugation demonstrated that PF2 is part of a large complex in wild-type and rescued strains, but sediments as a smaller complex in sup-pf-3 extracts. These results suggest that the missing PF2 domains in sup-pf-3 are critical for the assembly of the DRC. Dikarya rescue experiments with a PF2-GFP strain also indicated that the time course of DRC assembly is much slower than might be predicted simply on the basis of intrflagellar transport. (Supported by NIH).

1607 Reduced Inter-doublet Sliding and the Response to Calcium in Mouse Sperm Lacking the Central Apparatus Protein, SPA16L K. A. Leech, Z. Zhang, J. F. Strauss, C. B. Lindemann; Biological Sciences, Oakland University, Rochester, MI. "Obstetrics and Gynecology, Virginia Commonwealth University, Richmond, VA
A targeted disruption of the PF20 gene in mice affected spermatogenesis and resulted in sperm cells with reduced flagellar motility (Zhang et al., 2002, Mol.Cell.Biol. 22: 7993-8004). The same study determined that the defective protein associated with this mutation, spermatogenic-antigen 16 (SPA16L), is localized to the central apparatus of the flagellar axosome. To test the potential role of the SPA16L protein in sperm motility we compared the primary motor function of sperm from mice carrying this defect to sperm from age and strain-matched controls. Axonemal sliding disintegration was assayed by a method that utilized a 0.5% Triton X-100 / 2 mM DTT extraction buffer (pH 9.4) to remove the mitochondrial sheath. Sliding disintegration was initiated with 1mM ATP and the number of disintegrating cells vs. non-responders was counted and the pattern of disintegration recorded. Fewer SPA16L sperm cells showed disintegration than control cells (27% vs. 52%, p<0.01), and of the responding cells significantly fewer cells from the SPA16L strain extruded doublets from both sides of the axoneme (6% vs. 14%, p<0.05). We evaluated the ability of demembranated sperm to respond to Ca^2+ which is evident by a change in direction of flagellar curvature following exposure. Significantly less of the sperm from the mutant mice responded to Ca^2+ (37%) compared to the control sperm (74%), p=0.006. Furthermore, control sperm responded to 1mM Ca^2+ by acquiring a curlicue configuration (1.9 x 10^9 radians/meter) while the SPA16L sperm cells showed a significantly reduced curvature development in the midpiece region (2.5 x 10^9 radians/m; p<0.001). This suggests a reduced ability of sperm cells from SPA16L mice to develop bending torque. Taken together, our results implicate a central role for the SPA16L protein in primary motor function of sperm flagella. Supported by N.S.F. grant #MCB-0516181 and N.I.H. grant # HD37416.

1608 Lack of Cilia Is Permissive for Knock-down of the Microtubule-severing Protein Katanin M. Q. Rasi, L. M. Quarmby; Molecular Biology and Biochemistry, Simon Fraser University, Burnaby, BC, Canada
Katanin is a member of the AAA family of proteins. We have previously cloned and implicated Chlamydomonas katanin (ep60) in calcium-activated microtubule severing, a process which leads to the excision of flagella from the cell body (Lohret et al., 1999, Cell Mol. Cytoskeleton, 45: 221). Evidence from other model systems indicates that katanin plays a role in cell cycle regulation, but the precise role has been difficult to define. In this study we investigate the cell cycle status of katanin in Chlamydomonas reinhardtii. Repeated failure to isolate katanin mutants lead us to speculate an essential role for this protein. Consequently, we used RNA interference to knock down katanin. We screened over ~1500 stable katanin RNAi transformants for independent lineage-specific expansions of Nek family members. In order to identify subfamilies with potential ciliary functions, we are expressing representative eGFP-tagged cDNAs in ancestral Neks in both of the major eukaryotic lineages (unikonts and heterokonts), as well as in the last common ancestor of all eukaryotes. We find that various organisms have undergone phylogenetic analysis of this gene family. Neks are divergent enough to have resisted evolutionary analysis by traditional methods. We have used Bayesian inference to construct the gene family trees. The number of NIMA kinase genes in organisms is correlated with the presence of cilia on cells that divide (Quarmby & Mahjoub 2005. J. Cell Sci. 15:5161). Based on this correlation, and our

1609 Defining the Ciliary Targeting Domain of Chlamydomonas NIMA-related Kinase 2 (Cnk2p) B. Bradley, M. White, L. Quarmby; Simon Fraser University, Burnaby, BC, Canada
Cnk2p is an ankyrin repeat protein that plays a role in regulating flagellar length and cell cycle progression in Chlamydomonas (Bradley and Quarmby 2005. J. Cell Sci. 118:3317). In mouse IMCD-3 (inner medulla collecting duct) cells, transiently transfected eGFP-Cnk2p localizes to primary cilium and centrosomes suggesting that a mammalian Nek may perform similar functions. Because the ciliary localization of Cnk2p is conserved we were able to use mammalian cells to express truncation mutants of Cnk2p. We have determined that the ciliary targeting domain of Cnk2p contains a motif (LLQ/NTIK/RXP LPXXY) conserved in a ciliary subfamily of Tetrahymena NRK members but not in mammalian Neks (Wicigo et al. 2006. Mol. Biol. Cell 17:2799). The localization of eGFP-K40A-Cnk2p, carrying a mutation in the invariant lysine of the kinase domain, is the same as eGFP-Cnk2p indicating that kinase-activity is not required for localization. Like Cnk2p, eGFP-Cnk2p appears to be regulated via phosphorylation. Cnk2p migrates as a doublet on Western blots of sucrose density gradient fractions of KCl extracts from wildtype Chlamydomonas axonesmes. We propose that the second band represents a phosphorylated Cnk2p because we also observe differences in the electrophoretic mobility of 6xHis-tagged Cnk2p compared with kinase-dead Cnk2p expressed in E. coli. This observation also suggests that Cnk2p is capable of autophosphorylation. This work is supported by an NSERC operating grant to LMJ. BAB is supported by graduate fellowships from MSHFR and NSERC.

1610 Evolutionary Analysis of the NIMA-related Kinase Family B. Bradley, J. Parker, L. Quarmby; Simon Fraser University, Burnaby, BC, Canada
The number of NIMA kinase genes in organisms is correlated with the presence of cilia on cells that divide (Quarmby & Mahjoub 2005. J. Cell Sci. 15:5161). Based on this correlation, and our studies in Chlamydomonas which revealed that at least two different Neks in this organism serve both ciliary and cell cycle functions (Parker & Quarmby, 2005. J. Cell Biol. 169:707) we have hypothesized that the ancestral roles of this kinase family relate to the alternating use of centrioles as spindle poles and as basal bodies. In order to test this idea, we have undertaken a phylogenetic analysis of this gene family. Neks are divergent enough to have resisted evolutionary analysis by traditional methods. We have used Bayesian inference to construct the gene family tree of Nek kinase domains, using sequences from the genome projects of diverse eukaryotic organisms. We infer the number of ancestral Neks in both of the major eukaryotic lineages (unikonts and heterokonts), as well as in the last common ancestor of all eukaryotes. We find that various organisms have undergone independent lineage-specific expansions of Nek family members. In order to identify subfamilies with potential ciliary functions, we are expressing representative eGFP-tagged cDNAs in ciliated mouse kidney cells. We have identified several proteins with ciliary or centrosomal association. We anticipate that this work will contribute to our understanding of ciliopathies such as...
the cystic kidney diseases. For example, mutations in two mammalian Neks (Nek1 and Nek8) have been shown to be causal in the murine models of proliferative cystic kidney diseases and jck, respectively. The cellular roles of these Nek remain enigmatic. This project is funded by an NSERC Discovery Grant to LMQ. BAB is supported by graduate fellowships from MSFHR and NSERC.

1611 Architectural Analysis of the Chlamydomonas Intraflagellar Transport Complex B

B. F. Luckner,1 M. S. Miller,2 H. Qin,2 P. Blackman,3 M. Ferrell,1 J. Rosenbaum,2 D. G. Cole,1 1Microbiology, Molecular Biology and Biochemistry, University of Idaho, Moscow, ID, 2Biology, Texas A&M University, College Station, TX, 3Cellular, Developmental, and Behavioral Biology, Yale University, New Haven, CT

Intraflagellar transport (IFT) is a strongly conserved process required for the assembly and function of eukaryotic cilia and flagella. During IFT, large protein particles carry axonemal precursors to the distal tip of the organelle while axonomal turnover products are ferried back to the cell body. Excluding the IFT motors, IFT particles consist of multiple copies of two separate complexes, A and B. Here we report on our ongoing characterization of complex B architecture. Previously, we have found that several B subunits dissociate from the Chlamydomonas complex in moderate ionic strength (300 mM NaCl) revealing a salt-stable core containing IFT88, IFT81, IFT74, IFT72, IFT52 and IFT27. Using a combination of biochemical and yeast-two- and three-hybrid analyses we have found IFT81, IFT74, and IFT72 appear to form a higher order oligomer within the complex B core at a ratio of 2:1:1, respectively (Luckner et al., J. Biol. Chem. 280:27688). Here we report that yeast two-hybrid analysis indicates that IFT46 and IFT52 are able to interact, while chemical cross-linking reveals that IFT52 and IFT88 are neighboring subunits. Bacterial co-expression studies further support this finding by revealing that IFT46 can interact separately with either IFT52 or IFT88 and that all three subunits are capable of forming a ternary complex. Deletion analysis reveals that the C-termini of both IFT46 and IFT72 are important in mediating the IFT46-52 interaction. Lastly, in an effort to verify that the reconstituted IFT46 retains biologic activity, we electrofugally fluorescently labeled IFT46 into an ikt64 mutant. Within 8 h, the flagellar assembly phenotype was rescued in more than 50% of the cells. Furthermore, the labeled IFT46 was imaged in live cells and, as expected for IFT proteins, was found to localize near the basal bodies and within the flagella. Supported by GM16920 (DGC), P20RR016454 (DGC) and GM14642 (JL).
shifting to the restrictive temperature, the amount of PKD2 in flagella increased, suggesting that IPT is involved in the turnover of flagellar PKD2. Flagellar PKD2--GFP fusion protein was concentrated in puncta that may represent clustered channels. When visualized by time-lapse imaging, most of the puncta did not move along the flagellum; however, smaller PKD2--GFP particles did move bidirectionally along the flagellum. Several PKD2 RNA interference strains show defects in mating, suggesting that this cation channel is involved in coupling flagellar adhesion to the increase in flagellar calcium concentration required for gamete activation. Supported by NIH grants GM14642 to JLR and GM30626 to GBW.

1616 Guanine Nucleotide Exchange Factor Vav2 Couples VEGF Signaling to Rac1 in Human Endothelial Cells

T. A. Garrett, J. D. van Baal, K. Burridge; Cell and Developmental Biology, University of North Carolina at Chapel Hill, Chapel Hill, NC

Vascular endothelial growth factor (VEGF) signaling is critical for both normal and disease-associated vascular development. Dysregulated VEGF signaling has been implicated in ischemic stroke, tumor angiogenesis, and a host of other vascular diseases. VEGF signals through several intracellular effectors, including the Rac family of small GTPases. Rac1, a member of this family, promotes endothelial cell migration by stimulating lamellipodia and membrane ruffles. To form these membrane protrusions, Rac1 is activated by guanine nucleotide exchange factors (GEFs) that catalyze the exchange of GDP for GTP. Although VEGF has been shown to activate Rac1, the GEFs that promote activation have not been identified. Using a Rac1-nucleotide-free mutant (G15A Rac1), a mutant that has a high affinity for binding activated GEFs, we show that G15A Rac1 associates with Rac1 GEF Vav2 after VEGF stimulation. Conversely, G15A Rac1 fails to stably associate with Vav2 when VEGF receptor-2 (VEGFR-2) signaling is inhibited. We also show that depleting endothelial cells of endogenous Vav2 using siRNA prevents VEGF-induced Rac1 activation, suggesting Vav2 couples VEGF signaling to Rac1. Additionally, Vav2 is tyrosine phosphorylated upon VEGF stimulation. Tyrosine phosphorylation of Vav2 temporally correlates with Rac1 activation and requires VEGF-2 signaling and Src kinase activity. Finally, we illustrate that expression of Vav2 siRNA impairs endothelial cell chemotaxis towards VEGF and wound healing. Taken together, our results provide evidence that Vav2 acts downstream of VEGF to activate Rac1 and is important for endothelial cell migration that precedes blood vessel formation. Supported by an American Heart Association Predoctoral Fellowship (TAG) and NIH grant HL45100 (KB)

1617 Transition State for Actin Filament Elongation by Ena/VASP

F. Ferron,1 Y. Li,1 G. Rebowski,1 D. Chereau,1,2 R. Dominguez1; 1Department of Physiology, University of Pennsylvania, School of Medicine, Philadelphia, PA, 2Elan Pharmaceuticals, Inc, San Francisco, CA

The dynamics of the actin cytoskeleton and its regulation by a myriad of actin-binding proteins play a critical role in eukaryotic cell motility. The Ena/VASP family of actin-binding proteins has been implicated in actin-based motility during fibroblast migration, axon guidance, and T cell polarization. Members of this family share a common architecture, consisting of an N-terminal EVH1 domain, a central Pro-rich region and a C-terminal EVH2 domain. The EVH2 contains G- and F-actin-binding sites, known respectively as the GAB and FAB domains, and a tetramerization coiled-coil region. Ena/VASP is enriched at the tips of filopodia, where it is a critical component of the so-called elongation complex. VASP associates with the barbed ends of growing filaments, competing with capping proteins. VASP binds profilin via the central Pro-rich region, and this interaction plays a role in filament elongation. Despite intensive investigation, the molecular bases of actin-filament elongation by members of the Ena/VASP family remain a mystery. Based on a number of crystal structures and a throughout biochemical characterization of the many protein-protein interactions involved in this process, we propose a model for actin filament elongation by Ena/VASP. This model explains how the poly-pro-GAB module of Ena/VASP contributes to filament elongation by "processing" profilin-actin complexes for their incorporation onto the barbed end of growing actin filaments.

1618 The Involvement of Shp-2 in Rho/ROCK-mediated Cell Detachment

H. Lee, Z. Chang; Institute of Biochemistry and Molecular Biology, College of Medicine, National Taiwan University, Taipei, Taiwan

Rho protein-mediated cell interplay between cell adhesion and de-adhesion plays an important role in normal hematopoiesis. Dysregulation of cell migration behavior has been shown to be closely associated with hematopoietic disease. In this study we focused on unraveling the mechanism by which myeloid leukemia cells detach from the fibronectin (FN) matrix is regulated. We showed that RhoA activity was down-regulated in cells when adhering to FN matrix. Treatment of cell with dominant active form of TAT-RhoAV14 protein induced cell de-adhesion from FN matrix, accompanying by myosin light chain (MLC) phosphorylation in a ROCK-dependent manner. Interestingly, the RhoAV14-induced cell detachment was abrogated by the pretreatment of tyrosine phosphatase inhibitor or expression of catalytically inactive mutant of tyrosine phosphatase Shp-2(C/S). By in vitro kinase analysis, we demonstrated that ROCK kinase activity was decreased in cells expressing Shp-2(C/S). Further analysis showed that ROCK was tyrosine phosphorylated in the pervanadate-treated cells and the expression of Shp-2(C/S) enhanced the ROCK kinase activity. By in vitro kinase assay, we found that tyrosine phosphorylation decreased the binding affinity of ROCK to RhoA. Accordingly, we proposed that Shp-2-regulated dephosphorylation of ROCK modulates Rho/ROCK activating signal to control cell migration.

1619 Endogenous Arf6 Interacts with Rac1 upon Angiostatin II Stimulation to Regulate Membrane Ruffling and Cell Migration

M. Cotton,1 P. Boulay,1 T. Houndolo,1 J. A. Pitcher,1 A. Claing1; 1Pharmacologie, Université de Montréal, Montréal, PQ, Canada, 2MRC Laboratory for Molecular and Cellular Biology and Department of Pharmacology, University College London, London, United Kingdom

ARF6 and Rac1 regulate remodelling of the actin cytoskeleton. Here, we demonstrate that these GTPases are sequentially activated when HIEK 293 cells expressing the angiostatin type 1 receptor are stimulated with angiostatin II (Ang II). Following receptor activation the two small G proteins transiently form a complex. ARF6/Rac1 complex formation is, at least in part, direct and dependent upon the nature of the nucleotide bound to both small G proteins. ARF6-GTP preferentially interacts with Rac1-GDP. AT1R expressing HIEK293 cells ruffle, form membrane protrusions and migrate in response to agonist treatment and ARF6 and Rac1 localize to the actin rich protrusions. ARF6, but not ARF1, depletion using small interfering RNAs recapitulates the ruffling and migratory phenotype observed following Ang II treatment. These results suggest that ARF6 depletion or Ang II treatment are functionally equivalent and point to a role for endogenous ARF6 as an inhibitor of Rac1 signaling. Taken together, our findings reveal a novel function of endogenously expressed ARF6 and demonstrate that by interacting with Rac1, this small GTPase is a central regulator of the signaling pathways leading to actin remodelling.

1620 A Rho Family GEF GEFL Promotes Prosurvove Activity at the Leading Edge of Migrating Cells

J. M. Bristow, D. Webb; Dept of Biological Sciences, Vanderbilt University, Nashville, TN

The Rho family of small GT Pases, which includes Rho, Rac and Cdc42, are involved in many cellular processes, including cell migration. Because individual Rho GT Pases can have various effects on cells, the level of regulation for specific cellular functions, such as migration, is almost certainly controlled by upstream activators. Activation of the small GT Pases is tightly controlled by guanine nucleotide exchange factors (GEFs), which promote the exchange of GDP for GTP. When we identified a novel Rho family GEF in a proteomics screen for molecules that regulate cell migration, we saw this as an opportunity to gain insight into the function of these molecules. Our results indicate that this GEF is a strong activator of Cdc42, but only weakly activates Rac1. Further analysis showed that Cdc42-GTP is more effective than Rac1-GTP in regulating cell migration activity. Taken together, these results provide evidence that Cdc42 acts downstream of VEGF to activate Rac1 and is important for endothelial cell migration that precedes blood vessel formation. Supported by an American Heart Association Predoctoral Fellowship (TAG) and NIH grant HL45100 (KB)

1621 The IFN-γ-induced Murine Guanylate-binding Protein-2 Does Not Require GT Pase Activity but Must Be Inoprenylated to Inhibit Cell Spreading

A. F. Messmer, S. Balasubramanian, D. J. Vestal; Biological Sciences, University of Toledo, Toledo, OH

Interferon-γ (IFN-γ) can alter cell adhesion/spreading and migration in a variety of cell types. The Guanylate-Binding Proteins (GBPs) are a family of large GT Pases that are robustly induced by IFN-γ. The two most closely related murine GBP family members mGBP-2 and mGBP-1, are 80% identical and share complete identity through the first 160 amino acids. mGBP-2 inhibits

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cells spreading on fibronectin through inhibition of the small GTPase Rac1. This inhibition by mGBP-2 does not require GTPase activity. In absence of the N-terminal GTP binding domain, the C-terminal α-helices are sufficient to inhibit spreading. However, inoposyn is necessary for this inhibition. The type of inoposyn is not important, as shown by the ability of the normally geranylated geranylgeranyl mGBP-2 to inhibit cell spreading as efficiently when farnesylated. In contrast, mGBP-1 fails to inhibit cell spreading and is poorly inoposynylated, despite having an identical CaaX sequence. Together these results suggest that 1) there is a second signal within the C-terminus of mGBP-2 that directs efficient geranylgeranylation and 2) mGBP-2 inhibits cells spreading through interactions at the C-terminus rather than GTase activity. Using chimeric GTase proteins generated by exchanging regions of the C-terminus of mGBP-2 with the same regions of mGBP-1, we predict that the second signal for inoposynylase resides within the α-helices 10 and 11. Work is currently underway to further define the region proposed as the putative second signal.

1622 Modeling Membrane Diffusion with Virtual Cell: Application to Quantitative Analysis of FL
I. L. Novak, F. Gao, Y. Chu, D. Resasco, J. C. Schaff, B. M. Slepchenko; 2Cell Biology, University of Connecticut Health Center, Farmington, CT, 1Mathematics, University of Connecticut, Farmington, CT, 3Computer Science, Yale University, New Haven, CT

A new Virtual Cell capability, that allows one to model diffusion in cell membranes coupled to processes in the cytosol, is applied to a quantitative analysis of experiments with fluorescence loss in photobleaching (FLIP). The experiments involve G-protein Rac, which cycles between the cytosol and cell membrane (Moissoglu et al., Mol Biol Cell, 17, 2770 (2006)). Simulations, which are run on realistic 3D geometry based on experimental images, help validate, and determine the accuracy of, a simple method of obtaining parameters of the in vivo Rac-membrane interactions from FLIP data. The project is supported by NIH through grants P41 RR13186 and U54 GM64546.

1623 RLIP76 (RalBP1) Is an R-Ras Effector That Mediates Adhesion-dependent Rac Activation and Cell Migration
L. E. Goldfinger, C. Patak, E. D. Jeffery, J. Shahanawatz, D. F. Hunt, M. H. Ginsberg; 1Medicine, University of California, San Diego, La Jolla, CA, 2Advan Biosciences, Inc., Ithaca, NY, 3Chemistry, University of Virginia, Charlottesville, VA, 4Chemistry and Pathology, University of Virginia, Charlottesville, VA

The Ras family of small GTases regulates cell proliferation, spreading, migration and apoptosis, and malignant transformation by binding to several protein effectors. One such GTase, R-Ras, plays distinct roles in each of these processes, but to date identified R-Ras effectors were shared with other Ras family members (e.g. H-Ras). We utilized a new database of Ras-interacting proteins to identify RLIP76 (RalBP1) as a novel R-Ras effector. RLIP76 binds directly to R-Ras in a GTP-dependent manner, but does not physically associate with closely related paralogues, H-Ras and Rap1A. RLIP76 is required for adhesion-induced Rac activation and resulting cell spreading and migration, and for the ability of R-Ras to enhance these functions. RLIP76 regulates Rac activity through the adhesion-induced activation of Arf6 GTase and activation of Arf6 bypasses the requirement for RLIP76 in Rac activation and cell spreading. Thus, we identify a novel R-Ras effector, RLIP76, which links R-Ras to adhesion-induced Rac activation through a GTase cascade that mediates cell spreading and migration.

1624 Rac and Rho Regulation of Focal Adhesion Kinetics
M. K. Knowles, E. Rericha, C. M. Waterman-Storer; Physiology, Marine Biologicacl Laboratory, Woods Hole, MA

Rac and Rho are small GTases critical in regulating focal adhesion (FA) formation and maturation in cell migration. FA maturation is characterized by mechanically and/or biochemically induced growth of small focal complexes into FA. We hypothesize that growth/maturation is mediated by altering the balance of on and off rates of key structural proteins that bind to and dissociate from FA. To test this hypothesis, we analyzed the dynamics of GTP-fusion proteins in mouse embryo fibroblasts (MEF) cells using TIRF and FRAP. The size and FA turnover rates were assessed by TIRF microscopy and the turnover times of individual proteins within FA were measured with FRAP. We focused on paxillin, thought to be a key regulator of adhesion maturation. Rac and Rho activity were modulated by transfection with constitutively active (CA) and dominant negative mutants. As is established, CA Rac produced small focal complexes and the CA Rho induced an elongated mature FA. Rac and Rho affected the recovery rates of paxillin-GFP fluorescence after photobleaching in FA. The recovery curves are well fit by the sum of two exponentials, indicating two characteristic times for paxillin interaction with FA. The long time recovery (t∞ ~ 50 s) was independent of Rac and Rho conditions, however, the initial recovery was affected by Rho activation. In control cells, the short time paxillin recovery occurred with a t1/2 ~ 1.5 s, while for CA Rho conditions, paxillin recovered faster (t1/2 ~ 2 s). Thus, Rho promotes rapid dissociation of a portion of adhesion-associated paxillin. In addition, constitutive Rho activity caused anisotropy in the fluorescence recovery - the intensity filled in centripetally within the bleached zone. The complex kinetics of paxillin within FA are consistent with the established complexity of binding interactions and phosphoregulation for paxillin. These results suggest that Rho may be key in FA maturation.

1625 Rac1 Modulation of Ca2+ Mobilization in Neuronal Growth Cone: Novel Role for Rac1 in Promoting MT Dynamics and Sensitizing Ca2+ Release
X. Zhang, P. Forscher; MCDB, Yale University, New Haven, CT

Rac1, a member of the small Rho GTase family, promotes neuronal growth cone extension. Ca2+ exerts myriad effects on growth cone motility. Here we report that Rac1 activity modulates Ca2+ mobilization evoked by 5-HT or LPA in growth cones of Aplysia bag cell neurons. In CA Rac1 backgrounds, 5-HT/LPA evoked a sustained Ca2+ rise in greater than 78% of growth cones tested. In contrast, only 5-6% of growth cones responded to 5-HT/LPA with transient Ca2+ increases in DN Rac1 backgrounds. In controls, 13-17% of the growth cones manifested Ca2+ mobilization after 5-HT/LPA exposure. Inhibitors of PLC or IP3 receptors blocked 5-HT/LPA induced Ca2+ rise in CA Rac1 backgrounds, but ryanodine receptor inhibition had no effect on Ca2+ elevation triggered by 5-HT/LPA. Rac1 activation by 5-HT/LPA, suggesting that Rac1 enables a 5-HT/LPA-PLC/IP3-Ca2+ signaling cascade in growth cones. Analysis of MT and ER dynamics revealed that Rac1 promotes concomitant MT and ER extension into the growth cone periphery. Inhibition of MT dynamics selectively suppressed peripheral Ca2+ responses. On the other hand, assessment of reactive oxygen species (ROS) indicated that Rac1 enhanced ROS levels in growth cones. Inhibition of NADPH oxidase activity or addition of ROS scavengers abolished 5-HT/LPA evoked Ca2+ elevations in CA Rac1 backgrounds, whereas HO2 restored this Ca2+ response in DN Rac1 backgrounds. Taken together, these results suggest Rac1 regulates 5-HT/LPA evoked Ca2+ mobilization by: 1) regulating MT dynamics with subsequent effects on ER distribution and 2) ROS dependent sensitization of Ca2+ release from ER stores.

1626 Filamin A Is Required for R-Ras to Inhibit Breast Epithelial Cell Migration
R. K. Schmocker, L. Kwong, P. J. Keely, S. Gehler; Pharmacology, University of California, Madison, WI

R-Ras, a small GTase, is important for breast epithelial cell migration, which is necessary for cancer cell metastasis. It has been shown that constitutively active of R-Ras (38V) inhibits random cell migration and disrupts cells polarity on a collagen substratum (Wozniak et al., 2005). Furthermore, R-Ras mediates its migratory and adhesive effects through the n2β1 integrin (Keely et al., 1999). Filamin A, an actin binding protein, has been shown to associate with β1 integrin and plays an important role in melanoma cell migration, but it is unclear which signaling molecules regulate filamin A (Stossel et al., 2001; Cunningham et al., 1992; Calderwood et al., 2001). Since expression of R-Ras (38V) increases the association of filamin A with the n2β1 integrin, and filamin A and R-Ras both regulate cell migration, we hypothesize that filamin A is necessary for the inhibitory effects of R-Ras on the migration of breast epithelial cells. Migration was assessed by inducing a wound through a confluent cell monolayer and measuring the rate of wound closure at 24 and 48 hours. We have demonstrated that control cells expressing pZip vectors alone migrate twice as fast as cells expressing R-Ras (38V). Co-transfection of control cells with filamin A shRNA induced a slight increase in cell migration after 24 hours and a 35% increase after 48 hours. Interestingly, filamin A shRNA rescues the migratory phenotype of R-Ras (38V)-expressing cells by increasing migration 58% and 66% after 24 and 48 hours, respectively. These results suggest filamin A is required for the regulation of cell migration by R-Ras.

1627 A RasGEF Complex Coordinates Regulation of Activation of Distinct Ras Proteins during Chemotaxis in Dictyostelium
P. G. Charest, A. T. Sasaki, Z. Shen, S. Briggs, R. A. Firel; 1Division of Biological Sciences, University of California, San Diego, La Jolla, CA, 2Dep’t of Systems Biology, HMS, Div. of Signal Transduction, BDIMC, Boston, MA

Ras activation is one of the earliest polarized responses to directional chemotactant stimulation in Dictyostelium, which occurs at the leading edge of chemotactic cells. Dictyostelium cells lacking the Ras guanine exchange factor (GEF) Aimless (alet) were reported to display defects in gradient sensing and cAMP production. To further study the role and regulation of Ras

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signaling in chemotaxis, we identified Aimless interacting proteins using sequential affinity purification coupled to mass spectrometry. In addition to Aimless, the complex contained another RasGEF, RasGEFH, and a 150 kDa protein, DG1105. Developmental analysis of null mutants of these genes revealed that while DG1105 cells fail to aggregate, rasgefh cells exhibit delayed development but ultimately produce small fruiting bodies. The two null strains, as well as aleA, grow normally but display defects in both chemotaxis and random cell movement. Also, aleA, rasgefh and DG1105 present reduced basal tyrosine and cAMP-stimulated Akt activation and show impaired chemotaxis. The second peak of chemotactic-stimulated actin polymerization that is linked to pseudopod elongation, disruption of Aimless or RasGEFH produces strong effects on cAMP-induced Ras activation (using a pan-Ras antibody): total Ras activity is reduced in aleA whereas it is increased in rasgefh cells. Moreover, preliminary biochemical data indicate that Aimless and RasGEFH have different substrate specificities, regulating the activity of distinct, or distinct sets, of Ras proteins. Altogether, these results suggest that Aimless and RasGEFH have both distinct and shared functions. We hypothesize that a complex containing Aimless and RasGEFH, two RasGEFs with individual specificities, allows for the coordinated regulation of different Ras signaling pathways that control directed cell movement.

1629 Elevation of Plasma Membrane Cholesterol Content Impairs Macrophage Migration through Inactivation of RhoA
T. Nagao1, C. Qin1, I. Groshova1, R. F. Maxfield2, L. M. Pierini1; 1Biochemistry, Weill Medical College of Cornell University, New York, NY, 2Surgery, Weill Medical College of Cornell University, New York, NY
Atherogenesis generally begins as small accumulations of lipid-laden macrophages, known as foam cells, in the space just beneath the endothelial cells that make up arterial walls. Foam cells develop through unregulated uptake of subendothelial modified and aggregated LDL. The reason why foam cells remain in the atherosclerotic plaque rather than migrating out of the area is unclear. We tested the hypothesis that elevated membrane cholesterol content, which may result from interactions with aggregated LDL, affect macrophage migration. To investigate the effects of elevated membrane cholesterol content on macrophage migration, cholesterol-cholchit-substituted methyl-β-cyclodextrin was used to acutely increase membrane cholesterol levels. Functional responses to complement 5a (C5a) were examined for control and cholesterol-loaded J774 cells. Cholesterol loading decreased macrophage migration in transwell migration assays, even though cholesterol-loaded macrophages responded to a bath application of C5a with modified changes similar to control cells. In a microperitope polarization assay, cholesterol-loaded cells were able to polarize toward a C5a gradient, indicating that cholesterol-loaded cells maintained abilities to sense and respond to chemotactic gradients. Further analysis of J774 cells migrating through pores of a transwell filter revealed that cholesterol-loaded cells could extend lamellae through the pores but were unable to translocate their cell bodies. Since translocation of the cell body is thought to be dependent on contractile forces and regulated by the RhoA/Rho kinase pathway, we measured the activation status of RhoA in these two cell types. We found that cholesterol loading decreased GTP-bound active RhoA and phosphorylation of myosin light chain. These results suggest that increases in plasma membrane cholesterol content interfere with RhoA activation, resulting in inhibition of cell migration due to reduced contractile force generation. These findings provide one possible explanation for the retention of foam cells in atherosclerotic lesions.

1630 A Dictyostelium Rabgap Protein Regulates Chemotaxis by Mediating F-actin Polymerization and Cell Adhesion
F. Du, K. Edwards, Z. Shen, B. Sun, S. Lee, B. Briggs, R. A. Firtel; 1UCSD, La Jolla, CA, 2La Jolla Institute for Allergy and Immunology, La Jolla, CA
Several studies have demonstrated expression of B class Ephs and ephrins in a variety of tumors and suggested a functional relationship between their expression and tumor progression. Here we show that EphB receptors, and especially EphB4, regulate the migration of murine melanoma cells. Highly malignant melanoma cells express the higher levels of EphB4 receptor and migrate faster than less malignant melanoma cells. We further demonstrate that EphB4 receptor tyrosine kinase is actively engaged in signaling in the fast migrating SW1 melanoma cells and its inhibition by overexpression of a B class Ephb lacking the cytoplasmic portion or by treatment with competitively acting soluble Ephb2-Fc result in slower cell migration. In contrast, overexpression of full length Ephb4 or its activation with ephrinB2-Fc enhances cell migration. The effects of Ephb4 receptor on cell migration and cell morphology require its kinase activity because the inhibition of Ephb4 kinase activity by overexpression of kinase dead Ephb4 inhibits cell migration and affects the organization of actin cytoskeleton. Activation of Ephb4 receptor with its ligand ephrin-B2-Fc affects the morphology of melanoma cells and increases RhoA activity, while inhibiting Ephb receptor forward signaling decreases RhoA activity. Moreover, expression of dominant negative RhoA blocks the effects of active Ephb4 on cell migration and actin organization. These data suggest that Ephb4 forward signaling contributes to the high migratory ability of invasive melanoma cells by influencing RhoA-mediated actin cytoskeleton reorganization.

1632 hMena Works as a Negative Regulator of Rac1 GTPase at the Tip of Lamellipodia
C. Ishikawa, M. Higashi, K. Azuhata, H. Kawana, M. Kitagawa, K. Harigaya; Molecular and Tumor Pathology, Chiba University Graduate School of Medicine, Chiba, Japan
hMena, a member of Ena/VASP proteins, has been implicated in cell motility through regulation of the actin cytoskeleton, including lamellipodial protrusion. However the molecular mechanism and the signaling mechanism of hMena are not well understood. hMena (hMena) by siRNA causes cells to be highly spread with enormous lamellipodia, and to be highly motile. Increased motility of the cells with the siRNA can be reversed by introducing dominant negative form of Rac1, but not with LY294002, an inhibitor of phosphatidylinositol 3-Kinase (PI3-Kinase). With a Rac activation assay, hMena knockdown cells show increased Rac activation. hMena colocalizes with Rac1 at the tip of lamellipodia. These results indicate that hMena negatively regulates the activity of Rac1 at the leading edge.
SSX, New Molecular Target to Regulate Sarcoma Invasion
K. Isho, K. Yoshokawa; Biology, Osaka Medical Center for Cancer, Osaka, Japan

The SSX / SYT fusion genes were initially identified as fusion partners to the SYT 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. Using Nucleic Acid Sequence-Based Amplification, we have reported that the expression level in the malignant tumors (3.8±plasmn.1.4 copies/µg of total RNA in log10 order) was significantly higher than that in the benign tumors (2.4±plasmn.0.5, p<0.0001). In order to examine the biological function of SSX, we made stable transfectants with wild type SSX using human osteosarcoma cell line, Saos-2, which moderately expressed SSX. The expression of SSX was mainly localized in the nucleus with patched pattern. The SSX transfectants promoted colony formation in soft agar and tumor formation in nude mice, but showed little change in growth rate in 2D culture. The transfectants also increased motility, chemotaxis and invasiveness using scratch wound assay and Boyden chamber assay. Enhanced Rho activity and stress fibers were observed in these SSX overexpressed Saos-2 cells. By contrast, the lowering of the endogenous expression of SSX1 in fibrosarcoma HT1080 cells by the treatment with specific siRNA markedly decreased membrane ruffling, chemotaxis and invasiveness using Boyden chamber assay, but did not affect cell proliferation in 2D culture. Moreover, SSX1 deficient R1c-activation and myosin light chain phosphorylation. We also prepared wrapped liposome containing siRNA and introduced into nude mice bearing HT1080 cells. Preliminary results showed in vivo inhibitory effects of siRNA against SSX in tumor progression. Collectively, these data suggested that SSX protein regulated Rho-family small GTPases to the tumor invasion and progression, and thus could be a novel molecular target under clinical setting.

Integrin Signaling through Arg Activates p190RhoGAP by Promoting Its Binding to p120RasGAP and Recruitment to the Membrane
W. D. Bradley,1 S. E. Hernandez,1 J. Settleman,2 A. J. Koleske1,2,3; 1Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT, 2MGH Cancer Center, Massachusetts General Hospital, Harvard Medical School, Charlestown, MA, 3Interdepartmental Neuroscience Program, Yale University, New Haven, CT

The Rho family GT-Pases RhoA (Rho), Rac1, and Cdc42 are essential effectors of integrin-mediated cell attachment and spreading. Rho activity, which promotes formation of focal adhesions and actin stress fibers, is inhibited upon initial cell attachment to allow sampling of the new adhesive environment. The Abl-related gene (Arg) tyrosine kinase mediates adhesion-dependent inhibition of Rho through phosphorylation and activation of the Rho inhibitor p190RhoGAP-A (p190). p190 phosphorylation promotes its binding to p120RasGAP (p120). Here, we elucidate the mechanism by which p120 binding regulates p190 activation following adhesion. We show that p120 binding inhibits p190 activity in vitro. However, p120 binding is not sufficient to activate p190RhoGAP activity in vivo. Instead, activation of p190 requires its recruitment to the cell periphery. Integrin-mediated adhesion promotes relocalization of p190 and p120 to the cell periphery in wild type fibroblasts, but not in arg/−/− fibroblasts. A dominant-negative p120 fragment blocks p190 complex formation, prevents activation of p190 by adhesion, and disrupts the adhesion-dependent recruitment of p190 to the cell periphery. Our results suggest that integrin signaling through Arg activates p190 by promoting its association with p120, resulting in recruitment of p190 to the cell periphery where it inhibits Rho.

Regulation of Lipoma Preferred Partner in Cell Motility by c-Src-mediated Phosphorylation at Multiple Sites
C. Chen, Y. Lai, F. Lin; Cell Biology, UAB, Birmingham, AL

Lipoma Preferred Partner (LPP) is an actin-associated focal adhesion molecule that belongs to the LIM domain-containing zyxin family. Suppression of endogenous LPP expression reduces Lysophosphatic acid (LPA)-induced Rho GTPase activation and cell migration, suggesting an important role for LPP in cell motility. Previously we have shown that c-Src-mediated phosphorylation of TRIP6, another zyxin family member, regulates its function in the binding to Crk and LPA-induced cell migration. In this report, we further demonstrate that LPP, but not zyxin, is a substrate for c-Src in vitro and in cells. LPA induces tyrosine phosphorylation of LPP, which is virtually eliminated in Src-null fibroblasts. In contrast to TRIP6 that is specifically phosphorylated by c-Src at Tyr-55 residue, LPP is phosphorylated at multiple sites in the internal sequences. This phosphorylation does not affect the function of LPP in focal adhesion targeting or association with actin. However, mutation of these phosphorylation sites abolishes the association of LPP with Rac1 and reduces LPA-induced Rho GTPase activation. Consequently, the function of LPP in promoting LPA-induced cell migration is impaired. Taken together, c-Src phosphorylates LPP and TRIP6 at different sites, and regulates their functions in cell motility through distinct mechanisms.

Caspase Regulated Cytoskeletal Rearrangement in Macrophages for Chemotaxis and Fusion
T. Nihan,1 R. W. Liles,2 S. Schwartz; 1Pathology, University of Washington, Seattle, WA, 2Molecular Medicine, University of Toronto, Toronto, ON, Canada

Active caspases are the hallmark of a dying cell undergoing apoptosis. We observed that thioglycollate-elicited mouse peritoneal macrophage (MPM) adherence in vitro activate caspases in a Fas-dependent (CD95) manner without leading to cell death. Active caspases within living macrophages selectively cleaved a subset of caspase substrates, such as Rho kinase (ROCK-1). Functional assays of living macrophages with active caspases showed that caspases can regulate macrophage chemotaxis and fusion. Cell permeable inhibitors of caspases (zVAD) and ROCK-1 (Y27632) attenuated macrophage chemotactic response to monocyte chemotactic protein-1 (MCP-1). Caspases from MCF-7, T47D, BT20, MDA-MB-231 cells. By confocal laser-scanning microscopy, we examined localization of hamartin and tuberin in MCF-7 cells migrating in response to insulin-like growth factor 1 (IGF-1). Hamartin, but not tuberin, accumulated in activated-enriched lamellipodia of IGF-1-stimulated cells. Hamartin and tuberin co-precipitated; however, the phosphorylated hamartin (140 kDa) was absent from the complex. In MCF-7 cells, IGF-1 causes rapid but transient activation of a small GTPase Rap1, an essential regulator of adhesion receptors and actin dynamics. Based on in vitro findings of tumorIP activity towards Rap1, we hypothesized that tumorIP may act as Rap1GAP in cells. To test this hypothesis, we used 3 approaches: over-expression of tumorIP, siRNA inhibition of hamartin, and over-expression of tumorIP mutants that have potential to be dominant-negative towards endogenous tumorIP. We found that the N-terminus truncated mutant of tumorIP inhibited IGF-1-induced Rap1 activation. Together, these data implicated the phosphorylated hamartin in the regulation of lamellipodia and tuberin in modulation of Rap1 in moving breast cancer cells. These results suggest a new role for hamartin, tuberin and migration of breast cancer cells. (Sponsored by the University of Pennsylvania Research Foundation and the American Cancer Society IRG1/78-002026 to M.A.G.)

Dynamic Imaging of Human Tumor Invasion and Angiogenesis in an Novel Zebrasfish Xenograft Model
K. Stolcovo, O. Pertz, M. Holcomb, T. Ronai, R. Klemke; Pathology, UCSD, La Jolla, CA

We developed a novel human tumor xenograft model that takes advantage of transgenic Zebrasfish. When injected in immunosuppressed Tg(B1:EGFP) Zebrasfish fluorescently labeled human tumor cells home to the fish blood vessels where they form tumor-like aggregates and induce rapid angiogenic response. Transparency of Zebrasfish tissue allows us to perform high resolution multi-color in vivo imaging of dynamic interplay between the tumor invasion and angiogenesis for periods ranging from several hours to days. Using this model, we examined the role of the small GTPase Rac in tumor invasion. Rac is overexpressed in a highly metastatic form of breast cancer, however the mechanism by which it promotes tumor cell invasion is not known.
Simultaneous imaging of differentially labeled parental and RhoC overexpressing tumor cells revealed that while parental cells form tightly packed aggregates, RhoC overexpressing cells scatter within the tissue. Time lapse analysis revealed that while parental cells utilize mixed amoeboid and mesenchymal types of 3D cell migration RhoC switches cell migration to completely amoeboid. RhoC overexpression also increased tumor cell membrane dynamics leading to continuous shedding of small cellular fragments. RhoA overexpression had significantly less potent effect than RhoC with tumor cells being of intermediate morphology. Chemical inhibition of RhoC downstream effector ROCK reversed these processes. We also performed quantitative imaging of effects of two anti-cancer compounds: a VEGFR inhibitor, SU5416, and a Src inhibitor, PP1 on tumor progression. Analysis of 3D high resolution images of compound and mock treated animals revealed that SU5416 leads to shrinkage of tumor cells and tumor-induced vasculature while PP1 mainly affects tumor cell survival. Taken together, we designed a novel 3D in vivo tumor invasion and angiogenesis model that allows for high resolution quantitative imaging of these processes as well as for quantitative in vivo testing of pharmacological compounds.

Integrin α4β1-stimulated Neuroblastoma Cell Motility Requires Src and p130Cas but Not FAK

L. Wu,1 J. A. Bernard-Trifilo,1 Y. Lim,1 S. Lim,1 D. G. Stupack,2 D. D. Schlaepfer2; 1Immunology, The Scripps Research Institute, La Jolla, CA, 2Pathology, University of California San Diego, La Jolla, CA

The fibronectin binding integrins α5β1 and α6β1 generate signals pivotal for cell migration through distinct yet undefined mechanisms. Neuroblastoma (NB) cells are highly invasive and derive from α6β1-expressing neural crest cells. Here, we analyzed the role of α4-associated integrin signaling involved in NB cell motility using two representative human-derived α4 integrin-expressing NB cell lines, NB8 and SK-N-AS. We found that NB cells expressed high levels of fibronectin-binding integrins and that their migration on cellular fibronectin was mediated by both α4β1 and α5β1 integrins. It was further revealed that α4β1 integrin-mediated NB cell migration required both Src-family kinase and focal adhesion kinase (FAK) activities whereas α5β1 integrin-mediated NB cell migration involved Src but not FAK activity. By employing haptotaxis motility assay and time-lapse imaging analyses, we found that inhibition of p130Cas and c-Src expression with short-hairpin RNA (shRNA)-mediated interference decreased α4β1 integrin-mediated NB cell motility. Inhibition of FAK or paxillin expression was without effect on α4β1-stimulated NB cell motility whereas inhibition of c-Src expression selectively reduced α4β1-stimulated p130Cas tyrosine phosphorylation. In addition, α4β1 integrin-mediated cell spreading and motility of α130Cas-/- fibroblasts was enhanced by p130Cas re-expression. Abolishing α4 integrin expression in NB cells blocked α4β1 but not α5β1-stimulated cell migration. Further, α4β1 integrin-mediated NB cell motility and α4-stimulated Src activation was rescued by re-expression of wild type α4 but not by an α4 integrin mutant with a cytoplasmic domain deletion. These results show that α4 integrin plays an important role in promoting NB cell motility and that the α4 cytoplasmic domain is an important determinant for α4β1 integrin-stimulated Src activation, p130Cas tyrosine phosphorylation, and NB cell migration.

Visualization of Axonal Transport of α-synuclein in Slow Component-b (SCb)

S. Roy,1 M. J. Winton,1 M. M. Black,2 J. Q. Trojanowski,1 V. M. Y. Lee1; 1Center for Neurodegenerative Research, University of Pennsylvania, Philadelphia, PA, 2Anatomy and Cell Biology, Temple University Hospital, Philadelphia, PA

Overall rates of slow and fast axonal transport differ by several orders of magnitude. Slow axonal transport moves cytoskeletal and cytosolic proteins along the axon in fairly discrete compartments called slow component-a and -b (SCa and SCb) respectively. Mechanisms generating this slow overall movement are intriguing, as velocities of known molecular motors are much lower than the velocity of the slow component. Previously, we had shown that individual neurofilaments moving in SCa are transported rapidly at rates comparable to known motors, but the movement was infrequent and intermittent, making the overall population slow (Roy et al., J Neuroscience 2000, 20:6484-61). In this study, we visualized the axonal transport of an SCb protein α-synuclein (α-SYN) and directly compared it to synaptophysin (SYP), a transmembrane fast component protein. Using live cell imaging of fluorescent-tagged α-SYN and SYP we show that individual particles of both proteins are transported at similar average velocities (~1μm/s), α-SYN particles often pause during transit and their overall transport frequency is ~10 times lower than SYP. Simultaneous visualization of both proteins in the same axon by dual-cam video microscopy using red/green chimeras revealed that despite moving with vastly different frequencies, individual α-SYN and SYP particles can transiently merge during transit. By directly comparing a slow and fast component protein, these data show that α-SYN, an SCb protein is transported rapidly like fast component proteins, but the movement is infrequent with pauses, and suggest that all slow component proteins may use this “stop and go” mechanism for transport.

Effects of Crowding on Intracellular Diffusion

P. Kraikivski, I. L. Novak, B. M. Slepchenko; Center for Cell Analysis and Modeling, Department of Cell Biology, University of Connecticut Health Center, Farmington, CT

The interior of the cell is filled with polymer meshwork and intracellular membranes with intricate geometry. Recently, there have been a number of reports of anomalous subdiffusion in both in vitro and in vivo experiments. Here we use various modeling approaches to analyze how the intracellular structures affect diffusion of macromolecules in the cytoplasm. Three-dimensional simulations of a Brownian motion in the entangled filament networks reveal the anomalous time dependence of a particle mean square displacement on the time and spatial scales determined by the ratio of a particle radius and the network mesh size. This is consistent with experiments with tracer particles and earlier modeling studies of diffusion on lattices. Using these results, an accurate continuous approximation for the diffusive transport in crowded media is derived and tested numerically. The project is supported by NIH through the grant 1U54 RR022232.

In Vivo Motor Protein Interaction Revealed in Spatial and Temporal Dynamics of Vesicle Transport in Drosophila Segmental Nerve Axons

G. F. Reis,1 G. Yang,1 S. Shah,2 L. Szpankowski,3 G. Danuser,1 L. S. Goldstein; 1Neuroscience, UCSD, La Jolla, CA, 2The Scripps Research Institute, La Jolla, CA, 3Bioengineering, University of Maryland, College Park, MD, 4Bioinformatics, UCSD, La Jolla, CA, 5Cellular and Molecular Medicine, UCSD, La Jolla, CA

Proper functioning of microtubule-based bidirectional axonal transport is crucial for neuronal function and survival. We studied molecular mechanisms of neuronal transport by imaging and measuring high-resolution trajectories of axonal cargo motion in vivo. By altering the expression of specific motor proteins, we probed the regulation and coordination of the molecular transport machinery. Until recently, a major hurdle in obtaining an accurate quantitative analysis of single axon cargo trafficking hinged on development of reliable and efficient computational tools to track vesicle motion and generate complete spatial and temporal readouts for large datasets. The computational challenge lies in the physiology of vesicle transport, which is often comprised of dense anti-parallel motion with apparent particle crossing, merging, and splitting. We have developed reliable and efficient computational tools to recover complete trajectories of large numbers of cargos as well as characterize the spatial and temporal dynamics of vesicle transport. These tools include a novel multiple-hypothesis-testing (MHT) algorithm for resolving particle superposition. Human-machine interaction is used to complement automation, ensuring complete and accurate trajectory readouts. We are able to recover complete tracks of large numbers of cargos by validating an approach suitable for high-throughput processing. Experimentally, we imaged the bidirectional movement of motor-associated cargoes containing Yellow Fluorescent Protein (YFP) fused to the C-terminal of human amyloid precursor protein (APP-YFP) in a subpopulation of Drosophila segmental nerve axons. We imaged transport in wild type and mutants expressing a 50% genetic reduction in components of kinesin and dynein motor complexes, enabling comparative analyses of several transport parameters across 7 genotypes and over 560 movies. Our results reveal mutant-dependent changes in cargo populations and motility parameters, suggesting distinct modes of coordination and regulation for kinesin and dynein. Among other intriguing findings, we report a possible novel role for kinesin as a processivity factor for the retrograde motor dynein.

Regulated Bidirectional Transport of Drosophila Lipid Droplets Involves Functional Cooperation and Physical Interactions between Klar, LSD2, and the Novel Protein Sfo

D. Kim,1 M. Zapata,2 J. G. Ghimbar3, M. A. Wehe1; 1Rosenstiel Center, Brandeis University, Waltham, MA, 2Department of Biology, University of Richmond, Richmond, VA

How motor proteins are regulated in vivo is poorly understood. We study this issue using lipid droplets in Drosophila embryos; these organelles display developmentally controlled bidirectional transport along microtubules. Therefore this model system provides the opportunity to understand how plus- and minus-end directed motors are coordinately controlled in vivo. Klar to lipid droplets. Yet the complexes containing Klar, Sfo and/or LSD2 must have additional roles since double mutants of Klar and LSD2 or Sfo display stronger defects in embryonic development than single mutants. Using yeast two-hybrid screens, we identified the novel protein Sfo as a Klar binding partner. Indeed, the distribution of an Sfo-RFP fusion in embryos resembles that of Klar, and LSD2 interact physically in embryos. These interactions are apparently functionally important as embryos lacking Sfo show defective droplet transport; net plus-end motion is abolished. Sfo, Klar to lipid droplets and of Klar. In yeast, Sfo also binds to LSD2, and a central 98 aa region is sufficient for interaction with both LSD2 and Klar. By immunoprecipitation, we confirmed that Sfo, a central 98 aa region is sufficient for interaction with both LSD2 and Klar. By immunoprecipitation, we confirmed that Sfo, Klar, and LSD2 interact physically in embryos. These interactions are apparently functionally important as embryos lacking Sfo show defective droplet transport; net minus-end motion is impaired. In embryos doubly mutant for LSD2 and Sfo, but not in the single mutants, Klar fails to localize to lipid droplets; thus LSD2 and Sfo act in a partially redundant manner to recruit Klar to lipid droplets. Yet the complexes containing Klar, Sfo and/or LSD2 must have additional roles since double mutants of Klar and LSD2 or Sfo display stronger defects in embryonic axonal transport.
Zebrafish Melanophilin Implements Pigment Granule Dispersion by Suppressing the Frequency and Run Lengths of Dynein-driven Motility Events

L. Sheetz,1 D. G. Ransom,1 E. M. Mellgren,2 S. L. Johnson,3 B. J. Schnapp;4 Department of Cell and Developmental Biology, Oregon Health and Science University, Portland, OR, 2Washington University School of Medicine, St. Louis, MO

The coordination of different motors on the same organelle is crucial for many cell processes. An ideal system for investigating this is the fish melanocyte, which rapidly aggregates and disperses its pigment granules in response to cAMP. How cAMP actually regulates the melanin, dynein and myosin motors that direct granule transport is not understood. To elucidate these mechanisms we are using zebrafish because we can study pigment mutations. Here we report on the mutant normal, whose melanocytes disperse their granules only part way. We tracked single granules in wild-type and normal melanocytes during dispersion, and computed the kinetic parameters of granule transport along microtubules. Whereas the frequency and run lengths of minus-end motility events are suppressed in wild type cells, which causes net plus-end travel, these parameters remain high in normal, and the average travel distances in the plus- and minus-end directions remain, thereby, precisely balanced. Thus, the normal mutation unmasks a regulatory feature, which we term the “balancer”. The balancer appears to equalize the oppositely directed travel distances by adjusting the frequency and run lengths of only the minus-end, dynein-driven motility events. We propose that the product of the normal gene normally inhibits the dynein-based balancing of travel distances, and thereby triggers cAMP-induced dispersion by biasing granule movement toward the microtubule plus-end. Positional cloning of the normal gene reveals it is zebrafish melanophilin, a novel finding, as melanophilin and its homologues in mammals link myosins to organelles to facilitate their “hand-off” from microtubules to the cortical actin network. A microtubule-related function for melanophilin proteins has not been suspected or investigated. We propose that melanophilin proteins coordinate both actin and microtubule-based transport to determine an organelle’s position in the cell.

Adaptor Protein-2 Interaction with Arrestin Regulates N-formyl Peptide Receptor Post-endocytic Trafficking

B. M. Wagener, C. M. Revankan, N. A. Marjon, E. R. Prossnitz; Cell Biology and Physiology, University of New Mexico School of Medicine, Albuquerque, NM

G protein-coupled receptors (GPCRs) are integral to cellular function in nearly all physiologic and numerous pathologic processes. GPCR signaling is an intricate balance between receptor activation, inactivation (desensitization and internalization) and resensitization (recycling and reassembly). While much is known regarding the first two processes, the latter has not been as thoroughly studied. To better understand the process of GPCR post-endocytic trafficking, we focused on the N-formyl peptide receptor (FPR), a chemoattractant receptor found primarily on neutrophils and macrophages. Previous studies have demonstrated that, although FPR internalization occurs in the absence of arrestins, FPR recycling is blocked. Furthermore, FPR stimulation in the absence of arrestins leads to receptor accumulation in recycling endosomes and apoptotic signaling. In this study, we determined that the carboxy terminals of arrestin-2 is critical for intracellular receptor trafficking and performed scanning mutagenesis of this region to ascertain the mechanisms involved. Our results reveal that two arrestin-2 mutants (F391A and K397A/M399A/K400A), at sites known to be involved in AP-2 binding, fail to rescue the trafficking and signaling defects observed in the absence of arrestins. Further results demonstrate that AP-2 associates with the receptor-arrestin complex in recycling endosomes and is required for proper post-endocytic trafficking of the FPR. Finally, we observe that AP-1 is associated with the receptor-arrestin complex under recycling-competent conditions, suggesting a transfer of receptors from AP-2- to AP-1-associated vesicles. This is the first study to demonstrate a requirement for AP-2 in the post-endocytic trafficking of a GPCR and serves as a model for future studies in GPCR trafficking and resensitization.

Actin Filament-based Motility of Ntcp-containing Hepatocyte Vesicles

S. Sarkar, A. W. Volling, J. D. Murray; Anatomy and Structural Biology and Liver Research Center, Albert Einstein College of Medicine, Bronx, NY

The bile salt transporter moves between an intracellular vesicular pool and the hepatocyte plasma membrane. Vesicles must move on both actin microfilaments and microtubules. In previous studies, we reconstituted and characterized the microtubule-based motility in vitro. We now report reconstitution of the actin-based motility. A glass microcoated chamber was washed with rhodamine-labeled actin to which vesicles prepared from rat liver were bound. Ntcp was observed by staining with primary and fluorescently-labeled secondary antibodies. Nearly 20% of the actin-bound ntcp-containing vesicles (n=692) moved following addition of 1mM ATP but not buffer or ADP. Motility events were of two types: (1) 11.8% vesicles moved >2pixels (~420 nm) before detaching and (2) 8.2% immediately detached (“pop-off”). Addition of the myosin ATPase inhibitor, butane-dione monooxime (10mM) significantly decreased movement of >2pixels but not pop-off events whereas at 30mM it decreased both events by >50%. Immunoblot showed the presence of the actin-based motors, myosins Ila/b and Va/b but not myosin VI. Immunofluorescence analysis revealed that 50% and 87% of actin-containing vesicles co-localized with myosins Ila/la and Ila/la respectively while only 12% and 18% colocalized with myosin Ila/Ia respectively. While myosin Va/Va/Vb antibodies did not inhibit motility, myosin Ila/Ia antibody decreased motility by 30%. Interestingly, we have shown previously that >50% of the ntcp-containing vesicles are associated with microtubule-based motors kinesin-1 and dynein, suggesting that a single vesicle can be associated with microtubule and actin-based motors. This may facilitate transfer of vesicles between the two cytoskeletal systems. Other previous studies also showed the presence of PKCδ on these vesicles and inhibition of its activity by a highly specific pseudosubstrate inhibitor eliminated microtubule-based motility. This inhibitor decreased actin-based movements of >2pixels by 60%, suggesting that phosphorylation plays an essential role in the actin-based motility of these vesicles as well.

The Roles of Cytoskeletal and Membrane Proteins in Tip Growth in Aspergillus nidulans

N. Taheri-Talesh, T. Horio, X. Dou; S. A. Osmani, B. R. Oakley; Molecular Genetics, Ohio State University, Columbus, OH, 2Institute of Health Biosciences, University of Tokushima Graduate School, Tokushima, Japan

To clarify mechanisms of tip growth in Aspergillus, we have created fluorescent-protein-tagged cytoskeletal and membrane proteins and observed them in growing hyphae. Actin is in patches at the cell cortex. Many are clustered in a ring slightly behind the hyphal tip and there is an actin spot at the extreme apex. Actin Binding Protein 1 (ABP1) shows the same localization except that the apical spot is not apparent. A vesicular snare (SNC1 homolog) is in vesicles within the hypha and at the plasma membrane in a hemisphere extending from the extreme hyphal apex to the ring of actin-ABP1 patches. A target snare (SSO1 homolog) is located in the plasma membrane with a small region of higher concentration at the apex. These data suggest that vesicles with SNC1 fuse to the plasma membrane in a small zone at the extreme hyphal tip. Actin-ABP1 patches are likely sites of endocytosis that remove SNC1 from the plasma membrane, allowing it to be reused. When microtubules are depolymerized with benomyl, actin-ABP1 patches remain but lose their localization. Hyphal tip growth continues at a reduced rate, and is often abnormally curved. Additional sites of growth may form, creating branched hyphae. Actin-ABP1 patches cluster at the sites of new growth. These data indicate that microtubules play a role in maintaining the directionality of growth and preventing branching in the tip cell presumably by helping to maintain the localization of the polarisome at the apex of the hypha. Although microtubules are important for the localization of actin-ABP1 patches, a second microtubule-independent positioning mechanism also must exist. Cytocelalasin A causes all actin-containing structures to disappear and hyphal growth to stop completely. Supported by the National Institute of General Medical Science, the Deutsche Forschungsgemeinschaft and the Japan Society for the Promotion of Science.

Differential Association of Cas with Crk or BCA23 in a Cell-dependent Manner upon Insulin-stimulation

M. J. Oh, K. H. Kim, B. H. Juann; Nanomedical Engineering, Pusan National University, Miryang, Republic of Korea

Cas is known to associate with Crk and BCA23, a novel adaptor protein containing SH2-domain, but it is not investigated in the insulin-sensitive adiocytes and muscle cells. In the present study, we studied a functional role of Cas in insulin signaling and its association with Crk and BCA23. Microinjection of anti-Cas antibody inhibited the insulin-induced DNA synthesis and the membrane ruffling, suggesting that Cas is important in the insulin signaling. In HIRcB cells, Cas was associated with both Crk and BCA23, and dephosphorylated upon insulin stimulation resulting in the decreased association with Crk. But, the association of Cas protein with BCA23 was not changed. EGF and IGF-1 stimulation did not change the phosphorylation and association of Cas with BCA23. In 3T3-L1 adipocytes, Cas did not change the tyrosine phosphorylation of Cas. Crk association with Cas was increased in the 3T3-L1 adipocytes and L6 muscle cells. BCA23 association with Cas upon insulin stimulation was decreased in L6 muscle cells, but not changed in 3T3-L1 adipocytes. Our result show that p300 is involved in the insulin signaling and differentially associates with Crk or BCA23 in a cell-dependent manner upon insulin-stimulation.
Presenilin (PS), Amyloid Precursor Protein (APP) and Molecular Motors: Two Alzheimer’s Disease (AD) Proteins in the Kinesin Mediated Transport Pathway

S. Gunawardana, L. S. B. Goldstein; Cellular and Molecular Medicine, UCSD/HHMI, La Jolla, CA

Previously we suggested that APP and APP-like function as kinesin receptors in axonal transport. Recently, secretases (BACE and PS) responsible for the generation of pathogenic Ab were suggested to be present within APP vesicles transported by kinesin. Biochemical analysis indicates that APP cleavage occurs in Drosophila. Strikingly, PS influences both kinesin and APP-mediated transport pathways. Reduction of PS with excess APP causes the suppression of APP-mediated axonal blockages. Surprisingly, reducing the dose of nicastrin, APH-1 or PEN-2 with APP did not suppress blockages, suggesting that the PS-dependent suppression phenotype may not solely be due to PS’s gamma secretase activity. In vivo analysis of APP transport in RS cells revealed axon show increased anterograde transport of APP vesicles, and the velocities of APP vesicles traveling anterogradely and retrogradely increase compared to the transport of synaptogamin vesicles, suggesting that reduction of PS directly affects APP transport. One possibility is that PS mediates the transport of APP by aberrant cleavage of APP. Alternatively, PS may independently influence the transport of APP by directly affecting the transport machinery. To test this we increased the dose of PS with excess APP. Surprisingly, increasing PS also lead to the suppression of APP-mediated axonal blockages. Although excess PS alone had no effect, reducing the dose of kinesin (not dynexen) with excess PS caused blockages, suggesting that PS also affects kinesin-mediated transport. In vivo analysis of APP transport in PS increased axon also shows increased anterograde transport of APP vesicles, but a broad distribution of velocities for APP vesicles traveling anterograde or retrograde is observed. Perhaps excess PS may cleave APP to generate C-terminal fragments, which may be moving at different speeds. Thus we propose that while PS may also function in kinesin-mediated transport, APP cleavage may play a vital role in regulating APP vesicle movement within axons.

Shiga Toxin: A Model for Studying the Regulation of Dyneme-based Motility

H. A. Hetchly, M. Sambres; Physiology & Biophysics, University of Iowa, Iowa City, IA

The organization of cellular organelles and transport of intracellular vesicles require the cytoskeleton. We found that a bacterial exotoxin, Shiga toxin, requires microtubules for its retrograde traffic through the secretory system. Shiga toxin also increases the rate of microtubule and dynein dependent Golgi stack repositioning following nocodazole treatment. We have developed a permeabilized-cell system to study motor protein requirements during transport. Using permeabilized cells we found that Shiga toxin transport can be reconstituted in a cytosol- and ATP-dependent manner. Dynein inhibitors revealed that Shiga toxin utilizes dynein for transport to a juxtanuclear region. We have now tested whether other dynexin-dependent motility events can be studied in a permeabilized cell system. Following dispersal using nocodazole treatment, Golgi membranes can reassemble in permeabilized cells in the presence of cytosol and an ATP regenerating system. We are currently exploiting this reconstitution system to examine the role of Rho-family GTP-binding proteins, such as Cdc42, in regulating the motor-based intracellular motility of Shiga-toxin-containing transport intermediates and Golgi-derived vesicles. We hypothesize that some features of regulated dynein function in the secretory pathway will be shared among different trafficking steps.

Sequence-specificity of Plasmid Cytoplasmic Trafficking

E. E. Vaughan, D. A. Dean; Medicine, Northwestern University, Chicago, IL

Relatively little is known about how naked DNA moves through the cytoplasm to the nucleus during transfections and other forms of gene delivery. We have shown that in adherent cells, plasmid DNA requires intact microtubules to reach the nucleus (Vaughan and Dean, 2006, Molecular Therapy 13:422). Further, we have demonstrated that inhibition of dynein impedes this trafficking. However, it remains to be determined how plasmids interact with dynein and microtubules. In these studies, we used plasmids containing a promoter, a reporter gene, and a downstream DNA nuclear Targeting Sequence (DTS) that acts as a scaffold for transcription factors involved in DNA nuclear import. We envision two possible mechanisms for plasmid cytoplasmic trafficking. Either specific DNA sequences are required to bind to specific proteins that then interact with the dynein/microtuble complex, or no DNA sequence specificity exists and rather non-specific DNA-binding proteins (s) can act to bridge the DNA to the microtubules. To address this, we used a microtubule spin-down assay and quantitative PCR to determine if plasmas with or without various eukaryotic sequences were able to bind to microtubules in the presence of cell extract. We found that pBR322, a plasmid with no eukaryotic sequences did not interact with microtubules. By contrast, plasmids with a eukaryotic promoter were found to interact with the microtubules, suggesting that DNA-microtuble interactions are sequence dependent. These results are important in elucidating how naked DNA reaches the nucleus and an understanding of this process is imperative to improving the efficiency of gene transfer.

RhoB and the Mammalian Diaphanous-related Formin mDia2 Govern Actin Dynamics on Endosomes Necessary for Trafficking and Vesicle Fusion

A. D. DeWard, B. J. Waller, A. S. Alberts; Cell Structure and Signal Integration, Van Andel Research Institute, Grand Rapids, MI

Rho GTPases and the dynamic assembly and disassembly of actin filaments have been shown to have critical roles in both the internalization and trafficking of growth factor receptors. While all three mammalian Diaphanous-related (mDia1/2/3) formin GTPase effectors have been localized on endosomes, a role for their actin nucleation, filament elongation, and/or bundling remains poorly understood in the context of intracellular trafficking. In a study of a functional relationship between RhoB, a GTPase known to associate with both early- and late-endosomes, and formin mDia2, we show that 1) RhoB and mDia2 interact on endosomes; 2) inhibition of RhoB or mDia2 has no effect on internalization of growth factors; 3) GTPase - the ability to hydrolyze GDP - is required for the ability of RhoB to govern endosome dynamics; and 4) the actin dynamics controlled by RhoB and mDia2 is necessary for vesicle trafficking as well as maintaining their morphology. These studies further suggest that Rho GTPases significantly influence the activity of mDia family formins in driving cellular membrane remodeling through the regulation of actin dynamics.

Actin Filament Polarity in Retinal Pigment Epithelial Cells of Green Sunfish, Lepomis cyanellus

R. Vagnozzi, C. King-Smith; Department of Biology, Saint Joseph's University, Philadelphia, PA

The retinal pigment epithelium (RPE) lies between the retina and the choroid of the vertebrate eye. RPE cells from fish contain numerous pigment granules, or melanosomes, which undergo massive migrations in vivo to protect photoreceptors from bleaching. In the light, melanosome disperses from the RPE cell body out into long, thin apical projections. In the dark, melanosomes aggregate out of the projections back into the cell body. Melanosomes aggregation and dispersion are both actin-dependent. Previous research using in vitro motility assays has shown that melanosomes move towards the barbed end of actin filaments, presumably by myosin motors [McNeil et al., 2004 Cell Motil. Cytoskel. 58:71]. The purpose of the current research was to determine the polarity and organization of actin filaments in RPE cells using myosin S1 labeling. Dissociated RPE cells isolated from green sunfish were permeabilized in 10% saponin and incubated in 0.5 mg/ml myosin S1. Cells were then fixed in glutaraldehyde, embedded, sectioned, and examined using transmission electron microscopy. Both individual decorated actin filaments and actin filament bundles were observed in the cell bodies of RPE. Of the bundles analyzed, 69% had uniform polarity, 71% had completely random polarity, 21.4% showed 2/3 uniformity, and 7.1% showed ¾ uniformity. The presence of highly isotropic actin bundles in the RPE cell body suggests that melanosomes may independently influence the transport of APP by directly affecting the transport machinery. Thus we propose that while PS may also function in kinesin-mediated transport, APP cleavage may play a vital role in regulating APP vesicle movement within axons.
midbody. In addition, in mouse primary Schwann cells the two proteins also colocalize at the cell membrane. Our data indicates that the lack of merlin in the mouse primary Schwann cells drastically alters their microtubule cytoskeleton. Furthermore, merlin enhances tubulin polymerization both in vitro and in vivo in primary Schwann cells. This study describes a role for merlin as a microtubule organizing protein. It also suggests that merlin plays a key role in the regulation of Schwann cell microtubule cytoskeleton, possibly contributing to cytoskeletal defects observed in human schwannomas.

1655 Spindophilin Facilitates PPI-mediated Dephosphorylation of Pser297 Doublecortin in Microtubule Bundling at the Axonal "Wrist"
S. L. Bielas, F. Serno, M. Chechlas, T. Deerrink, P. Allen, M. Ellisman, J. Gleeson; 1Neurobiology, UCSD, La Jolla, CA, 2Neuroscience, UCSD, La Jolla, CA, 3National Center for Microscopy and Imaging Research, UCSD, La Jolla, CA, 4Psychiatry, Yale, New Haven, CT

The axon shafts of neurons contain bundled microtubules, extending through the transition zone may bundle microtubules during axon outgrowth. Among the candidates for mediating microtubule condensation is Doublecortin (Dcx), a microtubule-associated protein with a role in microtubule stabilization and bundling that when mutated in humans disrupts normal cortical development. Phosphorylation has been shown to regulate Dcx microtubule associated functions. We find that that Dcx is associated with Spindophilin (Spn), a protein- phosphatase 1 (PP1) targeting protein, selectively at the base of growth cones. Deletion of Spn results in mislocalization of inactive phosphorylated Dcx, and deletion of either Spn or Dcx results in defective microtubule bundling in axon shafts. Spn/PPI promotes Dcx dephosphorylation specifically at CdK5-phosphorylated S297, which restores the MT bundling effects of Dcx. We propose that the Spn/PPI/Dcx association is important for proper microtubule condensation at the base of the growth cone, leading to constriction of the growth cone and organized microtubule bundling in the axon shaft.

1656 Regulation of Microtubule Formation in Activated Mast Cells by Complexes of γ-Tubulin with Fyn and Syk Kinases
P. Draber, V. Sulimenko, E. Draberová, L. Macík, V. Richterová, P. Draber; 1Biology of Cytoskeleton, Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague, Czech Republic, 2Signal Transduction, Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague, Czech Republic

Aggregation of the high affinity IgE receptors (FceRI) on the cell surface initiates a chain of signaling events culminating in the release of allergy mediators. Although microtubules are involved in mast cell degranulation, the molecular mechanism that controls microtubule rearrangement after FceRI triggering is poorly understood. Here we show that the activation of bone marrow-derived mast cells (BMMCs) induced by FceRI aggregation or treatment with pervanadate leads to a rapid polymerization of microtubules. This polymerization was not dependent on the presence of Lyn kinase as determined by experiments with BMMCs isolated from Lyn-negative mice. One of the key regulators of microtubule polymerization is γ-tubulin. Immunoprecipitation experiments revealed that γ-tubulin from activated cells formed complexes with Fyn and Syk protein tyrosine kinases and several tyrosine phosphorylated proteins from both wild-type and Lyn− BMMCs. Pre-treatment of the cells with Src-family or Syk-family selective tyrosine kinase inhibitors, PP2 or piceatin, respectively, inhibited the formation of microtubules and reduced the amount of tyrosine phosphorylated proteins in γ-tubulin complexes, suggesting that Src and Syk family members are involved in the initial stages of microtubule formation. This notion was corroborated by pull-down experiments in which γ-tubulin interacted stably with the SH2 and SH3 domains of Fyn kinase. We propose that Fyn and Syk kinases are involved in the regulation of binding properties of γ-tubulin and/or its associated proteins, and thus modulate the microtubule nucleation in activated mast cells.

1657 RNA Depletion of γ-Tubulin as a Tool for Studying γ-Tubulin Role in Organization of Acentrosomal Cells
A. Doskociľová, J. Procházková, V. Cenklčová, P. Binarová; 1Institute of Microbiology, Prague, Czech Republic, 2Institute of Experimental Botany, Olomouc, Czech Republic

We studied the downregulation of γ-tubulin in Arabidopsis thaliana by inducible RNAi technique. The RNAi depletion of γ-tubulin led to serious distortions of development in A. thaliana seedlings. We found that cells with decreased levels of γ-tubulin could progress through mitosis, but late mitotic events and cytokinesis were strongly affected. Particularly, we observed that polar distribution of γ-tubulin during late mitosis was disturbed and the phragmoplast formation failed. In contrast to control cells where anaphase spindles were reoriented into the phragmoplast, long anaphase spindles persisted between separated nuclei in RNAi cells. Rearrangement of mitotic microtubules was delayed relative to the nuclear cycle. The cell plate formation sites were often misaligned. These discrepancies in late mitosis and cytokinesis often resulted in bin- or multi-nuclei cells and disruption of regular cell files was observed. The aberrant cytokinesis was also observed in cultured cells of expressing RNAi for γ-tubulin. It is well known that stomata development follows a strict developmental pattern. The stomata patterning was disrupted in RNAi expressing plants. Clusters of two to four stomata were observed in cells with reduced γ-tubulin levels while in plants with severe depletion of γ-tubulin, the cytokinetic defects of guard cells were found in addition to clustering. Cortical MTs of differentiating A. thaliana cells were sensitive to the reduction of γ-tubulin level by RNAi; γ-tubulin rapidly disappeared from its locations from its cortical MTs that became bundled and randomized. Ectopic root hairs formation was observed in cells with randomized MTs, anisotropic growth was disturbed. We suggest that some functions of γ-tubulin that are important for cytokinesis, cell specification and polar growth might be microtubule independent and require further analysis. Supported by grants A50209302 from GA CAS, grant PC 1545-MSMT CR and grant 204/05/I023 GACR.

1658 The Kinesin KIF1C and Microtubule Plus Ends Regulate Cytoskeletal Dynamics in Macrophages
P. Kopp, 1R. Lammers, 2M. Aepfelbacher, 2G. Woehlke, 1T. Rudel, 1N. Macy, 4W. Steffen, 1S. Linder; 1LMU, Munich, Germany, 2Universitaet Tubingen, Tuebingen, Germany, 3UKE, Hamburg, Germany, 4MPI f. Infektionsbiologie, Berlin, Germany, 5Med. Hochschule, Hannover, Germany

The recruitment of the kinesin KIF1C, a member of the Kinesin-3 family, a plus-end-enriched motor which targets regions of podosome turnover. Expression of mutation constructs or siRNA-/shRNA-based depletion of KIF1C resulted in decreased podosome dynamics and ultimately in podosome deficieny. Importantly, protein interaction studies showed that KIF1C binds to microtubules. The absence of microtubules leads to a defect in the podosome localization of KIF1C. In particular, KIF1C depletion led to a disorganization of podosome patterns, which correlates with an alteration of podosome function. This notion was corroborated by pull-down experiments in which γ-tubulin, the protein as a tool for studying γ-tubulin role in organization of acentrosomal cells. Spindle MTs of differentiating A. thaliana cells were sensitive to the reduction of γ-tubulin level by RNAi; γ-tubulin rapidly disappeared from its locations from its cortical MTs that became bundled and randomized. Ectopic root hairs formation was observed in cells with randomized MTs, anisotropic growth was disturbed. We suggest that some functions of γ-tubulin that are important for cytokinesis, cell specification and polar growth might be microtubule independent and require further analysis. Supported by grants A50209302 from GA CAS, grant PC 1545-MSMT CR and grant 204/05/I023 GACR.

1659 Force Regulation of Microtubule Dynamics in Fission Yeast
C. Tischer, D. Brunner, M. Dogterom; 1AMOLF, Amsterdam, The Netherlands, 2EMBL, Heidelberg, Germany

The microtubule cytoskeleton is crucial for generating long range order inside eukaryotic cells. To properly fulfill this function microtubules must adopt an appropriate length distribution, which is governed by their growth and shrinking rates as well as the rates of switching between growth and shrinkage (“catastrophe” and “rescue”). Here, we use the nodulated yeast (S. Pombe) to address the following fundamental questions regarding the organization of interphase microtubules: Are the rates that govern microtubule length spatially regulated? Do the rates adapt to changes in cell size? And, do compressive forces generated as microtubules grow against the cell boundary locally enhance the catastrophe rate? To address these questions thoroughly we developed an image analysis methodology for the fully automated detection of microtubule tips, catastrophes and growth events from movies of GFP-labeled microtubules. Unlike in previous investigations of actin microtubule dynamics, we find that the rate of catastrophe is insensitive to an increase (10%) of the external force acting on the cell surface distribution of microtubule tips and catastrophes makes it possible to directly map the local catastrophe rate constant (\( \text{Crate} \)) for each position within the cell. Analyzing \( >30000 \) catastrophe and growth events in \( \sim400 \) cells, we find that both cytoplasmic catastrophe rate constants and microtubule growth velocities are almost independent on cell size. Cytoplasmic factors, therefore, do not appear to adapt microtubule length to changes in cell size such as occurring during interphase. Furthermore, we find that \( \text{Crate} \) is approximately two-fold enhanced at the cell end regions. We provide evidence that compressive forces arising from growth of microtubules against the cell boundary contribute to this local enhancement of \( \text{Crate} \). Supported by grants A50209302 from GA CAS, grant PC 1545-MSMT CR and grant 204/05/I023 GACR.
Polarized cells, such as neuronal, epithelial and fungal cells, display a specialized organization of their microtubule (MT) cytoskeleton. In the rod shaped fission yeast, Schizosaccharomyces pombe, the interphase MT cytoskeleton has been extensively described by fluorescence microscopy. We have made a large-scale, electron tomography investigation of S. pombe, preserved by high-pressure freezing and freeze-substitution fixation. The results provide the first 3D reconstruction of a full eukaryotic cell volume at electron tomography resolution. Interphase MTs in fission yeast are commonly single or in pairs. Larger bundles of up to nine MTs can assemble, most frequently in bundles associated with the spindle pole body (centrosome), but only a single MT extends towards the cell's tip. Each time that a single MT contacted the plasma membrane, it did so with a fixed end. Such flares at one MT end, coupled with caps at the other, provide evidence about cytoplasmatic MT polarity, information that improves our understanding of MT organization in interphase fission yeast. Individual MT bundles are often composed of antiparallel MTs. Electron dense bridges between MTs themselves and between MTs and the nuclear envelope were frequently observed. We also found an intimate association between MTs and mitochondria. Our analysis shows that the combination of native cell preservation and electron tomography is ideally suited for describing fine ultrastructural details that were not visible with previous techniques.

Electron Tomography Reveals the Molecular Details by Which Fascin and Filbrin Bundle Actin Filaments

M. E. W. Janssen, E. Kim, S. Almo, P. Matsudaira, D. Hanein; 1The Burnham Institute for Medical Research, San Diego, CA, 2Albert Einstein College of Medicine, New York, NY, 3Whitehead Institute, Cambridge, MA

The three-dimensional organization of filamentous actin is essential for biological processes such as cell shape, division, motility, transport and signaling. Actin filaments can be organized in vivo into tightly ordered bundles, whose structural roles of packing appear to be determined by their respective actin bundling proteins. Two of the most abundant actin binders, fimbrin and fascin, are believed to induce actin bundles through crosslinking via a tandem repeat of actin binding domains. Although both proteins bundle actin filaments, they are not interchangeable. While fascin participates in formation of filopodia, filbrin participates in podosome formation at the leading edge of migrating cells. Our aim is to determine the molecular details of actin crosslinking by fimbrin and fascin. Due to the size and the helical nature of filamentous actin, conventional methods such as NMR spectroscopy and x-ray crystallography respectively are not suitable for studying our actin-crosslinker complexes. However, transmission electron microscopy combined with image reconstruction techniques is an excellent tool to determine the structure of these large molecular assemblies. We used electron tomography to obtain three-dimensional electron density maps of actin filaments crosslinked by fimbrin or fascin. Isolation of fascin or filbrin that crosslink actin filaments by various image analysis tools followed by docking of atomic models made it possible to determine the molecular details of the binding interfaces between actin and its respective bundler.

SmgGDS, a Guanine Nucleotide Exchange Factor, Regulates Myosin Organization and Cellular Contraction in Vascular Smooth Muscle Cells

R. Thill, E. L. Lorimer, C. L. Williams; Pharmacology and Toxicology, Medical College of Wisconsin, Milwaukee, WI

The guanine nucleotide exchange factor SmgGDS, promotes nucleotide exchange by several GTPases in both the Ras and Rho families, especially by RhoA. Although RhoA is an important regulator of vascular smooth muscle cell (VSMC) contraction, the expression and function of SmgGDS in VSMC have not been previously investigated. A7r5 cells transfected with SmgGDS siRNA or RhoA siRNA exhibited reduced [3H] thymidine incorporation suggesting that SmgGDS is likely involved in the regulation of cellular proliferation through a Rho or Ras GTPase signaling pathway. Cells with reduced SmgGDS expression have enhanced spreading and no longer exhibit the elongated shape that is characteristic of the VSMC morphology of A7r5 cells. In contrast, cells with reduced RhoA expression exhibit an elongated shape with only minor cell spreading suggesting that SmgGDS regulates VSMC morphology by controlling the activities of multiple small GTPases in addition to RhoA. Subsequent immunoblotting with a human monoclonal IgM antibody against myosin heavy chains revealed a disruption of the myosin filament organization in the cells with reduced SmgGDS expression, which is consistent with inactivation of the Rho signaling pathway. In order to determine whether the absence of SmgGDS affected myosin dynamics through the inactivation of Rho-kinase, the phosphorylation levels of Thr696 and Thr850 on myosin phosphatase (MYPT1) were evaluated following siRNA transfection. Although treatment of A7r5 cells with 3μM of the pharmacological Rho kinase inhibitor, Y27632, significantly inhibited the phosphorylation of Thr850, transfection of SmgGDS siRNA did not affect phosphorylation of MYPT1 at either Thr696 or Thr850. However, transfection of SmgGDS siRNA or RhoA siRNA resulted in an impaired ability of the A7r5 cells to undergo contraction following depolarization of the cell with 90mM K+. Taken together these results identify SmgGDS as a novel regulator of myosin organization and contraction in VSMC.

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Actin Dynamics and Contractility in Oocyte Wound Healing

B. Burkel, W. Bement; Zoology, University of Wisconsin, Madison, WI

F-actin and Myosin 2 play integral roles in both cytokinesis and the closure of cortical wounds. During these events, the cell rapidly assembles a dynamic contractile ring composed of these core proteins. While the importance of F-actin in these contractile structures has been established for some time, accurately assessing its temporal and spatial dynamics has been hindered in part by the shortcomings in available probes for imaging F-actin in vivo. These shortcomings range from perturbation of filament dynamics to the inability to distinguish F-actin from total actin core proteins. While the importance of F-actin in these contractile structures has been established for some time, accurately assessing its temporal and spatial dynamics has been hindered in part by the shortcomings in available probes for imaging F-actin in vivo. These shortcomings range from perturbation of filament dynamics to the inability to distinguish F-actin from total actin core proteins. Here we have characterized a fluorescent fusion protein comprised of the first 261 amino acids of the actin binding protein, utrophin, that overcomes these shortcomings. This fusion protein, Utu-125, accurately reports F-actin populations, but does not alter actin dynamics or impact myosin 2-based contractility. By using this probe in conjunction with various photoactivation techniques, we have established that these contractile structures are divided into discrete sub-populations that differ sharply with respect to their dynamics. Perturbation of actin dynamics through either dominant negative or pharmacological means dramatically impacts not only actin, but also myosin localization and overall contractility. Furthermore, the elimination of myosin-based contractility alters ring formation and myosin localization. Together the evidence supports proper maintenance of actin dynamics and myosin contractility is essential for efficient ring formation and subsequent wound closure.

The Molecular Mechanism of the Inagination and Oocyst Formation during Chick Embryos Development

X. Sai; Sensory Development, RIKEN CDB, Kobe, Japan

The inner ear is formed on the surface ectoderm of the embryos, first appeared denser than most of the surrounding ectoderm epithelium, as development proceeds it gradually forming a deeper depression, followed by internalization and closure. Although we observed this phenomenon easily, but the detail molecular mechanism including in this morphogenesis remain unknow. Here we evidenced that after anic fate decision of F-actin play an important role to convergence apical region and non-muscle myosin II play an important role to expansion the basolateral membrane of the auditory placode. They cooperation to invaginate and close up the otic primordium.

Elucidating the Roles of PIP5K Kinases in the Regulation of the Actin Cytoskeleton

A. M. Corgan, M. Yamaga, J. W. Wang, K. Luby-Phelps, H. L. Yin; Department of Physiology, The University of Texas Southwestern Medical Center, Dallas, TX

Phosphoinositide-4,5-bisphosphate (PIP2) is an important regulator of the actin cytoskeleton and plasma membrane functions. It is synthesized primarily by the type I phosphatidylinositol 4-phosphate 5 kinases (PIPKIs). Mammals have three PIP5K genes (PIP5Kα, PIP5Kβ, and PIP5Kγ), and the γ isozyme has two splice variants: a long 90 kDa and short 87 kDa form. We found that the depletion of each individual PIP5K isoform by siRNA in HeLa cells generated distinct changes in the actin cytoskeleton and signaling responses. The actin phenotype of the PIP5Kγ knockdown (using siRNA directed against both the 90 and 87 kDa isoforms) in HeLa cells is particularly striking: it results in increased actin stress fiber formation and decreased chemotaxis migration. Although PIP5Kγ has already been implicated in focal adhesion formation, its knockdown alone (using the long form specific siRNA) failed to produce these changes. Therefore, the actin phenotype may be primarily due to the depletion of PIP5Kγ. We sought to identify the molecular mechanisms of this actin phenotype. Immunofluorescent studies demonstrated that focal adhesions, as stained by anti-vinculin antibodies, are more abundant in the PIP5Kγ depleted cells. Using real-time interference reflection microscopy (IRM), we found that the turnover of focal adhesions occurs at a much more retarded rate in the PIP5Kγ depleted cells. These results indicate that the observed actin phenotype of PIP5Kγ knockdown cells may be due to an inability of these cells to disassemble focal adhesions, and that the PIP2 generated by PIP5Kγ regulates the uncoupling of the adhesion sites from the actin cytoskeleton.

2006 ASCB Annual Meeting Abstracts
1666 Focal Adhesion Kinase Modulates Tension Signaling to Control Actin and Focal Adhesion Dynamics

M. Schober,1 2 S. Raghaavan,2 A. Pasolli,2 M. Nikokova,2 L. Polak,1 H. E. Beggs,1 L. F. Reichardt,1 E. Fuchs2; 1 The Rockefeller University, New York, NY, 2 Columbia University School of Medicine, New York, NY, 3 Department of Physiology, University of California, San Francisco, CA

Integrins function as crucial transmembrane regulators of a diverse array of cellular processes, ranging from cell adhesion, basement membrane assembly and tissue homeostasis to cell migration and wound-healing. In response to αβ integrin signaling, transducers such as focal adhesion kinase (FAK) become activated, relaying to specific machineries and triggering distinct cellular responses. By conditionally ablating Fak in skin epidermis, we show that FAK is dispensable for keratinocyte adhesion and basement membrane assembly, both of which require αβ integrins. By contrast, we discovered a role for FAK downstream of αβ-integrin in regulating cytoskeletal dynamics and orchestrating polarized keratinocyte migration out of epidermal explants. The Fak-null keratinocytes display an aberrant actin cytoskeleton which is tightly associated with peripheral focal adhesions and microtubules. We show that in keratinocytes lacking FAK, FAs are not able to efficiently activate Src, p190 RhoGAP or PAK, which results in increased signaling by Rho/ROCK as well as myosin light chain kinase. The consequential elevated tension signaling accounts for many of the alterations in cytoskeletal organization and FA dynamics that we see in these cells. These findings have important implications for our understanding of the role of FAK in wound-healing and provide a molecular explanation for why the loss of FAK impairs skin tumorigenesis.

1667 A Mechanism for Regulating the Activity of Zyxin-VASP Complexes

M. D. H. Hansen, B. Adams, P. Bell, E. Bushnell, J. Moody; Physiology and Developmental Biology, Brigham Young University, Provo, UT

Zyxin localizes to sites of active actin polymerization in cells, including sites of cell adhesion. Zyxin contains ActA repeats, protein domains that bind actin regulators of the Ena/VASP family. Zyxin has been proposed to drive actin rearrangements as a scaffold protein: by recruiting VASP family members to cellular sites via ActA repeats. In this model, zyxin functions merely to dock VASP at sites of actin reorganization. However, domain analysis of zyxin suggests a novel mechanism for regulating VASP availability. Here we show that the zyxin αβLim domain inhibits actin binding of another actin binding site. This is demonstrated by expression of zyxin mutants lacking αβLim domains in MDCK cells, which results in increased incorporation of VASP into actin networks at cell-cell junctions. This effect is not observed with full-length zyxin and requires ActA repeats. Consistent with a role for the αβLim region in blocking VASP function in zyxin/VASP complexes, recombinant zyxin αβLim domains precipitate endogenous zyxin and VASP from cell extracts. While the mechanism by which αβLim regions block VASP activity remains elusive, our findings show a direct interaction between VASP and zyxin αβLim domains. Based on this, we propose an auto-regulatory system for controlling the activity of zyxin/VASP complexes. In this model, zyxin-VASP complexes exist in either active or inactive forms. In active complexes, VASP is bound to ActA repeats and is free to drive actin polymerization. In inactive complexes VASP is bound to ActA repeats and zyxin αβLim domains simultaneously blocking VASP from contributing to actin polymerization. A biochemical approach using in vitro reconstitution is employed to test this hypothesis.

1668 Regulation of Polarized F-Actin Organization in Chemotaxing Cells via Biased Delivery of Vesicles Containing WASP, CLP1/2, and RacC to the Leading Edge

J. W. Han, L. R. Leeper, J. S. Gruver, C. Y. Chung; Pharmacology, Vanderbilt University Medical Center, Nashville, TN

The actin cytoskeleton is highly polarized in chemotaxing Dictyostelium cells, with F-actin assembled predominantly in the anterior leading edge and to a lesser degree in the cell’s posterior. It has been demonstrated that WASP localizes on vesicles and these vesicles appear to be preferentially distributed at the leading edge and uropod of chemotaxing cells. We have examined the role of the F-actin organizing proteins, CLP1 and CLP2, in the regulation of polarization of WASP localization and F-actin organization. The F-actin organizing proteins include an amino-terminal FCH domain followed by a coiled-coil domain, proline-glutamic acid-serine-threonine-rich (PEST) sequences, and a SH3 domain. CLP1 and CLP2 appear to be functionally redundant and deletion of both CLP1 and 2 cause a loss of polarity and severe defects in chemotaxis. WASP and CLP1 are colocalized on vesicles and interactions between SH3 domain of CLP1/2 and the proline-rich repeats of WASP are required for vesicular localization. Polarized distribution of vesicles is absent in cells lacking CLP1/2 or RacC, a major regulator of WASP in cell migration. Our results suggest that polarized F-actin organization in chemotaxing cells is controlled by biased delivery of vesicles containing WASP, CLP1/2, and RacC to the leading edge and uropod presumably via interaction of FCH domain of CLP1/2 with microtubules.

1669 Role of Oligomerization of Cdc24, a Key Regulator of Budding Yeast Polarized Growth

C. Mironett; Centre de Biochimie, ISDBCNRS UMR6543, Nice, France

Summary: Polarized growth is a fundamental biological process critical for establishment and maintenance of cell asymmetry, necessary for morphological changes. Highly conserved small GTPases, in particular members of the Rho family, like Cdc42, play an essential role in actin cytoskeleton organization. Guanine nucleotide Exchange Factors activate G-proteins by catalyzing the exchange of GDP for GTP. Cdc24 is the unique GEF for Cdc42 in Saccharomyces cerevisiae, is part of the ubiquitous Dbll family defined by a Dbll Homology catalytic domain adjacent to a Plekstrin Homology domain. These GEFs are strictly regulated in space and time, in yeast, Cdc24 localization varies during the cell cycle. In mammalian cells, GEFs, such as Dbll and LARG, have been shown to homo-oligomerize. Dbl and LARG mutants which are unable to oligomerize, have altered transforming potentials, suggesting that this association is important for regulation of these G-protein activators. Using immunoprecipitation and in vitro binding assays, we have demonstrated that Cdc24 forms homo-oligomers. Analysis of different deletion mutants indicates that the catalytic DH domain of Cdc24 is both necessary and sufficient for homo-oligomerization. Examination of point mutants in the Cdc24 DH domain indicates that mutants defective for oligomerization allow growth as the sole copy and conversely mutants that are not viable as the sole copy, oligomerize normally. We have used the inducible oligomerization domain of Fkbp fused to Cdc24 to force oligomerization in vivo. Constitutive Cdc24 oligomerization resulting in a decrease in active Cdc42, determined by the CRIB pull down method. Using a Cdc24-FKBP-GFP fusion we examined whether constitutive oligomerization affects the localization of Cdc24, which normally shuttles between the sites of growth and division. Using a Cdc24-FKBP-GFP fusion we examined whether constitutive oligomerization affects the localization of Cdc24, which normally shuttles between the sites of growth and division. Our results suggest that constitutive oligomerization of Cdc24 affects the localization of Cdc24, which normally shuttles between the sites of growth and division.

1672 Dia2 Regulates Actin Dynamics and Organization in the Lamella for Efficient Epithelial Cell Migration

S. L. Guntup,1 K. Eisenmann2; 1 Cell Biology, Scripps Research Institute, La Jolla, CA, 2 Van Andel Research Institute, Grand Rapids, MI

A dynamically organized filamentous actin (F-actin) cytoskeleton is required for cell migration. In a migrating cell, the dynamic organization of F-actin is spatially modulated by distinct actin-associated proteins, which mediate polymerization, depolymerization, contraction, bundling, or engagement to the substratum through focal adhesions. Dia2 is a member of the Dia family of GTPases, which have been extensively studied in vitro, but their function in the cell has been less well characterized. Here we study the role of Dia2 activity in the dynamic organization of F-actin in migrating epithelial cells. We find that Dia2 localizes in the lamella of migrating epithelial cells, where it is involved in the formation of a stable pool of cortical actin and protection of free filament barbed ends at focal adhesions. Specific inhibition of Dia2 alters the turnover of focal adhesions and reduces migration velocity. We suggest that the protection of free barbed ends at focal adhesions may be necessary for the formation of a stable pool of cortical lamella actin and the proper assembly and disassembly dynamics of focal adhesions, making Dia2 an important factor in epithelial cell migration.

1673 Spatial-Temporal Observation of Stress Fiber Formation in Nonmuscle Cells and Possible Contribution of Filamin A

K. Kato,1 H. Haga,1 Y. Koyama,1 K. Ohishi,1 T. Ito,1 K. Kawahata; 1 Division of Biological Sciences, Graduate School of Science, Hokkaido University, Sapporo, Japan, 2 Department of Biochemistry, Graduate School of Medicine, Hokkaido University, Sapporo, Japan, 3 Department of Biology, Faculty of Science, Chiba University, Chiba, Japan, 4 Division of Biological Sciences, Graduate School of Science, Kyoto University, Kyoto, Japan

Spatial distribution of stress fibers in a nonmuscle cell is one of the most important factors that determine the mechanical behaviors of cells, such as cellular movement and the maintenance of its shape. We consider that filamin A (FLNa), one of the actin-binding proteins, regulates the formation of stress fibers. We previously compared the spatial distribution of stress fibers between FLNa-deficient human melanoma cells (M2 cells) and M2 derived cell line expressing FLNa (A7 cells). In A7 cells, dorsally formed stress fibers were observed, whereas in M2 cells were not. These results indicate that the expression of FLNa affects the spatial distribution of stress fibers. However, the process of stress fiber formation was not elucidated. The purpose of this study is to clarify the dynamics of stress fiber formation and the role of FLNa in this process. Time-lapse observations of stress fibers in A7 and M2 cells were performed immediately after cells were

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plated on a glass substrate. To observe the stress fiber formation, cells were transfected with an expression plasmid for GFP-tagged myosin regulatory light chain were examined using fluorescence confocal microscopy. As the first step of stress fiber formation, bundles of stress fibers were formed at the basal periphery in A7 cells. Then, some of stress fibers unraveled from these basal bundles and moved toward apices of the cells. This upward movement of stress fibers was not observed in M2 cells. We also performed RNAi experiments to specifically knock down FLNa in HT1080 cells, human fibrosarcoma cells. Specific knock down of FLNa in HT1080 cells resulted in the decline of the stress fiber bundles formed at basal periphery in the initial step of stress fiber formation. We consider that the FLNa-dependent formation of stress fiber bundles at the basal periphery is significant in the formation of dorsal stress fibers.

1674 Perturbation of Non-Muscle Myosin IIb by Chromophore-assisted Laser Inactivation (CALI) Leads to Severing of Actin Stress Fibers, Tearing of the Plasma Membrane, and Change of Overall Cell Cytoskeletal Structure

F. -. S. Wang,1 2 D. Lenz,2 J. P. Robinson,2,3,4 X. P. Zeng1; 1Biological Sciences, Purdue University Calumet, Hammond, IN, 2Bindley Bioscience Center, Purdue University, West Lafayette, IN, 3Dept. of Basic Medical Sciences, Purdue University, West Lafayette, IN, 4Weldon School of Biomedical Engineering, Purdue University, West Lafayette, IN

Actin stress fibers are thought to be actively tensed by myosins motors and to function as flexible and resilient cables that structurally reinforce the cytoskeletal structure of a cell. They are important for maintaining cell shape and regulating cell motility (Ingber, 2003; Kumar et al., 2006). Non-muscle myosins have been shown to display periodic distribution along stress fibers and are essential in stress fiber assembly and maintenance (Cramer et al., 1997; Hotulainen and Lappalainen, 2006). In this study, we describe the effect of perturbing non-muscle myosin IIb function by chromophore-assisted laser inactivation (CALI). CALI utilizes laser irradiation to inactivate a protein molecule bound by a specific chromophore-conjugated antibody. The specificity of target selection and the ability for instantaneous inactivation at a specific time and location make CALI a powerful tool in probing functions of biomolecules in physiological processes with a high spatiotemporal resolution. Chicken embryonic fibroblasts transiently expressing EFGP-actin were microinjected with malachite green-labeled anti-non-muscle myosin IIb monoclonal antibodies (Conrad et al., 1995). Selected areas of the cells were subjected to laser irradiation while being monitored by time-lapse video microscopy. Our results show that perturbation of non-muscle myosin IIb function by CALI causes an immediate diffusion of EGF fluorescence away from the center of the laser spot and an instant severing of actin stress fibers within the irradiated area. The severing of actin stress fibers correlates with the breakdown of cell cytoskeletal structures and the tearing of the plasma membrane or a complete disintegration of an entire cell. Maximum tearing of cell membrane is reached within 2 minutes following the severing of actin stress fibers while re-sealing of the plasma membrane is achieved in about 10 minutes. Our data demonstrated that non-muscle myosin IIb is essential in maintaining actin stress fiber integrity, membrane-cytoskeletal structure, and overall cell shape.

1675 Dynamics of Myosin II during Mitosis and Cytokinesis of Drosophila S2 Cells

J. Spudich, R. Uehara, G. Goshima, R. Vale; Physiology Course 2006, Marine Biological Laboratory, Woods Hole, MA

Myosin II is one of the earliest components of the contractile ring to appear in the equatorial cortex during mitosis. In Drosophila S2 cells, myosin is initially localized to the equatorial cortex by signals from rho1 and rho kinase. A second rho1-dependent pathway that leads to formation of a specific actin filament assembly involving formin and profilin is necessary to stabilize the myosin at the equatorial cortex (Dean, S.O., Rogers, S.L., Stueuman, N., Vale, R.D. and Spudich, J.A., Proc Natl Acad Sci USA 102:13473-13478, 2005). We have used FRAP analysis to examine the dynamics of the turnover of myosin II in the equatorial cortex at the earliest stage of recruitment, after stabilization into a contractile ring, and at various times after cytokinesis proceeds. We compared these dynamics to that of a “myosin II cloud” found in the spindle region just prior to initial myosin recruitment to the equatorial cortex. We found that the recovery rate of GFP-tagged myosin II regulatory light chain (GFP-RLC) in the equatorial cortex after photobleaching is slower than that of the myosin II cloud, and it dramatically decreases upon the metaphase-anaphase transition, and further decreases as constriction of the contractile ring occurs. Cells containing GFP-RLC-E20E21 (a phospho-mimic form of the RLC) show delayed fluorescence recovery at all stages compared to wild type cells, indicating the importance of rho kinase phosphorylation control on the myosin dynamics. FRAP of these cells was also used to explore whether the origins of the myosin in the contractile ring are from cortical flow or from the equatorial cytoplasm.

1676 Development of a Fluid Dynamical Model, the Cylinder-thinning Model, for Cytokinesis

E. M. Reichl,1 M. Delanoyx,1 J. Effler,2 D. N. Robinson3; 1Cell Biology, Johns Hopkins University, Baltimore, MD, 2Electrical and Computer Engineering, Johns Hopkins University, Baltimore, MD

Cytokinesis is the process by which a mother cell divides to form two daughter cells. Historically, this process was modeled as a constriction of concentric rings of actin and myosin (the Purse-string Model). Our studies of the cleavage furrows of dividing Dicystostelium cells by electron microscopy and fluorescent microscopy do not show the presence of a ring structure. Instead, actin filaments appear disordered or interpolar. Furthermore, Dicystostelium cells lacking myosin II are able to complete cytokinesis, but with altered bridge morphology and furrow-thinning kinetics. Addition of a 10-fold slower myosin II rescues the myosin II mutant morphology and kinetics of furrow-thinning, but not the stress modulus. The global actin crosslinkers dynamin and fimbrin also contribute to the dynamics and cortical mechanics of Dicystostelium cells. Removal of these crosslinkers increases the rate of furrow-thinning and reduces the stress modulus compared to wild type cells. These results suggest that an alternate model for the forces driving cytokinesis is necessary. Our data is being used to evolve the Cylinder-Thinning Model, which includes both passive and active properties of the cortex to account for cytokinesis. Overall, our structural and mechanical data are providing insight into the contractile mechanism of cell division.

1677 Interaction of the Calmodulin-like Domain of α-Spectrin with β-Spectrin and Its Regulation by Calcium

Z. Zhang,1 X. An,1 X. Gao,1 A. Baines,4 N. Mohandas; 1Red Cell Physiology, New York Blood Center, New York, NY, 2Department of Biosciences, University of Kent, Canterbury, Kent, United Kingdom

Erythrocyte spectrin is composed of α- and β-spectrin subunits which form anti-parallel dimers through lateral association, and tetramers through head-to-head association of dimers. The subunits are arranged in an anti-parallel fashion such that the N-terminus of β-spectrin is in apposition to the C-terminus of α-spectrin. At the C-terminus of the α-chain is a calmodulin-like domain (CLD) that has four EF hands. The N-terminal region of β-spectrin contains the actin/4.1 binding domain (ABD) and the first triple helical repeat (R1). In another member of the spectrin superfamily, α-actinin, the CLD interacts with the ABD-R1 region on the opposite subunit. We hypothesized a similar interaction in erythrophil spectrin. In this study, we construct a β-spectrin containing the N-terminal ABD through R1 (β CLD) that is able to interact with the α CLD domain. The interaction was observed by fluorescence energy transfer experiments suggested a conformational change in the CLD and EF 3-4 fragment upon calcium binding. The short linker in β-CLD-spectrin that joins the ABD to R1 contains a region predicted to form a basic amphiphilic α-helix, reminiscent of sequences likely to bind calmodulin. We tested the significance of this region by mutagenesis. Mutation of highly conserved valine residues to alanine or glutamic acid significantly reduced the interaction. We conclude that α-CLD binds to the region of β-CLD that links the ABD to R1 in a Ca2+-sensitive manner. Since residues critical for this interaction are conserved in many β-spectrins, this interaction is likely to occur in most, if not all, spectrins.

1678 Characterization of Cellular Titin in Human Megakaryocyte, Mouse Fibroblast, and Deer Fibroblast Cell Lines

P. J. Cavanr, S. G. Olenych, T. C. Keller; Department of Biological Sciences, Florida State University, Tallahassee, FL

We previously discovered a large titin-like protein - c-titin - in chicken epithelial brush border and human blood platelet extracts that binds alpha-actinin and organizes arrays of myosin II bipolar filaments in vitro. We have investigated the molecular relationship between c-titin and striated muscle titin by RT-PCR analysis of total RNA from human megakaryoblastic (CHRF-288-11) and mouse fibroblast (3T3) nonmuscle cells. Amplified products obtained contain sequences identical to known titin gene exon sequences that encode parts of the Z-line, I-band, PEVK domain, A-band, and M-line regions of striated muscle titins. Products containing Z-line exons encode the Z-repeat domains Zr1-3 and 7 and the Zq domain, which bind alpha-actinin in striated muscle titins. I-band region sequences encode the ZA2 exons, but none of the exons distinctive for the N2B or Novex I and II isoforms. Products from the A-band and M-line regions contain the long titin gene exon that encodes an Ig-FN3 domain superrepeat region important for myosin filament binding and the exon that encodes the kinase domain. We found two variants containing PEVK-encoding exons not known to be expressed in muscle. These sequences are differentially spliced in patterns not reported for any striated muscle titin isoform. The data suggest that rabbit polyclonal antibodies raised against expressed protein fragments encoded by the Z-repeat and kinase domain regions to investigate the relationship between the RT-PCR products and the c-titin protein. Both antibodies react with the c-titin band in Western analysis of platelet extracts and immunoprecipitate c-titin in whole platelet extracts. Immunofluorescent localization
reveals the majority of the c-titin colocalizes with alpha-actinin and actin in 3T3 and Indian Muntjac deer skin fibroblast stress fibers. Our results suggest that differential expression of titin gene exons in nonmuscle cells yields multiple novel isoforms of the protein c-titin which are incorporated with the actin stress fiber structures.

1679 Smitin Interactions with Smooth Muscle Contractile Apparatus Proteins
R. J. Chi, A. R. Simon, S. G. Olenych, T. C. S. Keller; Biological Science, Florida State University, Tallahassee, FL
The striated muscle Z-line is a complex network of proteins in which N-terminal domains of titin interact with the actin filament cross-linker alpha-actinin, the titin cross-linker telethonin/Tcap, and other proteins to establish and maintain the structural integrity of the sarcomere. We previously discovered a titin-like protein - smitin - in smooth muscle cells suggesting that similar interactions may exist in the alpha-actinin rich dense bodies and dense plaques that act as the smooth muscle equivalent of the Z-line. RT-PCR analysis of smooth muscle RNA and western blot analysis with titin domain-specific antibodies has revealed that smitin contains the alpha-actinin-binding Z-repeat 1-3, and Zq domains and telethonin/Tcap-binding Z1-Z2 domain encoded by the titin gene. We have investigated whether loops in the alpha-actinin R2 and R3 spectrin-repeat domains that are modeled to lie in proximity on the surface of the central rod and are highly conserved between smooth and striated muscle alpha-actinins constitute the binding site for the Zq domain. We have found the mutations A423A, T425A or T427D in the R2 loop and N586A in the R3 loop decrease binding to expressed Zq domain in GST-pulldown and solid phase binding assays. We also have found expressed Zq peptide migrates on HPLC size exclusion chromatography as an apparent helical dimer, further supporting our proposed model for the R2-R3 loop interaction with the Zq domains of titin and smitin. Further sensitive RT-PCR analysis of RNA from multiple smooth muscle sources revealed evidence for T-cap transcripts in commercially available human bladder, uterus and carotid artery smooth muscle RNA and in A7r5 rat aortic smooth muscle cell RNA. These results suggest T-cap may be present in smooth muscle cells, where it could provide additional stability to the smitin anchorage in the dense bodies, functionally similar to its role in striated muscle.

1680 Focal Adhesions: Structural and Signaling Platforms for Keratin Filament Precursor Formation
F. W. Flitney,1 K. M. Ridge,2 R. D. Goldman1; 1Cell & Molecular Biology, Northwestern University, Chicago, IL, 2Division of Pulmonary & Critical Care Medicine, Northwestern University, Chicago, IL
A Soluble Pool of Keratin 18 Is Incorporated into Endogenous Intermediate Filaments(IF) in Human Alveolar Epithelial Cells Under Flow
F. W. Flitney, K. M. Ridge,2 R. D. Goldman1; 1Cell & Molecular Biology, Northwestern University, Chicago, IL, 2Division of Pulmonary & Critical Care Medicine, Northwestern University, Chicago, IL
The keratin intermediate filament (IF) network in lung epithelial cells is severely disrupted when cells are exposed to shear stresses of ~30 dynes/cm² for up to 24 hr. Here we report on the effects of shorter periods of flow (<6 hr) at lower shear forces (7-15 dynes/cm²) on keratin IF in human A549 alveolar epithelial cells. Cells were grown on glass microscope slides and placed in a laminar flow chamber generating uniform shear stresses of 7 or 15 dynes/cm² for up to 6 hr. They were then either (a) fixed in formaldehyde or methanol and immunostained with a rabbit anti-keratin antibody, or (b) extracted briefly (5 min, on ice) with 0.5% Triton-X100 in an IF stabilization buffer, to examine the protein composition of TX-100 soluble and insoluble fractions. Control cells were not exposed to flow but were maintained under static culture conditions throughout. Immunofluorescence revealed an extensive but delicate meshwork of fine IF in control cells. Western blots showed that a significant quantity of K18 was present in the TX-100 soluble fraction in untreated cells (n = 9), amounting to ~ 9% of the total cellular K18. This pool of K18 decreased substantially (~60%) when cells were exposed to flow (p<0.02; n = 7). Immunostaining showed that the reduced solubility of K18 caused by shearing the cells coincided with the appearance of thicker, often ‘wavy’ bundles of keratin IF (tonofibrils). This was verified at higher resolution by electron microscopy. Our results suggest that shear stress causes the soluble pool of K18 to become integrated into the endogenous IF network. We postulate that the enhanced filament ‘bundling’ induced by flow serves to reinforce the network and improve the ability of cells to resist mechanical forces. Supported by NHLBI.

1684 Cytokine-controlled Organization and Scaffolding Functions of Keratin Filaments: Implications for MAP Kinases-regulated Migration
S. Osmanagic-Myers, M. Gregor, G. Walko, G. Burgstaller, S. Reipert, G. Wiche; Molecular Cell Biology, University of Vienna, Vienna, Austria
Plectin is a major intermediate filament (IF)-based cytokine protein that stabilizes cells and tissues mechanically, regulates actin filament dynamics, and serves as a scaffolding platform for signaling molecules. Our recent studies with primary and immortalized mouse keratinocytes showed that plectin deficiency causes an aberrant organization of the keratin cytoskeleton. As revealed by electron microscopy, keratin IFs in plectin-deficient cells lack orthogonal cross-bridges, leading to lateral bundling and increased mesh-size of IF networks. Such keratin networks...
were found to be more susceptible to osmotic shock-induced fragmentation from peripheral areas, and their okadac acid-induced disruption (paralleled by stress-activated MAP kinase p38 activation) proceeded faster. Furthermore, we found basal activities of MAP kinase Erk(1/2) and of the membrane-associated upstream protein kinases c-Src and PKCδ to be significantly elevated, and migration rates to be increased. The latter was assessed by in vitro wound closure assays and time-lapse microscopy of single cells. Forced expression of RACK1, which is the plectin-binding receptor protein for activated PKCδ, in wild-type keratinocytes elevated their migration potential close to that of plectin-null cells. These data establish a link between cytoskeleton-controlled cytoscape/substrate scaffolding of keratin IFs and specific MAP kinase cascades mediating distinct cellular responses.

1685 Monitoring of Caspase Activation Using Novel Keratin Fragment-specific Antibodies
G. Tao,1 M. Teivola,1 Q. Zhou,1 P. Strnad,1 N. Sandersona,1 D. H. Li,1 A. Hong,2 M. B. Ormay1; 1Palo Alto VA Medical Center and Stanford University, Palo Alto, CA, 2AnSpec, Inc, San Jose, CA
Keratins 18 and 19 (K18/K19) are epithelial type I intermediate filament (IFs) that form an intracellular fibrillar network with their type II IF partner, K8. Previous studies demonstrated apoptosis-induced caspase cleavage at the highly conserved K18 or K19 Asp237, but this cleavage can only be assessed using biochemical means such as immunoblotting. During apoptosis, K18 is initially cleaved at Asp396 followed by digestion at Asp373. In order to more effectively study K18 and K19 proteolysis during apoptosis, we generated antibodies that selectively recognize the K18 and K19 Asp373-cleaved products by immunizing rabbits with K18 and K19 peptides that expose the Asp373. The generated antibodies recognized only cleaved but not intact K18 and K19 as determined by blotting or immunofluorescence staining of cultured human HT29 cells treated with anisomycin or of livers isolated from mice injected with Fas to induce hepatocyte apoptosis. The antibodies to the K18 and K19 peptides both recognized the common VEVD motif as determined by immunoblotting lysates of cells transfected with K8/K18 or K8/K19. Specificity of the antibodies to the K8/K19 caspase motif was further confirmed by their inability to recognize the caspase-targeted sequence VEMD in the stratified epithelial keratins, K14-K17, as determined by immunoblotting purified K14 that was pre-incubated with caspase-3. Versatility of the antibodies was also verified by their ability to selectively immunoprecipitate cleaved K18 or K19 after induction of apoptosis in cultured cells. Apoptosis-induced sequential cleave of K18 at Asp396 then Asp373 was confirmed by blotting lysates of HT29 cells that were treated with anisomycin for various times. Therefore, the newly generated anti-K18/K19 Asp373 directed antibodies provide an important adjunct for studying K18 and K19 apoptosis in various human and mouse epithelia using biochemical or biophysical or tissue imaging modalities.

1686 Interaction of the C-terminal Kinesin KIFC3 with Keratins in Polarized MDCK Epithelial Cells
A. R. Belkin, D. Cohen, A. Munch; Tyson Vision Research Institute, Cornell Medical College, New York, NY
Epithelial cell polarization results in distinct apical and basolateral cellular domains and is accompanied by microtubule reorganization from a radial, centrosome-focused array into a non-centrosomal array. This microtubule organization is thought to facilitate polarized vesicular transport, and microtubule disruption inhibits the maintenance of epithelial luminal domains in vivo. Keratin intermediate filament array is abundant around the centrosome and connect to both the subapical and lateral cell cortex. Keratins are therefore good candidates to play a role in microtubule organization. We studied the function KIFC3, a minus-end directed microtubule motor of the kinesin-14 family, in polarized MDCK cells. Expression of a dominant negative KIFC3 (DN-KIFC3) lacking the microtubule motor domain results in a failure of cells to polarize. Cells form junctions, but fail to deliver normal amounts of an apical marker gp135/podocalyxin to the apical cell surface, fail to compact and grow to a normal size, fail to reorganize microtubules into a polarized array, and fail to form a lumen in a collagen overlay assay, an assay that mimics the formation of lumen by polarized epithelia in vivo. KIFC3 localizes to the centrosome and to desmosomes of polarized MDCK cells. In addition, puncta of KIFC3 colocalize with keratin 19 (K19). Expression of GFP-KIFC3 brought a fraction of K19 from the Triton-insoluble fraction into the soluble fraction. HA-tagged KIFC3 or DN-KIFC3 expressed in a GFP-K19 cell line that incorporates the recombinant keratin into filaments, efficiently co-immunoprecipitated with GFP-K19. Microtubule regrowth after cold depolymerization showed that microtubules grew from the centrosome into desmosomes. However, microtubule regrowth in cells expressing DN-KIFC3 was characterized by fewer microtubules that grew in a disorganized fashion without a central aster. We speculate that KIFC3 could be important for keratin organization along keratin filaments. Defects in cell polarity due to DN-KIFC3 could stem from a role of KIFC3 in microtubule organization.

1687 Relationship between Keratins and AKT Anti-apoptotic and FAS Pro-apoptotic Signaling Pathways in Human Hepatocellular Carcinoma Cells HepG2
I. Laboubu, A. Fortier, V. Villemagne, É. Asselin, M. Cadrin; Cellular and Molecular Biophysics Group, Departments of Chemistry and Biology, University of Québec at Trois-Rivières, Trois-Rivières, PQ, Canada
Keratin 8 and 18 (K8/18) are the only intermediate filament (IF) proteins present in hepatocytes. One of their functions is to protect hepatocytes from mechanical and non-mechanical stresses. The molecular mechanisms underlying this function are not yet fully understood. Hepatocellular carcinoma cells HepG2, which contain only K8/18 were used as a model system to investigate keratin regulation following a treatment with griseofulvin (GF) [0,07 and 0,2 μg/ml in 0.1% DMSO] for 8, 24, 48 and 72h. Because the serine/threonine kinase Akt/PKB (pAkt) plays a pivotal role in hepatocytes cell survival and that Fas receptor (Fas-r) is an important inducer of the extrinsic apoptosis signaling pathway we investigated if a relationship exist between K8/18 and these molecules. We first analyzed by Western blotting the effects of GF treatments on K8/18 phosphorylation. K8 pS73 and pS341 and, K18 pS52 were significantly increased following treatment with GF. K18 pS52 was increased but to a lesser extent. Treatment of cells with P53 inhibitors (Wortmannin and LY294002) inhibited Akt phosphorylation and K8 phosphorylation on S73 suggesting that K8 is a substrate for pAKT. By double immunofluorescence staining we show that Fas-r and pAkt co-localize with IF network in some cells. GF treatment increases Fas-r and pAKT rearrangement and they both re-localize at the plasma membrane to form a membrane cap. Phosphorylated keratins are associated with these structures. These results suggest that keratins accomplish their protective roles by interacting with pAKT a central player in cell survival. Keratins also associate with Fas-r an important receptor involved in transmission of death signal. Changes in K8/18 phosphorylation could be involved in the translocation and release of the regulatory molecules pAKT or Fas-r to the membrane. Keratins could act as a key regulator in maintaining the delicate balance between pro and anti-apoptotic signal molecules in hepatocytes. (Supported by NSERC)

1688 CAPC Is Associated with Intermediate Filament Cytokeratin18 and Expression of CAPC Stimulates Malignant Phenotypes In Vitro
X. Liu, T. Beri, D. Ha, Y. Man, B. Lee, I. P nasti; NC1, NIH, Bethesda, MD
CAPC is a recently discovered gene; its RNA is expressed at a high level in many cancers, including breast cancer, prostate cancer, colon cancer and pancreatic cancer. In normal tissues CAPC RNA is highly expressed in normal prostate and salivary gland, very weakly expressed in colon, pancreas, intestine and not detectable in other tissues. Using a polyclonal antibody to CAPC we have found that CAPC protein co-localizes with intermediate filaments and cytokeratins in the breast cancer MCF 7 cell line. We now further characterized CAPC by western blot analysis and confocal microscopy studies. CAPC protein is elevated during mitosis in many cancer cell lines including the pancreatic cancer cell lines HTB-79 and HTB85; ovarian cancer cell lines OVCAR3 and HTB77; prostate cancer cell line LNCAP and PC3; colon cancer cell lines Lovo and SW403; breast cancer cell lines MDA-231, BT20 and MCF7, but not in normal human fibroblasts, WI-38, and normal breast cell line MCF-10A. CAPC is highly enriched in the cytoskeleton fraction and by confocal microscopy co-localizes specifically with keratin 18, but not keratin19. Using a Boyden chamber assay stable over-expression of CAPC in MDA-MB-231 cells leads to increased cell migration and invasion compared to the parental cell line. Taken together, CAPC is associated with keratin 18 and appears to be involved in regulating cancer cell motility and invasion.

1689 The Functional Significance of Vimentin IF Expression during the Epithelial to Mesenchymal Transition (EMT) in Human Embryonic Stem Cells (hESC) and in Mammary Duct Epithelial Cells
M. G. Méndez, R. D. Goldman; Cell and Molecular Biology, Northwestern University, Chicago, IL
Pluripotent hESC are cuboidal epithelial cells that express keratins (Type I and II IF). We have found that vimentin (Type III IF) and nestin (Type IV IF) are also expressed during the EMT which represents the earliest stage of hESC differentiation. Expression of vimentin, and in some cases nestin, also accompany the EMT in metastatic cells of epithelial origin. Morphometric analyses show that the expression of vimentin and nestin are closely correlated with the alterations in cell shape and migratory behavior that take place during the EMT at the periphery of hESC colonies. Pluripotent hESC grow in tightly-packed colonies of cuboidal epithelial cells. The cells at the edges of these colonies are induced to undergo the EMT by cessation of daily medium changes, during which nestin is expressed as non-filamentous particles. Subsequently, vimentin is subsequently expressed as particles, which are converted into short IF that also contain nestin. As these short IF link up into long vimentin/nestin IF, the hESC acquire the typical shape and motile behavior of mesenchymal cells. Likewise, only keratin IF are expressed in MCF-7 mammary gland ductal epithelial cells. Following the microinjection of vimentin protein, particles, short and eventually long IF are assembled as these cells assume a more
messenchymal shape. Following transfection with vimentin cDNA, vimentin-IF expressing MCF-7 cells are mesenchymal in shape and more motile, as demonstrated by their accumulation at colony edges and their migration away from colonies. We have initiated experiments to determine the function of vimentin and/or vimentin/nestin in the changes in cell shape and motility seen in the EMT. We hypothesize that vimentin expression in epithelial cell lines introduces a new type of cytoskeletal crosstalk between IF, microtubules and their associated motors, which underlies the EMT. Funded by NIGMS, an NIH Stem Cell Center Award and an NCI Training Grant.

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Variant Isoforms of the Intermediate Filament, Synemin, Regulate Its Subcellular Location and the Function of Its AKAP Domain
J. P. Kerr,1 L. Lund,2 M. Russell,3 M. Bond,3 Department of Physiology, University of Maryland, Baltimore, Baltimore, MD, 4Department of Biological Sciences, Kent State University, Warren, OH

Synemin, an intermediate filament (IF) which is believed to crosslink Z-discs and myofilaments with the costamere and intracellular organelles, is able to bind the regulatory (RII) subunit of cAMP-dependent protein kinase A (PKA) through an amphipathic helical domain within its C-terminal tail, making synemin an A-kinase anchoring protein (AKAP). AKAPs regulate PKA distribution by binding RII to target PKA near substrates. Using plazqe display with a human heart library to identify novel RII-binding peptides, our lab identified synemin as a potential AKAP. In confirmation of these results, we further demonstrated that synemin binds RII via blot overlays and co-immunoprecipitates with RII from adult cardiomyocytes. In order to explore the functional relevance of synemin’s AKAP domain, we showed colocalization of synemin with RII at Z-lines and the perinuclear region using immunohistochemical studies with adult rat ventricular myocytes. Interestingly, in isolated rat neonatal CMs, synemin localized to the sarcolemma but did not colocalize with filamentous, cytoplasmic desmin. Furthermore, there are two known isoforms of synemin (α and β) that differ by a 932bp deletion in the C-terminal domain. Using antibodies specific to each isoform, we demonstrated that the two isoforms differ in the subcellular location and that α- and β-synemin preferentially distribute to cellular striations. A polyclonal antibody generated against α-synemin recognizes the 210KDa α isoform, and also recognizes a 100KDa band via western blots of cell lysates from cardiomyocytes, suggesting a new putative isoform. Using affinity-tagged synemin constructs with cultured cells, we are isolating the multimolecular protein complexes formed by each synemin isoform. We hypothesize that these isoforms will have variable subcellular locations and binding partners. We conclude that synemin isoforms have different functions and may act to target PKA near different substrates within the myocyte.

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Interactions of Intermediate Filament Protein Synemin Isoforms with Vinculin in Mammalian Muscle Cells
N. Sun,1 Z. Li,2 D. Paulin,3 D. R. Critchley,1 R. M. Robson,1 Muscle Biology Group, Iowa State University, Ames, IA, 2Pierre et Marie Curie University, Paris, France, 3Biologie Moleculaire de la Differentiation, Universite Paris, Paris, France, 4Department of Biochemistry, University of Leicester, Leicester, United Kingdom

Synemin is a large, unique member of the intermediate filament (IF) protein superfamily. Synemin with desmin, the major IF protein in striated muscle cells, comprise the heteropolymeric IFs that encircle and link the Z-lines of all adjacent myofibrils, and the Z-lines of the peripheral layer of cellular myofibrils to the costameres located periodically along and subjacent to the sarcolemma. Synemin is expressed as only one form within avian muscle cells, but as at least two isoforms, α and β, within mammalian muscle cells. α-Synemin, considered the ortholog of avian muscle synemin, is larger than β-synemin, containing an extra 312 amino acid insert near the end of the long C-terminal tail. Previous studies from our lab showed that tissue-purified α-synemin interacts with α-actinin and vinculin. However, primary sequence identity between avian and mammalian synemins is less than 37%. In this study we examined interaction of mammalian α- and β-synemin isoforms with both vinculin and the slightly larger isoform, metavinculin. Solid-phase protein-protein binding assays (blot overlays) demonstrated that the specific region within mammalian α-synemin (312 amino acid insert) interacts directly with both vinculin and metavinculin in vitro. Co-immunoprecipitation studies, using antibodies to vinculin, precipitated both vinculin and synemin from cultured rat smooth muscle (A-10 cells) lysates. Immunofluorescence staining of A-10 cells showed co-localization of synemin and vinculin within focal adhesions. Transient expression of the enhanced green fluorescent protein (EGFP) recombinant 312 amino acid insert of α-synemin in A-10 cells indicated this insert was specifically incorporated within focal adhesions and actin stress fibers. These results suggest that α-synemin may serve a critical cross linking role enabling desmin/synemin-containing heteropolymeric IFs to link the Z-lines of all adjacent myofilbrils and the Z-lines of peripheral layer of cellular myofibrils to the costameres located periodically along and subjacent to the sarcolemma. (Supported by USDA-CSREES-NRICGP Award 2003-35206-12823)

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A Model for the Mechanism of GFAP Mutation in Alexander Disease
R. Tian; Physiology, Columbia University, New York, NY

Most cases of Alexander Disease (AxD) are associated with mutations in glial fibrillary acidic protein (GFAP), an intermediate filament protein predominantly expressed in astrocytes. To determine the effects of GFAP mutations, we have developed an efficient and reproducible method to generate astrocytes stably expressing mutant GFAP (R239C GFAP) by retroviral gene expression and 8-bromo-cAMP (8C-cAMP) differentiation. RC2.E10 cells were derived from primary embryonic cells (E16) of the rat cortex and immortalized by stable retroviral infection with the temperature-sensitive mutant of the SV40 T antigen at 33 °C. Upon infection with retroviral construct expressing eGFP, wildtype GFAP-eGFP and mutant GFAP-eGFP respectively, green cells were selected by FACs and maintained at 33 °C. The stable clonal cells were then transferred to 37 °C to inactivate the SV40 T antigen activity and a cessation of proliferation. The astroglial differentiation was initiated by adding 8b-cAMP and continued by incubation at 37 °C for 4 days. Astrocytes derived in this manner are GFAP+, S-100β+, Vim+, GLT-1 and GLAST+, MBP+, Tuj1+, Pax6-, NeuN-, Oligo2-. Particularly, the heterogeneity in the GFAP expression mirrors a typical histopathological feature of astrocytes in brain tissues of Alexander patients. Thus, these transformed astrocytes appear to be an excellent experimental model for AxD.

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Aggregations of GFAP after Thermal Stress in C6 Glioma Cells
T. W. Tseng, K. Lu, W. Lee, C. Chien; Anatomy and Cell Biology, National Taiwan University, College of Medicine, Taipei, Taiwan

Some intermediate filament (IF) proteins expressed in the development of glia include nestin, vimentin, and glial fibrillary acidic protein (GFAP). However, GFAP is the major intermediate filament protein of mature astrocytes. To determine the organization of GFAP in glial cells, rat GFAP cDNA tagged with enhanced green fluorescent protein (EGFP) was transfected into the rat C6 glioma cell line. After selection, two stable C6-EGFP-GFAP cell lines were established. Stable C6-EGFP-GFAP cell lines with or without heat shock treatment were analyzed by immunocytochemistry, electron microscopy, and Western blot analysis. In the transient transfection study, EGFP-GFAP transiently expressed in C6 cells formed punctate aggregations in the cytoplasm right after transfection, but gradually a filamentous structure of EGFP-GFAP was observed. The protein level of nestin in the C6-EGFP-GFAP stable cells was similar to that in the pEGFP-C1 transfected C6 stable clones and non-transfected C6 cells, whereas the level of vimentin was reduced in Western blotting. Interestingly, the expression level of small heat shock protein 9B-crystallin in C6-EGFP-GFAP cells was also enhanced after EGFP transient expression. The aggregation of GFAP was dispersed as a fine filamentous structure. However, after heat shock treatment, GFAP formed IF bundles in C6-EGFP-GFAP cells. In the meantime, α-crystallin also colocalized with IF bundles of GFAP in C6-EGFP-GFAP cells. We have developed an efficient method to generate astrocytes stably expressing mutant GFAP (R239C GFAP) by retroviral gene expression and 8-bromo-cAMP (8C-cAMP) differentiation. RC2.E10 cells were derived from primary embryonic cells (E16) of the rat cortex and immortalized by stable retroviral infection with the temperature-sensitive mutant of the SV40 T antigen at 33 °C. Upon infection with retroviral construct expressing eGFP, wildtype GFAP-eGFP and mutant GFAP-eGFP respectively, green cells were selected by FACs and maintained at 33 °C. The stable clonal cells were then transferred to 37 °C to inactivate the SV40 T antigen activity and a cessation of proliferation. The astroglial differentiation was initiated by adding 8b-cAMP and continued by incubation at 37 °C for 4 days. Astrocytes derived in this manner are GFAP+, S-100β+, Vim+, GLT-1 and GLAST+, MBP+, Tuj1+, Pax6-, NeuN-, Oligo2-. Particularly, the heterogeneity in the GFAP expression mirrors a typical histopathological feature of astrocytes in brain tissues of Alexander patients. Thus, these transformed astrocytes appear to be an excellent experimental model for AxD.

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Proper Perinuclear Localization of the BLOC-1-linked TRIM-like Protein Myosynr Requires Its Binding Partner Desmin
A. Kouloumenta, M. Mavroidis, Y. Capetanaki; Cell Biology, Foundation for Biomedical Research, Academy of Athens, Athens, Greece

Desmin, the muscle specific intermediate filament protein, surrounds the Z-discs, interconnects them to each other and links the entire contractile apparatus to the sarcolemmal cytosome, cytoplasmic organelles and the nucleus. In an attempt to explore the molecular mechanisms of these associations we have performed a yeast two-hybrid screening of a cardiac cDNA library. Here we show that the domains (NS5a +103a) of desmin molecule binds to a 413KDa protein, called myosynr, which is expressed in cardiac and skeletal muscle. Myosynr has been found to interact with dystrobin, which is a component of the soluble fraction of Biogenesis of Lysosomes Related Organelles Complex 1 (BLOC-1) which is involved in protein trafficking and organelle biogenesis. Binding of desmin with myosynr was confirmed in vitro, with GST pull down assay and co-immunoprecipitation of in vitro transcribed/ translated myosynr and desmin. Deletion analysis revealed that only the NS5a +103a fragment of desmin can bind to myosynr and this association takes place through the 24a long C-terminal end of the SPRY domain of myosynr. Using an antibody against the COOH terminus of myosynr we determined that myosynr co-localizes with desmin at the periphery of the nucleus of mouse neonatal cardiomyocytes in addition to adult heart muscle, the two proteins co-localize, predominantly, in intercalated discs and costameres. Desmin seems to be essential for the appropriate perinuclear localization of myosynr since its pattern is altered in cardiomyocytes from desmin null neonatal mice. The association of myosynr with intermediate filaments, suggests a potential role of desmin in vesicle trafficking.
Nestin Mediates the Interaction between Intermediate Filaments and Insulin Degrading Enzyme

Y. Chou, W. Kuo, M. Rosner, R. Goldman; Cell and Molecular Biology, Northwestern University, Chicago, IL, Ben May Institute for Cancer Research, University of Chicago, Chicago, IL

Nestin, a type IV intermediate filament (IF) protein, is transiently expressed in proliferating progenitor stem cells. Nestin is a large IF protein with an extremely long C-terminal domain (over 1200 amino acids) of no known function. To begin to investigate the function of nestin, we expressed a peptide fragment (nestin 657-853) derived from the conserved 11 amino acid repeat region of the nestin tail and used it for affinity chromatography to identify potential binding partners from soluble frog egg extracts. To date, a 110 KD protein has been found to be specifically absorbed by this nestin fragment. Mass spectrometric and immunoblotting analyses identify this protein as the insulin degrading enzyme (IDE). When assayed with a fluorogenic peptide substrate, the highly purified egg IDE has an active enzymatic activity comparable to that of the recombinant human IDE expressed in and purified from insect cells. Neither egg nor recombinant human IDE possesses any detectable proteolytic activity toward vimentin, nestin or other protein components present in purified native IF preparations. Interestingly, the activity of both egg and human IDE is enhanced about 100% by nestin 657-853 or a longer nestin fragment 1-1177. By immunofluorescence, IDE does not appear to be associated with vimentin IF networks in interphase HeLa cells. However, a partial colocalization of IDE with disassembled vimentin IF (i.e., vimentin particles) is observed in dividing HeLa cells. We have reported previously that formation of vimentin particles in mitotic cells is a feature associated with progenitor cells expressing nestin. Taken together, our results suggest that IDE, an enzyme involved in regulating the turnover of many small peptide hormones, can be modulated spatially and temporally by its association with the IF protein nestin in progenitor cells. (supported by the NIGMS)

Autophagy Helps Clear Intracellular Mallory Body Inclusions in Mouse Liver

M. Harada, D. Toiviola, P. Strnad, M. B. Omary; Medicine, VA Palo Alto Health Care System and Stanford University, Palo Alto, CA, Medicine, Kurume University School of Medicine, Kurume, Japan

Intracellular inclusions in liver and neurodegenerative diseases consist of mixed proteins that may ultimately lead to cell death. Hepatocyte inclusions, termed Mallory bodies (MB), consist primarily of keratins 8 and 18 (K8/K18). A K8:K18 ratio is essential for MB formation, but the significance and turnover of MBs are poorly understood. Autophagy, which degrades cytosolic proteins and organelles, is a potential mechanism for eliminating neuronal inclusions, though its relationship to MB clearance is unknown. We hypothesized that autophagy plays an important role in clearing hepatocyte inclusions in vivo. Mouse MBs were induced by feeding a 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC)-containing diet for 3 months. K8-overexpressing transgenic mice, which develop inclusions spontaneously and have increased susceptibility for MB formation, were also treated with a proteasome inhibitor. Autophagic activity was determined by immunoblotting for light chain (LC3), and inclusions were identified by electron microscopy and immunofluorescence co-staining of K8/18 and ubiquitin. DDC-treated mouse livers develop numerous inclusions, in association with conversion of LC3-I to II which indicates activation of autophagy. Untreated K8-overexpressing mice had few inclusions that increased upon treatment with the proteasome inhibitor, and this increase was inhibited by induction of autophagy with rapamycin. Rapamycin also reduced the formation of spontaneous inclusions in K8-overexpressing mice. In contrast, transgenic mice treated with a proteasome inhibitor do not develop inclusions. Ultrastructural analysis showed that the inclusions consist of filamentous and electron-dense material that was also noted in the autophagic vacuoles and lysosomes of both mouse models. In conclusion, autophagy can play a significant role in eliminating hepatocyte inclusions. Manipulation of autophagy may be a useful strategy for various inclusion body associated diseases.

The SMRT Way to Resist Death

K. K. Tsai, C. Chatterjee, J. N. Lakins, V. M. Weaver; Department of Pathology, University of Pennsylvania, Philadelphia, PA

Apoptosis resistance plays a key role in malignant transformation, tumor metastasis and treatment response. While much is known about the pathways that regulate survival in non-differentiated cells, we know less about how apoptosis is regulated in three-dimensional (3D) multi-cellular tissues or tumors in vivo. Analogous to differentiated tissues in vivo, 3D reconstituted basement membrane-differentiated mammary acini are highly resistant to an array of exogenous apoptotic insults including gamma radiation, chemotherapy agents and immune receptor stimuli. Here we report that tissue morphogenesis and apoptosis resistance are associated with global repression of multiple stress-response genes, and that the enhanced survival phenotype of differentiated tissues is functionally-linked to active repression of stress response genes by the nuclear receptor corepressor SMRT (silencing mediator of retinoic acid and thyroid hormone receptor). We found that mammary epithelial cell (MEC) morphogenesis correlates with an up-regulation of SMRT that is associated with apoptosis resistance because stable knockdown of SMRT rendered 3D differentiated mammary tissues sensitive to exogenous death stimuli. Likewise we observed that phenotypic reversion of mammary tumor tissue was associated with a significant increase in SMRT expression and acquisition of apoptosis resistance. Consistently, ectopic expression of SMRT permitted acquisition of apoptosis resistance in 3D tumor tissues and isolated tumor cells. The SMRT-mediated death resistance was found to be functionally-linked to chromatin remodelling-mediated gene silencing through activation of the histone deacetylase (HDAC). HDAC3, because either treatment with trichostatin A or stable knockdown of HDAC3 could restore the death sensitivity of apoptotically-resistant SMRT-expressing MECs. Moreover, stable expression of a mutant SMRT (K449A) that neither recruits HDAC3 into nuclei nor permits its activation rendered MECs hypersensitive to exogenous death induction. The relevance of SMRT/HDAC3-dependent epigenetic regulation of apoptosis resistance to treatment resistant, recurrent cancers is now being actively explored. (Supported by DOD BCRP W81XWH-05-1-330 and NIH CA078731 to VMW)

The Breast Tumor Modulator HoxA9 Regulates BRCA1 and Integrin-mediated Adhesion

P. M. Gilbert, J. K. Mow, M. K. Gbegnon, J. N. Lakins, V. M. Weaver; Institute for Medicine and Engineering/Pathology and Lab Medicine, University of Pennsylvania, 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. We have been studying how altered expression of developmental regulators contributes to breast tumorigenesis and have specifically focused on investigating their influence on integrin expression and/or activity. Homeobox genes play a critical role in tissue development, are frequently lost in tumors, and can regulate integrin and ECM expression. We showed that HoxA9 is lost in invasive human breast tumors and that re-expressing HoxA9 in breast cancer cells decreases migration, invasion, and anchorage independent growth in culture. Additionally, HoxA9 re-expression reduces proliferation and drives phenotypic reversal of breast tumor cells grown within a 3D reconstituted basement membrane (rBM), but not on tissue culture plastic. HoxA9 represses the malignant phenotype of breast cancer cells in vivo as well as in 3D rBM and this reversion is coincident with BRCA1 induction and normalization of adhesion and integrin expression. Ablating BRCA1 function through expression of the delta exon-11b BRCA1 mutant or a BRCA1 R1105H disrupts rBM morphogenesis in normal MECs and inhibits reversion of HoxA9 re-expressing breast cancer cells. Accordingly, we are exploring functional and mechanistic links between HoxA9, BRCA1 and integrin dependent tissue behavior. In addition, we are studying the effects of altered HoxA9 expression on the biophysical properties of the stroma and how in turn, changes in the mechanical properties of the extracellular matrix during normal development and mammary tumorigenesis can influence HoxA9 expression. (Support: D1SS042047, DOD/RO1WXH-05-1-330, NCI CA078731 and DAMD17-01-1-0368)

IGSF9 Is Upregulated in Primary Colon Tumors and Mediates Tumor Cell Adhesion

R. Chalam, N. W. O'Brien, B. L. Colton, S. Ho, X. Cao, F. Huang, B. Shestowsky, J. M. Daniels, K. R. McLachlan; Biogen Idec, San Diego, CA, Path Inc., San Diego, CA

IGSF9 is a cell surface protein belonging to the Ig-cell adhesion molecule (Ig-CAM) family. It is expressed in the developing nervous system and thought to play a role in synapse formation in the rat. Other members of this family mediate adhesion interactions and are frequently dysregulated in cancer. IGSF9 was identified as a pan-tumor antigen through microarray data analysis and the protein is upregulated in various cancers including ovary, breast, colon, and lung. Overexpression of IGSF9 in a colon tumor cell line resulted in altered migration and adhesive properties of the cells. Some of these phenotypic changes were integrin mediated. Taken together, this data suggests that IGSF9 may play a complex role in tumor biology.
**Force and Malignant Transformation**

W81XWH-05-1-330 and NIH CA078731 to VMW).

Studies are currently underway to explore effects on tumor metastasis and to delineate molecular mechanisms for these effects. (Supp: NIH T32HL00795404 to KRJ; DOD

**PEG-based Synthetic Extracellular Matrices with Tunable Mechanical Properties That Regulate Smooth Muscle Cell Behavior**

S. R. Peyton, C. C. Rauh, V. P. Keskinur, A. J. Putnam, C. Chemical Engineering and Materials Science, University of California, Irvine, Irvine, CA, D. Biomedical Engineering, University of California, Irvine, Irvine, CA,

The mechanical properties of blood vessels are dramatically altered in a variety of cardiovascular pathologies due to extensive remodeling of the extracellular matrix (ECM). We hypothesize these mechanical changes may provide instructive cues to vascular smooth muscle cells (SMCs) in the medial layers of the vessel wall. To address this hypothesis we have adapted polyethylene glycol) (PEG)-based hydrogel model systems, in part, to the ease with which their mechanical and cell adhesive properties can be independently controlled. In 2-D, SMC attachment and spreading occurred on RGD and full-length matrix protein-modified PEG surfaces, but not on unmodified control surfaces or scrambled peptide sequences (RGD) after 24 hours of incubation. However, the extent of cell spreading was not significantly influenced by ECM mechanics across a wide range of elastic moduli (13.7 kPa - 423.9 kPa), nor by ligand density or identity (RGD, KQACD, fibronectin, or collagen-I). In contrast, quantitative analysis revealed that focal adhesion size and elongation were in fact dependant on substrate stiffness and ligand density. Moreover, the histological maturation of isolated SMCs was associated with increased proliferation and increased substrate stiffness, with increased proliferation and increased substrate stiffness. It is well known that unloading of skeletal muscle with spaceflight or tail suspension leads rat soleus muscle atrophy. However, it is unclear how muscle atrophy is caused and how muscles respond to microgravity. We addressed the response of collagen and its chaperone system to gravitational forces. We show here that expression of HSP47, a collagen-specific molecular chaperone, responds to gravitational changes, including microgravity and hypergravity, in vitro and in vivo. By using a method of hindlimb suspension of rats which mimics microgravity conditions, we demonstrated that the expression of Hsp47 mRNA decreased within one day and the mRNA levels of collagen types I and IV were subsequently reduced. In contrast, hypergravity stimulated HSP47 expression. Intriguingly, HSP47 mRNA levels in cultured myoblasts increased significantly with hypergravity treatment at 40G for 2 h, and decreased with microgravity treatment at almost 0G for 1-2 h. Collagen mRNA levels were also altered, although changes were slower and less pronounced compared with those for HSP47. Next, we examined the effect of MG on the mRNAs of TGFβ-1, which is known to induce Hsp47 expression, in cultured myoblasts by semi-quantitative RT-PCR. At 1h of MG treatment, the expression of TGFβ mRNA in myoblasts was significantly decreased. From these results, the gravity-regulated HSP47 may play a role in the maintenance of the extracellular matrix by modulating collagen production at the primary stage of adaptation.

**Extracellular Matrix Composition and Modulation Differs in Dystrophic and Control Primary Human Myotubes**

S. Zanotti, S. Saredi, A. Ruggieri, S. Romaggi, L. Morandi, M. Mora; Neuromuscular Diseases and Neuroimmunology, National Neurological Institute C. Besta, Milano, Italy.

In muscular dystrophies disease severity is generally related to the extent of muscle fibrosis. To determine whether extracellular matrix (ECM) components contribute to the severe fibrosis seen in Duchenne muscular dystrophy (DMD), we quantified RNA transcript levels of several ECM constituents by competitive PCR in primary myotubes from 7 DMD patients and 5 controls. We found a significant increase of basal MMP-2, TIMP-1 and TIMP-2 transcript levels, while no significant variation were observed for MMP-9 and TIMP-3. By zymographic analysis MMP-2 activity was significantly increased in DMD myotubes while there was no variation in MMP-9 activity. Transcript levels of fibronectin, vitronectin and vimentin were similar in pathological and control cells, while collagen I and VI transcripts were variably increased. Cell transfection with a decorin antisense oligonucleotide, determined the following mRNA level changes: TGFβ-beta increase in control and DMD fibroblasts, vimentin increase in control and no change in DMD fibroblasts, TIMP-2 increase in control and decrease in DMD myotubes. Our results suggest that the expression of several ECM components is different in the two cell populations, that the activity of some of them is also altered, and that there is a different response to decorin inhibition in DMD versus control muscle cells. This disregulation may contribute to the development of excessive fibrosis observed in DMD muscle.

**Protein Conformational Changes Occur during Cardiomyocyte Contraction on Rigid, but Not Flexible Matrices**


Cardiomyocytes lose function within hours to days when plated on traditional collagen-coated rigid substrates or when observed in disease models where fibrotic scarring impairs cardiac output. Recent work has shown prolonged palstic contralactility when cells are cultured in matrigel, but the mechanism behind this, however, is still poorly understood. Using polyacrylamide matrices, we demonstrate that compliant microenvironments, which mimic the developing myocardial microenvironment, e.g. cells on flexible matrices with an elasticity of 10kPa, promote a contractile phenotype with beating at 1Hz, optimal spread area and shape, and preserved actomyosin striations. Matrices with elasticity similar to stiffer external layers of perimyocardial fibroblasts are less desirable, while cells on more rigid matrices, even when clustered together, cannot preserve contractions and normal beat frequency for extended periods of time. Analysis of matrix deformation on more rigid matrices points to a potential mechanism: mismatched intracellular contractions and subsequent displacement fields observed at the cell-matrix interface suggest a putative mechanochemical transformation, or one resulting from an increased mechanical mismatch between myocytes and extracellular matrix (ECM).

β1 integrin (V737N) expression in pre-malignant MCF10DCIS-MECs promotes their malignant transformation in 3D bM culture and in vivo. Results showed that xenografts of pre-neoplastic MCF10DCIS-MECs with increased numbers of mature focal adhesions formed larger palpable tumor masses of significantly increased mass as compared to their DCTS controls. Histologically forced focal adhesion maturation promoted malignant transformation evidenced by tumor invasion, increased angiogenesis and enhanced tumor cell survival. Studies are currently underway to explore effects on tumor metastasis and to delineate molecular mechanisms for these effects. (Supp: NIH T32HL00795404 to KRJ; DOD W81XWH-05-1-330 and NIH CA078731 to VMW).

**βHsp47**
Affect of Substrate Rigidity on Force Generation and Sarcomeric Organization in Neonatal Rat Cardiac Myocytes

J. G. Jacot, S. G. Campbell, E. J. Howard, A. D. McCulloch, J. H. Omens; Bioengineering, University of California, San Diego, La Jolla, CA

Many cell types respond to the rigidity of their environment by altering their proliferation, migration, or differentiation. We hypothesize that substrate rigidity affects sarcomeric organization and force generation in neonatal rat cardiac myocytes (NRMCs), whose morphology and myofilibril architecture are less differentiated than that of adult cardiac myocytes. We plated primary NRMCs at 10,000 cells/cm² on polyacrylamide gels containing varying amounts of crosslinker to produce a shear modulus ranging from 400 Pa (soft) to 20 kPa (stiff), and coated with type I collagen. We measured the deformation of the gel during cell contractions by tracking fluorescent beads embedded in the gel, and transformed these deformations into a map of traction forces using custom software. Cells on soft gels formed sarcomeres apparent under phase-contrast microscopy at 48 hours after harvesting and about 20% of these cells beat spontaneously, while cells on stiff gels displayed no organized structure and did not beat spontaneously. By 7 days, sarcomeres were visible on both gel rigidities and both cell types beat spontaneously. NRMCs on stiff gels had an average sarcomere length around 2.1 microns while NRMCs on soft gels had an average sarcomere length of 1.7 microns. Cells on stiffer substrates generated 6 times as much force as cells on soft substrates. A numerical model incorporating sarcomere length-dependent myofilament interactions and single-cell mechanics predicts a similar 6-fold change in force generation based solely on the resistance to cell shortening. We thus conclude that, although NRMCs on soft substrates develop a distinct sarcomeric structure more quickly than those on stiff substrates, cells on either substrate generate a total force after 7 days in culture that is predictable based on the sarcomere length and resistance to shortening.

Matrix Elasticity Directs Stem Cell Differentiation through Non-muscle Myosin II

A. Engler, H. Lee Sweeney, S. Sen, D. Discher; University of Pennsylvania, Philadelphia, PA

Stem cells are known to differentiate into multiple soft tissue cell types, but the role of the physical microenvironment has not been assessed. We show that the distinct softness of various tissues strongly influence stem cell differentiation through the contractile activity of myosin (Engler et al, Cell 2006). Tissue elasticities are mimicked with a range of collagenated gels, and we show that Mesenchymal Stem Cells express non-muscle myosin isoforms that allow the cells to contract and respond distintively to matrix stiffness. Non-lineage-specific growth factors are needed - just physical cues from the microenvironment. Elasticity-specific up-regulation of key tissue markers (eg. MyoD for myogenesis, CBFα1 for osteogenesis) is similar in magnitude to expression induced by soluble factors typically added to induce differentiation. The results highlight the importance of the physical character of the microenvironment and have significant implications for developmental processes as well as stem cell repair and regeneration of soft tissues.

Jnk Activity Is Required to Suppress Erk-Dependent Cell Proliferation, Cell Survival, and Emt-like Changes during 3D-Mammary Epithelial Acini Formation

E. T. R. McArdle; UCD School of Biomolecular and Biomedical Research, Conway Institute, Dublin, Ireland

Hypothesis that one possible mechanism by which ECM compliance exerts its influence on osteogenesis is by modulating the mitogen activated protein kinase (MAPK) pathway. To address this hypothesis, MC3T3-E1 cells were cultured on dextran hydrogels evaluated on the basis of morphology and expression of SMC-specific markers, such as smooth muscle actin, myosin, caldesmon and calponin. The two latter proteins have been previously reported to be downregulated in traditional tissue culture systems, suggestive of phenotypic loss by SMCs under such conditions. Recovery and maintenance of the phenotype of cells ex vivo in tissue culture systems are essential for future applications in cell-based therapies.

The Regulation of Osteogenesis by Ecm Rigidity in MCT3-E1 Cells Requires Mapk Activation

C. B. Khatiwala, S. R. Peyton, J. J. Putnam; 1Chemical Engineering and Material Science, University of California, Irvine, Irvine, CA, 2Biomedical Engineering, University of California, Irvine, Irvine, CA

Recent efforts to develop synthetic biomaterials as extracellular matrix (ECM) analogs capable of controlling cell fate have begun to exploit the idea that mechanical cues in the microenvironment influence tissue development. In prior studies, we demonstrated that tuning ECM mechanics can directly regulate the behavior of pre-osteoblastic MC3T3-E1 cells. We hypothesized that one possible mechanism by which ECM compliance exerts its influence on osteogenesis is by modulating the mitogen activated protein kinase (MAPK) pathway. To address this hypothesis, MC3T3-E1 cells were cultured on poly(ethylene glycol) (PEG)-based model substrates with tunable mechanical properties, and their differentiation assessed over a period of 14 days. Alkaline phosphatase (ALP) levels at days 7 and 14 were found to be significantly higher in cells grown on stiffer substrates (423.9 kPa hydrogels and rigid tissue culture polystyrene (TCP) control) than on a soft hydrogel (13.7 kPa). Furthermore, osteocalcin (OCN) and bone sialoprotein (BSP) gene expression levels followed a similar trend. These differences correlated with significantly elevated MAPK activity in cells cultured on stiffer substrates at both time points. Inhibiting this activation pharmacologically, using PD98059, resulted in significantly lower ALP levels, OCN, and BSP gene expression levels on the hydrogels. Interestingly, the effectiveness of PD98059 was itself dependent on substrate stiffness, with marked inhibition of MAPK phosphorylation in response to stiff hydrogels, but less robust reduction on TCPs. Combined, these data confirm MAPKs involvement in the regulation of osteogenic differentiation by ECM compliance. Furthermore, the activation of Caspase-3 and Bcl-2 gene expression were not significantly affected by ECM compliance, indicating that MAPK pathway is not involved in the regulation of apoptosis in these cells.

Regulation of Jnk Activity by Matrix Stiffness Mediates Apoptosis Induction in a Mammary Epithelium

N. Zahir, J. L. Leigh, J. L. Lakins, B. Alston-Mills, K. K. Tsai, V. M. Weaver; 1Pathology, University of Pennsylvania, Philadelphia, PA, 2Department of Animal Science, North Carolina State University, Raleigh, NC

We show that Mesenchymal Stem Cells express non-muscle myosin isoforms that allow the cells to contract and respond distintively to matrix stiffness. Non-lineage-specific growth factors are needed - just physical cues from the microenvironment. Elasticity-specific up-regulation of key tissue markers (eg. MyoD for myogenesis, CBFα1 for osteogenesis) is similar in magnitude to expression induced by soluble factors typically added to induce differentiation. The results highlight the importance of the physical character of the microenvironment and have significant implications for developmental processes as well as stem cell repair and regeneration of soft tissues.
The composition and spatial organization (3D) of the extracellular matrix (ECM) influence apoptosis responsiveness of mammary epithelial cells (MECs) to cancer therapeutics (Weaver et al., Cancer Cell, 2002). Because the 3D organization of the ECM determines its mechanical properties, the question of whether and how matrix stiffness could modify therapeutic efficacy in a mammary epithelium was investigated. Distinct microenvironments were established by utilising basement membrane (BM) crosslinked polyacrylamide gels of calibrated stiffness (elastic modulus, E). Similar to normal mammary acini residing within a soft tissue in vivo (E = 170 Pascals), MECs grown on soft 3D BM-gels (E = 140 Pascals) formed polarized acini and exhibited resistance to Taxol, TRAIL and gamma radiation. In contrast, MECs grown on a 3D BM-gel with a similar stiffness to mammary tumors (E = 5000 Pascals) formed disorganized structures and displayed a marked sensitivity to exogenous apoptotic cues, despite growth rates similar to MEC matrix. Matrix stiffness resulted in an increase in the magnitude and duration of JNK activation. Moreover, JNK activity was necessary for cancer therapy-dependent apoptosis induction in 3D MEC tissues. Whether JNK activity is sufficient for apoptosis induction in 3D MEC tissues is now under investigation. These data illustrate how the mechanical properties of the tissue microenvironment could alter the efficacy of anti-cancer treatments through by modulating adhesion-dependent growth and survival signaling. (Funding: W81XWH-05-1-330 & CA087831 to V.W.M. & T32CA09677-13 to N.Z.)

RPTPa Is Required for Fibronectin-specific Rigidity Response at the Leading Edge of the Growth Cones in Hippocampal Neurons

A. Kostic, J. Jap,’ M. P. Sheetz;’ Department of Biological Sciences, Columbia University, New York, NY, ’Institute for Molecular Pathology, University of Copenhagen, Copenhagen, Denmark

Although the importance of mechanosensing has been long recognized, the molecular details of cellular response to the physical properties of the environment remain elusive. Based on work by our lab and others, receptor protein tyrosine phosphatase α (RPTPα) appears to be an essential component of force-dependent integrin signaling and rigidity response. Our previous studies in fibroblasts have shown that RPTPα-deficient mice has revealed no overt abnormalities apart from striking morphological and functional defects in the hippocampus. Further, RPTPα was implicated in neurite extension regulation, neuronal development, learning, memory, and anxious behavior. Here, we report that RPTPα is required for fibronectin rigidity response in hippocampal neurons. While the axon extension is inhibited on rigid fibronectin matrices in the presence of RPTPα, the neurons isolated from RPTPα knockout hippocampi fail to discriminate between the substrates of various rigidities. Moreover, axon differentiation seems to be dependent on both RPTPα-related activities and soft matrices. We speculate that RPTPα is required for the reinforcement of cytoskeleton-integron bonds, which is critical in the processes of neurite extension and differentiation. By using optical trap, we were able to confirm that RPTPα-/- neurons exhibit impaired reinforcement of FN-coated beads. Additionally, RPTPα dependence of rigidity response in neurons appears to be fibronectin-specific, and requires α5β1 integrin recruitment to the leading edge of the growth cones. Furthermore, the recruitment of downstream targets of RPTPα, in particular Src family kinases, and their substrate, p130Cas to the leading edge is also rigidity-dependent.

Dynamic Changes in Epithelial Organization and Interjunctional Cells during Mammary Epithelial Invasion and Morphogenesis

A. J. Ewald, M. Duong, Z. Werb; Anatomy, University of California, San Francisco, San Francisco, CA

Collective cell movements are required to build and remodel many vertebrate epithelial organs, yet relative little is known about how cells move and rearrange while remaining part of an epithelial sheet. We have focused on the cell behaviors extending under formation and bifurcation of mammary ductal epithelial tubes. We have taken a combined imaging, cell biological, and pharmacological approach to determine the cell behaviors and adhesive interactions underlying these movements. We cultured primary murine mammary epithelium in Matrigel. These explanted tissues begin as a partially polarized epithelium, with myoepithelial cells basal to luminal epithelial cells. Initially, they have multiple cell layers and solid cores. The cells then rearrange to constitute a bilayer of myoepithelial cells, then luminal epithelial cells surrounding a fluid filled lumen. Following lumen formation there is a growth factor- and matrix metalloproteinase-dependent change in luminal epithelial organization at discrete sites. This reorganization is characterized molecularly by a high level of F-actin, multiple luminal cell layers, and small pockets of ZO-1. Using long-term (100+ h), 3D confocal imaging we observed that new ducts extend from these sites through vigorous cellular rearrangements within a multilayered invasion front. These rearranging cells have E-cadherin at all luminal-luminal cell boundaries and ZO-1 at their apical surfaces. Polymerized actin is concentrated at the apical and lateral surfaces; the cell-matrix border (basal surface) is low in polymerized actin. Several cell diameters behind the front full epithelial polarity is re-established, with tight and adherens junctions, and a fluid filled lumen. Treatment with inhibitors for Rac1 and ROCK1 changes the final tissue architecture, cell behaviors and junctional status of these organ cultures and analysis of these differences has given us insights into the cellular and molecular mechanisms underlying this novel collective cell behavior. Supported by NCI CA057621 and by a CBCRP fellowship (11FB-0015).

Force-dependent Mammary Morphogenesis and Malignancy in a Tunable 3D Model System


Epithelial morphogenesis proceeds within a 3D tissue in which soluble, cellular and physical cues cooperate to direct tissue form and function. Although we understand much about the role of soluble factors and cell interactions in homeosis and tumorigenesis, we know little about the role of spatial organization and mechanical force in these processes. Key to understanding tissue behavior is the availability of tractable model systems in which biochemical, spatial and mechanical variables can be independently manipulated. Here we report the development, analysis and morphogenetic assessment of 2D and 3D gels for the study of mammary epithelial cell (MEC) behavior including: collagen I/reconstituted basement membrane (COL/rBM-gel), rBM polyacrylamide (rBM-PA-gels), self-assembling peptide/rBM (SAP/rBM-gel), and peptide-conjugated polyethyleneglycol (PEG-conj-gels). MEC morphogenesis can be recapitulated using rBM-gels, COL/rBM-gels, and rBM-PA-gels with elastic moduli similar to that of normal mammary gland. However, 3D models employing natural biomaterials (rBM-gels, COL/rBM-gels) are biomechanically and chemically non-uniform; thus, in these undefined systems it is difficult to derive definitive conclusions relating force, ligand density and epithelial behavior. Additionally, while synthetic biomaterials, e.g., rBM-PA-gels, permit precise control of matrix stiffness and ligand density, PA gels are incompatible 3D and in vivo. SAP/rBM-gels offer a versatile 3D matrix with calibrated stiffness and ligand presentation. Unfortunately SAP gels are not easily adapted for studies directed at 3D epithelial morphogenesis and migration/invasion because matrix stiffness depends upon increasing matrix density and the peptide backbone is not easily remodeled by embedded cells. Although PEG gels that can be mechanically and biochemically modified offer an attractive alternative, traditional chemistry has been optimized for relatively stiff materials, e.g., cartilage and muscle. Accordingly, we are investigating soft PEG-conj-gels with adhesive laminin-derived ligands and collagenase-digestible linkers by testing their suitability as a tractable biomaterial for reconstituted multi-cellular epithelial morphogenesis studies. Here we will summarize our strategy and biological characterization.

A Novel Human Biomatrix Model for Real-time Analysis of Angiogenesis

R. Singh, M. Belousova, E. Seales, D. Hamrick, M. Klinger; Vivo Biosciences Inc, Birmingham, AL

Current in vitro models of angiogenesis mostly employ animal-derived purified or reconstituted biomatrix substrates and therefore do not reliably predict the complex human tissue biology. For example, common Matrigel system contains many mitogenic and angiogenic agents (EGF, FGF). To overcome this limitation, we developed an improved angiogenesis bioassay incorporating a new human-derived biomatrix, named HuBiogel. Unlike Matrigel, it contains all major matrix components but is GF-free. Human umbilical vein endothelial cells (HUVEC) were seeded onto collagen I, HuBiogel or Matrigel, and both early (proliferation) and late-stage (sprouting & tube formation) angiogenesis events were quantitated by real-time morphologic analysis. EC on collagen I grew poorly (<60% viability). EC on HuBiogel grew and proliferated normally (~15% sprouting without differentiation), and maintained high viability out to 15 days. In contrast, Matrigel induced rapid, uncontrolled tube formation within 8 hrs. A focused microarray analysis showed higher expression of angiogenic markers in EC on Matrigel versus HuBiogel, e.g.; notch-4, ephrin-A1 and angiopoietins. To demonstrate the biological utility, we plated EC on HuBiogel enriched with purified bFGF (10-50 ng/ml). A time and dose dependent angiogenesis activity was observed. For mimic tumor angiogenesis, EC were co-cultured on HuBiogel with human glioma cells which secrete soluble VEGF. A positive correlation was found between secreted levels of VEGF and angiogenic activity in HuBiogel assays. Matrigel failed to distinguish these functional parameters. We therefore conclude that HuBiogel-based assay system allows precise evaluation of cellular & molecular signaling pathways and their biological endpoints. This defined or controllable bioassay will positively impact neovascularization and cancer research areas. (Supported by HL62736 & CA108118)
Pulmonary vasculogenesis, angiogenesis and normal alveolar development rely upon the paired-related homeobox gene transcription factor, Prx1, which is expressed in the embryonic lung mesenchyme. Mechanistically, Prx1 controls expression of tenasin-C (TN-C), an extracellular matrix glycoprotein required for normal vascular development and lung branching morphogenesis. To further understand how Prx1 controls lung development, we focused on tropoelastin and fibrillins (1 & 2): microarray and immunohistochemical studies involving tissue derived from wild type and Prx1-null fetal mouse lungs demonstrated that expression of these extracellular matrix genes relies upon Prx1. Also, since fibrillins regulate TGF-β activity, we compared expression of phosphorylated Smad-2 (P-Smad-2; a downstream effector of TGF-β signaling), as well as the levels of α-smooth muscle actin and TN-C (targets of TGF-β) in wild type and Prx1-null lungs. These studies revealed major deficits in TGF-β signaling (both in the embryonic lung epithelium and mesenchyme), as well as differences in TN-C expression and recruitment of α-actin-positive cells to the pulmonary vasculature. Currently, we are using an organotypic culture system involving de-cellularized fetal lung scaffolds derived from wild type and null mice to evaluate how Prx1 controls the expression of TN-C, tropoelastin and fibrillins, and how these extracellular matrix proteins control vascular and epithelial cell behavior in the developing lung.

During myogenesis in vitro, myoblasts undergo cell cycle arrest, migrate, and fuse with one another to form multinucleated myotubes. Extracellular matrix (ECM) molecules play critical roles during these processes. Matrix metalloproteinases (MMPs) are neutral endopeptidases capable of degrading ECM components. Although the role of MMPs in myogenesis has been investigated, almost nothing is known about the role of their inhibitors, the tissue inhibitor of metalloproteinases (TIMPs). Here, we report that TIMP-2, MMP-2, and MT1-MMP are differentially expressed during mouse myoblast differentiation in vitro. Furthermore, we show that TIMP-2/- myotube size is altered: when differentiated in horse serum-containing medium, TIMP-2/- myotubes are larger than control myotubes. In contrast, when serum-free medium is used, TIMP-2/- myotubes are much smaller than controls. Regardless of culture condition, myotube size is directly correlated with MMP activity and inversely correlated with beta integrin expression. The reduced TIMP-2/- myotube size can be rescued by co-culturing with wild-type myoblasts in close apposition, without direct contact, suggesting that soluble factor(s) are responsible. Furthermore, reduced myotube size can be rescued by treatment with either recombinant MMP-2 or TIMP-2. Elucidating the role of TIMP-2 in myogenesis in vitro may lead to new therapeutic options for myogenic defects in vivo.

Tenascin-C (TNC) is an extracellular matrix glycoprotein not detected in normal adult myocardium but expressed under various pathological conditions. To elucidate the role of TNC in myocardial fibrosis in hypertensive heart, we treated mice. Balb/c wild type (WT) mice and TNC knockout (TNKO) mice were treated with 560 ng/kg body weight/min AgII subcutaneously by osmotic minipump for 4 weeks. In both wild type and TNKO mice, AgII treatment increased blood pressure, heart weight/body weight ratio, and sizes of cardiomyocytes but no significant differences were detected between WT/AgII and TNKO/AgII mice. AgII treatment also caused increase of interstitial collagen fibers at perivascular regions. Increase of interstitial collagen fibers were less than those in WT/AgII. In both wild type and TNKO mice, relative mRNA level of collagen I, III and TGF-β1 gene expression were both significantly increased, but no significant differences were detected between WT/AgII and TNKO/AgII mice. In WT/AgII mice, accumulation of macrophages positive myotubes were observed at perivascular region but reduced in TNKO/AgII mice. Alcin Blue staining showed dense deposition of glycosaminoglycans (GAGs) at perivascular area in WT/AgII. In contrast, TNKO/AgII showed sparse and rough fibrillar staining pattern. Immunostaining demonstrated that deposition of PGM/versican, a chondroitin sulphate proteoglycan was reduced in TNKO/AgII mice. More PDGF-B positive cells were found that in TNKO/AgII at peri-vascular region. These results suggest that, TNC may promote fibrosis in hypertensive heart by enhancing macrophages accumulation and deposition of proteoglycans.

Deposition of Elastin to Microfibrillar Fibers in the Presumptive Dermis of the Chick Embryonic Tarsometatarsus.

Y. Yamazaki,1,2 K. Isokawa,2 M. Yuguchi,1 M. Shirai,3 K. Shinozuka,1 S. Usami1; 1Department of Anatomy, Nihon University School of Dentistry, Tokyo, Japan, 2Division of Functional Morphology in Dental Research Center, Nihon University School of Dentistry, Tokyo, Japan, 3Department of Oral and Maxillofacial Surgery, Nihon University School of Dentistry, Tokyo, Japan

Fibrillin microfibrils are integral components of elastic fibers and serve as a scaffold for elastin deposition. However, microfibrillar fibers (MFs) are not necessarily committed to develop into so-called elastic fibers. In dermis, elastin-free oxytalan MFs originating from the dermo-epidermal junction are continuous to elastin-type MFs (with a small amount of elastin) in the deeper papillary dermis, whereas the reticular dermis contains horizontally arranged elastic fibers, or MFs embedded largely in elastin. In this study, we have investigated temporospatial pattern of elastin deposition in the MFs of tarsometatarsal presumptive dermis. While the earliest expression of elastin was demonstrated immunohistochemically as early as embryonic day 4 (ED4) in the wall of cardiac outflow and pharyngeal arch arteries, its deposition in the tarsometatarsus was first detected at ED6 in the deeper or core mesenchyme and at ED13 in the subcuticular mesenchyme. In the latter tissue, MFs had become perpendicularly to the covering ectoderm by ED4, quite before an overt accumulation of collagenous matrix; the onset of accumulation was at about ED9-10 when examined by picrosirius-polarization method. Elastin deposition observed initially in a punctate manner at ED13 became linear along subectodermal MFs. During these processes. Matrix metalloproteinases (MMPs) are neutral endopeptidases capable of degrading ECM components. Although the role of MMPs in myogenesis has been investigated, almost nothing is known about the role of their inhibitors, the tissue inhibitor of metalloproteinases (TIMPs). Here, we report that TIMP-2, MMP-2, and MT1-MMP are differentially expressed during mouse myoblast differentiation in vitro. Furthermore, we show that TIMP-2/- myotube size is altered: when differentiated in horse serum-containing medium, TIMP-2/- myotubes are larger than control myotubes. In contrast, when serum-free medium is used, TIMP-2/- myotubes are much smaller than controls. Regardless of culture condition, myotube size is directly correlated with MMP activity and inversely correlated with beta integrin expression. The reduced TIMP-2/- myotube size can be rescued by co-culturing with wild-type myoblasts in close apposition, without direct contact, suggesting that soluble factor(s) are responsible. Furthermore, reduced myotube size can be rescued by treatment with either recombinant MMP-2 or TIMP-2. Elucidating the role of TIMP-2 in myogenesis in vitro may lead to new therapeutic options for myogenic defects in vivo.
Tarsometatarsal (TMT) skeleton of the avian leg is peculiar in development. In the chick embryo, the 2nd to 4th metatarsal bone primordia elongate and merge into a TMT skeleton with a long bone morphology. However, the chronology of TMT bone development has not been investigated in detail. In this study, progression in morphogenesis and ossification of the TMT bone has been examined in conjunction with ultrastructural and immunohistochemical surveys on the development of periosteal elastic microfibrils. Three cartilage rudiments in the metatarsus showed a rapid elongation from the embryonic day 5 (ED5). Their proximal ends incorporated tarsal cartilage(s) to form TMTs by ED10. Calcification from the forming ossified bone collar occurred by ED8 at the mid-diaphyseal region and extended cylindrically on the surface of cartilage rudiments in the proximal and the distal directions. The length of bone collar increased from about 2 mm at ED9 to more than 12 mm at ED17, but epiphyses remained cartilaginous even just before hatching. Thickening of bone collar resulted in lateral osseous fusion of the initially independent three TMTs. This fusion began by ED17 and TMTs became unseparable by ED20, while cavities of the bone marrow remained separated incompletely. Along with these changes, perichondrium at early stages was converted into periosteum, which wrapped TMTs individually and afterwards enmeshed an united TMT entirely. Perichondrium and peristeme were rich in fibrillin-positive microfibrils largely arranged parallel to the long axis of TMTs. Elastin deposition was detected immunohistochemically at ED10 and became distinct ultrastructurally by ED13. Morphometrically, the cross sectioned area of microfibrillar bundles increased 4 times during ED10-20. Our findings provide useful chronological landmarks for the developmental studies of TMT skeleton. Supported from the Promotion and Mutual Aid Corporation and by grants from the Sato Fund and Dental Research Center in NUSD.

1723
Molecular Biological Characterization and Expression of Female Specific Lecitin Involved Gamete Recognition of Marine Red Alga Agloathamnion oosumiense (Ceramidae Rhodophyte)

B. H. Jo, G. H. Kim; Department of Biology, Konkuk National University, Konkuk, Republic of Korea
Rhodobindin is a lecin protein involved in gamete recognition of a red alga, Agloathamnion oosumiense. This lecin was previously identified to be present on the female trichogyne and be involved in recognition with the male surface glycoproteins. This protein has specificity to N-acety1-d-galactosamine, it was purified by N-acety1-d-galactosamine affinity chromatography column. The protein is monomeric, with a molecular weight of 21 kDa. Result of 2DE analysis conformed that it was female-specific protein. The analysis of cDNA/ EST sequences was performed and the database for Agloathamnion oosumiense was constructed. Specific primer was produced based on the discovered amino acid sequence and coincided Cluster DNA sequence in ESTs database, then and the full sequence of the lecin was obtained. PCR amplification with an open reading frame (ORF) to the non-composite restriction enzyme site and expression vector for transformation into the E. coli competent cell was performed. Expression of the large quantity of Rhodobindin recombinant protein was induced in transformed E. coli by induction of IPTG. Thereafter the recombinant protein was purified by affinity chromatography column and Tagging protein was removed by protease. This study presents first report on both purification of red algal lecin and production of large scale recombinant protein.

1724
Lectin/Glycan Interactions Play a Role in Recognition in a Coral/Dinoflagellate Symbiosis

E. M. Wood-Charchori,1 L. L. Hollingsworth,2 D. A. Krakp,2 V. M. Wei,1 Zoology, Oregon State University, Corvallis, OR, 3University of Hawai, Hawai’i Institute of Marine Biology, Kamehameha Schools, Windward Community College, Kailua, HI
Cell-cell recognition is an important step in the establishment of highly specific mutualistic associations. Yet, for the majority of symbioses, very few of the mechanisms responsible for recognition between symbiotic partners are well-understood. In this study, we provide evidence that a lectin/glycan interaction is functioning as a cell-cell recognition mechanism during initial contact between larvae of the coral Fungia scutaria and their endosymbiotic dinoflagellate algae. Symbiont-free F. scutaria larvae developed from artificially fertilized gametes. Symbiotic algae were extracted from adult F. scutaria and incubated with enzymes to remove components of the algal cell surface or with FITC-lectins to bind glycans on the algal cell surface. Symbiont cell surface labeling by FITC-lectins was quantified using flow cytometry. Treated symbionts were used to infect symbiont-free larvae, and infection success was compared to control infections with untreated larvae. We determined that an intact larval cell surface was required for successful infection of F. scutaria larvae. Modification of the algal cell surface by enzymatic digestion with trypsin (cleaves at Arg and Lys) or N-glycosidase (cleaves N-linked glycoproteins) significantly reduced infection success, and implicated algal cell surface glycans in recognition. Using flow cytometry, FITC-lectin binding identified α-mannose/α-glucose and α-galactose residues as potential recognition ligands on the algal cell surface. Finally, inhibition of these cell surface glycans by lectins significantly reduced infection of F. scutaria larvae by the algae. We found that the expression of these surface glycans helps promote infection of F. scutaria larvae.

1725
Stochastic Analysis of Transcription Regulation in Lux-based Quorum Sensing

K. Timp1, W. Timp,1 U. Mirsadov,1 G. Timp,1 Y. Freycone,1 P. Matsudaira1; 1Molecular and Cellular Biology, University of Illinois at Urbana-Champaign, Urbana, IL, 2Electrical Engineering, Massachusetts Institute of Technology, Cambridge, MA, 3Electrical Engineering, University of Illinois at Urbana-Champaign, Urbana, IL, 3Biology, Massachusetts Institute of Technology, Cambridge, MA
In order to synchronize behavior between cells for biological computation, the noise and threshold of the signaling mechanism must be well characterized. In cell-cell signaling single molecules diffuse from one cell to another, and cause a reaction which can change the receiving cell behavior dramatically. Since this happens with just a few molecules, diffusing from one cell to another, it falls within the stochastic regime. We used flow cytometry and fluorescent microscopy to gather single-cell data on the transcriptional regulator portion of the lux system from V. Fischeri, luxR. Two different plasmids were transfected into E. coli, containing the lux promoter coupled to either GFP or GFP-LVA, a degradable form of GFP. The goal was to determine the dependence of activation threshold and noise on the incubation temperature. We found that the saturation threshold was shifted downward dramatically as the temperature was reduced, e.g. at 37°C the saturation threshold is a 100M [AII], but at 30°C the saturation threshold is only 10M. The noise was low in the saturation areas, but high in the switching area. There is a slight noise dependence on temperature, but not as dramatic as the observed threshold adjustment. When the degradation sequence in added to GFP, we found it reduced the leakage signal when the system is off. This is especially useful for synchronizing, as it will prevent background signal from climbing before the desired on switch. Cell-cell signaling of this kind is important for use in synthetic biology, as a mechanism for synchronizing behavior or logic in all bacterial cells of a population. Our quantification of the noise inherent in this bacteria system, allows us to use this signal in bacterial circuits. In addition the temperature information gives us the ability to easily shift the threshold dependent on the desired result.

1726
Functional Genomics during the Initial Onset of Coral/Algal Symbiosis

C. E. Schnitzler, V. M. Wei; Zoology, Oregon State University, Corvallis, OR
The mutualism between corals and their intracellular dinoflagellate algae represents a highly specific partnership of two eukaryotic organisms. Very little is known about the molecular and cellular mechanisms controlling the successful establishment of a stable relationship in the early stages of coral/alggal symbiosis. The planula larva of the solitary scleractinian coral Fungia scutaria and its dinoflagellate symbiont Symbiodinium sp. represents an ideal model for studying the onset of coral/alggal symbiosis due to the predictable availability of gametes, and the ability to raise non-symbiotic larvae and establish the symbiosis experimentally. The goal of this study is to identify genes differentially expressed in F. scutaria larvae during the initiation of symbiosis with its algal symbiont. Symbiotic larvae will be compared to non-symbiotic larvae using a blind cDNA microarray. The array was constructed with cDNA libraries from symbiotic larvae. Modification of the algal cell surface by enzymatic digestion with trypsin (cleaves at Arg and Lys) or N-glycosidase (cleaves N-linked glycoproteins) significantly reduced infection success, and implicated algal cell surface glycans in recognition. Using flow cytometry, FITC-lectin binding identified α-mannose/α-glucose and α-galactose residues as potential recognition ligands on the algal cell surface. Finally, inhibition of these cell surface glycans by lectins significantly reduced infection of F. scutaria larvae by the algae. We found that the expression of these surface glycans helps promote infection of F. scutaria larvae.

1727
T-cell Receptor Signaling Model Presents Multiple Activation Levels and Switching Mechanisms

J. M. Burk1, L. Willc,1 M. L. Kemp,1 D. A. Lauffenburger2; 1Biological Engineering, Massachusetts Institute of Technology, Cambridge, MA, 2Biology, Massachusetts Institute of Technology, Cambridge, MA
A properly functioning immune system requires ligand-appropriate T-cell responses. We theorize that T-cell responses (viral, self, and partial) to antigens are biologically controlled by two parameters: receptor-to-peptide ratio (ρ) and specific peptide affinity (α). We derived a mass-action ODE model describing this signaling process using minimal rate constant fitting. The model
is experimentally-verified from data found in literature. Simulations varying $\mu$ and $\kappa$ suggest the theoretical existence of multiple quasi-stable states corresponding with appropriate immune responses, implying possible switching mechanisms controlling T-cell receptor activation. Moreover, we show in model simulations that the presence of self peptide on antigen presenting cells increases the sensitivity of T-cell receptor detection of foreign peptides.

1728 Measurement of the Field Potential in Individual Cardiomyocytes by Multi-electrode Array with Agarose Microchambers

T. Kaneko,1,2 K. Kojima,1 Y. Sugio,1 I. Suzuki,1 K. Yassuda,1 Department of Biomedical Information, Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, Tokyo, Japan; 2Precursory Research for Embryonic Science and Technology, Japan Science and Technology Agency, Saitama, Japan

Extracellular recordings of electrical activity with multi-electrode array (MEA) enable non-invasive long-term monitoring of the cell activation. We tried to develop the agarose microchamber (AMC) on the MEA for control of the cell position and the connecting pattern. Then the individual-cell-based electrophysiological measurement system, combining MEA with AMC, has been developed for non-invasive long-term analysis of the cell network activity. We have applied cardiomycocytes to this AMC/MEA system for the analysis of the cell communication. The advantages of this AMC/MEA system are that it allows control of the cell positions on the electrodes and numbers for cultivation using agarose microchambers and control of the flexible connecting pattern of the cell-cell interaction through photothermal etching with a 1480 nm infrared laser. Using the AMC/MEA system, the field potential of the individual spontaneous beating cardiomyocytes arranged on the electrodes in the agarose microchambers was recorded. We have found that the field could be detected reproducibly in field potentials recorded on the same electrode within a single preparation. In two cardiomyocytes synchronization, the field potential of each cardiomyocyte was recorded simultaneously on the neighboring electrodes. The distance of each electrode is 50 μm and the delay time of electrical activity between two cardiomyocytes is 1 ms, and consequently the conduction velocity is 50 mm/s. These results provide a basis for the future analysis of the community effect for the number, kind, and patterning of the cell in the networks.

1729 Microengineered Substrates Reveal E-cadherin-mediated Regulation of Cell Polarity

R. A. Desai, L. Gao, C. S. Chen; Bioengineering, University of Pennsylvania, Philadelphia, PA

Cell polarity is orchestrated by a variety of extracellular cues and guides events such as chemotaxis, mitosis and wound healing. In scratch wound assays of cell monolayers, it has been demonstrated that the microtubule organizing centers (MTOCs) of wound edge cells orient toward the open wound. Several studies implicate the formation of new cell-extracellular matrix (ECM) adhesions as cells spread into the wound as the origin of this polarizing signal. Here we sought to investigate the role of cell-cell adhesions in the process of cell polarization. To eliminate the dynamic events of spreading and new adhesion following scratch wounding, we cultured cells onto large patterned islands of ECM such that groups of cells attached and formed a discrete monolayer on each island. As such, cells at the edge of the monolayer retain asymmetric cell-cell contacts, but do not experience dynamic spreading or new adhesion events associated with a wound scratch. Using this system, we observed normal rat kidney epithelial cells along monolayer edges polarized such that their MTOCs were located between the nucleus and the edge, suggesting that the MTOC may move to an equilibrium position farthest from cell-cell interactions in the absence of changes in cell spreading and cell-ECM adhesion. We then examined whether cell-cell contacts were sufficient to drive this orientation, by using microengineered substrates to generate pairs of cells with specified interfacial geometry between them. Indeed, a limited area of cell-cell contact was sufficient to induce the MTOC to orient on the opposite side of the nucleus from the cell-cell contact. Furthermore, expressing a dominant-negative E-cadherin using an adenoviral vector completely abolished this bias in MTOC location. These findings suggest a role for E-cadherin-mediated cell-cell adhesion in providing signals that drive the polarized cell structure seen during chemotaxis and wound healing.

1730 Human Phagocyte Activity Is Inhibited by SIRP-CD47

R. Tsai, S. Subrahmanyan, D. Fischer; University of Pennsylvania, Philadelphia, PA

The integrin-associated protein (IAP) or CD47, which is found in many tissues, inhibits phagocytic activity of many cell types. CD47 is a ligand for the transmembrane signaling protein, SIRPα, present on macrophages and neutrophils, and it is postulated that CD47 is a ‘marker of self’ through its interaction with SIRPα by signaling an inhibition of phagocytosis. Past studies using mouse knockouts have raised many questions, including whether other proteins are involved. To begin addressing such questions with a reductionist approach, we have coupled CD47 to synthetic particles for studies of phagocytosis. These modified particles were first shown to bind to the extracellular domain of SIRPα in mice. In tests of phagocytosis with opsonized particles and a mouse-derived macrophage cell line, mouse CD47/particle dramatically reduce phagocytosis. The CD47-particle was further explored in human phagocytes where we demonstrate that negative E-cadherin using an adenoviral vector completely abolished this bias in MTOC location. These findings suggest a role for E-cadherin-mediated cell-cell adhesion in providing signals that drive the polarized cell structure seen during chemotaxis and wound healing.

1731 RKIKK Motif in the Intracellular Domain Is Critical for Spatial and Dynamic Organization of ICAM-1: Functional Implication for the Leukocyte Adhesion and Transmigration

H. Oh, S. Lee, Y. Kim, H. Wee, C. Jun; Department of Life Science, GIST, Gwangju, Republic of Korea

No direct evidence has been reported that the spatial organization of ICAM-1 on the cell surface is linked to its physiological function in terms of leukocyte adhesion and transendothelial migration (TEM). Here we observed that ICAM-1 by itself directly regulates de novo elongation of microvilli and is thereby clustered on the microvilli. However, truncation of intracellular domain resulted in uniform cell surface distribution of ICAM-1. Mutation analysis revealed that the C-terminal 21 amino acids are dispensable, whereas a segment of 5 amino acids NRKIKK in the NH-terminal third of intracellular domain is required for the proper localization and dynamic distribution of ICAM-1, and the association with F-actin, ezrin, and moesin. Importantly, deletion of the 507RKIKK511 did not reduce firm adhesion, but significantly delayed LFA-1-dependent ring-shaped membrane projection and subsequent TEM. Collectively, these findings demonstrate that 507RKIKK511 is an essential motif for the micrervillous ICAM-1 presentation and further suggest a novel regulatory role for ICAM-1 topography in leukocyte TEM.

1732 Sustained Thy-1 Engagement of avß3 Integrin Stimulates Astrocyte Migration

N. Munoz,1 D. Munoz,1 A. F. G. Quest,1 K. Barridge,1 L. Leyton1; FonDAP Center for Molecular Studies of the Cell, Facultad de Medicina, Universidad de Chile, Santiago, Chile, 1Cell & Developmental Biology, University of North Carolina at Chapel Hill, Chapel Hill, NC

Astrocytes, the most numerous glial cells in the central nervous system contribute to a fibroblast-like morphology when exposed to blood borne factors upon brain injury. These so called ‘reactive astrocytes’ also proliferate and migrate to the damaged site to form the glial scar, which constitutes a major impediment to neuronal regeneration. Such morphological changes observed in astrocytes in vivo may be related to those triggered in vitro by Thy-1, an abundant antigen expressed on the surface of T lymphocytes. Here we hypothesized that Thy-1 interaction with avß3 integrin in astrocytes provoking dramatic changes in cell shape and increased cell adhesion. Thus, in this study we hypothesized that sustained Thy-1 engagement of avß3 integrin stimulates astrocyte migration. An astrocyte monolayer was scratched with a pipette tip and migration into the cell-free zone was monitored after treating the cells with soluble Thy-1-Fe-Protein A complexes. Thy-1, but not a control Fe-protein, induced the formation of filopodia, lamellipodia and also migration of astrocytes into the wounded area. Moreover, according to results obtained with MTS proliferation and Trypan blue exclusion assays, this finding was not due to cell proliferation. Our data indicate that binding of Thy-1 to avß3 integrin initially stimulates cell adhesion and changes in cell shape, followed by cell migration in a sequence similar to that described for astrogliosis.

1733 The C. elegans LICAM, LAD-2, Functions in Axon Pathfinding

W. Wang,1 T. Cheever,1 V. Schwarz,2 K. Opperman,1 H. Hutter,1 L. Chen1; GCD, University of Minnesota, Minneapolis, MN, 2Developmental Biology, Simon Fraser University, Burnaby, BC, Canada

LICAMs are highly conserved adhesion molecules that are involved in the immune system and in neural development. LICAM homologues in C. elegans, LAD-1, SAX-7 and LAD-2, also play key roles in the immune and nervous systems. Like other LICAM family members, LAD-2 has a short divergent cytoplasmic tail that lacks any recognizable motif, including the ankyrin-binding motif that is present in LICAMs. In C. elegans, the gene sax-7 is ubiquitously expressed, but transcriptional promoter analysis revealed that sax-7 is not expressed in the nervous system. Our data indicate that binding of Thy-1 to avß3 integrin initially stimulates cell adhesion and changes in cell shape, followed by cell migration in a sequence similar to that described for astrogliosis.
cases where apoptosis is blocked, extrusion could enable a carcinoma to exit its primary site within an epithelium, facilitating its metastasis to another site.

Contact the actin and myosin ring. This suggests a model whereby the plus ends of microtubules in the cells neighboring a dying cell contact and locally activate contraction in the basal portion of tissue lymphoma 1) in the signaling from PKC to the IκB activity.

We present results that activation of CaMKII regulates the interactions between the key scaffolding proteins CARMA1, BCL10 (B cell leukemia 10) and MALT1 (mucosa-associated lymphoid tissue lymphoma 1) in the signaling from PKC to the IκB kinase at the T cell immunological synapse. Our results support a model by which Ca2+ signals, through CaMKII activation of NF-κB, can be integrated at the T cell immunological synapse.

Microtubules Regulate the Polarized Contraction of Actin and Myosin That Drives Apical Extrusion of Apoptotic Cells

J. Rosenberg, G. Slattum, L. Cramer, K. McGee; 1Oncological Sciences, Huntsman Cancer Institute, Salt Lake City, UT, 2Cell Biology, LMCB University College London, London, United Kingdom, 3Division of Cell Biology, Institute of Ophthalmology, UCL, London, United Kingdom

We have previously found that to maintain the barrier function of an epithelial monolayer, cells within the epithelium extrude their dying cells. During extrusion, a dying cell signals its live neighbors that react by forming an actin and myosin ring that contracts to squeeze the apoptotic cell out. Apoptotic cells typically get extruded apically (into the lumen), but can also exit basally (to the mesenchyme/blood). As the eventual fate of an apoptotic cell depends on where it gets extruded, we want to determine what dictates the direction of apoptotic cell extrusion. We find that the extrusion polarity is driven not by where the actin/myosin extruding ring forms but rather by the direction that it contracts. Furthermore, we find that microtubules spatially regulate ring contraction. Disrupting microtubules with nocodazole blocks contraction and extrusion of most cells. By contrast, interfering with microtubule dynamics by taxol addition reverses the direction of extrusion. Disruption with either drug does not change the inherent polarity of the cells within the monolayer; rather, it disrupts the number of microtubule plus ends that contact the actin and myosin ring. This suggests a model whereby the plus ends of microtubules in the cells neighboring a dying cell contact and locally activate contraction in the basal portion of the actin/myosin ring; basal contraction then drives extrusion apically. These results may also have implications for tumor cells where cell death is blocked. Since extrusion can still occur in cases where apoptosis is blocked, extrusion could enable a carcinoma to exit its primary site within an epithelium, facilitating its metastasis to another site.

Up-regulation of Semaphorin 3A by EGF in Cultured Human Corneal Fibroblasts

J. Ko, 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. Semaphorin 3A (Sema3A), one of the most well-characterized members of this family, also up-regulates the expression of cell adhesion molecules and regulates cell-cell and cell-extracellular matrix interactions as well as cell motility in fibroblasts. Fibroblasts in the corneal stroma are surrounded by collagen fibers and are responsible for maintenance of transparency of the cornea. Epidermal growth factor (EGF) stimulates collagen synthesis by corneal fibroblasts and thereby contributes to maintenance and repair of the extracellular matrix of the corneal stroma. We have now investigated the effect of EGF on Sema3A expression in cultured human corneal fibroblasts. Reverse transcription and polymerase chain reaction analysis revealed that human recombinant EGF increased the amount of Sema3A mRNA in these cells in a concentration-dependent manner up to 50 ng/ml. At higher concentrations (100 ng/ml), however, EGF induced down-regulation of Sema3A mRNA. The maximal increase in the abundance of Sema3A mRNA induced by EGF 50ng/ml was apparent at 12 h. Immunoblot analysis showed that EGF also increased the amount of Sema3A protein in corneal fibroblasts in a concentration-dependent manner up to 10 ng/ml. An inhibitor of the tyrosine kinase activity of the EGF receptor, PD153035 (10 µg/ml), blocked the up-regulation of both Sema3A mRNA and protein by EGF. These findings indicate that EGF acts at its specific receptor to regulate the expression of Sema3A in cornal fibroblasts. This effect of EGF may play an important role in maintenance of corneal structure and repair of corneal damage.

Increased Basal Insulin Secretion from Isolated MIN6B1 Cells: A Consequence of Elevated Cytosolic Calcium

F. Jaques, A. Tomas, J. Jirginger, P.A. Halban; Medecine Genetic and Development, Medical Center Geneva, Geneva, Switzerland

Objective: Cell-connection is essential for proper function of islet-beta-cells. We showed previously that dispersed MIN6B1 cells are poorly responsive to glucose vs. confluent cells. The aim here was to understand why basal secretion is elevated in dispersed. Methods: After 48h in culture, cells were preincubated for 2h in KR-buffer at 2.8mM glucose and then incubated for 1h each at 2.8mM and 16.7mM glucose. In order to decrease intracellular calcium, a 10min period in KR-buffer without calcium supplemented with 0.1mM EGTA was performed at the beginning of the preincubation. Results: When cells were confluent (with extensive cell-cell interactions), basal secretion was low (0.45% content/h) and was stimulated 22-fold with 16.7mM glucose. Dispersed cells without cell-cell interaction showed only weak glucose-stimulation of insulin secretion (1.76fold that was due to greatly elevated basal secretion (3.8% content/h) and to a decrease in glucose stimulated insulin secretion (6.5% vs. 9.8%, dispersed vs. confluent cells). The use of various secretagogues showed that dispersed cells were not able to be stimulated by secretagogues that need a change in intracellular calcium level to allow exocytosis but were able to respond to secretagogues acting by a mechanism independent of changes in cytosolic calcium. Using confocal microscopy, we show that cytoskeleton organization in dispersed cells is disorganized as compared to confluent cells that exhibit a well polymerized cytoskeleton in basal conditions that remodels after glucose stimulation. Finally we show that decreasing intracellular calcium before the secretion test is enough to decrease the basal secretion of dispersed cells to that of confluent cells (0.87% vs. 0.73%, dispersed vs. confluent cells) and allow them to respond to glucose. Conclusions: Dispersed cells appear unable to regulate normally cytosolic calcium levels under basal conditions. This could be rectified by short-term (10min) privation of calcium in order to decrease cytosolic calcium in dispersed cells.

Integrin-dependent Actomyosin Contraction Regulates the Loss of E-cadherin-mediated Adhesion during Epithelial Cell Scattering

J. de Rooij, E. Spanjaard, A. Sonnenberg; Cell Biology, The Netherlands Cancer Institute, Amsterdam, The Netherlands

We are studying the role of the mitogen-activating protein kinases (MAPK) in the integrin-mediated actomyosin-contracting process. The MAPK-activating and -promoting growth factor that induces the loss of cell-cell junctions in MDCK cells, surprisingly did not downregulate the ability of E-cadherin to engage in the homotypic interaction that mediates cell-cell adhesion. Instead, HGF induced an increase in actomyosin-myosin dependent tension on cell-cell junctions, which was needed for their efficient breakdown as confirmed by the use of compliant substrates on which cells fail to produce tension. In addition, surfaces coated with ECM components that promote the reorganization of actomyosin showed more rapid and extensive cell-scattering in response to HGF compared to ECM components that did not induce cell-scattering. We can show that actomyosin contraction is critically involved in the breakdown of E-cadherin-mediated cell-cell junctions in response to HGF and that the observed crosstalk between integrins and cadherins depends on integrin-mediated regulation of actomyosin contraction in these cells. Apart from its apparent regulation by increased tension, a loss of tension also results in a destabilization of E-cadherin mediated adhesion. This implies that, like integrin-mediated focal adhesions, cadherin-mediated adhesions junctions are governed by a tight regime of cytoskeletal tension. The alteration of normal cellular tension, for instance during tumor progression, may thus result in the deregulation of junctional integrity. Currently, we are investigating which of the proteins present in the cadherin adhesion complex are responsible for the regulation by cytoskeletal tension. De Rooij J, Kerstens A, Danuser G, Schwartz MA, Waterman-Storm CR. Integrin-dependent acto-myosin contraction regulates epithelial cell scattering. J Cell Biol. 2005 Oct 10;171(1):153-64.
Phosphorylation as a readout, we are identifying upstream signaling pathways and kinase(s) that presumably act through PKC or a PKC-dependent kinase to directly modify p120 activity. Dephosphorylated by PKC activation. In contrast, S873 is not phosphorylated under basal cell conditions but becomes heavily phosphorylated upon phorbol ester treatment. Using S873 to report the generation and characterization of a novel monoclonal antibody to p120 serine 873 (S873), a C-terminal phosphorylation site. The antibody is p120-, phospho- and site-specific and thereby affects cell morphology, motility, proliferation, and ultimately tumor progression. The majority of p120 signaling events are controlled by an N-terminal 300 amino acid segment of p120-catenin (p120) which regulates cell-cell adhesion through its interaction with the cytoplasmic tail of cadherins. In addition to its link with cell adhesion, p120 modulates Rho-GTPase activities, thereby affecting cell morphology, motility, proliferation, and ultimately tumor progression. The majority of p20 signaling events are controlled by an N-terminal 300 amino acid segment of p20 termed the "regulatory domain". This region contains all but two of the known tyrosine (Y) and serine/threonine (S/T) phosphorylation sites and serves as the binding site for several tyrosine kinases and phosphatases. We have identified the major p120 Y and S/T phosphorylation sites and have generated phospho-specific monoclonal antibodies to several of them. Here, we next examined the effect of p120 loss on MDCK cell growth in soft agar, an assay generally used to predict tumorigenicity in vivo. Surprisingly, while morphological transformation by p120 depletion eliminated the adherens junction proteins E-cadherin, beta-catenin, and alpha-catenin (as reported in other cell lines), but cell-cell junctions were retained, possibly because desmosome and tight junction proteins were not affected. RhoA activity was constitutively elevated, resulting in enhanced formation of actin stress fibers. We next examined the effect of p20 loss on MDCK cell growth in soft agar, an assay generally used to predict tumorigenicity in vivo. Surprisingly, while morphological transformation by dominant active (DA)-Rac1 or v-Src was not obviously affected in p20-depleted cells grown in two-dimensional culture, Rac1- and v-Src-induced, but not H-Ras-induced, growth in soft agar was completely blocked in the absence of p20. Moreover, this effect was efficiently restored by selective inhibition of ROCK. Thus, RhoA activation in p20-depleted cells appears to block Rac1- or Src-induced growth in agarose via a ROCK dependent pathway. The data show that p20 plays an essential role in Rac1- and Src-mediated anchorage-independent growth by negatively regulating RhoA activity.
The Role of Serine/threonine Phosphorylation of P120: Does It Alter E-cadherin Dynamics at the Plasma Membrane?
Y. Fukumoto, Eppley Cancer Center, University of Nebraska Medical Center, Omaha, NE
p120-catenin is a membrane associated protein that regulates E-cadherin at the plasma membrane and was first identified as a substrate for oncogenic Src and receptor tyrosine kinases. In contrast to its well-studied transient tyrosine phosphorylation in response to growth factor stimuli, its relatively constitutive serine/threonine phosphorylation is less well understood. In this study we examined the function of serine/threonine phosphorylation of p120 in cadherin activity using cadherin-deficient MIAPaCa-2 and p120-deficient S2013 pancreatic cancer cells. Forced expression of cadherins in MIAPaCa-2 cells converted the otherwise dispersed cells into epithelial-like cells, localized p120 to sites of cell-cell contact, and induced p120 phosphorylation. Triple alanine mutant constructs of cadherins that couple p120 to the cadherin complex blocked p120 phosphorylation. Detergent extraction showed abundant phosphorylation of p120 in the insoluble fraction, and immunofluorescence confirmed membrane localization of phosphorylated p120. Modified E-cadherin constructs incapable of traveling to the plasma membrane did not induce serine/threonine phosphorylation of p120. However, an E-cadherin cytoplasmic domain that was artificially targeted to the plasma membrane did induce serine/threonine phosphorylation of p120. These data suggest that serine/threonine phosphorylation of p120 occurs independent of signals from cadherin-cadherin dimerization and independent of trafficking through the ER/Golgi. Forced expression of various p120 isoforms in S2013 cells influenced E-cadherin dynamics. Calcium switch and detergent solubility assays indicated that p120 isoform 3A stabilized E-cadherin at the plasma membrane relative to isoform 4A. Since the major phosphorylation domain of p120 is included in isoform 3A but not in isoform 4A these data suggest that serine/threonine phosphorylation of p120 influences the stability of the E-cadherin complex at sites of cell-cell contact.

Regulation of Junctional Integrity by Receptor Protein Tyrosine Phosphatase DEP-1
J. L. Salle, K. Burridge; Cell and Developmental Biology, University of North Carolina Chapel Hill, Chapel Hill, NC
Decreased endothelial cell barrier function and the subsequent increase in leukocyte transmendothelial migration have been associated with several chronic inflammatory disease states, such as asthma, multiple sclerosis, and atherosclerosis. An increase in tyrosine phosphorylation of junctional proteins correlates with a decrease in junctional integrity. Tyrosine phosphorylation of any protein reflects the balance between protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). DEP-1, a receptor PTP, is expressed in a variety of cells and has been found to localize to adherens junctions. DEP-1 binds p120ctn, γ-catenin (plakoglobin) and β-catenin in vitro in a phosphorylation-dependent manner. This localization pattern raises the possibility that DEP-1 regulates cell-cell adhesion. We have explored this possibility by DEP-1 expression via shRNA constructs. Depression of DEP-1 levels causes a reduction in electrical resistance across cell monolayers, suggesting that DEP-1 is important for the maintenance of junctional integrity. In addition, reduction of DEP-1 enhances phosphorylation of p120ctn during EGTA-induced junctional disassembly. Current work is aimed at testing the hypothesis that DEP-1 may be involved in the remodeling of cell-cell junctions during processes such as diapedesis. Supported by Department of Defense BCRP Predoctoral Traineeship Award BC50198 and NIH grant HL45100.

The Role of Alpha-catenin in Invadopodial Assembly in Human Breast Cancer
S. Mandal, Biochemistry, University of Nebraska Medical Center, Omaha, NE
Tumor formation and invasion are significant human health problems. Cell-cell and cell-matrix interactions are important for cellular migration and invasion. During malignant transformation, cells must co-ordinate changes in cell-cell and cell-matrix interactions. Migratory cells display dynamic plasma membranes and form adhesive structures, including invadopodia and podosomes, on their bottom surfaces that interact with the extracellular matrix. Invadopodia facilitate invasion by forming foci for increased extracellular protease digestion. We are interested in how the adherens junction plaque protein alpha-catenin co-ordinates cell-cell adhesion with the activity of invadopodia. Our preliminary data show that re-expression of alpha-catenin in the alpha-catenin null MDA-MB-468 human breast cancer cell line leads to disassembly of invadopodia. We also show that phosphorylation on ERK is dramatically reduced in alpha-catenin expressing MDA-MB-468 cells as compared to the parental cells. This suggests that cross-talk exist between the cadherin/ catenin adhesion complex and ERK signaling. However, the pathways that mediate crosstalk between ERK signaling and invadopodial assembly are not yet understood. Our long-term goal is to understand the role of alpha-catenin in ERK signaling and how this influences the assembly of invadopodia.

Allosteric Regulation of Alpha-catenin and Its Role in Cell-Cell Adhesion and Cytoskeletal Reorganization
J. Benjamin, W. Nelson; Biological Sciences, Stanford University, Stanford, CA
The adherens junction is dependent upon homophilic binding between cadherins from opposing cells and is mediated intracellularly through binding to β-catenin, which in turn binds α-catenin. While it was previously believed that cadherins are directly linked to the actin-cytoskeleton through α-catenin, recent data from our lab show that α-catenin cannot simultaneously bind the cadherin-β-catenin complex and actin filaments in vitro. This mutually exclusive binding is explained by allosteric regulation of α-catenin; monomer preferentially binds the cadherin-β-catenin complex whereas the homodimer binds actin filaments. While the in vitro data are compelling, the existence of a monomer-dimer transition and its effects on actin have not been demonstrated in vivo. One focus of our studies will be to perturb the localization of α-catenin, such as concentrating α-catenin at the membrane independent of cell-cell adhesion. To do this, we added a myristylation/ palmitoylation tag to the α-catenin binding domain of β-catenin. When transiently transfected into MDCK cells, the β-catenin fragment localizes to the plasma membrane. This construct has no effect on expression or localization of endogenous E-cadherin yet it is able to recruit and increase the amounts of endogenous α-catenin at the plasma membrane as compared to untransfected controls. Transfected cells will be tested for changes in actin organization and cellular dynamics to determine if increased concentration of α-catenin is sufficient for its dimerization. These data will allow us to determine the relative importance of α-catenin dimerization on the establishment and maturation of cell-cell adhesion.

Myosin VI Supports E-cadherin Adhesion and Integrity of the Zonula Adherens in Mammalian Epithelial Cells
M. P. Maddugoda, A. Shewan, A. S. Yap; Institute for Molecular Bioscience, University of Queensland, St Lucia, QLD, Australia
Adhesive cell-cell interactions are dynamic processes that determine tissue patterning and integrity during development and in post-embryonic life. The cell-cell adhesion receptor E-cadherin critically supports such morphogenetic events. In recent years it has become increasingly clear that a complex set of cytoplasmic components are involved in the stringent regulation of E-cadherin activity. Specifically, many components have been identified that regulate the functional interaction between E-cadherin and the actin cytoskeleton. Recently, Myosin VI was identified as an actin-based motor protein that affects E-cadherin-based morphogenesis in Drosophila (Geisbrecht 2002); its potential impact on cadherin activity in mammalian systems has not, to date, been identified. Here we report an association between Myosin VI and E-cadherin at mammalian epithelial cell-cell contacts. Myosin VI was recruited to cadherin adhesions in an E-cadherin-dependent fashion. Characteristically, this was a relatively late event in the biogenesis of confluent epithelial monolayers, that coincided with the formation of cohesive, linear contacts and the apparent formation of the zonula adherens. Depletion of Myosin VI by RNAi disrupted the morphological integrity of cell-cell contacts, perturbed the perijunctional actin cytoskeleton and significantly reduced cadherin surface adhesion. Cell-matrix adhesiveness, however, was not affected. The disruption of cohesive cell-cell contacts was also accompanied by perturbed integrity of tight junctions and desmosomes. Our findings then identify Myosin VI as a key cytoplasmic determinant that is recruited by E-cadherin adhesion to support the biogenesis of the apical junctional complex in epithelia.

Involvement of TRPV4 in the Formation of Cell-Cell Junctions in Skin
T. Fukami-Tominaga, T. Sotabe, S. Yonemura, M. Mizuno, M. Suzuki, M. Tominaga; 1Section of Cell Signaling, National Institute for Physiological Sciences, Okazaki, Japan, 2RIKEN Center for Developmental Biology, Kobe, Japan, 3Jichi Medical School, Minamikawachi, Japan
Barrier function of the skin is essential for terrestrial life to protect against both infection from environment and dehydration from inside. Keratinocytes in epidermis continuously proliferate and are stratified, generating the cornified cell layer to provide the hydrophobic barrier. In addition to cornified cell layer, tight junctions caused by maturation of cell-cell adhesion (adherence junction) also form a significant barrier. Differentiation and formation of these junctions need an increase in intracellular Ca2+, followed by reorganization of actin cytoskeleton. However, the molecular mechanism of Ca2+ signaling responsible for the keratinocyte differentiation remains poorly understood. TRPV4 is a Ca2+-permeable ion channel belonging to the transient receptor signal pathways that mediate crosstalk between ERK signaling and invadopodial assembly are not yet understood. Our long-term goal is to understand the role of alpha-catenin in ERK signaling and how this influences the assembly of invadopodia.
potential (TRP) ion channel super family and activated by various stimuli including warm temperatures (~33°C), and known to be expressed in skin keratinocytes. Because cytoplasmic region of TRPV4 was found to bind β-catenin in keratinocytes which is important for maturation of stratification, we examined the role of TRPV4 in the differentiation process of keratinocytes and barrier function by comparing TRPV4-deficient and wild type mice. Impaired barrier function together with thickened cornified layer was observed in TRPV4-deficient mice both in vitro and in vivo levels. Reorganization of actin cytoskeleton and formation of cell-cell junction in TRPV4-deficient keratinocytes was delayed and immature compared to that in wild type ones. Furthermore, increase in cytosolic Ca++ at 33°C upon high extracellular Ca++ was significantly higher in wild-type keratinocytes than in TRPV4-deficient ones. And the increase was inhibited by disruption of actin filaments in wild-type, but not in TRPV4-deficient keratinocytes. We propose that interaction of TRPV4 with β-catenin plays an important role for actin polarization by enhancing tightness of cell-cell adhesion upon Ca++ entry through the channel.

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Regulation of Phosphoinositides in Cell-Cell Adhesion
M. K. Iijima, M. Anai, T. Kodama, Y. Shibasaki; Laboratory for Systems Biology and Medicine, RCAST, The University of Tokyo, Tokyo, Japan
Cell-cell adhesion is essential for cell growth, morphogenesis, and development. Actin rearrangement is required for cell-cell adhesion and is regulated by phosphoinositides such as PtdIns(4,5)P2 (PIP2). Rho family small GTPases have been implicated in cell-cell adhesion and a constitutively active Rac (V12Rac) enhanced this process. However, the mechanisms and downstream effectors remain to be elucidated. We have shown that overexpressed phosphoinositides 5-kinase (PIP5K) γ, a PIP2 synthesizing enzyme, was localized in cell-cell adhesion in A431 epidermoid cells. In this report, we investigated the involvement of other isoforms of PIP5K in cell-cell adhesion. To this end, we have made isoform specific monoclonal antibodies against PIP5Ks and studied endogenous as well as exogenous PIP5Ks. By immunoprecipitation and Western blotting, each antibody detected band(s) with expected size. PIP5K was concentrated in cell-cell adhesion in addition to ruffling in Hela cells. V12Rac increased the accumulation of PIP5K at cell contact sites. PH domain of PLCγ connected to GFP (PH-PLCγ), an indicator for PIP2, and polymerized actin concentrated in contact sites. Our results are consistent with the model that PIP5K increased PIP2 and regulated actin polymerization at cell adhesion. But Rac did not coimmunoprecipitate with PIP5K, suggesting that Rac and PIP5K did not associate directly. PIP5Kγ has been known to be involved in cell-substratum adhesion. These results suggest that phosphoinositides mediated actin dynamics is important both for cell-cell adhesion and cell-substratum adhesion.

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Vitamin C Transport in Sertoli Cells: Functional Evidence for the Presence of Facilitative Hexose Transporters (GLUTs) and Sodium-dependent Vitamin C Transporters (SVCTs)
C. Angulo, M. A. Castro, R. Maldonado, A. J. Yahez, J. C. Sileve, J. C. Vera, I. I. Concha, Bioquimica, Universidad Austral de Chile, Valdivia, Chile, Fisiopatologia, Universidad de Concepcion, Concepcion, Chile
A central question for a better understanding of testicular function is how germ cells obtain vitamin C, considering that the hemato-testicular barrier limits the direct access of molecules from the plasma into the adenlular compartment of the seminiferous tubule. In this study we analyzed the molecular identity of the transporters involved in the transport of vitamin C in isolated Sertoli cells and 42GPAA Sertoli cell line. WT1 was used as Sertoli cells specific marker to further validate the use of 42GPAA cell line as a model system. The kinetic assays revealed that both, ascorbic acid transporters (GLUTs) and sodium-dependent vitamin C transporters (SVCTs), are functionally active in these cells. This data is consistent with the concept that Sertoli cells have the ability to take up vitamin C, a finding that may be of importance to the physiology of the germ cells. (FONDECYT 1060135 and 1020451, PhD thesis support grant CONICYT 2003, MECESUP AUS0006)

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Participation of Astrocytes in the Inhibition of Neuronal Glucose Uptake by Ascorbic Acid
M. A. Castro, C. Angulo, I. I. Concha, F. Nuñal; Biochemistry, Universidad Austral de Chile, Valdivia, Chile, Cell Biology, Universidad de Concepcion, Concepcion, Chile
Ascorbic acid (AA) is highly concentrated in the Central Nervous System due to sodium-dependent vitamin C transporters in cerebral cells. We have previously demonstrated that an increase in neuronal intracellular AA inhibits glucose utilization in neurons under synaptic glutamatergic activity. Because L-glutatmate is able to stimulate AA efflux from astrocytes, we postulate that astrocytes could play a fundamental role on neuronal glucose inhibition. We show here that L-glutamate stimulates 1H-AA exportation from astrocytes and exported AA inhibited 1H-deoxyglucose (1H-DG) uptake in neurons. The effect, elicited by L-glutamate, was concentration and time dependent. Finally, we observed that 1H-DG transport inhibition was dependent upon AA concentration used in each experiment and on neuronal AA uptake. Therefore, AA exportation from astrocytes to neurons stimulated by L-glutamate and inhibition of neuronal DOG uptake could represent a key element in a metabolic modulation on neurons under glutamatergic synaptic activity. (FONDECYT 1060115, PhD thesis support grant CONICYT 2003)

1754
Membrane Proteins Alkylated by Sulphur Mustard
N. M. Sayer, A. C. Green, J. Jenner, C. D. Lindsay; Biomedical Sciences, Defense Science and Technology Laboratory, Salisbury, United Kingdom
Although much research has been carried out on the vesicant and lung damaging agent Sulphur Mustard (HD) since its first use in World War I little is known about the mechanisms of action underlying its toxic effects. It is known that HD and related compounds have the ability to alkylate and cross-link biomolecules however the exact nature of the cellular target's that initiate HD-induced inflammation and blistering remain unknown. To understand the mechanisms of action of HD in binding to the skin and causing vesication we have investigated the interactions occurring between HD and cellular proteins. In this study we describe the labelling of cultured keratinocyte cell membrane proteins by HD and a related vesicant with the aim of identifying proteins important in HD pathology. Membrane fractions were prepared and analysed by Western blotting to confirm the distribution of sub-cellular fractions. These membrane fractions were incubated with 1H-C and the labelled proteins separated and detected using 1D and 2D electrophoresis and fluorography. Current studies show 1H-C labelling of a number of membrane proteins. Comparison of these results with those of 14C-hemi-HD, a compound with lower vesicant potential shows differences in labelling of a number of cell membrane proteins, especially those of higher molecular weight. These differences will be used to identify proteins alkylated selectively by HD and HD-related compounds with vesicant properties, using mass spectroscopy. Knowledge of these target proteins will benefit our understanding of the vesication process. © Crown Copyright 2006

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Multiple Regulatory Roles of Non-T Cell Activation Linker (NTAL/LAB) in Mast Cell Signaling
P. Draber, L. Draberova, G. M. Shaik, P. Volna, M. Tumova, P. Lebduska, P. Heneberg, J. Korb; Signal Transduction, Institute of Molecular Genetics, Prague, Czech Republic
The transmembrane adaptor NTAL [non-T cell activation linker, also called linker for activation of B cells (LAB)] assembles multiple proteins involved in immunoreceptor signaling. Previously, human mast cells with a decreased NTAL expression, by small interfering RNA, were shown to exhibit reduced Fce receptor I (FceRI)-mediated degranulation, whereas bone marrow derived mast cells cultured from NTAL-/- mice exhibited enhanced degranulation, compared to wild-type mice. To understand the effect of NTAL on mast cell signaling, we compared the FceRI-mediated activation events in rat basophilic leukemia 2H3 cells with enhanced or reduced NTAL expression. Overexpression of NTAL inhibited FceRI-induced tyrosine phosphorylation of the FceRI subunits, Syk kinase, LAT and Gab2 adaptor. Downstream events such as calcium mobilization and degranulation were also impaired. On the other hand, reduced expression of NTAL (by RNA interference) had no effect on phosphorylation of FceRI subunits, Syk and LAT, but it did inhibit calcium mobilization and secretory responses. The amount of cellular NTAL directly correlated with the uptake of extracellular calcium in thapsigargin-stimulated cells. Interestingly, thapsigargin did not induce enhanced NTAL tyrosine phosphorylation, suggesting a novel function of NTAL in immunoreceptor signaling. Formation of signaling assemblies on the plasma membrane, containing phosphatases and other signaling molecules, was also affected by enhanced or reduced expression of NTAL. These results suggest that NTAL regulates the FceRI-mediated activation events at multiple steps and by different mechanisms.

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Transmembrane Domains of the Sln1 Osmosensor Regulate Signaling
G. D. Tchou, M. C. Gastin; Biochemistry & Cell Biology, Rice University, Houston, TX
The ability to sense and adapt to changes in external osmotic pressure is an essential feature of cellular survival, particularly in yeast and other microorganisms. In Saccharomyces cerevisiae, the transmembrane osmosensor Sln1p is thought to act as the primary regulator of the osmotic stress response. During constant osmotic conditions, Sln1p participates in a multistep phosphorelay system that inhibits the HOG (high osmolarity glycerol) pathway and positively regulates genes involved in cell wall biogenesis and cell cycle progression. Exposure to increased...
external osmolarity inactivates Sln1p, leading to rapid phosphorylation of Hog1p and transcription of osmoadaptation genes. Putative osmosensors homologous to Sln1p are found in many bacteria, fungi, and plants, but the mechanism by which these proteins detect and respond to changes in external osmolarity is largely unknown. Previous work has indicated that the two transmembrane domains (TMDs) of Sln1p are important for osmotic signaling. In order to determine how the TMDs might be functioning in the Sln1p-mediated osmotic stress transduction mechanism, we targeted two potential dimerization regions within the TMDs for mutation. Interestingly, while replacement of TMD1 with sequences from topologically similar membrane proteins resulted in the loss of Osm1p stability within the two TMDs increased the activity of the receptor. These results suggest that helix-helix interactions between the Sln1p TMDs may play a central role in its osmotic signaling mechanisms.

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The Functional Regulation of Extracellular Calcium Sensing Receptor by Caveolin in Mouse Mesangial Cells
S. Cha, 1 J. Kwak, 1 J. Kwak, 1, 2Department of Pharmacology and Toxicology, Inha University, Incheon, Republic of Korea, 1Department of Physiology and Biophysics, Inha University, Incheon, Republic of Korea
The extracellular calcium sensing receptor (CaSR) belongs to the type III family of G-protein-coupled receptors, a family that comprises the metabotropic glutamate receptor. The CaSR plays an important role in calcium homeostasis in various cell types. In this study, we examined the expression of the CaSR in the mouse mesangial cells (MMC). Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed with CaSR-specific primers, and this was followed by nucleotide sequencing of the amplified product; this process identified the CaSR transcript in the MMCs. Moreover, CaSR protein was present in the MMCs as assessed by Western blot and immunocytochemical analysis using a polyclonal antibody specific for the CaSR. Functionally, [Ca2+]o-induced increment of the intracellular calcium concentration ([Ca2+]i) in dose-dependent manner. This [Ca2+]o increment by [Ca2+]o was attenuated by the pretreatment with a phospholipase C inhibitor (U73122) and also by a pretreatment with a CaSR antagonist (NPS 2390). The similar results also obtained in IP3 accumulation by [Ca2+]o. To investigate the physiological effect of the CaSR, the effect of the [Ca2+]o on cell proliferation was studied. The increased [Ca2+]o (up to 10 mM) produced a significant increase in the cell numbers. This mitogenic effect of [Ca2+]o was inhibited by the co-treatment with a CaSR antagonist. In addition, CaSR bind to caveolin and CaSR function in [Ca2+]o increment was significantly inhibited by the treatment with caveolin antisense oligodeoxynucleotide. From these results, the [Ca2+]o-induced [Ca2+]i elevation in the MMC is coupled with the extracellular calcium sensing receptor. Furthermore, [Ca2+]o produces a mitogenic effect in MMCs and upregulated by caveolin in normal physiological condition.

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Caveolins Bind to Renal Organic Anion Transporter 1 and Regulate Organic Anion Uptake
J. Kwak, 1 C. Su, 1 S. Cha, 1, 2Pharmacology and Toxicology, Inha University, Incheon, Republic of Korea, 3Physiology and Biophysics, Inha University, Incheon, Republic of Korea
The rat organic anion transporter 1 (rOAT1) has recently been identified as the first member of the organic anion transporter family. The mechanisms that regulate rOAT1 function remain to be elucidated. rOAT1 contributes to the transport of a number of organic anions across the basolateral membrane. However, the role of rOAT1 in the intracellular calcium signaling remains unclear. To address the relationship of these two proteins, we investigated the protein-protein interaction between rOAT1 and Cav-1 or -2. The rOAT1 mRNA and protein expression were observed in the rat kidney, and the expressions of Cav-1 or -2 mRNA and protein were also detected in the kidney. Confocal microscopy of the immuno-cytochemistry experiments using primary cultured renal proximal tubular cells showed that OAT1 and Cav-1 or -2 were co-localized at the plasma membrane, respectively. This finding was confirmed by Western blot analysis using isolated caveolae-enriched membrane fractions from the rat kidney and immuno-precipitation experimentation. When rOAT1’s synthesized cRNA of rOAT1 along with the antisense oligodeoxynucleotide of Xenopus Cav-1 or -2 were co-injected into Xenopus oocytes, the [3H] aminoohippuric acid uptake was significantly decreased. These findings suggest that rOAT1 and caveolin-1 or -2 share a cellular expression in the plasma membrane and Cav-1 and -2 regulate the organic anionic compound uptake via rOAT1 under normal physiological conditions.

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Molecular Identification and Functional Characterization of a Novel Organic Anion Transporter OATN1
T. Hira, 1 E. Babu, 1 X. He, 1 T. Ishikawa, 1 T. Soga, 1 N. Anzai, 1 H. Endou, 1 Y. Kana, 1 Pharmacology and Toxicology, Kyorin University School of Medicine, Tokyo, Japan, 3Research and Development, Human Metabolome Technologies, Inc., Yamagata, Japan, 2Institute for Advanced Biosciences, Keio University, Kanagawa, Japan
The kidney plays the important roles in the removal of harmful endogenous compounds and xenobiotics from the body and the reabsorption of the essential endogenous compounds. SLC22 organic ion transporter family is responsible for these organic ions transport across the epithelial cells in the renal proximal tubules. This family is composed of 3 subfamilies: organic anion transporter (OAT), organic cation transporter (OCT) and organic cation/wortmannin transporter (OCTN) subfamily. Recently, we identified a novel SLC22 family member, OATN1 (golv type organic anion transporter 1) from the genome database. OATN1 shows ~36% identity to other OAT members at amino acid level. Proximal tubule cell lines (S2 cells) stably expressing OATN1 were used for the functional study. OATN1 mediated transport of nicotinic acid, salicylic acid and prostaglandin D2. In contrast, OATN1 does not transport the typical substrates for OATs (para-aminobenzoate, estrone, estrone sulfate, taurocholate), OCTs (tetraethyl ammonium, choline) and OCTNs (carnitine). OATN1 mRNA was detected only in kidney. Immunohistochemical study showed that OATN1 protein was localized at the apical membrane of renal proximal tubules. Since the substrate selectivity and structure of OATN1 are different from those of other OAT family members in spite of the same localization, we speculate that OATN1 should denote a member of a new SLC22 subfamily and have a different physiological role in renal proximal tubules.

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Cell Migration and Signaling Specificity Is Determined by Ralc’s Phosphatidyserine Recognition Motif
C. V. Finkielstein, 1 M. Overduin, 2 D. G. S. Capelluto 3; 1Biological Sciences, Virginia Polytechnic Institute and State University, Blacksburg, VA, 2School of Medicine, CR-UK Institute for Cancer Studies - University of Birmingham, Birmingham, United Kingdom, 3Chemistry, Virginia Polytechnic Institute and State University, Blacksburg, VA
The Rho guanine triphosphatases (GTPases) control cell shape and motility and are frequently overexpressed during malignant growth. These proteins act as molecular switches cycling between active GTP- and inactive GDP-bound forms. Despite being membrane-anchored via their isoprenylated C-termini, Rho GTPases rapidly translocate between membrane and cytosolic compartments. Here, we show that the Rho GTPase Rac1 preferentially interacts with phosphatidyserine (PS)-containing bilayers through its polybasic motif (PBM). Rac1 isoprenylation contributes to membrane binding but is not critical for PS recognition. The similar protein Cdc42 (cell division cycle 42), however, only associates with PS when prenylated. Conversely, other Rho GTPases such as Rac2, Rac3 and RhoA do not bind to PS even when they are prenylated. Cell stimulation with PS induces translocation of Rac1 towards the plasma membrane and stimulates GTP loading, membrane ruffling and filopodia formation. This stimulation also promotes Cdc42 activation and phosphorylation of mitogen-activated protein kinase through Rac1/PS signaling. Consequently, the PBM specifically directs Rac1 to effect cytoskeletal rearrangement and cell migration by selective membrane phospholipid targeting.

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Characterization of the DHHC Protein Acyltransferase (PAT) Erf2/Erf4 That Palmitoylates Yeast Ras Protein
K. Ishizuka, L. Zhao, C. Budde, G. Ganeshan, H. Guan, T. Berg, D. A. Mitchell, R. J. Deschenes; Biochemistry, Medical College of Wisconsin, Milwaukee, WI
Palmitoylation is an important posttranslational modification of conserved polar residues with functional and regulatory roles for many proteins. Recently, our lab has identified protein acyl transferases (PATs) for yeast and mammalian Ras protein palmitoylation. In yeast, it is composed of Erf2 and Erf4, which colocalizes on the endoplasmic reticulum (ER) membrane. Both subunits are required for the Ras PAT activity, but the function of individual subunit is unclear. Erf2 contains four putative transmembrane domains (TMDs) and a conserved zf-DHHC (zinc-finger Asp-His-Cys) cysteine-rich domain, which is characteristic of the PAT family. On the other hand, Erf4 has no recognizable motif. To better understand the mechanism of palmitoylation by Erf2/Erf4, we began by determining the topology of Erf2. Analysis of Erf2 amino acid sequence predicted that N- and C-terminus and the loop between TMD 2 and 3, which contains the zf-DHHC domain, are in the cytoplasmic side of the ER membrane. The prediction was tested experimentally by inserting invertase Sac2, which is used as the reporter for the lumenal exposure indicated by glycosylation, into various positions. The results were consistent with the prediction. In addition, the association of Erf4 with Erf2 and/or the membrane has been investigated. Transcriptional mutants of Erf2 and Erf4 were constructed to determine the domain required for the Erf2-Erf4 interaction. Our co-immunoprecipitation results show that the region of Erf2 including a zf-DHHC domain is required for the association of Erf2 and Erf4. Detailed study of Erf2/Erf4 complex serves as a model for the growing list of zf-DHHC proteins that have been found in eukaryotes examined to date.
A Proteome-scale Screen Identifies Novel Phosphoinositide Binding Proteins

S. Lee, 1 M. Kerr, 1 R. Sim, 1 K. A. A. Galbraith, 2 S. Madharavanu, a C. E. Kennedy, b L. C. Cantley, 2 S. J. Field, 1 Endocrinology, University of California San Diego, La Jolla, CA, 2 Division of Signal Transduction and Department of Systems Biology, Beth Israel-Deaconess Medical Center and Harvard Medical School, Boston, MA

Proteins that bind phosphoinositides are critical regulators of membrane trafficking, cell proliferation, apoptosis, and metabolism. Although the number of proteins that are known to bind phosphoinositides has increased dramatically over the past several years, many more are likely to exist. We have devised a systematic and comprehensive approach to identify phosphoinositide binding proteins. We have used a high-throughput assay to screen ~4800 unique proteins (so far) for their ability to bind each of the phosphoinositides. The screen has identified many proteins that bind phosphoinositides. We will present data identifying a novel protein that we demonstrate binds Phosphoinositide 4P both in vitro and in vivo. This protein localizes to the Golgi due to its affinity for Phosphoinositide 4P. Further characterization of this protein will be presented.

Immunohistochemical Studies on the Isolation of Protein 4.1R during Erythropoiesis

W. Nunomura, a M. Hibiuchi, b H. Kato, c Y. Gao, c K. Sawada, a N. Mohandas, a Y. Takakawa a a Biochemistry, Tokyo Womens Medical University, Tokyo, Japan, b Internal Medicine III, Akita University, Akita, Japan, c Red Cell Physiology, NYBC, New York, NY

Two major isoform of protein 4.1R, 135kDa type (4.1R 135) and 80kDa type (4.1R 80) are expressed in erythroblast and in erythrocyte, respectively. Only one difference between these molecules is 4.1R 135 having additional 209 amino acids, named head-piece (HP) at the NH2 termini of 4.1R 80. Based on the in vitro binding assay, we suggested that the Ca2+ regulates the 4.1R 135 binding to membrane proteins through the calmodulin binding to HP. In the present study, we studied on the immunohistochemical localization of 4.1R isoform during the erythropoiesis. [Material and Methods] Isolation and culture of CD34 + cells and immunocytochemical methods were described in previous paper (Blood 2006 107:1366). We prepared the affinity purified rabbit antibodies to HP and to 4.1R. The antibodies to glycoporin A (GPA) and glycoporin C (GPC) were purchased from Sigma. The cell lysate was fractioned by ProteoExtract™ kit (CALBIOCHEM). To examine the effects of Ca2+ on the 4.1R distribution, the cells at D7 (7 days culture after the isolation) cultured in the presence of 5mM EGTA for 40 min. [Results] A. Immunocytochemistry: At D5, the 4.1R 135 broadly distributed in the cells. At D7, the 4.1R 135 localized in the cytoplasm and at the peripheral membrane. At D12, the 4.1R 135 was stained like a dot in the cytoplasm. The GPA and GPC localized at the peripheral membrane after D7. In the EGA treated cells, the 4.1R 135 was strongly stained at the peripheral membrane. B. Immunoblot: The immunoblot detected 4.1R 135 and 4.1R 80 in the membrane fraction at D7. At D12, the 4.1R 80 was more strongly detected in the membrane fraction. However, the band for 4.1R 135 was hardly seen in each fraction. [Conclusion] The localization of 4.1R isoform at peripheral membrane may change during the erythropoiesis. The Ca2+ may regulate the distribution of the 4.1R isoform.

Protein 4.1R Isolom in Blood Cells of Zebrafish (Danio rerio): Analysis of Membrane Binding Properties

W. Nunnoru a, Y. Takakaw a, K. Murata a a Biochemistry, Tokyo Womens Medical University, Tokyo, Japan, a Animal Science, California University at Davis, Davis, CA

Introduction: We have reported that the binding profiles of N-terminal 30kDa (so-called FERM, 4.1-ezrin-radixin-moesin) domain of zebrafish protein 4.1R (ZF4.1R) with human Band3, Glycoporin C (GPC) and p55. A complex of Ca2+ and calmodulin (CaM) did not regulate the 4.1R interactions with these membrane proteins (45 ASCB Meeting Abstract #2608 (2005)). In this study, we found novel isoform of 4.1R containing part of 30kDa domain named BL42 in the blood cells and studied on its binding to membrane proteins of zebrafish and CalMs. [Materials and Methods] Expression of recombinant proteins, BL42, ZF4.1R and GPC (GPCcyt) as a GST-fusion protein and purification of CalMs were performed according to previous reports (JBC 2000 275, 24540). The RNA was isolated from the blood cells with RNA isolation kit (STRATAGENE). RT-PCR was performed with ritanumMTM One-step RT-PCR™ kit (CLONTECH) following the industry instruction. Rabbit antibodies to 4.1R, GPA and p55 of zebrafish were prepared in our laboratory. Protein-protein interaction was measured using IAsys system based on the resonant mirror detection method. [Results] (1) BL42 contains 197 residues of N-terminal and 122 residues of C-terminal of 4.1R. (2) GPC, p55 and 4.1R were detected immunocytochemically in the red cells, (3) The K(D) values of BL42 binding to GPC was ~10 -7 M; (4) The K(D) value for BL42 binding to CalM was ~10 -11 M in the presence and the absence of Ca2+; (5) Ca2+-CalM did not reduce the binding affinity for BL42 to GPCcyt. [Conclusion] Our results suggest that BL42 binding to membrane proteins is not affected by Ca2+-CalM in erythrocytes regardless of Ca2+ level. [Foot Note] The authors WN and KM contributed in equivalent to this study. This study was supported in part by Grant-in-Aid for Scientific Research from the Ministry of Education Culture, Sport, Science and Technology of Japan (#15570123 for NW).

Tissue-specific Alternately Spliced Protein 4.1R Isoforms in the Heart and Blood of the Zebrafish, Danio rerio

K. Murata a, D. Chu a, E. Shimada a, G. Chen a, W. Nunomura a a Animal Science, California University at Davis, Davis, CA, a Environmental Toxicology and Nutrition, Bodega Marine Laboratory, University of California at Davis, Bodega Bay & Davis, CA, a Biochemistry, Tokyo Women’s Medical University, Tokyo, Japan

Introduction: Protein 4.1R (4.1R) has been identified as the major component of the erythrocyte membrane cytoskeleton involved in maintaining the erythrocyte shape and controlling membrane mechanical properties. We have demonstrated the existence of the 4.1R isoform in zebrafish using immunological analysis (ASCB Meeting Abstract #2608, 2005). Particularly in heart tissue, different isoforms are localized in the ventricle and atrium respectively. [Objectives & Methods] To identify the 4.1R isoforms, several CDNs were isolated using RT-PCR. [Results] Full length CDNA encoding zebrafish p4.1R (ZF4.1R) (NM_175084) is composed of putative functional domains that correspond to the human 4.1R N-terminal 30kDa FERM (four-one, ezrin, radixin, moesin) domain, a spectrin and actin binding domain (SABD) and a C-terminal domain (CTD), two unique domains (UD) and an amino acid repeated domain. Five DNA fragments from the blood samples and four from heart sample were amplified. Blood3 encoded the entire FERM domain plus truncated UD and CTD. The Blood4 encoded the FERM domain missing the Ca2+-insensitive calmodulin (CaM) binding site, truncated SABD and CTD missing the p55 binding site. Blood5 encoded a truncated FERM domain missing both CaM binding sites, truncated SABD and truncated CTD. Of note, the Heart3 encoded only truncated UD and whole SABD and CTD. [Conclusions] In the erythrocyte, three unique clones were obtained and their gene products may compensate each other for maintaining the cell shape and its physiological function. The Heart3 gene product may be involved in stabilizing cardiomyoctye structure in the atrium. This suggests that the hetero dimer of spectrin is important in stabilizing the plasma membrane and only SABD in the human 4.1R molecule is essential to bind with hetero dimer of spectrin. These results supported our hypothesis that in the zebrafish, each tissue-specific alternatively spliced gene product of 4.1R will have different functions in different tissues.

Apoptosis and Blood-Testis Barrier during the First Spermatogenic Wave in the Puberlal Rat

J. Cavicchia, 1 A. Morales, 2 F. Mohamed 2 Cuyo Medical School, Institute of Histology and Embryology (IHEM), Mendoza, Argentina, 1 Universidad Nacional de San Luis, Facultad de Quimica, Bioquimica y Farmacia, San Luis, Argentina

In previous works we have found in several animal species that the classically called blood-testis barrier (BTB) or more properly termed Sertoli cell barrier develops in close proximity to zygotene-pachytene spermatocytes. This barrier isolates all developing germ cells from this stage on. It is interesting the exploration of the initial assembling of the BTB during puberty because of a massive physiological apoptosis in the first meiotic cells. Fragments of testsis from 14 to 20 day-old rats were fixed in buffered glutaraldehyde followed by conventional transmission electron microscope techniques. Lanthanum hydroxide was used as an intercellular tracer. TUNEL assay was undertaken in paraffin embedded sections to confirm apoptotic death by light microscopy. When the germ cell line reaches the zygotene-pachytene spermatocyte level and the BTB was not established yet, these cells undergo apoptosis. After the BTB becomes competent, the solid cords turn into the seminiferous tubules and the spermatogenic process continues only with sporadic apoptotic death. The interruption of the sperm cells destined to be isolated in the adluminal compartment prior to BTB assembling may lead to germ cell apoptosis diminishing the efficiency of the first round of spermatogenesis. These events emphasize the importance of a competent BTB in maintaining the progress of spermatogenesis during puberty.

2006 ASCB Annual Meeting Abstracts
A Novel Serine Palmitoyltransferase Enzyme Isolated from the Emiliania huxleyi

G. Han,1 L. Yan,1 K. Gable,2 W. Wilson,3 J. Harmon,1 T. Dunn3;1 Biochemistry, Uniformed Services University of the Health Sciences, Bethesda, MD, 2Microbiology and Immunology, Uniformed Services University of the Health Sciences, Bethesda, MD, 3Plymouth Marine Laboratory, Plymouth, United Kingdom

Serine palmitoyltransferase (SPT), the committed enzyme of sphingolipid synthesis, is a member of the subfamily of pyridoxal 5'-phosphate (PLP) utilizing enzymes known as the α-oxoamine synthases. Most enzymes in this family, including the prokaryotic SPT protein, are soluble homodimers. In contrast, eukaryotic SPT is an ER-associated heterodimer comprised of the Lcb1p and Lcb2p subunits. Previous studies indicated that there is a single active site at the interface of the heterodimer with critical PLP-binding residues residing in both subunits. The stability of the Lcb2p subunit is dependent on Lcb1p. Furthermore, optimal activity of the yeast Lcb2p-Lcb1p heterodimeric enzyme requires the 80-aa Tsc3p protein. However, the basis of stabilization of Lcb2p by Lcb1p and the role of Tsc3p are not well understood. Remarkably, the recently sequenced genome (Wilson et al. (2005) Science 309, 1090-1092) of E. huxleyi (a virus which infects the marine algae Emiliania huxleyi) revealed the presence of a gene predicted to encode a fusion protein containing of an Lcb2p-like domain fused to an Lcb1p-like domain, hereafter called v-SPT. This v-SPT fusion protein was expressed in yeast and was found to complement the lethality of yeast Δlcb1Δlcb2Δ double mutant. Analysis of the sphingoid base profiles from the v-SPT-repressed Δlcb1Δlcb2Δ yeast mutant raised the possibility that the v-SPT preferentially utilizes myristoyl-CoA (C14) rather than palmitoyl-CoA (C16). This was confirmed in enzymatic assays of the v-SPT. Based on the studies of the v-SPT enzyme, we have constructed and undertaken the expression of yeast Lcb2p-Lcb1p and Lcb1p-Lcb2p fusion proteins. The study of these fusion proteins and their interactions with the yeast Lcb1p, Lcb2p and Tsc3p proteins is providing important insights into the structure and activity of the SPT enzyme, as well as into the role of the Tsc3p protein.

Proteomics for Functional Complexes: Rapid and Direct Access to Intracellular Protein Clusters
C. Lin, Fundamental Science, Pacific Northwest National Lab, Richland, WA

To preserve and regulate biological/physiological functions, intracellular proteins often cluster into biologically active complexes comprised of numerous subunits. Identification of all subunits within complexes is important in understanding the mechanisms and structural requirements of biochemical processes. Membrane-bound proteins are often known to be integral parts of biological-active complexes. Isolation and purification of these proteins are usually tedious and inefficient. In order to minimize dissociation between subunits, and lose of some unknown subunits in many complexes, we have developed a co-fractionation strategy to localize and separate complexes under native states, followed by direct MS/MS analysis of the digested protein fractions. For demonstration, clones of recombinant His-tag ATP synthase were expressed in Shewanella oneidensis MR-1. Membrane proteins were solubilized and separated under native conditions, and separated in order of ionic strength on a Mono Q column. Fractions collected were trypsin digested and forwarded to LC MS/MS (Thermo-Finnigan LCQ). Results of the peptide analysis (Sequest) revealed that not only were ATP synthase subunits eluted in identical fractions, but also proteins within other complexes co-eluted in fractions of different ionic strengths, suggesting the presence of a cluster protein pattern. In parallel, we could detect in-gel ATP hydrolysis activity at the molecular size of the synthase complex (> 450 kD) when membrane fractions were subjected to electrophoresis in native conditions. Two dimensional electrophoresis images of the dissociation-free subunits ranging from 20 kD to 60 kD. We suggest that co-fractionation and electrophoretic separation of membrane proteins in conjunction with powerful mass-spectrometric analysis is a valid and rapid way to gain insight about intracellular protein complexes.

Truncation of Connexin43 Protein Changes Number and Size of Cardiac Gap Junctions
K. Maass1, S. E. Chase1, K. Willecke2;1 Pharmacology, SUNY Upstate Medical University, Syracuse, NY, 2Genetik, Universitaet Bonn, Bonn, Germany

INTRODUCTION: Recent in vitro studies have shown that the carboxy terminal region of the gap junction protein connexin43 (Cx43) acts as a regulatory domain, influencing the interactions with other proteins as well as channel gating. To analyze the functional role of Cx43CT on cardiac gap junctions, mice expressing a mutant isoform lacking most of the Cx43CT (Cx43K258stop) in place of wild type Cx43 have been used in this study. RESULTS: Western blot analysis of whole-cell lysates of adult ventricular tissue revealed a 75% reduction in total Cx43K258stop protein compared to controls. Yet Triton solubility experiments indicated that the mutated protein was more prevalent in the Triton-insoluble fraction when compared to wild-type. Only 14.6% (±3.5%) of Cx43 protein was found in the Triton-insoluble fraction in controls (n=4), whereas 49.2% (±3.7%) of Cx43K258stop protein was detected in the Triton-insoluble fraction (n=3). This seems consistent with previous in vitro data showing a prolonged half-life of truncated Cx43. Co-immunofluorescence analysis of the cardiac intercalated discs further showed a segregation of Cx43K258stop gap junction plaques from adherens junctions or desmosomal proteins. Accordingly, only the wild-type Cx43 protein, but not the truncated mutant (Cx43K258stop), could be co-immunoprecipitated with antibodies against proteins of the mechanical cell junctions or the scaffolding protein ZO1. Both immunofluorescence and TEM analysis revealed a significant reduction in the number of gap junctions in Cx43K258stop hearts (TEM per 100 μm2: 0.14 vs 0.03; n=5 vs. n=3 in controls) while the average size of individual gap junctions was increased and showed a higher scattering (TEM 0.78 μm ±0.11 vs 0.5 μm ±0.02 in controls). TEM analysis further revealed that the overall area covered by gap junctions in Cx43K258stop cardiomyocytes was decreased to 40.8% of the area detectable in controls. CONCLUSION: Cx43CT is essential for regulating stability, localization and size of cardiac gap junctions in vivo.

Site-Directed Mutations Improve High-Level Production and Homogeneous Purification of an Affinity-Tagged Chimeric Human Vitamin K 2,3-Epoxide Reductase- Green Fluorescent Protein Construct (hVKORC1-EGFP) in Pichia pastoris for Structure/Function Analysis
C. G. Bevans,1 H. Tran,2 W. Haase,3 M. Huenberg,2 A. Schauhausen,3 S. Rost,3 A. Fregin,4 C. R. Mueller,2 C. Reinhardt5;1 Structural Biology Department, Max Planck Institute of Biophysics, Frankfurt/Main, Germany, 2Institute of Human Genetics, Biocenter - University of Wuerzburg, Wuerzburg, Germany, 3Molecular Membrane Biology Department, Max Planck Institute of Biophysics, Frankfurt/Main, Germany, 4Institute of Experimental Hematology and Transfusion Medicine, University Clinic Bonn, Bonn, Germany

We recently reported high-level expression and purification of a protein construct (α-flag-his6-pVKORC1-biotag) including enzymatically functional human vitamin K 2,3-epoxide reductase (hVKORC1) (Mol. Biol. Cell 18512). To achieve more rapid and sensitive detection of the construct during production in P. pastoris and purification, we replaced the C-terminal biotag (biotinylation domain, J. Biol Chem 265(18):10327-10330) with a C-terminal StrepIITag-labeled green fluorescent protein (α-flag-his6-pVKORC1-EGFP). Although this protein exhibited vitamin K epoxide reductase (VKOR) activity similar to wild-type hVKORC1, we found evidence of proteolysis by endogenous Pichia proteases and intracellular localization suggesting potential dimerization as previously reported for EGFP (Science 296:913). Site-directed mutagenesis of four amino acids resulting in the new construct α-flag-his6-pVKORC1-EGFPmut eliminated endogenous proteolysis during purification, eliminated dimerization, and maintained wild-type enzymatic activity and sensitivity to the specific VKOR inhibitor warfarin. Biochemical analysis of potential post-translational modifications revealed that the alpha factor (α) targeting sequence, but not glycosylation of the hVKORC1 domain. A total production level of 6.6 mg/L in shaker flask culture was achieved as assessed by EGFP fluorescence relative to commercially available EGFP standard protein.

hScrib: A Key Protein in Cell Polarity
C. Navarro1, N. Vitale1, S. Audebert1, S. Nola1, J. Borg2;1 Randall Division of Cell and Molecular Biophysics, King's College London, London, United Kingdom, 2Centre de Neurochimie, Unité Propre du Centre National de la Recherche Scientifique 2356, Strasbourg, France

α(ΔhVKORC1) (exhibited vitamin K epoxide reductase (VKOR) activity similar to wild-type hVKORC1, we found evidence of proteolysis by endogenous Pichia proteases and intracellular localization suggesting potential dimerization as previously reported for EGFP (Science 296:913). Site-directed mutagenesis of four amino acids resulting in the new construct α-flag-his6-pVKORC1-EGFPmut eliminated endogenous proteolysis during purification, eliminated dimerization, and maintained wild-type enzymatic activity and sensitivity to the specific VKOR inhibitor warfarin. Biochemical analysis of potential post-translational modifications revealed that the alpha factor (α) targeting sequence, but not glycosylation of the hVKORC1 domain. A total production level of 6.6 mg/L in shaker flask culture was achieved as assessed by EGFP fluorescence relative to commercially available EGFP standard protein. Purification is achieved by two-step affinity chromatography using Ni-NTA resin (binding the N-terminal polyhistidine tag of the protein constructs) followed by streptactin resin (binding the C-terminal StrepIITag) to ensure recovery of only full length protein construct. Thin section electron microscopy of immunogold labeled protein constructs in P. pastoris cells confirmed localization of α-flag-his6-pVKORC1-EGFP and α-flag-his6-pVKORC1-EGFPmut in different subcellular compartments in stacked membranes that can be easily and efficiently separated from most other cellular organelles before solubilizing with detergents, eliminating contaminants including proteases and enzymes that could post-translationally modify the protein constructs. Functional analysis of VKOR enzymatic activity of affinity-purified α-flag-his6-pVKORC1-EGFPmut following various washing protocols before elution suggests lipid, bound protein or cofactors are essential to maintain enzymatic function. Support: National Genome Research Network Cardiovascular Diseases (BMWF-DLR-01GS0424) & Baxter Germany
spectrometry analysis of selected bands. We discovered several new protein complexes associated with Sec6: ZO-2 and betaPIX/GIT1/Pak1. Moreover, we were able to demonstrate that the PDZ domains of Sec6 are essential to mediate these interactions. Furthermore, we uncovered a function for the Sec6/betaPIX/GIT1 complex in regulated exocytosis and in neuronal transmission. Moreover, this complex mediates vesicular recycling of Thyrotropin Receptor (TSHR) in thyroid cells and is required for the signalling capacities of the receptor. Taken together, these results suggest a role of Sec6 in various aspects of cell polarity and receptor signalling.

1773 Homologues of Oysterol-binding Proteins Affect Cdc42p- and Rho1p-mediated Cell Polarization in S. cerevisiae

K. G. Kozmikowski, G. Allaró, S. Dighe, C. Behl, Biology and Cell Biology, University of Virginia, Charlottesville, VA, 1Molecular Biology and Biochemistry, Simon Fraser University, Burnaby, BC, Canada, 2University of Virginia, Charlottesville, VA.

Polarized cell growth requires the establishment of an axis of growth along which secretion can be targeted to a specific site on the cell cortex. Disrupting the ordered progression of these processes causes a failure in polarized growth, leading to cells with aberrant morphologies and impaired function. How polarity establishment and secretion are choreographed is not fully understood, though Rho and Rab GTPase-mediated signaling is required. Superimposed on this regulation are the functions of specific lipids and their cognate binding proteins. In a screen for S. cerevisiae genes that interact with Rho-family RalA homologous to mammalian oxysterol-binding protein (OSBP). Other yeast OSH genes (OSBP homologues) had comparable genetic interactions with CDC42, implicating OSH genes in the regulation of CDC42-dependent polarity establishment. Loss of OSH genes (OSHI-OSH7) disrupted cell polarization. However, many proteins involved in the polarized organization of the actin cytoskeleton localized at the bud site independently of OSH gene function. We found that the OSH gene family promotes cell polarization by maintaining the proper localization of the Rho GTPases Cdc42p and Rho1p, and the Rab GTPase Sec4p. The OSH gene family is also necessary for proper septin ring assembly. Disruption of all OSH gene function caused specific defects in polarized exocytosis (e.g., block of Bgl2p-associated secretion), indicating the Osh proteins are collectively required for a secretory pathway implicated in the maintenance of polarized growth. In contrast to the above results, mcOsy OSH gene expression, especially that of OSH4, antagonized a rho1 growth defect. The OSBP1 and OSBP2 domains of Os4p (and other Oshs) differentially affected CDC42- and RHOA-regulated cell polarization, respectively. We propose that Osh proteins collectively mediate transitions between early and late cell polarization events.

1774 Molecular Mechanisms for a Non-mechanical Role of Intermediate Filaments in Apical Polarization

A. S. Ono, F. A. Wuld, A. H. Langshaw, P. J. Salas, Cell Biology and Anatomy, Univ. of Miami School of Medicine, Miami, FL, Pediatrics, Univ. of Miami School of Medicine, Miami, FL.

Intermediate filaments (IFs) are apically polarized in simple epithelia. Such a polarization precedes that of microtubules and actin filaments. Previous studies indicate that keratin 8 (K8) null mice villus enterocytes, fully depleted in IFs, display a complex apical phenotype that includes disorganization of the microtubular architecture and redistribution of microtubule-organizing centers (MTOCs), as well as deocalization of apical membrane proteins. Likewise, overexpression of K8 in transgenic mice abolishes the formation of the brush border. The present study was performed to gain understanding of the molecular mechanisms underlying those phenotypes. We analyzed binding in vitro and in the yeast-two hybrid model between keratins and components of the apical actin scaffold (ezrin) and the MTOC (GCP6). Various mutants of these proteins were studied to identify the binding sites in each molecule, as well as the possible regulation by signaling pathways. The results confirm the localization of the keratin binding site in GCP6 within the C-terminal domain, the presence of a physiological Cdk-1 phosphorylation site, and point at the localization of the corresponding binding site in K8 and K19. The binding sites for dormant ezrin were also identified. In addition, and as a by-product of these studies, the data from the microtubule nucleation also support a model of microtubules by non-centrosomal MTOCs, with centrosomes inactive in 70 % of Caco-2 cells in interphase. The results are consistent with a non-mechanical role of apical keratins in the assembly of apical cytoskeletal scaffolds, specially during the acquisition of epithelial polarity.

Ganglioside GM1 and GM3 Highlight Distinct Lipid Microdomains within the Apical Plasma Membrane of Polarized Epithelial Cells

P. Janich, D. Corbeil, Tissue Engineering Laboratories, BIOTECH, Technical University of Dresden, Dresden, Germany

The domain of epithelial cells is composed of distinct subdomains such as plasma membrane protrusions (microvilli and cilium) and a non-protruding region. It contains as well a variety of lipid species that have distinct physico-chemical properties. Using the cholesterol-binding protein prominin-1 as a specific marker of plasma membrane protrusions we have previously proposed the coexistence of different cholesterol-based lipid microdomains (lipid rafts) within the apical plasma membrane, which might underlie the generation and maintenance of subdomain structures (Kopf et al., NatCellBiol. 2000). This hypothesis was based in part on detergent extraction experiments where prominin-1 was found to be soluble in Triton X-100 but insoluble in another non-ionic detergent, Lubrol WX. However, concerns about the ability of this latter detergent to selectively solubilize membrane proteins and thus discriminate between those associated or not with lipid microdomains have been raised. Here we have re-investigated the issue whether the microvillar plasma membrane contains a distinct set of lipids compared to the planar, non-protruding portion of the apical domain by analyzing the distribution of prominin-1 and ganglioside GM1 and GM3 by fluorescence microscopy. Interestingly, GM1 was found to co-localize with prominin-1 on microvilli irrespective of the staining procedures and fixation methods. On the other hand, the major ganglioside of MDCK cells, GM3, was segregated from prominin-1/GM1-positive microdomains suggesting its localization in the planar region. Regarding the cilium overlapping fluorescent signals of GM1 and prominin-1 were observed. Remarkably, GM3 immuno-reactivity was also observed in the cilium. Taken together our data corroborate the hypothesis that the microvillar plasma membrane subdomains are composed of a lipid microdomain that differs from that found in the non-protruding region, and reveal surprisingly that two plasma membrane protrusions with different structural bases (actin for the microvillus and tubulin for the cilium) are composed of distinct types of lipids.

Species-specific Interactions among β-subunit of the Na+K+-ATPase

V. M. Castro Villella, M. Hidalgo, M. Roldán, R. Fiorentino, E. Del Oso Agustín, R. G. Contreras, M. Cereijido, L. Shoshani; Physiology, CINVESTAV-IPN, Mexico City, Mexico

Due to its peculiar polarized expression, Na+K+-ATPase is responsible for the ionic balance of the cell, as well as for the transcellular movements of biologically important substances. We have previously shown that this polarisation is due to specific adhesion between the β-subunits of two Na+K+-ATPases present in neighboring cells (Shoshani, L. et al. Mol. Biol. Cell 16: 1071-1081, 2005). This interaction is a highly specific one. Thus MDCK cells never express Na+K+-ATPase at contacts with CHO cells, except when these express β1-subunit (i.e. same animal origin than MDCK cells). In the present work we pursue the study of this β1-β1 interaction. Using a mammalian two hybrid assay, we observe that the β1-β1 interaction is a direct one, so that an intermediate molecule may not be required. The high specificity of this interaction is revealed by the fact that transfecting CHO cells with β1-subunit from the rat kidney (CHO-β1a) which has a 97% homology with the dog one, does not achieve the correct polarized distribution of the pump. This is confirmed by studies with NRK-E52 which naturally express rat β1-subunit. However, when these NRK-E52 cells are transfected with dog β1-subunit (NRK-β1b), MDCK cells do express their Na+K+-ATPase in the correct polarized position. Taken together, our results indicate that epithelial cells like MDCK express Na+K+-ATPase in its borders contacting another (i.e. at the lateral membrane), because this is the only position where the β-subunits of their Na+K+-ATPases can interact with each other.

Sec5 and Exo84 Mediate Distinct Aspects of RalA/Exocyst-dependent Cell Polarization

K. E. Gokay, D. J. Sturgill, C. Yeaman; Anatomy & Cell Biology, University of Iowa, Iowa City, IA

Spatial regulation of the secretory machinery and the cytoskeleton is essential for development of cell polarity. Yet mechanisms linking polarized exocytosis and cytoskeletal remodeling are incompletely understood. Here, we show that polarized growth of invasive cell protrusions in prostate tumor cells is dependent on the Exocyst, an octameric complex involved in tethering secretion, indicating the Osh proteins are collectively required for a secretory pathway implicated in the maintenance of polarized growth. In contrast to the above results, multicopy OSH gene expression, especially that of OSH4, antagonized a rho1 growth defect. The OSBP1 and OSBP2 domains of Os4p (and other Oshs) differentially affected CDC42- and RHO1-regulated cell polarization, respectively. We propose that Osh proteins collectively mediate transitions between early and late cell polarization events.

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RaLα to limit lamellipodium growth. These results suggest that RaLα-binding to different Exocyst subunits orchestrates activation and inactivation of actin regulatory factors necessary for maintaining cell polarity and persistent migration.

1778 Fission Yeast Type I Myosin Regulates Sterol-rich Membrane Organization through the TH1 Tail Domain
M. A. Ellis, F. Chang; Microbiology, Columbia University, New York, NY

The rod-shaped fission yeast Schizosaccharomyces pombe exhibits distinct membrane domains enriched in sterols at growing cell tips and at the medial region of the cell during cytokinesis. We are interested in how these membrane domains are generated and localized. Our lab recently identified myosin type I (myo1p) as a protein needed for membrane organization (Takeda and Chang, Current Biology 2005). Deletion of myo1p results in loss of distinct sterol-rich domains, while myo1p over-expression causes the formation of ectopic membrane domains. Here, we examine what domains of myo1p are needed for this function, focusing our attention on the polybasic TH1 domain. A myo1 mutant lacking the TH1 domain does not rescue the membrane defects in a myo1-deletion mutant. Overexpression of the TH1 domain alone, but not other tail domains of myo1, partially rescues sterol-rich membrane organization and morphology defects in a myo1 deletion background. Overexpression of the TH1 domain can also cause ectopic sterol-rich membrane formation in wild-type cells. In other myosin type I, the tail domain has been shown to interact with acidic phospholipids such as PI2P. We are currently testing if the TH1 domain of myo1p can bind and cluster membrane components to organize a membrane domain in vitro and in vivo.

1779 Lateral Distribution of Proteins and Lipids within Plasma Membrane Is Triggered by Membrane Potential
G. Grossmann,1 M. Opekavara,2 J. Malinsky,1 I. Weig-Meckl,1 W. Tanner,1 Lehrstuhl für Zellbiologie und Pflanzenphysiologie, Universität Regensburg, Regensburg, Germany, 2Laboratory of Cell Reproduction, Institute of Microbiology CAS, Prague, Czech Republic, 3Department of Microscopy, Institute of Experimental Medicine CAS, Prague, Czech Republic

Two non-overlapping lateral raft-based membrane compartments (RMC) within the yeast plasma membrane have recently been described. The first one (RMC P) is occupied by the H- ATPase, Pma1p (Malinka K et al., Mol Biol Cell 18:4427-36, 2003). The other (RMC C) houses Sar1p, and the proton symporters Can1p, Fur4p (Malinka K et al., J Cell Sci 117:6031-41, 2004), and HUP1, the heterologously expressed hoxose/H+ symporter from Chlorella (Grossmann G et al., Eukaryot Cell 5:945-53, 2006). Here we present evidence that ergosterol, the main lipid constituent of the yeast plasma membrane, together with another H+-symporter, tryptophan permease Tat2p, accumulate within RMC C. We also document that the composition of RMC C depends on the membrane potential. After plasma membrane depolarization, H+-symporters (Can1p, Fur4p, HUP1, Tat2p) together with ergosterol leave RMC C within minutes in a reversible manner, whereas Sar1p is not affected. Depolarization in RMC P is not affected either. Depolarized cells are considerably less sensitive to detants, possibly due to the more disorder structure of their plasma membrane. The reported results provide direct evidence for the dynamic character of yeast plasma membrane compartmentation. Financial support of DFG (Schwerpunkt 1108) and of Fonds der Chemischen Industrie is acknowledged. M.O. was also supported from LC545 and GA CR (204/06/0009), and J.M. from AS CR (AV0Z 5039512) and GA CR (304/05/0374).

1780 Transbilayer Distribution of Sphingomyelin Studied by Freeze-fracture Immunoelectron Microscopy
M. Murate, K. Ishii, T. Kobayashi; Sphingolipid Functions Laboratory, RIKEN, Wako, Japan

Little is known about the transbilayer distribution of lipids in biomembranes. The combination of freeze-fracture replica technique with immunocytochemical labelling (Fujimoto et al. J. Cell Sci. 109, 2453 (1996)) is a promising method to directly examine lateral membrane lipid distribution in both model- and biomembranes under electron microscope. In this study, we examined the lateral and transbilayer distribution of sphingomyelin-rich membrane domains in red blood cells and human skin fibroblast. We have employed the sphingomyelin-specific toxin, lysenin, as a probe (Kiyokawa et al. J. Biol. Chem. 280, 24072 (2005)). In red blood cells, sphingomyelin was distributed exclusively in the outer leaflet of the membrane. Interestingly, in human red blood cells, sphingomyelin is 12-15 % of total phospholipids sphingomyelin-rich domain whose radius was around 30 nm was observed. In contrast, in sheep erythrocytes (sphingomyelin comprises 40-50 % of total phospholipids), sphingomyelin was randomly distributed. Unlike red blood cells, sphingomyelin was distributed both in the outer and the inner leaflet of the plasma membrane from human skin fibroblasts, although majority of the lipid was in the outer leaflet. On the outer leaflet, sphingomyelin formed domains with 30 nm radius. Interestingly, sphingomyelin forms distinct clusters in the inner leaflet of the plasma membrane. Our results thus indicate that sphingomyelin exists both in the inner and outer leaflet of the plasma membranes in fibroblasts but not in red blood cells.

1781 Clustering of Raft-associated Proteins in the External Membrane Leaflet Modules Internal Leaflet H-Ras Diffusion and Signaling
S. Eisenberg,1 D. E. Shvartsman,2 M. Ehrlich,3 Y. J. Hemi,1 Neurobiochemistry, Tel Aviv University, Tel Aviv, Israel

One of the least-explored aspects of cholesterol-enriched (rafts) in cells is the coupling between such domains in the external and internal monolayers and its potential to modulate transbilayer signal transduction. Here, we employed FRAP to study the effects of antibody-mediated patching of influenza hemagglutinin (HA) proteins (raft-resident wild-type HA and glycosylphosphatidylinositol-anchored HA, or the nonraft mutant HA(2A520)) on the lateral diffusion of internal-leaflet raft and nonraft Ras isoforms (H-Ras and K-Ras, respectively). Our studies demonstrate that clustering outer-leaflet or transmembrane raft-associated HA proteins (but not their nonraft mutants) retards the lateral diffusion of H-Ras (but not K-Ras), suggesting stabilized interactions of H-Ras with the clusters of raft-associated HA proteins. These modulations were paralleled by specific effects on the activity of H-Ras, but not of the nonraft K-Ras. Thus, clustering raft-associated HA proteins facilitated the early step whereby H-Ras is converted to an activated, GTP-loaded state, but inhibited the ensuing step of downstream signaling via the Mec/Erk pathway. We propose a model for the modulation of transbilayer signaling by clustering of raft proteins, where external clustering (antibody or ligand-mediated) enhances the association of internal-leaflet raft proteins with the stabilized clusters, promoting either enhancement or inhibition of signaling.

1782 Competitive Recruitment to the Mga5/Galectin Lattice and Caveolin Raft Domains Regulates EGFR Signaling and Tumor Growth
P. Lajoie,1 E. A. Parttridge,2 G. Guay,1 S. Nim,2 J. G. Goetz,1 J. Pawling,2 A. Lagana,4 J. W. Dennis,2 I. R. Nabi1

1Cellular and Physiological Sciences, University of British Columbia, Vancouver, BC, Canada, 2Mount Sinai Hospital, Samuel Lunenfeld Research Institute, Toronto, ON, Canada, 3Pathologie et Biologie Cellulaire, Université de Montréal, Montréal, PQ, Canada, 4Biotechnology Research Institute, National Research Council of Canada, Montreal, PQ, Canada

Goïgi N-acetylgalactosaminyltransferase V (Mga5) generates galectin-binding β1,6GlcNAc-branched N-glycans on receptors that promote surface residency and cytokine responsiveness. Cav1 levels in tumors from PyMT transgenic mice correlate inversely with tumor growth rates in Mga5- mice but not Mga5- littermates, suggesting that downregulation of Cav1 relieves growth restrictions imposed by Mga5 deficiency. Cav1 is a broad-specificity negative regulator of cytokine signaling and lipid raft endocytosis, and a putative tumor suppressor. Reduced Cav1 expression in Mga5- mammary tumor cells restores sensitivity to EGF, but not sensitivity to TGF-β, epithelial-mesenchymal transition, or fibronectin fibrillogenesis, that are all rescued by Mga5 expression in Mga5- cells. Cav1 inhibited raft-dependent endocytosis and diffusion of cell surface GM1-bound cholera toxin. Galectin binding promotes surface residency of EGF in a dynamic microdomain-specific available for ligand activation that is dominant to Cav1-dependent sequestration and negative regulation. These results support Cav1 as a conditional tumor suppressor, whose loss is advantageous when β1,6GlcNAc branched N-glycans are below a threshold for optimal lattice formation. Supported by the Canadian Institutes of Health Research (CIHR)

1783 Association of Caveolin-1 with Gap Junctions in Chicken Lens Fiber Cells
S. Biyas1, C. Zhou,1 W. Peng,1 W. Lo2,3

1Anatomy & Neurobiology, Morehouse School of Medicine, Atlanta, GA, 2Ophthalmology, Emory University, Atlanta, GA

Caveolin-1 is a signature protein of caveolar lipid rafts in many cell types. Our preliminary study showed that a large amount of Cav-1 was found in lens fiber cells of various species studied. Since caveolae are rarely observed in lens fiber cells, we wanted to determine what non-caveolar domains are associated with Cav-1 in fiber cells of chicken embryonic and adult lenses. Immunoblotting analysis showed that Cav-1 has a molecular weight of ~24 kDa containing and β forms in fiber cells. Immunofluorescence study revealed that Cav-1 was expressed early in the lens pit and lens vesicle during lens formation at E2.4. Although Cav-1 was also labeled in the anterior epithelium, the elongating posterior epithelial cells (i.e., nuclear fiber cells) exhibited a significant dotted labeling pattern along cell membranes at E5.7. At E8-20, and in the adult lens, the unique dotted pattern of Cav-1 labeling was more prominent in middle cortical fiber cells.

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as they underwent the maturation process from superficial to deep cortical regions of the lens. We hypothesize that Cav-1 is associated with gap junctions because both Cav-1 and connexins show similar dotted labeling pattern in the specialized ball-and-socket interlocking domains as well as flat membrane of fiber cells. Double immunofluorescence labeling showed that Cav-1 was partially co-labeled with Cx43 and Cx36 in fiber cells, suggesting that Cav-1 may be selectively associated with different gap junction populations in the lens. By using freeze-fracture immunogold labeling (FRIL) for high resolution localization, Cav-1 was indeed localized in many, but not all, gap junctions of the ball-and-socket domains and flat membrane examined. Since Cav-1 is in close proximity to the heterogeneous distribution of cholesterol in fiber gap junctions, it is currently under investigation with filipin cytochemistry in conjunction with freeze-fracture TEM and FRIL. Supported by NEI/NIH grant EY05314.

1784 Correlating Membrane Nanostructure with IgE Receptor Crosslinking in Mast Cells: An Ultrafast Dynamics Approach
A. M. Davey,1, E. D. Sheets,2,3 and A. A. Heikal2,3; 1Department of Chemistry, The Pennsylvania State University, University Park, PA; 2The Huck Institutes of the Life Sciences, University Park, PA; 3Department of Bioengineering, The Pennsylvania State University, University Park, PA

Cholesterol-rich microdomains (or “lipid rafts”) within the plasma membrane have been hypothesized to exist in a liquid-ordered phase and play important roles in cell signaling. However, these microdomains are difficult to examine using conventional imaging in living cells under physiological conditions. Here, we exploit the exquisite sensitivity of excited-state dynamics and anisotropy to probe the correlation between IgE crosslinking and the nanostructure of lipid microdomains in suspended RBL mast cells. Our experimental approaches encompass integrated biophotonic techniques such as confocal microscopy, two-photon fluorescence lifetime imaging (FLIM), and fluorescence polarization anisotropy for imaging the dynamics of the lipid marker, dil-C18, and Alexa 488-labeled IgE. Extensive crosslinking of the high affinity receptor for IgE (FcRII) triggers co-redistribution of molecules associated with cholesterol-rich microdomains (e.g., saturated lipids [the lipid analog dil-C18] and lipid-anchored proteins) with crosslinked FcRII in well-defined patches. Upon IgE-FcRII crosslinking, we observe an enhanced fluorescence lifetime with reduced segmental mobility of both probes, which indicates a restrictive and highly ordered membrane environment within these patches. Our results support the hypothesis that FcRII moves into more ordered membrane domains for further signaling and establishes excited-state dynamics imaging as an effective approach for probing local membrane heterogeneity and nanostructure. Currently, we are investigating the structure-function relationships between the plasma membrane of adherent mast cells and fluorescently labeled signaling molecules during IgE stimulation under physiological conditions.

1785 Immobilization of the Type XIV Myosin in Toxoplasma Membranes Is Cholesterol-dependent
T. M. Johnson, Z. Rajfur, K. Jacobson, C. Beckers; Cell and Developmental Biology, University of North Carolina at Chapel Hill, Chapel Hill, NC

Toxoplasma gondii is an intracellular protozoan parasite of animal cells that can cause severe disease or death in fetuses and immunocompromised individuals. Movement of the parasite within the infected animal as well as actual host cell penetration is believed to be facilitated by the interaction of F-actin, associated with a cell surface adhesin of the parasite, and myosin-A, a type XIV myosin. Myosin-A is found in a complex, called the glideosome, with three additional subunits: a myosin light chain (MLC1) and two accessory proteins, GAP45 and GAP50. The latter is an integral membrane glycoprotein that is required for the movement of the inner membrane complex (IMC), a membrane system underlying the parasite plasma membrane. In order for net movement to be produced by this motility system it is critical that the glideosome is firmly anchored within the plane of the IMC. Using FRAP analysis of YFP-tagged GAP50 we demonstrate here that this is indeed the case. While YFP-tagged cytoplasmic proteins or proteins associated with the IMC through acylation demonstrate a rapid recovery after photobleaching, we could not detect recovery of photobleached GAP50-YFP, suggesting that this protein is immobilized within the plane of the IMC membrane. This immobilization is not due to the interaction of GAP50 with other IMC proteins as judged by failure to detect these by chemical cross-linking or co-immunoprecipitation. The use of different detergent extraction approaches reveals that the glideosome, as well as the whole IMC membrane, is solubilized by Triton X-100 in a temperature-dependent fashion. We find that the IMC contains a significant proportion of cholesterol and that an redistribution of the glideosome is sensitive to depletion of cholesterol. These findings suggest that the glideosome is found in a cholesterol-rich membrane fraction that may constitute a large portion of the IMC.

1786 The Involvement of Lipid Microdomains in the Activation of Sperm from the Nematode Caenorhabditis elegans
J. J. Fraire-Zamora, C. C. Castillo, A. Cortez, H. Miyata, R. A. Cardullo; Biology, UC Riverside, Riverside, CA

In the nematode Caenorhabditis elegans fertilization takes place in the spermatheca of hermaphrodites. Subsequent to ovulation, or following ejaculation from a male inside the hermaphroditic uterus, the round and sessile spermatids undergo spermiogenesis resulting in the extension of a pseudopod and the initiation of motility. Although nematode sperm lack both a flagellum and an acrosomal vesicle, it has been hypothesized that these cells must undergo similar physiological changes to acquire motility and the ability to fertilize an egg. We are particularly interested in the recruitment and activation of signaling molecules with lipid microdomains (lipid rafts) in the plasma membranes of nematode gametes as these have been implicated in sperm from other taxa, most notably deuterostome lineages. In C. elegans, the current genetic model for spermiogenesis suggests a physical interaction of both membrane and cytoplasmic proteins to form a multi-component complex. Specifically, novel proteins involved in spermatic activation and the acquisition of motility (SPE-6, SPE-8, SPE-12, SPE-19, SPE-27 and SPE-29) are thought to play a central role in the signal transduction pathways that represses or triggers spermiogenesis. Using large-scale isolation methods and in vitro activators, we have demonstrated that caveolin is found in the plasma membrane of spermatids. The presence of caveolin in the plasma membrane suggests the involvement of lipid microdomains in spermiatic activation.

1787 Coordinated Volume and Lateral Cell Surface Reduction Are Linked with Isochoric Lumen Initiation in MDCK Three-Dimensional Cell Culture
A. Velgodszky, A. Ferrari, R. Korschewski; Institute of Biochemistry, ETH Zurich, Zurich, Switzerland

Epithelial cells embedded in collagen matrices can proliferate and differentiate into cysts, lumen-enveloping growth arrested monolayers of polarized cells. Despite the fact that lumen formation is an essential and conserved process during metazoan ontogeny, the cellular mechanisms causing the formation of a lumen and thus a novel cell free compartment and apical surface are unresolved. Therefore we investigated the conditions upon which lumens between epithelial cells are generated. Lumens in the MDCK model system were always initiated in aggregates containing 2 to 10 cells. Solid aggregates of more than 10 cells had no residual probability to initiate a lumen. Lumen initiation, where the lumen was smaller than a single cell volume, occurred in a solid aggregate and was followed by cell proliferation and lumen enlargement. Formation of initial lumens was isochoric as it occurred without changes in the total aggregate volume. The reasons for this were reductions of volume and specifically of the cell-cell contact surface of individual cells in the aggregates. The increases of cells in the aggregate did not change during lumen initiation. Thus, we exclude some possibilities to form a lumen and demonstrate that predominantly the reduction of the cell-cell contact surface and cell volume are linked with lumen initiation. We conclude that the novel apical surface was formed at cost of the cell-cell contact surface and that initial lumen filling involves membrane trafficking. Thus neither apoptosis nor exclusively lipase pumping caused lumen initiation indicating the presence of a discrete trafficking dependent step in lumen formation during cystogenesis.

1788 Suppression of dru2Δ Cold-Sensitive Growth and Ergosterol Localization Defects by kes1Δ
B. Muthusamy, T. R. Graham; Biological Sciences, Vanderbilt University, Nashville, TN

Dru2p is a lipid binding protein, its relation with the heterogeneous distribution of cholesterol in fiber gap junctions is currently under investigation with filipin cytochemistry in conjunction with freeze-fracture TEM and FRIL. Supported by NEI/NIH grant EY05314.

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CD21 Expression in B-cells Inhibits Uptake of αCD19 Immunconjugate Therapy of Lymphomas

G. S. Ingle, 1 P. Chan, 1 J. P. Stephan, 1 J. Elliott, 3 C. Nelson, 1 M. Kljavin, 1 L. Giere, 4 W. Chang, 5 S. J. Scales 1; 1Molecular Biology, Genentech, Inc., South San Francisco, CA, 2Assay and Automation Technology, Genentech, Inc., South San Francisco, CA, 3Pathology, Genentech, Inc., South San Francisco, CA, 4Protein Chemistry, Genentech, Inc., South San Francisco, CA, 5Translational Oncology, Genentech, Inc., South San Francisco, CA

CD19 and CD21 function as co-receptors to enhance signaling through the B cell receptor in response to antigen. CD19 and CD21 are also expressed in various B-cell cancers including Non-Hodgkin’s lymphoma, diffuse large B-cell lymphoma, and follicular lymphoma. An ideal immunconjugate for therapy of such lymphomas could comprise a B-cell specific antibody conjugated to a cytotoxic drug such that the drug would only be released inside the B-cells rather than non-specifically into the circulation, but this requires that the immunconjugate be sufficiently internalized to be efficacious. We therefore performed internalization experiments using immunofluorescence and FACS in various B-cell lines to evaluate the suitability of antibodies to CD19 and CD21 for immunconjugation. We found that αCD19 antibodies do not significantly internalize, which could explain the lack of efficacy of certain αCD19 conjugates in clinical trials. We observed that αCD21 antibodies are efficiently internalized, which could explain the efficacy of αCD21 conjugates in clinical trials. We also observed that αCD19 antibodies are efficiently internalized, which could explain the efficacy of αCD21 conjugates in clinical trials. We also observed that αCD21 antibodies are efficiently internalized, which could explain the efficacy of αCD21 conjugates in clinical trials. We also observed that αCD21 antibodies are efficiently internalized, which could explain the efficacy of αCD21 conjugates in clinical trials. We also observed that αCD21 antibodies are efficiently internalized, which could explain the efficacy of αCD21 conjugates in clinical trials. We also observed that αCD21 antibodies are efficiently internalized, which could explain the efficacy of αCD21 conjugates in clinical trials. We also observed that αCD21 antibodies are efficiently internalized, which could explain the efficacy of αCD21 conjugates in clinical trials. We also observed that αCD21 antibodies are efficiently internalized, which could explain the efficacy of αCD21 conjugates in clinical trials. We also observed that αCD21 antibodies are efficiently internalized, which could explain the efficacy of αCD21 conjugates in clinical trials. We also observed that αCD21 antibodies are efficiently internalized, which could explain the efficacy of αCD21 conjugates in clinical trials. We also observed that αCD21 antibodies are efficiently internalized, which could explain the efficacy of αCD21 conjugates in clinical trials. We also observed that αCD21 antibodies are efficiently internalized, which could explain the efficacy of αCD21 conjugates in clinical trials. We also observed that αCD21 antibodies are efficiently internalized, which could explain the efficacy of αCD21 conjugates in clinical trials. We also observed that αCD21 antibodies are efficiently internalized, which could explain the efficacy of αCD21 conjugates in clinical trials. We also observed that αCD21 antibodies are efficiently internalized, which could explain the efficacy of αCD21 conjugates in clinical trials. We also observed that αCD21 antibodies are efficiently internalized, which could explain the efficacy of αCD21 conjugates in clinical trials. We also observed that αCD21 antibodies are efficiently internalized, which could explain the efficacy of αCD21 conjugates in clinical trials. We also observed that αCD21 antibodies are efficiently internalized, which could explain the efficacy of αCD21 conjugates in clinical trials. We also observed that αCD21 antibodies are efficiently internalized, which could explain the efficacy of αCD21 conjugates in clinical trials. We also observed that αCD21 antibodies are efficiently internalized, which could explain the efficacy of αCD21 conjugates in clinical trials. We also observed that αCD21 antibodies are efficiently internalized, which could explain the efficacy of αCD21 conjugates in clinical trials. We also observed that αCD21 antibodies are efficiently internalized, which could explain the efficacy of αCD21 conjugates in clinical trials. We also observed that αCD21 antibodies are efficiently internalized, which could explain the efficacy of αCD21 conjugates in clinical trials. We also observed that αCD21 antibodies are efficiently internalized, which could explain the efficacy of αCD21 conjugate therapy of lymphomas.
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Ligand-dependent Cellular Trafficking of Lactoferrin Receptor

V. Lopez, S. L. Kelleher, B. Lonnerdal; Nutrition, UC Davis, Davis, CA

Lactoferrin (Lf) is an iron-binding protein secreted by macrophages to modulate diverse cellular functions. We have previously demonstrated that Lf binds to its receptor in human placenta cells; however, downstream effects of ligand stimulation on lactoferrin receptor (LfR) have not been investigated. We hypothesized that Fe-saturated-(holo) Lf and Fe-saturated-(apo) Lf, which have profoundly different conformations, will elicit different cellular events resulting from their interaction with LfR. BeWo cells were treated with holo-Lf or apo-Lf. For siRNA, mastoparan and cycloheximide studies, 125I- apo- and holo-Lf uptake was measured. Plasma membrane proteins were biotinylated and LfR abundance was assessed by SDS-PAGE. Biotinylated apo- and holo-Lf, Lamp1, a lysosomal marker, and LfR localization were visualized with confocal microscopy. Knockdown of LfR protein significantly decreased 125I-apo-Lf uptake and 125I-holo-Lf to a lesser extent compared to controls. Mastoparan significantly decreased 125I- apo- and holo-Lf uptake compared to controls. Cell surface abundance of LfR protein significantly increased in the presence of apo-Lf, while holo-Lf had no effect. Cycloheximide significantly increased 125I- apo-Lf uptake after 3 h, compared to cells without cycloheximide. However, 125I-holo-Lf uptake was significantly decreased with cycloheximide compared to untreated cells. After 20 min, LfR co-localized with Lamp1 when stimulated with holo-Lf and to a lesser extent with apo-Lf. At 1 h, LfR co-localized with biotinylated apo- and holo-Lf and by 2 h biotinylated apo-Lf co-localized with the nucleus whereas biotinylated holo-Lf was absent. In summary, LfR mediated apo-Lf uptake, potentially due to intracellular degradation by the lysosomal compartment, whereas apo-Lf was transported to the nucleus. Our data suggest that internalization of apo- and holo-Lf was clathrin-dependent and changes in plasma membrane abundance were ligand-dependent. De novo LfR protein synthesis was a requisite for holo-Lf uptake, potentially due to intracellular degradation by the lysosomal compartment, whereas apo-Lf was transported to the nucleus. Our data suggest that internalization of apo- and holo-Lf mediates subsequent LfR compartmentalization, which in turn may elicit different cellular responses. Supported by NIH HD43240.

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Ferritin Induces Degradation of the Transferin Receptor

L. Horonchik, M. Westling-Resnick; Genetics and Complex Diseases, Harvard School of Public Health, Boston, MA

Previous studies from our laboratory identified “ferritinian” (NCI #306711) as an inhibitor of transferrin (Tf) mediated iron uptake [Brown et al. (2004) Chem Biol 11: 407-416]. To further characterize the drug’s mechanism of action, rates of Tf uptake and recycling were studied in HeLa and HEK293T cells. Neither trafficking pathway was influenced by ferritinian, but [125]Tf and [55Fe]-Tf binding experiments showed that the number of cell surface Tf receptors was significantly reduced after 4 h incubation with the drug. Western blot analysis revealed this effect was due to a time- and dose-dependent loss in receptor protein levels and that the presence of the ligand holo-Tf protected against ferritinian-induced Tf receptor degradation. Immunofluorescence microscopy experiments showed a corresponding decrease in Alexa-red Tf uptake after pre-treatment with ferritinian, while internalization of FITC-dextran and D-glucosamine was unaffected, confirming that endocytosis was not disrupted by the drug and supporting its specificity of action towards the Tf receptor. Ligand-independent Tf receptor degradation induced by ferritinian was blocked by inhibitors of both proteosomal (MG132, epoxomicin) and lysosomal (bafilomycin A1, leupeptin, chymostatin) function. Filipin also interfered with ferritinian-induced Tf receptor degradation, suggesting that a clathrin-independent pathway is involved. The properties associated with ferritinian inhibition of iron uptake point to a ligand-independent pathway for receptor down-regulation and degradation that could potentially be exploited to therapeutically limit iron acquisition by cancer cells.

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Differential Endocytic Sorting of Myelin Proteins

C. Winterstein, A. Handwerk, W. Moebius, K. Nave; Biology, Molecular Cell Biology, Johannes Gutenberg-University, Mainz, Germany, 3Max-Planck-Institute for Experimental Medicine, Goettingen, Germany

Myelin is a sophisticated compartmentalised membrane system produced by oligodendroglial cells, which is reminiscent of the segmented membrane domains of polarised cells. Development and maintenance of the myelin sheath requires coordination of protein and lipid synthesis and directed trafficking towards the axon. To unravel membrane trafficking mechanisms we have established assay systems to resolve vesicular transport pathways of myelin components. Using confocal microscopy and subcellular fractionation, we separated oligodendroglial membrane compartments and made use of SNAREs as markers to map oligodendroglial trafficking pathways. Since the major myelin protein PLP appears to be enriched in a lysosome-like compartment, we hypothesised that endocytosis plays a role in the sorting of myelin components. Cell-surface biotinylation and antibody uptake assays performed with oligodendroglial cells demonstrate that myelin proteins are indeed subject to endocytosis. Interestingly, the endocytic route followed by PLP seems to be distinct from that of other myelin proteins, such as MAG and MOG. We have dissected these endocytic pathways and are addressing whether re-association of endocytosed PLP and other myelin components to the plasma membrane (myelin) occurs.

1797

GIPC Is Recruited by APPL to Peripheral TrkA Endosomes and Regulates TrkA Trafficking and Signaling

T. Varsano, M. Dong, L. Niesman, H. Gacula, X. Lou, T. Ma, J. R. Testa, J. R. Yates, M. G. Faragher; Cellular and Molecular Medicine, University of California San Diego, La Jolla, CA, 2Human Genetics Program, Fox Chase Cancer Center, Philadelphia, PA, 3Department of Cell Biology, Scripps Research Institute, La Jolla, CA

GIPC is a PDZ protein located on peripheral endosomes. Previously we showed that it binds to the juxtamembrane region of the TrkA NGF receptor and that overexpression of GIPC interferes with NSF gating (Lou et al. MBC 12: 615, 2001). To shed light on the mechanism by which GIPC affects TrkA signaling we used IPs and mass spec to identify additional GIPC-binding proteins. We found that endogenous GIPC binds to the C-terminus of APPL, a Rab5 binding protein, which is a marker for signaling endosomes. When TrkA is activated, GIPC and APPL translocate from the cytoplasm and bind to incoming, endocytic vesicles carrying TrkA concentrated at the tips of the cell processes. GIPC, but not APPL, dissociates from these peripheral endosomes prior to, or during their trafficking from the juxtanuclear region where they acquire EEAP. GIPC’s interaction with APPL is essential for recruitment of GIPC to peripheral endosomes and for TrkA signaling, because a GIPC PDZ domain mutant that cannot bind APPL, or APPL knockdown with siRNA inhibits NGF-induced GIPC recruitment, MAP kinase activation and neurite outgrowth. GIPC is also required for efficient endocytosis and trafficking of TrkA because depletion of GIPC slows down endocytosis and trafficking of TrkA and APPL to early EEAP endosomes in the juxtanuclear region. We establish that GIPC, following its recruitment to TrkA by APPL, is required for efficient trafficking of TrkA and APPL to early endosomes and for optimal TrkA signaling from peripheral endosomes. The ability of GIPC to form homodimers and bind to APPL, TrkA, and several additional receptors (TrkB, IGF, TGFβIII, dopamine D2, LH and β1Adrenergic receptors) suggests that GIPC might play a similar role in the assembly of protein complexes required for endocytosis and signal transduction of other receptors.

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A Screen of Rab GTPases Reveals Different Endocytic Pathways of Human Adenovirus Type 2 and Type 35

M. Gastadelli, N. Linka, B. T. Amstutz, F. DiGenmaro, M. Havenga, K. Boucke, U. F. Greber; 2Department of Zoology, University of Zurich, Zurich, Switzerland, 2Crucell Holland BV, Leiden, The Netherlands

Human Adenoviruses (Ads) are nonenveloped DNA viruses causing respiratory or gastrointestinal diseases. The species B adenovirus serotype Ad35 and the species C serotypes Ad2 and Ad5 interact with different cellular receptors. Hematopoietic cells are largely refractory to species C Ad infection due to low levels of the attachment receptor CAR. In epithelial cells, Ad2 and Ad5 attach to CAR by virtue of protruding fiber proteins, and bind integrins with the proximal penton base proteins stimulating clathrin-mediated viral uptake, and accessory macrophagocytosis. In contrast, the fibers of Ad35 attach to the membrane cofactor CD46. CD46 attachment leads to endocytosis of Ad35 into both epithelial and hematopoietic cells, independent of dynamin 2, a large GTPase involved in the regulation of clathrin-mediated endocytosis, caveolar endocytosis and phagocytosis. In epithelial cells, Ad35 endocytosis was blocked by amiloride and protein kinase C inhibitors, which inhibit macrophagocytosis. Endocytic vesicles containing Ad35 acquired lysobisphosphatidic acid and lysosomal associated membrane protein 1 (Lamp1) which are markers of late endosomes and lysosomes. In contrast, vesicles containing Ad2 and Ad5 were deficient of early and late endosomal markers. An infectious virus entry screen of Rab GTPases revealed that the infection of HeLa cells was independent of Rab5, which is implicated in the regulation of membrane traffic from the plasma membrane to early endosomes. One particular Rab protein was found to regulate the entry of both species B and C Ads, indicating that there is at least one common trafficking knoll of clathrin-dependent endocytosis and macrophagocytosis. These results allow us to explore the mechanistic links between different endocytic trafficking routes in the context of viral infections.

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The Intracellular Fate of Campylobacter jejuni

R. O. Watson, J. E. Galan; Yale University-School of Medicine, Section of Microbial Pathogenesis, New Haven, CT

Campylobacter jejuni is the leading cause of bacterial food-borne illness in the United States and one of the most common causes of diarrhea worldwide. Both in vivo and in vitro studies have demonstrated that C. jejuni invades intestinal epithelial cells. Although C. jejuni internalization is believed to be important in campylobacter pathogenesis, little is known about the intracellular fate of C. jejuni.
Cytostome/cytopharynx. The described lipid raft/caveolae profiles of mammal cells. In this work we have demonstrated the occurrence in electron microscopy and immunofluorescence microscopy. Expression of clathrin and flotillin in (LIT with 10% inactivated fetal calf serum). Five-day-old culture parasites were submitted to protein and lipid extraction. The protein extract was used in Western blotting and dot blotting.

The EGF family member ErbB2 is overexpressed in 25-30% of human breast cancers. While ErbB2 is normally highly concentrated on the cell surface, the antibiotic geldanamycin (GA)

Clathrin-independent Endocytosis of ErbB2 in SKBr3 Cells

N. Porat-Shliom,1 Y. Kloog,2 J. Donaldson1; 1NHLBI/LCB, National Institutes of Health, Bethesda, MD, 2Neurobiochemistry, Tel-Aviv University, Tel-Aviv, Israel

pathway is inhibited by amiloride that characterizes it as macropinocytic in nature. These observations, plus the fact that CD94/4/NKG2A endocytosis functions independently of dynamin affect the cell surface levels or internalization of CD94/NKG2A, suggesting an involvement of a non-clathrin dependent endocytic pathway. Nystatin treatment had no effect on its endocytosis.

CD94/NKG2A receptors are internalized into early EEA-1+ and Rab5+ endosomes, but failed to co-localize with transferrin receptor, Rab-4, -7, or -11. We then investigated if any of the CD94/NKG2A, but does reduce cell surface expression, suggesting that the actin cytoskeleton is required for CD94/NKG2A exocytosis but not endocytosis. Immunostaining revealed that the CD94/NKG2A protein containing the translocation and the catalytic domain of DT but an alternative receptor binding domain causes the same phenotype. These large Rab5 positive vesicles are early endosomes as demonstrated by the colocalization with the Rab5 effector EEA1. It was shown that a Rab5 mutant, S34N, inhibits fusion between early endosomes and its overexpression induces the accumulations of very small endosomes. After treatment of GFP-Rab5-S34N positive cells with DT, only small punctate endosomes were present demonstrating that large endosomes induced by DT are excessively fused. Ammonium chloride and bafilomycinA, a specific inhibitor of the vacuolar proton ATPase, increases the endosomal pH in cells and blocks DT translocation. We demonstrate that in Rab5 positive endosomes, ammonium chloride and bafilomycinA block the toxin effect on endosome fusion. In HeLa cells, nocardazole blocks the transport from ECV to late endosomes but it doesn’t change the morphology of the Rab5 positive endosome treated with DT. We can conclude that the DT translocation domain promotes endosome fusion following membrane insertion and may be coupled with toxin entry into the cytosol.

A Novel Clathrin- and Lipid Raft Independent Macropinocytic Pathway for Internalization of the CD94/NKG2A Inhibitory Receptor in Natural Killer Cells

M. Masilamani, M. E. Prieto, F. Ilorrego, J. E. Coligan; Receptor Cell Biology Section, Laboratory of Allergic Diseases, NIAID, NIH, Rockville, MD

An adequate immune response must be tightly regulated to avoid poor responses on one hand or excessive inflammation leading to autoimmunity on the other. This is achieved through a balance of activating and inhibitory signals that control immune cell function. Natural Killer (NK) cells are poised to kill any cell that they encounter without prior sensitization. CD94/NKG2A is an inhibitory receptor expressed by NK cells that recognizes HLA-E expressed by normal cells, but often downregulated on diseased cells. CD94/NKG2A expression needs to be constantly maintained, as any ligand-induced downregulation of CD94/NKG2A would permit activation receptor(s) induced killing of normal bystander cells. The mechanism by which CD94/NKG2A continuously recycles to and from the cell surface maintains steady state levels as has been described. We show that latrunculin A does not affect the internalization of cell surface CD94/NKG2A, but does reduce cell surface expression, suggesting that the actin cytoskeleton is required for CD94/NKG2A exocytosis but not endocytosis. Immunostaining revealed that the CD94/NKG2A protein is internalized into early EEA-1+ and Rab5+ endosomes, but failed to co-localize with early EEA-1, Rab-4, -7, or -11. We then investigated if any of the known endocytic pathways are involved in the internalization of CD94/NKG2A. Hypertonic treatment of cells with sucrose and expression of dominant negative dynamin K44A mutant did not affect the cell surface levels or internalization of CD94/NKG2A, suggesting an involvement of a non-clathrin dependent endocytic pathway. Nystatin treatment had no effect on its endocytosis suggesting that lipid raft dependent pathways are not likely involved. The CD94/NKG2A vesicles co-localized with endocytosed dextran indicating involvement of a pinocytic pathway. This pathway is inhibited by amiloride that characterizes it as macropinocytic in nature. These observations, plus the fact that CD94/NKG2A endocytosis functions independently of dynamin indicate that CD94/NKG2A utilizes a previously described endocytic pathway.

H-Ras Traffics through and Regulates Clathrin-independent Endocytosis

N. Porat-Shliom,1 Y. Kloog,2 J. Donaldson1; 1NHLBI/LCB, National Institutes of Health, Bethesda, MD, 2Neurobiochemistry, Tel-Aviv University, Tel-Aviv, Israel

Despite their high sequence homology and their ability to bind and activate the same down stream effectors in vitro, the biological outputs in vivo of Ras proteins are distinct. Accumulating data indicates that these differences are a result of diverse post translational modifications of Ras isoforms, and differential localization within the cell that serve as platforms for different down stream effector activation. For example, H-Ras, but not K-Ras, was shown to activate Raf1 from endosomes in the clathrin-dependent pathway. However little is known about the role of the clathrin-independent pathway in H-Ras trafficking and signaling. The clathrin-independent pathway includes the internalization of distinct cargo proteins such as the Major Histocompatibility Complex I (MHC). Clathrin-independent derived cargo enters in distinct endosomes that then fuse with early endosomes that contain clathrin-dependent derived cargo, e.g., transferrin. In HeLa cells, at this stage, cargo might be targeted for degradation or recycled back to the plasma membrane via the characteristic recycling tubular endosomes. We sought to identify whether H-Ras use the clathrin-independent pathway as an additional platform for trafficking and signaling. Using immunofluorescence and localization studies, we demonstrate that H-Ras co-localizes with the clathrin-independent pathway markers, Arf6 and MHC1. Furthermore, we were able to show that the dominant active mutant of H-Ras (G12V) stimulates the clathrin-independent pathway and increases the levels of MHC1 internalization, while it has no effect on transferrin internalization. These results establish the clathrin-independent pathway as a route for H-Ras trafficking not only as a cargo molecule but also as a potential regulator. Since signaling molecules such as Src and Erk are also present on these endosomes, our next goal is to examine whether this pathway could provide an additional unique platform for Ras signaling.

Clathrin-independent Endocytosis of ErbB2 in SKBr3 Cells

D. J. Barr, A. G. Ostermeyer-Fay, D. A. Brown, Biophysics and Cell Biology, SUNY Stony Brook, Stony Brook, NY

The EGFR family member ErbB2 is overexpressed in 25-30% of human breast cancers. While ErbB2 is normally highly concentrated on the cell surface, the antibiotic geldanamycin (GA) triggers internalization and downregulation of the receptor. It is known that GA treatment of cancer cells overexpressing ErbB2 leads to transport to early endosomes and multi-vesicular bodies. However, the initial internalization pathway remains uncharacterized. In the present study we examined GA-induced internalization and trafficking of ErbB2 in SKBr3 human breast cancer cells. We found that GA-induced ErbB2 internalization was not blocked by dominant negative forms of dynamin or eps15. Further, internalization was not inhibited by the clathrin
inhibitor chlorpromazine, and ErbB2 did not colocalize with transferrin during initial internalization. Internalization was blocked by filipin treatment, and the receptor colocalized with cholera toxin beta subunit and GPI-anchored proteins after 5 minutes of internalization. The receptor did not accumulate in enlarged endosomes induced by constitutively active ARF6. Taken together, these results suggest ErbB2 was internalized by a non-clathrin mediated pathway. After 30 minutes of internalization, ErbB2 colocalized with markers of early and late endosomes and lysosomes, confirming earlier reports. In summary, we showed that in GA-treated cells, ErbB2 is internalized by a clathrin-independent mechanism, but soon merges with the classical endocytic pathway.

1806
Active Arf6 Recruits ARNO/cytohesin GEFs to the PM through an Interaction with Their PH Domains Leading to Sequential Arf Activation
L. A. Cohen,1 P. Varani,1 A. Houda,2 T. Balla,2 J. Donaldson,1 Laboratory of Cell Biology, NIH/NHLBI, NIH, Bethesda, MD, 1Endocrinology and Reproduction Research Branch, NICHD, NIH, Bethesda, MD
ARNO is a guanine nucleotide exchange factor (GEF) for the Arf family of small GTP binding proteins. Although in biochemical assays ARNO prefers Arf1 as a substrate over Arf6, its localization in cells suggests an interaction with Arf6 at the plasma membrane (PM). In this study we examined the relationship between ARNO and its potential substrates Arf1 and Arf6. In HeLa cells, ARNO activation of Arf6 was twice as efficient as activation of Arf6. By contrast, an unambiguous exchange factor for Arf6, EFA6, was two times more effective at activating Arf6 than Arf1. These results suggest that ARNO prefers Arf6 as a substrate when expressed in HeLa cells. On the other hand, co-expression of Arf6 and ARNO in HeLa cells led to a dramatic recruitment of ARNO onto the PM and endocytic structures. Curiously, the Q67L (GTP locked) mutant of Arf6 was also capable of recruiting ARNO onto cellular membranes, whereas ARNO’s substrate mimic, the T27N (GDP bound) mutant, could not. Remarkably, ARNO co-immunoprecipitated with Arf6-GTP, and this binding was mediated not by the sec7 domain, but through the PH domain of ARNO. The interaction with Arf6 is conserved in other ARNO/cytohesin family members, and requires both inositol phospholipids and GTP. There is ample evidence for involvement of "Golgi-associated" Arfs at the PM as we and others have observed that expression of ARNO and Arf1 in COS-7 cells induces membrane ruffles and marcupinosomes where both proteins are transiently associated. Hence, this study reveals a novel downstream effector for Arf6-GTP, which is the recruitment of ARNO family GEFs capable of activating cytosolic Arfs such as Arf1 in a sequential cascade at the PM.

1807
The Arf6 GEF BRAG2 Regulates Cell Adhesion by Controlling Endocytosis of β1 Integrins
J. L. Dunphy,1 P. Melancon,1 J. E. Casanova1; 1Cell Biology, University of Virginia, Charlottesville, VA, 1Cell Biology, University of Alberta, Edmonton, AB, Canada
Accumulating evidence indicates that Arf6 regulates post-endocytic trafficking of a subset of membrane proteins, including β1 integrins. Integrins have been reported to enter an Arf6-positive endosomal compartment that is morphologically and functionally distinct from early endosomes, and dominant negative Arf6 mutants impairs recycling of these proteins to the plasma membrane, suggesting that this process requires active Arf6. BRAG2 is a recently described guanine nucleotide exchange factor (GEF) with specificity for Arf6 in vitro. We have identified a second, longer isoform of BRAG2 (BRAG2b), which like BRAG2a is ubiquitously expressed. Both isoforms activate Arf6 in vivo, as determined using a pulldown assay for Arf6-GTP. To determine the role of BRAG2 in Arf6 function, we performed an RNAi analysis using sh1 integrin trafficking as a readout. Knockdown of Arf6 itself led to a 30% reduction in surface expression of β1 integrin and impaired both attachment and spreading of cells on fibronectin. Surprisingly, knockdown of BRAG2a and BRAG2b resulted in a 50% increase in surface β1 and a corresponding enhancement of both attachment and spreading. This effect was specific for integrin, as surface levels of transferrin receptor remained unchanged, and was also specific for BRAG2, as knockdown of a different Arf6 GEF, ARNO, had no impact on adhesion. Intriguingly, BRAG2b contains a functional clathrin box near its N-terminus, suggesting that it interacts directly with the clathrin endocytic machinery. Together these results indicate that Arf6 regulates both integrin internalization and recycling and that BRAG2 selectively regulates the endocytic step.

1808
Regulation of CXC4 Receptor Trafficking and Signaling by ARF6
C. C. Moore, J. L. Benovic; 1Biochemistry and Molecular Biology, Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA
CXCR4, a chemokine G protein-coupled receptor essential for neuronal, cardiovascular, and hematopoietic cell migration towards stromal-derived factor (SDF), has recently emerged as a critical player during cancer cell migration. This receptor is involved in the vigorous pursuit of factors that regulate the CXCR4-SDF axis. We demonstrate here that ARF6 regulates cell surface levels of CXCR4 following SDF stimulation, as measured by an ELISA assay. Expression in HEK293 cells of either wild-type ARF6 or a GTPase-deficient ARF6 mutant (Q67L) inhibits CXCR4 receptor internalization, whereas a GDP/GTP exchange-deficient ARF6 mutant (T27N) promotes CXCR4 internalization. Consistent with these data, agonist-dependent CXCR4 internalization is altered following modulation of endogenous ARF6 by EFA6, an ARF6-selective guanine nucleotide exchange factor (GEF), or by ACAP1, an ARF6-selective GTPase activating protein (GAP). In addition to regulating CXCR4 receptor trafficking at the cell surface, ARF6 regulates SDF-promoted MAPK signaling at the membrane, in both HEK293 and MDA-MB-361 cells, a metastatic breast cancer cell line. These data demonstrate ARF6 regulation of the CXCR4-SDF axis, and thus may have implications on dysregulated CXCR4 trafficking and signaling during cancer metastasis.

1809
Cholesterol Controls Own Endocytosis through Rab11
M. Takahashi,1 M. Fukuda,2 A. Otta,3 T. Koyabashii; 1Molecular Membrane Biology Lab., RIKEN, Saitama, Japan, 1Spinophilin Functions Lab., RIKEN, Saitama, Japan, 2Research Center, Tohoku University, Miyagi, Japan, 3Department of Biotechnology, University of Tokyo, Tokyo, Japan
The endocytic pathway of cholesterol is not well understood. Recently we have shown that fluorescent poly(ethylene glycol)-derivatized cholesterol (PEG-Chol) is preferentially partitions to cholesterol-rich membrane domains (Sato et al. J. Biol. Chem. 279, 23790 (2004)). Membrane impermanent nature of PEG-Chol made it possible to follow the endocytic route of cholesterol-rich domains of the plasma membrane in living cells. In this study, we examined the internalization of PEG-Chol in subconfluent and confluent Chinese hamster ovary (CHO) cells. In subconfluent cells, PEG-Chol was accumulated to the recycling endosomes whereas in confluent cells the fluorescence was observed in the early endosomes. Endocytosis of fluorescent sphingomyelin but not lactosylceramide was similarly affected, suggesting that the endocytosis of a limited number of cargos are affected by cell confluency. Our biochemical and histochemical analysis showed the dramatic increase of cellular cholesterol in confluent cells. The crucial role of cellular cholesterol in cell-confluency dependent endocytosis was confirmed by the observation that the fluorescent sphingomyelin was transported to the recycling endosomes when cell surface cholesterol was depleted in confluent cells. In order to understand the mechanistic network(s) of cell confluency- and cholesterol-dependent endocytosis, we examined intracellular distribution of small GTPases. Our results indicate that rab11 but not rab4, altered intracellular localization in a cell confluency-associated manner and this alteration was dependent on cell cholesterol. In addition, the expression of a constitutive active mutant of rab11 changed the endocytic route of PEG-Chol from early to recycling endosomes. These results thus suggest that cholesterol controls endocytic routes of certain cargos including cholesterol itself through rab11.

1810
EHD1 Regulates LDL Internalization and Cholesterol Homeostasis
N. Nalavsky,1 J. Rahajeng,1 D. Rapaport,2 M. Horowitz,2 B. G. Coon,3 R. C. Aguilar,3 P. L. Sorgen,1 S. Caplan1; 1University of Nebraska Medical CenterDept. of Biochemistry and Molecular Biology, University of Nebraska Medical Center, Omaha, NE, 2Dept. of Cell Research and Immunology, Tel Aviv University, Ramat Aviv, Israel, 3Department of Biological Sciences, Purdue University, West Lafayette, IN
Endocytic transport is a key mechanism for controlling the subcellular distribution of free cholesterol and the endocytic recycling compartment (ERC) is an important organelle that stores cholesterol and regulates its transport. Rab11, which regulates transport via the ERC, regulates the exit of cholesterol from this organelle. EHD1, a member of the C-terminal EH-domain family of proteins that regulates recycling and coordinates transport via the Rab11 pathway, has also been reported to play a role in free cholesterol transport through the ERC. To directly assess the role of EHD1 on cholesterol homeostasis and cellular distribution, we utilized mouse embryonic fibroblasts derived from EHD1 knockout mice (MEF +/-). Surprisingly, these cells displayed reduced levels of cellular free and esterified cholesterol, an effect that could be rescued by overexpression of wild-type EHD1. To understand the reduction in intracellular cholesterol in the absence of EHD1, we turned our focus to low density lipoprotein (LDL) and its receptor, LDLR, a major source of cellular cholesterol intake. We observed higher levels of LDLR on the plasma membrane of MEF +/- cells, yet LDL itself was internalized at a slower rate in these cells. Furthermore, in cells lacking EHD1, lipopid droplets appeared greatly reduced in size, suggesting that less esterified cholesterol and triglycerides were present in lipid droplets. Two-hybrid binding assays and NMR spectroscopy suggest that the EH-domain of EHD1 interacts

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with Epsin, a well-characterized component of the endocytic pathway that contains NPF motifs and regulates receptor internalization. Our data indicates that EHDI affects cholesterol homeostasis and lipid droplet biogenesis by controlling internalization of LDL receptor, possibly through interactions with NPF-containing endocytic regulators such as Epsin.

1811
Rab8-dependent Recycling Promotes Endosomal Cholesterol Removal in Normal and Sphingolipidosis Cells
M. D. Linder, 1 R. Uronen, 1 M. Hölttä-Vuori, 2 P. van der Sluijs, 2 J. Peränen, 3 E. Ikonen 1; 1Institute of Biomedicine, University of Helsinki, Helsinki, Finland, 2Department of Cell Biology, University Medical Center Utrecht, Utrecht, The Netherlands, 3Institute of Biotechnology, University of Helsinki, Helsinki, Finland

The mechanisms by which low-density lipoprotein (LDL)-cholesterol exits the endocytic pathways are not well understood. The process is defective in Niemann-Pick type C (NPC) disease in which cholesterol and sphingolipids accumulate in late endosomal compartments. This is accompanied by defective cholesterol esterification in the endosomal reticulum and impaired ATP-binding cassette transporter A1 (ABCA1)-dependent cholesterol efflux. We show here that overexpression of the recycling/exocytic Rab GTPase Rab8 rescued the late endosomal cholesterol deposition and sphingolipid mistrafickling in NPC fibroblasts. Rab8 redistributed cholesterol from late endosomes to the cell periphery and stimulated cholesterol efflux to the ABCA1-ligand apolipoprotein A-I (apoA-I) without increasing cholesterol esterification. Depletion of Rab8 from wild-type fibroblasts resulted in cholesterol deposition within late and recycling endosomal compartments. This cholesterol accumulation was accompanied by impaired traffic of LDL-cholesterol from endocytic compartments to apoA-I, and could not be bypassed by liver X receptor activation. Our findings establish Rab8 as a key component of the regulatory machinery that leads to ABCA1-dependent removal of cholesterol from endocytic circuits.

1812
Multiple Degradation Pathways for Misfolded Mutants of the Yeast Plasma Membrane ATPase, Pma1
Y. Liu, S. Sirazade, A. Chang; Molecular, Cellular, and Developmental Biology, University of Michigan, Ann Arbor, MI

To understand protein sorting and quality control in the secretory pathway, we have analyzed intracellular trafficking of the yeast plasma membrane ATPase, Pma1. Pma1 is ideal for such studies because it is a very abundant polytopic membrane protein, and its localization and activity at the plasma membrane are essential for cell viability and growth. We have tested whether the cytoplasmic amino and carboxy terminal domains of Pma1 carry sorting information. As the sole copy of Pma1, mutants truncated at either N- or C-termini are targeted at least partially to the plasma membrane and have catalytic activity to sustain cell viability. In addition, the mutants are detected to be degradation pathways. Strikingly, N- and C-terminal Pma1 mutants are differentially recognized for degradation at distinct cellular locales. C-terminal mutants are recognized for destruction by ER-associated degradation. By contrast, N-terminal mutants escape detection by ERAD entirely, and undergo endoplasmic degradation after apparently normal cell surface targeting. Both N- and C-terminal mutants are conformationally abnormal, as revealed by increased sensitivity to trypsin cleavage, but are able to assemble to form oligomers. We propose that different quality control mechanisms may assess discrete domains of Pma1 rather than a global conformational state.

1813
Overcoming the KDEL Sequence and Escaping the ER: A Lumenal Golgi Complex Protein
A. N. Perry, 1 Z. Wang, 2 D. Russel, 2 Z. Cabarova, 2 P. E. Scherer, 3 E. L. Snapp 2; 1Syracuse University, Syracuse, NY, 2Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, NY

Cellular proteins localize to specific organelles through a combination of targeting sequences and their corresponding receptors. For example, proteins with the lumenal endoplasmic reticulum (ER) retrieval sequence, KDEL, interact with the KDEL receptor in post ER compartments to be returned with the KDEL receptor to the ER. Here, we report a lumenal protein with a KDEL sequence that is inefficiently retrieved to the ER and exhibits a predominantly Golgi complex localization pattern. We used immunofluorescence and GFP-fused constructs to characterize protein localization. The steady-state distribution the protein is not due to protein overexpression or saturation of the KDEL receptor machinery. A co-expressed ER-RFP-KDEL protein does not escape the ER. Truncation and chimera experiments reveal that trafficking of the protein to the Golgi complex is mediated by a previously uncharacterized sorting sequence. The sequence is recognized by several cell types, consistent with a general machinery that overrides the KDEL sequence and creates a steady-state Golgi complex localization pattern for lumenal proteins.

Our results may help explain how a number of resident lumenal ER proteins escape into the secretory pathway and suggest that escape is a regulated event.

1814
Transition of Galactosyltransferase 1 from Trans-Golgi Cisterna to the Trans Golgi Network (and Back) Is Signal Mediated
B. Schaub, B. Berger, E. Berger, J. Rohrer; Physiology, University of Zurich, Zurich, Switzerland

The Golgi apparatus (GA) is the organelle where complex glycan formation takes place. In addition, it is a major sorting site for proteins destined for various sub-cellular compartments or for secretion. Here we investigate β1,4-galactosyltransferase 1 (galT) and α2,6-sialyltransferase 1 (siaT), two trans-Golgi glycosyltransferases, with respect to their different pathways in monensin treated cells. Upon addition of monensin galT dissociates from siaT and the GA and accumulates in swollen vesicles derived from the trans-Golgi network (TGN), as shown by co-localization with TGN46, a specific TGN marker. We analyzed various chimeric constructs of galT and siaT with confocal fluorescence microscopy and time lapse video-microscopy as well as Optiprep density gradient fractionation. We show that the first 13 amino acids of the cytoplasmic tail of galT are necessary and sufficient for its localization to swollen vesicles induced by monensin. We also show that the monensin sensitivity resulting from these 13 amino acids can be conferred to siaT, which leads to the rapid accumulation of the galT-siaT chimera in swollen vesicles upon monensin treatment. Based on these data we suggest that cycling between the trans-Golgi cisterna and the trans-Golgi network of galT is signal mediated.

1815
Identification of Go Palmitoyl Acyl Transferase
R. Tsutsui, 1 Y. Fukata, 1 M. Fukata 1; Laboratory of Genomics and Proteomics, National Institute for Longevity Sciences, Aichi, Japan

Palmitoylation is the post-translational lipid modification of proteins and regulates subcellular trafficking and function of proteins. Several a subunits of trimeric G proteins (Gα) including Gαi, Gαq and Gαs are palmitoylated and their membrane localization and functions are dynamically regulated by this reversible palmitoylation. However, the responsible enzymes for palmitoylation of Gα (G-PAT) remain unclear. Here we report the enzymes that promote Gα palmitoylation in vivo. HEK293 cells were co-transfected with the cDNA for Gα and twenty-three cDNA clones encoding DHHC palmitoyl transferase family members. Only DHHC3 and 7 enhanced the incorporation of [3H]palmitate into Gαq and Gαs. We also found that DHHC3, 7, 8 and 21 enhanced Gαq palmitoylation. Mutations at cysteine residues 9 and 10 of Gαq abolished the increased palmitoylation by DHHC3 and 7. Overexpression of DHHC3 or 7 relocated Gαs and Gαq to the peri-nuclear region, suggesting that DHHC-3 and 7 regulate subcellular localization of Gαs and Gαq through palmitoylation. Time-lapse imaging revealed that treatment with 2-bromomalpinolate, an inhibitor of thioacylation, dispersed Gαq from the plasma membrane to cytoplasm, indicating that palmitate on Gαq dynamically turns over. These observations suggest that several DHHC proteins are physiological G-PATs, regulating Gα subcellular localization and functions.

1816
Activity-dependent Regulation of PSD-95 Palmitoylation by P-PAT
J. Noritake, 1,2 Y. Fukata, 1 M. Fukata 1; Laboratory of Genomics and Proteomics, National Institute for Longevity Sciences, National Center for Geriatrics and Gerontology, Obu, Japan, 2Department of Cell Pharmacology, Nagoya University Graduate School of Medicine, Nagoya, Japan

PSD-95 palmitoylation is the posttranslational lipid modification of proteins and regulates the membrane targeting and function of proteins. Palmitoylation is unique in that it is reversible and dynamically regulated by specific extracellular signals. Several studies have reported that proteins containing a DHHC cysteine-rich domain mediate palmitoyl acyl transferase activity in yeast. We recently isolated 23 mammalian DHHC proteins and found that a subset of DHHC proteins, P-PAT subfamily, regulate synaptic function through PSD-95 palmitoylation. However, the regulatory mechanism of PSD-95 palmitoylation by P-PAT remains to be clarified. Here, we monitored palmitoylation level of endogenous PSD-95 in cultured hippocampal neurons by several methods including acyl-biotin-exchange and metabolic labeling with [3H]palmitate. Treatment with 2-bromomalpinolate, an inhibitor of palmitoylation, reduced PSD-95 palmitoylation, but not Gαq palmitoylation, suggesting that PSD-95 palmitoylation dynamically turns over in neurons. We also found that inhibition of glutamate receptor activity by kynuretate, APV or CNQX robustly enhanced PSD-95 palmitoylation. This enhancement was significantly blocked by the dominant negative form of P-PAT, suggesting that P-PAT activity is negatively regulated downstream of glutamate receptors. Also, PSD-95 dynamics examined by TIRF (Total Internal Reflection Fluorescence) imaging will be discussed.
The Cdc50p-Drs2p Putative Aminophospholipid Translocase and the Arf GAP Gcs1p Are Involved in Vesicle Formation in the Retrieval Pathway from Yeast Early Endosomes to the TGN

H. Sakane,1 T. Yamamoto,2 K. Tanaka2; 1Institute for Genetic Medicine, Hokkaido University, Sapporo, Japan, 2Institute for Genetic Medicine and Life Science, Hokkaido University, Sapporo, Japan

Drs2p, the catalytic subunit of the Cdc50p-Drs2p putative aminophospholipid translocase, has been implicated in conjunction with the Arf1 signaling pathway in the formation of clathrin-coated vesicles (CCVs) from the TGN. Herein, we explore another vesicle transport pathway in which Cdc50p-Drs2p is involved, we searched for Arf regulator genes whose mutations were synthetically lethal with cdc50Δ, and identified the Arf GAP gene Gcs1. Most of the examined transport pathways in the Cdc50p-depleted gcs1Δ mutant were nearly normal, including endocytosis to vacuoles, carbonylase Y sorting, and the processing and secretion of invertase. In contrast, this mutant exhibited severe defects in the early endosome-to-TGN transport pathway; proteins that are transported via this pathway, such as the v-SNARE Snc1p, the t-SNARE Tlg1p, and the clathrin synthase III subunit Chs3p, accumulated in TGN-independent aberrant membrane structures. Electron microscopy and immunolabeling revealed that the Cdc50p-depleted gcs1Δ mutant accumulated large abnormal double-membrane structures containing Snc1p. We extended our analyses to clathrin adaptors and found that the Cdc50p-depleted gcs1Δ gga2Δ mutant and the gcs1Δ apl2Δ (the β1 subunit of AP-1) mutant exhibited growth defects and intracellular accumulation of Snc1p-containing membranes. These data suggest that Gga1pGga2p and AP-1 were also involved in the early endosome-to-TGN transport pathway. Interestingly, Ap4lp-GFP, the γ subunit of AP-1, was mislocalized in the Cdc50p-depleted gcs1Δ mutant, suggesting that Cdc50p-Drs2p and Gcs1p are involved in the AP-1 recruitment to endosomal membranes. Our results suggest that Cdc50p-Drs2p plays an important role in the Arf1p-mediated formation of CCVs via AP-1 for retrieval from early endosomes to the TGN.

Endocytic Recycling in Yeast Is Regulated by Putative Phospholipid Translocases and the Ypt31p32p-Rcy1p Pathway

N. Furuta, K. Fujimura-Kamada, T. Yamamoto, K. Saito, K. Tanaka; Institute for Genetic Medicine, Hokkaido University, Sapporo, Japan

Most cell types display an asymmetric distribution of phospholipids across the plasma membrane bilayer. The regulation of this asymmetry is believed to require the action of phospholipid translocases (PLTs). In budding yeast, putative PLTs are encoded by the DRS2 gene family of type 4 P-type ATPases. The homologous proteins Cdc50p, Lem3p, and Cre1p are potential non-catalytic subunits of Drs2p, Dnr1p, and Dnt2p, and Dnt3p, respectively, and these putative putative PLTs share an essential function for cell growth. To explore the cellular function(s) in which PLTs are involved, we constructed and dissected the cdc50-30 lem3Δ cre1Δ temperature-sensitive mutant (cdc50-30 mutant). The temperature-sensitive growth of the cdc50-30 mutant was suppressed by overexpression of YPT31/32, two genes that encode Rab family small GTases that are involved in both the exocytic and endocytic recycling pathways. In the cdc50-30 mutant, the recycled exocytic v-SNARE Snc1p intracellularly accumulated in an aberrant structure, whereas it was predominantly localized to the plasma membrane in the wild-type. Additionally, TGN/early endosome t-SNARE Tlg1p accumulated in a similar structure. This aberrant compartment is probably derived from early endosomes, since the accumulated Snc1p colocalized with the endocytic tracer FM4-64 after a short incubation. Furthermore, abnormal membranous structures containing Snc1p were observed in the cdc50-30 mutant by electron microscopy. In contrast, the cdc50-30 mutant did not exhibit defects in the exocytic pathways. Genetic studies suggested that the YPT31/32 effectors RCT1 and CDC50 function in the same signaling pathway, and simultaneous overexpression of CDC50 and DRS2 restored growth as well as the plasma membrane localization of Gfp-Snc1p in the rct1Δ mutant. In addition, Rcy1p coimmunoprecipitated with the Cdc50p-Drs2p complex. We propose that the Ypt31p32p-Rcy1p pathway regulates putative PLTs to promote formation of vesicles destined for the TGN from early endosomes.

Synaptophysin I Selectively Specifies the Exocytic Pathway of Synaptobrevin2/VAMP2

D. Bononomi,1 L. Rusconi,1 C. Colombo,1 F. Benfenati,2 F. Valorta1; 1Department of Neuroscience, San Raffele Scientific Institute and "Vita-Salute" University, Milan, Italy, 2Department of Neuroscience, The Italian Institute of Technology and Department of Experimental Medicine, Genova, Italy, 3The Italian Institute of Technology, Research Unit of Molecular Neuroscience, Milan, Italy

Biogenesis and recycling of synaptic vesicles are accompanied by sorting processes that preserve the molecular composition of both the vesicles and the compartments from which these organelles are generated. However, the mechanisms underlying selective protein sorting during synaptic vesicle assembly remain elusive. In this study we address the targeting of synaptobrevin2/VAMP2, a critical component of the synaptic vesicle fusion machinery, in a heterotypic context where its sorting is not confounded by the presence of other neuron-specific molecules. Ectopically-expressed synaptophysin 1 controls the intracelluar distribution of VAMP2 with no detectable effects on the targeting of other membrane proteins, including exogenous synaptotagmin I and markers of the endosomal recycling pathway. Along the secretory pathway synaptophysin I ret總是 VAMP2 to vesicles exhibiting a low propensity for constitutive exocytosis. These results indicate that interfacial interactions between the sorting determinants of synaptic vesicle proteins can operate independently of a neuronal context and implicate the association of VAMP2 with synaptophysin I in the specification of the pathway of synaptic vesicle biogenesis.

Identification of a Novel Protein Trafficking Regulator in Saccharomyces cerevisiae

L. Tu, D. K. Banfield, Biology, Hong Kong University of Science and Technology, Hong Kong

We identified VPS754 as a multi-copy suppressor of the lethality associated with the loss of SFT1— which encodes an essential Golgi SNARE protein in Saccharomyces cerevisiae. Although Vps74p is a non-essential yeast protein it shares ~40% amino acid sequence identity with two mammalian Golgi phosphoproteins: GM3x3alpha/GDP34 and GM3x3beta/GDP34 and recognizable homologues have been identified in many eukaryotes - with the exception of plants. We have determined that Vps74p contains both cytoplasmic and Golgi-membrane associated pools and that it binds to the Golgi coat protein complex COP (companion) as well as to certain SNAREs. In addition, null mutants of VPS74 are sensitive to calcitran white, indicating that the cell walls of these cells are defective. Consistent with this, processing of the GPI-anchored protein Gas1p, which is required for proper cell wall assembly and morphogenesis, was altered in vps74 deletion cells. We have established that the Gas1p processing defect observed in vps74 deletion cells is attributable to the mislocalization and rapid degradation of a subset of mannose transferases. Our current data suggests that one of the roles of Vps74p may therefore be to function as a protein sorting receptor for COP1. Acknowledgements This work was supported by grants from the Hong Kong Research Grants Council to DKB (HKSUST440/02M and HKSUST607/05M).

The Role of Protein Acylation in Cell-Surface Transport of Multi-spanning Membrane Proteins

K. K. Y. Lam,1 M. Davey1, N. G. Davis,1 E. Combea1; 1centre for Molecular Medicine and Therapeutics, Department of Medical Genetics, University of British Columbia, Vancouver, BC, Canada

Many cell surface proteins, such as ion channels, transporters, and receptors, are trafficked from intracellular storage to the plasma membrane in response to specific extracellular cues. This process of regulated trafficking is crucial to cellular function, but it is not well characterized. A number of multi-spanning membrane proteins in the yeast Saccharomyces cerevisiae undergo regulated trafficking, thus providing models to study the underlying mechanisms. These proteins include several amino acid permeases (AAPs) and the chitin synthase Chs3p, which are transported to the cell surface in response to external nutrient conditions and cell stress, respectively. We have previously identified Pfa4p as a genome-wide fluorescence screen for mutants that missort Chs3. Pfa4p is a DHHC palmitoyltransferase responsible for modifying Chs3 at the ER and facilitating Chs3 export from this compartment (Lam et al., 2006. J Cell Biol 174: 19). To complete this work we have recently performed aкрыл mutagenesis screen for mutants that affect the trafficking of Chs3. Our current data suggests that one of the roles of Pfa4p may therefore be to function as a protein sorting receptor for COP1. Acknowledgements This work was supported by grants from the Hong Kong Research Grants Council to DKB (HKSUST4105/02M and HKSUST4607/05M).

Moving Transporters into Intracellular Storage: Identifying New Components of the Early Endosome Sorting Machinery in Saccharomyces cerevisiae

Y. Tam, M. Davey, E. Combea; Medical Genetics, University of British Columbia, Vancouver, BC, Canada

Many cell surface proteins, such as ion channels, transporters, and receptors, are trafficked from intracellular storage to the plasma membrane in response to specific extracellular cues. This process of regulated trafficking is crucial to cellular function, but it is not well characterized. A number of multi-spanning membrane proteins in the yeast Saccharomyces cerevisiae undergo regulated trafficking, thus providing models to study the underlying mechanisms. These proteins include several amino acid permeases (AAPs) and the chitin synthase Chs3p, which are transported to the cell surface in response to external nutrient conditions and cell stress, respectively. We have previously identified Pfa4p as a genome-wide fluorescence screen for mutants that missort Chs3. Pfa4p is a DHHC palmitoyltransferase responsible for modifying Chs3 at the ER and facilitating Chs3 export from this compartment (Lam et al., 2006. J Cell Biol 174: 19). To further characterize this lipid modification, the 22 cysteine residues in Chs3 that can serve as potential palmitoylation sites were mutated. Our data suggest that Chs3 is palmitoylated at an internal cysteine. Interestingly, Pfa4p has also recently been shown to modify several AAPs at a C-terminal consensus sequence that is not present on Chs3 (Roth et al., 2006. Cell 125:1003). Moreover, through genome-wide screens of the yeast deletion collection, we find that Pfa4p may influence the cell surface activity of one of these AAPs. A thorough comparison between AAP and Chs3 trafficking may therefore elucidate whether Pfa4p-mediated palmitoylation plays a more general role in regulating the transport of multi-spanning membrane proteins. The similar lipid modifications of AAPs and Chs3 could direct common sorting events that may also be relevant for transporter translocation in higher cells.
In the yeast *Saccharomyces cerevisiae*, the chitin synthase Chs3 is maintained in Golgi/endosome-derived intracellular pool and is transported to the plasma membrane under extracellular stress. Transport of Chs3 to the cell surface requires Chs6. In chs6Δ cells, cell surface translocation of Chs3 can be restored by mutations in the clathrin adaptor complex, AP-1. This suggests that AP-1 normally sorts Chs3 into intracellular compartments. However, how AP-1 regulates Chs3 transport or whether other factors are involved in this process is still unknown. To uncover genes that are specific for the transport of Chs3 between early endosomes and the TGN, we have deleted the CHS6 gene in yeast knockout collections and screened for double deletion mutants that have restored translocation of Chs3 to the plasma membrane, using a novel fluorescence assay. This approach has identified known AP-1 components, endosomal transport factors as well as uncharacterized genes. To delineate functionally related genes that work in the AP-1 and/or other pathways, epistatic miniarray profiling and hierarchical clustering have been performed on the set of top mutants. We have characterized two novel factors, Cby1 (Chs6 bypass suppressor 1), and Cby2, which interact with AP-1 and participate in the AP-1-dependent retrograde transport of Chs3.

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An AP-1-interacting Sorting Signal Directs Intracellular Transport of Yeast Chitin Synthase III

T. Starr; University of California, Berkeley, Berkeley, CA

Sorting signals present in proteins that traverse the secretory pathway are essential for the proper localization of these proteins within the cell. The composition and location of these signals within proteins determine act on the signals and thus determine where the proteins are delivered. We identified a sorting signal that is required for the intracellular retrieval of yeast Chitin Synthase III (Chs3p). This signal is similar to signals found in other proteins that are known to interact with clathrin adaptor protein complexes (APs). Tandem affinity purification (TAP) of AP-1 revealed an interaction between AP-1 and Chs3p; mutation of the signal reduces this interaction. To determine the direction in which the sorting signal may transport Chs3p, we made fusions between a region of Chs3p containing the sorting signal and the vacuolar protein alkaline phosphatase (ALP) to make Chs3-ALP. The Chs3-ALP fusion protein is transported to the vacuole and this event is dependent on the specificity of the AP-1-interacting signal. We are currently working towards understanding the role of the compartment(s) of the signal sorting directs Chs3-ALP en route to the vacuole.

1824

Di-leucine Motifs Bind to the Gamma/Sigma1 Hemicomplex of Adaptor Protein-1 (AP-1) and Expose the Yxxφ Binding Site on the Mu1 Subunit

I. Lee, B. Dorney, S. Kornfeld; Internal Medicine, Washington University School of Medicine, St. Louis, MO

The adaptor protein AP-1, a heterotetramer containing γ1/μ1/ε1 subunits, is known to bind cargo proteins via two types of motifs: tyrosine-based Yxxφ and di-leucine-based E(D)XXLL motifs. While it is well established that Yxxφ motifs bind to the μ1 subunit, di-leucine motifs have been reported to bind to μ1, β1, and, based on the yeast-three hybrid technique, to the γ1/ε1 hemicomplex (Janvier et al., JCB, 163:1281-1290, 2003; Coleman, et al., J. Virology, 79:2066-2078, 2005). To approach this issue from a biochemical standpoint, we have expressed each subunit of AP-1 individually as well as the β1/μ1 and γ1/ε1 hemicomplexes in insect cells and used GST-pulldown assays to determine the binding properties of these subunits. We find that the γ1/ε1 hemicomplex binds the di-leucine motifs of several proteins quite strongly, whereas binding to β1 and μ1 is extremely weak by comparison. Under these binding conditions, there was no interaction with GST alone. The binding to γ1/ε1 does not require the hinge or appendage domain of γ1, implicating the trunc region as the site of interaction. Peptide competition experiments indicate that the various di-leucine motifs bind to the same site on γ1/ε1. We have also found that binding of a di-leucine motif-containing peptide to intact cytosolic AP-1 results in enhanced accessibility of the μ1 subunit, as determined by increased sensitivity to tryptic cleavage. This is associated with increased binding of Yxxφ peptides. In the case of CI-MPR cytoplasmic tail, which contains both types of motifs, we found that binding of the Yxxφ motif to the μ1 subunit of cytosolic AP-1 is totally dependent on the presence of the di-leucine motif. We conclude that di-leucine motifs bind the γ1/ε1 hemicomplex of AP-1 and induce a conformation change in the heterotetramer that facilitates binding of Yxxφ motifs to the μ1 subunit.

1825

 Trafficking and Retention of a Plasmodium Falciparum Protein, Pfs16, in the Parasitophorous Vacuole Membrane

S. Eksi, L. Ma, M. T. Mcintosh, D. Elliott, J. Gibbons, K. C. Williamson; 1Biology, Loyola University Chicago, Chicago, IL, 2Medicine, Yale University, New Haven, CT, 3Biological Sciences, University of Illinois at Chicago, Chicago, IL

Red blood cell invasion and parasitophorous vacuole (PV) formation in *Plasmodium falciparum* is critical for the development and pathogenesis of malaria, a continuing global health threat. As the intraerythrocytic parasite grows from 1um to 5um diameter there is a corresponding increase in the size of the PV membrane (PVM). Since mature RBC lack internal organelles and no longer actively synthesize membranes, the production and trafficking of lipids and proteins required for the expansion of the PV is orchestrated by the parasite. Pfs16, a 16kDa integral PV membrane protein was chosen as a model for studying trafficking of material from the parasite across the PV space to the plasma membrane. Pfs16 synthesis begins right after RBC invasion and continues to be expressed on the PVM as P. falciparum develops through the 5 morphologically distinct stages of gametocytogenesis. Green fluorescent protein (GFP) was used as a reporter to test the ability of distinct regions of Pfs16 to direct trafficking to the PV. The combined presence of the 25aa amino-terminal secretory signal and a 42aa region, made up of the predicted 22aa transmembrane domain (TM), Pfs16 aa105-126 and the next 20aa (Pfs16 aa127-146), were sufficient to target expression to the PV. The 20aa tail contains 3 sections with distinct charge profiles. Section 1 is the first 6aa following the TM domain and includes 4 lysines (K). Section 2 is the next 8aa and contains 3 tandem aspartates (D) followed by K-glycine-D. Section 3 is the remaining 6aa which is made up of 3 tandem repeats of KD. Although, wt Pfs16 contains an additional 11 C-terminal aa (Pfs16 aa147-157) including 4 Ds, these aa are not required for PVM targeting. The 44aa PVM targeting region identified by this work provides an important probe for further evaluation of this unique parasite to PVM trafficking pathway.

1826

Traffic of *Salmonella typhi* Cytochalal Distending Toxin within Host Cells

S. Spano, J. E. Galan; Section of Microbial Pathogenesis, Yale University School of Medicine, New Haven, CT

*Salmonella typhi*, the agent of typhoid fever, encodes CdbB, a toxin that induces G2 cell cycle arrest and cellular distortion by infiltrating limited host-cell DNA damage. This toxin is delivered to the host cell by a novel pathway that depends on bacterial internalization into host cells. Expression of the toxin occurs only 2 to 3 hours after S. typhi internalization into the host cell, when the bacterium has reached a highly specialized membrane-bound compartment. In order to be directed to its cellular target CdbB must traffic from the Salmonella-containing vacuole to a subcellular compartment where retro-translocation to the cytosol can occur. The aim of this study is to dissect this novel pathway and identify the molecular mechanisms that govern it. We have been able to detect epitope-tagged CdbB in infected epithelial cells and follow its transport within the host cell by microscopic and biochemical techniques. Secretion of CdbB from the bacterium starts 3 to 5 hours after infection. At this time the toxin is localized on the surface of intracellular bacteria, within the Salmonella-containing vacuole. At later times after infection, the toxin is also detected in small puncta throughout the cell, seemingly emanating from the Salmonella-containing vacuole. We hypothesize that these are intermediate compartments that may transport the toxin to the site of retro-translocation. The number and fluorescent intensity of these structures gradually increase up to 20 hours after infection. The nature and dynamics of these CdbB transport intermediates are currently being investigated.

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Expression and Localization of the Mammalian Facilitative Glucose Transporter GLUT12 in Renal Epithelial Cells

A. L. Wilson-O'Brien, L. DeHaan, S. Rogers; 1Medicine St. Vincent's, The University of Melbourne, Melbourne, Australia, 2Department of Genetics, The University of Melbourne, Melbourne, Australia

Glucose homeostasis is a major cause of diabetic complications, including diabetic nephropathy. Our studies have shown GLUT12 expression in the distal tubules of rat fetal and human adult kidney, with subcellular localization to the apical membrane. In addition, renal GLUT12 expression was upregulated in an animal model of diabetic nephropathy. We aimed to elucidate the mechanisms that regulate expression and localization of GLUT12 in the kidney in response to factors associated with diabetic nephropathy. Regulated expression of GLUT12 in response to AngII and TNFα was studied in cultured cell models using real time PCR and Western blotting. Regulated targeting of GLUT12 was studied with fluorescence-tagged constructs, retroviral transfection and confocal microscopy in response to acute and chronic high glucose (25mM). In Human Embryonic Kidney 293T cells, GLUT12 RNA and protein levels remained unchanged after TNFα and AngII treatments. Transfection of renal epithelial Madin Darby Canine Kidney (MDCk) cells with a fluorescence-tagged GLUT12 construct demonstrated a perinuclear localization in basal conditions. Acute treatment with high glucose had no effect on GLUT12 localization. However, chronic high glucose treatment resulted in GLUT12 movement from a perinuclear position to the apical membrane. MDCk polarization was demonstrated by the expression of the ZO1 junctional marker, with apical localization of GLUT12 confirmed by Z-section imaging and co-localization with GLUT3. The functional role of GLUT12 was examined using glucose uptake assays and transwell filters. Our study localizes GLUT12 to the apical

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surface of renal epithelial cells in response to chronic elevated glucose. This novel finding suggests a role for GLUT12 in reabsorption of glucose in the late nephron, implicating GLUT12 in the progression of diabetic nephropathy.

1828 Nucleocytoplasmic Shuttling of Leukemia-associated RhoGEF
E. Grabocka, P. B. Wedegaertner; Department of Biochemistry and Molecular Biology, Thomas Jefferson University, Philadelphia, PA
Leukemia-Associated-RhoGEF (LARG), together with PDZ-RhoGEF and p115RhoGEF, also known as RGS-RhoGEFs, function in linking heterotrimeric G-protein signaling to the monomorphic RhoGTPase. As such they are important regulators of several pathways controlling cell growth and differentiation. Here we provide the first evidence that LARG undergoes nucleocytoplasmic shuttling regulated by its dimerization. Thus, deletion of the C-terminus (ΔC), previously demonstrated to be involved in the dimerization of RGS-RhoGEFs, causes LARGΔC, but not PDZ-RhoGEFΔC or p115RhoGEFΔC, to localize to the nucleus as indicated by immunofluorescence and nuclear fractionation assays. FRAP analysis reveals LARGΔC to be actively imported into the nucleus, and nuclear import is Ran dependent. We identify a nuclear localization sequence (NLS), 29PTDKKQ35, necessary for the nuclear targeting of GFP-LARGΔC. The NLS of LARG, however, requires an additional N-terminal stretch of amino acids to target an unrelated protein to the nucleus. Our data suggests that dimerization of LARG blocks the exposure of both nuclear localization and nuclear export sequences (NES). Thus, using the rapamycin inducible FRB/FKBP dimerization system, otherwise nuclear GFP-LARGΔC fused to the FRB or FKBP domains shows a cytosolic localization when expressed in the presence of rapamycin. We identify a predicted coiled-coil region (a.a. 1508-1522) in the extreme C-ter of LARG as a regulator of dimerization and nucleocytoplasmic shuttling. We demonstrate that whereas GFP-LARG is not sensitive to the CRM1 nuclear export pathway inhibitor leptomycin B (LMB), a LARG mutant lacking the coiled-coil region shows a nuclear localization upon LMB treatment. Taken together, our results indicate that LARG may play a signaling role in the nucleus and that nucleocytoplasmic shuttling of LARG may be a mechanism regulating its signaling functions.

1829 Expression Patterns of Lectins in Cardiovascular System
P. Chu, S. Jung, A. Wu, Medicine, Chang Gung Memorial Hospital and University, Taipei, Taiwan, 2Pathology, Chang Gung Memorial Hospital and University, Taipei, Taiwan, 3Glyco-Immunochromepstry Research Laboratory, Chang Gung University, Taipei, Taiwan
Objective: A variety of common cardiovascular disorders are characterized by the changes of lectin, such as P-selectin on the activated endothelium in the reperussed area and L-selectin on the leucocytes have a vital role in mediating the initial steps of this cascade. Lectins or glycoproteins are simply defined as proteins which specifically bind (or crosslink) carbohydrates. The existence of glycoproteins in cardiovascular system has not been demonstrated completely. The purpose of this investigation was to elucidate the expression patterns of lectins in cardiovascular system.
Methods: This study analysis of micro-tissue array composing of 80 patients with cardiac myxoma that were surgically excised. The expression patterns of 21 glycoproteins in cardiomyocytes and vessels were elucidated by immunohistochemical analysis. Results: All heart containing vessels were analyzed detailed. In the membrane of cardiomyocytes, only wheat germ agglutinin (WGA, 95%), Ricinus communis (RCA, 85%), and Cysteine-rich antifungal protein 2B (M2B, 60%) were expressed. There were more lectins expressed in the nucleus of the cardiomyocytes, including RCA, M2B, RCA, ricin, Bandeiraea (Griofinia) simplicifolia lectin-I, isolectin B4, Agaricus bisporus agglutinin, Bandeiraea (Griofinia) simplicifolia lectin-I, isolectin A4, Abra-A, BHG3, Jacalin, and M2B. The existence of glycoprotein in the endothelium is more ubiquity except Vicia villosa B4(4). Conclusion: The characteristics of expression patterns of glycoprotein in cardiovascular system imply the important role of cardiovascular diseases.

1830 Nicotine Decreases Pregnenolone Synthesis and Reduces STAR Import into Mitochondria
H. S. Bose, M. Bose, D. Deb Nath, C. Huang; Physiology, University of Florida, Gainesville, FL
The rate-limiting step in steroidogenesis is the transport of cholesterol from the outer to inner mitochondrial membrane, which is facilitated by steroidogenic acute regulatory protein (STAR). STAR is a 37 kDa cytoplasmic phosphoprotein processed as 32 kDa and mature 30 kDa protein. STAR activity is proportional with its residency time at the outer mitochondrial membrane. Exposure of 1 mM nicotine to the COS-1 cells transfected with STAR/F2 decreased pregnenolone synthesis and reduced expression of 37 kDa and 32 kDa STAR by 50%, and 2.5 mM nicotine ablated STAR expression and activity. Incubation of biosynthetic STAR to isolated mitochondria with 1 mM nicotine reduced pregnenolone from 600 to 285 ng/ml. Mitochondria pretreated with 1 mM nicotine inhibited STAR import and stopped completely with 2.5 mM, but similar doses of nicotine had no effect on the import of signal sequence substituted STAR (SCC/N-62STAR). 355-Star imported onto isolated mitochondria formed one single complex on digitonin extraction. Density gradient ultracentrifugation showed that 37 kDa OMM associated complex was degraded with nicotine but not the imported 30 kDa STAR complex. Fluorescence emission maxima of N-62 STAR at 337.2 ±0.2 was unchanged but the free energy of unfolding 2.46 changed to 3.14 kcal/mol with 1 mM nicotine. Thus we conclude that the transport process of STAR at the OMM is specific and sensitive to environmental factors, like nicotine, resulting interruption in cholesterol fostering into mitochondria, which ultimately decreases pregnenolone synthesis.

1831 Structural Requirements That Determine Tail Anchoring of Myotonic Dystrophy Protein Kinase (DMPK) Isoforms to ER or Mitochondria
R. J. A. Oude Ophuis, J. Fraenen, B. Wieiringa, D. G. Wansink; Cell Biology, Nijmegen Centre for Molecular Life Sciences, Nijmegen, The Netherlands
DMPKs, alternatively spliced isoforms of the gene mutated in myotonic dystrophy type 1, are members of the AGC group of kinases, to which also MRCKs, Rho kinases, and citron kinase belong. DMPK appears to be an evolutionary "recent" protein, occurring only in mammals. Long DMPK isoforms A and C of mouse contain distinct C-terminal tails that anchor the protein selectively to the ER or the mitochondrial outer membrane (MOM), respectively. Human DMPK A and C, although highly homologous, both target to the MOM. We here report on the precise structural requirements that determine DMPK's behaviour as a tail-anchored protein. Deletion mutation analysis, protein tagging and transfection studies in muscle and neuronal cell types revealed that only the tails of DMPK A and mDMPK A are necessary and sufficient for correct membrane targeting. For human and mouse DMPK C, additional presence of a short upstream coiled coil region was required for selective binding. Mutation of single amino acid positions revealed that charged residues (arginines) in close proximity of a putative transmembrane domain in the tail determined MOM or ER selectivity. Interestingly, expression of mDMPK C or mDMPK C, which also bind to the MOM. This effect resides in the tail domain only and is not dependent on kinase activity of the protein. We will report on the delineation of the precise structural and cell-type conditional requirements for selective MOM anchoring and the induction or absence of associated biological effects. Also data on membrane topology determinants for tail anchoring of DMPK will be presented. 1. Groene, P.J. et al. HUMG 9, 605-16 (2000). 2. Wansink, D.G. et al. MCB 23, 5489-501 (2003). 3. van Herpen, R.E. et al. MCB 25, 1402-1404 (2005).

1832 Identification of the Yeast R-SNARE Nyp1p as a Novel Longin Domain-containing Protein
L. Chen, ' W. Wen, ' H. Wu, ' X. Sun, ' M. Zhang, ' D. K. Banfield; ' Biology, Hong Kong University of Science and Technology, Hong Kong, 'Biochemistry, Hong Kong University of Science and Technology, Hong Kong
Using NMR spectroscopy we establish that the N-terminal domain of the yeast vacuolar R-SNARE Nyp1p adopts a longin-like fold similar to those of Sec22p and Ykt6p. Nyp1p is sorted to the limiting membrane of the vacuole via the AP3 adaptin pathway and we show that its longin domain is sufficient to direct transport to this location. In contrast, we found that the longin domains of Sec22p and Ykt6p were not sufficient to direct their localization. A YXXPHI-like adaptin-dependent sorting signal (Y31GTI34) unique to the longin domain of Nyp1p mediates interactions with the AP3 complex in vivo and in vitro. We show that amino acid substitutions to Y31GTI34 (Y31QI34Q) resulted in mislocalization of Nyp1p as well as reduced binding of the mutant protein to the AP3 complex. Also, the longin domain of vacuolar r-SNAREs is dependent upon the Y31GTI34 motif, and Y31 in particular, our finding is consistent with structure-based amino acid substitutions in the mu chain (Apm3p) of yeast AP3 suggest a mechanistically distinct role for this subunit in the recognition of YXXPHI-like sorting signals. Acknowledgements:This work was supported by grants from the Hong Kong Research Grants Council to KDB (HKUST6015/02M and HKUST6047/05M) and MZ (HKUST6125/02M, HKUST6138/03M and HKUST6125/04M).
It has recently been proposed that the TBC (Tre2/Bub2/Cdc6) domain functions as a GAP (GTPase-activating protein) domain for small GTPase Rab. Because of the large number of Rab proteins in mammals, however, most TBC domains have never been investigated for Rab-GAP activity. In this study we established panels of the GTP-fixed form of 60 different Rabs constructed in pGAD-C1, a yeast two-hybrid bait vector. We also constructed a yeast two-hybrid prey vector (pGIDU-C1) that harbors the cDNA of 40 distinct TBC proteins. Systematic investigation of 2400 combinations of 60 GTP-fixed Rabs and 40 TBC proteins by yeast two-hybrid screening revealed that seven TBC proteins specifically and differentially interact with specific Rabs (e.g., OAT1 interacts with Rab2A; FLJ12085 with Rab5A/B/C; and Evi5-like with Rab10). Measurement of in vitro Rab-GAP activity revealed that OAT1 and Evi5-like actually possess significant Rab2A- and Rab10-GAP activity, respectively, but that FLJ12085 do not display Rab5A-GAP activity at all. These results indicate that specific interaction between TBC protein and Rab would be a useful indicator for screening for the target Rabs of some TBC/Rab-GAP domains, but that there is little correlation between the Rab-binding activity and Rab-GAP activity of other TBC proteins.

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**Screening for Target Rabs of TBC (tre-2/bub2/cdc6) Domain-containing Proteins Based on Their Rab-binding Activity**

T. Itoh, M. Fukuda,1,2, RIKEN, Wako, Japan, 3Developmental Biology and Neurosciences, Tohoku University, Sendai, Japan

It has recently been proposed that the TBC (Tre2/Bub2/Cdc6) domain functions as a GAP (GTPase-activating protein) domain for small GTPase Rab. Because of the large number of Rab proteins in mammals, however, most TBC domains have never been investigated for Rab-GAP activity. In this study we established panels of the GTP-fixed form of 60 different Rabs constructed in pGAD-C1, a yeast two-hybrid bait vector. We also constructed a yeast two-hybrid prey vector (pGIDU-C1) that harbors the cDNA of 40 distinct TBC proteins. Systematic investigation of 2400 combinations of 60 GTP-fixed Rabs and 40 TBC proteins by yeast two-hybrid screening revealed that seven TBC proteins specifically and differentially interact with specific Rabs (e.g., OAT1 interacts with Rab2A; FLJ12085 with Rab5A/B/C; and Evi5-like with Rab10). Measurement of in vitro Rab-GAP activity revealed that OAT1 and Evi5-like actually possess significant Rab2A- and Rab10-GAP activity, respectively, but that FLJ12085 do not display Rab5A-GAP activity at all. These results indicate that specific interaction between TBC protein and Rab would be a useful indicator for screening for the target Rabs of some TBC/Rab-GAP domains, but that there is little correlation between the Rab-binding activity and Rab-GAP activity of other TBC proteins.

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**PAT-1 Confers Increased Neutral Lipid Storage Capacity in Cultured Cell Lines**

W. E. Ackerman,1 S. R. Bartholomew,2 E. M. Hlavina,3 T. L. Summerfield,1 J. T. Tansey1, Obstetrics and Gynecology, The Ohio State University, Columbus, OH, Chemistry and Biochemistry, Otterbein College, Columbus, OH

Lipid storage droplets (LSDs) serve an essential cell role in neutral lipid storage and trafficking within most eukaryotic cells. A family of proteins sharing homology in the N-terminal PAT domain has been implicated in the regulation of mammalian LSDs. In addition to the perilipins, adipose differentiation-related protein (ADRP), tail-interacting protein of 47 kDa (TIP-47), and S3, a novel mammalian PAT paralog (termed PAT-1 or myocardial LD protein [MLDP]) has been identified. To explore the function of PAT-1, an epitope-tagged fusion construct of this recently characterized protein (PAT-1-3xFLAG) was ectopically expressed in two cell lines (CHO-K1 and NIH 3T3) lacking endogenous PAT-1 expression. The recombinant protein partitioned with ADRP in the basolateral LD fraction following sucrose gradient centrifugation, and localized to the perimeter of neutral-lipid containing organelles. PAT-1-3xFLAG localized to both ADRP- and TIP-47-encocated vesicles of varying sizes, from microlipid droplets to enlarged LSDs. Unlike ADRP, the expression of PAT-1-3xFLAG was not destabilized by lipid deprivation. Moreover, ADRP was not destabilized in the presence of epitope-tagged PAT-1, suggesting that, unlike perilipin A, PAT-1 does not displace endogenous PAT proteins from LSDs. Finally, stable expression of PAT-1-3xFLAG enhanced the capacity of CHO-K1 cells to accumulate cholesterol and triglycerides following oleic acid supplementation. These results suggest that PAT-1 represents a bona fide LD coat protein that acts in association with ADRP and TIP-47 to confer increased neutral lipid storage capacity in cultured cells.

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**Computational Modeling of Gab Family PH Domains**

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

The Grb-2 associated binder (Gab) adapter/scaffolding molecules have no enzymatic activity, but they contribute to the localization and/or amplification of signal transduction pathways. The Gab family currently comprises mammalian Gab1, Gab2, Gab3, the Drosophila homolog Daughter of seventimes less, and the Caenorhabditis elegans homolog Suppressor of Clear1- (SoC-1). There are many studies to date that link the Gab family proteins to oncogenic transformation. Gab1 has been implicated in breast, thyroid, and leukemia tumors. Gab2 has been shown to play a role in Bcr-Abl transformation, which causes chronic myelogenous leukemia (CML) and breast cancer. It has been suggested that Gab1 transforming potential may be activated by the loss of its pleckstrin homology (PH) domain, which is the most conserved region within various Gab family members. Many PH domains bind membrane phosphoinositides but the exact role of PH domain is still unknown. However, it is apparent that mutation or deletion of the PH domain has a drastic effect on the functioning of Gab proteins, leading to aberration in signaling cascades and oncogenesis. We have characterized 21 Gab family PH domains by 1) predicting secondary structures; 2) creating homology models using a number of different approaches, i.e. homology modeling, protein threading, multiple sequence and structure alignment, sequence to profile alignment, and loop refinement; 3) generating the electrostatic potential contours; 4) searching for a consensus sequence, which predicts high affinity binding to PI3-Kinasic enzymes. We present the detailed characterization of these PH domains and the analysis of their physical interactions with membrane phosphoinositides. On-line supplementary information is available at http://userhome.brooklyn.cuny.edu/ssingh/gabs/gabs.html

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**Halofuginone Is a Small Molecule Regulator of a Novel Transcriptional Complex That Controls Extracellular Matrix Gene Expression**

Y. G. Kamberov,1 R. Mazitschek,2 M. R. Whitman1; Developmental Biology, Harvard Dental School, Boston, MA, Chemical Biology Program, Broad Institute, Cambridge, MA

Halofuginone (HF) is a potent and specific regulator of ECM gene expression and is unique in its therapeutic potential. The basis for HF effects on the ECM is unknown but ultimately results in the transcriptional control of a number of ECM components including Type I collagen (Pines M and Nagler A 1998, Gen. Pharmacol. 30, 445-450). The aim of this study was to identify the molecular mediators of HF function and regulation of the complex by which the compound exerts cell-type specific effects. We have synthesized active and inactive variants of HF, and have used derivitized HF as the basis for a novel polymer based-affinity purification technique to identify the first specific HF binding proteins. We find that HF interacts with a novel complex of nuclear chromatin remodeling factors, and we have mapped the domains necessary for HF association with this complex. Our work with the HF protein complex suggests a new approach to small molecule based regulation of the ECM transcriptional program in vivo. In addition, our affinity purification system provides an improvement over existing approaches for the identification of novel cellular targets of small molecules. Knowing the targets of HF allows the development of more specific and better tailored therapeutics for regulating ECM disorders, and provides a powerful tool for elucidating ECM biology on the sub-cellular level.

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**Expression of Membrane Type 2 - Matrix Metalloproteinase during Avian Development**

R. A. Patterson,1 V. Cantemir,2 P. R. Brauer,1 M. V. Reedy1, Department of Biology, Creighton University, Omaha, NE, Department of Biomedical Sciences, Creighton University, Omaha, NE

Matrix metalloproteinases (MMPs) comprise a family of zinc-binding proteolytic enzymes that play essential roles in tissue remodeling, cell proliferation, migration, differentiation, apoptosis, and degradation of the extracellular matrix. Inhibition of MMP activity results in birth defects and developmental anomalies. Membrane Type-MMPs (MT-MMPs) are membrane-bound forms of MMPs that can direct to specific regions on their cell surface to localize proteolytic activity. We used whole mount in situ hybridization to determine the expression pattern of MT2-MMP mRNA during various stages of avian embryonic development (HH stages 9-22). After hybridization, embryos were embedded in tissue freezing medium and sectioned in order to better evaluate the distribution pattern of MT2-MMP-expressing cells. At stages 10-14, MT2-MMP expression was detected in the notochord and in the posterior neural folds prior to fusing to form the neural tube. At stages 11-15, MT2-MMP expression was found in the intermediate mesoderm, tailbud, and in blood islands of the extraembryonic tissue. MT2-MMP expression was also detected in the otic placodes spanning stages 12-22. At stages 17-22, MT2-MMP expression was also found in the pharyngeal arches, ventral myocardium of the developing heart, dorsal limb bud mesoderm, and dermomyotome. These observations suggest that MT2-MMP plays multiple roles in the embryogenesis of several organs. This research was supported by the American
Mechanical Stress Induces Involvement Associated Events in Mammary Epithelial Cells

A. Quaglino, T. B. Tanos, O. A. Coso, E. C. Kordon; IFIBYNE-CONICET, FCCEyN - Universidad de Buenos Aires, Buenos Aires, Argentina

It has been demonstrated that local factors are responsible for the initiation of mammary gland involution after weaning. Results from different groups, including our own, indicate that during this process there is transcription induction of specific genes, as if, c-fos and tnf-a, and activation of different quinases and transcription factors as Erk1/2 and Stat3. It has been speculated that milk-stasis and the consequent stretching of lobular acini could be the earliest stimuli for triggering this process. However, up to now there has been no report indicating whether mechanical stress could exert any effect on mammary epithelial cells. Therefore, our goal was to determine whether mammary cell stretching, could be an early signal that triggers biochemical changes that in turn would lead to epithelial apoptosis. Then, a novel device that applies controlled radial stretching to cells growing in monolayer was designed for mimicking the physical tension generated in the mammary acini when milk efflux is interrupted. Mammary epithelial HC1 cells were seeded on flexible silicone sheets previously coated with collagen. When cells reached confluence, silicone sheets were stretched up to 20% during different times (2min - 4hrs). By semiquantitative and quantitative RT-PCR assays we found that cells exposed to sustained stretching (30 min) showed if, c-fos and tnf-a transcription induction. In addition, by Western blot analysis we found that mechanical stress also induced Erk1/2 serine and Stat3 tyrosine phosphorylation after 5 minutes. In addition, after 1h a strong increase in c-Fos protein levels was detected. Interestingly, stretched cells showed a much higher content of this protein in the cell nuclei than controls, suggesting that mechanical stress not only induced c-Fos expression but also its activation. Therefore, these results are the first direct evidence showing that mammary cell stretching could be relevant in triggering events leading to cell death during post-lactational involution.

Casein Expression in Heterotopic Organs of Lactating Mice

T. Kanazawa, A. Takagai, H. L. Hosick;1 Department of Bioresource Science, Ibaraki University, College of Agriculture, Ibaraki-ken, Japan, 2School of Biological Sciences, Washington State University, Pullman, WA

Casein is accepted to be a milk-specific protein that is synthesized in mammary glands. Messenger RNA for casein is, however, detected in some other tissues of the mouse, including thymus, tongue and pancreas. These findings raise the question of whether casein is expressed specifically in mammary glands. We have therefore surveyed casein in a variety of organs of the mouse at various reproductive stages, by using Western blotting, immunohistochemistry and RT-PCR techniques. Western blotting analysis, using a mouse whole casein-specific antisemur and mouse β-casein-specific monoclonal antibody as probes, revealed casein-related bands in the homogenates from skin of abdominal-, dorsal- and tail-portion, tongue, salivary glands, liver, pancreas, brain, kidney, thymus, uterus, ovary, lung and cardiac tissues of lactating mice, at levels less than that of mammary glands. No casein-related bands were detected in these organs of virgin and expressing in heterotopic organs of mice during lactation, and that casein expression in these heterotopic organs is also mediated through prolactin stimulation. We interpret these findings to indicate that casein is not milk-specific, although the amount of casein expressed in heterotopic tissues is not high as in lactating mammary glands in mice.

Further Analysis of HLA-G and HLA-E Expression in Human Placental Tissue

M. Greimel, M. Siwetz, A. Blaschitz, G. Dohr, H. Hutter; Institute of Cell Biology, Histology and Embryology, Medical University of Graz, Graz, Austria

The human MHC class I genes HLA-G and HLA-E are referred to as non classical or class 1b molecules. They are distinguished from the classical HLA class I molecules by a low allelic polymorphism and they differ by specific patterns of transcription, protein expression and immunotolerant functions. HLA-G and HLA-E proteins may play a central role in the development of tolerance between fetal and maternal tissue at the feto-maternal interface in the human placenta. The aim of this work was to further analyze the expression pattern of HLA-G and HLA-E in human placental tissue during the first trimester of pregnancy. Using a set of specific monoclonal antibodies (HC2A, 4H84, MEM-G1/1, MEM-G9, MEM-E2, and MEM-E/8) and different biochemical and immunohistochemical methods (e.g. Western blotting, single/double immunostaining and immunofluorescence), we detected a strong expression of HLA-G in extravillous cytrophoblast as well as a weaker expression of HLA-E in the same placental cell population. Moreover a strong expression of HLA-G in endothelial cells of blood vessels and endometrial glands of the decidua was detected. The cell specific expression of HLA-G and HLA-E in human placental tissue may suggest an interacting functional role of these proteins with Killer Cell Inhibitory Receptors (KIR) of decidual NK cells which may result in down regulation of the immune response and a successful maintenance of pregnancy.

Parathyroid Hormone-related Protein (PTHRP) Regulates Tumor-Related Genes in Breast Cancer Cells

J. Dittmer, A. Dittmer, M. Vetter, D. Schunke, P. N. Span, F. Sweep, C. Thomssen;1 Clinic for Gynecology, University of Halle, Halle, Germany, 2Department of Chemical Endocrinology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands

PTHRP is expressed by the majority of breast cancer. It is responsible for hypercalcemia of malignancy and is involved in the regulation of biological activities of cancer cells. By interacting with the PTH1 receptor, PTHRP is able to activate protein kinase A and C pathways. PTHRP is also capable of traveling into the nucleus, an activity that is regulated by cdc2. To identify genes that are regulated by PTHRP, we suppressed endogenous PTHRP expression of MDA-MB-231 breast cancer cells by PTHRP-specific siRNA (siPTHRP) and subjected the RNA from these cells to microarray analysis. Three independent experiments were performed and evaluated. Of the genes whose expression was significantly altered relative to control siRNA, some were chosen for further analysis. The expression of more than 200 genes were affected by siPTHRP. Interestingly, siPTHRP interfered with cdc2 activity by increasing its expression and the expression of its activator cdc25B and cdc25B's regulator -Towe. PTHRP peptide 1-34 and forskolin downregulated cdc2 and cdc25B expression suggesting that PTHRP regulates these genes via PTH1R. In primary breast cancer, higher expression of cdc2 and cdc25B was found at low PTHRP levels. siPTHRP downregulated integrin alphait (ITGA6), Kias-1 and plasminogen activator inhibitor-1 (PAI-1). In primary breast cancer, ITGA6 RNA levels were positively correlated with those of PTHRP. siPTHRP inhibited migration and proliferation. These siPTHRP effects could be mimicked by a combination of siRNAs directed against ITGA6, PAI-1 and Kias-1 suggesting that PTHRP may exert its migratory, proliferative and motogenic effects through these genes. The results suggest that, by controlling cdc2 activity, PTHRP may regulate its own nuclear activity. The data also imply, that by interfering with the expression of oncogenes, such as ITGA6 and PAI-1, and tumor suppressor genes, such as KISS-1, PTHRP contributes to the regulation of proliferation and migration of breast cancer cells.

Prostate Tumor Cells Near and Distant from Focally Disrupted Basal Cell Layers Have Different Gene Expression Profiles

Y. Man, X. Liu, J. Mason, S. Prabhakar, B. Wang, X. Zeng, M. Stamatakis, W. Gardner;1 Armed Forces Institute of Pathology and American Registry of Pathology, Washington, DC, 2Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD, 3Superarray Bioscience Corporation, Frederick, MD

Our previous studies revealed that a subset of pre-invasive breast tumors contained focally disrupted myoepithelial (ME) cell layers that were surrounded by leukocyte infiltration. Compared to adjacent cells within the same tumor, but distant from disruptions, tumor cells overlying these focal disruptions showed a significantly higher frequency of tumor invasion-related genetic alterations. As the prostate basal cell layer is structurally and functionally comparable to the ME cell layer, and that similar focal basal cell layer disruptions and leukocyte infiltration are seen in a subset of pre-invasive prostate tumors, this study attempted to assess whether these two organs would share a similar pattern of genetic alterations. Consecutive sections were prepared from primary breast cancer, and from multiple areas of primary breast cancer. Tumor gene expression profiles were analyzed in a subset of prostate tumors with similar pathology. The data was compared with a panel of prostate gene expression profiles. siRNA were chosen for further analysis. The expression of more than 200 genes were affected by siPTHRP. Interestingly, siPTHRP interfered with cdc2 activity by increasing its expression and the expression of its activator cdc25B and cdc25B's regulator -Towe. PTHRP peptide 1-34 and forskolin downregulated cdc2 and cdc25B expression suggesting that PTHRP regulates these genes via PTH1R. In primary breast cancer, higher expression of cdc2 and cdc25B was found at low PTHRP levels. siPTHRP downregulated integrin alpha3 (ITGA6), Kias-1 and plasminogen activator inhibitor-1 (PAI-1). In primary breast cancer, ITGA6 RNA levels were positively correlated with those of PTHRP. siPTHRP inhibited migration and proliferation. These siPTHRP effects could be mimicked by a combination of siRNAs directed against ITGA6, PAI-1 and Kias-1 suggesting that PTHRP may exert its migratory, proliferative and motogenic effects through these genes. These results suggest that, by controlling cdc2 activity, PTHRP may regulate its own nuclear activity. The data also imply, that by interfering with the expression of oncogenes, such as ITGA6 and PAI-1, and tumor suppressor genes, such as KISS-1, PTHRP contributes to the regulation of proliferation and migration of breast cancer cells.

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The Role of the 3UTR in Regulating BMP2 Gene Expression in Lung Cells

S. Jiang, D. T. Fritz, M. B. Rogers; Biochemistry and Molecular Biology, Graduate School of Biomedical Science, UMDNJ-New Jersey Medical School, Newark, NJ

Bone morphogenic protein 2 (BMP2) is an essential growth factor that regulates cell proliferation, apoptosis and differentiation. BMP2 expression is abnormal in various tumors. The BMP2 protein stimulates angiogenesis and promotes lung tumor growth. Mis-regulated BMP2 expression in lung tissue may promote tumor formation. Using northern blots and ELISA, we detected BMP2 mRNA and protein in A549 cells (human lung adenocarcinoma cells) but not in BEAS-2B cells (immortalized human bronchial epithelial cells). This mirrors the finding that BMP2 is over-expressed in lung tumors compared to normal tissues. Mycoplasma infection induced BMP2 in BEAS-2B cells and further induced BMP2 in A549 cells. Additionally, mycoplasma induced a novel transcript in both cells. Chronic mycoplasma infection reduced contact inhibition and increased the proliferation of BEAS-2B cells. Noggin, a BMP2 antagonist, abrogated the increased proliferation. The incidence of mycoplasma infection is elevated in lung cancer patients relative to controls. An influence of mycoplasma on BMP2 expression may influence the onset and progression of lung cancer. We hypothesized that an ultra-conserved sequence in the BMP2 3′ untranslated region contains cis-regulatory elements that regulate BMP2 expression via specific RNA-protein interactions. The ultra-conserved sequence induced gene expression in cells that express BMP2 (A549), but repressed expression in cells that do not express BMP2 (BEAS-2B). RNA oligonucleotide linking experiments and RNase protection assays demonstrated that cell-type specific proteins bound to the BMP2 RNA. Some are Al-rich element binding proteins, well-known factors that regulate RNA stability and translation. RNAs with different alleles of a common human SNP in the ultra-conserved region bound different proteins and decayed differently. We are now using proteomics to identify protein complexes that associate with the ultra-conserved sequence. Understanding the post-transcriptional mechanisms that regulate BMP2 expression is necessary to elucidate why certain cancer cells express BMP2 aberrantly and may lead to novel lung cancer treatments.

Identification of CPEB in Light and dark-adapted Octopus Retinas

S. Kelly, H. Yamamoto, L. J. Robles; Biology, California State University, Dominguez Hills, Carson, CA

CPEBs are trans-acting elements known to bind to the CPE regions located with the 3′ untranslated regions of mRNAs, resulting in the temporal regulation of translational repression and activation of the mRNA in Xenopus (Mendez and Richter, 2001; Stelbins-Boaz et al., 1999; Cao and Richter, 2002). In addition to regulating the mRNA localization of vertebrate and invertebrate maternal mRNAs, CPEB up-regulation is also indicated in translational regulation occurring in hippocampal neurons and mouse synapses, such as the activated synapses in Aplysia (Si et al., 2003). We have previously reported the differential expression and translation of S-crystallin and α-tubulin mRNAs and proteins in light- and dark-adapted octopus retinas. We believe that the increases in S-crystallin and α-tubulin mRNA expression in the light and the subsequent increase of protein synthesis in the dark may result from mRNA masking and unmasking. Here we investigate the presence of a CPEB in light- and dark-adapted octopus retinas. Equal concentrations of total protein isolated from light- and dark-adapted octopus retinas were subjected to SDS-PAGE and western blot analysis, using a CPEB antibody directed against the amino acid residues 545-562 of human CPEB and that also recognizes recombinant mouse CPEB. The detection of bands at ~62 kDa and 28 kDa confirmed the presence of CPEB in light- and dark-adapted retinas, with preliminary analysis revealing higher concentrations of CPEB present in the light-adapted retinas. Increased concentrations of CPEB in light-adapted octopus retinas may be involved in the activation of masked S-crystallin and α-tubulin mRNAs, resulting in increased protein synthesis in dark-adapted octopus retinas. Supported by NIH/NIGMS/MRBS GM08156.

Pathology and Nuclear Abnormalities in Hearts of Transgenic Mice Expressing M371K Lamin A Encoded by an LMNA Mutation Causing Emery-Dreifuss Muscular Dystrophy

Y. Wang,1 A. J. Herron,2 H. J. Worman;1 Departments of Medicine, and Anatomy and Cell Biology, College of Physicians and Surgeons, Columbia University, New York, NY, 2Institute of Comparative Medicine and Department of Pathology, College of Physicians and Surgeons, Columbia University, New York, NY

Mutations in LMNA, which encodes nuclear lamin A and C, cause a broad range of diseases, including autosomal dominant Emery-Dreifuss muscular dystrophy and related disorders with a predominant cardiomyopathy phenotype. While homologous Lmna model “knock-in” and null mice develop cardiac hypertrophy, heterozygous mice do not. Overexpression of lamin A mutants that cause cardiomyopathy in cultured cells induces morphological abnormalities in the nuclear envelope and lamina; however, effects on tissue and organ pathology have not been determined. We used the heart-selective α-myosin heavy chain promoter to drive transgenic mice of human wild type and M371K lamin A, which causes Emery-Dreifuss muscular dystrophy. Mice expressing M371K lamin A were born at approximately 0.07 of the expected frequency and those born typically died at 2 to 7 weeks of age. Histological analysis showed increased eosinophilia and fragmentation of cardiomyobasts, nuclear pyknosis and edema without fibrosis or significant inflammation, indicative of acute or subacute injury. Expressing human wild type lamin A were born at only slightly less than the expected frequency and had normal life spans. Confocal immunofluorescence microscopy demonstrated abnormal nuclear envelopes with intranuclear foci of lamins in cardiac cells expressing M371K lamin A. Electron microscopy revealed extensively convoluted nuclear envelopes, intranuclear inclusions and chromatin clumps in cardiomyocyte nuclei. These results suggest that expression of a lamin A mutant that alters mutations in nuclear morphology can cause tissue and organ damage in mice with a normal complement of wild type lamin.
Role of Transcription Factor NFI in Regulation of Human Osteoblast IGF Binding Protein 5 Gene Expression

L. A. Perez-Casellas, 1,2 G. Gutierrez, 3 D. D. Strong, 1,2 T. A. Linkhart 2,3 Biochemistry and Microbiology, Loma Linda University, Loma Linda, CA; Loma Linda VAMC, Loma Linda, CA

IGF Binding Protein-5 (IGFBP-5) is expressed in many cell types and has complex effects on IGF activities depending on cell type and localization. IGFBP-5 stimulates osteoblast proliferation and differentiation and inhibits osteoblast apoptosis in vitro, while stimulating bone formation in vivo. Glucocorticoids (GCs) inhibit IGFBP-5 expression in normal human osteoblasts (hOBs), suggesting that decreased IGFBP-5 production contributes to GC inhibition of bone formation. We found that GC inhibition of IGFBP-5 gene promoter activity was mediated by a composite response element containing a Nuclear Factor I (NFI) binding site. Four homologous mammalian NFI genes are important in development, but their role in osteoblasts is unknown. To determine whether NFI might regulate IGFBP-5 expression in human osteoblasts, effects on IGFBP-5 transcription were compared by transient transfection with NFI expression vectors and an IGFBP-5 promoter Luciferase reporter in TE-85 osteosarcoma cells. NFI-B and NFI-X stimulated promoter activity 10 fold and 3 fold respectively, but NFI-A and NFI-C were not active. NFI-B increased promoter activity 10 fold in MG-63 osteosarcoma cells, and this induction was inhibited by GC treatment. NFI mRNA levels were determined by quantitative RT-PCR in TE-85, MG-63 and U-2 OS human osteosarcoma cell lines and hOB cultures. NFI-C was most abundant in all cells except MG-63, in which NFI-B was 16 fold higher than NFI-C. NFI-B may thus be the most important activator of IGFBP-5 expression in MG-63 cells. When NFI-B expression was inhibited with specific siRNA oligonucleotides in MG-63 cells, NFI-B mRNA and protein levels were decreased by 55% and 33% respectively, compared to control siRNA. Similar effects of NFI-B knockdown were observed with hOBs. These results suggest an important role for NFI-B in modulating IGFBP-5 expression in human osteoblasts and thus in regulating proliferation, differentiation and apoptosis.

High Throughput/High Content Assay of Lipid Droplets and Perilipin in Human Adipocytes

R. M. Agustin, 1 B. M. Bauer, 2 D. Stricker, 3 N. L. Prigozhina, 1 E. A. Hunter, 1 I. Mikic, 1 S. Callaway, 1 J. H. Price, 2 P. M. McDonough 3 The Burnham Institute for Medical Research, La Jolla, CA, ZenBio, Research Triangle Park, NC, Biological Studies, Vala Sciences Inc, La Jolla, CA

Obesity is an important health problem, particularly, in the US where 30% of adults have a body mass index ≥ 30. Adipogenesis (the formation and differentiation of adipocytes) is therefore of high interest to the biomedical community. We report here the development of automated imaging and analysis techniques to enable high throughput/high content screening of test compounds on lipid droplets and associated proteins. Primary human pre-adipocytes from liposuction procedures, were plated in 96-well plates for 15 days in the presence of rosiglitazone (rosi), a PPARγ agonist which promotes adipogenesis, then fixed and stained for lipids and nuclei. To quantify the number and size of the lipid droplets, an algorithm was developed that identifies circular staining patterns within cell-based images. The number of lipid droplets, as quantified by the algorithms, increased 6-fold, in a dose-dependent manner for cells exposed to rosi (maximal at 1 μM), with a Z’ value of 0.47. Lipid droplet size was also increased by rosi. The dose-response relationship for rosi vs. lipid droplet number was very similar to that obtained for overall triglyceride content, as assayed by traditional techniques. A related algorithm was developed to quantify the association of perilipin with lipid droplets. Perilipin is involved in beta-adrenergic stimulation of lipid droplet metabolism and was visualized in a separate fluorescent channel. For adipocytes cultured in maintenance medium, perilipin was associated with < 5% of the droplets, whereas it increased to 75% for rosi-treated cells. Thus, both lipid droplet formation and the association of proteins with the droplets can be assayed in cultured human adipocytes in a high throughput manner which will enable discovery of drugs that can modify fat deposition and metabolism.
It is known that condensed chromosomes contain higher Ca2+ concentrations while decondensed chromosomes contain lower concentrations, which range 20-32 mM. We have shown recently that the nucleolus of mammalian cells contains numerous vesicular inositol 1,4,5-trisphosphate (IP3)-sensitive Ca2+ stores that consist of the IP3 receptor/Cal2+ channels, Ca2+ storage protein chromatin B, and phospholipids. These Ca2+ store vesicles have an average diameter of ~50 nm. Here we show localization of these IP3-sensitive Ca2+ store vesicles, which were labeled with IP3 receptor and chromatin B, in close association with several round structures that have diameters of ~11 nm. These round structures were labeled with histone H3 and considered to be nucleosomes. Each Ca2+ store vesicle was surrounded by several nucleosomes that appear to be enwrapped by DNA strands. Localization of these vesicles in close proximity with the nucleosomes suggests a close functional coupling between the nucleosomes and the Ca2+ stores, and strongly suggests the Ca2+ stores’ indispensable roles in controlling chromatin structure.

1856

The Role of Smu1 in Splicing Is Conserved through Evolution
K. Sugaya, E. Hongo, Y. Ishihara, H. Tsujii; Radiation Effect Mechanisms Research Group, National Institute of Radiological Sciences, Chiba, Japan

Temperature-sensitive CHO-K1 mutant cell line tsTM18 exhibits chromosomal instability and cell cycle arrest at S and G2 phases with decreased DNA synthesis at the nonpermissive temperature, 33°C. We were severely affected by the depletion of PR-Set7, suggesting that PR-Set7-dependent mono-methylation plays an important role in cell proliferation. We investigated the in vivo function of PR-Set7-dependent methylation by studying the cell cycle in mutant neuroblasts. Neuroblasts are diploid and their cell cycle progression has been well documented. We found that in PR-Set7 mutants, both the mitotic and the S phase indices were reduced, progression through early mitosis was delayed, and cyclin B was downregulated by APC/C proteolysis. In a double mutant of PR-Set7 and mei-41 (the fly ATR ortholog), the abnormalities of cell cycle progression and protein level of cyclin B were restored, indicating that in PR-Set7 the DNA damage checkpoint is activated, and that this activation results in mitotic abnormalities. We also observed abnormal chromosome condensation in PR-Set7 that was enhanced when the checkpoint-arrest was abolished in the double mutant, suggesting that the defect in chromosome condensation is independent of the checkpoint activation. Most histone methyltransferases have generally been considered to be regulators of transcription. However, since the expression of genes involved in the DNA damage checkpoint is normal in PR-Set7, control of gene expression is not involved in activation of the checkpoint. We propose that mono-methylated H4K20 is involved in the maintenance of proper higher order structure of DNA and is consequently essential for chromosome condensation. A function in DNA damage response has been shown for methylated histone H4K20 in S. pombe. Interestingly, the function of mono-methylation of H4K20 in Drosophila is clearly different. Our results suggest that mono-methylation of H4K20 has a more global effect on chromatin structure than described so far.

1857

Aberrant Mono-methylation of Histone H4 Lysine 20 Activates the DNA Damage Checkpoint in Drosophila
A. Sakaguchi, R. Stewart; Molecular Biology and Biochemistry, Waksman Institute, Rutgers University, Piscataway, NJ

PR-Set7 is a histone methyltransferase that specifically mono-methylates histone H4 lysine 20 (H4K20). In Drosophila larvae, tissues with higher rates of cell divisions, such as imaginal discs, are severely affected by the depletion of PR-Set7, suggesting that PR-Set7-dependent mono-methylation plays an important role in cell proliferation. We investigated the in vivo function of PR-Set7-dependent methylation by studying the cell cycle in mutant neuroblasts. Neuroblasts are diploid and their cell cycle progression has been well documented. We found that in PR-Set7 mutants, both the mitotic and the S phase indices were reduced, progression through early mitosis was delayed, and cyclin B was downregulated by APC/C proteolysis. In a double mutant of PR-Set7 and mei-41 (the fly ATR ortholog), the abnormalities of cell cycle progression and protein level of cyclin B were restored, indicating that in PR-Set7 the DNA damage checkpoint is activated, and that this activation results in mitotic abnormalities. We also observed abnormal chromosome condensation in PR-Set7 that was enhanced when the checkpoint-arrest was abolished in the double mutant, suggesting that the defect in chromosome condensation is independent of the checkpoint activation. Most histone methyltransferases have generally been considered to be regulators of transcription. However, since the expression of genes involved in the DNA damage checkpoint is normal in PR-Set7, control of gene expression is not involved in activation of the checkpoint. We propose that mono-methylated H4K20 is involved in the maintenance of proper higher order structure of DNA and is consequently essential for chromosome condensation. A function in DNA damage response has been shown for methylated histone H4K20 in S. pombe. Interestingly, the function of mono-methylation of H4K20 in Drosophila is clearly different. Our results suggest that mono-methylation of H4K20 has a more global effect on chromatin structure than described so far.

1858

The Effect of Overexpression of H1 Variants- H1c and H1e on Global Gene Expression
S. Bhan, W. May, S. Warren, D. B. Sittman; 1Biochemistry, University of Mississippi Medical Center, Jackson, MS, 2Preventive Medicine, University of Mississippi Medical Center, Jackson, MS

Histone H1 is an essential component of chromatin that binds to the linker DNA between nucleosomes and facilitates the formation of a compact chromatin fiber. Multiple nonallelic variants of H1 have been preserved through evolution. Extensive literature suggests that these variants are heterogeneous and may play distinct roles in the organization of chromatin. However, it is not yet clear what unique functional roles these variants serve. Previously, our lab has attempted to identify functional differences between two H1 variants- H1c and H1e, by developing a system for inducible overexpression of these variants in stably transfected 3T3 fibroblasts. Overproduction of individual variants perturbs the normal H1 stoichiometry and can, thus, help to understand their functional significance. In these studies, we found that these variants have differential effects on gene expression. We have studied the effects of these two variants on global gene expression using the same system of inducible overexpression. The effects of these H1 variants on gene expression were studied at two early time points in G1 phase of the cell cycle- 3h and 6h. Expression of numerous genes was found to be affected by either one of the variants of H1. This is suggestive of unique or specific roles of H1 variants. However, there were several genes that were affected by both the type of variants. Interestingly, the expression of these genes was affected similarly by both the variants. This suggests that besides having unique or specific functions, the H1 variants may have common roles in the organization of chromatin. Also, as expected, more number of genes was affected by the variants at the later time point of 6h. Identification of genes affected by H1s can help decipher the functional differences between the H1 variants as well as specific roles played by the class of H1 proteins.

1859

Regulation of Chromatin Structure and Gene Expression by JIL-1 Kinase-mediated Histone H3S10 Phosphorylation in Drosophila
H. Deng, X. Bao, W. Cai, M. J. Blacketer, J. Girton, J. Johansen, K. M. Johansen; Biochemistry, Biophysics & Molecular Biology, Iowa State University, Ames, IA

Epigenetic regulation of gene expression and chromatin structure is an important aspect of both disease and normal development. In order to gain a molecular understanding of these processes we have identified a histone H3S10 kinase, JIL-1, that localizes specifically to gene-active interband regions of polytene chromosomes. We have recently demonstrated that a major function of the JIL-1 histone H3S10 kinase is to prevent H3K9-dimethylation and heterochromatization from occurring on inappropriate euchromatic targets (Zhang et al., Development 133:229, 2006). This is underscored by the finding that JIL-1 loss-of-function lethality is restored by a reduction in histone dimethyl-transfase activity. Furthermore, analysis of hypomorphic JIL-1 alleles shows that loss of JIL-1 activity results in major alterations in chromatin structure and gene expression. These results suggest a model where JIL-1 mediated phosphorylation of histone H3S10 at interphase functions to antagonize heterochromatization by regulating a dynamic balance between repressing and activating factors of gene expression. To directly test the hypothesis that changes in chromatin structure can be caused by the phosphorylation state of histone H3S10 we have used a lac-tethering system to target JIL-1 ectopically to different chromatin environments. The results show that when lac-JIL-1 is tethered to a banded polytene chromosome region the chromatin attains an interband morphology with the band appearing "split in two" and that this "split" is directly correlated with ectopic upregulation of histone H3S10 phosphorylation. Furthermore, we show that neither viability, heterochromatic spreading, nor polytene chromosome morphology is rescued by introduction of a full-length "kinase dead" construct into JIL-1 null animals. Thus, taken together these experiments provide strong support for the hypothesis that chromatin structure and packaging in Drosophila are regulated by JIL-1 kinase-mediated histone H3S10 phosphorylation. (Supported by NIH grant GM62916).

1860

Relationships of Drosophila RECQ5/QE to Cell Cycle Progression and DNA Damage
K. Kowalski, M. Nakayama, K. Nakano, S. Murayama, F. Ito, K. Kato 1, S. Miyazaki 1, S. Kato; Developmental Genetics Group, The National Institute for Basic Biology, Hitachinaka, Japan

Regulation of cell cycle progression is a fundamental process in all eukaryotic organisms. The mammalian cell cycle is regulated by a complex network of proteins that are involved in cell cycle entry, cell proliferation, and maintenance of chromosomal stability. Members of the RecQ family of proteins are regulators of this network and are involved in response to DNA damages. For example, BLM protein is expressed in late S and G2. DNA-damaging agents induce BLM by a p53- and ATM- independent mechanism (Bischof et al (2001) J Cell Biol 153: 367-380). However, little is known about RecQ5, another member of RecQ family. How RecQ5 is involved in cell cycle regulation and in response to DNA damage is still unclear. To investigate the cell cycle regulation of RecQ5, we analyzed the RECQ5/QE protein in Drosophila S2 cells. The amount of RECQ5/QE protein was almost constant during cell and can be induced by irradiation with UV or MMS. The amount of RECQ5/QE protein in S2 cells. These results suggest that RECQ5/QE protein is regulated in response to DNA damages. To elucidate the biological functions of RECQ5/QE in vivo, we have generated transgenic flies that overexpress RECQ5/QE in the developing eye primordia. These flies showed mild roughening of the normally smooth ommatidial lattice. The phenotype of RECQ5/QE overexpression was enhanced by DNA-damaging agents and mei-41 mutation. Overexpression of RECQ5/QE...
perturbed the cell cycle progression in response to DNA damages in the eye imaginal discs. These results suggest that RECQ5/QE interacts with cell cycle progression in response to DNA damages.

1861

Repetitive Element 1360 as a Target for Heterochromatin Formation in Drosophila melanogaster

A. Figueroa-Clarevega, K. Haynes, K. Huisinga, S. Elgin; Department of Biology, Washington University in St. Louis, St. Louis, MO

Drosophila melanoguster has served as an excellent model for the study of chromatin packaging, which impact levels of gene expression. Proximity to heterochromatin, the form of packaging associated with gene silencing, has the ability to induce PEV (Position Effect Variegation), in which a reporter gene is silenced in some of the cells where it is normally active. Earlier work has suggested that the repetitive element 1360, which is enriched in heterochromatic domains, can serve as an initiation site for heterochromatin formation in the fourth chromosome (Sun et al. 2004. Mol. Cell Bio. 24: 8210). To test this idea, we generated several fly lines carrying a transposable P-element containing one or four copies of 1360 upstream of an hsp-70 white reporter. Without the 1360 element upstream of hsp-70 white, only 1% of lines recovered from previous P-element mobilizations screens are variegating. We have observed that with one copy of 1360, 3% of the lines are variegating; with four copies of 1360, 11% of the recovered lines are variegating. These results suggest that increasing copies of 1360 increase the likelihood of initiating heterochromatin formation. Pericentric, telomeric and chromosome four heterochromatin are known to induce PEV. We are currently mapping the distribution of the insertion sites to determine whether increased 1360 copy number allows ectopic heterochromatin formation outside of these regions. Supported by a Wu/Hmmi Fellowship to A.F.C. and NIH grants GM68388 and GM73190 to S.C.

1862

Induction of Protracted Genomic Instability in Fission Yeast after Brief Exposure to Cr (VI) Ions

I. Papageorgiou, 1 C. J. Norbury, 2 C. P. Case 2; Orthopaedic Surgery, University of Bristol, BIRC Lab, Bristol, United Kingdom, 3Sir William Dunn School of Pathology, Department of Cell Biology & Pathology, University of Oxford, United Kingdom

Genomic instability occurs in a variety of cells after exposure to low doses of radiation and metal ions including chromium (VI). We have recently shown that many but not all aspects of genomic instability may be prevented through ectopic expression of the catalytic component of telomerase (Glaviano et al., 2005). In this study we investigated whether short-term exposure to Cr (VI) ions could lead to a genomic instability as assayed by minichromosome loss in the fission yeast Schizosaccharomyces pombe. Four hours of exposure to Cr (VI) ions caused a dose dependent increase in minichromosome loss, scored by the appearance of red (ade-) sectors in otherwise white (ade+) colonies. Subsequent measurement of further minichromosome loss events in cells derived from the ade- sectors of such colonies suggested that a long-lived state of chromosomal instability had been induced by the prior exposure to Cr(VI). Control experiments ruled out the possibility that the appearance of ade- progeny was due to events other than minichromosome loss, and furthermore showed no evidence for bystander effects such as those previously reported in mammalian cells. We conclude that fission yeast provides a convenient model system in which to investigate the genetic determinants of long-term chromosomal instability induced by metal ions.

1863

The Chromodomain Protein, Chromator, Interacts with the JIL-1 Kinase and Regulates the Structure of Drosophila Polytenic Chromosomes

Y. Ding, U. Rath, H. Deng, H. Qi, X. Bao, W. Zhang, J. Girton, J. Johansen, K. M. Johansen; Biochemistry, Biophysics & Molecular Biology, Iowa State University, Ames, IA

The chromodomain protein, Chromator, was originally identified in a yeast two-hybrid screen as an interaction partner of the putative spindle matrix component, Skeletor, and localizes to the spindle and the centromeres during mitosis (Rath et al., J. Cell. Biochem. 93:1033, 2004). However, Chromator's localization to polytenic interbands suggests it also may play a functional role in maintaining chromatin structure during interphase. In this study we have generated two new hypomorphic Chromator alleles by EMS mutagenesis and analyzed the consequences of reduced Chromator protein function on polytenye chromosome structure. We show that in chrom/chrom mutants the polytenye chromosome arms were coiled and compacted with a disruption and misalignment of band and interband regions and with numerous ectopic contacts connecting non-homologous regions. Furthermore, we demonstrate that Chromator co-localizes with the JIL-1 kinase and that the two proteins interact within the same protein complex. The JIL-1 kinase localizes specifically to euchromatin interband regions of polytenye chromosomes and is the predominant kinase regulating histone H3S10 phosphorylation at interphase. That both proteins are necessary and may function together is supported by the finding that a concomitant reduction in JIL-1 and Chromator function synergistically reduces viability during development. Overlay assays and deletion construct analysis suggested that the interaction between JIL-1 and Chromator is direct and that it is mediated by sequences in the ade- sectors of such colonies suggested that a long-lived state of chromosomal instability had been induced by the prior exposure to Cr(VI). Taken together these findings indicate that Chromator and JIL-1 interact in an interband-specific complex that functions to establish or maintain polytenye chromosome structure in Drosophila. (Supported by NSF grant MCB0445182 and NIH grant GM62916).

1864

Molecular Characterization of a Novel, CCCH Zinc Finger Protein, UKp83

M. Hará, 1 S. Miyakura, 1 S. Watabe 2; 1Department of Cellular and Environmental Biology, Tokyo Medical and Dental University Graduate School, Tokyo, Japan, 2Department of Laboratory Sciences, Faculty of Health Sciences, Yamaguchi University School of Medicine, Ube, Japan

UKp83 was first isolated as a cDNA of candidate gene of chromatin associated protein whose expression in gene screening was detected by antisera against isolated chromatin from sonicated interphase nuclei obtained from rat liver cells. To investigate the function and relation of this protein to chromatin, we have characterized some properties. The deduced primary structure of UKp83 indicates that it has a nuclear localization signal and five CCCH zinc finger motifs, and is 83k of molecular weight. Cellular immunofluorescence demonstrated that the UKp83 was localized within euchromatic regions in the interphase nuclei and similar to SC35 as a known marker for nuclear speckles, which represent subnuclear compartments enriched in splicing factors. However, when the nuclear envelope was disintegrated at the prometaphase of mitosis, UKp83 located on the chromosomes, though a part of the protein was dispersed into the cytoplasm. An in vitro RNA/DNA binding assay indicated that UKp83 was bound to ssDNA firstly, and less to both RNA homopolymers and dsDNA. GFP-UKp83, which produces a GFP and UKp83 fusion protein in cultured cells by transfection of the expression vector, was not seen to affect any cell function, as the same as GFP-Luc (luciferase). On the other hand, GFP-AS, which produces antisense-RNA of UKp83, warped nuclear and cellular shape of the expressed cells. The ways of reduction of both GFP-Luc and GFP-UKp83 producing cells were almost the same after transfection. However, the ratio of those cells was sharply down in GFP-AS comparing with both GFP-Luc and GFP-UKp83, and it was indicated that expression of antisense-RNA for UKp83 could induce cells to be in death. The results obtained from these experiments suggest that UKp83 seems to play important roles in a construction of the chromatin higher-order structure and in a cellular function and to be essential for cell viability.

1865

Functional Dissection of the Sc35/SMC2 Protein in Chicken Di40 Cells

F. Lai, D. Hudson, W. Earnshaw; Institute of Cell Biology, Wellcome Trust Centre for Cell Biology, Edinburgh, United Kingdom

The mechanism by which genomic DNA is compacted nearly 10,000-fold to form the characteristic mitotic chromosome structure remains poorly understood. One factor thought to have a key role in this process is condensin, a pentameric complex of non-histone proteins composed of the SMC2 and SMC4 ATPases plus three auxiliary subunits (CapG/G2, CapD2/D3 and CapH/H2). Our lab carried out the first conditional knockout of one essential condensin subunit (Sc35/SMC2) in vertebrate cells. By quantitative Real-Time RT-PCR and immunofluorescence microscopy, we showed the down regulation of Sc35/SMC2 gene expression after addition of doxycycline. In cells lacking SMC2 protein, chromosomes can reach near normal levels of condensation but they are structurally abnormal and lack structural integrity. Furthermore, when isolated chromosomes are fractionated, the chromosome scaffold fraction appears to be largely missing. To investigate the role of ATPase domains function, we generated ATPases domains mutants deficient in ATP binding or hydrolysis. The analysis of these lines will allow us to understand how the ATPase function of SMC2 contributes to the assembly of the condensin complex and to the structure of mitotic chromosomes.

1866

Single-stranded Oligonucleotide-mediated Gene Repair in Mammalian Cells Has a Mechanism Distinct from Homologous Recombination Repair

Z. Wang, 1 Z. Zhou, 1 D. Liu, 1 J. Huang 1; 1The University of Hong Kong, Hong Kong, 2Peking Union Medical College, Beijing, China
Single-stranded DNA oligonucleotide (SSO)-mediated gene repair has great potentials for gene therapy and functional genomic studies. However, its underlying mechanism remains unclear. Previous studies from other groups have suggested that DNA damage response via the ATM/ATR pathway may be involved in this process. In this study we measured the effect of two ATM/ATR inhibitors caffeine and pentoxifylline on the correction efficiency in SSO-mediated gene repair. We also checked their effect on double-stranded break (DSB)-induced homologous recombination repair (HRR) as a control, which is well known to be dependent on the ATM/ATR pathway. We found these inhibitors could completely inhibit DSB-induced HRR, but could only partially inhibit SSO-mediated process, indicating SSO-mediated gene repair is not dependent on the ATM/ATR pathway. Furthermore, we found that thymidine treatment promotes SSO-mediated gene repair, but inhibits DSB-induced HRR. Collectively, our results demonstrate that SSO-mediated and DSB-induced gene repairs have distinct mechanisms.

1867
An EGFP-based Transgenic Mouse for Studying Single-Stranded Oligonucleotide-mediated Targeted Gene Repair
L. Lu, 1 K. S. E. Cheah, 1 D. Liu, 1 J. Huang; 1Biochemistry, The University of Hong Kong, Hong Kong, China, 2Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (CAMS) & Peking Union Medical College (PUMC), Beijing, China
Targeted gene repair using single-stranded oligonucleotide is a promising alternative to current gene therapy strategies. However, the detailed mechanism of this method remains elusive, and the repair efficiency has to be raised before this technique could be used clinically. Currently, almost all studies of this technique are carried out in immortalized cell lines, which may not be true reflections of the cells in normal conditions. In our study, an EGFP-based transgenic mouse was made to study single-stranded oligonucleotide-mediated targeted gene repair in normal cells. The mutated EGFP gene, driven by beta-actin promoter, contains an A to T mutation at the start codon. The reporter was integrated in mouse chromosome eight through gene targeting and the expression of the reporter was ubiquitous. We have tested the technique in embryonic stem cells and primarily cultured embryonic fibroblasts, however the repair events were rarely detected. Besides being a convenient tool for studying gene repair in various primarily cultured cells, this reporter mouse line would also be useful for studying gene repair in vivo.

1868
Multiple Condensin Protein Complexes Build, Express, and Segregate the Genome: Evidence for Condensin I in the Worm
K. Sparh, 1 C. Sparks, 1 M. Child, 1 J. Carey, 1 T. Tabachn, 1 H. Liu, 1 M. McLeod, 1 J. R. Yates, 2 B. J. Meyer, 1 K. A. Hagstrom; 1Program in Molecular Medicine & Program in Cell Dynamics, University of Massachusetts Medical School, Worcester, MA, 2Department of Cell Biology, The Scripps Research Institute, La Jolla, CA, 1HMMI and Department of Molecular and Cell Biology, UC Berkeley, Berkeley, CA
Building proper chromosome structure is essential for genome maintenance, impacting processes from gene regulation to chromosome segregation. We are studying how conserved protein complexes called condensins govern chromosome organization. A canonical condensin complex contains five proteins: a heterodimeric core of structural maintenance of chromosomes (SMC) proteins, and three chromosome associated polypeptide (CAP) subunits. Previous work showed that C. elegans has two distinct condensin complexes, one with a conserved function in chromosome segregation during mitosis (condensin II), and another specialized for X chromosome gene regulation during dosage compensation (condensin I). These complexes were shown to share one core SMC subunit, while other subunits were distinct or as yet undefined. We report here the identification of the missing CAP subunits from each condensin complex, and present evidence that the core SMC subunits of condensin II may associate with the CAP subunits of condensin I to form yet a third condensin complex (condensin I). Evidence that certain subunits of the two known condensin complexes “mix-and-match” to form a third complex includes the finding that these subunits co-immunoprecipitate, co-localize, and perform common meiotic and mitotic functions. We propose that this third complex is analogous in structure and function to the human mitotic condensin I, resolving the previous paradox that C. elegans is the only higher eukaryote appearing to lack a mitotic condensin I. These findings pave the way to study unique or redundant activities among condensin complexes, and the mechanisms that regulate their differential localization, function, and cell cycle timing.

1869
The C-terminus of Stn1, A. cerevisiae Telomere Protein, Is Required for Telomere Length Regulation and Capping
R. Petreaca, H. Chiu, C. Nugent; University of California, Riverside, CA
The function of telomeres is twofold: cap the chromosome ends to protect against fusions and illegitimate recombination, and facilitate complete replication of linear eukaryotic chromosomes. In the budding yeast S. cerevisiae, STN1 is an essential gene with a critical role in these processes. The Stn1 protein is known to interact with three additional essential proteins: Ten1, a protein critical for negative regulation of telomere length, Cdc13, a single-strand telomere binding protein with roles in activating and inhibiting telomere elongation, and Pol12, the regulatory subunit of primase. To better understand the role of Stn1 in telomere protection and replication, we analyzed the regions of STN1 that are essential for viability. We found that strains with deletions covering the carboxyl terminal half of Stn1, stn1-t186 and stn1-t281, which do not encode amino acids from 187-494 or 282-494 respectively, were viable but have greatly elongated telomeres. Neither of these alleles retain an interaction with Cdc13p. Surprisingly, smaller deletions of the carboxyl terminus also destabilize the interaction with Cdc13p but such alleles are not viable. Telomere elongation in stn1-t186 and stn1-t281 is dependent on telomerase; moreover, viability of the strains requires elongated telomeres. Examination of telomere structure showed the presence of abnormally high levels of single-stranded TG3s. In addition, these strains displayed large chromosome end-to-end fusions. Thus, loss of the STN1 carboxyl terminus leads to defective chromosome end protection that allows fusion of elongated telomeres. These strains proliferate with reasonably high plating efficiency, and few cells activate the DNA damage checkpoint, although the checkpoint remains responsive to other types of damage, indicating that this telomere damage is not recognized as DNA damage. We propose that the elongated telomeres allow the formation of a terminal structure that masks the single-stranded telomeric sequences from the checkpoint surveillance machinery.

1870
RNA-mediated Nucleoid Architecture in Bacteria
R. L. Ohnwa, 1 K. Monkawa, 2 J. Kim, 1 T. Ohtia, 1 C. Wada, 1 K. Takeyasu; 1Graduate School of Biostudies, Kyoto University, Kyoto, Japan, 2Graduate School of Comprehensive Human Medicine, Philadelphia, PA
We have previously reported that, when Escherichia coli, Staphylococcus aureus and Clostridium perfringens was lysed under physiological salt condition, the fibers with the width of ~30 nm and ~80 nm, but not DNA, could be observed by atomic force microscopy (AFM). In E. coli, the loop structure composed of 80 nm fiber also appeared under the same lysis condition. These results suggest that the step-wise folding of DNA, in which 30 nm fiber built from naked DNA turns into 80nm fiber, is present in each loop from the central core. In the present study, we show that a nascent single strand RNA is involved in maintaining the step-wise folding of the nucleoid. In Escherichia coli, RNase A, but not RNase III or RNase II, broke down the thicker fibers into thinner fibers whose dimensions are ~10 nm. The nucleoid in Staphylococcus aureus also turned into 10-nm fiber from the thicker fibers with the treatment of RNase A. Rifampicin or chloramphenicol treatment to E. coli cells changed the majority of the nucleoid fibers into 10-nm one. These results indicate that RNA resistant 10-nm fiber is present in bacteria, and that the thicker fibers than 30 nm are built up from the 10-nm fiber with help of single strand RNA coupled with transcription and translation.

1871
SNP-based Oligonucleotide Microarrays Detect Loss of Heterozyosity (LOH) in a Child with Multiple Congenital Anomalies
T. G. Charles, 1 E. Geiger, 1 M. Vaddi, 1 T. Shaikh; 1Biology, Morgan State University, Baltimore, MD, 2Departments of Pediatrics and Genetics, University of Pennsylvania School of Medicine, Philadelphia, PA
Chromosomal rearrangements like microdeletions and microduplications have been shown to be the underlying cause of a significant proportion of human genetic diseases. We hypothesized that many children with various congenital anomalies (MCA) have submicroscopic microdeletions or microduplications. We use SNP-based oligonucleotide microarrays to detect copy number changes, which result from microdeletions or microduplications in patients with MCA. Here, we report a child with MCA of unknown etiology who was tested with the microarray. Her phenotype includes cranio-facial abnormalities, hypotonia, and global development delay. We did not detect any copy number changes in her genome. Surprisingly, we detected a large region (~20 Mb) on chromosome 16 with copy number neutral homozygosity. We hypothesize that this apparent loss of heterozygosity (LOH) is the result of the uniparental inheritance of this region of chromosome 16 (16q21-q22.1). We are currently testing the patient and her mother with microsatellite markers to further validate the microarray results. If we can confirm the LOH, this will strongly suggest that the phenotype seen in our patient is the result of uniparental disomy (UPD). UPD can cause disease either by unmasking a recessive mutation or due to imprinting of the region involved. Thus, our approach allows us to detect both copy number variation as well as LOH, that may be the underlying cause of genetic diseases.
Nuclear Translocation Levels Regulate Cell Cycle Dynamics of Condensin Distribution in the Yeast Genome

B. Wang,1 P. Butylin,1 M. Basral,1 M. Lichten,1 A. Strunnikov;1 1Laboratory of Gene Regulation and Development, NIH/NICHD, Washington, DC, 2Genetic Branch, Center for Cancer Research, NIH/NCI, Washington, DC, 3Laboratory of Biochemistry, Center for Cancer Research, NIH/NCI, Washington, DC

Mitotic chromosome condensation is established and maintained by condensin complex. However, the molecular mechanisms regulating condensin binding to the specific genomic sites remain largely unknown. In budding yeast, the nuclear organizer (NOR or rDNA) has been identified as the major target site for condensin binding in vivo. To understand the molecular pathways that determine condensin binding to nuclear chromatin, we analyzed the properties of condensin-bind sites within rDNA repeat during the cell cycle. The time course experiments revealed that condensin is enriched in rDNA repeat in a cell cycle dependent manner, which is negatively correlated with 35S rDNA transcription levels. Parallel analysis of RNA polymerase I (Pol I) transcription levels and condensin binding in wild type and fis1Δ mutants with full or minimal copies of rDNA further demonstrated that the bulk of mitotic condensin binding to 35S rDNA is drastically reduced or eliminated when Pol I transcription is maximized. Conversely, when rDNA transcription is repressed either by rapamycin treatment or by promoter shut-off, condensin binding to rDNA is increased. Thus, constitutive and/or periodic repression of Pol I transcription could be a crucial factor in determining the segregation proficiency of rDNA. In cells with an episonal NOR, where rDNA is actively transcribed, the whole-genome analysis showed that condensin released from nucleolus re-distributed to the secondary sites in the genome, with particular enrichment at subtelomeric regions, suggesting a genome-wide regulation of condensin control by rDNA transcription.

Chromatin (VD) Induces a Telomerase-dependent Bystander Effect to Cause Genomic Instability in Human Cells

N. M. Cogan,1 F. Lyng,1 C. Mothersill,1 C. P. Case;2 Clinical Science at North Bristol, University of Bristol, Bristol, United Kingdom, 2Chemical and Pharmaceutical Science, Dublin Institute of Technology, Dublin, Ireland, 3Medical Physics and Research, Juravinski Cancer Centre, Hamilton, ON, Canada

There is current interest in the role of the bystander effect in chromosomal and genomic instability. The bystander effect has so far been shown to occur following exposure to ionizing radiation. Cells directly exposed to radiation induce genomic instability in neighboring ‘bystander’ cells either through secretion of factors into the growth medium or through transfer of a signal via gap junctions. A previous study (Glaviano et al 2006) demonstrated that short term (24 hour) exposures to chromium and vanadium lead to an increase in genomic instability up to 30 days after exposure. The ectopic expression of hTERT protects against this instability. In the current study, we have addressed whether, like radiation, metals are able to induce a bystander effect that would cause chromosomal instability. To do this we treated BJ human fibroblast cells for 24 hours with 0.4 μM potassium dichromate (K₂Cr₂O₇). The cells were washed 5 times to remove any trace of metal ions and incubated in fresh growth medium for 1 hour. This medium was filtered and transferred to non-metal-exposed BJ cells for 24 hours. Our results show that there was an increase in breaks, nucleolar, nucleolccc, and γ-HAX foci in cells subjected to a medium transfer bystander treatment. We also demonstrated that ectopic expression of hTERT protects cells from the bystander response. In conclusion, we demonstrate for the first time, a metal-induced bystander effect that causes genomic instability and is reduced in the presence of telomerase. Glaviano et al (2006) Oncogene 25 3424-3435

Novel Insights into the Location of the P62 Complex and Its Role in Nuclear Import

K. Schwarz-Herion,1 T. Guan,2 B. Maco,1 U. Sauder,1 L. Gerace,2 B. Fahrenkrog;1 1Structural Biology, MHH Biozentrum Basel, Basel, Switzerland, 2Department of Cell Biology and Department of Molecular Biology, The Scripps Research Institute, La Jolla, CA

The nuclear pore complex (NPC) perforates the nuclear envelope (NE) of the eukaryotic cell and regulates the transport of proteins and RNA across the NE. One major component of the NPC is the p62 complex, which is located at the central pore of the NPC and has previously been described to be involved in protein import. The proteins of the p62 complex contain flexible domains called FG-repeats, which function as docking sites for transport receptors, and coiled-coil domains, which anchor the p62 complex to the NPC. Using immuno-electron microscopy with domain-specific antibodies and epitope-tagged proteins we could show how the coiled-coil domains of the p62 complex are anchored at the cytoplasmic side of the pore, whereas the flexible FG-repeat domains can traverse to the nuclear face of the NPC. Furthermore we studied the effect of distinct antibodies against the different compounds of the p62 complex on the protein import. For this purpose we used isolated nuclei of Xenopus oocytes to study the import of gold-labeled nucleoplasmin into the nuclei on electron microscopy level and analyzed the import of GFP-labeled nucleoplasmin into the nuclei of HeLa cells on fluorescence level. We could show that import of cargo was inhibited by certain combinations of antibodies against the p62 complex, whereas individual antibodies against single compounds of the p62 complex had minor effects on the import.

Topology of Yeast NDC1: Predictions for the Human NDC1 Homologue

C. K. Lau, V. A. Delmar, D. J. Forbes; Biology, University of California, San Diego, La Jolla, CA

The nuclear pore complex is the predominant structure in the nuclear envelope that spans the double nuclear membranes of all eukaryotes. Yeasts have an additional organelle that is embedded in the nuclear envelope: the spindle pole body, which functions as the microtubule organizing center. The only protein known to localize to and be important for the assembly of both these yeast structures is the integral membrane protein, Ndc1p. Here, we identify and analyze NDC1 homologues that are conserved throughout evolution. We show that the overall topology of these homologues is conserved. Each contains six transmembrane segments in its N-terminal half and has a large soluble C-terminal half of ~300 amino acids. Charge distribution analysis infers that the N- and C-termini are exposed to the cytoplasm. Limited proteolysis of yeast Ndc1p in cellular membranes confirms the orientation of its C-terminus. Although the functional domains of vertebrate NDC1 are not yet known, the human homolog contains three FG repeats in the C-terminus, a feature of many nuclear pore proteins. Moreover, a small region containing mutations which affect assembly of the nuclear pore in yeast is highly conserved throughout evolution. Hence, the NDC1 homologues are expected to function at nuclear pores.

A Novel Nuclear Pore/Kinetochore Protein Required for Correct Nuclear Pore Assembly

B. A. Rasala, A. Orjalo, Z. Shen, S. Briggs, D. Forbes; Biology, UC San Diego, La Jolla, CA

Nuclear pores span the nuclear envelope and act as gated aqueous channels to regulate the transport of macromolecules between nucleus and cytoplasm -- from individual proteins and RNA to entire viral genomes. By far the largest subunit of the nuclear pore is the Nup107-160 complex, which itself consists of at least nine proteins. As the major subunit, this complex is critical for nuclear pore assembly. At mitosis, the Nup107-160 complex in part localizes to the kinetochores, suggesting that it may also function in the process of chromosome segregation. To investigate the dual roles of the Nup107-160 complex at the pore and during mitosis, we set out to identify binding partners for the complex using both interphase and mitotic Xenopus laevis egg extracts and mass spectrometry. Only a small number proteins were identified to specifically co-immunoprecipitate with the Nup107-160 complex, most of which bind in both interphase and mitotic extracts, including a putative transcription factor. Like the Nup107-160 complex, this large protein localizes to nuclear pores in interphase and kinetochores in mitosis in mammalian cells. Importantly, its depletion by RNA interference leads to a severe nuclear pore disruption in the nuclear envelope and an increase in cytokinesis defects. We conclude that these identified and unexpected members of the nuclear pore and kinetochore that functions in both nuclear pore assembly and faithful cell division.

Different Lamin A/C Mutations Are Associated with Distinct Defects in Nuclear Mechanics

J. Lammerding,1 J. Y. Ji, L. G. Fong,1 A. Muchir,1 H. J. Worman,2 C. L. Stewart,2 G. S. Young,2 R. T. Lee;1 1Medicine, Brigham & Women's Hospital, Harvard Medical School, Cambridge, MA, 2Medicine, University of California, Los Angeles, Los Angeles, CA, 3Medicine and Anatomy and Cell Biology, Columbia University, New York, NY, 4Cancer and Developmental Biology Lab, National Cancer Institute, Frederick, MD

Mutations in LMNA (which encodes the nuclear envelope proteins lamin A and C) cause a variety of human diseases, including Emery-Dreifuss muscular dystrophy, familial partial lipodystrophy, and Hutchinson-Gilford progeria syndrome. At the cellular level, many of these mutations lead to characteristic defects in nuclear shape and morphology, but it remains unclear if these changes affect nuclear function and directly contribute to the disease mechanism. Here, we investigated the effect of specific lamin mutations associated with distinct laminopathies on nuclear mechanics by measuring nuclear deformations in fibroblasts subjected to biaxial strain. Normalized nuclear strain, defined as the ratio of nuclear to applied membrane strain, was assessed in fibroblasts from human subjects with Emery-Dreifuss muscular dystrophy, familial partial lipodystrophy, and Hutchinson-Gilford progeria syndrome patients, as well as healthy control subjects and from mouse models of these diseases. We found that mutations associated with muscular dystrophies resulted in decreased nuclear stiffness, potentially rendering cells more susceptible to physical damage in mechanically stressed tissues such as skeletal and cardiac muscle. In contrast, cells from familial partial lipodystrophy patients had normal nuclear
stiffness. Interestingly, skin fibroblasts from Hutchinson-Gilford progeria syndrome patients initially had normal nuclear stiffness, but developed increasingly stiffer nuclei with increasing passage number as the mutant prelamin A (progerin) accumulated at the nuclear envelope. These results indicate that not all nuclear shape abnormalities associated with LMNA mutations are “created equal;” instead, they can arise either from a functional loss of lamins A/C (as in many of the muscular dystrophies) or from the accumulation of a mutant protein at the nuclear envelope (as seen in progeria), with each scenario resulting in distinct changes in nuclear shape and mechanics. These alterations in nuclear mechanical properties could adversely affect cellular mechanotransduction signaling and cell viability under strain and contribute to the specific pathophysiology of these diseases.

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Post-translational Modification and Nuclear Localization of Lamin A/C in Mouse Embryonic Fibroblasts (MEFs)

B. Liu, Z. Zhe,; Department of Biochemistry, The University of Hong Kong, Hong Kong

Lamin A/C is one of the major components of nuclear envelope and nuclear matrix. Lamin A is firstly synthesized as pre-form with 18 extra amino acids, followed by farnesylation of the cysteine (-CSIM) by FT, proteolysis to release -SIM (-AAX) by Zmpste24/Rce1, methylation of the cysteine by ICMT, and lastly proteolysis to remove 15 amino acids by Zmpste24. Recently, LMNA mutations were identified in Hutchinson-Gilford Progeria Syndrome (HGPS) patients. Mouse models deficient for Zmpste24 also showed similar early ageing phenotypes as HGPS. In both human and mouse, the unprocessed prelamin A retains farnesy group in its C-terminus, which may interfere the integrity of nuclear lamina. Illustration of the complex post-translational proteolysis of prelamin A protein may help to better understand the possible molecular mechanism of laminopathy-based premature ageing. In our current study, we constructed pEGFP-prelamin A-3 and pEGFP-prelamin A-18, and transfected them into Zmpste24+/− or wild-type MEFs. Fluorescent microscopic and immunoblotting study suggested that (1) farnesyl or methyl group is not required for the nuclear envelope targeting of prelamin A; (2) Zmpste24 involves in the second cleavage to release 15 amino acids; (3) methylated, but not farnesylated cysteine is required for the second cut of Zmpste24; (4) farnesylcysteine is required for first proteolysis by Zmpste24/Rce1. Our results illustrate the biological relevance of different modification steps during the maturation of Lamin A, this may help to better understand the underlying mechanism of premature ageing caused by abnormal Lamin A.

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Regulation of the Inner Nuclear Membrane Protein Emerin by Tyrsoine Phosphorylation

K. E. Trifl, K. L. Wilson; Cell Biology, Johns Hopkins, Baltimore, MD

Emerin-Dreifuss muscular dystrophy (EDMD) is characterized by weakening of selected skeletal muscles, contractures of major tendons, and cardiac conduction defects. Loss of the inner nuclear membrane protein emerin causes X-linked EDMD. Emerin regulates gene expression, potentially via direct binding to transcriptional regulators like BAF, GCL, Lmo7, Bf, and mRNAs splicing factor YT521B. Emerin also binds structurally proteins (lamin A, actin, and Nesprins) suggesting additional roles in nuclear structure. The binding sites in emerin for many partners overlap, suggesting that emerin’s interactions are regulated. Twelve sites of tyrosine phosphorylation in emerin have been identified in five independent proteomic studies of cells treated with tyrosine phosphatase inhibitors. We propose that tyrosine phosphorylation of emerin regulates interactions with specific partners and hence its function. Supporting this hypothesis, BAF binding to emerin is significantly decreased by tyrosine phosphorylation. This effect is partly alleviated by a Src kinase inhibitor suggesting that Src kinases regulate BAF-emerin binding. We made single missense mutations (Y93A or Y93F) at the twelve tyrosine phosphorylation sites in emerin to identify their physiological roles in binding to specific partners in vivo. Mutations at either Y19, Y41, Y461, and/or Y167 disrupted binding to BAF in vivo suggesting that phosphorylation at these sites might inhibit binding to BAF. Residues Y19 and Y41 are located in the LEM domain which is sufficient to bind BAF. Interestingly, Y161 and Y167 did not disrupt BAF binding in previous in vitro studies suggesting that these residues either are part of a second region in emerin that binds to BAF or may affect BAF binding indirectly in vivo. We are also investigating the roles of three tyrosine kinases (Her2, EGF, and Src) in emerin regulation. Understanding how emerin is regulated is crucial to understanding emerin function and determining the molecular mechanism of EDMD.

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The Role of Kap95 during Nuclear Pore Complex Assembly

S. B. Rabelo, R. McAndrew, K. J. Ryan; Biology, Texas A&M University, College Station, TX

Nuclear pore complexes (NPCs) are large, proteinaceous structures spanning nuclear envelope (NE). They allow for the passive diffusion of small metabolites and proteins and the selective transport of large proteins and RNAs between the nucleus and cytoplasm. Proteomic studies have found that NPCs are assembled from multiple copies of approximately 30 individual proteins collectively termed nucleoporins or Nups. In higher eukaryotes, both the NE and NPCs are disassembled at the onset of mitosis and must be reassembled following chromsome segregation. Cells continue to assemble NPCS into a closed NE during interphase. In organisms where many of the differentiated cells are either slowly dividing or post-mitotic, NPCs assembled during interphase are likely to account for the majority of nucleocytoplasmic transport capacity. To study the molecular mechanism of NPC assembly into an intact NE, we are using the yeast Saccharomyces cerevisiae and are focused on a cytoplastic function of the karyopherin Kap95 during this process. Cells depleted of Kap95 fail to assemble NPCs and accumulate extended sheets of cytoplasmic membranes. To identify factors interacting with Kap95 at this stage, we are directly testing the kap95-E126K allele for enhanced fitness defects with mutant alleles of NUPs and genes involved in membrane fusion. So far, we have observed interactions between kap95-E126K and a subset of Nups. We have also conducted a multi-copy suppressor screen with kap95-E126K. Both NIC96 and NUP170 are able to suppress the growth phenotype of kap95-E126K when expressed from high copy plasmids. These two NUPs also displayed enhanced lethality with the kap95 allele. In addition to NIC96 and NUP170, we have isolated at least 3 other genomic inserts capable of suppressing the growth defect of kap95-E126K. Further characterization of these genetic interactions will provide insight into the role of Kap95 during NPC assembly.

1881

Functional Domains of Sun1

Q. Liu, K. J. Roux, M. Crisp, M. Elsaage, B. Burke; Anatomy & Cell Biology, University of Florida, Gainesville, FL

The nuclear envelope (NE) forms the boundary between the nucleus and cytoplasm. It features biochemically distinct inner and outer nuclear membranes (INM and ONM) separated by a perinuclear space (PNS). The INM proteins Sun1 and 2 function as tethers for nesprin proteins in the ONM. This involves the interaction of the Sun/nesprin lumenal domains within the PNS. Since Sun proteins are associated with the nuclear lamina and nesprin proteins interact with components of the cytoskeleton, the Sun/nesprin complexes represent links in a molecular chain that connect the nuclear skeletton to the cytoskeleton. We have now sought to further characterize the Sun1 molecule which functions as a tether for nesprin 2 Gant. In this study we made use of several naturally occurring splice isoforms along with engineered deletion constructs to define domains required for Sun1 localization, membrane integration and homotypic oligomerization. We observed that the nucleoplasmic and luminal domains of Sun1 were each sufficient for NE targeting, suggesting at least two independent INM retention mechanisms. The largest isoform of Sun1 has four distinct hydrophobic sequences each of which could function as a transmembrane domain. We hypothesize that Nvj1p is anchored in the ONM through interactions with the vacuole membrane protein Vac8p to form nucleus-vacuole (NV) junctions. Nvj1p remains also lost in reporters with mutations that mildly reduce the hydrophobicity of the N-terminal sequence. These results indicate that not all nuclear shape abnormalities associated with LMNA mutations are “created equal;” instead, they can arise either from a functional loss of lamins A/C (as in many of the muscular dystrophies) or from the accumulation of a mutant protein at the nuclear envelope (as seen in progeria), with each scenario resulting in distinct changes in nuclear shape and mechanics. These alterations in nuclear mechanical properties could adversely affect cellular mechanotransduction signaling and cell viability under strain and contribute to the specific pathophysiology of these diseases.
Degradation, nuclear import was dramatically reduced. These results indicate that degradation of Nup98 is not sufficient to inhibit nuclear import. To determine if the viral 2A protease (2Apro) was responsible for degradation of NPC proteins, we incubated HeLa whole-cell lysates with purified rhinovirus 2Apro. The addition of 2Apro was sufficient to cause degradation of Nup153, p62 and Nup98, when levels of Nup98 were almost undetectable, nuclear import was indistinguishable from mock-infected cells. In contrast, at late times post-infection when Nup98, Nup153 and p62 were all disrupted (e.g. by individual point mutations) lead to tissue specific diseases. In 2003, a subtractive proteomic analysis of liver NEs extracted with either salt/detergent or alkaline solutions identified 67 novel putative NE transmembrane proteins (NETs). Subsequent analysis of the expression profiles of proteins in this dataset indicated that many had a clear preference for expression in liver, suggesting that each cell type or tissue may have a subset of unique NETs. Recently we performed a similar proteomic analysis of alkali-extracted lymphocyte NEs from either resting or activated cells and we are in the final stages of processing and analyzing the NE proteome of salt/detergent-extracted lymphocyte and muscle NEs. In our first dataset of expression in liver, suggesting that each cell type or tissue may have a subset of unique NETs. Recently we performed a similar proteomic analysis of alkali-extracted lymphocyte NEs from either resting or activated cells and we are in the final stages of processing and analyzing the NE proteome of salt/detergent-extracted lymphocyte and muscle NEs. In our first dataset of expression in liver, suggesting that each cell type or tissue may have a subset of unique NETs. Recently we performed a similar proteomic analysis of alkali-extracted lymphocyte NEs from either resting or activated cells and we are in the final stages of processing and analyzing the NE proteome of salt/detergent-extracted lymphocyte and muscle NEs. In our first dataset of expression in liver, suggesting that each cell type or tissue may have a subset of unique NETs. Recently we performed a similar proteomic analysis of alkali-extracted lymphocyte NEs from either resting or activated cells and we are in the final stages of processing and analyzing the NE proteome of salt/detergent-extracted lymphocyte and muscle NEs. In our first dataset of expression in liver, suggesting that each cell type or tissue may have a subset of unique NETs. Recently we performed a similar proteomic analysis of alkali-extracted lymphocyte NEs from either resting or activated cells and we are in the final stages of processing and analyzing the NE proteome of salt/detergent-extracted lymphocyte and muscle NEs.
Defining the Cellular Populations in the Adult and Developing Heart

I. Banerjee, J. W. Fuseler, B. K. Thomas, T. A. Baudino; CDBA, University of South Carolina School of Medicine, Columbia, SC

The cell types in the adult myocardium function in a dynamic fashion to regulate the heart’s biomechanical, electrical and structural properties. The cardiac fibroblasts, myocytes, endothelial cells and vascular smooth muscle cells interact to regulate the function of the heart as a pump. What is critical to understand is the effect that developmental and pathophysiological signals have on cellular function in the heart. Alterations in number and location of the various cell types can cause altered cardiac function. Studies performed in the 1970’s established the cell populations in the heart as consisting of 30% myocytes and 70% non-myocytes, i.e. fibroblasts and endothelial cells (Nag 1979, Zak 1974). These studies were performed without the use of molecular markers and were largely based on electron microscopy morphological analysis and centrifugation studies. In this study, we make use of immunospecific markers and Fluorescence Activated Cell Sorting (FACS) analyses to quantify the cell populations during development, homeostasis and pathology in the murine heart. Analysis of the heart at birth demonstrated cellular populations of 10% fibroblasts, 60% myocytes, 3% endothelial and 27% smooth muscle cells. Physiological perturbations observed during the early neonatal period did not cause significant fluctuations in myocyte populations, but did cause significant increases in cardiac fibroblasts. The increase in cardiac fibroblasts was significant in each day observed postpartum, with the greatest increase in the early neonatal period. These data indicate that cardiac fibroblasts respond significantly to the biomechanical load in development. These studies also establish that during homeostatic in the adult heart, the cellular constituents consist of 26% fibroblasts, 55% myocytes, 7% endothelial and 10% vascular smooth muscle cells. Furthermore we have confirmed these results using morphometric and FACS sectional analyses. Taken together, these data taken will enable us to establish a model for defining the cellular constituents of the myocardium.

Protein Knockdown of Edg2 Inhibit Heart Contraction and Blood Circulation in Zebrafish

S. Hayashi, A. Inoue; Biology, Osaka University, Toyonaka, Japan

The cardiac myocytes have striated fibrous structures called myofibrils that contain characteristic myocyte-specific proteins such as cardiac myosin. In early embryonic stage, the heart has already been beating and the size of heart increased dramatically in embryo. Revealing the proliferation mechanism of cardiac myocytes is important not only for understanding the development of heart but also for the medical treatment of heart disease. But it has not known how differentiation and proliferation of cardiac myocytes occur in developing heart. In murine adult heart, it was reported that cardiac progenitors or stem cells exist, and it is considered that non-differentiated proliferative cardiac progenitors can proliferate but terminally differentiated beating cardiac myocytes cannot proliferate. However, there is a possibility that cardiac myocytes proliferate during early stage of embryo. We found that the cardiac myocytes obtained from chick day 7 embryos, expressing cardiac myosin heavy chain, a marker of differentiated cardiomyocytes, proliferated in dispersed culture. This observation suggests that the differentiated cardiac myocytes have the proliferative potential. By administration of bromodeoxyuridine, it was confirmed that embryonic cardiomyocytes passed through DNA synthesis. The cell cycle of cardiomyocytes in culture was estimated to be about 4 days and almost all cardiomyocytes proliferate in culture. Immunocytochemically, the sarcomeric cardiomyocytes were observed in embryonic chicken heart and also embryonic murine heart. We conclude that almost all differentiated cardiomyocytes enter the cell cycle and contribute the enlargement of functional heart in chick embryo.

A DNA Repair Protein Required for Normal Heart and Blood Development

Y. Wang, C. C. Shupenko, L. F. Melo, M. P. R. Strauss; Biology, Northeastern University, Boston, MA

Abasic (AP) sites in DNA are produced spontaneously and by many genotoxic and endogenous agents. The repair of such damage is initiated by AP endonucleases, which are highly conserved from E. coli to man. AP endo1, which constitutes 95% of the AP cleavage activity in mammals, is an embryonic lethal in mice. We are examining its role in embryogenesis in zebrafish. Zebrafish contain two genomic copies (zfAPEX1a and b) with identical coding sequences. zfAPEX1b lacks introns. Recombinant protein (ZAP1) is highly homologous with and has the same enzymatic properties as its human ortholog. ZAP1 is highly expressed throughout development. Embryos microinjected with morpholinoligonucleotide (MO) targeting the translation start site (TS) die at the end of the embryo. They are rescued with mRNA for human WT APEX1 but not APEX1 encoding endonuclease defective protein. Rescued embryos develop dysmorphic hearts, pericardial edema, few erythrocytes, small eyes and abnormal notochords. Embryos microinjected with MO targeting zfAPEX1a intron/exon junctions also pass the MBT with similar abnormalities. We conclude that AP endonuclease 1 is involved in both repairing DNA and regulating specific early stages of embryonic development. This remarkable result could lead to novel therapies in repairing fetal heart defects. Supported by NIH CA72702 and funds from Northeastern University.

Regulation of Ventricular Compaction; Laser Capture Microdissection and Microgenomic Analysis

Y. Li, M. Daniel, P. J. Gruber; Children's Hospital of Philadelphia, Philadelphia, PA

During embryonic heart development, the process of compaction plays a critical role in cardiac morphogenesis. Myocytes in trabecular (inner) layer are more differentiated, while those in compact (outer) layer are more proliferative. The overall objective of these experiments is to better understand the mechanisms that underlie ventricular morphogenesis. We specifically sought to generate new hypotheses to test through a combination of methods new to the study of ventricular compaction. We used microgenomic amplification of laser capture microdissected (LCM) ventricular myocytes to profile expression patterns of compact compared to trabecular myocardium. In brief, E14.5 murine embryos were cryosectioned (8um) at the midpapillary level of the ventricle. Trabecular and compact layers were separated by LCM. RNA was isolated and two rounds of amplification of the mRNA fraction from total cellular RNA were performed from these two cell populations and cRNA analyzed by Affymetrix 430 Mouse Genome chip microarrays. From 50 genes that have at least 2-fold change in each group, the top 5 previously unstudied genes in each group were further analyzed by a combination of qPCR and in situ hybridization. Two candidate genes were selected, Tangerin and Pde1c, both of which are highly up-regulated in trabecular myocardium. In situ hybridization analysis in e14.5 embryos confirmed that Tangerin and Pde1c are highly expressed in compact layer. These results confirm our hypothesis that these genes contribute to the compaction of the ventricular myocardium and have a significant role in the regulation of ventricular morphogenesis.

Knock Down of Edg2 Inhibit Heart Contraction and Blood Circulation in Zebrafish

T. Chan,1 K. Tsao,1 T. Chen,1 H. Lee,2 S. Lee1; 1Institute of Zoology, National Taiwan University, Taipei, Taiwan, 2Department of Life Science & Institute of Zoology, National Taiwan University, Taipei, Taiwan

Unsaturated lysophosphatidic acid (LPA) has been shown to induce vascular remodeling in vivo. However, targeted deletion of LPA receptor genes, edg2 and edg4, in mice resulted in no significant cardiovascular defect that makes the signal transduction of LPA-mediated vascular remodeling in puzzle. To better understand the regulation of LPA-dependent vascular remodeling, we have cloned edg2 and edg4 genes in zebrafish. Whole mount in situ hybridization showed that edg2 is specifically expressed at the pharyngeal arches and in close vicinity to heart in 3 day embryos. Similarly, edg4 is also observed in Edg4, but a reduced expression was evident in other organs including brain and trunk. Embryos injected with 5 ng per embryo edg2 antisense morpholinoligos (MO) at 1-cell stage appeared normal during the first 3 days post injection, but the blood circulation was gradually reduced and even stopped at around Day 5 post injection. In contrast, edg4 MO had no effect on circulation at 10 ng per embryo. To examine the effect of edg4 MO on the vasculature, we used the fli-egfp embryos, which reveal bright fluorescence in vessels. No significant abnormality in vessels was observed in edg2 MO-injected embryos. However, we found that the contraction of heart was gradually weaker that may contribute to the reduced flow in circulation. These results suggested that LPA may mediate the heart contraction via its receptor Edg2, but not Edg4 in developing zebrafish larvae.

Glomeruli Tissue Engineering by Niche Dynamics Utilizing Type V Collagen Fiber

Y. Masuoka, P. Miyata; Institute of Applied Biotechnology, University of Tsukuba, Tsukuba City, Japan

Complicated nephron cannot be completed by current tissue engineering. One barrier is that artificial tissue derived from renal cells can hardly fuse to patient tissue and both blood and urine flows cannot form after transplantation. Particularly, regeneration of glomeruli from tissue fused between microvascular and epithelial ureter is difficult. Although recent tissue engineering improved generation of renal tubes from ureteric buds at development stage, it can neither regenerate glomeruli nor induce microvascular formation within epithelial tissue. This study is to
develop a method using type V collagen fiber to regulate niche dynamics and induce kidney morphogenesis to balance vascular and epithelial formation. Our experiment showed type V collagen fiber existed ubiquitously at early metamorphoses, which incorporated into mesenchymal cap at ureteric bud branching tip and leded connection between glomeruli and ureteric bud. It existed at immature glomeruli while collagen IV at mature ones. This suggest phase changes of ECM occurred. Only V collagen induced vascular morphogenesis from mesenchyme. Experiment culturing adult glomeruli with metanephros showed glomeruli fused to ureteric buds tip to from a complete nephron when it was cultured in medium with V fiber. Microgravity was applied to culture system and result showed V fiber induced glomeruli formation with microvessel. Glomeruli requires both epithelial and vascular formation. But in vitro culture showed interaction of cells and factors for epithelial and vascular morphogenesis inhibited each other during renal development and caused failure formation of glomeruli. Separating cell and factor spatially is important. Type V works as a mediator not only keeping synergic excellerul environment but also allowing dynamics for phase changes. This niche dynamics induced mesenchyme aggregation and separately localized different tissue morphogenesis with harmony. In summary, V fiber ECM induces microvascular and glomeruli formation, and fusion between glomeruli and ureteric bud.

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**exc-9, a LIM-domain Protein That Regulates Tubular Structure**

X. Tong, M. Buechner; Molecular Biosciences, The University of Kansas, Lawrence, KS

We are interested in the formation and maintenance of tubular structure. The excretory canal cell of the nematode *C. elegans* provides a simple single-celled model for this study. Tube formation can be studied without considering cell-cell interactions by using this model. The EXC proteins regulate apical diameter of the excretory canal; mutations in exc genes alter the diameter of excretory canal lumen and outgrowth of the canal. Exc-9 mutants show shortened cystic excretory canals. In addition to canal defects, hermaphrodite exc-9 mutants show non-penetrant tailtip defects, and males exc-9 mutants show non-penetrant ray defects. We cloned the exc-9 gene, and found that it encodes a small protein containing a single LIM domain. The LIM domain is a zinc binding, cysteine rich motif consisting of two zinc fingers. LIM domains mediate protein-protein interactions. Exc-9 shows high homology to CRIP (Cysteine-Rich Intestinal Protein), first identified as a cytosolic protein from the mammalian intestine. An exc-9 reporter construct is expressed in several nematode tissues including the excretory canals, the tailtip, the distal tip cells, several neurons, and the ureter cell seam. Overexpression of the reporter construct in wild-type background sometimes arrests the worms at the 2-fold stage of embryogenesis. This phenotype is similar to the phenotype of mutants in the homeobox gene *coh-6*, and suggests that coh-6 activates exc-9 transcription. Low levels of expression of a translational exc-9:gfp fusion construct partially rescue the cystic canal phenotype. High expression levels disrupt canal shape and length. This phenotype is similar to that of overexpression of a translational construct of exc-5, which encodes a guanine exchange factor for CDC42. Preliminary genetic interactions suggest that exc-9 functions downstream of exc-4 (CLC channel) and exc-2 proteins, but upstream of EXC-5. A yeast-two-hybrid assay is being performed to determine possible binding partners of EXC-9.

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**Searching for Foxes in the Zebrafish Foregut**

L. Hammonds-Odie, K. Young, D. McGarity, T. Collins; Biology Department, Spelman College, Atlanta, GA

The genetic programs required for mesodermal interactions with endodermal cells in development, particularly as they relate to the formation of organs of the digestive system, have been the subjects of intense study in recent years. During embryogenesis, endoderm-dependent organ formation is accomplished through regional signaling pathways that specify the gut tube to form the thyroid, lungs, liver, pancreas, and gall bladder of vertebrates. Several transcription factors have been implicated in the development of the liver and pancreas. However, frequently in studies of the biliary system or pancreatic development, the gall bladder is completely ignored, despite the proximity. Transcription factors expressed in the foregut endoderm or in the surrounding mesoderm have potential roles in the divergence of the presumptive gallbladder from the hepatic diverticulum. The goal of this study is to document the expression of the zebrafish homologs of transcription factors for which a role in murine gall bladder development has been demonstrated. More specifically, we have examined the expression patterns of the forkhead transcription factors, FoxA2, FoxA3 and FoxF1 in staged zebrafish embryos using in situ hybridization. This work was supported by NIGMS/NIH GM008241-22052, NIH RIMI MD000215-01 and MIE NASA NCC8-227.

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**Barx2 Regulates Lachrymal Gland Development**

C. W. Tsai, ’R. Meech,’ K. N. Gonzalez,’ H. Makarenkova,’ T. Collins; Neurobiology, The Scripps Research Institute, La Jolla, CA

A healthy eye is dependent on functions of ocular surface components, which include the cornea, conjunctiva and lacrimal gland (LG). “Dry eye” is a very common condition and is a result of defective function of the LG. Defects in the LG can be congenital or result from autoimmune diseases or aging. “Dry eye” can lead to severe problems and result in a variety of eye diseases, including cataracts, corneal ulcerations, and even blindness. Despite of the commonality of the dry eye condition, only recently have molecular genetic studies of LG development been initiated. Our studies have been centered on the homeodomain transcription factor Barx2 to identify its function in lacrimal gland development. The normal mouse LG at E19.0 consists of a long duct with two branching structures including extra-orbital and intra-orbital branches. In the Barx2 null mouse, the LG was poorly developed or entirely absent. In most cases the lacrimal gland was vestigial, close to the eye, and characterized by ectopic branching from the duct. Unwaveling, down-regulation of Barx2 in LG explants cultures using antisense oligonucleotide also resulted in branching defects. Moreover, gain of Barx2 function experiments show that Barx2 promotes cell migration, and regulates the expression of MMPs and cell adhesion molecules. Together these data suggest that Barx2 plays an essential role in regulating branching morphogenesis of the LG, possibly by modulating the extracellular matrix and cell-cell adhesion.

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**BIM Regulates Apoptosis during Mammary Ductal Morphogenesis and Its Absence Reveals Alternative Cell Death Mechanisms**

A. A. Mauleux,’ M. Overholtzer,’ T. Schmiede,’ P. Bouillet,’ A. Strasser,’ J. S. Brugge,’ Cell Biology, Harvard Medical School, Boston, MA; ’The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia

The adult virgin mammary gland is a highly organized tree-like structure formed by ducts with a hollowed lumen. Although lumen formation during puberty development appears to involve apoptosis, the molecular mechanisms that regulate this process are not known. Here we demonstrate that the BH3-only pro-apoptotic protein BIM is required for apoptosis during pubertal development and disruption of the Bim gene in mice prevents clearing of the lumen in terminal end buds (TEB). However, cells that escape apoptosis in the Bim-/- TEB are eventually cleared from the adjacent ducts by a caspase-independent death process. Interestingly, these cells, which are deprived of matrix attachment, undergo squamous differentiation prior to cell death. Squamous differentiation also takes place in vitro when immortalized mammary epithelial cells are detached from matrix, suggesting that this differentiation program is in fact a direct response to loss of attachment. These data provide important mechanistic information on the processes involved in sculpting the mammary gland in vivo and demonstrates that BIM is a critical regulator of apoptosis associated with this event.

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**Regulation of Mucin 1 in Pancreatic Ductal Epithelium: Identification of a Required Minimal Promoter Region**

S. Scott, B. Ghosh, S. D. Leach, Surgery, Johns Hopkins University, Baltimore, MD

The pancreas is composed of two main components: the exocrine tissue that produces digestive enzymes and the endocrine tissue that produces insulin and glucagon. The main components of the exocrine system are responsible for producing glucose regulating hormones, and an exocrine compartment consisting of acinar cells and an elaborate system of ducts. Although pancreatic ductal epithelium is altered in many human diseases, including pancreatic cancer, little is known regarding how ductal differentiation is regulated. We have used immunofluorescent staining to confirm the ductal specification of *Muc1* (*Muc1*) throughout development, and have observed subsequent restriction to the terminal ductal elements in the murine adult pancreas. In order to better understand the regulation of *Muc1* expression and to identify regulatory elements responsible for cell type-specific expression, we created constructs with two different 5' flanking regions fused to the lacZ reporter gene. When 2200bp and 756bp of the *Muc1* 5' flanking sequence were used to drive luciferase (2200bpG3L3 and -756bpG3L3, respectively) in human and murine pancreatic cell lines, activity was observed only in cell lines that were established from pancreatic ductal epithelium, with no expression in islet or acinar cell lines and no quantifiable difference between the activity of the two promoter regions. This indicates that regulatory elements necessary for the transcriptional regulation of *Muc1* may reside within the 756bp 5' flanking region of the gene. We are currently generating a number of constructs of varying lengths in order to more definitively map this region in hopes of identifying novel trans-acting regulators of ductal differentiation. Our results will also enable us to potentially create transgenic mice with EGF expression under the control of a specific and defined *Muc1* regulatory element. This will provide a system whereby we will be able to study the growth and differentiation of the pancreatic ductal system.
Prenatal Development of the Pancreas Is Normal in Shwachman-Diamond Syndrome Mouse Models

L. L. Chen, S. Zhang, P. R. Durie, J. M. Rommens: Program in Genetics & Genomic Medicine, Hospital for Sick Children, Toronto, ON, Canada; aGraduate Department of Molecular and Medical Genetics, University of Toronto, Toronto, ON, Canada; bDepartment of Pediatrics, Hospital for Sick Children, Toronto, ON, Canada; cDepartment of Pediatrics, University of Toronto, ON, Canada.

Shwachman-Diamond syndrome (SDS) is an autosomal recessive disorder characterized by exocrine pancreatic dysfunction, and hematological and skeletal abnormalities. It is caused by mutations in the SBDS gene, a member of a highly conserved protein family involved in RNA processing and/or ribosome biogenesis. Exocrine pancreatic insufficiency (as measured by serum enzyme levels), presents in infancy with maldigestion, poor growth and failure to thrive. Imaging studies show pancreata with reduced size, and autopsy results show extensive fatty replacement with intact ducts and islets, but sparse or no acinar cells. SDS disease models were generated with gene targeting technologies, to obtain inbred mouse strains that are homozygous for the hypomorphic R126T disease allele (SBd/+126T/126T) or heterozygous, with hypomorphic and null allele combinations (SBd/+126T). These mice strains display severe growth retardation and die perinatally. The murine pancreas experiences rapid growth during the course of its development, in the periods before birth and around the time of weaning. Further, some digestive enzymes are produced prior to birth. Early pancreatic development was examined using markers to evaluate cell morphology, differentiation, and enzyme production from E14.5 to perinatal stages. The prenatal development of the pancreas of the mutant mice appears comparable to their wild type siblings. Acinar lobules with peripherally positioned nuclei were readily distinguishable with interspersed clusters of islet cells. Further, immunohistochemical staining of pancreatic sections reveal intact islet and acinar components of the mutant pancreas with production of insulin, glucagon, and amylase. These findings suggest normal development during the embryonic period, such that loss of SBds affects the pancreas only postnatally. While postnatal models are required for more detailed analysis, we conclude that the onset of exocrine pancreatic dysfunction in SDS patients may occur as late as postnatal periods, with the regression or apoptosis of previously differentiated exocrine cells.

An Analysis of Cellular Processes Involved in Organogenesis of the Drosophila Embryonic Gonad

J. W. Weyers, A. B. Mihalitschov, Y. Takeda, M. Yan Doren; Biology, Johns Hopkins University, Baltimore, MD.

The successful formation of organs and tissues requires different cell types to cooperate while engaging in several cellular processes, such as migration, cell-cell adhesion, and cell-cell signaling. One model system in which to study these cellular processes and interactions is the early formation of the Drosophila embryonic gonad. Early gonad formation in flies requires several distinct steps, each of which involves different cellular events. There are two cell types in the embryonic gonad, germ cells (GCs) and somatic gonadal precursors (SGPs). To start, these cells must be properly specified; GCs form very early in embryogenesis, while the SGPs are specified out of the mesoderm in three different segments of the embryo. The GCs are passively carried into the gut during gastrulation, but then actively migrate to join the SGPs. As the germ cells approach and contact the SGP clusters, individual SGPs will undergo cell shape changes to completely ensheath each GC. Concurrently, in what is called SGP cluster fusion, the individual SGP clusters merge together into a band of cells stretching across three segments of the embryo. From there, the cells will coalesce into a properly structured round ball that is the embryonic gonad. The cellular mechanisms for ensemaphate, SGP cluster fusion, and coalescence are mostly unknown. To better understand the processes involved, we have performed a mutagenesis screen to find new genes involved in these mechanisms. Our screen identified 21 different complementation groups, representing new genes, that play a role in these events of gonad formation. An early description of these gene’s roles in the cellular events required for proper gonad formation will be presented.

The Study of Dolichol Phosphate Mannose Synthase I (DPMI) Homolog Y66H1A.2 in C elegans

D. Kim, J. Cho; Department of Biology, Yonsei University, Seoul, Republic of Korea.

Dolichol phosphate mannose synthase is one of the multiple enzymes involved in N-glycan assembly in ER. It catalyses the synthesis of dolichylphosphatemannosyl(Dol-P-Man) from GDP- mannose and dolichylphosphate. This enzyme is composed of a catalytic subunit DPMI and regulatory subunits DPM2, DPM3. It was reported that partial deficiency in human DPMI biosynthesis causes CDG(Congenital disorders of glycosylation) type Ie. We found that dpm1 homolog(y66h1a.2) in C elegans was mainly expressed in hypodermis and intestine. Functional block of dpm1 homolog by RNA interference caused developmental delay, formation of huge embryos or unfertilized oocytes, abnormal cleavage of embryos, egg-laying defect, and enlargement of intestinal lumen. The intestinal lumen enlargement was defined to be associated to dysfunction of digestion. We confirmed problems on ovary occurs when the funtion of DPM1 is inhibited; by mating wild type male and hemaphrodite, they could not produce any progenies. These results indicates that C.elegans DPM1 have important roles involved development.

The RhoGAP Crossveinless-c Links Trachealess and EGFR Signalling to Cell Shape Remodelling in Drosophila Tracheal Invagination

V. Brod, J. Casanova; Institute for Research in Biomedicine (IRB), Barcelona, Spain.

Organ formation requires coordinated changes in cell populations in terms of their proliferation, migration, differentiation and shape. These synchronized changes are controlled by the genes specifying cell fate and by the ability of cells to respond to extracellular cues. A major issue in morphogenesis is to understand how the activity of genes specifying cell fate affects cytoskeletal components that modify cell shape and induce cell movements. We have approached this question by investigating how a group of cells from an epithelial sheet initiate invagination to form the vertebrate eye. We are currently exploring the mechanisms of epithelialization in the eye and the acquisition of columnar epithelial morphology by the neural retina. We use the zebrafish, in which eye formation occurs rapidly, between 10 and 24 hours post fertilization (hpf). In addition, its unparalleled optical accessibility permits continuous observation of all of the cell movements during these events. Our screen revealed a novel role for the RhoGAP Crossveinless-c in regulating the activity of the trachealess patterning gene and the EGFR signalling pathway. Altogether, our results unveil a developmental pathway, linking genes specifying cell fate and signalling pathways with cytoskeleton modifications that underlie the early tracheal cell shape remodelling.

Tissue-specific Loss of BMP Signaling from the Pharyngeal Region Affects Thymus and Parathyroid Organogenesis

J. Gordon, S. R. Patel, N. R. Manley; Genetics, University of Georgia, Athens, GA.

The thymus and parathyroids originate from the third pharyngeal pouches that form as endodermal outpocketings on embryonic day 9 (E9.0) of mouse development. We have previously shown that Foxn1 and Gcm2 define the thymus and parathyroid domains within the primordium prior to organ formation. We recently reported that Bmp4 expression is confined to the thymus domain of the third pouch from E10.5, while its antagonist Noggin is expressed in a non-overlapping domain within the parathyroid region. Here, we have used a series of conditional gene knockouts to delete Bmp4 or Bmp8A from the pharyngeal endoderm and/or the surrounding mesenchyme using Fosq1Cre or Wnt1Cre mouse strains. Deletion of Bmp4 from the endoderm and mesenchyme prior to the onset of Foxn1 expression did not affect patterning of the primordium or initial organ differentiation, but did cause significant morphological defects, suggesting that the two processes are independently controlled. The thymus and parathyroid, which normally separate from each other at E12.5, remained closely associated at E13.5. In addition, we observed a reduction in condensing mesenchyme around the epithelium, resulting in partial disruption of the capsule and thinning of the basement membrane around the thymic primordium. This may in turn be responsible for the disruption migration of the thymus observed in the mutants at E12.5. These results suggest a common BMP-mediated mechanism may be responsible for mesenchymal condensation, capsule formation and separation of the parathyroid from the thymus during mouse embryogenesis. Deletion of Bmp4 from neural crest cells did not affect early thymus and parathyroid organogenesis, suggesting a requirement for reciprocal BMP-mediated signals between the pharyngeal endoderm and mesenchyme. Furthermore, deletion of Bmp4 after the onset of Foxn1 expression did not produce the same phenotype, providing evidence for temporal variation in the role of Bmp4 signaling during thymus and parathyroid organogenesis.

Cellular Mechanisms of Vertebrate Eye Morphogenesis

K. M. Kwan, C. Chien; Neurobiology and Anatomy, University of Utah, Salt Lake City, UT.

The vertebrate eye develops as an evagination from the neuroepithelium of the brain. This epithelial tissue undergoes a series of striking cell and tissue rearrangements to form initially the optic vesicle, and later, the optic cup. The goal of this project is to develop a cellular and molecular understanding of the morphogenetic movements responsible for generating the vertebrate optic cup. We are currently exploring the mechanisms of epithelialization in the eye and the acquisition of columnar epithelial morphology by the neural retina. We use the zebrafish, in which eye formation occurs rapidly, between 10 and 24 hours post fertilization (hpf). In addition, its unparalleled optical accessibility permits continuous observation of all of the cell movements occurring during eye morphogenesis. We are tracking these movements via timelapse confocal microscopy, using a variety of fluorescent proteins. Among these is the photactivatable protein, which allows us to follow movements of individual cells through time.

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fluorophore Kaede, with which we can observe single cell behaviors during eye morphogenesis. Surprisingly, we find that neural retina cells in the early eye (10-16 hpf) display amoeboid behaviors, with active lamellipodial protrusions on both apical and basolateral surfaces. These protrusions have ceased by the time the optic cup is formed (24 hpf), by which point the cells achieve a columnar epithelial morphology. Protrusions cease in an asymmetric manner, with apical lamellipodia lost prior to basolateral lamellipodia. Interestingly, we find that apicobasal polarity is established early: at the onset of optic vesicle evagination, Par-3 is localized to the apical surface, and shortly after, centrosomes are oriented apically. Our current hypothesis is that formation of apicobasal polarity functions to suppress epithelial cell behaviors on the apical surface, while extracellular matrix, specifically laminin, is required to suppress lamellipodia formation on the basolateral surface. We are testing this using genetic mutants and morpholino knockdown combined with live imaging.

1907 Cytomegalovirus-induced Embryopathy: Mouse Submandibular Sialog gland Epithelial-Mesenchymal Ontogeny as a Model M. Melnick, 1 E. S. Mocarski, 2 G. Abichaker, 3 J. T. Tashjian, 1 Lab Developmental Genetics, University of Southern California, Los Angeles, CA, 3Dept Microbiology and Immunology, Stanford University, Stanford, CA

Human studies suggest, and mouse models clearly demonstrate, that cytomegalovirus (CMV) is dysmorphic to early organ and tissue development. CMV has a particular tropism for embryonic salivary gland and other head mesenchyme. CMV has evolved to co-opt cell signaling networks so to optimize replication and survival, to the detriment of infected tissues. It has been postulated that mesenchymal infection is the critical step in disrupting organogenesis. If so, organogenesis dependent on epithelial-mesenchymal interactions would be particularly vulnerable. In this study, we chose to model the vulnerability by investigating the cell and molecular pathways of CMV-infected mouse embryonic submandibular salivary glands (SGMs). We infected E15 SMG explants with mouse CMV (mCMV). Active infection for up to 12 days in vitro results in a remarkable cell and molecular pathology characterized by atypical epithelial hyperplasia, apparent epithelio-mesenchymal transformation, oncocytic-like stromal metaplasia, β-catenin nuclear localization, and upregulation of NFκB, Relb, Iκb, Stat3, and Cox2. Rescue with an antiviral nucleoside analogue indicates that mCMV replication is necessary to initiate and maintain SMG dysmorphogenesis. Our results indicate that mCMV infection of embryonic mouse explants results in dysplasia, metaplasia, and, possibly, anaplasia. The molecular pathology appears to center around the activation of canonical and, perhaps more importantly, noncanonical NFκB. Further, COX-2 and IL-6 are important downstream effectors of embryopathy. At the cellular level, there appears to be a consequential interplay between the transformed SMG cells and the surrounding extracellular matrix, resulting in the nuclear translocation of β-catenin. From these studies, a tentative framework has emerged within which additional studies may be planned and performed. NIH grants R01 DE014555 (TJ/MM) and ROI A130363 (ESM).

1908 Detection of Apoptotic Cells in Early Developmental Stages of Three Bivalves K. Perry, J. Lynn; Biological Sciences, Louisiana State University, Baton Rouge, LA

In general, bivalve larvae progress through several early life history stages before transitioning into juvenile and adult forms. Morphological alterations in early life stages likely require apoptosis for differentiation of tissues. Embryonic apoptosis events are not well documented for bivalve broadcast spawners with external fertilization. The occurrence of apoptotic blastomeres and cells were followed at 4 hour intervals through embryonic and larval stages for 24 hours after fertilization in two invertebrates, Dreissena polymorpha (zebra mussel) and Mytilus galloprovincialis (Mediterranean mussel), and one indigenous bivalve, Crassostrea virginica (eastern oyster). Apoptotic cells detected using the fluorescence-based TUNEL assay in cleavage (4-64 cells), ciliated blastula, and trochophore stages were compared. During cleavage stages, D. polymorpha had no detectable apoptotic cells similar to observations by Vega and Epel (2004, Mar Environ Res 58:799) in the sea urchin, however C. virginica and M. galloprovincialis embryos exhibited apoptosis in cleavage stages. There was a marked increase in apoptotic cells during the transition from the ciliated blastula stage to the trophophore stage in all three species. M. galloprovincialis embryos were exposed to increasing concentrations of the molluscicide Bayluscide® at fertilization and ending at 4 hrs and 24 hrs of exposure. Both the 4 hr and 24 hr exposures of Bayluscide® (3.9 µg/mL and 0.1 µg/mL assay concentrations respectively) induced significant numbers of apoptotic cells relative to controls. The stress-induced increases in the early stages of C. virginica and M. galloprovincialis development were in contrast to findings of Vega and Epel (2004). Using this data, it may be possible to monitor apoptosis as a consequence of environmental stress, signifying susceptibility in an ecosystem.

1909 Gene Expression as Artemia franciscana Embryos Encyst and Enter Diapause Z. Qiu, 1, S. Tsoi, 1 T. H. MacRae 2; 1Biology, Dalhousie University, Halifax, NS, Canada, 2OBstetrics and Gynecology, University of Arkansas for Medical Sciences, Little Rock, AR

Ooviviparous embryos of the crustacean Artemia franciscana develop into swimming larvae, whereas oviparous embryos cease development as gastrulae, encyst and enter diapause, a form of suspended animation. The metabolic activity of cysts, as these embryos are called, is reduced drastically and their ability to withstand stress is markedly enhanced. Subtractive hybridization was employed to analyze gene expression two days post-fertilization as Artemia embryos enter diapause. Of the 264 cDNA clones examined, 85 were similar to sequences in GenBank and these fell into four functional categories including environmental information processing, cellular processes, genetic information processing and metabolism. p26, a previously characterized small heat shock protein which is up-regulated in cysts was detected, providing confidence in the subtractive methodology. Analysis by semi-quantitative RT-PCR confirmed that seventeen genes were up-regulated and four down-regulated. Genes encoding a homolog of the human co-transcription factor p6, two new small heat shock proteins, putative cell cycle suppressor proteins, and metabolic enzymes were among those up-regulated. The co-transcription factor p8 may influence gene expression and it is potentially one of many regulatory proteins involved in establishing the diapause developmental program. The BRCA 1 associated protein (Bap-1) and functionally related proteins may modulate cell growth during transition into diapause, small heat shock proteins, protect Artemia embryos from physiological stress, and chitin related proteins contribute to cyst wall construction. This study represents the first systematic molecular characterization of early diapause in a crustacean embryo. The synthesis within oviparous Artemia embryos of proteins such as molecular chaperones, which have been detected in other organisms undergoing diapause, was established. In addition, novel differentially expressed genes were identified, thereby increasing the number of proteins known to be modified during early diapause in Artemia, and by extrapolation, in other organisms.

1910 Artemia, a Ferritin Homologue from Artemia franciscana, Is a Developmentally Regulated Molecular Chaperone T. Chen, 1 T. S. Villeeuneve, 2 K. A. Garant, 2 R. Amons, 2 T. H. MacRae 2; 1The College of Animal Science and Technology, Hunan Agricultural University, Changsha, China, 2Biology, Dalhousie University, Halifax, NS, Canada, 3Molecular Cell Biology, Leiden University, Leiden, The Netherlands

Encysted embryos (cysts) of the crustacean, Artemia franciscana, exhibit a level of stress tolerance seldom seen in metazoans, a property thought to depend partly on the regulated synthesis of artemin, a ferritin homologue. Artemia is an abundant heat stable protein produced in cysts which forms oligomers equal in molecular mass to those assembled by ferritin but fails to bind iron. The objective of this study was to better understand artemin function and to this end the protein was synthesized in transformed Escherichia coli and purified to apparent homogeneity with TALON™ affinity resin. Purified artemin consisted of oligomers approximately 700 kDa in molecular mass which dissociated into monomers and a small number of dimers upon SDS polyacrylamide gel electrophoresis. Artemin inhibited heat induced aggregation of citrate synthase in vitro, an activity characteristic of molecular chaperones. Apoferritin and ferritin also exhibited chaperone activity approximately equal to that shown by artemin, suggesting protection of cells by mechanisms other than iron sequestration. Transfected mammalian cells synthesizing artemin were more resistant to heat and H2O2 than were cells transfected with vector only. The data support the proposal that artemin is a structurally modified ferritin arising from either a common ancestor gene or by duplication of the ferritin gene. Divergence, including acquisition of a carboxy-terminal extension and ferroxidase center modification, eliminated iron sequestration, however chaperone activity was retained. Consequently, because artemin accumulates in large amounts, it has the potential to protect Artemia embryos against physiological stress during encystment and diapause without adversely influencing iron metabolism. This work expands the range of activities attributed to artemin, a protein reported previously to bind proteins and metabolic enzymes were among those up-regulated. The co-transcription factor p8 may influence gene expression and it is potentially one of many regulatory proteins involved in establishing the diapause developmental program. The BRCA 1 associated protein (Bap-1) and functionally related proteins may modulate cell growth during transition into diapause, small heat shock proteins, protect Artemia embryos from physiological stress, and chitin related proteins contribute to cyst wall construction. This study represents the first systematic molecular characterization of early diapause in a crustacean embryo. The synthesis within oviparous Artemia embryos of proteins such as molecular chaperones, which have been detected in other organisms undergoing diapause, was established. In addition, novel differentially expressed genes were identified, thereby increasing the number of proteins known to be modified during early diapause in Artemia, and by extrapolation, in other organisms.

1911 Mirror-imaged Doublets of Sterkiella histriorumascorn: Implications for the Development of Left-Right Asymmetry A. J. Bell, P. Satir; Anatomy/Structural Biology, Albert Einstein College of Medicine, Bronx, NY

Ciliated protozoa possess cellular axes reflected in the arrangement of their ciliature. Upon transverse fission, each daughter cell develops an identical ciliary pattern, ensuring perpetuation of the cellular axis. Cilia are generally spatially coordinated, forming a single columnar epithelial sheet. These spatially coordinated cilia may be visualized using ultrathin sections, some of which are capable of intracellular propagation and regeneration after excision. One such phenotype in the ciliate Sterkiella histriorumascorn (formerly Oxytricha falkas/trifilas) is the mirror-imaged doublet. These cells possess two distinct sets of cilia, juxtaposed on the surfaces, arranged in mirror-image symmetry, with a common anterior-posterior axis. We have examined whether individual ciliary components in mirror-image doublets of Sterkiella are themselves mirror-imaged. Ultrathin section analysis indicates that despite the global mirror-imaging of the ciliate, the detailed organization of the membranebilayer is reversed in the mirror-image doublet. This suggests that, like the cells from which they are derived, the mirror-image doublets are themselves mirror-imaged. Ultrastructural analysis indicates that despite the global mirror-imaging of the ciliate, the detailed organization of the membranebilayer is reversed in the mirror-image doublet.
oral apparatus, such that the effective stroke of the cilia propels food away from the mouth. Assembly of compound ciliary structures of both oral apparatuses starts out identically, but as the structures associated with the mirror-image oral apparatus continue to form, its primordial set of oral membranelles undergoes a 180° rotation in the plane of the ventral surface relative to the same structures in the typical oral apparatus. The overall symmetry of the oral apparatus thus appears to be obtained from the more localized assembly of individual basal bodies. “True” mirror imagery of the membranelles would require an enantiomorphic form of the individual ciliary components, particularly the basal bodies, which is never observed. These observations suggest a mechanistic hypothesis with implications for the development of left-right asymmetry not only in ciliates, but perhaps development of left-right asymmetry in general.

1916 The Paromycin Sensitive Aminopeptidase PAM-1 Regulates Centrosome Dynamics and Chromosome Segregation in C. elegans
S. Marshall, L. Washam, J. Kerschner, R. Lyceaz; Biology Department, Ursinus College, Collegeville, PA
PAM-1 is a C. elegans aminopeptidase that is highly related to mammalian PSA. We have identified mutations in this aminopeptidase in C. elegans and have discovered roles for this enzyme in regulation of meiotic exit, anterior-posterior polarity and chromosome segregation. We are analyzing the polarity and chromosome defects in detail in the early embryo.
Determination of Intercellular Forces during Drosophila Embryogenesis

X. Ma, P. Scully, M. S. Hatson; Physics & Astronomy, Vanderbilt Institute for Integrative Biosystem Research & Education, Vanderbilt University, Nashville, TN

Differences in intercellular tensions along cell-cell boundaries have been hypothesized as a driving force for cellular movements during morphogenesis. We have developed a method to measure these tensions in living fruit fly (Drosophila) embryos. The method combines confocal imaging of embryos expressing GFP-chimeras with laser-microsurgery. In the regime of low Reynolds number, the tension along a cell-cell boundary before an incision is well-approximated by the product of the viscous damping constant and the initial recoil velocity of adjacent cell edges after an incision. To measure this recoil velocity, a Q-switched Nd: YAG laser is used to cut a single cell edge while line-scan kymographs of adjacent cell edges are recorded at time resolutions as fast as 2 μm. The time-dependent cell edge positions are extracted from the kymographs using custom Imaged plugins based on the Lucas-Kanade algorithm. The post-incision recoil velocities are then estimated using either a double exponential fit or a Savitzky-Golay filter. Finally, a power spectrum analysis of cell-edge position fluctuations is used to determine the viscous damping constant. This technique has been applied to simulate kymographs to evaluate the robustness of the algorithm and to three distinct stages of Drosophila development - germ band retraction, early and late dorsal closure - that show differences in cellular tensions.

Integrins and TGFβ Family Member Dpp Interact during Drosophila Dorsal Closure

J. W. Bloore; Department of Biosciences, University of Kent, Canterbury, United Kingdom

Dorsal closure dorsal closure describes a morphogenetic event that occurs in the embryo when two epithelial sheets initially positioned laterally sweep up to the dorsal midline where they fuse to form a continuous epidermis. This process involves physical interaction between the epithelial sheets and the amnioserosa, another cell sheet that initially occupies the embryonic dorsal surface. It is contraction of the amnioserosa together with contraction of a supra-cellular actomyosin pursestring formed in the leading cell row of each epithelial sheet, which drives dorsal closure. Dpp is also expressed in the cells of the leading edge. It is not involved in pursestring formation, but does appear to have a role in promoting leading edge filopodia, the structures that knit the two epithelial sheets together at the dorsal midline. Integrins also function during dorsal closure, where they have been shown to mediate survival of the amnioserosa - in the absence of integrins the cells of the amnioserosa enter anoikis. We will present evidence for an additional role for integrins: the promotion of functional leading edge filopodia. Moreover we show, through live embryo imaging, that the dorsal closure phenotypes of integrin and Dpp pathway mutants are remarkably similar. These observations lead us to the hypothesis that integrins and Dpp act together to promote epithelial fusion at the dorsal midline. In support of this hypothesis we show that integrin and Dpp pathway mutants interact genetically. Currently we are attempting to determine whether there is a physical protein-protein interaction between the two. We suggest that they do and that this provides a novel way of generating a local Dpp signal.

A New Family of Nucleases: The Uracil-DNA Specific Nuclease of Pupating Insects

B. G. Vertessy, A. Bekesi, V. Maha, M. Pukanszky, I. Leveles, I. Zaguya; Institute of Enzymology, Budapest, Hungary

Uracil in DNA may arise by cytosine deamination or thymine replacement and is removed by members of the uracil-DNA glycosylase (UDG) superfamily. The surprising lack of the major UDG-superfamily member UNG from the fruitfly genome may allow the fly to tolerate thymine replacing incorporation of uracil in its DNA. Such incorporation is usually prevented by the enzyme dUTPase that removes dUTP from the DNA polymerization pathway. Simultaneous lack of dUTPase and UNG leads to biosynthesis and stable presence of uracil-substituted DNA in mutant E. coli strains. However, dUTPase is present in all multicellular organisms and is usually up-regulated in cells with active DNA synthesis. Fruitfly larvae present a surprising exception to this general rule: dUTPase is confined to the imaginal disks while larval tissues, associated with active DNA synthesis, do not contain any detectable dUTPase (Bekesi et al, 2004 J Biol Chem 279(21):23262-70). Here we asked if the putative presence of uracil-DNA in larval tissues, potentially accumulated due to lack of both UNG and dUTPase, might trigger any physiological response. Such response would require macromolecular (e.g. protein) factors specifically recognizing uracil-DNA; we therefore set out to identify such proteins from Drosophila extracts. We show that the most intensive hit of uracil-DNA pull-down experiments is a uracil-DNA specific nuclease (UDE) with no detectable homology to other proteins except a group of sequences present in genomes of other pupating insects. UDE protein is strongly up-regulated right before pupation by an edysone-controlled mechanism potentially involving betaFTZ-F1. These data imply the existence of a novel DNA nuclease family specific for uracil-DNA with a possible role in cell death during metamorphosis.

Functional Importance of Polymerization and Localization of Calsequstrin in Caenorhabditis elegans

J. Ahn, J. Cho, K. Ko, G. Singaruvelu; Life Science, Gwangju Institute of Science and Technology, Gwangju, Republic of Korea

Dual role of calsequstrin being the ‘Ca2+ donor’ and ‘Ca2+ acceptor’ makes it an excellent Ca2+ buffering protein within the sarcoplasmic reticulum (SR). We have isolated and characterized of the calsequestrin (csq-1) in C. elegans. To our surprise, csq-1(jh108) mutant showed no gross defect in muscle function as well as muscle development. However, csq-1 mutant is highly sensitive to perturbation of Ca2+ homeostasis. The rational of homology modeling, we investigated the domains of calsequstrin which are important for polymerization and cellular localization required for its proper buffering functions. The in vivo patterns of polymerization and localization of several mutated CSQ-1 were observed and then were correlated with the structures of different mutations for their proper polymerization and localization as well as their effectiveness for the rescue in Ca2+ perturbed conditions. Our results suggest that polymerization of CSQ-1 is essential, but not sufficient, for proper cellular localization and function of CSQ-1. In addition, the direct interaction between calsequstrin and myonidine receptor (RYR), is found by in vitro binding assay, suggesting that the cellular localization of calsequstrin is indeed modulated by the RYR via physical interaction in C. elegans.

Axis Specification during First Division in Primates: Pronuclear Orientation, Not the Second Polar Body (2Pb), Defines the Cleavage Plane

C. Simlerly, E. Jacoby, D. McFarland, C. Navara, G. Schuett, Pittsburgh Development Center, Pittsburgh, PA

In mammalian embryos, existence of oocyte polarity as a determinant of cleavage axis formation remains controversial. Time-lapse video microscopy (TLVM) of fertilized mouse zygotes suggested conflicting evidence regarding spatial cues (2Pb, sperm incorporation site) near the animal pole that may directly orient division planes. Alternatively, perhaps no specification of axis exists in the early oocyte and orientation of the two opposing pronuclei predicts cleavage planes. Here, in primate zygotes, TLVM was employed to observe pronucleus formation, migration, alignment and cytokinesis after fertilization or artificial activation. After intracytoplasmic sperm injection (ICSI), zygotes demonstrate both radial (157(294)) and tangential (137(294)) orientation pronuclei in the cytoplasm. The cortical site of cleavage furrow formation begins near opposed pronuclei and proceeds through the long axis of the ‘docked’ parental genomes regardless of 2Pb position. If the 2Pb is outside of the cleavage furrow, a massive cortical rotation positions it between the daughter blastomers. Following in vitro fertilization (IVF), cortical residing pronuclei are nearly always oriented radially near the site of sperm penetration and the first mitotic spindle aligns to permit the cleavage furrow to pass near the 2Pb. Following artificial activation, the single formed female pronucleus migrates randomly in the cytoplasm- the cleavage furrow forms nearest the adjacent cortex and passes through the site of the 2Pb. Conversely, after ICSI into enucleated oocytes (androgenesis), the male pronucleus migrates to a random cortical region and cytokinesis occurs irrespective of the 2Pb region. Collectively, cleavage furrow initiation in fertilized NHP zygotes depends on mitotic spindle orientation and equatorial chromosome alignment. Although the 2Pb may not be a rigid fiduciary mark of cytokinesis, a dynamic and complex signaling mechanism, with feed-back loops, between the cortex, central cytoplasm, mitotic spindle and chromosomes appears to underlie the supramolecular mechanism specifying the first cleavage axis in primates.

A Cell Biological Checkpoint for Development: Does the Spindle Checkpoint Regulate Asymmetric Spindle Positioning?

E. K. McCarthy, B. Goldstein, Biology, University of North Carolina at Chapel Hill, Chapel Hill, NC

The mechanisms that regulate asymmetric cell division are not well understood in any animal system. The first mitotic division of 2Pb may not be a rigid fiduciary mark of cytokinesis, a dynamic and complex signaling mechanism, with feed-back loops, between the cortex, central cytoplasm, mitotic spindle and chromosomes appears to underlie the supramolecular mechanism specifying the first cleavage axis in primate embryos.
anaphase transition, we disrupted proteasome activity by either treating embryos with a proteasome inhibitor or depleting embryos of a proteasome regulatory subunit. These treatments delay the metaphase to anaphase transition, as expected, and we found that these treatments also delay asymmetric spindle positioning. These results indicate that proper proteasome function is required for timely spindle positioning. As proper proteasome function is required for checkpoint signaling, we are currently further testing whether the spindle checkpoint directly regulates asymmetric spindle positioning. To test this, we are using the timing of spindle positioning after early inactivation of the checkpoint and also after constitutive activation of the checkpoint.

2023

Phosphoinositides Are Required for Male Germ Cell Polarity and Sperm Tail Formation in Drosophila

H. Wei,1 G. Polevoy,1 J. Rollins,2 C. Buzin,2 J. A. Brill,1 1Developmental and Stem Cell Biology, Hospital for Sick Children, Toronto, ON, Canada, 2Biological Sciences, St. John's University, Jamaica, NY

Phosphoinositides (PIPs) are well established as important determinants in signal transduction, vesicle trafficking, cell polarity and actin assembly in cultured cells. However, the role of PIPs in the differentiation of multicellular organisms is less well understood. Here we describe a novel approach to studying PIPs in vivo. To investigate the role of PIPs in Drosophila spermatogenesis, we introduced a potent Salmonella PIP phosphatase SigD into developing spermatids. Germline cells expressing high levels of SigD lack detectable plasma membrane phosphatidylinositol 4,5-bisphosphate (PIP2) and accumulate phosphatidylinositol 4-phosphate (PIP4). In the cytoplasm. Spermatids exhibit defects in sperm tail formation, including accumulation of wavelike structures and absence of axoneme assembly. Low level SigD expression has only mild effects on PIP levels. In addition, sperm tail formation appears normal. However, spermatids lack the F-actin-based investment cones required to individualize mature sperm. Moreover, stable intercellular bridges (ring canals) that normally connect developing spermatids are absent and cell polarity appears disrupted, as nuclei are found at both ends of elongating spermatids. This suggests PIP levels are critical for actin cone assembly, ring canal stability and spermatid polarity. Expression of a YFP tagged PIP 5-kinase (YFP-Skil), which generates PIP, from PIP4, suppresses the membrane and axoneme defects caused by high levels of SigD, indicating that the observed phenotypes are due to altered PIP levels. As YFP-Skil localizes to ring canals at the distal end of elongating spermatids, we propose that PIP biosynthesis at the growing end is important for cell polarity and efficient flagellar outgrowth during sperm development.

2024

Ankyrin-B and Ankyrin-G Determine the Physiological Segregation of Ion Channels between the Inner and Outer Segments of Photoreceptors

K. Kizhatil,1 N. Sandhu,2 N. S. Peachey,1 V. Bennett1 1Cell Biology and HHMI, Duke University, Durham, NC, 2Ophthalmic Research, Cole Eye Institute, The Cleveland Clinic Foundation, Cleveland, OH, 1Ophthalmic Research, Cole Eye Institute, The Cleveland Clinic Foundation and Cleveland VA Medical Center, Cleveland, OH

Vision requires the constant flow of a current between the photoreceptor inner and outer segments in the dark. The phototransduction cascade culminates in cessation of the "dark current" established between the photoreceptor inner and outer segments. Generation of the current requires the proper localization of distinct sets of ion channels between the inner and outer segments of photoreceptors. Specifically, localization of the cyclic nucleotide gated channel to the outer segment and the NaKATPase to the inner segment is critical for the current generation. These proteins are localized to the plasma membrane of the photoreceptor. We report that in photoreceptors of humans and mice ankyrin-B and ankyrin-G localize to the inner and outer segments in a mutually exclusive manner. We show that the inner segments of photoreceptors in the ankyrin-B haploinsufficient retina show a marked reduction of NaKATPase and the Na/Ca exchanger in a manner analogous to ankyrin-B haploinsufficient cardiomyocytes. These results show that ankyrin-B is the physiological binding partner of NaKATPase as well as Na/Ca exchanger in the inner segment of the photoreceptor. In addition, using coimmunoprecipitation and a cellular membrane recruitment assay we show that ankyrin-G interacts with the beta subunit of the cyclic nucleotide gated channels. Together these results show that ankyrin-B and ankyrin-G define distinct membrane domains of the photoreceptor essential for vision.

2025

Subcellular Compartmentalization of Drosophila Neurons

M. M. Rolls,1 D. Satoh,2 T. Uemura,2 C. Q. Doe1,2 1Institute of Neuroscience, University of Oregon, Eugene, OR, 2Kyoto University, Kyoto, Japan, 1Howard Hughes Medical Institute, Eugene, OR

It has been well-established that vertebrate neurons are divided into subcellular compartments with distinct functions and protein compositions: the soma, axons, and dendrites. It has been unclear how much of this organization is shared by invertebrate neurons. In order to establish whether Drosophila neurons are a good system in which to study subcellular compartmentalization of neurons, we have investigated whether they are organized in the same ways as vertebrate neurons. Using localization of tagged markers in Kenyon cells in the larval brain, we find that many features of vertebrate neuronal organization are also present in Drosophila. We have determined that: (1) a marker of glutamatergic synapses is exclusively localized to dendrites, while synaptic vesicles are localized to both dendrites and axons (as they are in many vertebrate neurons that perform higher order processing); (2) the machinery responsible for protein synthesis is predominantly localized to the soma; (3) the microtubule cytoskeleton preferentially binds different markers in axons and dendrites, and (4) the proximal part of the neurite shares several features with the vertebrate axon initial segment. In addition, we have investigated microtubule organization in more detail in the Drosophila peripheral nervous system. By tracking EB1-GFP movements in neurons, we find that microtubule polarity is strikingly different in axons in dendrites, as is the case in vertebrate neurons. We conclude that the subcellular organization of Drosophila neurons is very similar to that of vertebrate neurons, and that this system will be a very powerful one in which to study neuronal polarity.

2026

Cell Polarity: Can Wnt Act as a Positional Cue?

M. Roh,1 A. Mikels,2 R. Nusse,3 B. Goldstein,1 1Biology, University of North Carolina, Chapel Hill, NC, 2Developmental Biology, Stanford University, Stanford, CA

Establishing cell polarity is essential for normal development. Cell polarity requires intricate cell-cell signalling and remodeling of the underlying cytoskeleton - all phenomena that remain under-explored. The Wnt pathway is a conserved pathway, known to play a role in establishing cell polarity in a variety of organisms. Although this is a well-studied pathway, an outstanding question that remains is whether a Wnt signal can act as a positional cue. To address this, we are studying the four-cell stage C. elegans embryo. At this stage, signals from one cell to its neighbour result in polarization of the responding cell such that the responding cell divides asymmetrically. Two signals, MES-1 and MOM-2/Wnt, are sent from the signalling cell to polarize the responding cell and align the mitotic spindle as well as regulate gene expression. Although both pathways are required for polarized division, cell manipulation experiments suggest that it is the position of the Wnt signal that dictates spindle positioning (Goldstein et al., 2006). However, it remains elusive whether this is a direct effect of Wnt, or an indirect downstream effect. To address this issue, we use a novel approach in which beads coated with purified MOM-2/Wnt proteins are used to manipulate the position of the MOM-2/Wnt source on an isolated responding cell. We have also constructed a transgenic strain containing GFP-tagged tubulin in a novel approach to studying PIPs in vivo. To investigate the role of PIPs in Drosophila spermatogenesis, we introduced a potent Salmonella PIP phosphatase SigD into developing spermatids. Germline cells expressing high levels of SigD lack detectable plasma membrane phosphatidylinositol 4,5-bisphosphate (PIP2) and accumulate phosphatidylinositol 4-phosphate (PIP4). In the cytoplasm. Spermatids exhibit defects in sperm tail formation, including accumulation of wavelike structures and absence of axoneme assembly. Low level SigD expression has only mild effects on PIP levels. In addition, sperm tail formation appears normal. However, spermatids lack the F-actin-based investment cones required to individualize mature sperm. Moreover, stable intercellular bridges (ring canals) that normally connect developing spermatids are absent and cell polarity appears disrupted, as nuclei are found at both ends of elongating spermatids. This suggests PIP levels are critical for actin cone assembly, ring canal stability and spermatid polarity. Expression of a YFP tagged PIP 5-kinase (YFP-Skil), which generates PIP, from PIP4, suppresses the membrane and axoneme defects caused by high levels of SigD, indicating that the observed phenotypes are due to altered PIP levels. As YFP-Skil localizes to ring canals at the distal end of elongating spermatids, we propose that PIP biosynthesis at the growing end is important for cell polarity and efficient flagellar outgrowth during sperm development.

2027

Drosophila Tao-1 Gene Is Required for the Cortical Microtubule Nucleation in the Oocyte

W. Yang, V. Riechmann; Institute for Developmental Biology, Universitity of Cologne, Cologne, Germany

In the Drosophila oocyte, microtubules (MTs) nucleate from the anterior and lateral cortices, with the exception of the posterior pole. These cortical MTs are organized in a gradient from anterior to posterior, with the highest density in the anterior part. Along this MT network, transcripts of axis determinants, bicoid, oskar and gurken mRNA are precisely transported to distinct locations. Thus, the polarized MT network determines the anterior/posterior axis. Although cortical MT nucleation is essential for polarizing the oocyte, how this is regulated is unknown. Many mutants affecting the polarity of the MT network have been identified, but most of these mutants show only partially impaired polarity and do not affect cortical MT nucleation. This is reflected by the distribution of bicoid, oskar and gurken, which localize aberrantly, but which are still transported in a polar manner in these mutants. In contrast, a mutant affecting cortical MT nucleation would completely abolish polar mRNA transport. Here we report such a novel MT phenotype affecting cortical MT nucleation. This phenotype is caused by a mutation in the gene encoding the conserved Ser/Thr kinase Tao-1. Tao-1 protein localizes at the oocyte cortex. In tao-1 mutants, TuRC+, y tubulin and Dgrip, are not enriched in the oocyte cortex but dispersed throughout the ooplasm. This is accompanied with a strong reduction of cortical MT nucleation and a strong accumulation of randomly oriented MTs within the ooplasm. Consistently, MT polarity markers are also present throughout the ooplasm rather than at the anterior and posterior poles. Therefore, polarity of the MT network is completely lost in tao-1.
Asymmetric cell division is a fundamental process to generate cell diversity during development. We use the early *C. elegans* embryos to study this process. In the one cell embryo the conserved PAR-proteins (partitioning defective) govern the establishment and maintenance of cell polarity. PAR-3, PAR-6 and PKC-3 form a protein complex (PAR-3/6/3 complex) and localize to the anterior cortex while PAR-2 is enriched on the posterior cortex. In order to define how the extracellular environment affects cell polarity, we analyzed the organization of individual cells plated on defined micro-patterned substrates imposing cells to spread on various combinations of adhesive and non-adhesive areas. The reproducible normalization effect on overall cell morphology and orientation quantification of the spatial organization of the actin network and associated proteins; the distribution of microtubules; and the positioning of nucleus, centrosome and Golgi apparatus. By using specific micropatterns and statistical analysis of cell compartments positions, we demonstrated that ECM geometry determines the orientation of cell polarity axes. The nucleus-centrosome orientations were reproducibly directed towards cell adhesive edges. The anisotropy of the cell cortex in response to the adhesive conditions did not affect the centrosome positioning at the cell centroid. Based on the quantification of microtubule plus end distribution we propose a quantitative model that accounts for these observations. In conclusion, we show that LARP-1 and NOS-3 have a novel role in cell polarity by regulating the expression of PAR-6 in the embryo.

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Cell polarity is a shared feature of numerous cell types from bacteria to mammals. Strikingly, while cells exhibit various shapes and perform different functions, the core proteins establishing cell polarity are common to all eukaryotic cells types. *Drosophila* epithelia exhibit 2 types of polarity: an Apical/Basal (A/B) polarity and a second along the plane of the epithelium (Planar Cell Polarity or PCP). Biochemical and genetic evidence indicates that some A/B polarity and PCP factors can be in fact involved in both types of cellular polarization. Scribble, a protein that is identified through its requirement in A/B polarity of epithelial cells, form a complex with Dlg (Discs large) and Lgl (Lethal Giant larvae). Our results show that this polarity complex, physically and genetically interacts with Strabismus, a core PCK gene, to regulate epithelial patterning in *Drosophila* tissues.

### 1934

**Hepatocyte Nuclear Factor 4α Provokes Microvillus Formation via Induction of ERM-binding Phosphoprotein 50**

H. Chiba, N. Sakai, M. Murata, M. Osanai, K. Takashi, N. Sawada; Department of Pathology, Sapporo Medical University School of Medicine, Sapporo, Japan

Microvilli are actin-based organelles on apical plasma membranes and involved in nutrient uptake and transduction of various stimuli. Numerous microvillus components, including ezrin/radixin/moesin (ERM) proteins, which upon phosphorylation link filamentous actins to transmembrane proteins, have been identified, but it is unclear which signals drive microvillus biogenesis. Here we show, using two distinct cell lines, that conditionally or ectopically expressed hepatocyte nuclear factor 4α (HNF4α) provokes EBP-binding phosphoprotein 50 (EBP50) expression and de novo formation of microvilli on cells expressing HNF4α alone. Notably, RNA interference in EBP50 inhibited these changes and microvillus development. Furthermore, we employed wild-type and RXRa/RXb-deficient F9 cells, and demonstrated that these retinoid receptors activated ezrin expression but hardly contributed to microvillus organization. These findings indicate that HNF4α, but not retinoid receptors, acts as a powerful morphogen to trigger microvillus formation via induction of EBP50.

### 1935

**Teacher Professional Development Programs - Key to Student Success**

M. A. Sognier, C. W. Houston; Universities Space Research Association, NASA Johnson Space Center, Houston, TX, 2Educational Outreach, University of Texas Medical Branch, Galveston, TX

To inspire the next generation of STEM-based career workers, UTMB has implemented: 1) Teacher professional development programs and 2) student science enrichment pipeline programs (Mol Biol Cell 15:231a). To meet the needs of area teachers, the UTMB Office of Educational Outreach provides six programs. 1) The Annual Science Teacher Conference is a one-day event where K-12th grade teachers experience workshops provided by leading educational experts and view science education vendor exhibitors. 2) The Galveston County Regional Collaborative for Excellence in Science teaching is part of a state-wide network providing high intensity sustained teacher workshops where educators in grades K-12 receive more than 100 contact hours/year of standards-based content with accompanying kits/supplies for classroom implementation. 3) The teacher Institute for the Advancement of Space Biomedical Science provides 4-12th grade teachers with a one-week immersion experience in space sciences followed by another week to develop space-related, standards-aligned classroom science activities. These activities, published as the Space Connections book, are also now available in Spanish. 4) The High School Summer Research Program provides teachers with a biomedical sciences immersion experience. Teachers spend 8 weeks in a faculty mentor’s research lab learning state-of-the-art science and technology while performing a project that is presented in a formal poster session and also developing an experience-based classroom activity. 5) The Science Education Resource Center loans educational kits (FOSS, GEMS, etc.), supplies, and equipment for classroom use. This is an essential resource for many teachers where district funding cannot provide needed supplies to effectively teach content. Participation in these high-quality, high-intensity professional development programs increases teachers content knowledge, provides necessary supplies, establishes an effective teacher network, and enhances their ability to inspire students to pursue future STEM-based careers. Supported by grants from the Howard Hughes Medical Institute, National Space Biomedical Research Institute, and Texas Regional Collaboratives.

### 1936

**A Study of the Impact of an Informal Science Education Program on Middle School Students’ Science Knowledge, Science Attitude, STEM High School and College Course Selections, and Career Decisions**

M. A. Ricks; Baytown, TX

The Summer Science Camp (SSC) I at the University of Texas Medical Branch (UTMB) is an informal summer science education program for 7th and 8th grade pre-college students. The SSC I program engages participants in authentic hands-on laboratory-based scientific experiments and STEM (science, technology, engineering and mathematics) related experiences. The SSC I program expected to increase participants’ science knowledge and affect a positive influence on their attitudes and outlook toward science careers and issues. This study has a twofold purpose represented respectively by a quantitative and qualitative approach. Study approach one intended to assess improvements and changes in participants’ science knowledge and science attitudes based on their SSC I experiences. Data from participants in the 2005 UTMB 7th and 8th grade SSC I were used to address this perspective and assess the program’s impact on changes in students’ science knowledge and science attitudes. Study approach two assessed information about decisions previously (1993-1999) participants may have made in selecting advanced STEM courses, and career decisions as a result of participation in the SSC. This second study perspective used data from questionnaires, interviews, and program evaluation forms collected from the 1993-1999 SSC participants. The findings were statistically significant for Study approach one, showing an increase in participants’ science knowledge and science attitude. Study approach two also determined statistical significance through contextual descriptions about the STEM selections of former SSC I participants from a retrospective point of view. The outcomes of this study suggest that the innovative hands-on learning activities, problem-solving experiences, and use of authentic scientific tools were important elements for students’ meaningful understanding and appreciation of science concepts. The study proposes that the elements of this informal program are important and recognizable forces that impact students’ science learning, science attitudes, interests, and decisions.

### 1937

**Pre-college Education: Basic Research Is an Excellent Educational Tool**

M. C. Fields; Neuroscience Research Course, Sidwell Friends School, Washington, DC

The Neuroscience Research Class at Sidwell Friends School (SFS) is an elective which allows students to participate in a course that replaces the traditional method of teaching with a research approach. Summer reading of scientific articles supplied by the instructor provides the necessary background for students to begin a three-month investigation in the fall. Collaborating with scientists via the internet and in person exposes participants to scientific methods, research, and analysis. This course promotes critical thinking, problem solving, and data analysis through statistical methods. Students gain an appreciation of scientific research while they master concepts from the core of biology, including but not limited to immunology, neuroscience, cytology, developmental biology, genetics, protein synthesis, and DNA replication. Modern research techniques used in neuroscience and molecular biology are investigated and performed. Students are no longer the passive recipients of knowledge, but acquire it actively and through collaborative effort to complete the research and produce a scientific poster for presentation at a scientific meeting (SSC I, Biological Society, Society for Neuroscience). Current projects include studying the neurobiology and behavior of weakly electric fish, the developmental biology of zebrafish, researching slow wave sleep patterns in crustaceans, and tectemicroscopy. Student-designed protocols for these projects as well as data and conclusions are included in this poster. This poster also presents the origins and history of the course as well as the mechanics for conducting such a class.

### 1938

**Innovative Use of Assessment Rubrics Promotes Successful Scientific Collaboration in the High School Biology Classroom**

E. M. Stone; Berkeley High School, Berkeley, CA

Alternates of assessment are especially useful for measuring learning for students who do not perform well using traditional assessment methods. In a long-term effort aimed at creating different types of assessment that are effective for all students in the high school classroom, a computer-based curriculum called “Cell Reproduction & Human Diseases” was designed in the Web-based Inquiry Science Environment (WISE; http://wise.berkeley.edu). Different methods for assessing what students know and understand pre/post test drawings of cell division, embedded ongoing assessment questions, modeling tasks, multiple-choice test items, and a final poster presentation on a disease related to mitosis or meiosis. Previously the poster has been assessed for understanding of cell division defects and mechanisms of disease and treatment. Those student teams that are more successful at working together to research and present their poster have been able to produce a higher quality final product; however, the collaborative effort was not explicitly assessed. Thus a Group Collaboration Rubric was designed to measure this essential component of the scientific process. The rubric assesses components that categorize successful collaboration in the research laboratory: sharing ideas, distributing work, using time efficiently, and decision-making. When using the rubric, students are asked to provide specific evidence that describes the extent to which they met criteria for effective scientific collaborations. Evaluation of the use of this rubric in the classroom indicates that the value of collaboration becomes more transparent to students. Moreover, students demonstrate an ability to honestly and
critically reflect on what they can do to improve teamwork with their classmates. Ideas will be presented for how the different assessment tools developed in this work can be applied to other learning contexts and across secondary, undergraduate, and graduate curricula.

1939

Teaching Scientist Volunteers about K-12 Science Education, Pedagogy, and Partnership

P. Caldera, J. MacCormack; University of California, San Francisco, San Francisco, CA

Many scientists today see opportunities to share their commitment to science with the wider community and are interested in lending their expertise to teachers and their students with a view to improving science education. Scientists are enthusiastic about sharing their science knowledge and skills, but they generally have had limited experience in K-12 classroom settings. Based on our experience with scientist volunteers working in K-12 classrooms, the Science and Health Education Partnership (SEP) at the University of California, San Francisco, developed a workshop series to support scientist volunteers in their teaching efforts. Utilizing hands-on, interactive science activities, this workshop series is designed to equip scientist volunteers with teaching strategies that will enable them to: focus on the concrete in their teaching; involve all students in classroom lessons; get to know learners through pre-assessment; incorporate inquiry-based methods in their approaches; consider all aspects of lesson-planning (e.g., setting specific lesson goals, managing materials, addressing language needs); and work in partnership with teachers by acknowledging and relying on teachers' expertise in areas such as classroom management and cognitive development. First offered ten years ago, the workshop series has evolved over the years to better address the needs of scientist volunteers. Scientist volunteers who have taken part in the workshop series consistently rate the workshops very highly, report improvement in their teaching skills, and have integrated many of the ideas and strategies in their work in K-12 classrooms.

1940

Transformation of a Traditional Biology Laboratory Course into a Research Experience

E. Vasquez, C. Ricart, M. Echegaray, R. Chiesa; Biology, University of Puerto Rico at Cayey, Cayey, Puerto Rico

The traditional laboratory exercises of a General Biology course have been substituted with research projects where students go through the process of identifying a problem, developing an experimental design, analyzing data, and interpreting results to reach meaningful conclusions. In this approach, the professor becomes a mentor and the students, who are mostly freshmen, become researchers. Our data demonstrate that this strategy is highly successful by empowering students with scientific knowledge acquired through a hands-on experience. It has also developed a keen interest in most participants to pursue advanced degrees in science. The effect that this experience may have in retention, overall academic performance, and skills is being assessed through a longitudinal study. So far we have impacted seventy students in two years. Thirty research projects have been completed, many of which have been presented at scientific meetings. This learning model allows for deep understanding of fundamental scientific concepts and the true nature of the scientific endeavor.

1942

Engaging Undergraduates in the Emerging Field of Synthetic Biology

A. M. Campbell, E. E. Zwick, K. A. Haynes, S. Simpson, L. J. Heyer, W. L. Harden, S. N. Rosenmold, T. T. Eckdahl, A. D. Brown, T. L. Butner, L. H. Heard, E. L. Jensen, K. J. Mallory, B. J. Ogden, J. Poest, M. L. Broderick; Department of Biology, Davidson College, Davidson, NC, Department of Business, Davidson College, Davidson, NC, Biology, Hampton University, Hampton, VA, Department of Biology, Missouri Western State University, St. Joseph, MO, Department of Mathematics, Missouri Western State University, St. Joseph, MO, Research experiences that are multidisciplinary, challenging, and team-oriented provide undergraduates with the training they need to be successful scientists. We found that the emerging field of synthetic biology offers ideal research training. Synthetic biology uses mathematics, biology, and the principles of engineering to program cells to carry out new functions. We competed in the 2006 International Genetically Engineered Machines (iGEM) Synthetic Biology Jamboree at MIT, along with 36 other teams from around the world. Our multi-institutional team included sophomore and junior undergraduates majoring in biology and mathematics with little or no previous research experience. The team defined a good mathematical problem—the pancake problem (sorting by flipping). These devices allow us to design appropriate DNA devices for these applications (e.g., control recombination of genetic elements) and DNA computing (e.g., evolution of syntonic chromosones). The project involved creating simulations, investigating pancake graphs, modeling kinetins, searching databases for components (i.e., genes and enzymes), using the iGEM registry for some parts, constructing our own parts, and engineering bacteria to carry out inversions. We found synthetic biology to be an inexpensive and enjoyable way to learn, teach, share, collaborate, and conduct original research with undergraduates.

1943

Cell Biology and Genetics Laboratory Modules Incorporating the Desert Tree Lizard as a Model Organism

P. A. Marshall, C. E. Deutch, D. Dennis; Integrated Natural Sciences, Arizona State University at the West Campus, Phoenix, AZ

The Department of Zoology and the Desert Natural Science Institute at the University of Arizona have adopted a new curriculum at the West Campus that leading to a B.S. degree in the Life Sciences. This curriculum includes two semesters of General Biology, core courses with laboratories in Fundamentals of Genetics, Cell Biology, and Ecology, and clusters of upper-level electives in Cellular, Molecular, and Physiological Biology, Organismal Biology, and Integrative Systems Ecology. An innovative feature of this curriculum is the inclusion of experimental work with the desert tree lizard (Urosaurus ornatus) in many different courses to link them together thematically. While the experiments in the General Biology course focus primarily on the morphology and behavioral ecology of these organisms, those in the required courses in Fundamentals of Genetics and Cell Biology are designed to introduce students to techniques in modern cell biology. In the laboratory for the Fundamentals of Genetics course, students carry out a two-week project using non-denaturing gel electrophoresis in agarose gels to study the occurrence of alleles of acosta hydratase and the NADP-dependent L-malate dehydrogenase in different individuals in captive populations. In the laboratory for the Cell Biology course, students perform a two-week project using SDS-polyacrylamide gel electrophoresis to compare the proteins found in various lizard organs including heart and liver. In both cases, the students gain experience in casting and running gels, in staining and imaging the gels digitally, and in analyzing the images quantitatively. Additional ideas will be presented for how the different assessment tools developed in this work can be applied to other learning contexts and across secondary, undergraduate, and graduate curricula.

1944

Biotechnology for Science-Phobes

T. L. Beaty; Chemistry & Physics, Le Moyne College, Syracuse, NY

The majority of college non-science majors who must take a science course to graduate are wary and nervous about this curricular requirement. “Biotechnology: Wonder Drugs to Mutant Bugs” is an introductory biotechnology course designed for these students. They learn the basic science concepts underlying several important fields of biotechnology, including DNA fingerprinting, genetic screening, recombinant DNA, nuclear transfer cloning, and bioprospecting. Due to the students’ resistance to learning science, care is taken to ensure their understanding of essential concepts (i.e., molecular polarity and gene expression) and techniques (i.e., gel electrophoresis and fermentation), which are important to a wide range of biotechnology applications. Historical relevance and societal implications are integrated into each unit to help students gain an appreciation of the “big picture.” By the end of the course, students can interpret popular media reports of biotechnology, discuss the general product line of an active biotech company, and debate the ethical considerations of specific applications.

1945

How Does Using Critical Literacy Help Teachers Understand Implications of the Human Genome Project?

M. L. Gleason, 1 E. E. Zwack, 1 K. L. M. Kleine 1; Biological and Environmental Sciences, Georgia College & State University, Milledgeville, GA, 1Early Childhood and Middle Grades Education, Georgia College & State University, Milledgeville, GA

In a reading- and writing-intensive course taught in the summer of 2006 at a small liberal arts university we used a critical literacy approach (Alvermann and Hagood, 2000) to help teachers challenge their subtle attitudes, values, and beliefs about the ethical, legal, and social implications (ELSI) of issues arising from advances in human genetics. Sixteen in-service middle and high school teachers (half each from science and English) were collaboratively taught by a biologist, a linguist, and a science educator, with the assistance of two graduate students. Learning was measured by 1) pre- and post- assessments of content acquisition; 2) change in depth of students’ oral responses over time; 3) number of genetic practices and ELSI concerns identified by students upon a reviewing of the film GATTACA (Nichol, 1997), 4) formal essays written during the course, and 5) a final journal entry completed about the meaning and applicability of
critical literacy as a pedagogical tool. In a concept mapping post-assessment 100% used all terms correctly compared with 50% in the pre-assessment; similarly 88% provided propositions that accurately established relationships between concepts, compared with only 38% before. Early oral responses showed that only two teachers had some familiarity with prenatal genetic diagnosis options compared with 16 by course’s end. Identification of genomic practices and ELSI concerns by teachers during the second viewing of GATTACA ranged from 3 to 24 (mean = 13) practices and 5 to 26 issues (mean = 18). Gains as evidenced through writings and journal entry were difficult to enumerate, but were enlightening and, with the aforementioned data, supported our premise that the personalized focus on the integration of thoughtful reading and writing in this class enhanced the teachers’ professional and intellectual development, preparing them to provide a critical literacy approach in their classrooms this fall.

1946 
Representing Time and Space in Animations of Molecular Machines
J. E. Honts; Biology, Drake University, Des Moines, IA
The sarcoplasmic calcium pump/ATPase is an example of a macromolecular machine that is frequently depicted in textbook and teaching resources in terms of its structure and mechanism. We have initiated a study that compares the pedagogical effectiveness of various representations of this ion-pumping molecular machine. In spring 2006, 26 students participated in a study comparing 9 models of representing change in the structure and function of this pump over time. In one of these, motion was continuous over the pumping cycle (a “fluid animation”). In the other, key frames were selected from the fluid animation, so that time was represented in a discontinuous manner (a series of static “snapshots”). Participants were randomly assigned to view one of the two presentations. A short written pre-viewing evaluation was administered to assess the students’ understanding of the structure and mechanism of the calcium pump. Students were also asked to draw a diagram representing the structure and function of the pump. Upon completion of these tasks, students were asked to view their assigned presentation for ten minutes (sufficient time for several repetitions of the presentations). Following viewing, students were asked to repeat the written evaluation, draw a new diagram, and answer several questions concerning the presentations. An analysis of scores on the pre- and post-viewing evaluations revealed no statistically significant difference between the two groups with respect to scores obtained on the post-viewing evaluation. Both groups did, however, show a significant improvement in post-viewing over pre-viewing scores. The student responses also revealed that a small number of students developed specific misconceptions as a result of viewing the presentations. While these largely relate to minor aspects of the structure and mechanism, they will be investigated further so that they can be remedied in revisions of these presentations and others under development.

1947 
Student Mental Models of Cellular Structure: A Non-Majors’ Perspective
I. Ecarma-Robinson, J. Robinson, M. L. Casem; Biological Science, California State University, Fullerton, Fullerton, CA
The objective of this research was to investigate the mental models of cellular structure held by undergraduates outside of the biology major and to examine the impact of instruction on student perceptions. Students were prompted to draw and label a cell. Drawings were collected post-instruction from students enrolled in a lower division, general education biology survey course. Student-generated images were evaluated using a calibrated rubric that assessed the diversity of elements within the drawing, and the correlation between the image and the label. The majority of students represent a cell as being eukaryotic (containing a nucleus) and round or oval in shape. Non-majors were equally likely to label the primary line of their drawing as cell wall versus membrane (30% vs 39%). Non-majors’ drawings did not include details of the phospholipid bilayer. The average student-generated drawing included 5.3 ± 0.6 distinct items and 5.0 ± 0.25 labels; however, the match between label and image averaged 2.7 ± 0.08. The most common labels were nucleus, mitochondria, and ribosome, while the most common images reasonably represented the nucleus, ribosomes, and generic vesicular structures. The general biology course is taught in multiple sections by multiple instructors. A statistical relationship exists between the course instructor and performance of students as regards the complexity of their drawings, the number of labels used in the drawing, and their ability to correctly associate the name of a structure (label) and its appearance (mental model). The results of this research indicate that undergraduates completing a non-majors’ general survey course in biology possess a mental model of the cell that is relatively similar and simple to the mental models reported by beginning biology majors. The variation in student drawings as a function of instructor may reflect differences in the emphasis placed on cellular structure or instructional strategies employed by diverse faculty members.

1948 
Student Mental Models of Cellular Structure: A Biology Major’s Perspective
A. Nazarain, M. Shin, S. Shirzadegan, M. L. Casem; Biological Science, California State University, Fullerton, Fullerton, CA
The objective of this research was to gain insight into student mental models of cellular structure, to examine the impact of instruction on student perceptions, and to identify misconceptions. Students were prompted to draw and label a cell. Drawings were collected pre- and post-instruction from students enrolled in a lower division cellular biology course. Drawings were also collected from graduating seniors. Student-generated images were evaluated using a calibrated rubric that assessed the diversity of elements within the drawing, and the correlation between the image and the label. The majority of all students represent a cell as being eukaryotic (containing a nucleus) and round or oval in shape. Only 37% of beginning students label the cell’s plasma membrane and only 4% provide details of the phospholipid bilayer compared with graduating seniors of whom 72% labeled the membrane and 32% represented the bilayer structure. Student drawings are significantly more complex post-instruction (7.2 ± 0.09) compared with pre-instruction (3.84 ± 0.12), and remain at about the same level for graduating seniors (6.83 ± 0.16). At least half of all beginning biology majors tested included a nucleus that assessed the diversity of elements within their initial drawing. Post-instruction, or at graduation, over half the students tested also included the rough ER, smooth ER, Golgi apparatus, ribosomes, and vesicles in their drawings. Graduating seniors used more labels in their drawings compared with post-instruction students (7.78 ± 0.17 vs 7.13 ± 0.13) and significantly more of their drawings matched between label and image (3.7 ± 0.02). Misconceptions related to cell walls, membranes, and chloroplasts were identified. Results of this study indicate that student mental models of a cell are enhanced by instruction and that gains made early in the major are retained at graduation. Student perception of a cell favors eukaryotic over prokaryotic and animal over plant cells, possibly indicating an instructional bias.

1949 
Incorporation of an Original Research Theme into Three Advanced Undergraduate Laboratory Classes in Cell Biology, Molecular Biology, and Biochemistry
C. M. O’connor, M. J. Pietelli, A. R. Wyman; Biology, Boston College, Chestnut Hill, MA
An integrated group of three advanced undergraduate laboratory classes in cell biology, molecular biology and biochemistry were designed to introduce students to original research, using the fission yeast, Schizosaccharomyces pombe, as an experimental model. Each of the three courses studies discrete aspects of a common research theme with discipline-specific tools. Common research foci are protein repair enzymes and enzymes that protect cells from reactive oxygen species (ROS). These enzymes were chosen for study because of their strong evolutionary conservation and because of growing evidence that these enzymes protect organisms against a variety of degenerative diseases. After an introduction to the biology of ROS, students chose to study thioredoxin peroxidase, glutathione peroxidase, thioredoxin and superoxide dismutase. Students used genomics resources to guide the construction of molecular clones and yeast strains. Students worked in teams to develop hypotheses, design and conduct experiments, and analyze and present experimental data. Cell biology students constructed yeast strains that overexpressed the ROS protective enzymes and monitored the effects of enzyme overexpression on resistance to oxidative stress. Students in the molecular biology class studied the expression of the same enzymes under a range of controlled environmental conditions and developed new expression vectors. Biochemistry students overexpressed the enzymes in bacteria, purified the enzymes, and developed enzymatic assays used to biochemically characterize the yeast enzymes. Integration of research activities among the three classes provided students with an interdisciplinary perspective to the research problems and built a learning community of students and faculty. A common website, the Boston College Biology Commons (BC2), provides a central forum with links to class data, experimental protocols, tutorials and database links. Students responding to questionnaires express a sense of greater competency and a higher level of satisfaction with these project-based classes compared with more scripted laboratory classes.

1950 
Courses to Integrate Biology, Computer Science, and Physics for Undergraduate Science Majors
R. P. Donaldson,1 S. Church,1 R. Sinha,2 M. E. Reeves; 1Biological Sciences, George Washington University, Washington, DC; 2Computer Science, George Washington University, Washington, DC, 1Physics, George Washington University, Washington, DC
Our objective is to develop courses that bring together quantitative-computational skills and physical principles with the concepts of molecular biology in the first year of a university education. A pilot course was developed and taught this year to 16 students to test the feasibility of this approach. We implemented a series of active learning modules in two courses, Biology of Proteins and Introduction to Bioinformatics. The first course included solving problems on the effects of pH on amino acids, enzyme kinetics, computational simulations of chemical reactions, networks and differential equations, the principles of entropy applied to proteins, 3D visualization of protein structures, and group research projects on selected protein families. Formative assessments were weekly quizzes, homework problems, and written and oral reports. The second course involved the use of online databases, understanding basic bioinformatics algorithms such as sequence alignment, 358

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implementing gene prediction methods, and creating phylogenetic trees. Students were expected to formulate questions and explore various methods of analysis based on data available in the databases. Students engaged in projects on complex questions that often revealed the limits of available data relative to the types of questions that interested the students. For the freshmen, their development as scientists was apparent based on increasing complexity of their projects as well as the solutions proposed when database or computational limits hindered their research projects. We observed that freshmen were more adventurous than upperclassmen in their choice of projects and willingness to conform database or computational limits in their research. A significant challenge in implementing the courses was in selecting appropriate students. We assessed learning and attitude outcomes for the students who took these courses in the 2005-06 academic year. Three of the 12 freshmen in the courses did research internships the following summer. Supported by grants from HHMI and the GWU CCAS Teaching Initiative.

1951
Isolation and Characterization of Yeast Mutants Defective in Chromosome Segregation in an Undergraduate Genetics Research Laboratory Course
H. Sleister; Drake University, Des Moines, IA
In an effort to expose undergraduate students to scientific inquiry, an upper-level genetics project-based laboratory course was developed. Students are immersed in a research project with the ultimate goal of identifying proteins important for chromosome transmission in mitosis. After mutagenizing yeast Saccharomyces cerevisiae cells, students implement a genetic screen that allows for visual detection of mutants with an increased loss of an ADE2-marked yeast artificial chromosome (YAC). Students then genetically characterize the mutants and begin efforts to identify the defective gene in these mutants. While engaged in this research project, students learn a variety of technical skills in both classical and molecular genetics. Furthermore, they learn to collaborate and gain experience in sharing scientific findings with others in the form of written papers, poster presentations, and oral presentations.

1952
Report of Observation-, Experience-, and Exercise-based Understanding Class of Cell and Human Biological Systems for Integration of Cell Biology and Health Education at the University of Tokyo
T. Sakurai, Y. Atomi, Department of Life Sciences, The Graduate School of Arts and Sciences, The University of Tokyo, Tokyo, Japan
Understanding of own cell and life system which work in his/her body may contribute to cell biology education as well as health education. At the 44th Annual Meeting, we made a presentation of report of the class of cell biology education and health education in physical exercise class for undergraduate students. We named the class “Sport Science Course.” In this course, students learn cell and body systems through dynamism at different of self-individuals, functional tissues in mammalian, and cells in mammalian tissues and cultured cells on dishes. Self-recognition, exercise physiology for self body are effective for extracting motivation of learning and understanding self biological system of the body. In the physical exercise course for freshmen of the University of Tokyo, we give five contents 1) Integrative brain system and disagreement of his/her intention and the output, 2) Measuring of his/her own body alignment and flexibility, 3) Supporting system of endurance running from heart rate and respiratory responses to gravity, linear and non-linear phenomena, respectively, 4) Observations of mammalian tissues, beating cultured primary heart cells, muscle cells during differentiation process from myoblasts to myotube and neuronal cells, and 5) Information of basic life support for accident in physical exercises. In observation of tissue and cells, use of fluorescent dyes helps us to perceive our body from cellular basis. Especially beating heart cells and a process of muscle differentiation are appropriated for understanding automanaged, self-propagating chemical system constituted by fibrous protein cytoskeleton and motor system developing tension from cell to human body. A principle of a use dependency in brain and muscle function at cellular level is educable hypothesis for self-oriented health education. This class orients to the understanding of our own body and cell systems by observation-, experience-, and exercise-based learning methods.

1953
Integrating Technology in the Teaching of the Undergraduate Molecular Cell Biology Curriculum
D. B. Taylor; College of Science, Benedictine University, Lisle, IL
Structural biology and bioinformatics are common tools of the modern day cell biologist. How do faculty members teaching Molecular Cell Biology in a post-genomics era engage students in interactive learning about macromolecular structure/function relationships in an undergraduate (UG) curriculum? A fundamental problem is that of visualization: •High-end molecular modeling software requires dedicated expensive workstations and is best used for individual research projects •Chalkboard drawings do not illustrate the intricacies of macromolecular structure/function •The web-based exhibits allow the simultaneous viewing of molecules while reading descriptive text •The exhibits are investigatory •The exhibits allow students to learn about macromolecular structure at their own pace •Another tool ideal for the integration of technology in the teaching of Molecular Cell Biology is the multimedia software tool The Dynamic Cell •Available at Springer-Verlag 2000. The Dynamic Cell depicts the cell in a series of animated 3-D scenes providing instructor and student with an interactive visual tour of the cell demonstrating structure/function relationships of complex cellular processes in a dynamic fashion (http://www.springer.de). The advantages of using The Dynamic Cell as a tool to integrate visualization of 3-D structures of macromolecules with cellular processes in the teaching of Molecular Cell Biology will be discussed. Supported in part by the Scholl Endowed Chair in Health Sciences.

1954
Making Connections: An Inquiry-based Cell Biology Course That Integrates Biology and Chemistry
C. H. Sullivan, C. J. Brubaker, N. Greg Little, V. Pratt, J. C. Robison; Biology, Grinnell College, Grinnell, IA
In a restructuring of the biology curriculum to better emphasize investigative learning and to support a new Biological Chemistry major, the core course, Biology 251 Molecules, Cells, and Organisms was developed. BIO 251 is an intermediate course that predominantly serves two student audiences. Biology majors will proceed to the Organisms, Evolution and Ecology course while biological chemistry majors follow BIO 251 with Introduction to Biological Chemistry. All students entering BIO 251 have taken at least one semester of introductory chemistry and an introductory biology course that emphasizes an inquiry-based approach to studying biological questions related to a specific research topic. Therefore our students have a common set of analytical skills: reading primary literature, defining good questions, designing experiments, analyzing and presenting data, and writing scientific papers. BIO 251 is organized to capitalize on these skills by allowing laboratory work to drive the content and order of lectures and other classroom activities. The course consists of four modules: mutagenesis, gene expression, metabolism, and organismal movement using two species of bacteria, several plant species, and Caenorhabditis elegans. Each module teaches the students basic techniques that can be expanded into a student-designed project. Students keep a laboratory notebook and write two scientific papers based on their independent projects. Pre-test/post-test comparisons demonstrate significant improvement in the areas of factual information, data interpretation, and experimental design. A second important goal of our core curriculum is to emphasize the connections between biology and chemistry, and we require organic chemistry 1 as a co-requisite for Bio 251. Workshops for biology and chemistry faculty have produced a student manual (Investigations) that emphasizes common approaches used in teaching biology and chemistry courses at Grinnell. The second outcome of this workshops is an archive of lecture examples, problem sets, and exam questions that students will encounter in both courses.

1956
Student Mobility Profile: An Instrument for Measuring the Effectiveness of Efforts to Improve Teaching and Learning
W. S. Bradshaw; J. Nelson, 2 J. D. Bell; 1Microbiology and Molecular Biology, Brigham Young University, Provo, UT; 2Physiology and Developmental Biology, Brigham Young University, Provo, UT
The process of educational renewal, including efforts to improve the learning of cell biology, should incorporate assessment - a rigorous evaluation of whether or not changes made to a course actually achieved their intended goals. The Student Mobility Profile (SMP) is a novel statistical instrument designed to facilitate such an assessment. The SMP will process data obtained from the beginning and end of a scholastic interval, scores from a first and final exam, or from a pre and post semester problem-solving exercise, for example. An important advantage is that it can be applied post hoc allowing one to utilize existing data from past versions of a course for comparison to recent offerings in which innovations have been attempted. The results of the analysis indicate the presence or absence of systematic factors leading to either improvement or decline in performance of the student population over the time period being assessed. Consequently, instead of indicating whether students have simply acquired the factual information from the course, the analysis reveals the extent to which they have improved in their ability to learn. For example, the teacher might discover whether students have developed additional expertise in practicing an important scientific skill, such as drawing valid conclusions from experimental data. The theory and statistical procedures that underlie the instrument are described, along with examples of its application in university science courses. The results of computer simulations to determine the range and limitations of this instrument will also be reported.

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In the absence of a laboratory fundamental issue in biology are still accessible to students by asking simple, creative questions. Here we queried developmental challenges between mouse and fly using standard computational genomic approaches. The genetics of Drosopha has identified a number of genes involved in oogenesis and early development. In Mus genetic screens are more difficult to perform; as a result the genetics of oogenesis and early development are not as well characterized. We utilized Drosophila as a genetic screen for use in Mus by establishing a computational search for Mus homologs of Drosophila genes involved in oogenesis and early development. Conserved genes are functionally significant, while genes which are apparently absent in mouse may identify divergent strategies for solving the same developmental challenge. Using Flybase as a starting point we generated a list of over 100 target genes; in particular genes which function in germ-line stem cells (such as vasa and nanos), oocyte determination and anterior-posterior axis formation (gurken and pamilo), dorsal group genes (mago nashi and cappucino), terminal genes (torso and torshilde) and posterior group genes (oskar and staufen). In a progressive fashion we searched for homologs by gene name at SWISS-PROT and Entrez Gene, by protein sequence similarity at SWISS-PROT and MGI, and by conserved protein domains using ProDom. For protein sequence similarity searching we used BLAST and PSI-BLAST to find bi-directional best hits. Our results discovered "better-fit" homologs of previously identified homologs, new homologs, and an apparent absence of homologs. Interestingly, most of the missing homologs are genes involved in polarity establishment in Drosophila oogenesis, a process which appears to be fundamentally different in Mus. The next steps are to refine the meaning of a negative result and determine when in evolution the "missing" homologs dropped out.

**Trends in Histology Laboratory Teaching in US Medical Schools**

R. A. Bloodgood,1, 2 R. W. Ogilvie; 1Cell Biology, University of Virginia School of Medicine, Charlottesville, VA, 2Cell Biology and Anatomy, Medical University of South Carolina, Charleston, SC

Laboratory instruction for U.S. medical students has been undergoing dramatic change. Between the 1960-61 and 1985-86 academic years, total laboratory teaching hours at the Case Western Reserve University School of Medicine declined by 65% (Guth et al., 1992, Acad. Med. 67:203-206). Nationwide, hours of laboratory instruction in all the anatomical sciences declined by 52% between 1967 and 2003 (Gartner, 2003, Clinical Anatomy 16:434-439). Factors contributing to this decline probably include increased emphasis on NIH funded research, managed care, changes in medical practice, reductions in total hours allotted to basic science teaching, and advances in computer-aided instruction. To identify recent trends in histology laboratory instruction in U.S. medical schools, a detailed web survey was administered to histology course directors, with about two-thirds of U.S. medical schools responding. Consistent with the long term trend of declining total laboratory teaching hours in U.S. medical schools, there is an ongoing reduction in the number of hours of faculty-directed histology laboratory instruction that each medical student receives, with a concomitant reduction in hours of faculty time devoted to histology laboratory instruction. In terms of the tools used in the histology laboratory, there has been a dramatic increase in the use of various forms of computer-aided instruction (including Virtual Slides). Contrary to expectations, this increase in the use of Computer-Aided Instruction (CAI) (and especially Virtual Microscopy) has not been accompanied by an equivalent elimination of the use of microscopes and glass slides. Rather, the clear trend has been toward a blending of the new computer-based instruction technologies with the long-standing use of microscopes and glass slides. Many of the course directors surveyed here argue that the intimate involvement of trained faculty is the key to the quality of histology laboratory education, rather than the choice of instructional tools.

**Teaching Postdoctoral Fellowship: A Means of Faculty Development and Curriculum Enhancement**

D. C. Barnard, M. S. Ledbetter; Biology, College of the Holy Cross, Worcester, MA

We have developed and pilot tested a "teaching postdoctoral fellowship" as a means to enrich the experience of future faculty members, enhance the curriculum of an undergraduate institution, and provide research expertise not otherwise available to the research program of an undergraduate department. Dr. Barnard, a molecular biologist with expertise in bioinformatics, has had a two-year appointment at Holy Cross, supported by College funding budgeted for sabbatical replacement and an NSF grant to Dr. Ledbetter. He taught three courses each year, including a specialty seminar in Bioinformatics (twice), a laboratory section of General Biology (once), and a general education course on the Human Genome Project (three times). He assisted Dr. Ledbetter and her research students in computer assisted microscopic studies and guided two students of his own in the summer and during the academic year. And he participated fully in lab meetings, weekly journal club, and department activities while developing research and teaching collaborations with two other faculty members. He also fulfilled the requirements of the Certification Program in Undergraduate Teaching offered through the Worcester Consortium of Higher Education, which assisted Dr. Ledbetter in mentoring his teaching. The experience of juggling the demands of both teaching and research taught him crucial skills in both areas, as well as time management. He will continue his career at Worcester State College in the fall, one of several positions he was offered. Through this experiment Holy Cross tested interest in Bioinformatics as a potential curriculum addition, and Dr. Ledbetter benefited from Dr. Barnard's expertise in an area of research unfamiliar to her. Our experience has been completely satisfactory. We recommend it as a way to combine faculty development, curriculum enhancement, replacement of faculty on leave, and research initiatives to the benefit of all four.

**The Binding of a 60S Ribosomal Subunit Is Sufficient to Open the Pore of a Translocon in the Endoplasmic Reticulum**

D. C. Barnard, M. S. Ledbetter; Biology, College of the Holy Cross, Worcester, MA

We have previously reported that the release of a nascent chain from a translationally-active, ribosome-bound translocon (RBT) creates an open pore through which small molecules can cross the membrane of the rough endoplasmic reticulum (RER) (JBC, 276:22655; JBC, 278:4397). The pore of a translocon remains open as long as a ribosome remains bound, closing only after its removal by the addition of high salt or EDTA. The goal of our present study was to determine if a translocon pore closed by the removal of ribosomes can be re-opened by the addition of purified 60S ribosomal subunits. We have measured the permeability of the RER in CHO-K1 cells to 4-methylumbelliferyl-alpha-D-glucopyranoside (4MG), whose entry can be detected when it is hydrolyzed by lumenal alpha-glucosidase II. Polysomes isolated from CHO-S cells were disassembled with puromycin and high salt, and 60S subunits were purified by sedimentation on 10-35% sucrose gradients. The purified 60S subunits exhibited no alpha-glucosidase II activity. The addition of 60S subunits to EDTA- stripped ER membranes produced a saturable, concentration-dependent increase in permeability, with an apparent Km of about 20nM. In contrast, the addition of 60S subunits to puromycin-treated, unstripped membranes did not produce a significant increase in permeability. The lack of effect with unstripped membranes was consistent with the binding sites for the 60S subunits being occupied unless the membranes were previously stripped with EDTA. Heating the 60S subunits to 90C for 5 min. eliminated their ability to increase the permeability. We conclude that the binding of a 60S subunit, alone, is sufficient to open the pore of a translationally-active translocon. Other components of the protein translation machinery, such as the signal sequence of a nascent chain, are not required to open the pore and increase its permeability to small molecules.

**Early Post-natal Death of Knock-in Mice Expressing a Mutant BIP**

M. Mimura,1, 2 M. Kasai,3 H. Jin,1 K. Kimura,1, 2 S. Tanabe,1 T. Aoe1; 1Department of Anesthesiology, Chiba University Graduate School of Medicine, Chiba, Japan, 2Department of Medicine and Clinical Oncology, Chiba University Graduate School of Medicine, Chiba, Japan

The endoplasmic reticulum (ER) provides a folding environment where secretory and transmembrane proteins interact with chaperones. Some membranous imperfect proteins are stably retained in the ER, while other membranous and soluble ones require transport to the Golgi complex and retrieval for ER quality control. BIP is one of the most abundant ER chaperones, assisting in protein translocation, folding and degradation. When secreted from the ER in the company of imperfect proteins, BIP is recognized with a carboxyl terminal Lys-Asp-Glu-Leu (KDEL) sequence and retrieved by the KDEL receptor from the post-ER compartment. Yeast studies show that the deletion of retrieval motif from BIP Kar2 is dispensable in a single cell. In order to investigate the physiological significance of BIP and its retrieval in multi-cellular organisms in vivo, we established knock-in mice expressing a mutant BIP with the retrieval sequence deleted by homologous recombination. The embryonic fibroblasts derived from the homozygous mutant embryo expressed the mutant BIP, which was enhanced by ER stress instead of the wild-type one. We found that a fraction of the mutant BIP was secreted in the medium in the resting state and that loss of the mutant BIP was compensated by the active UPR as seen in the yeast studies. The mutant BIP embryos grew apparently as well as the wild ones. However, the homozygous mutant BIP neonates died early after birth, indicating that the retrieval of BIP is essential for survival of mammalian.
The Endoplasmic Reticulum Stress Response Mediates a Component of Cellular Defense in Caenohabditis elegans Against Bacillus thuringiensis Pore-forming Crystal Toxins

L. J. Bischof, R. V. Arioa, Biological Sciences, University of California, San Diego, La Jolla, CA
B. thuringiensis produces crystal (Cry) toxins that are invertebrate specific pore-forming toxins that target intestinal epithelial cells. Specific Cry proteins intoxicate nematodes, including C. elegans. To address how an epithelial cell may mount a defense against these toxins, C. elegans mutants that are hypersensitive to intoxication by Cry5B have been identified. This approach has found endoplasmic reticulum stress response genes as being important components of this cellular defense. The three ER stress signaling pathways in C. elegans are ire-1, pek-1, and atf-6. The ire-1 gene and its downstream target, xbp-1, were found to confer a hypersensitive phenotype to Cry5B by using both mutants and RNA interference. This pathway is activated when the worm ingests Cry5B as shown by ire-1 mediated alternative splicing of xbp-1. Also, a GFP fusion gene controlled by xbp-1 is upregulated specifically in intestinal cells upon Cry5B ingestion. Lethal concentration toxicity assays indicate that while these mutants are hypersensitive to Cry5B, they are not more sensitive to intoxication by the heavy metal CeSO4 or the oxidative stress agent H2O2. This data argue for a specific role for the ire-1 pathway in defense against Cry toxins. Intestinal specific expression of xbp-1 rescues the Cry5B hypersensitive phenotype indicating this pathway is required in the cell targeted by the toxin. In addition to the ire-1 pathway, atf-6 mutants are also hypersensitive to Cry5B but less so than ire-1. Investigations of the pek-1 pathway are in progress, but current data does not indicate a significant role in cellular defense against Cry5B. Therefore, while all 3 pathways are involved in the ER stress response, the ire-1 pathway is most specific for mediating a cellular response to Cry5B. These results reveal a new physiological role for the ER stress response pathway, namely in cellular defense against bacterial pore-forming toxins.

Subcellular Localization and an Enzyme Activity of Fatty Acid Transport Protein 5 in the Liver

J. Manaka, N. Takahashi, K. Toyama, M. Ohno, K. Higashi, K. Motojima; Biochemistry, Meiji Pharmaceutical University, Tokyo, Japan
The liver plays a central role in fatty acid metabolism including energy utilization, incorporation into lipoproteins and membrane lipids, and storage. Uptake of fatty acids into hepatocytes is a key step in the metabolism and mediated by several transport proteins. However, the physiological role, mechanism of the uptake, control of expression, and functional regulation of each transporter have not been elucidated. In this study, we examined these issues by focusing mostly on one of the fatty acid transport proteins (FATPA). FATP5 that is uniquely expressed in the rodent liver.

Protein-tyrosine phosphatase 1B (PTP1B) is an endoplasmic reticulum (ER)-bound phosphatase that is a key regulator of receptor tyrosine kinase signaling (such as epidermal growth factor receptor and insulin receptor). The objective of this study is to map and quantify the dynamics of PTP1B movement ex vivo, and correlate it with PTP1B-substrate(s) interaction. Photoinactivation, fluorescence recovery after photobleaching (FRAP) and “substrate-bleaching” approaches were utilized to study the dynamics of PTP1B movement. Wild type PTP1B exhibited fast dynamics and mobility on the ER as revealed by photoinactivation and FRAP studies. Recovery of PTP1B fluorescence was observed within seconds (~20 seconds) after photobleaching.

Regulated Expression by PPARα and Unique Localization of 17β-hydroxysteroid Dehydrogenase Type 11 in Mouse Intestine and Liver

Y. Yokoi, Y. Horiguchi, R. Shirai, K. Higashi, K. Motojima; Biochemistry, Meiji Pharmaceutical University, Tokyo, Japan
We recently identified 17β-hydroxysteroid dehydrogenase type 11 (17β-HSD11) as a peroxisome proliferator-activated receptor α (PPARα)-regulated gene in the intestine (Motojima, 2004, Eur J Biochem 271:4141). 17β-HSD11 was also induced in the liver and intestine by feeding mice with various plant seeds and grains (Motojima and Hirai 2006 FEBS J 273: 292). In this study, we characterized the 17β-HSD11 at the protein level to obtain a clue about its physiological role in the intestine and liver. For this purpose, specific antibodies against the 17β-HSD11 recombinant protein and the synthetic peptide were obtained. Western blotting analysis showed that administration of a PPARα agonist induced 17β-HSD11 protein in the jejunum but not in colon much higher than in the liver of mice. Immunohistochemical analysis using tissue sections showed that 17β-HSD11 was induced mostly in intestinal epithelia and hepatocytes with heterogeneous localization both in the cytoplasm and vesicular structures. The subcellular localization study using Chinese hamster ovary cells and the green fluorescence protein-tagged 17β-HSD11 suggested that it mostly localized in the endoplasmic reticulum (ER). However, under the conditions where lipid droplets were formed in the cells, most GFP signals shifted from ER to around the lipid droplets, suggesting that the localization of 17β-HSD11 changes according to the physiological conditions. Dual localization of endogenous 17β-HSD11 in the liver and intestine was confirmed by subcellular fractionation followed by Western blotting and immunohistochemical analysis of the tissue sections containing lipid droplets. Taken together, these data suggest that 17β-HSD11 associated with lipid droplets is not a mere ER-contaminant, but a bona fide protein comprising the membrane component of both intracellular compartments, and 17β-HSD11 may play an unknown role to cope the exogenously overloaded lipids from the natural diets.

Identification of the Targeting Domain in the Foremost Lipid Droplet Lipase Family

E. B. Goldberg, W. J. Brown; Molecular Biology and Genetics, Cornell University, Ithaca, NY
Lipid droplets (lipid bodies, adiposomes) are highly conserved storage organelles for neutral lipids and cholesterol esters. Recently, a new family of cytoplasmic lipases named Pataxin-like phospholipid transfer proteins (PNPLA) was identified. In the regulation of lipid droplet metabolism, here we report the first identification of a targeting domain for a PNPLA family member. Transient transfection experiments of truncated versions of PNPLA5 (GS2-Like) fused to GFP revealed that the N-terminal two-thirds of PNPLA5 contains the Pataxin-homology domain, and was cytoplasmic and diffusely distributed. Conversely, a construct containing only the C-terminal third of the protein was localized to lipid droplets. Additional truncation mutants revealed a crucial C-terminal 25 amino acid domain for lipid droplet localization. Within this domain, there is homology with other members of the PNPLA family, such as ATGL (Adipose Triglyceride Lipase, or PNPLA2) and Adiponutrin (PNPLA3), suggesting a conserved lipid droplet targeting motif (LTM).

Probing the Dynamics of the Neuronal Endoplasmic Reticulum with a Novel Lentiviral Vector

V. C. Jones, L. McKeown, A. Verkhovsky, O. T. Jones; Faculty of Life Sciences, University of Manchester, Manchester, United Kingdom
The endoplasmic reticulum (ER) is an extensive, complex endomembrane system, containing channels and receptors that allow its lumen to act as a dynamic calcium store. In response to electrical or chemical stimulation, ER calcium can be released under strict spatiotemporal control to regulate processes ranging from synaptic plasticity to growth cone guidance. Currently, there is much controversy over the nature of the ER in neurons and how this intersects with calcium signalling. We hypothesize that in neurons the ER is a dynamic structure with a response to electrical or chemical stimulation, ER calcium can be released under strict spatiotemporal control to regulate processes ranging from synaptic plasticity to growth cone guidance. We hypothesize that in neurons the ER is a dynamic structure with a

Dynamics of Cellular Protein-Tyrosine Phosphatase 1B Movement and the Effects of Substrate Interaction on Phosphatase Mobility

F. G. Haj, V. Roukos, S. V. Peever, B. Neel, P. Bastaean; Medicine, Harvard University/Beth Israel Deaconess Medical Center, Boston, MA; European Molecular Biology Laboratories, Heidelberg, Germany
Protein-tyrosine phosphatase 1B (PTP1B) is an endoplasmic reticulum (ER)-bound phosphatase that is a key regulator of receptor tyrosine kinase signaling (such as epidermal growth factor receptor and insulin receptor). The objective of this study is to map and quantify the dynamics of PTP1B movement ex vivo, and correlate it with PTP1B-substrate(s) interaction. Photoinactivation, fluorescence recovery after photobleaching (FRAP) and “substrate-bleaching” approaches were utilized to study the dynamics of PTP1B movement. Wild type PTP1B exhibited fast dynamics and mobility on the ER as revealed by photoinactivation and FRAP studies. Recovery of PTP1B fluorescence was observed within seconds (~20 seconds) after photobleaching.
regions of the ER. Altering microtubules by treating cells with nocodazole dramatically decreased PTP1B mobility, which could be restored to normal levels after drug withdrawal. In addition, cells expressing the "substrate-trapping" PTP1B D181A (PTP1B D/A) mutant exhibited increased tyrosine phosphorylation at discrete cellular locations that correlated with trapping of substrate(s) at these sites. Interestingly, the dynamics of PTP1B D/A movement in the ER was significantly decreased compared to wild type. Moreover, using immunofluorescence and electron microscopy we showed that areas of cell-cell contact are regions of extensive substrate-trapping. The enhanced substrate interaction at these regions correlated with, and most probably caused the decreased mobility of PTP1B D/A at these areas. These studies provide major insight into the dynamics of PTP1B movement and the effects of substrate(s) interaction on this process.

1969

Bax Inhibitor-1 Contributes to the Homeostasis of Calcium and pH

H. Kim,1 K. Ha,2 G. Lee,2 D. Kim,2 S. Chae,2 H. Chae2; 1Department of Dental Pharmacology, School of Dentistry, Wonkwang University, Iksan, Republic of Korea, 2Department of Pathology and Cardiovascular Research Center, Medical School, Chonbuk National University, Jeonju, Republic of Korea

It was the aim of this study to investigate the effect of the anti-apoptotic protein-Bax inhibitor-1 on the regulation of cellular Ca\textsuperscript{2+} homeostasis. The expression of BI-1 decreased the amount of Ca\textsuperscript{2+} that could be released from intracellular stores. Analysis of the kinetics of Ca\textsuperscript{2+} store depletion revealed that BI-1 increased the permeability of the ER membrane. In BI-1 cells, Ca\textsuperscript{2+} leaks passively at lower rates from stores with IP3R inhibitor-IP3 or RyrR abrogator, but the kinetics of leaky calcium remains higher in BI-1 cells in the presence or absence of the calcium channel inhibitors relatively to Neo cells. The C-terminal is a highly conserved sequence that is present in all BI-1. The data indicate that the full length of BI-1 is capable of self-tetramerization. Deleting the final 9 amino acids from the full-length human BI-1 resulted in an inactive channel, attributed to impaired assembly of a tetrameric BI-1 complex. Based upon the Bioinformatic search data, we have investigated the effect of BI-1 on [Ca\textsuperscript{2+}]i and pH, using the fluorescent indicators Mag-fura-2 and BCECF, respectively. In BI-1 cells, acidic condition evoked a much larger and faster [Ca\textsuperscript{2+}]i increase than in Neo cells. The pHi was also much more decreased in BI-1 cells in the presence of calcium. In summary, BI-1 function revealed a significant regulation in ER Ca\textsuperscript{2+} homeostasis and pH regulation.

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1970

Contribution of a Sub-Region of the Endoplasmic Reticulum in ER-mediated Phagocytosis

E. Gagnon,1 L. Morrow,2 S. Duclos,1 J. Bergeron,3 M. Desjardins1; 1Pathologie et Biologie Cellulaire, Université de Montréal, Montreal, PQ, Canada, 2Anatomy and Cell Biology, McGill University, Montreal, PQ, Canada, 3Department of Pathologie et Biologie Cellulaire, Université de Montréal, Montreal, PQ, Canada

We have shown recently that the endoplasmic reticulum (ER) participates in phagosome biogenesis, a process that we referred to as ER-mediated phagocytosis. The contribution of ER to the phagosome membrane was further shown to provide functional properties favoring the cross presentation of exogenous peptides on MHC class I molecules. The ability of ER and phagosomes to interact has been recently challenged (Touret et al., Cell 123:157-170, 2005). In the present study, we used a variety of approaches, including some of the tools developed by Touret and colleagues, to show that a sub-region of the ER participated in phagocytosis. Western blot analyses indicated that phagosomes isolated from macrophages expressing the ER tracer KDEL-GFP contained several ER proteins but no detectable GFP. Furthermore, immunofluorescence microscopy of the ER protein calnexin and the overexpressed ER marker KDEL-GFP, indicated that these two molecules co-localized only to a sub-region of the ER. ER regions displaying a strong labeling for calnexin in the cell periphery, as well as the nuclear envelope, were devoid of KDEL-GFP. These results indicate that the overexpression of the tracer KDEL-GFP in macrophages results in the accumulation of this molecule in a sub-region of the ER that does not participate in phagocytosis. The use of a sub-region of the ER for phagocytosis might favor a rapid recruitment of membrane from an internal source while minimizing the potential deleterious effect of this process on other key functions of the ER.

1971

Proteomics Characterization of Endoplasmic Reticulum Proteins with Unknown Function

M. D. Jain,1 H. Nagaya,2 A. Gilchrist,1 C. Au,3 J. Paiement,3 J. J. M. Bergeron1; 1Department of Anatomy and Cell Biology, McGill University, Montreal, PQ, Canada, 2Department of Pathologie et Biologie Cellulaire, Université de Montréal, Montreal, PQ, Canada

Based on the analysis of 140 000 tandem mass spectra we have characterized 350 proteins of unknown function assigned to the ER and Golgi apparatus of rat liver parenchyma. Using a proteomics clustering tool as well as the kinetics of interaction between these expressed in BHK cells and were therefore predicted to be newly uncovered molecular chaperones. Both the proteomics clustering tool as well as the kinetics of interaction between these newly uncovered chaperones and tyrosinase predicted that two were functional in the rough ER and likely in proximity to the translocon. The third chaperone was predicted to be localized to the smooth ER with markedly later kinetics of interaction with tyrosinase. One of these proteins has been named previously as nicalin, which we characterize as a likely translocon-associated chaperone. The second also co-distributed with translocon proteins, and the third, more distally located chaperone we have named EDEX.

1972

Molecular Characterization and Functional Analyses of a Unique, Class-I, Secretory Nuclease from the Human Pathogen, Leishmania donovani

M. B. Joshi,1 D. M. Dwyer; Cell Biology Section/LPD, NIAID/NIH, Bethesda, MD

Leishmania donovani, a primitive protozoan pathogen of humans, resides and multiplies in highly restricted, microenvironments within their hosts (i.e. in the gut lumen of their sandfly vectors and in the phag-lysosomal compartments of infected mammalian macrophages). Like other tyrosinomatisated parasites, they are purine auxotrophs and are totally dependent upon salvaging these essential nutrients from their hosts. In that regard, we identified and characterized the biochemical and functional properties of a unique, 35 kDa, nuclease from these organisms. Our studies demonstrated this enzyme was constitutively released/secreted by both in vitro grown promastigotes and axenic amastigotes as well as, by in vivo-derived amastigote developmental forms of the parasite. Using a molecular approach, we identified, characterized and epistemically expressed the gene, LdNuc which encodes this new Class I nuclease family member from these organisms. Results of both structural and functional analyses showed that the LdNuc expressed-enzyme had properties identical to the native, wild-type, parasite released/neclease. Further, both LdNuc mRNA and secretory nuclease activity were differentially up-expressed by amastigotes >> promastigotes. Biochemical analyses showed that the LdNuc secreted nuclease could hydrolyze a variety of synthetic polynucleotide substrates as well as, both single- and double-stranded DNAs and RNAs. Based on these observations, we hypothesize that this leishmanial "secretory" nuclease could act, at a distance away from the parasite, to hydrolyze host-derived nucleic acids to satisfy the essential purine requirements of these organisms. Thus, LdNuc must have essential roles in facilitating the survival, growth and development of this important human pathogen.

1973

Identification, Biochemical and Molecular Characterization of Secreted Lipases in the Human Pathogen Leishmania

A. M. Shakarian,1 G. Meguans2, M. Joshi,1 D. M. Dwyer1; 1Biology and Biomedical Sciences, Salve Regina University, Newport, RI, 2Laboratory of Parasitic Diseases, NIAID/NIH, Bethesda, MD

The human pathogen Leishmania, found in the tropics and subtropics are investigated as models of human parasitism. Emphasis is placed on characterizing both the biochemical functions and gene structure of secreted proteins toward defining their roles in parasite survival and development. Lipases are ubiquitous enzymes that hydrolyze the ester linkages of fats to form glycerol and fatty acids. These enzymes are involved in biological activities ranging from antigenic variation and cell signaling to nutrient acquisition and have been implicated as virulence factors in some pathogens. Culture supernatants of Leishmania promastigotes were incubated with various 4-nitrophenyl fatty acids. Results of these assays identified lipase activities from in vitro culture supernatants of Leishmania. Subsequently, a PCR-based strategy identified a candidate lipase gene from Leishmania. The L. major genome database revealed 3 putative secretory lipases. The homologue of one of these, designated LIP3, was cloned. Sequence analysis of LIP3 revealed an ORF of 927 bp and a deduced protein of 306 aa with a predicted molecular mass of 33.0 kDa. Further analysis showed a putative 24aa signal peptide and the absence of an anchor motif, both consistent with a secretory molecule. Moreover, an active enzyme active site was identified. Southern analysis revealed the presence of more than one copy of this gene in the Leishmania genome. RT-PCR revealed that this gene is expressed in both promastigotes and amastigotes. Interestingly, amastigotes preferentially use fatty acids as an energy source. To further characterize this lipase, an epitope-tagged construct was epistemally expressed. Western
analysis revealed that the expressed protein was secreted by transfected *Leishmania*. Taken together, our data supports the hypothesis that lipase activity is secreted by *Leishmania*. Further characterization of this gene and protein will lead to a better understanding of the role of lipases within the biology of the important group of human pathogens.

1974

Protozoan Parasite Exit May Be Preceded by Fusion between the Parasitophorous Vacuole Membrane and the Host-Cell Membrane

S. León,1, J. Diaz;2 A. Corrales,2 G. Cortés,4 M. Caballero,1 1Departamento de Biología, Facultad de Ciencias, Universidad Nacional de Colombia, Bogota, Colombia, 2Departamento de Biología, Facultad de Ciencias, Universidad Nacional de Colombia, Bogota, Colombia, 3Laboratorio de Biofísica, Centro Internacional de Física, Bogota, Colombia, 4Departamento de Biología, Facultad de Ciencias, Universidad Nacional de Colombia, Bogota, Colombia, 5Grupo de Microscopía y análisis de Inmunes, Instituto Nacional de Salud, Bogota, Colombia

Introduction: Protozoa like *Leishmania* and *Plasmodium* are intracellular parasites confined to a compartment known as the parasitophorous vacuole. Two main models have been postulated for parasite exit. One supports the idea that parasite exit requires lysis of the parasitophorous vacuole membrane and the host membrane. The other model supports the idea that parasite exit is preceded by fusion between the parasitophorous vacuole membrane and the host membrane. We present data that supports the second model. Methodology: Two models were studied: infection of the macrophage-like cell line J774.A1 with *Leishmania amazonensis* and infection of human erythrocytes with *Plasmodium falciparum*. The infection cycle was followed in vitro and percentage of infection, parasite number and viability were measured in the presence of inhibitors of membrane fusion. Fluorescence microscopy, electron microscopy and electrophysiology were used to determine membrane capacitance, an indirect measurement of membrane area. Results: Leupeptin and NEM, two inhibitors of membrane fusion alter the decrease in parasite number observed in the *Leishmania* infection cycle in macrophages suggesting retardation of parasite exit whereas cytchalasin-B, an inhibitor of the cytoskeleton had no effect. There is a progressive increase in membrane capacitance of macrophages infected with *Leishmania* up to 72 hours post infection, with a drop at 96 hours. DilC16 labeling of erythrocyte membrane is transfer to intracellular membranes upon *Plasmodium* exit. Labeling with NBD-PE and RHO-PE macrophages and erythrocytes are altered by *Leishmania* and *Plasmodium* infection respectively. Conclusions: The changes in membrane capacitance of the host-cell membrane support the idea of fusion between the parasitophorous vacuole membrane and the host membrane.

1975

Malaria Transmission-blocking Antigen, Pfs230, Mediates Human Red Blood Cell Binding to Exflagellating Male Parasites and Oocyst Production

S. Eksi,1 B. Czesny,1 G. van Gemert,1 R. W. Sauerwein,2 W. Eling,2 K. C. Williamson,1 Biology, Loyola University Chicago, Chicago, IL, 2Medical Microbiology, University of Nijmegen, Nijmegen, The Netherlands

Exflagellation of male *Plasmodium* parasites can be stimulated by simulating conditions in the mosquito midgut, one of the most visually dramatic events in the lifecycle of the malaria parasite. Following emergence from the red blood cell (RBC), male parasites undergo rounds of nuclear division and form axonemes which extend out from the body of the parasite. The extended axonemes move with a vigorous whipping motion before releasing as individual motile microgametes to find and fertilize a female gamete. Before the microgametes are released he can associate with uninfected RBCs in the bloodmeal and draw the RBCs in toward the body of the parasite where the RBCs are retained. In wild type parasites this results in a cluster of 2-13 uninfected RBCs, called an exflagellation center. *Plasmodium falciparum* transmission-blocking vaccine candidate and gamete surface antigen, Pfs230, was found to be important for both exflagellation center formation and subsequent oocyst formation. Two clones distinct Pfs230 gene disruptions (D1.356 and D2.560) and a Pfs48/45 disruptant that result in Pfs230-minus Pfs48/45-plus and Pfs230-minus Pfs48/45-minus gametes, respectively, successfully emerge from RBCs and males exflagellate producing microgametes. However, exflagellating Pfs230-minus males, in the presence or absence of Pfs48/45, are unable to interact with RBCs and form exflagellation centers. Oocyst production and mosquito infectivity is also significantly reduced, 96-972% and 76-71%, respectively. In contrast, the expression pattern of other known sexual-stage specific antigens, including the Pfs230 paralog (PfsMB5), Pfs16, Pfs47 or Pfs25, were not altered. These results suggest that Pfs230 is the surface molecule on males that mediates RBC binding and plays an important role in oocyst development, a critical step in malaria transmission.

1976

Plasmodium Falciparum Ring-infected Erythrocyte Surface Antigen (RESA) Stabilizes Rhysbyocyte Spectrin Tetramer and Reduces Malaria Invasion

X. Pei,1 X. An,3 X. Guo,1 R. Coppell,1 N. Mohandas,3 Red Cell Physiology Laboratory, New York Blood Center, New York, NY, 1Department of Microbiology, Monash University, Victoria, Australia

Erythrocyte surface antigen (RESA) of *Plasmodium falciparum* is the first malaria protein shown to interact with erythrocyte skeletal protein, spectrin. However, the structural basis and functional sequel of this interaction remain unknown. In the present study, we first defined the binding motifs involved in the interaction. We show that spectrin bound to a 107 amino acid fragment of RESA (residues 663-770) and that this RESA fragment bound specifically to only one of the 37 spectrin repeats, repeat 16 of spectrin. Repeat 16 of spectrin has been previously shown to play a major role in regulating spectrin tetramer formation. In the present study, we demonstrated that binding of RESA fragment to spectrin stabilizes spectrin tetramers both in vitro and in situ. Importantly, resealing of the RESA fragment into erythrocyte ghosts resulted in both increased resistance of cells to heat-induced vesiculation and to enhanced membrane mechanical stability. Surprisingly, the invasion of RESA fragment ressealed-erythrocytes by malaria parasite, *Plasmodium falciparum*, is significantly diminished compared to control cells. Based on these findings, we conclude that association of RESA with repeat 16 of spectrin stabilizes spectrin tetramers, which in turn leads to the stabilization of erythrocyte membrane skeleton. Our data further suggest a role of spectrin tetramers in regulating thermal stability of erythrocyte membrane and in malarial parasite invasion.

1977

Three-dimensionally Cultivated Cardiomyocytes Infected by *Trypanosoma cruzi*: Possible New Model of In Vitro Chagasic Chronic Cardiomyopathy

D. Adesse, E. M. Azzam, M. I. Rossi, B. Borovjevic, M. N. Meirelles, L. R. Giaroni; Ultraestructura e Biologia Celular, Fiocruz-Brasil, Rio de Janeiro, Brazil

Chagas disease, caused by *Trypanosoma cruzi*, is endemic in Latin America, affecting 16-18 million people and 90 million are at risk of becoming infected. Chronic Chagasic Cardiomyopathy (CCC) is considered a progressive disease in which patients present myocarditis with inflammatory infiltrate, fibrosis and myocardial hypertrophy. Persistence of infection, presence of parasite antigens at the inflammatory site or autoimmune processes are hypotheses proposed to explain the pathological lesions in the hearts of infected patients. Moreover, it was demonstrated that cardiomyocytes can respond to in vitro *T. cruzi* infection by expressing TNF-α and IL-1β mRNA thereby demonstrating an inflammatory response. We recently developed a functional cardiac microtissue that mimics the in vivo situation once the cells secrete Extracellular Matrix (ECM) and self-organize in a 3D architecture, forming a spherical structure. The cardiac microtissue produces growth factors including vascular endothelial growth factor (VEGF) and when co-cultured with endothelial cells, vascular structures are produced, improving the contractile activity of microtissue. We now have this cardiac microtissue as a new 3D model of *T.cruzi*-cardiac tissue interaction and show that it reproduces in vitro important aspects observed during the chagasic cardiomyopathy. Our results show that *T. cruzi* successfully infects and completes its intracellular cycle inside cardiac microtissue, causing alterations in ECM, increasing the cardiomyocyte area and then total cardiac microtissue volume - aspects that may reflect the cardiomagnetically observed during Chagasic disease. Moreover, *T. cruzi* infection induces migration of inflammatory cells throughout the microtissue. Taken together, our results show that this new approach that more closely reflects what happens in vivo at flattened cultures and is likely to bring new insights in important associated aspects related to chagasic cardiomyopathy.

1978

Active Uptake of Host Eukaryotic Functions by the Intracellular Pathogen *Toxoplasma* J. D. Romano, N. Banó, I. Coppens; Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD

The obligate intracellular parasite *Toxoplasma gondii* actively invades mammalian cells, forming a specialized parasitophorous vacuole (PV) upon entry. From within this niche, the parasite redirects various host organelles to the PV in order to divert nutrients from them and to interfere with host immunity. Using time-lapse confocal microscopy we observed that shortly after invasion the PV moves directionally towards the host cell's nucleus. Surprisingly, we found that specific microtubules and actin destabilizing compounds have no effect on the migration of the PV to the cell center. However, several hours post-infection, the network of host microtubules is completely reorganized to surround the PV. To better understand the association of host microtubules with the PV, the dynamics of the host microtubule-organizing center (MTOC) was studied in *Toxoplasma*-infected cells. Early during infection, evaginations of the PV membrane were observed to extend towards the host MTOC in a subset of PVs. After 24 h of infection, the host MTOC was stably anchored to the PV surface. Concomitantly, host Golgi localized near the PV. Encelated host cells were then used to determine the respective contribution of the host cell and the parasite to the association of host structures post-infection. PV membrane formation, recruitment of host structures (i.e. ER, mitochondria, PV, microtubules), cholesterol scavenging from host endocytic organelles and parasite multiplication were unaffected in cytoplasts as compared to nucleated cells. These data implicate that *Toxoplasma* is actively involved in usurping many host eukaryotic functions and that new host transcripts are not essential to the early development of this parasite.

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1979

MicroRNA-mediated Post-transcriptional Gene Silencing Is Involved in Cholangiocyte Defense against Cryptosporidium parvum Infection
X. Chen, P. Splinter, S. O’Hara, N. LaRusso; Mayo Clinic College of Medicine, Rochester, MN

Our previous studies demonstrated that microRNA (miRNA), Let-7i, regulates TLR4 translation and infection of biliary epithelial cells (cholangiocytes) with Cryptosporidium parvum, a parasite that causes intestinal and biliary disease, decreases Let-7i expression and is accompanied by an increased expression of both TLR4 and associated production of the antimicrobial protein, human β-defensin 2 (HBD2). Here, we further explored: i) the mechanism by which C. parvum infection suppresses expression of Let-7i; and ii) the significance of Let-7i-mediated TLR4 translation in epithelial defense. Using an in vitro model of biliary cryptosporidiosis, we found that C. parvum infection suppresses Let-7i transcription 4-fold, a process blocked by the specific NF-κB inhibitor, SN50. Furthermore, using a mobility shift assay, we observed a shift in the Let-7i promoter fragment, which contains a NF-κB consensus motif, following C. parvum infection. Using a pGL3-Basic luciferase construct that contains the promoter elements of Let-7i, we determined found that C. parvum infection decreased Let-7i promoter-driven luciferase expression. In contrast, no decrease of luciferase expression was detected in infected cells in the presence of SN50. Consistent with our previous studies, exposure to C. parvum induced a two-fold (p<0.05) increase in HBD2 expression in biliary epithelial cells. HBD2 expression was decreased up to 85% (p<0.05) in cells with forced expression of Let-7i; in contrast, in contrast, forced suppression of Let-7i increased expression of HBD2 up to 45% (p<0.05). Moreover, we detected a significantly higher parasite burden (20%) increase) in cells with forced Let-7i expression. In contrast, a significantly lower parasite burden (40% decrease) was detected in cells with forced Let-7i suppression. Taken together, our results suggest that C. parvum suppresses Let-7i expression in cholangiocytes via activation of the NF-κB pathway, a process that is involved in cholangiocyte defense against C. parvum infection, possibly via regulation of TLR4 expression to mediate HBD2 production.

1980

Cholesterol in Intracellular Microsporidians and Host Cells Measured with Fluorescent Probes
E. Weinrider, H. Hale-Donze, D. Burkh; Biological Sciences, Louisiana State University, Baton Rouge, LA

Apicomplexa and most other intracellular parasites rely on the host cell for cholesterol since the parasites are devoid of sterol synthesis facility. How does it work in microsporidian parasites? In this study, the distribution of cholesterol in the parasite was investigated. Two questions were asked. (a) What is the distribution of cholesterol in different stages of microsporidian parasites; and, (b) what is the distribution of the cholesterol that the microsporidians have the capacity to produce cholesterol from precursors in the absence of host cells? For cholesterol distribution in parasites or in the host cells, fluorescent probes were used. Dehydroepiandrosterone (DHEA), a fluorescent cholesterol analog and the polypeptide antibiotic filipin were the primary probes used. The filipin-binding results indicate the microsporidian spores of Encephalitozoon and Nosema have cholesterol confined to the plasma membrane; however, the discharged invasive sporoplasm cell lacks cholesterol in the outer membrane. This supports other published data which indicates the outer membrane of the discharged sporoplasm comes from the membrane of the extrusion apparatus during spore discharge. To determine whether microsporidian parasites can make cholesterol, purified sporoforms from discharged spores were incubated on coverslips in medium with 500 nmol of mevalonate or 300 nmol of HMG CoA for 6 h. The cells were subsequently examined for cholesterol synthesis with the use of the probe filipin.

1981

Apoptosis as a Host Defense Mechanism in Crassostrea virginica and Its Modulation by Perkinsus marinus
S. Greweal, I. M. Sokolova, F. M. Hughes; Biology, University of North Carolina at Charlotte, Charlotte, NC

In several organisms apoptosis is an important defense against intracellular parasites in order to contain the infection and limit the spread. Meanwhile, parasites often attempt to suppress host cell apoptosis to extend their survival and increase their proliferation. Perkinsus marinus is an intracellular protozoan parasite of the eastern oyster, Crassostrea virginica. Perkinsus infects hemocytes (oyster immune cells) and is responsible for large scale mortalities. In this study we investigate the hypothesis that hemocytes increase apoptosis as a host defense against Perkinsus and that Perkinsus counters by suppressing this response. To investigate this hypothesis, acute effects were examined in vitro while longer-term responses were investigated in vivo. Initial light microscopic analysis of circulating hemocytes indicated that granulocytes predominate (~70%). Following in vitro infection (MOI 1:1), phagocytosis increased in a time-dependent manner. At 15 minutes, approximately 40% of hemocytes had either phagocytosed, or were closely associated with, Perkinsus; whereas by two hours this had increased to 65%. In response to infection, hemocyte apoptosis (Annexin V) increased within 15 min and was significantly enhanced by 2 hours, suggesting the induction of apoptosis as a host defense. To examine a more chronic response, oysters were infected in vivo (2x10⁵ Perkinsus/gram) for 16 days. At day-1, apoptosis was elevated, accompanied with a significant decrease in circulating hemocytes. However, by day-2, apoptotic levels returned to baseline and remained there, despite light to medium infections being established (Mackin scale). These results suggest that Perkinsus counteracts the initial hemocyte apoptotic response to ensure proliferation and spread within the host. Together these results indicate that apoptosis is one of the defense mechanisms employed by the oyster host to control the infection and that Perkinsus suppresses this response. Future studies will begin to investigate the molecular mechanisms of this interaction.

1983

Salmonella Induced Filaments and Salmonella-containing Vacuoles Display Dynamic Movement in Host Cells
J. Szeto, 1 A. Namolovan, 1 M. Ohlson, 2 S. Miller, 2 J. Brumell; 1Cell Biology, Hospital for Sick Children, Toronto, ON, Canada, 2Department of Microbiology, University of Washington, Seattle, WA

Salmoneella enterica serovar Typhimurium (S. Typhimurium) occupies a membrane-bound niche in host cells during infection, the Salmonella-containing vacuole (SCV). From here, S. Typhimurium uses its Salmonella Pathogenicity Island 2 (SPI-2)-encoded type III secretion system (T3SS) to inject bacterial effector proteins into the host cell to modulate various activities. Several hours after infection, membranous tubules termed Salmonella-induced filaments (Sif) arise from the SCV. Here we used GFP-Lamp1 to visualize Sif dynamics using live cell imaging. Our studies revealed that Sifs undergo rapid extension, retraction, branching, and swelling. Live cell imaging also revealed that SCVs exhibit intracellular movement independently of Sifs. For statistical analysis, we documented the localization of Lamp1 SCVs in fixed infected HeLa cells. We determined that wild-type SCVs gradually migrate to a perinuclear, Golgi-associated position; however, the discharged invasive sporoplasm cell lacks cholesterol in the outer membrane. This supports other published data which indicates the outer membrane of the discharged sporoplasm comes from the membrane of the extrusion apparatus during spore discharge. To determine whether microsporidian parasites can make cholesterol, purified sporoplasm from discharged spores were incubated on coverslips in medium with 500 nmol of mevalonate or 300 nmol of HMG CoA for 6 h. The cells were subsequently examined for cholesterol synthesis with the use of the probe filipin.

1984

Sema3E Enhances Metastasis of Ovarian Endometrioid Carcinoma in a Plexin D1-dependent Way
P. Huang, 2 M. Tsou, 2 S. Hsu, 1; 1Graduate Institute of Pathology, College of Medicine, Taipei, Taiwan, 2Department of Pathology, National Taiwan University & University Hospital, Taiwan

Malignant ovarian endometrioid carcinomas, usually accompanied with metastasis upon diagnosis, account for 10 to 20% of epithelial ovarian cancers (EOCs) and are refractory to chemotherapy. Consequently, ovarian endometrioid carcinomas can serve as a promising model to study cancer progression originated from ovaries. Using two well-established human endometrioid carcinoma cell lines with different ability in invasion (P0 cells: low invasiveness, P4 cells: high invasiveness), we found that among all class 3 semaphorins, Sema3E was significantly over-expressed in the high-invasive endometrioid carcinoma cells. However, the receptor for Sema3E, Plexin D1 and/or neuropilin, showed no difference. Statistical analysis of 39 patients with ovarian endometrioid carcinomas diagnosed from 1999 to 2005 at NTUH demonstrated positive correlation between tumor grade and Sema3E expression level assessed by immunohistochemistry. When xenografts Sema3E was stably introduced into the low-invasive P0 cells, Sema3E augmented cellular invasiveness and migration in a concentration-dependent way as assessed by wound healing and Transwell chamber assay in vitro. In vivo, xenografted P0 cells with stably expressed Sema3E resulted in multi-focal tumor metastasis in bilateral lungs of SCID mice, in strong contrast to non-metastasis of P0 cells without Sema3E expression. We further revealed that furin-cleaved p61-Sema3E, not unprocessed p87-Sema3E, enhanced cellular invasiveness and migration. When Plexin D1 was functionally knocked-down by siRNA in the presence of over-expressed Sema3E, Sema3E no longer promoted cellular invasive and distant metastasis. Our study strongly argues for the pivotal role of Plexin D1 signaling pathway triggered by over-expressed Sema3E in the regulation of invasiveness and migration ability of ovarian endometrioid carcinoma via a paracrine and/or autocrine manner.

364
Filamin A is a Novel Cavelin1-dependent Target of Insulin-like Growth Factor-I Signaling: Role in Cancer Cell Migration

D. Ravidi,1 R. Reich,1 M. Liscovitch1 Biological Regulation, Weizmann Institute of Science, Rehovot, Israel, 2Pharmacology, Hebrew University, Jerusalem, Israel

Cavelin-1 (Cav1) is a 52-kDa transmembrane protein constitutively expressed on the surface of many cell types. It has been suggested to be involved in cell migration but its exact role and mechanism of action remain obscure. We have previously reported that expression of cavelin-1 is up-regulated in human breast cancer (MCF-7/Cav1) cells results in up-regulation of a phosphorylated high molecular weight putative Akt substrate, provisionally designated pp340. We now show, using differential detergent extraction, SDS-PAGE and mass spectrometry, that the major protein in the pp340 band is the actin filament cross-linking protein filamin A. The identity of filamin A was confirmed by mass spectrometry and western blotting. Notum is expressed at high levels in mouse embryos and in various adult organs including liver, lung, kidney, ovary and brain. No expression was detected in the thymus, heart and spleen. Finally, by using semiquantitative RT-PCR, we found that Notum is expressed at high levels in mouse embryos and in various adult organs including liver, lung, kidney, ovary and brain. No expression was detected in the thymus, heart and spleen. Furthermore, our results suggest that filamin A may mediate the effects of cavelin-1 on IGF-I-induced cancer cell migration.

Expression and Purification of the Recombinant Bryohealins (Bryopsis plumosa lectins) Produced in Escherichia coli Cells

K. S. Yoon, T. A. Klosekova, K. Y. Ko, G. H. Kim, K. P. Lee Biology, Konkuk National University, Konkuk, Republic of Korea

Bryohealins, a N-acetyl-D-glucosamine and N-acetyl-D-galactosamine specific lectins from the marine green alga Bryopsis plumosa, were produced in Escherichia coli from a cDNA clones subcloned to a various expression vectors. The recombinant lectins (rBryohealin-1 and rBryohealin-2) were purified by a combination of affinity chromatography and gel filtration. By SDS-PAGE and Western blot, rBryohealin were highly pure with an apparent molecular mass of 27 kDa and 12 kDa, respectively. The primary amino acid sequence of the recombinant Bryohealin were found to be identical to the native proteins. The purified recombinant protein could agglutinate red blood cells of mice, and the agglutination activity was inhibited by N-acetyl-D-glucosamine and N-acetyl-D-galactosamine. These results suggest that the recombinant Bryohealin 1-protein is a potent agglutinin to have a structure and function similar to that of native protein.

Identifying the Role of Cortactin Phosphorylation in Breast Cancer Invasiveness

C. C. Mader, K. Machida, B. J. Mayer, A. J. Koleske1 Cell Biology, Yale University, New Haven, CT, 2Molecular Biophysics and Biochemistry, Yale University, New Haven, CT, 3Genetics and Developmental Biology, University of Connecticut Health Center, Farmington, CT

Cortactin is known to promote actin driven cell motility and has increased expression levels in invasive breast cancers. Originally shown to be a substrate of the Src oncogene, cortactin is also a substrate of Abl and Nrcot receptor tyrosine kinases. The role that phosphorylation of cortactin plays in breast cancer invadopodia is unclear. The goal of this study is to identify potential binding partners of Abl/Arg phosphorylated cortactin and the potential role which this phosphorylation plays in promoting breast cancer invasiveness. We find that Arg binds cortactin with a Kd of >3μM while a truncation mutant lacking the SH3, SH2 and kinase domains binds with higher affinity (Kd=0.5μM). However, Arg mutants lacking the PxxP motifs do not bind to cortactin. This suggests that cortactin binds to Arg via the internal PxxP motifs and this binding is regulated by the Arg N-terminus. Interestingly, phosphorylactin interacts selectively with the Arg SH2 domain and not the SH2 domains from Abl, Src, Nck, and Grb. A nonphosphorylatable cortactin mutant did not show this interaction. This shows that phosphorylation of cortactin may act as a switch causing interaction with Arg through two different binding motifs depending on cortactin’s phosphorylation state. Using breast cancer cell lines we show that phosphorylated cortactin levels are significantly increased in highly invasive lines. To determine if phosphorylation of cortactin affects interaction with downstream actin regulatory binding partners, we are examining the binding interaction of different truncation and phosphorylated forms of cortactin with the Arp2/3 activator N-WASP. We are also performing colocalization studies to show the effects of cortactin phosphorylation on N-Wasp colocalization in breast cancer invadopodia. Overall, our results suggest that Arg-mediated tyrosine cortactin phosphorylation plays a role as a switch to promote more motile or invasive cellular phenotypes.

Expression of 15-Hydroxyprostaglandin Dehydrogenase in Gastric Cancer

A. Thiel,1 A. Ganesan,1 A. Nykänen,1 H. Tai,2 C. Haglund,3 T. V. Petrova,4 A. Ristimäki1,4 1Molecular and Cancer Biology Research Program, University of Helsinki, Biomedicum, Helsinki, Finland, 2Department of Pathology, Helsinki University Central Hospital, Helsinki, Finland, 3Department of Oncology, Helsinki University Central Hospital, Helsinki, Finland

We have previously shown that expression of cyclooxygenase-2 (COX-2) is elevated in 55% of gastric carcinomas and also is an independent prognostic factor in this disease. COX-2 is critical for the conversion of arachidonic acid to prostanooids, especially to prostaglandin E2 (PGE2), the most abundant prostaglandin in gastrointestinal tumors, which is thought to play a major role in carcinogenesis via modulation of apoptosis, proliferation, angiogenesis. Now we report that the expression of 15-hydroxyprostaglandin dehydrogenase (15-PGDH) is reduced in gastric cancer. This enzyme catalyzes the inactivation of PGE2 by converting the 15-OH to a 15-koeto group. We showed on a cancer profiling array which contained 27 DNA samples from gastric tumor and corresponding normal tissues from individual patients that the expression of 15-PGDH was five-fold lower in tumor vs. normal samples (P<0.0001). Furthermore we used immunochemistry to stain 133 gastric tumor specimens with rabbit polyclonal 15-PGDH and found that only 18% of the tumors expressed 15-PGDH. In gastric cancer cell lines treatment with interleukin-1 reduced the expression of 15-PGDH mRNA and protein levels. We will use a 15-PGDH-promoter construct to study whether this downregulation is due to reduced activation of the promoter. Our results suggest that downregulation of 15-PGDH is an important step in gastric tumorigenesis, which contributes to the high tumor levels of PGE2 and tumor progression.
interaction between integrin beta4 and FAK. Indeed, we demonstrated that in several tumor cell lines, such as MDA-MB-231, HCT116, and B16 but not in MCF7 and HeLa, integrin beta4 could co-immunoprecipitate FAK protein endogenously. It implicated the participation of this interaction in tumorigenesis. Moreover, the interaction required an 11-amino-acids motif within FAK’s amino-terminus. Among them, 2 out of 11 amino acids exhibited a critical role in interaction with integrin beta4. Our data resolved, in the first time, a physical interaction between integrins and FAK, suggesting a strong link regarding integrin-FAK signaling events. Future work will decipher the signaling pathway(s) and biological significance through the integrin beta4-FAK interaction, which will shed a light on better strategies for cancer therapies.

1991

Identification of Genes Potentially Involved in Initiation and Progression of Epithelial-Mesenchymal Transition (EMT) in the Non-Tumorigenic Human Mammary Epithelial Cell Line MCF10A

P. D. Gascard, K. L. Andrarawea, M. H. Barcellos-Hoff; Cancer Biology, LBNL, Berkeley, CA

Epithelial-Mesenchymal Transition (EMT) is a hallmark of tumorigenesis. It is characterized by a loss of epithelial architecture and acquisition of a mesenchymal phenotype with change in cell shape and increased motility. We have developed a unique EMT model in the non-tumorigenic human mammary epithelial cell line MCF10A. MCF10A cells were subjected to a 24h irradiation 5 hours after plating at clonal density and subsequently cultured for 8 days in presence of 400pg/ml recombinant transforming growth factor-beta (TGF-beta). In these conditions, cells got disorganized and displayed a concomitant loss of expression of E-cadherin and the tight junction protein ZO-1 and a gain of expression of N-cadherin and vimentin. Strikingly, neither irradiation nor TGF-beta alone recapitulated the EMT phenotype. As a first step to elucidate the mechanisms responsible for the intriguing synergy between irradiation and TGF-beta in initiating EMT, we compared transcript expression levels of sham, irradiated, TGF-beta-treated and irradiated-TGF-beta-treated MCF10A cells. Ten EMT signature genes, whose expression was mostly altered in double-treated cells, were identified. Among the eight up-regulated genes, the most significant included the recently characterized interleukin IL-32/NK4, whose up-regulation may explain over-expression of other interleukins, such as IL6 and IL8. Other up-regulated genes included: the transcription factor TCF7/LEB1 and the fibroblast growth factor FGF2/FGF7, which have been both previously reported to down-regulate E-cadherin transcription; the ecdy-nucleotidase NTSE/CD73, whose over-expression has been documented in tumor cells; the radiation-inducible immediate early response gene IER3/IEX-1; activin A (INHBA), cyclo-oxygenase 2 (COX2/PTGS2); and the nucleoporin-like protein NUP1. The scavenger receptor SCARB2 and the secreted frizzled-related protein 1 gene SFRP1 were the two genes significantly down-regulated in double-treated cells. Taken together, these data suggest that MCF10A cells shift towards a tumorigenic phenotype upon EMT. The biological significance of these gene expression alterations in EMT initiation and progression remains to be investigated.

1992

UVB-Induced Dephosphorylation of Stat3 by T-Cell Protein Tyrosine Phosphatase (T-PTP)

D. J. Kim, J. DiGiovanni; Department of Carcinogenesis, The University of Texas M.D. Anderson Cancer Center, Science Park-Research Division, Smithville, TX

Ultraviolet B radiation (UVB) can cause DNA damage, induce gene mutations and modulate intracellular signal transduction contributing to the development of skin cancer. Signal transducer and activator of transcription 3 (Stat3) regulates various physiological functions including apoptosis, cell cycle regulation, and tumor angiogenesis through regulation of gene expression, and its constitutive activation is associated with human epithelial cancers. Recent studies indicated that Stat3 was rapidly dephosphorylated in keratinocytes after UVB irradiation. Vanadate treatment desensitized keratinocytes to UVB-induced apoptosis with the recovery of phosphorylated Stat3 protein levels, implying that a tyrosine phosphatase is involved in this mechanism. In the current work, the mechanism whereby UVB exposure leads to rapid dephosphorylation of Stat3 was examined. Western blot analysis showed that the nuclear form of T-PTP, which is known to dephosphorylate various signaling molecules, was constitutively expressed in cultured mouse keratinocytes. After UVB exposure, activated stat3 was rapidly reduced, whereas the level of T-PTP was not changed. Further analysis revealed that translocation of T-PTP from cytosol to nucleus was increased in response to UVB irradiation. Consistent with western blot analysis, dephosphorylation of Stat3 by T-PTP was rapidly increased after UVB irradiation of cultured keratinocytes. Stat3 target genes, such as cyclin D1 and c-Myc, were also down-regulated following UVB exposure. In mouse epidermis in vivo, the level of phosphorylated Stat3 was initially decreased, following by a significant increase at later time points after UVB exposure. The expression patterns of cyclin D1 and c-Myc followed the changes in activated Stat3. In addition, dephosphorylation of Stat3 by T-PTP was initially up-regulated, and then decreased in vivo after UVB exposure. These results suggest that T-PTP is responsible for the rapid dephosphorylation of Stat3 following UVB and may serve as part of an initial protective mechanisms against UV skin carcinogenesis. (Supported by NCI grant CA76520, J.D.)

1993

ECTO-NOX Proteins, a Family of Cell Surface NADH Oxidases, Are Labeled by Biotin and Selectively Isolated from Plasma Membrane Preparations

S. C. Dick, Z. Jiang, V. D. Kane, D. M. Morre, D. J. Morre; Foods and Nutrition, Purdue University, West Lafayette, IN, 1Medicinal Chemistry and Molecular Pharmacology, Purdue University, West Lafayette, IN

Biotinylation of plasma membranes and cell surfaces was performed to further purify ECTO-NOX proteins, a group of proteins with hydroquinone oxidase activity as well as disulphide-thiol interchange activity (Morre and Morre. 2003. Free Radical Research 37:795-808). Soybean plasma membranes contain the constitutive form of ECTO-NOX known as CNOX. Previous studies using immunoprecipitation and gel slice analyses of partially purified proteins suggested the molecular weight of soybean CNOX to be 54 ±2 kDa. When a soybean plasma membrane preparations were biotinylated, reactive bands of 57 and 68 kDa were observed on Western blots using anti-CNOX antibodies. HeLa S (a cervical carcinoma cell line) cells contain the constitutive form CNOX as well as the tumor-specific nCNOX. Biotinylation the cell surface of HeLa S cells produced labeled proteins of 68, 52, and 34 kDa on Western blots using a nCNOX-specific anti-peptide antibody. The proteins at 52 and 34 kDa corresponded to molecular weights previously ascribed to nCNOX (Chueh et al. 1997. Arch. Biochem biophys. 342:32-47; Yantiri et al. 2001. Jbid 391:149-159), and the 29 kDa band corresponded to a protamine resistant fragment of nCNOX (Kelker et al. 2001. Biochemistry 40: 7351-7354). Biotinylation of HeLa S plasma membranes produced labeled proteins of 68, 52, and 34 kDa using avidin-alkaline phosphatase as a probe, and a protein of 68 kDa using the anti-nCNOX peptide antibody. When probed with anti-CNOX antibodies, a protein at 57 kDa was revealed suggesting the biotinylated protein of 57 kDa represents the polypeptide chain of CNOX.

1994

Predicting Breast Cancer Metastasis Using Protein Networks

H. Chuang, E. Lee, T. Ideker; 1 Bioinformatics, UCSD, San Diego, CA, 2Bioengineering, UCSD, San Diego, CA, 3Biosystems, Korea Advanced Institute of Science and Technology, Daejeon, Republic of Korea

Accurate prediction of metastatic potential is vital to determining the appropriate therapy for breast cancer patients. Current strategies analyze gene expression profiles to identify disease-correlated markers, without utilizing or revealing information on how genes interrelate within a larger functional network. To address this limitation, we apply a network-based approach that classifies expression profiles using pathway modules extracted from a huge human protein-protein interaction network consisted of 57,235 interactions among 11,203 proteins. Modules are sub-networks which have discriminative activities between good and poor prognosis patients. The quantitative activity of a defined module was inferred from the expression values of its member genes. A logistic regression based classifier is built based not on expression levels of individual genes but the activity levels of network modules. We show that network-based classification has two major advantages over previous approaches. First, it improves 8% of the performance when classifying metastatic versus non-metastatic breast tumors in terms of ROC AUC (Area Under Curve). Second, the discriminative network modules provide high-level models of the molecular mechanisms underlying metastasis. Many known breast cancer susceptibility genes such as BRCA1, TP53 and ESR1, which are not expression responsive, are included in our network modules but not able to be detected by previous approaches. The network-based approach integrating protein interactions with breast cancer expression profiles leads to increased classification accuracy and, simultaneously, provides an implicit view of the biological processes underlying metastasis.

1995

Computational Analysis for Splicing Sequences of Tumor-associated NADH Oxidase (iNOX) Exon4

V. D. Kane, X. Tang, C. P. Kuntz, S. C. Dick, D. J. Morre, D. M. Morre; Foods and Nutrition, Purdue University, West Lafayette, IN, 1Medicinal Chemistry and Molecular Pharmacology, Purdue University, West Lafayette, IN

Pre-mRNAs subject to alternative splicing contain conserved exonic sequences that direct the spliceosome to either include or exclude that exon in the final product. These sequences have been classified as Exonic Splicing Enhancers (ESEs) which promote exon inclusion, or Exonic Splicing Silencers (ESSs) which direct exon exclusion (Eisenstein, M. Nature. 2005). Serine/Arginine-rich proteins (SR proteins) and heteromultimeric ribonucleoproteins (hnRNPs) bind ESE and ESS sequences, respectively (Chen, C.D. et al. 2005: Genes & Development 13:593-606). Our laboratories have demonstrated that exon4 is not present in the final mRNA transcript for tumor-associated NADH oxidase (iNOX) in cancer cells (X. Tang, unpublished). In this study, the gene sequence for iNOX, located on the X chromosome, was analyzed via Genscan (genes.mit.edu/GENSCAN) for the presence of possible ESE and ESS sequences (Chew, S.L. et
A Model System for Cancer-specific Expression of tNOX Exon4-minus Splice Variant mRNA

C. P. Kunz, X. Tang, C. C. Kane, D. M. Morris, D. J. Morre; Foods and Nutrition, Purdue University, West Lafayette, IN

A cancer-specific (pancancer) cell surface NADH oxidase with protein disulfide-thiol interchange activity designated as tNOX, is a member of a larger family of related cell surface NADH oxidases or ECTO-NOX proteins. Expression of tNOX at the cell surface results from an exon4-minus splice variant mRNA transcript (X. Tang, unpublished). To investigate the mRNA-processing events leading to the splice variant, 3T3 (mouse) and HUVEC (human vascular endothelial) cells were inoculated with SV-40 (ATCC pml-2) and characterized post-infection for tNOX presence at the cell surface by Western blot analysis and presence of drug- (capsaicin)-inhibited NADH oxidase activity, a defining characteristic of tNOX protein. With SV-40-infected 3T3 cells, tNOX was absent on day 3 post-infection but strongly present on day 6 post-infection. With SV-40-infected HUVEC cells, the kinetics of tNOX appearance were similar with 32 kDa and 34 kDa bands indicated by Western blot analysis being absent on day 3 post-infection and increasingly evident on days 5 and 7 post-infection. The SV-40-infected HUVEC cells exhibited proteins cross-reactive with anti-tNOX antibodies at 32 and 34 kDa plus a protein, also cross-reactive with anti-tNOX antibody, corresponding to a previously described INOX-specific protein with a Mr of 52 kDa (Yantiri and Morre, 2001. Arch. Biochem. Biophys. 391:149-159). Control (uninfected) 3T3 and HUVEC cells lacked tNOX presence. The findings suggest that either model system, SV-40-infected 3T3 cells or SV-40-infected HUVEC cells, offer a relatively narrow window post-infection (between day 3 post-infection and days 5 to 7 post-infection) for exon4-minus mRNA initiation and delivery of tNOX to the cell surface. The 3T3 system is presently under investigation to test the involvement of exonic splicing silencer sequence-binding proteins as initiators of the cancer-specific expression of the tNOX protein.
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IX) and Src (PP2) inhibitors. The cells were then stained for beta-actin and results were quantified via robotic microscopy and an image analysis algorithm optimized for images derived from confluent cell cultures. MDCK cells were exposed to concentrations of the GSK 3 inhibitor IX ranging from 0.6 to 10 micromolar with approx. 1250 cells analyzed per well. GSK inhibitor IX led to a dose-dependent 3.4-fold increase in Total Cytoplasmic Area, a 3.3-fold increase in Total Cytoplasmic Intensity (Z’ = 0.63), and a 1.8-fold increase in Total Nuclear Intensity. For HeLa cells, beta-cell-permeable PP2 increased 2.2-fold by 5 micromolar GSK-3 inhibitor IX. Similarly, Total and Median Nuclear Intensity (MNI) increased 2.6- and 2.3-fold and the ratio Median Nuclear/Median Membrane Intensity was increased 39% at 10 micromolar (Z’ = 0.70). In contrast, 20 micromolar PP2, reduced nuclear beta-actin by 27% and blocked the ability of GSK 3 inhibitor IX to induce nuclear localization of beta-actin. Thus-beta-actin distribution can be quantified utilizing high-throughput/high content microscopy, and Src activity may be essential for Wnt induced nuclear translocation of beta-actin in HeLa cells.

2001

Comprehensive Analysis for the Acquisition of Invasiveness in Mammary Epithelial Cells

S. Hashimoto, S. Wei, A. Hashimoto, K. Miura, H. Sabe; Molecular Biology, Osaka Bioscience Institute, Suita, Japan

Metastases are the major cause of human cancer death and correlate well with invasive properties of the tumors. In the case of primary tumors of the human breast, genome analyses have so far suggested that most of specific genetic alterations in the invasive ductal carcinoma have also been found in the ductal carcinoma in situ, which is apparently non-invasive. Gene expression profiles have also shown that these pathologically discrete stages of breast carcinomas are highly similar at the transcriptosome levels. Therefore, what cellular changes evoke invasive phenomena during progression of human breast carcinomas remains largely unknown. Both hypoxia and epithelial-mesenchymal transition (EMT) have been highly implicated in invasive, as well as metastatic activities of different types of cancer cells. NMuMG mouse mammary gland epithelial cells undergo EMT upon TGFB treatment. We isolated several clones from NMuMG cells, that respond both to TGFB and hypoxia (3% O2) to become highly invasive in a Matrigel chemoinvasion assay. By gene expression profiling of these cells under nonoxia, hypoxia and TGFB-treatment, we identified genes whose expression is common to both TGFB and hypoxia and EMT in mammary epithelial cells as compared with those under normoxia. Among them, we found that expression of NM23-M1, as well as its protein, is drastically downregulated under hypoxia and EMT. Forced expression of NM23-M1 inhibits matrigel invasion of NMuMG cells under hypoxia and EMT. A human homolog of NM23-M1, namely NM23-H1, was previously reported to be a tumor suppressor in melanoma. We will show a mechanism by which hypoxia and EMT commonly downregulate expression of NM23-M1. Our results suggest an aspect in which epigenetic events are deeply involved in processes acquiring invasiveness in breast carcinomas.

2002

A Novel Three-Dimensional Model to Quantitatively Study the Radial and Vertical Invasion of Melanoma

C. M. Gharai, V. Suresh, S. R. Peyton, C. B. Rauh, S. C. George, A. J. Putnam; 1Biomedical Engineering, University of California, Irvine, Irvine, CA, 2Chemical Engineering & Materials Science and Biomedical Engineering, University of California, Irvine, Irvine, CA

Melanoma, which accounts for 80% of all skin cancer deaths, is characterized by the aberrant growth of melanocytes within the epidermis (radial growth phase) and eventual penetration of transformed cells through the basement membrane into the underlying dermis (vertical growth phase). To study this progression and quantify the 3-D invasion of melanoma in vitro, we have developed a 3-D culture system in which cells coated onto microcarrier beads are embedded within a fibrin hydrogel with an over-coated dermal fibroblast monolayer. In particular, beads were coated with human epidermal neonatal melanocytes (HEM), radial growth phase melanoma cells (M14s), or highly metastatic melanoma cells (C8161,9) and observed over a 7-day period to monitor 3-D cell proliferation and penetration into the surrounding matrix. An image processing algorithm developed to quantitatively assess cell invasion revealed that the in vitro invasion of each cell type recapitulated its in vivo phenotype. Specifically, the radial invasion of C8161s was nearly three times greater than M14s, which were over twice as invasive as HEMs. Removing the fibroblast monolayer did not significantly affect C8161 and HEMs invasion, but led to far more aggressive and uncoordinated invasion of M14s. A confocal technique developed to study vertical invasion in this system confirmed that HEMs and M14 invasion remained planar while C8161 vertical penetration, regardless of fibroblast presence, was dramatically more extensive. Similar results were also obtained in matrices containing interstitial type-I collagen, except that the radial invasion of C8161s, in addition to M14s, became uncoordinated in the absence of fibroblasts. Collectively, these data demonstrate the utility of this novel 3-D model of melanoma invasion to quantitatively assess radial and vertical invasion in vitro. This system may be useful to screen the efficacy of pharmacologic inhibitors on melanoma growth, and may provide new mechanistic insights regarding the progression of melanoma.

2003

Evaluation of the Protective Effect of Fish Oil and Selenium on the Lung and Serum of 1,1'-Dimethyl-4,4'-Bipyridinium Dichloride Intoxicated Rats


1,1'-Dimethyl-4,4'-Bipyridinium Dichloride (paraquat) is a broad-spectrum herbicide known to produce lung injury via oxidative stress-mediated mechanisms. Different pharmacological strategies have been explored to reduce the formation of these reactive oxygen species and/or prevent their toxic effects in the treatment of paraquat poisoning. The present study was carried out to investigate the possible protective effects of fish oil (20 mg/kg) and selenium (0.26 mg/kg) against the toxic effects of paraquat. The present study was carried out to investigate the possible protective effects of fish oil (20 mg/kg) and selenium (0.26 mg/kg) against the toxic effects of paraquat. Eighty adults' albino rats were used in this study; they were classified into eight groups. Control, fish oil, selenium, paraquat (10 mg/kg), selenium + fish oil, paraquat + fish oil, paraquat + selenium and paraquat + selenium + fish oil. Selenium and fish oil were given orally for 12 consecutive days and paraquat was given intraperitoneally 24 hours before decapitation of the animals. Fish oil by itself increased lipid peroxidation and HDL-cholesterol markedly, catalase while LDL-cholesterol and triglycerides were decreased. Paraquat increased serum and lung lipid peroxidation and decreased catalase, and glutathione levels. Selenium alone or when it combined with fish oil pretreatments ameliorated these disturbances in prooxidant-antioxidants balance. These results indicate that antioxidant pretreatment prior to paraquat may have some beneficial effects against paraquat-induced lung injury. Also the beneficial effects of fish oil are improved when it provided with selenium.

2004

TRPV1 Induces a p53-dependent Apoptosis of Human Bladder Cancer Cells

R. Luciarrini, C. Amanti, M. Mosca, M. Rabini, S. Caprondi, A. Di Spilimbergo, M. A. Cardarelli, G. Mammana, G. Santoni; 1Experimental Medicine and Public Health, University of Camerino, Camerino, Italy, 2Pathological Anatomy and Cytodiagnostic, ASUR 9, Macerata, Italy, 3Pathological Anatomy and Cytodiagnostic, ASUR 9, Camerino, Italy, 4Urology Operation Unit, ASUR 9, Macerata, Italy

The p53 tumor suppressor gene is the most frequent mutated gene in human cancer. The present study was undertaken to analyse the expression and role of TRPV1 in human normal urothelial cells and bladder TCC lines expressing both p53 wild-type and p53 mutations. Thus, we evaluate by qRT-PCR, the expression of TRPV1 in human RT-4, TCCSUP, J82 and EJ cell lines. As normal control urothelial cells (HUC) and normal bladder tissues were utilized. As evaluated by quantitative real-time PCR analysis, TRPV1 mRNA is up-regulated in low grade, p53 wild-type expressing RT-4 cells, and unchanged or markedly down-regulated in high-grade TCCSUP, J82 and EJ cells harboring p53 mutations. By cytofluorimetric, confocal microscopy and western blot analyses we found that the expression of TRPV1 protein in normal HUC, and RT-4, TCCSUP, J82 and EJ cells, parallels the mRNA expression with low-grade RT-4 cells showing higher expression; in addition TRPV1 protein localizes on the plasma membrane and cytoplasm in RT-4 and TCCSUP cells, whereas negligible or negative TRPV1 expression is found in J82 and EJ cells. The TRPV1 channel is functional in RT-4, but not TCCSUP, J82 and EJ cells, as evaluated by its ability to induce increase of [Ca2+] in response to the TRPV1 agonist, capsaicin (CPS) stimulation. CPS treatment, also reduces in a dose-dependent manner, RT-4, TCCSUP, J82 and EJ cell viability, with RT-4 showing the higher sensitivity. Moreover, CPS exposure induces, increase in PS externalization, caspase-3 activation, Go/G1 subdiploid oligonucleosomal DNA fragmentation and apoptosis on RT-4, but not TCCSUP, J82 and EJ cells. The CPS-induced apoptosis of RT-4 cells, is associated to a time-dependent increase of p53 protein level, and induces p53 phosphorylation at the Ser-15, -20 and -392 residues. Overall, these findings suggest that bladder cancer cells expressing the p53 wild-type and TRPV1 are highly susceptible to CPS-induced apoptosis.

2005

Inhibition of NFkβ by a Novel Curcumin Analogue

A. L. Kasinski, Y. Du, S. Thomas, A. Sun, J. Snyder, D. Liotta, H. Fu; 1Department of Pharmacology, Emory University, Atlanta, GA, 2Graduate Program in Genetics and Molecular Biology, Emory University, Atlanta, GA, 3Graduate Program in Molecular Systems Pharmacology, Emory University, Atlanta, GA, 4Department of Chemistry, Emory University, Atlanta, GA

NFkβ is a transcription factor which is activated in response to various stimuli and has been shown to play a critical role in regulating inflammatory and immune responses. However, constitutive activation of NFkβ has also been associated with the development of various diseases, including cancer. Therefore, there is a need for the development of new and specific inhibitors of NFkβ. In our study, we have developed a novel curcumin analogue which is selectively and effectively inhibits NFkβ. The analogue has been shown to inhibit NFkβ activity at micromolar concentrations and has been shown to be safe in cell-based assays. Our findings suggest that this novel curcumin analogue has potential as a therapeutic agent for the treatment of diseases associated with NFkβ activation.
degradation. The inhibition of IκBα degradation was due to the inability of the inhibitor of kappa B kinase β (IKKβ) to phosphorylate IκBα, suggesting that EF24 may interfere with IKKβ activity. Using recombinant IKKβ and IκBα in an in vitro kinase assay, we show that EF24 but not curcumin effectively inhibited the ability of IKKβ to phosphorylate IκBα. These data suggest NFKB as a major target of EF24. Characterization of such small molecules with low to no toxicity will yield new avenues to explore the treatment of various forms of cancer as well as to unravel additional molecular mechanisms involved in cellular pathways.

Dynamics On Cancer Interrelated P53-MDM2 Feedback Loop Regulated By P19ARF

H. Liu, T. Zhan, Y. Han, Y. Z. Zhao, T. J. Zhao 2, Hebei University of Technology, Tianjin, China, 2China Institute of Atomic Energy, Beijing, China

The p53 tumor suppressor protein plays a key role in preventing the development of cancer and is inactivated in many human malignancies. Because some of the cellular effects of activated p53 can be irreversible, keeping p53 function under tight control in normal cells is critical. A key player in the regulation of p53 is the Mdm2 protein. This duality defines a negative feedback loop, which is widely recognized. P19ARF is another important new tumor suppressor protein, it has its own independent promoter. The P19ARF protein can increase the level of p53 by neutralizing Mdm2 which destabilize p53, ultimately play a role in suppressing cancer. During the years, several models addressing p53 in the context of statistical theories of multistage tumorigenesis have been proposed. We now present a dynamical model of the p53-Mdm2 feedback loop regulated by P19ARF both in individual cell and in population of cells. In our attempt to capture the gross mechanisms of p53-Mdm2 interactions regulated by P19ARF, we have investigated numerically how different parameters can shape the types of behavior that the system can exhibit. In particular, we show that specific assumptions characterizing the interactions between p53 and Mdm2 regulated by P19ARF lead to an oscillatory behavior of p53, Mdm2 and p19 protein levels after a sufficiently strong damage signal. Such oscillation may enable the more effective execution of a reversible p53 response. In agreement with this prediction, the levels of three proteins are proved to satisfactorily fit experimental results reported in lung cancer cells. The dynamical model of cancer interrelated P53-MDM2 feedback loop regulated by P19ARF and its numerical results will help to understand the origin of cancer and oscillatory behavior of p53, Mdm2 and P19ARF more deeply and rationally.

The Human Microneta Mir-21 Modulates Tumor Sensitivity to Chemotheraphy In Vivo by Modulating PTEN Dependent Signaling

R. Henson, F. Meng, H. Wehbe, T. Patel; Scott and White Clinic, Temple, TX

miRNAs (miRNAs) are endogenous regulatory RNA molecules that can mediate gene expression. Increased expression of selected miRNAs such as miR-21 occurs in several types of human cancer. We have recently shown that miR-21 can modulate PTEN expression in human cholangiocarcinoma cells in vitro (Gastroenterology 130(7):2113-29). Altered miR-21 expression could therefore modulate cell survival pathways and contribute to resistance to chemotherapy. Thus, our aim was to assess the role of miR-21 in the regulation of chemoresensitivity in human cholangiocarcinoma in vivo. Mz-Cha-1 human cholangiocarcinoma cell xenografts were established in eight week old male athymic nu/nu lines. Once tumor volume exceeded 200 mm³, mice were injected intra-tumorally (4 ng/mouse tumor) with either anti-miR-21 (n=6) or control anti-miRNA (n=6). The chemotherapeutic agent gemcitabine (120 mg/kg) was administered i.p. every three days for a total of 3 doses, and tumor volumes measured by serial measurements. Tumor growth in response to gemcitabine was decreased by 24-48 ± 12.18 % in xenografts receiving a single injection of anti-miR-21 whereas it was only reduced by 1.34 ± 5.40 % in controls after 10 days. Expression of potential downstream targets of miR-21 was assessed by immunoblot analysis of tumor cell xenograft homogenates. An increase in expression of PTEN along with decreased phosphorylation of Akt was observed in tumors treated with anti-miR-21 compared to controls. Thus, targeting miR-21 dependent signaling pathways may be a useful strategy to improve therapeutic responses for highly treatment refractory tumors such as cholangiocarcinoma. Regulation of PTEN dependent cell survival pathways may represent an important mechanism by which miR-21 modulates tumorigenesis and chemoresistance in cholangiocarcinoma and other cancers.

Modulation of E-cadherin and Actin in Colon Cancer Cells

C. P. Bennett, G. S. Marchetti, A. F. Witts, K. Book, R. Tripathi, S. Parikh; University of the Witwatersrand, Johannesburg, South Africa

During the phase of epithelial to mesenchymal transition (EMT) cancer cells acquire migratory phenotypes and become invasive. Linked to EMTs are changes in cadherin expression and regulation of the actin cytoskeleton. In cell migration/wound assays we show that treatment of human adenocarcinoma HT29 cells with the immunomodulatory drug levamisole, results in the inhibition of cell migration in a dose dependent manner with an associated alteration of cell morphology. As levamisole concentration increased from 0.1 to 5 mM, migration declined. At 5 mM levamisole, the wound edge remained smooth with very little evidence of cell migration. In control cultures, cells migrated extensively and in such cells, confocal microscopy showed cytoplasmic-localisation of E-cadherin and actin in a punctate pattern. However, when cells were cultured with levamisole, E-cadherin was polarised to the cytoplasm to a distinct peri-nuclear cytoplasmic accumulation, with additional nuclear associated E-cadherin expression. In association with increasing concentrations of levamisole (ranging from 0.1 to 5 mM), cells began to lose their typical fibroblast-like morphology, displaying reduced cytoplasmic processes with cytoplasmically localised actin stress fibres. Actin was also associated with the peripheral edges of filopodia. At 5 mM levamisole, cells retracted their cytoplasmic processes and rounded-up, with the distinct formation of cytoplasmic actin stress fibres, and with an actin band around the cell periphery. In the absence of levamisole, control cultures lacked stress fibres and cells retained a fibroblast-like morphology with extensive cellular processes. The changes in cytosolic E-cadherin and cellular actin distribution suggest that levamisole may modulate small Rho-GTPase signalling, thus influencing migratory cell behaviour.

Measurement of Oncoproteins in Preclinical and Clinical Specimens Using a Nano-fluidic High Throughput Approach


Studying changes in oncogene protein expression and activation is increasingly important in the development of molecularly targeted therapeutics. Serial tumor sampling to assess biological endpoints is severely limited by the invasiveness of procedures required to acquire adequate numbers of cells for investigation. Here we demonstrate the use of an automated nano-fluidic system to measure changes in the expression of oncoproteins in preclinical and clinical tumor specimens. We measured the levels of MYC, BCL2, and AKT in serial fine needle aspirates of haematopoietic tumor cell populations obtained from patients with Burkitt’s lymphoma and chronic lymphocytic leukemia. We have previously shown that inactivation of MYC sustains tumor regression using our conditional model of MYC-induced lymphoma. Hence, targeting the inactivation of oncogenes may be useful for the treatment of cancer. Tumor-derived cell lines generated from conditional transgenic mouse models of BCL-2/MYC-induced lymphoma were injected subcutaneously into syngeneic mice. Upon BCL-2/MYC inactivation, serial fine needle aspirations (FNAs) of tumors were performed. Using this technology BCL-2/MYC protein levels were found to decrease in the serial FNAs. This approach was validated by measuring the levels of MYC, BCL2, ERK and AKT proteins in the lymph nodes obtained from patients with Burkitt’s, mantle cell and follicular lymphoma. MYC was overexpressed in Burkitt’s and BCL2 was overexpressed in mantle cell and follicular lymphoma patients. In parallel, traditional Western blots were performed to confirm BCL-2/MYC levels. Thus, we have developed a technique with the ability to quickly assess the levels of key signaling/onco proteins from small preclinical transgenic mouse models and human clinical lymphoma samples. This enables the possibility of monitoring tumor response to targetted therapies. Additionally, this approach has wide applications: (1) high throughput strategy for preclinical characterization of tumors (2) clinical analysis of tumor specimens and (3) for use in molecular diagnostic assays to improve the treatment of cancer.
2010
Caspase-6 in Alzheimer Disease
A. C. LeBlanc, S. Albrecht, T. Petzke; Pathology, McGill University, Montreal, PQ, Canada

While amyloid and neurofibrillar tangles have been widely investigated in Alzheimer disease, relatively little is known about the underlying molecular mechanisms of cell death and degeneration in these brains. Using neuropilhepros for the active p20 subunit of Caspase-6 and to Tau protein cleaved by Caspase-6, we have identified strong activity of Caspase-6 in senile plaques, neurulps and neurofibrillar tangles in hippocampal from mild, moderate, severe and very severe Alzheimer disease individuals. Interestingly, Caspase-6 activity is already present and strong in the entorhinal cortex of mildly cognitively impaired aged individuals. In contrast, there is no active Caspase-6 in the hippocampi from young individuals. The neurons that are affected do not display the morphological characteristics of apoptotic cells. A proteomic study of caspase-6-mediated cleavage of neuronal proteins indicated thirty-three proteins proteolysed by the addition of active Caspase-6 in cytosolic or membrane proteins from primary cultures of human neurons. Thirty percent of these proteins represent cytoskeleton or cytoskeleton-associated proteins. Drehem, an actin-binding protein of post-synaptic densities, and alpha-tubulin, are both directly cleaved by active Caspase-6 in vitro. These results indicate that Caspase-6 could alter learning and memory through the disruption of the neuronal cytoskeleton very early in Alzheimer disease.

2011
Tau Redistribution, Truncation and Colocalization with Tubulin Accompanies Dendritic Degeneration in an In Situ Cellular Model of Tauopathy
W. Kim, C. Jung, G. F. Hall; Biological Sciences, U. Mass. Lowell, Lowell, MA

Drosophila Giant neurons (ABCs) in the brain of the larval sea lamprey that have been microinjected with plasmids expressing human tau (htau) isoforms accumulate filamentous tau aggregates and undergo neurofibrillary degeneration in a manner similar to that seen in human familial tauopathies and Alzheimer's Disease. We have used this model to investigate the cellular mechanisms underlying the neurodegeneration induced by abnormal accumulation of htau. In particular, we have focused on the progressive distal to proximal dendritic degeneration that occurs with prolonged (10–40+ days) of htau expression. In this study, we expressed the longest htau isoform (tau40) containing the P301L mutation in ABCs for 10, 20 and 30 days post plasmid injection (ppi). Ten micron sections through htau-expressing cells were co-labeled with a mAb (tau 12) specific for the htau N terminal and a mAb (C3) specific for the tau species truncated at residue 420. Other sections were co-labeled with tau1 (the mAb specific alpha tubulin (D1M1A). We show that tubulin levels are progressively reduced over time in htau-expressing ABCs, while dendritic beading and fragmentation are accompanied by the co-localization of tubulin and htau in perineuronal aggregates. In addition, we found that 420-truncated htau (C3 tau) was generated in most htau-expressing cells by 20 days ppi. C3 tau was particularly prominent in high density htau deposits in ABC dendrites and axons. In summary, we identify changes in dendritic tubulin and htau distribution that are correlated with dendritic degeneration in an in situ cellular model of htau-induced neurofibrillary degeneration. We also show that these changes are associated with the C terminal truncation of overexpressed htau, which recent studies suggest may be a key event in the generation of filamentous htau. Further studies of this model thus appear likely to shed light on the cellular mechanisms underlying human tauopathies.

2012
Pre-fibrillar β-Amyloid Leads to Tau Dependent Microtubule Disassembly in Neuronal and Non-Neuronal Cells
M. E. King, H. Kan, P. Bazz, A. Eris, C. G. Gable, G. S. Bloom; Biology, University of Virginia, Charlottesville, VA, Neurobiology and Anatomy, Drexel University College of Medicine, Philadelphia, PA, Psychology, University of Virginia, Charlottesville, VA, Molecular Biology and Biochemistry, University of California at Irvine, Irvine, CA

Alzheimer’s Disease (AD) is defined histopathologically by extracellular β-amyloid (Aβ) fibrils plus intraneuronal tau filaments. Studies of transgenic mice indicate that AD is caused by a pathological cascade in which Aβ lies upstream of tau, but that the steps which connect Aβ to tau have remained undefined. To seek evidence for how Aβ signals to tau, various forms of Aβ were provided extracellulary to primary neurons that expressed endogenous tau or were treated with siRNA to suppress tau protein levels to ~5% of normal, or to non-neuronal cells that co-expressed tau-CFP plus GFP-tubulin. To assay effects of the Aβ, the neurons were analyzed by a quantitative biochemical assay for polymerized and unassembled tubulin, and the non-neuronal cells were monitored by live cell, time lapse fluorescence microscopy. For both cell types, we found that tau confers acute hypersensitivity of microtubules to pre-fibrillar Aβ, which comprises monomers and small oligomers of β-amyloid peptides. Pre-fibrillar Aβ42 was active at submicromolar concentrations, several-fold below those required for equivalent effects of pre-fibrillar Aβ40, and microtubules were insensitive to fibrillar Aβ. Microtubule disassembly typically occurred abruptly within 1.5 hours of exposing cells to pre-fibrillar Aβ, and transfection of non-neuronal cells with fluorescently tagged MAP2C, MAP2C-tau chimeras and tau fragments established that the active region of tau is localized to an N-terminal domain that does not bind microtubules and is not part of the region of tau that assembles into filaments. Surprisingly, tau-dependent microtubule disassembly induced by pre-fibrillar Aβ was not accompanied by AD-like phosphorylation of tau. Taken together, these results highlight the most dramatic, rapid and sensitive link between Aβ microtubules and is not part of the region of tau that assembles into filaments. Surprisingly, tau-dependent microtubule disassembly induced by pre-fibrillar Aβ was not accompanied by AD-like phosphorylation of tau. Taken together, these results highlight the most dramatic, rapid and sensitive link between Aβ microtubules and is not part of the region of tau that assembles into filaments. Surprisingly, tau-dependent microtubule disassembly induced by pre-fibrillar Aβ was not accompanied by AD-like phosphorylation of tau. Taken together, these results highlight the most dramatic, rapid and sensitive link between Aβ microtubules and is not part of the region of tau that assembles into filaments. Surprisingly, tau-dependent microtubule disassembly induced by pre-fibrillar Aβ was not accompanied by AD-like phosphorylation of tau. Taken together, these results highlight the most dramatic, rapid and sensitive link between Aβ microtubules and is not part of the region of tau that assembles into filaments. Surprisingly, tau-dependent microtubule disassembly induced by pre-fibrillar Aβ was not accompanied by AD-like phosphorylation of tau.
2016

Alcadein, a Novel Cargo Receptor to Kinesin I, Regulates APP Metabolism
T. Suzuki, Y. Araki, T. Yamamoto, T. Kawano, M. Kinjo; Graduate School of Pharmaceutical Sciences, Laboratory of Neuroscience, Hokkaido University, Sapporo, Japan, *Research Institute for Electric Science, Hokkaido University, Sapporo, Japan

Amyloid b-protein (Ab) is generated by consecutive cleavages of APP with BACE and g-secretase complex, and aggregation and deposition of Ab are closely related to Alzheimer’s disease (AD) pathology. APP in neuron forms a tripartite complex with Alcadein (Alc), a type I membrane protein similar to APP, through their cytoplasmic interaction with the adaptor X11-like protein (X11L). Formation of a tripartite APP/X11L/Alc complex stabilizes intracellular metabolism of both APP and Alc. When X11L dissociates from the complex, APP and Alc are simultaneously cleaved in a coordinated fashion. Thus, naked APP generates Ab (or p3) and naked Alc generates b-Alc peptide. We therefore considered a possibility that Alc may be similar to APP in function as well as its metabolism. We found that Alc directly associates to KLC of kinesin I motor, assembles the motor complex, and drives cargo to neuron terminals functioning as a novel cargo receptor. The transport of Alc cargos compete with that of APP-containing vesicles for the kinesin I motor, and disruption of APP-containing vesicle transport increase Ab generation. It is known that APP interacts with kinesin I through JIP1b, but in vivo, Alc transport is largely independent of APP with different transport velocities. Thus, APP and Alc are separately transported to nerve-terminal, and then exposed on the plasma membrane. The three proteins, APP, X11L, and Alc may form a complex either prior to leaving the Golgi apparatus, at the plasma membrane, or during the endocytic process. Inappropriate connection of APP- and Alc-containing vesicles to kinesin I motor suggested in Alzheimer generation, suggesting vesicular transport system regulated by kinesin I motor with Alc and JIP1b is important for the physiologic metabolism of APP and Alc.

2017

Involvement of Phosphatidyl Serine as a Surface Membrane Receptor for the Alzheimer’s Disease Aβ Peptide
O. Simakova, N. J. Arispe; Anatomy, Physiology and Genetics, Uniformed Services University, Bethesda, MD

The toxic effect of exogenous Aβ on cells is initiated when Aβ directly interacts with components in the plasma membrane resulting in disruption of the membrane permeability. The interaction of Aβ with the membrane is selective to some cells, and to cells on specific regions of the Alzheimer Disease brain, suggesting that membrane components may be acting as receptors for Aβ. The identity of a membrane receptor for Aβ has been a quest for many years. Several candidates cell surface receptors have been considered, but recent works and our own preliminary results, have provided information implying the phospholipids phosphatidylserine (PS) as one of the possible membrane surface receptors. To provide irrefutable data supporting this finding different types of cells lines and ex-vivo neurons were exposed to fluorescent annexin V and to Aβ and sorted with Facs/Aria/BD cell sorter on the basis of their surface membrane Aβ binding. We found that cells within the same cell line can successfully be grouped and sorted in subpopulations based in their affinity for Aβ. These subpopulations of cells showed distinct morphological and compositional characteristic. The degree of cell affinity for Aβ binding suffered of a much higher induction of apoptosis when exposed to Aβ. We concluded that these distinct cell indicators may be the basis for the selective attack of Aβ on some cells in culture, and also observed on cells in specific regions of the AD brain.

2018

Cellular Indicators Correlated with the Cell Specific Binding and Cytotoxic Levels of the Alzheimer’s Aβ Peptide
O. Simakova, N. J. Arispe; Anatomy, Physiology and Genetics, Uniformed Services University, Bethesda, MD

Despite the Aβ peptide normal existence in healthy individuals, abundant evidence has shown that external application of Aβ on cells, either in cell cultures or in animal brains, has toxic effects. However, in Alzheimer’s disease Aβ selectively attacks on specific regions of the brain, and experiments in vitro show that not all cells within the same cell line are equally affected by external addition of Aβ, and some cells are even found to be resistant. To study this phenomenon we characterized specific features and stages of the cells that make them exceptionally sensitive or resistant to the effect of Aβ. Different types of cells lines and ex-vivo neurons were exposed to Aβ conjugated to a fluorescent probe (Aβ-FITC), and sorted with Facs/Aria/BD cell sorter on the basis of their surface membrane Aβ binding. We found that cells within the same cell line can successfully be grouped and sorted in subpopulations based in their affinity for Aβ. These subpopulations of cells showed distinct morphological and compositional characteristic. The degree of cell affinity for Aβ correlated with the levels of phosphatidylserine on the cell membrane surface and the cell cycle stage. The sorted subpopulations of cells with higher affinity for Aβ binding observed after externalizing PS with the apoptosis inducer camptotecin, and by the increased Aβ-induced mortality and LDH release on sorted cells with higher surface PS. The selectivity of the Aβ binding to surface PS was confirmed with anti-PS antibody. Our data convincingly confirm the involvement of PS as a membrane receptor for Aβ.

2019

Coordination with the Histidines Residues in the Aβ Molecule Affects Aβ Channel Activity and Cytotoxicity
J. C. Diaz, N. J. Arispe; Anatomy, Physiology and Genetics, Uniformed Services University, Bethesda, MD

The Aβ peptide associated with Alzheimer’s disease interacts with neurons to generate a potentially toxic increase in the intracellular calcium concentration. We have shown that the mechanism of the intracellular calcium increase is by the Aβ peptide itself forming an independent ion channel that conducts the calcium into the cell, disturbing the intracellular homeostasis. We recently showed that peptide segments, chosen from regions around the putative mouth of the polymeric membrane-bound Aβ channel model, effectively block the Aβ channel activity in planar lipid bilayers. We also showed that the presence of histidine residues in these peptides were essential for blocking the channel and for blocking Aβ cytotoxicity. We proposed that coordination between adjacent histidine-containing compounds and histidines in these peptides might have been established, thus blocking the entrance of the pore, the flow of current through the channel, and consequently blocking Aβ cytotoxicity. To test this proposition we studied the effect of compounds that potentially coordinate with the histidines in the Aβ molecule, on the activity of Aβ channels formed in the surface membrane of cells. Nickel ions, the histidine-reactive chain imidazole and a series of histidine-related compounds were studied on Aβ ion channels incorporated into planar lipid bilayers and on cells in culture exposed to Aβ. The results showed that the efficiency of these compounds to block the Aβ current activity in planar lipid bilayer was correlated with their capacity to react with the histidine residues in Aβ. This reactivity was also correlated with the magnitude of the prevention of cell surface membrane and mitochondrial disruption induced by Aβ. These data provide further evidence that not only the interaction with histidine residues are essential for blocking the Aβ channels, but that the Aβ ion channel activity contributes significantly to the cytoxic properties of exogenous Aβ.

2020

In Situ Aβ Pores in Neurons in the Brain with Alzheimer’s Disease: Ultrastructural Demonstration
S. Inoue; Anatomy and Cell Biology, McGill University, Montreal, PQ, Canada

According to the amyloid pore hypothesis, pores formed in the membrane by small oligomers of misfolded amyloidogenic proteins cause unregulated rapid influx of ions such as calcium ions which interferes with intracellular ion homeostasis resulting in cell death. Pores of various amyloid proteins have been reconstituted in vitro in lipid bilayer membranes and their ultrastructural features were examined by transmission electron microscopy (TEM) or atomic force microscopy. However, the presence of in situ amyloid pores in amyloid tissues has not yet been ultrastructurally demonstrated. In this study the attempt was made to ultrastructurally identify in situ pores of β amyloid protein (Aβ) in vivo with TEM in the neuronal cell membrane in the brain of patients with Alzheimer’s disease. Within the tissue of β amyloid all unbound Aβ identified by immunolabeling, was localized in the form of 2-3 nm wide helices (“helical rods”) formed by the tight coiling of 1 nm wide Aβ filaments. In the cell membrane of neurons the pore-like structures were randomly distributed. They were round entities of the diameter of 16 nm with a rim of the thickness of 3 nm and a 10 nm wide lumen. The rim was made up of a cylindrical sheet of laterally associated Aβ helical rods 11 nm in length. Since no other pore-like structures were found in the membrane it is proposed that these 16 nm wide short columnar structures perforating the cell membrane are toxic Aβ pores. Also, since Aβ helical rods were the only conformation taken by unbound Aβ, it is proposed that this characteristic helical conformation is the ultrastructural representation of misfolded Aβ. Thus, the demonstration of the presence of in situ Aβ pores in this study in the amyloid tissue would further strengthens the amyloid pore hypothesis for the mechanism of β amyloid pathogenesis.

2021

Seeding of Neuritic Plaques by Oligomeric Amyloid-β That Accumulates in Cortical and Hippocampal Projections of Brainstem Neurons: A Hypothesis
Z. Muresan, V. Muresan; Physiology and Biophysics, Case Western Reserve University, Cleveland, OH

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Our goal is to understand the pathogenesis of amyloid-β (Aβ) deposition in the Alzheimer's disease (AD) brain. It is thought that neuritic plaques that form in the hippocampus and cerebral cortex are initiated by a “bad seed” of oligomized Aβ, but the origin of this seed is unknown. We established a cell culture system, where brainstem-derived, catecholaminergic, neuronal cells (CAD) produce and accumulate within their processes large amounts of Aβ peptide, similar to what is believed to occur in brain neurons, in the initial phases of AD. Using this system, we show that accumulation of Aβ begins within neurites, prior to any detectable signs of neurodegeneration. Neuritic accumulation of Aβ is restricted to a small population of neighboring cells that express normal levels of amyloid-β precursor protein (APP), but show redistribution of β-secretase to the processes, where it co-localizes with Aβ and markers of late endosomes and autophagic vacuoles. Invariably, cells that accumulate Aβ appear in isolated islets, suggesting their clonal origin from a few cells that show propensity to accumulate Aβ. We further show that, unlike cultured cortical and hippocampal neurons, the brainstem-derived CAD cells accumulate oligomized Aβ primarily at the terminals of their processes. Since brainstem neurons innervate many brain regions, including the cerebral cortex and the hippocampus, they could provide the “bad seed” that nucleates plaques. These results suggest that Aβ accumulation is initiated in a small number of brainstem neurons by intracellular determinants that alter APP metabolism. We propose that plaque formation in the cerebral cortex and hippocampus is seeded by oligomized Aβ that accumulates at the terminals of projections of such brainstem neurons. CAD cells appear to recapitulate the biochemical processes leading to Aβ deposition, thus providing an experimental in vitro system for studying the molecular pathobiology of AD.

2022
Development of a Human Model System for the Study of Axonal Transport Defects
R. L. Nolan, J. D. Filippin, E. A. Davis, L. S. Goldstein; University of California—San Diego, La Jolla, CA
Recent work from our lab suggests that defects of individuals affected by Alzheimer's disease (AD) are likely to contain axonal defects. These defects are enhanced in Drosophila (Gunnawarden & Goldstein 2001) and mouse (Stokin et al 2003) AD models expressing reduced levels of the microtubule-based motor protein kinesin-1. To model these defects in human cells, we generated human embryonic stem cell (hESC) lines with Cre-regulatable RNA interference against the kinesin-1 subunit KLC1 (kinesin light chain 1). We transduced HUES9 hESC with lentivirus packaged to carry Cre-dependent expression of a small hairpin RNA sequence directed against KLC1. We then isolated and propagated individual colonies containing infected cells and characterized their progeny. We are now using these cell lines to reduce kinesin levels and to model cellular transport defects in human neurons differentiated in vitro.

2023
Pathological Consequences of VCP-Mutations on Human Striated Muscle: Nuclear and Cytoplasmic Inclusions, a Distinct Form of Dilatative Cardiomyopathy, and a Novel Link to VCP-Glycoprotein Interactions
C. U. Hübers,1 A. Hofmann,2 A. Bödicker,1 D. R. Thal,4 R. Schröder,4 C. S. Clemen1; 1Institute of Biochemistry I, University of Cologne, Cologne, Germany, 2Institute of Structural and Molecular Biology, University of Edinburgh, Edinburgh, United Kingdom, 3Department of Neuroproteomics, Max-Delbrück-Center for Molecular Medicine, Berlin, Germany, 4Institute for Neuropathology, University Hospital Bonn, Bonn, Germany
Mutations in the valosin containing protein (VCP, p97) gene cause a late-onset form of autosomal dominant Inclusion Body Myopathy associated with Paget disease of the bone and Frontotemporal Dementia (BMPF/D). VCP is an ubiquitously expressed member of the AAA-ATPase family with a tripartite structure comprising an N-terminal (Cdc48) domain involved in ubiquitin-binding, and two central D1- and D2-domains which hydrolyse ATP. VCP assembles into functional hexamers with a central cylinder formed by the D-domains surrounded by the N-domains. VCP has been associated with a wide variety of essential cellular processes including the ubiquitin proteasome protein degradation system and along with its co-factors Ufd1, Npl4, and Derlin-1 with the endoplasmic reticulum associated protein degradation (ERAD). We report on the pathological consequences of three heterozygous VCP-mutations (R93C, R155H, R155C), located in the N-terminal Cdc48 domain of the VCP protein) on human striated muscle in vivo and in vitro. IBMPF/D skeletal muscle pathology is characterized by degenerative changes and filamentous VCP- and ubiquitin-positive cytoplasmic and nuclear protein aggregates. Furthermore, mutant VCP leads to a novel form of dilatative cardiomyopathy with inclusion bodies. In contrast to post-mitotic striated muscle cells aggregate proteinopathy was neither detected in primary IBMPF/D myoblasts nor in transiently and stably transfected cells using wildtype-VCP and the VCP mutants. Pull-down experiments showed that all three VCP mutations do not affect the binding to Ufd1, Npl4, and ataxin-3. Structural analysis demonstrated that R93 and R155 are both surface-accessible residues located in the center of cavities that may enable ligand binding. Mutations at R93 and R155 are predicted to induce changes in the tertiary structure of the VCP protein. The search for putative ligands to the R93 and R155 cavities resulted in the identification of cyclic sugar compounds providing a novel link to VCP carbohydrate interactions in the complex pathology of IBMPF/D.

2024
CMT1A Neuropathies Caused by Partial Duplication of 17p11.2-p12 Region
J. S. Yu,1 J. K. Kim,1 B. O. Choi,2 K. Chung1; 1Biological Science, Kongju National University, Kongju, Republic of Korea, 2Neurology, Ewha Womans University, Seoul, Republic of Korea
Charcot-Marie-Tooth disease (CMT) is the most common motor and sensorimotor peripheral neuropathy. Tandem duplication of chromosome 17p11.2-p12 including PMP22 gene is the most frequent cause of CMT1A. It has been suggested that the mechanism of CMT1A duplication is remarkably uniform, however, two CMT1A families (FC85 and FC116) with partial duplication were identified as rare cases. The microsatellite genotyping analysis suggests that the proximal part of 17p11.2-p12 region which includes the PMP22 gene was partially duplicated in both families. These partial duplications were also confirmed by real-time PCR. It appears that this first (FC85 family) had a de novo mutation of paternal origin due to intrachromosomal rearrangement at meiosis. The other case was also regarded to be caused by intrachromosomal rearrangement between sister chromatids. Thus, it appears that the partial duplications might occur by a different mechanism from the general mechanism of duplication due to the interchromosomal unequal crossover during meiosis. The electrophysiological and MRI patterns of affected individuals with partial duplications were typical of CMT type 1 with somewhat mild tendency. The exact position of the duplication junction and the cytogenetic mechanism should be determined by further analysis.

2026
In Vitro Efficacy and In Vivo Pharmacokinetic Studies with BLX-1060, a New Chemical Entity with Anti-IL-12 Activity
A. P. Sen, A. Mukherjee, A. Nag, D. Dey, P. Neogi, B. Nag; Bexel Pharmaceuticals, Union City, CA
BLX-1060 is a small molecule and hydroxamic acid derivative. It does not show any cytotoxicity in hepG2 and human peripheral blood mononuclear cells (hPBMC) at 100 μM concentration. Here we report on its anti-inflammatory property as an inhibitor of IL-12, a biomarker for inflammatory bowel disease (Ulcerative colitis and Crohn’s disease), in in vitro cell-based assays and in vivo pharmacokinetic studies in rats. In in vitro cell based assays, BLX-1060 inhibited IL-12p40 increase in hPBMC cells and in mouse peritoneal macrophages by 40%. BLX-1060 also inhibited anti-CD40L induced IL-12 mRNA by 20% and IFN-g by 40%. For the pharmacokinetic study, BLX-1060 was administered at 100 and 200 mg/kg, PO, and 10 mg/kg, IV to male rats. Blood was collected at predetermined time points for up to 24 hours. Plasma was obtained from blood and analyzed with LC-MS. The table below gives the pharmacokinetic parameters. BLX-1060 also inhibited anti-CD40L induced IL-12 mRNA by 20% and IFN-g by 40%. For the pharmacokinetic study, BLX-1060 was administered at 100 and 200 mg/kg, PO, and 10 mg/kg, IV to male rats. Blood was collected at predetermined time points for up to 24 hours. Plasma was obtained from blood and analyzed with LC-MS. The table below gives the pharmacokinetic parameters. BLX-1060 also inhibited anti-CD40L induced IL-12 mRNA by 20% and IFN-g by 40%. For the pharmacokinetic study, BLX-1060 was administered at 100 and 200 mg/kg, PO, and 10 mg/kg, IV to male rats. Blood was collected at predetermined time points for up to 24 hours. Plasma was obtained from blood and analyzed with LC-MS. The table below gives the pharmacokinetic parameters.

2027
The Antiviral Effect of Proteins and Peptides Derived from Potatoes (Solanum tuberosum L.) is Effective Against PVY Infection
Y. Park, G. Raj Tripathi, C. Lee, H. Choi, H. Cheong; Department of Biotechnology, Chosun University, Gwangju, Republic of Korea
Potato Viruses (PVY) are a谈 type member of the genus potyvirus in the family Potyviridae is a widespread virus leading to severe damage in Solanaceae, which may lose crop yield up to 75% in a small number of plant species by intracellular determinants that alter APP metabolism. We propose that plaque formation in the cerebral cortex and hippocampus is seeded by oligomized Aβ that accumulates at the terminals of projections of such brainstem neurons. CAD cells appear to recapitulate the biochemical processes leading to Aβ deposition, thus providing an experimental in vitro system for studying the molecular pathobiology of AD.
2029

Zebrafish, an In Vivo Model for Screening of Whitening Agents

T. Choi, J. Kim, E. Jeon, C. Kim, J. Lee, K. Kim, T. Yoon; 1Department of Dermatology, School of Medicine, Chungnam National University, Daejeon, Republic of Korea, 2Department of Dermatology, Gyeongsang National University College of Medicine, Jinju, Republic of Korea

Although it is an aquatic animal, zebrafish has most of organs and tissues that can be seen in human, such as heart, kidney, pancreas, bones and cartilage. Especially, it has pigments in the epidermal portion, giving colors on the surface of organism. Therefore, it can be used as a phenotype-based screening model for whitening agents. We tested the effect of several candidate materials on the pigmentation of zebrafish embryo, in a 96-well platform. The chemicals tested include arbutin, kojic acid, ascorbic acid, 2-mercaptobenzothiazole, salicylhydroxamic acid, and tranylcypromine. Our results showed that arbutin, kojic acid, 2-mercaptobenzothiazole decreased the pigmentation of zebrafish embryo. To identify molecular target modulated by whitening agents, we performed whole-mount in situ hybridization with various pigmentation markers, including nacre/mif and sox10. In addition, we determined the effects of whitening agents on the melanin content and tyrosinase activity. This study is the first approach to validate the feasibility of screening model for whitening agents using zebrafish.

2030

Ultrastructure of Hepatoma Cells Following Administration of Orthovanadate

W. A. Dabros; Department of Pathomorphology, Jagiellonian University MC, Krakow, Poland

This study presents the investigation of the effect of sodium orthovanadate (V(IV)) - in the range of 0.5-20.0 mM - on the rat hepatoma cell line H35-19. The cells were tested with crystal violet and counted in a Bürker chamber to determine their rate of proliferation, while the survival level was established with neutral red and MTT [methylthiazolyltetrazo]. We found a progressive growth inhibition of rat hepatoma cell line H35-19 within the range of 0.5-20.0 mM Na3VO4. At the concentration of 5 mM Na3VO4 in the medium, the majority of the investigated cells showed approximately a 50% inhibition of proliferation and reduction of viability (c. 40%). As compared with metavanadate or vanadyl sulphate and especially organic vanadium derivatives previously studied by us in similar experimental conditions, the obtained effect was intermediate. The results were confirmed with electron microscopic examinations. For electron microscopy observation the cells were centrifuged at 400 rpm for 5 min and 4% solution of glutaraldehyde in incubation medium was added for fixation, next cell were processed in standard way. Cells exposed to sodium orthovanadate demonstrated different appearance than control hepatoma cell line H35-19. They become better differentiated. The vanadium compounds have toxic influence on the cells. Thus in groups with high concentration of orthovanadate only single cells survived. The cytoplasm of the surviving cells was rather scant, what may suggest that the cells were young, but they already contained numerous myelin-like structures originating from degraded membranous structures, pleomorphic condensed mitochondria and infrequent, narrow canaliculi of the rough endoplasmic reticulum. We can to conclude: Orthovanadate affects the rate of proliferation of rat hepatoma cells cultured in vitro. The compound seems to normalize the structure of liver hepatoma cell line H35-19. The structure of the orthovanadate treated cells is similar to the structure of control hepatoma cell line H35-19. However, orthovanadate is less toxic.

2031

Inhibition of Platelet Aggregation by Anthrax Edema Toxin

S. Alam, M. Gupta, R. Bhatnagar; Center for Biotechnology, Jawaharlal Nehru University, New Delhi, India

Edema toxin is a key virulence determinant in anthrax pathogenesis that causes potentiation of cAMP inside host cells. This exotoxin has been implicated in facilitating bacterial invasion by impairing host defenses. Here, we report for the first time that edema toxin plays an important role in suppression of platelet aggregation; an effect that could be of vital significance in anthrax. We tested the effect of several candidate materials on the pigmentation of zebrafish embryo, in a 96-well platform. The chemicals tested include arbutin, kojic acid, ascorbic acid, 2-mercaptobenzothiazole, salicylhydroxamic acid, and tranylcypromine. Our results showed that arbutin, kojic acid, 2-mercaptobenzothiazole decreased the pigmentation of zebrafish embryo. To identify molecular target modulated by whitening agents, we performed whole-mount in situ hybridization with various pigmentation markers, including nacre/mif and sox10. In addition, we determined the effects of whitening agents on the melanin content and tyrosinase activity. This study is the first approach to validate the feasibility of screening model for whitening agents using zebrafish.

2032

Characterization of Histidine 351 mutants of Bacillus anthracis Adenylate Cyclase

M. Gupta, S. Alam, R. Bhatnagar; Center for Biotechnology, Jawaharlal Nehru University, New Delhi, India

Edema factor (EF) is a calmodulin-dependent adenyl cyclase (AC) secreted as one of the primary exotoxins by Bacillus anthracis. We introduced mutations in histidine residue at position 351 of full-length EF. Various studies enunciate an indirect role of this residue in catalysis. Alanine (H351A), asparagine (H351N) and phenylalanine (H351F) variants of this residue were constructed with the objective of carrying out comprehensive biochemical, biophysical and toxicity studies. Spectral analysis of variants displayed no gross structural deformities. Kinetic analysis showed that AC activity of H351N and H351F decreased 34- and 40-fold respectively whereas H351A completely lost activity. Kinetic constants for ATP, pH activity profiles and especially organic vanadium derivatives previously studied by us in similar experimental conditions, the obtained effect was intermediate. The results were confirmed with electron microscopic examinations. For electron microscopy observation the cells were centrifuged at 400 rpm for 5 min and 4% solution of glutaraldehyde in incubation medium was added for fixation, next cell were processed in standard way. Cells exposed to sodium orthovanadate demonstrated different appearance than control hepatoma cell line H35-19. They become better differentiated. The vanadium compounds have toxic influence on the cells. Thus in groups with high concentration of orthovanadate only single cells survived. The cytoplasm of the surviving cells was rather scant, what may suggest that the cells were young, but they already contained numerous myelin-like structures originating from degraded membranous structures, pleomorphic condensed mitochondria and infrequent, narrow canaliculi of the rough endoplasmic reticulum. We can to conclude: Orthovanadate affects the rate of proliferation of rat hepatoma cells cultured in vitro. The compound seems to normalize the structure of liver hepatoma cell line H35-19. The structure of the orthovanadate treated cells is similar to the structure of control hepatoma cell line H35-19. However, orthovanadate is less toxic.

2033

Self-fermentation: A Useful Tool to Improve Quality and Effects of Extract from Pinus densiflora Sieb. Et Zucc.

G. Park, D. P. Paudyal, H. Lee, J. Jung, H. Cheong; Biotechnology, Chosun University, Gwangju, Republic of Korea

Pine needle (Pinus densiflora Sieb. et Zucc.) extract has been used to improve cardiovascular disorders, detoxification of nicotine, the infirmities of age and curing diseases of unidentified symptoms. To find out effects of pine needle extract and self-fermented pine needle extract, we performed antibacterial, antioxidant and fibrinolytic assay and the composition of the extract was also identified. The extract has various useful components including amino acids, vitamin C, terpenoids and chlorophyll. The composition changes when the extract is allowed for self-
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2034 The Cytosplasmic Tail of Polycystin-1 Functions as a Switch between the Stat1 and Stat6 Pathways to Regulate Proliferation in Renal Epithelial Cells

S. Mukherjee, S. Vasanth, S. Low, T. Obara, M. Kinter, T. Weimbs, 1Department of Molecular, Cellular, and Developmental Biology, University of California Santa Barbara, Santa Barbara, CA. 2Department of Medicine, MetroHealth Medical Center, Case Western Reserve University, Cleveland, OH. 3Department of Cell Biology, The Cleveland Clinic, Cleveland, OH.

Autosomal-dominant polycystic kidney disease (ADPKD) is caused by mutations in polycystin-1 (PC1), a large membrane protein of uncertain function. We show that polycystin-1 plays a major role as a regulatory switch between STAT1 and STAT6 pathways. The cytosplasmic tail of PC1 is proteolytically cleaved and undergoes nuclear translocation. The cleaved, complete tail or the C-terminal half alone stimulate STAT6 transcriptional activity in response to activation of IL-4 receptors. A fragment corresponding to the C-terminal half of the PC1 tail is highly expressed in ADPKD kidney tissues but not in normal tissues. Expression of the PC1 tail alone or activation with IL-4 stimulate proliferation in MDCK cells. The PC1 tail also regulates STAT1 activity but by two different modes. The membrane-anchored PC1 tail - but not the cleaved tail - directly activates STAT1. The cleaved tail, however, dramatically stimulates STAT1 activity in response to IFN-γ activation. This stimulation requires the full-length PC1 tail and does not occur with the C-terminal half. Inhibitor studies suggest that Jak3 is the major regulator of the PC1 mediated STAT1 and STAT6 pathways. These results suggest that PC1 acts as a switch between STAT1 and STAT6 activity. Uncloned PC1 provides a constitutive STAT1 signal. In contrast, the cleaved PC1 tail sensitizes renal epithelial cells to input from cytokines, providing a STAT6-mediated proliferative signal in response to IL-4 and a STAT1-mediated anti-proliferative signal response to IFN-γ. We propose that abberant proliferative signaling is involved in renal cyst growth in ADPKD.

2035 Activation of Polycystin-2 (or Pkd2) through Mammalian Diaphanous 1 (mdia1)-dependent Voltage Gating

C. Bai, S. Kim, L. Tsiokas; Cell Biology, University of Oklahoma, Oklahoma City, OK.

Autosomal dominant polycystic kidney disease (ADPKD) is one of the most common genetic diseases affecting 1 in every 400 to 1,000 individuals. Polycystin-2 or Pkd2 belongs to the transient receptor potential family of ion channels. Mutations of polycystin (PC) 1 or 2. However, the mechanisms that trigger cyst formation in ADPKD are still unclear. We have found that tumor necrosis factor-alpha (TNF-α), an inflammatory cytokine abundant in cyst fluid, plays an important role in promoting cystogenesis. TNF-α induces the production of a scaffold protein, FIP2, which interacts with the C-terminus of PC2 and recruits PC2 into an aberrant complex with a Rab GTPase. This prevents the correct localization of PC2 to the plasma membrane and the cilium and leads to disruption of the PC1-PC2 complex, a receptor-calcium channel complex in the primary cilia that responds to fluid flow stimulation. Directly supporting a role for TNF-α in cyst formation, we found that treatment of mouse embryonic kidney organ cultures with TNF-α results in formation of cysts, and this effect is enhanced with embryonic kidneys heterozygous for Pkd2, but not Pkd1 null mutations. Furthermore, analysis of human kidney cysts fluids revealed a correlation of TNF-α production with disease progression. Additionally, the levels of FIP2 and TNF-α receptor are significantly elevated in cystic kidney subtypes. These results indicate that TNF-α secreted in response to cyst-forming stimuli is a key factor in cyst formation in ADPKD.

2036 Alterations in Wnt/wg Gene Signaling in Cell Lines with Mutations in Polycystin-1


Department of Medicine/Nephrology, Indiana University School of Medicine, Indianapolis, IN.

Autosomal dominant polycystic kidney disease (ADPKD), a common hereditary disorder characterized by cystic dilation of the kidney tubules, results from heterozygous mutations affecting polycystin (PC) 1 or 2. However, the mechanisms that trigger cyst formation in ADPKD are still unclear. We have found that tumor necrosis factor-alpha (TNF-α), an inflammatory cytokine abundant in cyst fluid, plays an important role in promoting cystogenesis. TNF-α induces the production of a scaffold protein, FIP2, which interacts with the C-terminus of PC2 and recruits PC2 into an aberrant complex with a Rab GTPase. This prevents the correct localization of PC2 to the plasma membrane and the cilium and leads to disruption of the PC1-PC2 complex, a receptor-calcium channel complex in the primary cilia that responds to fluid flow stimulation. Directly supporting a role for TNF-α in cyst formation, we found that treatment of mouse embryonic kidney organ cultures with TNF-α results in formation of cysts, and this effect is enhanced with embryonic kidneys heterozygous for Pkd2, but not Pkd1 null mutations. Furthermore, analysis of human kidney cysts fluids revealed a correlation of TNF-α production with disease progression. Additionally, the levels of FIP2 and TNF-α receptor are significantly elevated in cystic kidney subtypes. These results indicate that TNF-α secreted in response to cyst-forming stimuli is a key factor in cyst formation in ADPKD. We propose that abberant proliferative signaling is involved in renal cyst growth in ADPKD.

2037 Control of Polycystin 2 Trafficking by Tumor Necrosis Factor-Alpha: A Potential Epigenetic Genetic Switch for Autosomal Dominant Polycystic Kidney Disease

X. Li, W. Bosi, B. S. Magenheimier, S. Xia, D. Wallace, J. Calvet, R. Li.

Stowers Institute for Medical Research, Kansas City, MO. 2Children's Hospital and Harvard Medical School, Boston, MA.

Autosomal dominant polycystic kidney disease (ADPKD), a common hereditary disorder characterized by cystic dilation of the kidney tubules, results from heterozygous mutations affecting polycystin (PC) 1 or 2. However, the mechanisms that trigger cyst formation in ADPKD are still unclear. We have found that tumor necrosis factor-alpha (TNF-α), an inflammatory cytokine abundant in cyst fluid, plays an important role in promoting cystogenesis. TNF-α induces the production of a scaffold protein, FIP2, which interacts with the C-terminus of PC2 and recruits PC2 into an aberrant complex with a Rab GTPase. This prevents the correct localization of PC2 to the plasma membrane and the cilium and leads to disruption of the PC1-PC2 complex, a receptor-calcium channel complex in the primary cilia that responds to fluid flow stimulation. Directly supporting a role for TNF-α in cyst formation, we found that treatment of mouse embryonic kidney organ cultures with TNF-α results in formation of cysts, and this effect is enhanced with embryonic kidneys heterozygous for Pkd2, but not Pkd1 null mutations. Furthermore, analysis of human kidney cysts fluids revealed a correlation of TNF-α production with disease progression. Additionally, the levels of FIP2 and TNF-α receptor are significantly elevated in cystic kidney subtypes. Based on these findings, we hypothesize that a regulatory circuit connecting TNF-α, PC2 localization and cystogenesis, which harbors two double negative feedback loops, could function as an epigenetic "second hit" promoting autosomal dominant disease progression. Unraveling epigenetic control pathways that potentially function as a "second hit" in ADPKD may provide hopeful targets for therapeutic intervention of disease initiation and progression.

2038 The Role of Ei3 in Diabetic Nephropathy

M. Matsoura, T. Tomonaga, H. Ahe, T. Doi.

Clinical Biology and Medicine, Course of Biological Medicine, Graduate School of Medicine University of Tokushima, Tokushima, Japan.

The purpose of this study is to elucidate the role of Ei3 in diabetic nephropathy in vitro and in vivo. We have recently demonstrated that TGFbeta-1 Smad signaling pathway played a key role for development of diabetic nephropathy. TGFbeta1 directly phosphorylates TGFbeta type II receptor (RII) and transduces the signal into intracellular factors. However, detailed mechanisms for the regulation of RII expression have been remained. Therefore, we focused on RII regulation in diabetic conditions. Ei3, a member of the Ets transcription factor family, is well known to transcriptionally regulate TGFbeta type II receptor (RII) expression. Ei3 expression was upregulated by advanced glycation end products (AGEs) stimulation in cultured mouse mesangial cells. Western blot and Northern blot analyses showed that AGEs increased both protein and mRNA levels of Smad1, type IV collagen, and type I collagen. Many reports have shown TGFbeta-1 plays a critical role in AGEs stimulation. However, TGFbeta-1 did not induce Ei3 in mouse mesangial cells. We have recently generated transgenic mice expressing inducible nitric oxide synthase (iNOS) under the control of insulin promoter (iNOS Tg mice), which showed both typical type1 diabetes and diabetic glomerulosclerosis. These mice show improved blood circulation and could be a good source of functional food development.
remarkable sclerotic region resembles human diabetic glomerulosclerosis. Immunohistochemical examination demonstrated manifest expression of type IV and type I collagen in sclerotic regions. Also, phosphorylated Smad1 (pSmad1), RII, and ElF3 were consistently observed in sclerotic glomeruli in iNOS Tg mice. On the other hand, these proteins (pSmad1, RII, ElF3) were hardly expressed in normal glomeruli. These results suggest that ElF3 involves in the progression of diabetic neuropathy through regulation of RII expression.

2039 Alterations of Uromodulin Biology: A Common Denominator of the Genetically Heterogeneous FJHN/MCKD Syndrome M. Kublova,1 P. Vyletel,1 M. Kalcbova,2 K. Hodanova,1 V. Barsova,2 B. Stibulkova,1 J. Sikora,2 H. Hulкова,2 J. Zivny,3 J. Majewski,1 A. Simmonds,4 J. Fryms,4 G. Venkat-Raman,4 M. Ellender,4 S. Knock,1 Institute for Inherited Metabolic Disorders, 1st School of Medicine, Charles University, Prague, Czech Republic, Department of Pathophysiology, 1st School of Medicine, Charles University, Prague, Czech Republic, Department of Human Genetics, McGill University and Genome Quebec Innovation Center, Montreal, PQ, Canada, Parina Research Unit, GKT, Guy’s Hospital, London, United Kingdom, 3Center for Human Genetics, University of Leuven, Leuven, Belgium, 4Renal Unit, Queen Alexandra Hospital, Portsmouth, United Kingdom Autosomal dominant hypertension, renal, and glomerular disease and progressive renal insufficiency are hallmarks of a disease complex comprising familial juvenile hyperuricicemic nephropathy (FJHN), medullary cystic kidney diseases type 1 (MCKD1) and type 2 (MCKD2). In some families the disease is associated with mutations of the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR), whereas in others no mutations in this gene have been found. Our results show that various genetic defects interfere with UMOD biology, which could lead to the development of the common FJHN/MCKD phenotype. "Uromodulin associated kidney diseases" (UAKD) may be a more appropriate term for the FJHN/MCKD syndrome. Urinary UMOD testing is a best method for identification of additional UAKD families and linkage mapping is a tool to reveal still unknown UAKD genes. Their identification may help to clarify the exact biological roles of UMOD, provide better diagnostic techniques and suggest potential therapeutic targets and therapeutic approaches.

2040 Aberrant Expression of laminin-γ3 with Congenital Autosomal-dominant Polycystic Kidney Disease in Mice H. Lin,1 S. Jiang,2 C. Lin,1 M. Tang,1 Y. Chou1; 1Department of Pediatrics, National Cheng Kung University Medical Center, Tainan, Taiwan, 2Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan, 3Institute of Medical Research, Chang Jung Christian University, Tainan, Taiwan, 4Department of Physiology, College of Medicine National Cheng Kung University, Tainan, Taiwan. Autosomal dominant polycystic kidney disease (ADPKD) has an incidence of 1 in 1000 live births. It is the most common life-threatening inherited cystic kidney diseases characterized by the development of gradually enlarging renal cysts and a progressive loss of normal renal tissue that can lead to chronic renal failure and hemodialysis. Finding of PKD1 and PKD2 genes were major milestones in studying of this specific kidney disease. ADPKD is recognized as a monogenic disorder caused by mutation in two genes, PKD1, accounting for approximately 85% - 90% of cases, and PKD2, accounting for approximately 10% of cases. Polycystin-1 is a large (~460 KD) membrane protein of about 4300 amino acids with 11 transmembrane domains. Its extensive extracellular N terminus contains a number of adhesive domains that implicate polycystin-1 in cell-cell and matrix-cell reactions. Their functions include cell proliferation, regulation of cell survival, altered tubular cell polarity, fluid secretion, and reaction with extracellular matrix. In our preliminary data, renal tubular cystic epithelium was noted to have a tendency to detach from extracellular matrix. Meanwhile, in renal tissue of CDNA microarray analysis, laminin γ3 upregulated also was noted. About this reason, our purposes will focus on the role of laminin γ3, element of ECM, in the developmental process of renal cystic formation and the interaction between extracellular matrix abnormalities and PKD disease. That uses critical information gained from this genetically manipulated mouse models for the purpose of aberrant expression of laminin γ3. Overall, in homzygous mutant, the distribution aberrant of lamininγ3 translocated from basement to apical side at day 1 and the expression of lamininγ3 was increased at day 30. Meanwhile, the expression of laminin γ3 and polycystin-1 protein is colocalized in either cytoplasm or apical side of congenital ADPKD mice.

2041 Par-4 Is a Novel Mediator of Renal Injury J. Xie, Q. Guo; Department of Physiology, The University of Oklahoma Health Sciences Center, Oklahoma City, OK Ischemia/reperfusion-induced renal injury (IRI) is clinically important because it typically damages renal tubular epithelial cells and glomerular cells, and is the most common cause of acute renal failure (ARF). Par-4 (prostate apoptosis response-4) is a leucine zipper protein initially linked to apoptotic cell death in prostate cancer and neuronal tissues. RNA interference (RNAi) induces very effective sequence-specific silencing of gene expression when small interfering RNAs (siRNAs) enter a multimeric nuclease complex and induce targeted destruction of mRNAs. We now report that, in a well-characterized and widely used in vitro model of renal IRI, levels of Par-4 in human renal proximal tubule HK-2 cells started to increase within 4 hours following chemical ischemia-reperfusion. Mitochondrial dysfunction, caspase-3 activation, and significant amount of apoptotic cell death in HK-2 cells were observed following ischemia-reperfusion, which was significantly ameliorated by siRNAs targeted against Par-4 mRNA, while non-silencing control siRNAs were ineffective. These results identify Par-4 is an early critical link in the chain of events leading to the initiation of apoptosis in human kidney proximal tubular cells, and indicate that targeted knockdown of Par-4 expression by RNAi may provide a novel cytoprotective mechanism against ischemia/reperfusion-induced renal injury.

2042 AATF as a Cytoprotective Factor against Renal Injury Q. Guo, J. Xie; Department of Physiology, The University of Oklahoma Health Sciences Center, Oklahoma City, OK AATF (apoptosis antagonizing transcription factor) is a leucine zipper domain containing protein that has anti-apoptotic properties. Human AATF has an open reading frame of 560 amino acids, and is expressed in several organs and tissues including the kidney. AATF may participate in inhibition of pro-apoptotic pathways and/or activation of anti-apoptotic pathways. Ischemia/reperfusion-induced renal injury (IRI) is clinically important because it typically damages renal tubular epithelial cells and glomerular cells, and is the most common cause of acute renal failure. We now report that AATF is expressed in human kidney proximal tubule (HK-2) cells, and its levels of expression were significantly altered in these cells in a well-established in vitro model of ischemia-reperfusion induced renal injury. Overexpression of AATF in transfected HK-2 cells preserved mitochondrial function, suppressed accumulation of superoxide and peroxynitrite, and alleviated lipid peroxidation and oxidative damage induced by IRI. RNA-mediated silencing of AATF exacerbated, while overexpression of the full-length AATF ameliorated, caspase-3 activation and apoptotic death induced by IRI in proximal tubule cells. These results identify AATF as a novel cytoprotective factor against oxidative and apoptotic damage in renal tubular cells. AATF may represent a potential candidate for therapeutic application in ischemia/reperfusion-induced renal injury.

2043 Second-hand Cigarette Smoke Inhibits Wound Healing of the Cornea by Stimulating Inflammation and Delaying Corneal Re-epithelialization C. Ma, M. Martins-Green; Cell Biology and Neuroscience, University of California Riverside, Riverside, CA Environmental cigarette smoke is known to delay skin wound healing, but very little is known about its effects on healing of corneal wounds. Corneal re-epithelialization is a key process in preventing corneal corneal healing and immunosuppression. Identification of factors that promote re-epithelialization, and the mechanism of action, will provide insights into how to improve and accelerate corneal epithelial healing. To address this issue, we exposed mice to cigarette smoke in an established system that mimics second-hand cigarette smoking by humans. We injured the corneal epithelium using a trephine and a scalpel and exposed the mice to smoke during the healing process. We observed that the epithelium at the front edge of the wound did not adhere to the basal matrix and as a result epithelial cell migration was also inhibited. Immunohistological studies showed that in the mice exposed to smoke, many of the ECM molecules critical for epithelial cell migration were not present and that there was an accumulation of superficial basement membrane, inflammatory cytokines, such as IL-1, IL-6, TNF-α, and GRO-alpha increase in the corneal stroma and epithelium. Local treatment to the wounded cornea with dexamethasone (an anti-inflammatory agent) resulted in increased healing rate and infiltration of fewer neutrophils into the wounded area. In conclusion, second-hand cigarette smoke delays corneal re-epithelialization by attracting more neutrophils to the wound site,
which, in turn, produce enzymes that degrade the ECM and consequently inhibit corneal epithelial cell adhesion and migration. These findings have implications not only for the exposure to second hand smoke and its effects on the healing of injuries to the cornea but also for the treatment of the most common type of corneal wounds.

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Proteomic Analysis of Lung Epithelial Cell Surface Molecules Recognized by Autoantibodies in SARS-associated Coronavirus Infection

Y. Fang,1 C. Lin,1 Y. Kuo,2 P. Liao,3 H. Lei,1 Y. Lin;1 Microbiology and Immunology, National Cheng Kung University Medical College, Tainan, Taiwan, 1Cell Biology and Anatomy, National Cheng Kung University Medical College, Tainan, Taiwan, 1Environmental and Occupational Health, National Cheng Kung University Medical College, Tainan, Taiwan

Severe acute respiratory syndrome (SARS) is a lower respiratory tract disease characterized by severe atypical pneumonia. Some of the patients have clinical worsening which is related to abnormal immune responses, such as the imbalance of immune cells and the production and dysregulation of cytokines and chemokines. In our study, we found the presence of antibodies (Abs) in SARS patient sera which cross-reacted with human lung epithelial cells A549 and caused cytotoxicity. By competitive binding assay, the cross-reactive Abs in SARS patient sera against SARS-CoV spike protein domain 2 (S2) were identified. By proteomic analysis using 2D gel electrophoresis and mass spectrometry, several candidate proteins, including annexin A2, glyceraldehyde-3-phosphate dehydrogenase, albumin, α-antitrypsin, aldo-keto reductase, and transferrin, recognized by SARS patient sera had been identified. Using immunofluorescence staining and ELISA analysis, we showed that annexin A2 proteins were recognized by anti-S2 Abs or SARS patient sera in A549 cells. Previous studies reported the high levels of inflammatory cytokines, such as IL-6 and IFN-γ, in SARS patient sera. We found that IL-6 and IFN-γ could increase annexin A2 expression in A549 cells. Interestingly, we also found the localization of annexin A2 on plasma membrane lipid-raft structure after IL-6 and IFN-γ stimulation. The elevated expression of annexin A2 by cytokine stimulation also increased the binding activity of anti-S2 Abs to A549 cells. Finally, these cross-reactive Abs could directly cause A549 cell cytotoxicity. Therefore, these results suggest that the regulation of annexin A2 expression by SARS-associated cytokines and the production of autoantibodies (anti-S2 and anti-annexin A2) may have implications in SARS pathogenesis.

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“Second-Hand” Cigarette Smoke Causes Lipid Accumulation in the Liver and in the Small Blood Vessels of the Heart

H. Yuan, L. S. Wong, M. Bhattacharya, C. Ma, M. Zafarani, M. Yao, M. Martins-Green; Cell Biology and Neurosciences, University of California, Riverside, Riverside, CA

Cigarette smoke, a mixture of more than 4,000 chemicals, is one of the major factors responsible for many diseases. Atherosclerosis, one of the pathological conditions severely affected by cigarette smoke, contributes to nearly 50% of the deaths in western societies. The early events in atherogenesis are stimulated, on the one hand by cytokines that chemotact attract leukocytes, and on the other hand, by decrease in circulation of molecules that protect endothelial cells (ECs) from injury. To study the effects of “second-hand” smoke on atherogenesis, we used a smoking machine that closely simulates exposure of humans to second-hand smoke, and mice transgenic for human ApoE<sup>−/−</sup>, a model system that closely mimics the human conditions that lead to atherosclerotic plaque formation. We found that smoke decreases the HDL level in the blood and also decreases the ratios between HDL and LDL, HDL and triglyceride (TG) and HDL and total cholesterol (TC). This change in lipid profiles causes not only lipid accumulation in the aortic walls, but also many of the smaller vessels of the heart are filled with lipids. In addition, mice exposed to smoke have decreased levels of an EC-protective protein called adiponectin. Furthermore, cytokine arrays revealed that smoking mice are in a permanent state of pro-inflammation. That is, the Th1 (pro-inflammatory) cytokine profile that develops when the mice are initially exposed to second-hand smoke does not switch to a Th2 (adaptive) response. In addition, TG levels increase significantly in the liver of smoke-exposed mice, thus leading to fatty liver disease. In conclusion, long-term exposure to second-hand smoke creates a state of permanent inflammation and an imbalance in the lipid profile that leads to lipid accumulation in the liver (potentially leading to non-alcoholic fatty liver disease) and in the blood vessels of the heart, potentially leading to heart attacks.

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Further Support for the Common Variants in Complement Factor H (Y402H) and LOC387715 (A69S) Genes as Major Risk Factors for the Exudative Age-related Macular Degeneration

B. S. Shastry, Biological Sciences, Oakland University, Rochester, MI

Age-related macular degeneration (AMD) is a progressive degenerative disorder of the retina. It causes changes in the macular region that is responsible for visualizing fine details clearly. The condition is the leading cause of irreversible visual loss in the elderly population in industrialized countries. The presence of large soft drusen (extracellular protein and/or lipid deposits) and retinal pigment epithelial abnormalities are considered to be early signs of the disease. When the disease progresses to an advanced stage, it is characterized by the presence of either geographic atrophy (dry AMD) or choroidal neovascularization (wet AMD). It is a clinically heterogeneous and epidemiologically complex disorder involving the interaction between genetic and environmental factors. Among environmental factors, age and smoking are consistently suggested to be the strongest risk factors. Much progress has been made previously in identifying a number of candidate genes. However, these can explain only a small proportion of AMD cases and none of them accounts for a substantial number of ARMD patients. Recently, several family-based whole genome linkage scans consistently identified two variants in two major susceptibility genes for this disorder. In order to replicate and to determine the overall prevalence of these variants, we have analyzed two unrelated families both having exudative ARMD. Our analysis has identified the same common polymorphism (Y402H) in the CFH gene in one family and the A69S polymorphism in the LOC387715 gene in the second family. These changes are heterozygous in both families, segregating in the LOC387715 gene in the second family. These changes are heterozygous in both families, segregating in the LOC387715 gene in the second family. The alternate localization of these proteins correlates with the dianged phenotype in present in infected mice. Mice that recover from the infection at 28-35 days post inoculum regain their normal membrane AQP localization. The altered localization of AQPs is partially dependent on the bacterial type III effector proteins EspF and EspG. We conclude that altered AQP localization may be a contributing factor to diarrhea during bacterial infection.

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Aquaporins Are Altered during Attacking and Effacing Pathogen-induced Diarrhea

J. A. Gutman, F. N. Samji, Y. Li, W. Deng, A. E. Lin, B. B. Finlay; Michael Smith Laboratories, University of British Columbia, Vancouver, BC, Canada

The attaching and effacing (A/E) pathogens, enterohemorrhagic E. coli (EHEC) and enteropathogenic E. coli (EPEC), cause serious global health problems. These bacteria colonize the gastrointestinal system, attach to intestinal epithelial cells, efface (collapse) infected cell microvilli and cause overt diarrhea that can result in host. Although pathogenically induced diarrhea is a significant global health issue, the molecular mechanisms that underlie this disease remain largely unknown. A natural murine infection model, using the A/E pathogen Citrobacter rodentium, has been helpful in studying the diseases in the mouse. As this disease progresses, infected mice develop a diarrhea phenotype. Aquaporin (AQP) water channels have been proposed to play a role in the normal dehydration of fecal contents. Here we examine whether C. rodentium infected mice may alter aquaporin localization in colonocytes. We demonstrate that during infection, AQP2 and AQP3 are mis-localized from their normal location along cell membranes to the cell cytoplasm. The change in localization of these proteins correlates with the diarrhea phenotype present in infected mice. Mice that recover from the infection at 28-35 days post inoculum regain their normal membrane AQP localization. The altered localization of AQPs is partially dependent on the bacterial type III effector proteins EspF and EspG. We conclude that altered AQP localization may be a contributing factor to diarrhea during bacterial infection.

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Overexpression of the E3 Ligase Rnf5 Is Associated with Early Onset of Muscular Disorders in Mice

A. Delaunay,1 K. D. Bromberg,2 C. Didier,1 S. A. Lira,1 Y. K. Hayashi,4 G. D. Shelton,4 Z. Romi1;1 Signal Transduction Program, The Burnham Institute for Medical Research, La Jolla, CA, 1Mount Sinai Hospital, New York, NY, 2Department of Immunobiology, Mount Sinai Hospital, New York, NY, 3Department of Neuroimmunology, National Institute of Neuroscience, Tokyo, Japan, 4Department of Pathology, UCSD, San Diego, CA

Rnf5 is a membrane anchored ubiquitin ligase conserved from C. elegans to humans. In C. elegans, Rnf5 localizes and participates to the organization of the dense bodies, a muscle structure similar to muscle Z-lines in mammals. To investigate the role of Rnf5 in mammals, we have constructed a mouse transgenic model to conditionally overexpress Rnf5 upon doxycyclin treatment. We have shown that Rnf5 overexpression leads to a very severe phenotype associated with early onset of kyphosis, muscle weakness, ultimately leading to death. Histopathological analysis of skeletal muscles from Rnf5 transgenic animals revealed a severe alteration of muscle structure with both occurrence of atrophic fibers and high level of regeneration. Analysis of Rnf5 expression in tissue samples from human dystrophic patients revealed Rnf5 expression is elevated in some unknown types of Limb-Girdle Muscular Dystrophy (LGMD), suggesting that Rnf5 is a new player in dystrophic diseases.
Glucoma-causing Mutations in Myocilin Lead to an Abnormal Association with Peroxisomal Targeting Signal Type 1 Receptor (PTSR1) and Elevated Intracellular Pressure (IOP)

T. Dechate1, 2, T. Shim1, S. A. Adam1, A. E. Goodman1, A. Rusinol3, M. S. Sinensky3, R. D. Goldman3, 2, Cell and Molecular Biology, Northwestern University, Feinberg Medical School, Chicago, IL, 1, Biochemistry and Molecular Biology, East Tennessee State University, JI Quillen College of Medicine, Johnson City, TN

IOP is most commonly caused by a point mutation in the lamin A (LA) gene generating a cryptic splice site. This results in the expression of a mutant LA (LAD50) lacking 50 amino acids within its C-terminal containing a proteolytic cleavage site necessary for the maturation of LA. This prevents the removal of the farnesylated/methylated C-terminus as demonstrated biochemically using base release and immunoblotting assays. Therefore LAD50 is permanently farnesylated/carboxymethylated, which has a negative impact on the properties of the mutant protein during mitosis. GBP-LAD50, when expressed in mitotic HeLa cells, abnormally colocalizes with the integral membrane protein emerin and with lamin B (LB), which is normally farnesylated and methylated, in membrane structures. In contrast the endogenous WT LA is found uniformly distributed throughout the cytoplasm during mitosis and cytokinesis until it begins to accumulate in daughter cell nuclei. As treatment of mitotic HeLa cells with a farnesyltransferase inhibitor leads to a uniform cytoplasmic localization of LAD50 identical to WT LA it can be assumed that the farnesyl group is responsible for the abnormal association of the mutant protein with membranes. Association of GBP-LAD50 with membranes during mitosis is also revealed in biochemical fractionation studies. FLIP experiments performed in late prometaphase to early anaphase show a clear difference in the dynamic behavior of GBP-LAD50 compared to GBP-LA. During nuclear reassembly GBP-LAD50 relocates exclusively to the nuclear periphery whereas GBP-LA and endogenous LA are concentrated within the nucleoplasm as revealed by live imaging. In addition, LAD50 is delayed with respect to completion of nuclear recolorization compared to GBP-LA. In fibroblasts derived from GBP patients LA together with LB and emerin are also abnormally structured in the cytoplasm in early G1. These delays in nuclear relocalization could explain some of the defects responsible for premature aging in HGPS.

Guanylate Binding Protein 5, a Novel Phagosomal Protein, Plays a Role in Salmonella Induced Cytotoxicity in Raw 264.7 Macrophages

A. Rupper1, D. Einhaus2, S. Donahoe3, A. Aderem1, J. Cardelli1, 1, Microbiology and Immunology, LSUHSC-Shreveport, Shreveport, LA, 2, The Institute for Systems Biology, Seattle, WA

In order to discover unknown proteins that might play a role against intracellular pathogens in macrophages, we performed a proteomic analysis of purified latex bead containing (LBC) phagosomes from interferon (IFN)-γ treated macrophages compared with purified LBC phagosomes from unstimulated macrophages. Both Lrg-47 and guanylate binding protein 5 (GBP-5) were found to be highly enriched on LBC phagosomes from IFN-γ stimulated cells. Lrg-47 and GBP-5 belong to a family of interferon induced large GTPases with structural similarity to dynamin. Lrg-47, a member of a p47 family, is required for resistance to intracellular pathogens in mice, while little is known about the function of GBP-5, a p67 family member (Taylor et al. Nat Rev Immunol, 2004). To determine if GBP-5 played a role in resistance to infection by S. typhimurium, Raw 264.7 cells were prepared that exhibit stabile expression of 3x Flag epitope tagged GBP-5 or mutant forms of this protein. 3x Flag-GBP-5 was found to rapidly localize with actin cups upon phagocytosis of ezymoan and was highly enriched in purified LBC phagosomes by western blot. Surprisingly, expression of tagged GBP-5 resulted in increased susceptibility of Raw 264.7 cells to S. typhimurium induced cytotoxicity as measured by release of lactate dehydrogenase from infected cell cultures. Expression of a putative slow cycling Gtpase mutant of GBP-5 (D183N) did not result in increased cytotoxicity from S. typhimurium, suggesting that wild type Gtpase activity was required for this function. GBP-5 might play a role in regulation caspase-1 activity in response to S. typhimurium infection and expression of GBP-5 might account for the increase in S. typhimurium induced cytotoxicity demonstrated in IFN-γ stimulated Raw 264.7 macrophages (Monack et al., PNAS, 1996).

Chemical Genetic Analysis of Protein and Lipid Kinases in Disease and Normal Physiology

K. Shokat; University of San Francisco, San Francisco, CA

Our laboratory has developed a systematic means to engineer each protein kinase to be inhibitable by a bio-orthogonal small molecule inhibitor that does not inhibit any wild-type protein kinases. Using this approach of combining chemistry and genetics we can analyze pathways with unparalleled specificity and rapidity revealing phenotypic effects often missed by traditional genetic studies. Using a more traditional pharmacological approach for the study of the much smaller lipid kinase family we synthesized a structurally diverse set of drug-like molecules based on their potential to serve as potent inhibitors of one or more enzymes involved in FfIP3 generation. Our goal was to produce different chemotypes with distinct target selectivities as probes of lipid kinase signaling. We have applied this matrix of inhibitors to both normal physiological pathways such as the insulin pathway and also disease pathways such as cancer.

Making and Shaping the Foregut

S. Mango; Huntsman Cancer Institute, University of Utah, Salt Lake City, UT

The goal of our research is to understand how the foregut is formed during development. Our focus is C. elegans, whose digestive tract is a simple, linear tube. We would like to understand how cells are specified to become one of seven cell types within the foregut and through aboral cell fates. Genetic studies have shown that the aboral cell has a deleterious gain-of-function. Depending on the mutation, glaucoma caused by mutant myocilin has a varying degree of onset and degree of severity of elevated IOP, a hallmark of primary open angle glaucoma (POAG). Yeast-two-hybrid studies showed myocilin associated with PTS1R, but only as the mutant form or if presented as a C-terminal fragment. Myocilin contains a cryptic peroxisomal targeting signal on the C-terminus, but is normally a secreted protein. Immunoprecipitation and fluorescent colocalization studies confirmed that only the mutant form of myocilin interacts with PTS1R and may cause a disruption in normal peroxisomal function of trabecular meshwork (TM) cells. Adenoviral vectors encoding wild-type and mutant myocilin were used to determine the effect of myocilin on IOP in mice. Wild-type adenoviral vectors did not cause elevated IOP when injected into mouse eyes, however, mutant myocilin adenoviral vectors caused elevated IOP which correlated with the severity of the mutation in humans. These data confirm the affinity of mutant myocilin for the PTS1R thus disrupting the normal function of myocilin as a secreted protein. As a result of the aberrant association for PTS1R, normal TM cellular function is compromised thus leading to a disease phenotype. This is the first demonstration of a disease resulting from mutation-induced exposure of a cryptic signaling site that causes mislocalization of mutant protein to peroxisomes and the first disease-gene based animal model of human POAG.

Decipher Mammalian Biology by Insertional Mutagenesis Screens in Mice

T. Xu1, 2; 1, Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, CT, 2, Institute of Developmental Biology and Molecular Medicine, Fudan University, Shanghai, China

Forward genetic screens have played a pivotal role in our understanding of modern biology in lower organisms including bacteria, yeast, flies, worms, plants, and zebrafish. However, the lack of comparable genetic screens in mammals has impaired our ability to understand many aspects of human biology and disease. The current approaches of gene inactivation in the mouse, the piggyBac (PB), a DNA transposon from the cabbage looper moth, and showed that it can efficiently transpose in human cells and in the mouse germline, providing a powerful tool for transgenesis and insertional mutagenesis in mammals. We are in the process of producing the first genome-wide set of mouse insertional mutants, which will allow scientists around the world to utilize forward genetic screens to decipher the genetic basis of mammalian biology and disease.
2056
Teaching Scientist Volunteers about K-12 Science Education, Pedagogy, and Partnership
P. Caldera, J. MacCormack; University of California, San Francisco, San Francisco, CA

Many scientists today are seeking opportunities to share their commitment to science in the wider community and are interested in lending their expertise to teachers and their students with a view to improving science education. Scientists are enthusiastic about sharing their science knowledge and skills, but they generally have had limited experience in K-12 classroom settings. Based on our experience with scientist volunteers working in K-12 classrooms, the Science and Health Education Partnership (SEP) at the University of California, San Francisco, developed a workshop series to support scientist volunteers in their teaching efforts. Utilizing hands-on, interactive science activities, this workshop series is designed to equip scientist volunteers with teaching strategies that will enable them to: focus on the concrete in their teaching; involve all students in classroom lessons; get to know learners through pre-assessment; incorporate inquiry-based methods in their approaches; consider all aspects of lesson-planning (e.g., setting specific lesson goals, managing materials, addressing language needs); and work in partnership with teachers by acknowledging and relying on teachers’ expertise in areas such as classroom management and cognitive development. First offered ten years ago, the workshop series has evolved over the years to better address the needs of scientist volunteers. Scientist volunteers who have taken part in the workshop series consistently rate the workshops very highly, report improvement in their teaching skills, and have integrated many of the ideas and strategies in their work in K-12 classrooms.

2057
Modeling Hematopoietic Transplantation with ES Cells
G. Q. Daley1,2; 1Division of Pediatric Hematology/Oncology, Children's Hospital, Boston, MA, 2Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA

Differentiation of embryonic stem (ES) cells in vitro yields abundant hematopoietic progenitors, but achieving stable blood engraftment of irradiated mice has proven difficult. ES cell differentiation chiefly recapitulates the earliest yolk sac stages of blood development, when progenitors have limited developmental potential and fail to productively engraft adults. In an effort to characterize factors that promote formation of definitive Hematopoietic Stem Cells (HSCs), we have shown that activation of a genetic pathway involving the homeodomain proteins cd46 and hesb4 promotes the differentiation of blood progenitors that engraft stable lymphoid-myeloid hematopoiesis in irradiated mice. Our studies in the blood forming system are aimed at establishing principles applicable to generating engraftable HSCs from human ES cells.

2058
Stem Cells and Their Lineages in Skin
E. Fuchs, C. Blanpain, H. Nguyen, V. Horsley, H. Rhee, M. Rendl, T. Lechler, G. Gudas, W. Lowry, J. Nowak; Howard Hughes Medical Institute, The Rockefeller University, New York, NY

The ability of stem cells (SCs) to maintain their growth and differentiation inhibited state while in the niche relies upon a balance of intrinsic and extrinsic factors. The niche must then be stimulated in order to mobilize SCs to their specialized microenvironment. A central issue in SC biology is the relation between SCs and their niche, and how these interactions govern self-renewal and SC activation. Skin is an excellent system for exploring these processes. In postnatal life, skin sets aside multipotent epithelial SCs within each hair follicle. In response to wounding, these SCs become activated and move upward to repair the epidermis. With each hair cycle, specialized mesenchymal-epithelial cross-talk activates SCs, producing rapidly dividing progeny that differentiate to regenerate the follicle to produce new hair. This growth phase is followed by destructive and resting phases, leading to loss of the old hair and completing the cycle. To understand the fascinating process by which multipotent skin SCs respond to these stimuli, change their transcriptional program and select specific cell fates, we developed strategies to isolate specialized mesenchyme and SCs in quiescent and activated states. Transcriptional profiling, biochemical studies and functional analyses have advanced the unraveling of key steps in SC lineage commitment. Questions that we now address include: 1) How does microenvironment render directionality to SC movement and permit SC replenishment within the niche? 2) How do follicle SCs change their gene expression program upon activation? 3) Do unipotent SCs reside within the specialized skin epithelial compartments? 4) How and when are different lineages fated? By addressing these questions, we hope to learn how external stimuli elicit transcriptional, cytoskeletal and adhesive changes that orchestrate the assembly of cells into a tissue. Our ultimate goal is to link the basic biology of our research to issues of human medicine.

2059
Control of Stem Cell Fate by Oriented Mitotic Division through Asymmetric Centrosome Behavior
M. T. Fuller1, Y. Yamashita2; 1Departments of Developmental Biology and Genetics, Stanford University School of Medicine, Stanford, CA, 2Department of Developmental Biology, Stanford University School of Medicine, Stanford, CA

Adult stem cells divide to both renew stem cells and produce cells that initiate differentiation. This choice must be highly regulated to replenish the stem cell population throughout life, yet retain in stem cell proliferative capacity of stem cells. The Drosophila germ line provides a powerful system to study mechanisms that regulate stem cell self-renewal and differentiation. Drosophila male germ line stem cells (GSCs) attach to adherens junctions to somatic hub cells, which comprise the stem cell niche. Hub cells express Usp, which activates the JAK-STAT pathway in adjacent germ cells to specify GSC self-renewal. The normally asymmetric outcome of stem cell divisions in the Drosophila male germ line is specified by stereotyped orientation of the mitotic spindle perpendicular to the junctional complex that attaches the stem cell to the niche. As a result, one daughter inherits the adherens junctions, retains contact with the niche and self-renews, while the other daughter is displaced away and initiates differentiation. GSCs orient toward the niche throughout the cell cycle. The single centrosome in G1 is next to the niche. After duplication, one centrosome stays next to the niche while the other migrates to set up the oriented mitotic spindle. Strikingly, differences between mother and daughter centrosomes appear to underlie this stereotyped behavior: the mother centrosome remains next to the niche, while the daughter centrosome consistently migrates to the opposite side of the nucleus. This programmed asymmetric behavior of mother versus daughter centrosomes requires wild type function of the centromosomal protein cnn, suggesting that attachment to astral microtubules helps anchor the mother centrosome to the junction with the niche. Thus, adult male GSCs execute specialized cytoskeletal programs to govern centrosomal positioning so that the spindle is set up perpendicular to the niche. As a result, stem cells in the niche maintain a centrosomal Eve.

2060
Dynamics of the Endocytic Coat Module in S. cerevisiae Regulated by an ArfGAP and Synaptotagmin
C. P. Toret, M. Kaksonen, Y. Sun, D. Drubin; Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA

Recent studies have revealed a highly choreographed recruitment of endocytic proteins to sites of clathrin/actin-mediated endocytosis. How these precise dynamics are achieved remains unresolved. Earlier, we used quantitative live-cell imaging of budding yeast to uncover a modular organization of the endocytic machinery. Here, we investigated possible roles for candidate disassembly factors in regulation of the dynamics of the endocytic coat module proteins. Components of the coat module include clathrin, Slap2p, Enl1p, Enl2p, Slap1p, Pan1p, and Enl3p, each of which has a mammalian homologue. Live-cell imaging analysis revealed that the putative ArfGAP, Gts1p, and the synaptotagmin, Imp52p, arrive at endocytic sites late in the internalization pathway, consistent with a role in coat disassembly. ArfGAPs and synaptotagmins have been implicated as disassembly factors in other cellular trafficking pathways. Analysis of mutants of the genes encoding Gts1p, and the Arf6 homologue Arf3 that it is presumed to regulate, and Imp52p, implicate these proteins in disassembly of the endocytic coat module, with Gts1p/Arf3 and Imp52p each regulating a subset of the coat components. Loss of both Gts1p/Arf3p and Imp52p activities together results in pronounced uncoating defects. Our results implicate two cooperative pathways, one involving GTP hydrolysis stimulated by ArfGAP, and the other involving lipid turnover catalyzed by synaptotagmin, in separate pathways for the uncoating of endocytic vesicles.

2061
Adaptors and Sorting Signals for Clathrin-coated Vesicles
M. S. Robinson; University of Cambridge, Cambridge, United Kingdom

Adaptors select cargo for transport in clathrin-coated vesicles (CCVs) between the TGN, endosomes, and the plasma membrane. We have investigated the functions of some of the binding sites and domains of the AP-2 adaptor complex, which acts at the plasma membrane, by stably transfecting cells with siRNA-resistant constructs and then knocking down the endogenous protein. These studies reveal a certain degree of functional redundancy, because no single mutation completely abolishes AP-2-dependent transferrin uptake; however, attaching a C-terminal GFP tag to the alpha subunit renders it non-functional. To investigate clathrin-mediated intracellular trafficking, we have been isolating CCVs from control and siRNA-treated HEla cells. Using a comparative proteomics approach, we have identified 63 bona fide CCV components. One of these proteins, CVAK104, is a novel regulator of AP-1-dependent trafficking, which is required for the sorting of two SNAREs, syntaxin 8 and vti1b, and for normal embryonic development in Xenopus tropicalis. EpisnR is another CCV component required for vti1b sorting, which acts as an alternative adaptor. We have mapped the binding site on vti1b for episnR to a folded domain, and we have solved the structure of this domain by X-ray crystallography.
Endocytosis is a key mechanism for plasma membrane protein downregulation, yet the intrinsic diversity of protein cargos and extracellular stimuli presents a challenge to achieving specificity. By lowering the abundance of specific plasma membrane proteins (e.g., sensors, receptors and channels), cells can reduce their sensitivity to certain environmental cues. Here we report a new family of proteins (ART, for Arrestin Related Trafficking adaptors) in *S. cerevisiae* that may play a pivotal role in cargo-specific endocytosis. Art proteins reside primarily in the cytoplasm, but they translocate to the plasma membrane in response to stress or extracellular nutrients. Two members (Art1p and Art2p) of the family were first discovered in our genome-wide chemical-genetic screens for yeast mutants defective in the endocytosis of specific 12-transmembrane amino acid transporters. Further screen analysis revealed seven additional homologs (Art3p to Art9p) in the yeast genome. The Art proteins are related to mammalian arrestins, and each additionally contains multiple Pro-X-Tyr (PY) motifs in the C-terminal region. We provide evidence that these PY motifs are required for recruiting the Nedd4-like Rsp5 ubiquitin ligase to the plasma membrane, where it ubiquitinates protein cargos as well as the Art proteins themselves. As a result, ubiquitinated protein cargos are targeted to the endocytic pathway followed by degradation in the vacuole/lysosome. Our data also suggest that a plasma membrane protein cargo can recruit distinct Art proteins in response to different extracellular signals. We propose that these Art proteins represent a new family of adaptors that target the Rsp5 ubiquitin ligase to specific membrane cargos in response to extracellular signals, resulting in their endocytosis and downregulation.

### 2063 Characterization of Yusunin, a Novel Component Required for TGN to Cell Surface Transport

C. Bossard, Y. Maceda, V. Malhotra; *Cell and Developmental Biology, UCSD, La Jolla, CA, Department of Immunoregulation, Osaka University, Osaka, Japan

Protein kinase D (PKD) is required to form trans-Golgi network (TGN) to cell surface transport carriers. PKD is recruited to the TGN by a specific pool of dicycloyglycerol (DAG) and activated by Gβγ and the Golgi apparatus-associated PKCγ. Inhibition of PKD inhibits membrane fission and resulting in the formation of large cargo filled tubules that remain attached to the TGN. Its overactivation, on the other hand, regulates tubules. PKD and its interactors therefore regulate events leading to separation, by fission, of the Golgi to cell surface transport carriers. We have identified a new downstream target of PKD and called it Yusunin. Our findings reveal that Yusunin - a cytosolic protein - is recruited to the TGN through binding to PKD. Yusunin interacts with inactive PKD and is released from PKD by phosphorylation. Inhibition of PKD-dependent phosphorylation of Yusunin abolishes Golgi to cell surface transport. Yusunin does not effect the recruitment of PKD to the TGN but inhibits TGN to cell surface transport. Yusunin, therefore, is a key component of the machinery involved in the formation of Golgi to cell surface transport carriers.

### 2064 Regulation of the Drs2p-dependent Flippase Activity in Golgi Membranes by Phosphatidylinositol 4-Phosphate

T. Graham, P. Natarajan, K. Liu; *Department of Biomedical Sciences, Vanderbilt University, Nashville, TN*

Phospholipid translocases (PLTs) flip fluorescent or spin-labeled derivatives of phospholipids from the exoplasmic leaflet of a membrane bilayer to the cytosolic leaflet, and are thought to establish asymmetry of endogenous phospholipid in biological membranes. The best candidates for PLTs are the Drs2p/ATPase II (ATP7A) subfamily of P-type ATPases (P4 ATPases). Drs2p from budding yeast localizes to the trans-Golgi network (TGN) and is required for a flippase activity measured with purified TGN membranes using a 7-nitro-2,1,3-benzoazadiazol-4-yl (NBD) phosphatidylinositol (PS) domain of Npt3p to destroy phosphoinositides markedly reduced Drs2p flippase activity. In contrast, Wortmannin, a PI 3-kinase inhibitor, had no effect. Moreover, Drs2p activity is substantially reduced in TGN membranes from pik1ts and 1p3p and stimulated the Drs2p-dependent NBD-PS flippase activity in these membranes. Further addition of the Sac1p (phosphoinositol phosphate) domain of Inp53p to destroy phosphoinositides markedly reduced Drs2p activity. In contrast, Wortmannin, a PI 3-kinase inhibitor, had no effect. Moreover, Drs2p activity is substantially reduced in TGN membranes from pik1ts incubated at the nonpermissive temperature (37°C). These results indicate that PI4P stimulates Drs2p-dependent NBD-PS translocase activity in TGN membranes. Therefore, we propose that Drs2p is a critical effector of PI4P at the yeast TGN.

### 2065 Golgin-160 Is Required for the Golgi Membrane Sorting of the Insulin-responsive Glucose Transporter GLUT4 in Adipocytes

D. Williams, S. W. Hicks, E. M. Madam, J. E. Fessin; *Department of Pharmacological Sciences, Stony Brook University, Stony Brook, NY, Department of Cell Biology, Johns Hopkins University, Baltimore, MD, Department of Microbial Pathogenesis, Yale University, New Haven, CT*

The peripheral Golgi protein golgin-160 is induced during 3T3L1 adipogenesis and is primarily localized to the Golgi cisternae distinct from the TGN in a general distribution similar to p115. siRNA mediated reduction in golgin-160 protein in an increase accumulation of the insulin-responsive aminopeptidase (IRAP) and GLUT4 protein (both endogenous and transfected) at the plasma membrane and enhanced glucose uptake in the basal state. The redistribution of GLUT4 was rescued by expression of a siRNA resistant golgin-160 cDNA. The basal state accumulation of plasma membrane GLUT4 occurred due to an increased rate of exocytosis without any significant effect on the rate of endocytosis. This GLUT4 trafficking to the plasma membrane in the absence of golgin-160 was independent of GLUT4 sorting, as it was no longer inhibited by the expression of a dominant-interfering GGA mutant. Moreover, expression of the amino terminal head domain (amino acids 1-393) had no significant effect on the distribution or insulin-regulated trafficking of GLUT4 or IRAP. In contrast, expression of carbonyl alpha helical region (393-1498) inhibited insulin-stimulated GLUT4 and IRAP trafficking but had no effect on the sorting of constitutive membrane trafficking proteins, the transferrin receptor or vesicular stomatitis virus G (VSV-G) protein. Taken together these data demonstrate that golgin-160 plays an important role in directing insulin-regulated trafficking proteins toward the insulin-responsive compartment in adipocytes.

### 2066 Direct Visualization of Prokaryotic Cytoskeletal Filaments and Other Ultrastructural Elements in Intact Cells by Electron Cryotomography

G. Jensen; *Division of Biology, California Institute of Technology, Pasadena, CA*

Using state-of-the-art electron cryotomography, we have now directly visualized ~10 distinct cytoskeletal filament types in the bacterial species Caulobacter crescentus, Magnetospirillum magnetcum, Mycoplasma pneumoniae, and Mesoplasma florum. Thus while the absence of any cytoskeleton was once thought to be a distinguishing characteristic of prokaryotes, it is now clear that the cytoskeleton is an important, complex, and widespread component of bacterial cell biology. In the case of *C. crescentus*, six distinct filament bundles have been characterized and efforts are underway to identify each through structural signatures, mutational analysis, and correlated fluorescence and electron microscopy. FtsZ2 in particular was seen to form half-rings and stacked helices with characteristic spacings. In the case of Magneticum, MamK was identified as a new actin-like protein filament used by the cell to organize the organelle-like magnetosome chain. In *M. pneumoniae*, the filamentous "attachment organelle" has been characterized in detail, prompting new hypotheses about its role in motility and infectivity. In *M. florum*, a cellular process known as "nuring" occurs as the cell porks across the cytoplasm. In addition to revealing novel cytoskeletal filaments, this work has also allowed us to visualize complete, unperturbed flagellar motors, chemoreceptor bundles, and polysomes in-situ, albeit at only medium resolution (~5 nm). This work has been a mixture of technology development and biological and, illustrates how electron cryomicroscopy is opening a new window into prokaryotic ultrastructure.

### 2067 Tension-dependent Regulation of Microtubule Dynamics at Kinetochores Can Explain Metaphase Congression in Yeast

M. K. Gardner, C. G. Pearson, B. L. Sprague, T. R. Zarzar, K. Bloom, E. D. Salmon, D. J. Odde; *Department of Biomedical Engineering, University of Minnesota, Minneapolis, MN, Division of Biological Sciences, University of California, San Diego, School of Medicine, La Jolla, CA, Department of Biomedical Sciences, University of California, San Diego, School of Medicine, La Jolla, CA, Bunting and Best Department of Medical Research and Department of Medical Genetics and Microbiology, University of Toronto, Toronto, ON, Canada*

Magnetosome chain. In clear that the cytoskeleton is an important, complex, and widespread component of bacterial cell biology. In the case of *M. K. Gardner, 1 C. G. Pearson, 2 B. L. Sprague, 3 T. R. Zarzar, 4 K. Bloom, 4 E. D. Salmon, 4 D. J. Odde 1; 1Department of Biomedical Engineering, University of Minnesota, Minneapolis, MN, 2Department of Biological Sciences, University of California, San Diego, School of Medicine, La Jolla, CA, 3Bunting and Best Department of Medical Research and Department of Medical Genetics and Microbiology, University of Toronto, Toronto, ON, Canada*
During mitosis, dynamic kinetochore-attached microtubules (kMTs) mediate the proper segregation of chromosomes into nascent daughter cells. In budding yeast, kinetochore-bearing microtubules play a significant role in the separation of chromosomes. As a result, the intervening chromatin is stretched and the chromosomes are centered at the spindle equator, a process known as congression. To understand how kMT plus end self-assembly dynamics are regulated to achieve congression, we used quantitative fluorescence microscopy combined with computational modeling to simulate (1) the extent of kinetochore-stabilized plus-end attachment during metaphase as measured via fluorescence recovery after photobleaching (FRAP) of Cse4-GFP; (2) kinetochore-associated fluorescence distribution during metaphase; (3) kMT plus-end self-assembly dynamics as measured via FRAP of GFP-Tubulin, and (4) kinetochore-associated fluorescence distribution during metaphase in a replication deficient mutant lacking tension generated from chromatin stretch. The only model that explained all four experiments was one in which kMT plus-end dynamics are regulated both by a spatial catastrophe gradient leading to kMT depolymerization at the spindle equator, and by attachment site tension that promotes net kMT assembly with increasing chromatin stretch. In this model, the catastrophe gradient establishes rudimentary bi-orientation, and tension between sister kinetochores corrects remaining errors to robustly achieve congression. Many other models were tested and failed to explain at least one experiment, establishing a method for systematic hypothesis testing via computational modeling.

2068 Structure of the Kinetochore Dam1 Complex and Its Assembly on Microtubules
1Pathology and Immunology, Washington University School of Medicine, St. Louis, MO, 2Microbiology and Immunology, Hamamatsu University School of Medicine, Hamamatsu, Japan

Multi-photon microscopy is providing crucial insight into the cell dynamics that underlie antigen presentation and T cell activation. However, published studies have used model systems that elicited immune responses with protein immunizations or peptide-pulsed dendritic cells (DCs). During an infection, host-pathogen interactions are likely to impact antigen recognition and it is unclear how well model systems can recapitulate physiological levels of inflammation, antigen abundance or peptide-MHC processing. Using multi-photon microscopy and immunofluorescence of thin spleen sections, we examined the fate of GFP-expressing Listeria monocytogenes (Lm), the distribution of macrophages and DCs and the kinetics of Lm-specific antigen presentation during infection. After i.v. challenge, bacteria were captured rapidly (~1h) by sessile marginal zone macrophages (MZM) and cleared from the marginal zone by 6h. In contrast, bacteria that infected DCs avoided destruction and were carried into the T cell area or periarteriolar lymphoid sheath (PALS). Paradoxically, the replication of Lm in the PALS induced the widespread apoptosis of lymphocytes and DCs at 24h-48h, yet Lm entry into PALS has been shown to be required for long-lived immunity. To understand how antigen recognition occurs in the face of pathogen counter measures, we imaged adoptively transferred TCR transgenic T cells as a probe for the presentation of Lm-derived antigens in situ. The CD8 T cell response was remarkably rapid and efficient; 9h after challenge large clusters of Lm-specific cells were seen on DCs and by 24h many lymphoblasts were visible migrating through the PALS. Strikingly, CD4 T cell activation occurred after CD8 activation, was comparably weak and occurred within a wider distribution in the PALS. Our studies suggest that during infection, Lm-induced tissue-remodeling transports pathogen-derived antigens to specific tissue microenvironments and that initial CD8 and CD4 T cell responses show distinct spatiotemporal dynamics.

2069 Spleen Remodeling and Antigen Presentation Dynamics during Listeria monocytogenes Infection
M. J. Millie, T. Aoshi, Y. Koide, F. F. Ruegg, E. B. Unanue
1Pathology and Immunology, Washington University School of Medicine, St. Louis, MO, 2Microbiology and Immunology, Hamamatsu University School of Medicine, Hamamatsu, Japan

Near-molecular Resolution of Intracellular Fluorescent Proteins Using Photoactivated Localization Microscopy (PALM)
E. Betzig, G. Patterson, R. Sougrat, M. Davidson, S. Olender, W. Lindwasser, J. Bonifacino, J. Lippincott-Schwartz, H. Hess, Janelia Farm Research Campus, Howard Hughes Medical Institute, Ashburn, VA, 2Cell Biology and Metabolism Branch, National Institute of Child Health and Human Development, Bethesda, MD, 3National High Magnetic Field Laboratory, Florida State University, Tallahassee, FL

In conventional biological imaging, diffraction places a limit on the minimal xy distance that two marked objects can be discerned. Consequently, imaging of target proteins within cells is typically coarser by two orders of magnitude than the molecular scale at which the proteins are spatially distributed. Here, we introduce a method capable of optically resolving selected subsets of fluorescently-tagged proteins within cells at mean separations of a few nanometers, overcoming the diffraction barrier in fluorescence microscopy. Terned photoactivated localization microscopy (PALM), the method involves serial photoactivation and subsequent bleaching of numerous sparse subsets of photoactivated fluorescent protein molecules. Individual molecules are localized at near molecular resolution by determining their centers of fluorescent emission via a statistical fit of their point-spread-function. The aggregate position information from all subsets is then assembled into a super-resolution image, in which individual fluorescent molecules are isolated at high molecular densities (up to 10^10 molecules per micron^3). PALM imaging of intracellular structures (including lysosome, Golgi apparatus and mitochondria) in cryo-prepared thin sections is presented. In addition, imaging of vinculin and actin by PALM in fixed cells with TIRF excitation is shown. Finally, correlative PALM/transmission electron microscopy images are presented of a mitochondrial marker protein, illustrating the feasibility of relating the nanometer scale distribution of a specified protein at high molecular density (i.e., 5500 molecules per mitochondrion) to cellular ultrastructure.

2070 Electron Tomography of Cells
R. McIntosh; Department of Molecular/Cell/Developmental Biology, University of Colorado, Boulder, CO

Improvements in electron microscopes, digital cameras, computers, and software have made electron tomography (ET) a powerful method for structural cell biologists. Specimen details can be visualized in 3-D with 4 - 6nm resolution and the volumes reconstructed can be big enough to include the entirety of an organelle or even a small cell. Large volume work is best accomplished with rapidly frozen, freeze-substitution fixed, stained, and plastic-embedded material, which facilitates the accumulation of montaged images from adjacent areas and the construction of serial tomograms from successive thick sections. The resulting evidence about the connectivity of cytoplasmic membranes and cytoskeletal elements is important. High-resolution work on fixed, stained samples is, however, hampered by the possibility of fixation artifacts and by the imaging of stain, rather than the macromolecules themselves. Cryo-ET of frozen-hydrated samples solves these problems, but resolution is now limited by poor image signal-to-noise, due to low contrast and the low electron dose that is tolerated by these radiation sensitive specimens. Image signal-to-noise can be improved when tomograms contain multiple copies of a given object; these 3-D structures can be excised, aligned, and averaged, essentially as in high-resolution cryo-EM of other kinds. Now signal-to-noise can be good enough that molecular shapes are distinct and informative, allowing atomic or other high-resolution structures to be docked into their cellular location. Examples of both kinds of data will be shown and discussed, along with evidence about the key outstanding problem in cellular ET, the identification and localization of specific macromolecules.
Neuromuscular transmission defects and muscle weakness are common in desminopathy, a disease caused by mutations in the intermediate filament (IF) protein desmin. These mutations can affect various muscle types, including skeletal, cardiac, and smooth muscle. The number of identified pathogenic mutations in the IF protein desmin, which lead to desminopathy of skeletal, cardiac, as well as smooth muscle, has grown considerably in the last few years. We have investigated, both in vitro and in vivo, 25 disease-causing mutations in order to obtain insight into the molecular and structural characteristics of desmin IFs accompanying tissue pathogenesis. In comparison to the wild-type protein, many of the mutant desmins variants exhibited distinct assembly defects at various stages of assembly. In particular, both tetramer and unit-length filament formation as well as filament elongation and IF maturation were affected. For further characterization of the viscoelastic properties of desmin IFs, we performed nanomechanical techniques employing the scanning force microscope. Thereby we demonstrated that IFs from mutated desmins were on average not mechanically weaker than wild-type IFs. In parallel, we expressed the proteins both in cellulo of cytokinetic intermediate filaments (human SW13) and into those containing an extensive vimentin network (mouse 3T3). The in vivo assembly properties matched - and by large - those determined in vitro. In summary, despite the ability to form apparently normal IFs by about half of the mutant desmins, in the tissue the mutated residues obviously interfere negatively with intrinsic cellular factors engaged in the establishment and maintenance of a functional myocyte architecture.

**2073 Assembly Competence, Biomechanical Properties, and Network Forming Ability of Desmin Disease Mutants**

H. Herrmann, S. Sharma, L. Kreplak, H. A. Katus, U. Aebi, H. Bär; Molecular Genetics, German Cancer Research Center, Heidelberg, Germany, M. E. Müller Institute, Biozentrum, Basel, Switzerland, Department of Cardiology, University of Heidelberg, Heidelberg, Germany

We have discovered a spontaneous mutation in the Sprague Dawley rat with a novel and unusual eye phenotype that we have named Nuc1. Nuc1 is inherited as a single Mendelian locus with viable homozygotes and with an intermediate phenotype in the heterozygotes. Heterozygotes exhibit nuclear cataracts, while homozygotes have microphthalmia, retinal abnormalities and rupture of the lens capsule shortly before birth. The Nuc1 locus was mapped to rat chromosome 10; a 27 nucleotide insertion in a beta-crystallin gene results in the Nuc1 phenotype. In Nuc1 homozygotes, the secondary lens fibers elongating from opposite directions exhibit abnormal migration and orientation and fail to iterdigitate to form normal sutures. We have observed a marked decrease in filensin in developing fiber cells of Nuc1 homozygotes both by immunohistochemical and confocal microscopy starting at embryonic day 19. Western blots using anti-filensin polyclonal antibody demonstrate decreased filensin in Nuc1 heterozygotes and homogygotes relative to wild-type lenses. Filensin is a constituent of the beaded filament, a lens-specific cytoskeletal element. Our yeast two-hybrid studies do indicate physical interactions between beta/crystallins and cytoskeletal proteins suggesting that crystallins may have a stabilizing function on the cytoskeletal structure. Finally, metabolic labeling studies with 35S amino acids in organ cultured lenses indicated that filensin synthesis was decreased in mutant lenses. Our results clearly demonstrate that the programmed remodeling of the lens fibers that is essential to maintain transparency is dependent on crystallins. We also provide evidence that crystallins are not merely structural proteins but have important regulatory functions in maintaining the cytoskeletal structure and thereby serve an important role in health and disease.

**2074 A Spontaneous Mutation in a Beta-Crystallin Gene Affects the Remodeling of Lens Fibers Cells**

D. Sinha, S. Kisse, S. Schubert, P. K. Ho, M. Can, O. Sandin, E. Ratovitski, P. Zelenka, K. W. Bromman, J. S. Zigler; Ophthalmo, Wilmer Eye Institute, The Johns Hopkins University School of Medicine, Baltimore, MD, The National Eye Institute, National Institutes of Health, Bethesda, MD, Otolaryngology-Head and Neck Surgery, The Johns Hopkins University School of Medicine, Baltimore, MD, Bios-statistics, The Johns Hopkins University, Baltimore, MD

We have discovered a spontaneous mutation in the Sprague Dawley rat with a novel and unusual eye phenotype that we have named Nuc1. Nuc1 is inherited as a single Mendelian locus with viable homozygotes and with an intermediate phenotype in the heterozygotes. Heterozygotes exhibit nuclear cataracts, while homozygotes have microphthalmia, retinal abnormalities and rupture of the lens capsule shortly before birth. The Nuc1 locus was mapped to rat chromosome 10; a 27 nucleotide insertion in a beta-crystallin gene results in the Nuc1 phenotype. In Nuc1 homozygotes, the secondary lens fibers elongating from opposite directions exhibit abnormal migration and orientation and fail to iterdigitate to form normal sutures. We have observed a marked decrease in filensin in developing fiber cells of Nuc1 homozygotes both by immunohistochemical and confocal microscopy starting at embryonic day 19. Western blots using anti-filensin polyclonal antibody demonstrate decreased filensin in Nuc1 heterozygotes and homogygotes relative to wild-type lenses. Filensin is a constituent of the beaded filament, a lens-specific cytoskeletal element. Our yeast two-hybrid studies do indicate physical interactions between beta/crystallins and cytoskeletal proteins suggesting that crystallins may have a stabilizing function on the cytoskeletal structure. Finally, metabolic labeling studies with 35S amino acids in organ cultured lenses indicated that filensin synthesis was decreased in mutant lenses. Our results clearly demonstrate that the programmed remodeling of the lens fibers that is essential to maintain transparency is dependent on crystallins. We also provide evidence that crystallins are not merely structural proteins but have important regulatory functions in maintaining the cytoskeletal structure and thereby serve an important role in health and disease.

**2075 Normalization of Differentially Expressed Genes in Keratin 8-null Colonoocytes in Response to Antibiotic Treatment**

D. A. D. Brooks, E. C. Butcher, M. B. Omary; Medicine, VA Palo Alto Health Care System and Stanford University Digestive Disease Center, Palo Alto, CA, Applied Biosystems, Foster City, CA, Pathology, VA Palo Alto Health Care System and Stanford University Digestive Disease Center, Palo Alto, CA

Keratin 8 (K8) is the major type II intermediate filament protein present in colonic epithelial cells. K8-null mice develop colonic hyperplasia, diarrhea, and chronic spontaneous Th2-type colitis amenable to broad-spectrum antibiotic treatment. The aim of this study was to identify genes that are differentially expressed as a result of K8 absence in colonic epithelial cells. Microarray analysis was used to examine differences in gene expression between colonocytes freshly isolated from K8 wild-type and K8-null mice. We also examined whether the differentially expressed genes were related to the presence of luminal bacteria by examining the gene expression profile following antibiotic treatment. The majority of the highly altered genes in untreated K8-null versus wild-type colonoocytes were related to apoptosis, growth and differentiation. Among these genes, we confirmed which were recognized by RT-PCR, tranquillatine-3 (14.4-fold by RT-PCR), clusterin (3.4-fold), and peroxiredoxin (2.2-fold) were highly down-regulated whereas survivin (5.7-fold) was up-regulated in K8-null colonoocytes. These alterations provide a mechanistic insight regarding the colon hyperplasia of K8-null mice and the involvement of keratin in modulating epithelial cell susceptibility to apoptosis. Notably, nearly all of the differentially regulated genes in K8-null versus wild-type colonoocytes were normalized following antibiotic treatment, thereby supporting the importance of colonoocyte interaction with the luminal bacterial milieu in manifesting the intestinal phenotype of K8-null mice.

**2076 Insights into the Role of Nuclear Laminas in DNA Replication: lamin Interactions with PCNA**

D. K. Shumaker, T. Shimi, L. Solimando, M. Cardoso, R. D. Goldman; Cell Molecular Biology, The Feinberg School of Medicine, Northwestern University, Chicago, IL, Max Delbruck Center for Molecular Medicine, Berlin, Germany

When nuclei are exposed to mutant laminas lacking their N-terminus, the nuclear lamin network is disassembled and DNA replication is blocked. Under these conditions, laminas are present in nuclear foci with the DNA replication elongation factors, PCNA and RFC, which normally associate with chromatin during S-phase. Based upon these observations, we have examined the relationship between laminas, B2, C and PCNA in more detail using S-phase HeLa cells and in vitro assembled Xenopus nuclei. The overall organization of Lamin A (LA), LB2 and LC is similar, but not identical. We have found that during S-phase in HeLa cells, PCNA foci are either associated with nucleoplastic lamin foci or the lamin at all stages of replication. The relationship between PCNA and lamin was further examined in live cells using EGFP and mCherry tagged proteins. Under these conditions, PCNA foci were also associated either with the nucleoplastic laminas or the nuclear lamin. Our results suggest that laminas play a role in regulating DNA replication through its interactions with PCNA. To determine the nature of the interactions between PCNA and laminas, various fragments of Xenopus LB3 and human LA were expressed and purified. Using affinity chromatography, we find that the C-terminal domain of both Xenopus LB3 and human LA bind PCNA in Xenopus egg and HeLa cell extracts. Further analyses show that this binding involves the highly conserved Ig-fold of the lamin C-terminus. To determine whether PCNA interacts directly with lamin fragments, immunoprecipitation assays using purified proteins and gel overlays with radioactively labeled PCNA were performed. The results show that PCNA can directly interact with the lamin Ig-fold domain. Supported by the NCI, The Ellison Foundation and the Progeria Research Foundation.
A Farnesylated Mutant Lamin A Affects the Extracellular Matrix and Cell Proliferation in a Model of Hutchinson-Gilford Progeria

K. Roux,1 L. Hernandez,2 B. Burke,2 C. L. Stewart1; 1Anatomy and Cell Biology, University of Florida, Gainesville, FL, 2UCBL, National Cancer Institute, Frederick, MD

The laminopathies is a group of diseases caused by mutations in the LMNA gene that encodes the A-type lamins. One of the most striking laminopathies is the premature aging disease Hutchinson-Gilford Progeria Syndrome (HGPS). The most prevalent mutation associated with HGPS is a base change in codon 608 that introduces a premature splice donor site into LMNA resulting in a 50 amino acid deletion within the C-terminal nonhelical domain of lamin A. Posttranslational modification of lamin A includes farnesylation of a terminal CaaX motif, followed by proteolytic processing that removes the terminal 18 amino acids. The HGPS deletion eliminates the cleavage site within lamin A, resulting in a truncated yet permanently farnesylated form of the molecule (Progerin). How Progerin causes HGPS is an area of intense investigation. Previously we described a mouse line in which Exon 9 of Lmna was deleted (Δ9 mice, Mounkes et al Nature 2003 423; p298). These mice exhibited phenotypes similar to many of the pathologies associated with HGPS. Here we show that the Exon 9 deletion results in a truncated form of lamin A that remains farnesylated suggesting that this mouse line may provide a model to uncover the etiology of HGPS. To this end we find that embryonic fibroblasts from the Δ9 mice, despite abnormally shaped nuclei, show normal growth parameters and gene expression. In contrast postnatal fibroblasts exhibit severe growth retardation associated with reduced expression of many extracellular matrix (ECM) proteins, as well as components in the FGF and TGFβ signaling pathways. Growth can be rescued by maintaining the Δ9 cells on ECM from normal cells or by treatment with FGF18. Our results support the notion HGPS maybe caused by developmentally regulated defects in ECM production that affects cell proliferation/maintenance in specific tissues such as cartilage, skeleton, and cardiovascular system.

2077

Molecular Details of Tubulin Assembly Measured with Optical Tweezers

E. Munteanu,1 J. W. J. Kerssemakers,2 T. Nötzel,1 M. Dogterom1; 1Bio-Assembly and Organization, FOM Institute AMOLF, Amsterdam, The Netherlands, 2MPI-CBG, Dresden, Germany

Although microtubules are known to self-assemble from tubulin dimers both in vivo and in vitro, the underlying sequence of molecular events remains poorly understood. We perform in vitro experiments aimed at understanding the molecular details of the assembly process of microtubules and the molecular mechanism by which microtubule associated proteins regulate microtubule dynamics. We use a combination of micro-fabrication techniques and optical tweezers to observe the growth process of individual microtubules at molecular resolution. We find that microtubules can near-instantaneously increase their overall length by amounts exceeding the size of single dimers (8 nanometers). When the microtubule-associated protein XMAP215 is added, this effect is markedly enhanced and fast length increases up to 60 nanometers are observed, which corresponds to the size of the XMAP215 protein itself. These observations show that small tubulin oligomers can add directly to growing microtubules and that XMAP215 speeds up microtubule growth by facilitating the addition of long oligomers.

2078

Structural Basis for the Activation Mechanism of Microtubule Assembly by Plus-End Tracking Proteins

I. Hayashi, M. Ikura; Department of Medical Biophysics, University of Toronto, Ontario Cancer Institute, Toronto, ON, Canada

A group of proteins, such as EB1, CLIP-170 and the dynein/dynactin complex, has been found to associate specifically with the microtubule growing ends in vivo. These proteins, termed “plus-tracking proteins (+TIPs)”, are thought to form a plus-end complex and guide microtubule growth toward specialized destinations by local influences on microtubule assembly. Despite much recent progress in the field, many key mechanistic questions remain unanswered. Previously we determined the crystal structure of EB1 and the dynactin subunit p150Glued, and demonstrated that EB1 alone has an activity to assemble microtubules whereas p150Glued plays a role as an allometric activator of EB1. CLIP-170 is structurally related to p150Glued. They both have the CAP-Gly domains in their N-terminal region which can interact with EB1. Furthermore, CLIP-170 has two putative zinc-binding motifs at the C terminus to control its intramolecular interaction by binding to the N-terminal CAP-Gly domains. In the present study, we have investigated the crystallographic, NMR and mutational analyses of EB1, p150Glued and CLIP-170. Our data reveal that EB1 and CLIP-170 recognize the same elements of p150Glued, and that these interactions are critical for the activation of EB1 and CLIP-170. We further show the regulatory interplays between these three +TIPs by in vitro binding analysis. Taken together, we propose a possible activation mechanism of microtubule assembly by EB1, CLIP-170 and p150Glued.

2080

Coordination of Microtubule Plus-End Binding Proteins

M. Raycroft, L. Casaletti, K. T. Vaughan; Department of Biological Sciences, University of Notre Dame, Notre Dame, IN

A growing number of microtubule binding proteins display some preference for the plus-ends of growing microtubules. Because these proteins are implicated in different functions, it remains unclear if plus-end localization reveals overlapping function or regulated microtubule binding. Focusing on the p150Glued subunit of dynactin, we used three approaches to perturb plus-end binding proteins and tested the impact on other members of this family. First we depleted p150Glued using siRNA and assessed the effect on EB1 and CLIP-170. Loss of p150Glued resulted in the loss of EB1 at plus-ends. Expression of p150Glued rescue constructs re-established plus-end labeling of both dynactin and EB1. This suggests that EB1 is somewhat dependent on dynactin for plus-end localization. To determine the order of plus-end binding, we coexpressed combinations of EB1, p150Glued and CLIP-170 and visualized GFP these proteins by live-cell imaging. Dual imaging during time-lapse microscopy revealed initial localization of EB1 to plus-ends, followed by p150Glued, followed by CLIP-170. This suggests an order of loading inconsistent with EB1 depending on p150Glued for binding. Finally we utilized mTOR manipulation to test for phosphorylation effects on plus-end binding. Inhibition of mTOR with rapamycin led to dramatic decoration of microtubules with EB1, but a loss of p150Glued from plus-ends. No effects on CLIP-170 were detected. Overexpression of active mTOR had the opposite effect; EB1 was lost from plus-ends, but p150Glued decorated microtubules along their lengths. To assess the basis of p150Glued modulation, we performed 2-D gel analysis on rapamycin-treated cells. Rapamycin increased the phosphorylation complexity of p150Glued consistent with phosphorylation at two sites. Together these results suggest that several key plus-end binding proteins influence each other and that phosphorylation is at the root of plus-end function.

2081

Microtubule Plus End Attachment to the Cell Cortex: Mechanisms and the Role in Vesicular Traffic

A. Akhmakova,1 I. Grigoriev,1 G. Lansbergen,1 S. Spangler,2 C. C. Hoogenraad,1 N. Galjart,1 F. Grosveld1; 1Department of Cell Biology and Genetics, Erasmus Medical Center, Rotterdam, The Netherlands, 2Department of Neuroscience, Erasmus Medical Center, Rotterdam, The Netherlands, 1KAN Research Institute, Kyoto, Japan

The organization of the microtubule network strongly depends on the interaction of microtubule tips with the cell cortex. In many types of mammalian cells, microtubule plus ends face the cell periphery and can be linked to the actin cytoskeleton or the plasma membrane by different plus end tracking proteins (+TIPs). One such microtubule-cortex attachment is formed by CLASPs - evolutionary conserved +TIPs - and the proteins LL5β and ELKS, which form relatively stable clusters at the cortex of HeLa cells and the leading edges of motile fibroblasts. Using RNA interference-mediated protein depletion we show that CLASPs, LL5β and ELKS are necessary to maintain the high density and stability of the microtubule network. We isolated additional protein components essential for the formation of LL5β-ELKS clusters with the aid of mass spectrometry-based approaches. We also investigated the function of the identified cortical complexes in vesicular transport. Since ELKS (also known as Rab6 Interacting Protein 2) binds directly to the small GTPase Rab6 (Monier et al., 2002, Traffic 3 (4), 289-97), we have focused our attention on the trafficking of Rab6-positive vesicles and found that ELKS is involved in catalysing the fusion of stable microtubules, suggesting Kif4 is necessary for microtubule stabilization. Endogenous Kif4 localized to the ends of stable microtubules while GFP-tagged Kif4 tracked the plus-ends of microtubules and accumulated at the
cell periphery where it might form part of a capping complex. Ki67 interacted with EB1 both by endogenous co-immunoprecipitation and directly by pull-down using recombinant proteins showing Ki67 interacts with other factors involved in microtubule stabilization. Together our results show that Ki67 contributes to microtubule stabilization and has properties consistent with it being part of a plus-end capping complex.

2083 Interactions between Microtubule Plus Ends and Dynein Cortical Receptor Numl in Budding Yeast

W. Lee; Biology Department, University of Massachusetts, Amherst, MA

Mitotic spindle positioning plays an essential role in determining where replicated chromosomes are deposited at the end of cell division. In the budding yeast S. cerevisiae, the site of division is at the bud neck, and a protein Num1 accumulates in patches along the membrane and this protein correlates with the point of attachment of microtubules that anchors the mitotic spindle. Genetic interactions and localization studies have indicated that Num1 functions as a receptor protein for dynein to mediate cortical sliding of microtubules in the dynein pathway of spindle positioning. Here, we have used one- and two-color time-lapse analyses to investigate microtubules and Num1 interactions during spindle movements. We observed that the plus end of a single cytoplasmic microtubule can stably attach to a Num1 patch at the cortex. Speckle analysis showed that microtubules can elongate at the plus end while remaining attached to Num1. To investigate components mediating microtubule attachment at Num1 patches, we used a split-ubiquitin two-hybrid assay to test for interactions between Num1 and dynein pathway candidates. We found that Num1 interacts with dynein HC/Dyn1, dynein LIC/Dyn3, dynactin Arp1, and a recently identified dynein pathway candidate Y1049w (Ye et al., 2005, Mol Syst Biol 1: 2005.0026). These results revealed a novel role for Num1 patches in microtubule capture, which may be a prerequisite step for cortical sliding of microtubules along the cortex.

2084 How Is Cell Polarity Intrinsically Acquired in Bacteria?

C. Jacobs-Wagner, H. Lam, W. Schofield, G. Ebersbach; Molecular, Cellular & Developmental Biology, Yale University, New Haven, CT

Cell polarity is one of the most fundamental principles in biology. It is now abundantly clear that even primitive organisms such as bacteria exhibit cell polarity. How bacterial cell polarity is acquired, translated into polarized cell functions, and transmitted to progeny remains largely unknown. The bacterium Caulobacter crescentus provide a good model system to address these questions because in this organism, cell polarity is readily apparent by the presence of polar organelles and by the asymmetric cell division which produces two daughter cells of different size, morphology and fate. Cell polarity is also important for regulatory proteins that couple development to the cell cycle. We have recently identified a cell polarity determinant (TipN) that serves as a spatial cue from the preceding cell cycle to set up the correct polarity of C. crescentus. Our findings suggest that TipN specifies the site of the most recent division by identifying the new pole. In the absence of the landmark protein TipN, the cells often misorient their polarity axis, resulting in misplacement of polarity markers. In addition, the division apparatus is often mispolarized in the absence of TipN, yielding progeny cells of reversed size asymmetry. Ectopic localization of TipN along the lateral side of the cell creates new axes of polarity as manifested by cell branch formation and occupation of competent cell poles at the ectopic TipN sites. Evidence suggests that TipN acts upstream of the bacterial actin homolog MreB in regulating cell polarity.

2085 Spatial Regulation of Exocytosis by Rho GTPases

P. Brennwald, O. Roumanie, H. Wu; Department of Cell and Developmental Biology, University of North Carolina, Chapel Hill, NC

Rho3 and Cdc42 are members of the Rho family of small GTPases in yeast and both proteins play important but distinct roles in regulating polarized growth. We have suggested a model by which these two GTPases function in exocytosis by locally activating the Exocyst complex at sites of polarized growth. We have previously shown that Cdc42 is critical to exocytic function during early bud emergence, when Cdc42 itself is highly polarized. In contrast, Rho3 appears to continue to function in regulation of exocytosis throughout bud growth. Consistent with its distinct function in exocytosis, we find that the localization of Rho3 is quite distinct from that of Cdc42; utilizing a novel mAb against Rho3, we find that Rho3 localization appears as dispersed plasma membrane staining which is not concentrated at bud tips of small budded cells. In order to understand the molecular basis for the specific functions and localization of these proteins in polarized exocytosis, we created chimeric forms of Rho3 and Cdc42 by exchanging specific regions within each GTPase. Surprisingly, we find that exchange of the N-terminal domain of Cdc42 with that of Rho3 will convert Cdc42 into a protein which fully complements a rho3 deletion. Localization studies on the chimeric proteins suggest that the N-terminal domain of Rho3 is both necessary and sufficient for the Rho3-like staining pattern strongly suggesting that the specific localization of Rho3 is critical to its function in exocytosis. Recent genetic analyses from our laboratory strongly support the notion that Rho3/Cdc42 engagement results in “activation” of the Exocyst complex in a manner that is similar to other well-characterized Rho-effector interactions. This suggests that the exocytic machinery has evolved in such a way that its intrinsic activity is highly sensitive to regulation by external spatial cues such as those generated by Rho/Cdc42 GTPases.

2086 Asymmetric Cell Division of Drosophila Neuroblasts

C. Q. Doe; Institute of Neuroscience, HHMI/University of Oregon, Eugene, OR

Drosophila neuroblasts have become an attractive system to investigate the establishment of cell polarity, the mechanism of spindle orientation, and the process of asymmetric cell division. I will first summarize work from many labs on neuroblast cell polarity and asymmetric cell division, and then focus on recent work from our lab on one or more of the following topics: (1) microtubule-induced cortical polarity via the kinesin heavy chain 73 (KHC73) pathway; (2) the role of a NuMA-like protein in aligning the mitotic spindle with the axis of cortical polarity; or (3) asymmetric cortical proteins such as Brain tumor (Brat), Prospero (Pros) and newly identified proteins that regulate neuroblast self-renewal.
Chromatin-based Signals Control Cortical Polarity during Mammalian Oocyte Meliotic Divisions

M. Deng, S. Praveen, R. Li; Stowers Institute for Medical Research, Kansas City, MO

Cortical polarity is critical for many developmental processes, such as directional cell migration and asymmetric cell division. Although in most systems, extra-cellular signals provide the cue for the establishment of cortical polarity, in mouse oocytes undergoing asymmetric meiotic divisions, the cue appears to come from the maternal chromosomes. Symmetry breaking occurs through migration of the chromosomes from the cell center to a subcortical region where significant cortical reassortments in preparation for polar body extrusion. We report here that the chromatin cues induce assembly of a cortical domain that is enriched in actin and Par3/aPKC polarity complex and surrounded by an actomyosin contractile ring during the metaphases of meiotic divisions. Remarkably, the chromatin-induced cortical fission can be fully reconstituted by cortical injection of DNA-coated beads into metaphase II-arrested eggs. The DNA beads induce polar actomyosin structure is dependent on the proximity of the beads to the cortex and number of beads injected. Microtubules are neither necessary nor sufficient for the DNA bead-induced cortical assembly. Instead, a Ran GTPase dependent pathway is required for the chromatin-cortex communication, as both the dominant negative and constitutively active Ran blocked the DNA-induced cortical response. Interestingly, myosin-II appears to be a critical effector of this signaling pathway, since blocking myosin-II assembly prevented the accumulation of actin filaments and Par3 at the polar cortex. Inhibition of myosin-II contractility, on the other hand, did not affect establishment of cortical polarity but prevented contractile ring formation. These results suggest that cortical polarity during mammalian oocyte maturation is governed by a unique chromatin-based signaling pathway that directly controls actomyosin cytoskeleton organization at the plasma membrane.

Myosin 19 (Myo19) Is a Novel Unconventional Myosin Involved in Mitochondrial Movements

O. A. Quinteiro,1 M. B. Kortan,1 K. M. Denefy,1 L. B. Case,1 A. J. Lier,2 R. E. Cheney2; Biology Department, Franklin and Marshall College, Lancaster, PA, 1Department of Cell and Molecular Physiology, University of North Carolina-Chapel Hill, Chapel Hill, NC

Mitochondria are central to many cellular functions. Though alterations in mitochondrial function have been implicated in a number diseases, little is known about the mechanisms by which vertebrate mitochondria are properly localized. Previous studies have shown that mitochondria move on both actin and microtubules in neuronal cells (R. Morris and PJ Hellenbeck, JCB, 1995), thus, until recently only microtubule motors have been identified in association with vertebrate mitochondria. We have identified a novel myosin, myosin 19 (Myo19), that we hypothesize is involved in mitochondrial motility. Antibodies against human Myo19 detect a protein of ~110kD in multiple cell lines, and also colocalize with MitoTracker via immunohistochemistry. Amino acids 866-912 are necessary but insufficient for mitochondrial localization, as GFP constructs that contain this region localize to mitochondria, but a GFP construct of this region alone fails to label mitochondria. Placement of the GFP tag at the c-terminus of the Myo19 tail construct fails to block localization to mitochondria, suggesting that Myo19 is not a tail-anchored mitochondrial outer membrane protein. However, FRAP suggests that Myo19 is tightly associated with mitochondria, as GFP-Myo19 constructs require several minutes to recover. In A549 cells, overexpression of full length GFP-Myo19 leads to a striking phenotype, resulting in cells where the majority of mitochondria are moving at velocities of approximately 80 μm/s, and these mitochondria taper with the leading end of the organelle wider than the trailing end. These movements were insensitive to 15μM nocodazole, but in the presence of 1μM cytochalasin D, the movements ceased, and the mitochondria lost their "tailpole" shape. In unindifferentiated CAD cells overexpressing GFP-Myo19, mitochondria could also be seen to move in the actin-rich lamella. These data suggest that Myo19 is an unconventional myosin involved in mitochondrial movements. Supported by a SRE postdoctoral training grant NIH/GM06078 to OAQ, and NIH/NIDCD DC03299 to REC.

Function of the C1 Inserted Isoform of Nonmuscle Myosin II-C in Tumor Cell Lines and Mice

S. S. Jana, R. S. Adelstein; Laboratory of Molecular Cardiology, National Institutes of Health, Bethesda, MD

Nonmuscle myosins IIIs play an important role during cytokinesis, cell migration and in establishing cellular polarity. Three isoforms of nonmuscle myosin heavy chain II (NMHC) have been identified in vertebrates, NMHC II-A, II-B and II-C. We report here on the role of an alternatively-spliced isoform, NMHC II-C1, which includes an α amino acid insertion in loop1 of NMHC II-C. We recently reported that expression of this isoform is markedly increased in a number of tumor cell lines and that inhibiting expression leads to a delay in cytokinesis, resulting in a decrease in cell proliferation in the A549 human lung tumor cell line (Jana et al. J. Biol. Chem. 2006 PMID: 16790446). We now report on a second tumor cell line showing increased expression of NMHC II-C1, the human breast cell line MCF-7. Unlike A549 cells which are adhesive to the underlying matrix, MCF-7 cells retain a rounded shape during cytokinesis and appear to be loosely attached to the surface. Decreasing NMHC II-C1 expression in MCF-7 cells leads to marked prolongation in a late stage of cytokinesis, from 3.5 to 5.5 h for control siRNA treated cells to 15-17 h for the NMHC II-C1 depleted cells, similar to our findings with A549 cells. This prolongation results in a 6-8 fold decrease in cell number at 120 h. Although A549 and MCF-7 cells differ morphologically during cytokinesis, in both cases inhibition of NMHC II-C1 leads to a delay in cell division. For in vivo studies of nonmuscle myosin II-C1 function we generated hypomorphic knockout mice using homologous recombination. These mice expressed decreased amounts of myosin II-C1 all of which lacked the C1-insert. Some of these mice show evidence for a cardiomyopathy as determined by echocardiography and histopathologic analysis. Of note is that expression of NMHC II-C1 in the heart is restricted to the embryonic stage.

Molecular and Functional Analysis on the Role of Myosin VI in Exocytic Membrane Trafficking Pathways

J. Au, C. Thut, D. Abraham, N. D. Ardell, J. Kendrich-Jones, F. Buss; MRC Laboratory of Molecular Biology, Cambridge, United Kingdom, 1Cambridge Institute of Medical Research, University of Cambridge, Cambridge, United Kingdom

The actin cytoskeleton and myosin motor proteins are essential for the efficient functioning of vesicular traffic and membrane dynamics. Myosin VI, the focus of our research, is a unique motor that moves backwards towards the minus end of actin filaments. We have localised myosin VI in a variety of different intracellular compartments including the Golgi complex and vesicles concentrating in the perinuclear area on the trans side of the Golgi. Our functional studies using siRNA knock down approaches, over expression of dominant negative myosin VI deletion constructs as well as stable cell lines derived from the myosin VI knock out mouse (Snell’s waltzer mouse) have established that myosin VI plays an important role in exocytic membrane trafficking pathways. In Snell’s waltzer fibroblasts myosin VI is required for efficient secretion of alkaline phosphatase (SEAP) to the plasma membrane and for the maintenance of Golgi morphology. In these non-polarised cells myosin VI is linked to the Golgi complex and to the secretory pathway by binding to optimin, which also binds the small GTPase Rab8. Myosin VI and Rab8 colocalise around the Golgi complex and in vesicles at the plasma membrane. Overexpression of constitutively active Rab8Q67L recruits myosin VI onto Rab8 positive structures. Therefore since we have shown that myosin VI in non-polarised cells is involved in the secretory pathway and is linked via optimin to Rab8, which is known to regulate basolateral transport in polarised epithelial cells, we are currently investigating the role of myosin VI in the sorting and delivery of newly synthesised proteins to the apical and basolateral domains in polarised MDCK cells.

A α-helical Domain of Melanophilin Is Essential for Myosin Va Recruitment and Melanosome Transport in Melanocytes

A. N. Hume,1 A. K. Taraldter,3 J. S. Ramalho,2 E. V. Sviderskaya,1 M. C. Seabra; 1Molecular and Cellular Medicine, Imperial College London, London, United Kingdom, 2Centre of Ophthalmology, University of Coimbra, Coimbra, Portugal, 3Department of Basic Medical Sciences, St. George’s, University of London, London, United Kingdom

Melanophilin (Mlp) regulates retention of melanosomes at the peripheral actin cytoskeleton of melanocytes, a process essential for normal mammalian pigmentation. Mlp has been proposed to be a modular protein the binding melanosome-associated protein Rab27a, Myosin Va (MyoVa), actin and microtubule end-binding protein (EB1), via distinct N-terminal Rab27a binding domain. The α-helical region together with R27BD and EFBD regions of Mlp play an important role during cytokinesis, cell migration and in establishing cell polarity. Three isoforms of nonmuscle myosin heavy chain II (NMHC) have been identified in vertebrates, NMHC II-A, II-B and II-C. Here we report on the role of an alternatively-spliced isoform, NMHC II-C1, which includes an α amino acid insertion in loop1 of NMHC II-C. We recently reported that expression of this isoform is markedly increased in a number of tumor cell lines and that inhibiting expression leads to a delay in cytokinesis, resulting in a decrease in cell proliferation in the A549 human lung tumor cell line (Jana et al. J. Biol. Chem. 2006 PMID: 16790446). We now report on a second tumor cell line showing increased expression of NMHC II-C1, the human breast cell line MCF-7. Unlike A549 cells which are adhesive to the underlying matrix, MCF-7 cells retain a rounded shape during cytokinesis and appear to be loosely attached to the surface. Decreasing NMHC II-C1 expression in MCF-7 cells leads to marked prolongation in a late stage of cytokinesis, from 3.5 to 5.5 h for control siRNA treated cells to 15-17 h for the NMHC II-C1 depleted cells, similar to our findings with A549 cells. This prolongation results in a 6-8 fold decrease in cell number at 120 h. Although A549 and MCF-7 cells differ morphologically during cytokinesis, in both cases inhibition of NMHC II-C1 leads to a delay in cell division. For in vivo studies of nonmuscle myosin II-C1 function we generated hypomorphic knockout mice using homologous recombination. These mice expressed decreased amounts of myosin II-C1 all of which lacked the C1-insert. Some of these mice show evidence for a cardiomyopathy as determined by echocardiography and histopathologic analysis. Of note is that expression of NMHC II-C1 in the heart is restricted to the embryonic stage.

Localization of Myosin to the Cleavage Furrow of Drosophila S2 Cells in the Absence of Cortical Flow

R. Vale, M. Zuccollo, M. Bettencourt-Dias, G. Goshima, J. Spudich; Physiology Course 2006, Marine Biological Laboratory, Woods Hole, MA
The localization of myosin to the cleavage furrow establishes the contractile machinery for cell division. The most widely cited hypothesis for how myosin relocates during the metaphase-anaphase transition is that cortical flow drives myosin filaments from the poles to the equatorial region. Here, we have investigated the mechanism of myosin localization during cytokinesis by visualizing myosin (regulatory light chain fused to GFP) in *Drosophila* S2 cells spread on concanavalin A coated surfaces using total internal reflection microscopy. By this method, single myosin filaments on the cortex can be imaged and tracked with time. During interphase, prominent actin-mediated retrograde flow of myosin filaments is observed. However, when myosin is accumulating at the cleavage furrow during anaphase, we find that cortical myosin filaments are largely stationary and do not flow towards the equatorial region. Instead, the accumulation of myosin at the cleavage furrow results from two processes. First, cortical myosin filaments rapidly disappear from beneath the centrosomes as they separate during anaphase B, causing the depletion of myosin from the poles. This process does not require conventional asters, as centrosomal poles (created by centrosomin RNAi) separating during anaphase also caused the disappearance of myosin filaments, resulting in a normal timing of cytokinesis. A similar result was observed with 3-D sectioning of dividing S2 cells by spinning disk confocal microscopy. Second, after the depletion of myosin filaments at the poles had begun, new myosin filaments appeared at the equator, a process that was correlated with the appearance of stable microtubules at the equatorial cortex (observed by TIRF microscopy of Cherry-tubulin). Our observations suggest that high densities of dynamic microtubules cause the disassembly of myosin filaments, while low densities of dynamic microtubules coupled with the formation of stable microtubules promote the stabilization and potentially active transport of myosin filaments to the equatorial cortex.
An essential aspect of nervous system development is the extension of axons, which find their correct partners and form synapses, enabling neurons to communicate. Although the actin and microtubule cytoskeletons are crucial to nervous system development and stability, their roles in axon growth and maintenance are not fully understood. Another class of cytoskeletal filament-forming proteins, the septin family of GTP-binding proteins, is demonstrated to interact with both the actin and microtubule cytoskeletons. Furthermore, septins are implicated in neurodegenerative diseases, including Parkinson's and Alzheimer's diseases. Understanding how septins function in neurons is therefore critical to understanding axonal dynamics in the developing nervous system and in disease. We recently described a novel role for the only two worm septins, UNC-59 and UNC-61, in ventral cord motor neuron axon migration. To further define the role of septins in the nervous system, we are using primary cultures of C. elegans embryonic neurons as a system where cell biological analysis can be combined with the powerful genetics of this model system. Additionally, the simplicity of the worm septin system gives C. elegans a clear advantage over other animal systems for the dissection of basic, conserved septin functions in animals. We find that neurons cultured from septin mutant embryos are defective in extension of processes, and that they display abnormalities in both their actin and microtubule cytoskeletons, suggesting that septins may regulate neuronal cytoskeleton assembly. Using RNAi directed against each septin, we observe defects in process outgrowth and maintenance. Furthermore, a drug reported to affect septin organization causes a rapid, reversible loss of neuronal processes in cultured neurons, and results in uncoordinated locomotion of treated worms. We hypothesize that septins are involved in establishment and maintenance of the axonal cytoskeleton, which is necessary for axon outgrowth and stability.

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Inhibition of Integrin Signaling by Amino-Nogo
F. Hu, S. Siritrimitr; Neuroscience, Yale University School of Medicine, New Haven, CT

Nogo is a myelin-derived protein that limits axonal regeneration after CNS injury. A short hydrophilic Nogo-66 loop between two hydrophobic domains of Nogo binds to NgR to inhibit axon outgrowth. A second Nogo domain, termed Amino-Nogo, inhibits axon outgrowth and cell adhesion through unknown mechanisms. Here we present evidence indicating that Amino-Nogo inhibits integrin signaling. Adhesion of COS-7 cells to fibronectin but not laminin or collagen is inhibited by Amino-Nogo, suggesting that Amino-Nogo affects the function of a certain subset of integrins. Amino-Nogo inhibition can be partially overcome by antibodies that activate integrin β1. Furthermore, FAK activation in response to fibronectin dependent adhesion is reduced in the presence of Amino-Nogo. Analysis of responses of various cell lines to Amino-Nogo revealed that αvβ3, αvβ5 and αvβ integrins are inhibited by Amino-Nogo. The inhibition of neuronal outgrowth by Amino-Nogo is modulated by the ECM substrates, suggesting that Amino-Nogo inhibition of axon outgrowth also involves inhibition of integrin signaling. Amino-Nogo inhibition of both cell adhesion and axon outgrowth can be overcome by the addition of Mn2+, a known activator of integrins. All these data indicate that perturbation of integrin signaling is a crucial aspect of Amino-Nogo inhibition.

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TGFβ Signaling Suppresses Axon Growth Ability in Sensory Neurons
Y. Chen,1 K. Liu,2 G. Yiu,1 H. Hasegawa,3 A. K. Wagner,3 S. Kaneko,1 K. Kuwako,1 M. Kaneko,1 Y. Zhang,5 F. Wang,2 Z. He,1 Division of Neuroscience, Children's Hospital, Harvard Medical School, Boston, MA, 2Department of Cell Biology, Duke University Medical Center, Durham, NC, 3Laboratory of Cellular and Molecular Biology, Center for Cancer Research, NCI, National Institutes of Health, Bethesda, MD

The failure of injured axons to regenerate in the adult central nervous system (CNS) has been partly attributed to the diminished ability of mature axons to regrow after injury. The mechanisms responsible for this decline in intrinsic regenerative capacity over the course of development, however, remain largely unknown. In this study, we find that dorsal root ganglion (DRG) sensory neurons exhibit a gradual age-dependent decrease in axonal elongation ability after their peripheral branches have established contacts with targets such as the skin. We further show that activation of FAK that are expressed in skin tissues could reduce axon elongation ability in both embryonic and adult DRG neurons through a canonical Smad signaling pathway. Interruption of the retrograde transport of target-derived signals by a signal from peripheral axons, which can promote axonal regeneration in axotomized neurons, also inactivates this pathway in the adult. Furthermore, local administration of a TGFβ inhibitor increases the axonal growth ability of the adult DRG neurons. Thus, our results suggest that the age-dependent loss of intrinsic axonal growth capacity is at least in part triggered by target-derived activin/TGFβ signaling, pointing to a novel direction for designing therapeutic strategies to promote axonal regeneration after neural injury.

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Deriving Insulin-producing Cells from the Embryonic Progenitor for Treatment of Type 1 Diabetes
G. Li,1 R. Luo,1 F. Xie,1 J. Zhang,1 O. L. Kon,2 M. Salto-Tellez,2 S. K. Lim,3 1National University Medical Institutes, National University of Singapore, NUS, Singapore, 2Division of Medical Sciences, National Cancer Centre, Singapore, Singapore, 3Dept of Pathology, National University of Singapore, NUS, Singapore, 4Stem Cell Biology, Genomic Institute of Singapore, Singapore, Singapore

Limited supply of cadaveric pancreata has restricted the therapeutic application of transplantation of human pancreatic islets for treatment of type 1 diabetes. Therefore, alternative sources and concomitantly the LacZ reporter gene. Neural-derived, LacZ-positive cells expressed mammary-specific functions including milk protein synthesis in secretory acini. Some adopted epithelial outgrowths suggests the following scenario: mammary epithelial cells recombine to assemble growth-competent cellular microenvironments, chimeras are formed which include neural stem cells; development proceeds. The neural cells not only contribute significantly to mammary growth but also facilitate during pregnancy activating the WAP-Cre gene and concomitantly the LacZ reporter gene. Neural-derived, LacZ-positive cells expressed mammary-specific functions including milk protein synthesis in secretory acini. Some adopted

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MicroRNA-mediated Control of Stem Cell Specification and Function in Arabidopsis thaliana
M. Barton; Carnegie Institution of Washington, Stanford, CA

Land plants rely heavily on stem cells for the generation of new leaf, stem and root segments. The stem cells are principally found within apical meristems located at the tips of roots and shoots. In the shoot apical meristem, stem cell descendants are pushed outward, where they participate in leaf primordium formation, or downward, where they participate in stem formation. The PINHEAD gene is required for efficient meristem formation and, when its expression is artificially shifted within the shoot apex, can cause the formation of extra, ectopic meristems. PINHEAD is a member of the ARBOGATA family of genes and therefore acts to mediate microRNA-directed regulation of target RNAs. Enhancers of the pinhead mutant phenotype have profound effects on meristem formation but these effects vary. Some enhancers cause scores of new meristems to develop from the stem surface while others cause the main shoot apical meristem to become greatly enlarged. The molecular identification of these enhancers and studies of their effects on targeted RNAs will be discussed.

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The Mammary Stem Cell Niche Modifies Neural Stem Cell Repertoire
C. A. Boulanger,1 A. Androutsellis-Theotokis,2 B. W. Booth,1 D. L. Mack,1 R. McKay,2 G. H. Smith,1 NCI, NIH, Bethesda, MD, 2NINDS, NIH, Bethesda, MD

Evidence for somatic stem cell niches has been clearly defined in several biological systems. To determine if stem cells from other organs would conform to signals from the mouse mammary gland environment, we mixed wild type mammary epithelial cells with bona fide neural stem cells isolated from a WAP-Cre/R26R embryo at a 1:1 ratio and inoculated them into the cleared inguinal fat pads of immune-compromised Nu/Nu female hosts. The host mice were bred 6-8 weeks later and examined 20-30 days post inoculation. This approach allowed for growth of mammary tissue, transient activation of the WAP-Cre gene, recombination and constitutive expression of LacZ from the Rosa 26 promoter. Whole mount analysis was done on the inguinal glands from the injected mice. Neural-derived cells were LacZ-positive in chimeric mammary glands and contributed robustly to the outgrowth. No LacZ-positive cells were observed in epithelial outgrowths collected from non-breeding hosts indicating that pregnancy was essential for activation. The presence of neural-derived cells in significant numbers throughout mammary glands from the injected mice. Neural-derived cells were LacZ-positive in chimeric mammary glands and contributed robustly to the outgrowth. No LacZ-positive cells were observed in
myoepithelial cell fates. We conclude that neural stem cells capable of self-renewal and multiple cell fates, enter mammary epithelium-specific niches and adopt the function of similarly endowed mammary cells. These results imply the tissue-dominance of stem cell niches.

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A Role for esgarcot in Stem Cell Niche Maintenance
J. Voog, 1 G. Hime, 2 M. Rocha, 1 M. Boyle, 1 M. T. Fuller, 1 L. Jones; 1 Laboratory of Genetics, The Salk Institute for Biological Studies, La Jolla, CA, 2 Department of Anatomy and Cell Biology/ARC Centre for Excellence in Biotechnology and Development, University of Queensland, Melbourne, Australia, 3 Departments of Developmental Biology and Genetics, Stanford University School of Medicine, Stanford, CA

Upon cell division, adult stem cells can generate both daughter stem cells (self-renewal) and cells that differentiate along a specific lineage. Removal of stem cells from their regulatory microenvironment, or niche, often results in loss of the capacity to self-renew, suggesting that niches play a major role in regulating stem cell fate. The somatic apical hub at the tip of the Drosophila testis is a primary component of the male germ-line stem cell niche. Hub cells secrete the ligand Unpaired (Upd), which activates the JAK-STAT pathway in adjacent germine stem cells (GSCs) to specify stem cell self-renewal. We have characterized an allele of the Snail family transcriptional repressor esgarcot called shutoff (sho). In adult esgarcot males we observe a loss of multiple hub cell markers, GSCs, and somatic cyst progenitor cells (PCPs). Loss of expression of upd and shothor (sho), the gene that encodes DE-cadherin, was observed in esgarcot testes from larval (L3) males. These findings suggest that hub cells may be lost and niche function compromised during development in esgarcot males. Previous work has demonstrated DE-cadherin expression is necessary for stem cell maintenance and anchorage to niche support cells. We hypothesize that stem cell loss in esgarcot males is due to loss of DE-cadherin expression between hub cells and between hub cells and stem cells. Germ-line stem cell clones homozygous mutant for sho were not capable of long-term self-renewal, supporting the thought that DE-cadherin expression is necessary for stem cell maintenance. In Drosophila, esgarcot has been shown to act genetically upstream of sho in the trachea. Mammalian Snail family members have also been shown to directly regulate E-cadherin expression. We then hypothesize that esg regulates sho expression within hub cells. This work suggests a genetic program exists which regulates maintenance of the Drosophila germ-line stem cell niche.

2106

Live Cell Imaging of Interactions between Hematopoietic Progenitor Cells and Osteoblastic Cells
J. M. Gillette, 1 F. Pruefer, 2 J. Zhou, 2 J. Lippincott-Schwartz; 1 Laboratory of Genetics, The Salk Institute for Biological Studies, La Jolla, CA

Hematopoietic stem cells localize to a complex niche within the bone marrow where they interact specifically with the bone forming osteoblasts. Despite knowledge of the importance of osteoblast contact for hematopoietic stem cell maintenance, how hematopoietic progenitor cells find osteoblasts within this microenvironment and what role the ensuing interaction between these cells plays in hematopoietic stem cell renewal and differentiation is not well understood. Here, we investigate the cellular and molecular interactions that occur between hematopoietic progenitor and osteoblastic cells using live-cell imaging techniques. KG1a and primary CD34+ hematopoietic progenitor cells were used in co-cultured experiments with osteoblastic cells. Through microscopy techniques, we observed a dynamic, amoeboid motility by the progenitor cells that was directed to the osteoblastic cells. The progenitor cells formed a specialized uropod domain that was enriched in lipid raft molecules (GPI-GFP and the cholera toxin B subunit) and served as the contact point between the progenitor and osteoblastic cells. Additionally, stem cell markers CD34 and CD133 were found to coalesce at the uropod contact site along with quantum dot nanocrystals. The quantum dots localized specifically at the cell surface of the progenitor cell uropod and could be disrupted by cholesterol sequestration. Remarkably, the quantum dot loaded uropod appeared to transfer and be taken up by the osteoblasts and preliminary interaction and may implicate a potential signaling mechanism mediated by the release and/or up-take of specific proteins within the hematopoietic/osteoblastic niche. Future studies will be directed at determining the mechanism of this molecular transfer and the potential downstream functions of this cell-cell interaction.

2107

Transcriptional Regulation of Cxcl12 and Stem Cell Engraftment during Wound Repair
K. A. Mace, N. Boudreau, D. M. Young; University of California San Francisco, San Francisco, CA

Adult stem cell migration, engrafment and differentiation in response to injury, is a tightly regulated process, although the mechanisms controlling the role of stem cells in wound repair and regeneration are largely unknown. Previously we have demonstrated that the HOXA3 transcription factor functions during wound repair to promote cell migration and angiogenesis, in part by regulating genes such as urokinase plasminogen activator receptor (upAR), which mediates extra cellular matrix remodeling. Microarray analysis of genes expressed during HOXA3-mediated wound repair revealed that Cxcl12 is also regulated by HOX A3. Cxcl12 is a cytokine that mediates incorporation of bone marrow derived cells (BMDCs) into injured tissue. In particular, Cxcl12 has been shown to recruit endothelial cell precursors to sites of angiogenesis. Our preliminary data suggests that HOX A3 function may enhance BMDC incorporation into injured tissue. Analysis of GFP bone marrow chimeras will be presented to evaluate the role of HOX A3 in the wound microenvironment and its impact on recruitment and engrafment of circulating BMDCs.

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The EphB4 Receptor Tyrosine Kinase as an Inhibitor of Breast Cancer Cell Tumorigenicity
N. K. Noren, G. Foss, C. A. Hauser, E. B. Pasquale; Burnham Institute for Medical Research, La Jolla, CA

Recent evidence suggests that EphB receptor tyrosine kinases have a tumor suppressor role in colorectal and prostate cancer. This is an activity that is unlike other families of receptor tyrosine kinases, which generally promote tumorigenesis. Here we report that the EphB4 receptor can act as a tumor suppressor in breast cancer when activated by its ligand, ephrin-B2. Activation of EphB4 in vivo by soluble ephrin-B2 Fc inhibits the growth of tumors in a mouse xenograft model of breast cancer. Furthermore, we have identified a novel Eph receptor signaling pathway involving the Abl family of tyrosine kinases. Through Abl-mediated phosphorylation and inactivation of the adaptor protein Crk, ligand-activated EphB4 inhibits cell viability and growth as well as cell migration and invasion. Additionally, EphB4 downregulates expression of the pro-invasive matrix metalloprotease MMP-2, which is a metalloprotease that has been correlated with a poor breast cancer prognosis. Consistent with these effects, in non-transformed mammary epithelial cells EphB4-ephrin-B2 interaction maintains high Abl activity and Crk phosphorylation. In these cells, EphB4 and ephrin-B2 are localized to cell-cell junctions and disruption of their interaction disturbs EphB4 and ephrin-B2 localization and the integrity of cell-cell junctions. Furthermore, reduced EphB4 signaling induces some characteristics of cell transformation. These data suggest that the EphB4-Abl-Crk pathway promotes an epithelial phenotype in normal cells and suppresses tumorigenesis in breast cancer cells.

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Ezrin Promotes Src-dependent Functions Associated with Malignancy
L. Heisku, 1 F. Zhao, 1 I. Saotome, 2 A. I. McClatchey, 3 O. Carpen; 1 Department of Pathology, University of Helsinki, Helsinki, Finland, 2 MGH Cancer Center and Harvard Medical School of Pathology, Charlestown, MA, 3 Department of Pathology, University of Turku and Turku University Hospital, Turku, Finland

Ezrin, a linker molecule between plasma membrane and actin cytoskeleton, has been associated to tumorigenesis and metastatic processes with unknown mechanisms. It is implicated e.g. in PTEN and Rho signalling pathways. Ezrin is a known target for oncogenic Src kinase, and both proteins are elevated or activated particularly in advanced cancers and metastases. To elucidate ezrin’s role in oncogenesis, we employed embryonic fibroblasts (MEFs) from the ezrin knock-out mouse to take advantage of a model without endogenous ezrin expression. To dissect Src-dependent functions, constitutively active Src was expressed. While the introduction of wild-type ezrin to ezrin-/MEFs expressing active Src had no effect on cell behaviour in normal growth conditions, it promoted malignant phenotype in three-dimensional growth conditions, i.e. cell growth and invasion in Matrigel. In addition, wild-type ezrin promoted cell growth in suspension. Upon reversion of the Src target amino acid on ezrin was mutated to phenylalanine, expression of the mutant ezrin protein did not provide cells any selective advantage compared to the parent MEFs. Moreover, when the cells were grown inside Matrigel, wild-type ezrin was locally concentrated to patches or specific regions on the membrane, whereas mutated ezrin was diffusely distributed. The data indicate that Src-dependent ezrin phosphorylation has a specific role in three-dimensional environment which is possibly linked to tumorigenesis and metastasis formation.
Activation of Hepatocyte Growth Factor/c-Met Signaling Contributes to Genomic Instability via Centrosome Hyperamplification and/or Changes in Mitotic Kinases

H. Nam,1,2 Y. Park,1,2 L. Min-woo,1,2 J. Lee1,2; 1Biochemistry and Molecular Biology, Ajou University School of Medicine, Suwon, Republic of Korea, 2Chronic Inflammatory Disease Research Center, Ajou Research Institute, Suwon, Republic of Korea

Genomic instability is accepted as one of the main pathways in human carcinogenesis. Since aberrant HGF/c-Met signaling has been known to cause human cancers, we have examined whether activation of HGF/c-Met signaling causes genomic instability. In order to address this question, we have established cells with constitutive HGF/c-Met signaling by transfection with M1268T mutant of Met. Indeed, in both stably and transiently M1268T expressing cells, increase in the cell population showing too few or too many chromosomes was observed. In addition, centrosome hyperamplification and increase in multinucleated cells were observed, evidencing positive effect of HGF/c-Met signaling in causing genomic instability. Among the possible down-stream signaling molecules, phospho-Erk and phospho-Akt levels were elevated in M1268T expressing cells compared to mock-transfected cells. LY294002, a PI3K inhibitor, but not U0126, an MEK inhibitor, could abolish both centrosome hyperamplification and multinucleated cell generation. Moreover, transfection with either dominant-negative Akt mutant or pTEN clearly inhibited both phenotypes whereas those were increased by WT-Akt overexpression, suggesting that activation of PI3K-Akt axis is both necessary and sufficient. Therefore, it is demonstrated that constitutive HGF/c-Met signaling induces the centrosome hyperamplification and the increase of aneuploidy cells via PI3K pathway. Interestingly, constitutive activation of HGF/c-Met signaling also resulted in the changes in expression and/or activity of some mitotic kinases. In M1268T expressing cells, the amount and activity of Aurora-A kinase increased while activity of Aurora-B kinase decreased. The changes in these kinases might induce genomic instability through aberrant mitosis via centrosome hyperamplification and/or obliteration of check point activity based on previous reports. In conclusion, abnormal HGF/c-Met signaling induces genomic instability through means of centrosome hyperamplification via PI3K pathway as well as changes in expression and activity of Aurora-A and Aurora-B kinases.

Identification of Statmin as a Novel Substrate of Aurora-A

J. D. Wu; Division of Cancer Research, National Health Research Institutes, Taipei, Taiwan

Identification of the downstream substrate of a protein kinase is an essential step to provide better understanding of its uncharted functions. We have previously identified several potential substrates for Aurora-A protein kinase via small pool screening. By using alanine-scanning mutagenesis of three peptides substrates of Aurora-A, we demonstrate that [K/R]-X-[S/T]-[I/L/V] are the substrate specificity determinants for Aurora-A, similar to PKA. This finding raises the possibility that Aurora-A and PKA might phosphorylate a subset of common mitotic targets. Moreover, phosphorylation sites of centrosome proteins were identified at two different criteria, overexpression in cancer cells and location in centrosome and mitotic spindle, were used to prioritize the potential targets, resulting in the identification of statmin as one of the potential substrates of Aurora-A. In vitro kinase assay reveal that both Aurora-A and PKA phosphorylate statmin at the same sites. Overexpression or knockdown Aurora-A affects the phosphorylation status of statmin-Ser13 at mitotic phase. Finally, Aurora-A might cooperate with Statmin to against Taxol-induced alpha-tubulin polymerization. Together, this systematic search for the downstream substrates and substrate specificity determinants of Aurora-A protein kinase may provide novel insights toward the functionality study of Aurora-A.

The Ring Finger Domain of MDM2 Is Essential for MDM2 Mediated TGF-β Resistance

C. Kannemeier, R. Liao, P. Sun; Molecular Biology, The Scripps Research Institute, La Jolla, CA

TGF-β is a multifunctional cytokine that inhibits the proliferation of normal and early-stage tumor cells. TGF-β induced growth inhibition is frequently evaded in late-stage, metastatic tumors with increased production of TGF-β, resulting in enhanced malignancy due to the pro-metastatic functions of TGF-β. We have shown previously that increased expression of MDM2 is a potential genetic alteration that leads to resistance to TGF-β induced growth arrest in tumor cells. In the current study, we attempted to gain insights into the molecular mechanism underlying MDM2 mediated TGF-β resistance. Our results demonstrated that MDM2 rendered cells refractory to TGF-β by overcoming a TGF-β induced G1 cell cycle arrest. In addition, since the TGF-β resistant phenotype was reversible upon the removal of MDM2, MDM2 was likely to confer TGF-β resistance by directly targeting the cellular machinery involved in the growth inhibition by TGF-β, rather than by promoting secondary mutations that in turn cause the resistance. Investigation of the structure-function relationship of MDM2 revealed three elements that were essential for MDM2 to confer TGF-β resistance, the C-terminal half of the p53 binding domain, the nuclear localization of MDM2 and the zinc coordination residues involved in the E3 ubiquitin protein ligase activity of the RING finger domain. In contrast, the interaction of MDM2 with B5, p21/WAF1, p19/p14ARF and ribosomal protein L5 was not required for TGF-β resistance. The ability of MDM2 to bind to nucleotides and RNA was also dispensable. Furthermore, MDM2 mediated TGF-β resistance could be uncoupled with its ability to inactivate p53, suggesting that MDM2 might confer TGF-β resistance through a p53 independent mechanism.

Mdm2/p53 Interaction Studied Using a Novel Self-labeling Protein Tag

K. Muenter,1 S. Roesti,1 L. Gedge,1 A. Brecht,2 T. Gibbs1; 1Marketing, Covalys Biosciences AG, Witterswil, Switzerland, 2R and D, Covalys Biosciences AG, Witterswil, Switzerland

In living cells DNA damage causes an increase in p53 levels and stabilizes the protein which contributes to cessation of cell growth, senescence and apoptosis. Inactivation of p53 is a common feature of transformed cells leading to unregulated cell proliferation, thus it presents a pharmaceutical target of considerable interest. Mdm2 (p53 specific E3 ubiquitin ligase) is the chief cellular antagonist of p53. Monoubiquitination of p53 by mdm2 targets p53 for degradation by the proteasome. In its turn p53 activation also induces mdm2 expression. We present the use of a novel protein-tag, the SNAP-tag, to study p53/mdm2 localization and interactions. This protein tag labels itself covalently and stably with a wide range of benzyl guanine-derived substrates carrying fluorescent or affinity labels. Using this approach, SNAP-mdm2 could be labeled with a cell-permeable fluorescent substrate and was shown to localize to the nucleus. The ability to decouple expression of the fusion protein and labeling permits approaches such as pulse chase imaging to follow successive generations of the fusion protein population using a sequential labeling approach. Elucidation of interacting protein complexes allows candidate interacting proteins to be evaluated in downstream functional assays. Recombinant SNAP-p53 could be rapidly covalently coupled to benzyl guanine coated Sepharose resin in physiological conditions for use as a bait to pull down Mdm2. Having confirmed interaction by pull down, the p53/Mdm2 interaction could then be characterized further using a TR-FRET interaction assay. This self-labeling SNAP-tag approach permits a whole program of experiments using labeled p53 ranging from localization to interaction studies. This should facilitate identification of potential modulators of the p53/mdm2 interaction.

Cyclin-dependent Kinase p16/INK4A Rescue of Neoplastic Phenotype in Canine Mammary Tumor Cells

R. Bird, P. Delmonocete, P. Agarwall; Pathobiology, Auburn University, Auburn, AL

Spontaneous canine mammary cancer (CMC) is the most common malignancy of dogs and a highly homologous model of human breast cancer. We developed a well characterized panel of cell lines independently derived from neoplastic canine mammary carcinomas. Analysis of gene expression associated with neoplastic potential and loss of proliferative control revealed frequent defects in 2 tumor suppressor genes: p16/INK4A and p53. p16/INK4A expression is defective in 2 CMT cell lines (no mRNA detected by rt-PCR), with or without concomitant p53 defects, and abundant in a third CMT cell line. Phenotype rescue was attempted by transfecting human p16/INK4A cDNA, in expression constructs (3 Modiano), into CMT cell lines followed by

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G418/neo selection, single cell cloning by cell sorting into 96-well plates and p16/INK4A expression analysis. Transfected cell clones (3-5 per parental CMT cell line) were selected for analysis of characteristics typical of neoplastic transformation. Clones with reduced growth rates were recovered only from CMT cells lacking p16/INK4A. Cell cycle phase distribution was only slightly altered including some reduction in G1 phase fraction in p16/INK4A-transfected cells with reduced growth rates. Many of the clones with reduced growth rates also reacquired substrate-dependent growth as assayed on soft-agar plates. At least 1 CMT clone also became senescent/post-proliferative by ~25 passages post-transfection. Changes in morphology were also observed consistent with rescue of the transformed phenotype. This data suggests multiple mechanisms by which p16/INK4A expression influences exit/entry pathways and kinetic rate of the cell cycle, promotion of the differentiated phenotype and cellular senescence. Data suggests that each of these characteristics strongly influences neoplastic transformation and many characteristics can be rescued independently. Thus, p16/INK4A offers a promising therapeutic target that contributes to multiple pathways promoting the transformed and immortalized phenotype in this spontaneous model of breast cancer. (Supported by NIH & Scott-Ritchey Research Center)

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Comparative Analysis of In Situ DNA Binding Sites of p53 and p73 in Response to Hydroxyurea

V. Huang, Y. Kwon, Y. Jiang, X. Fu, Y. J. Wang; Division of Biological Sciences and Moores Cancer Center, University of California, San Diego, La Jolla, CA, 2School of Medicine, University of California, San Diego, La Jolla, CA

The p53 gene family regulates DNA repair, cell cycle checkpoints and apoptosis in response to stress signals. The three members of the p53-family - p53, p63 and p73 encode sequence-specific transcription factors which share significant identity in their DNA binding domains. However, phenotypic defects of the p53, p63 and p73 knockout mice suggest that they may regulate similar and distinct sets of genes. In this study, we have compared the promoter occupancy profiles of p53 and p73 in a human colorectal cancer cell line that does not express p53. The in situ binding sites of p53 and p73 were examined in response to hydroxyurea (HU) using a modified chromatin immunoprecipitation (ChIP) and oligonucleotide-based microarray analysis, or ChIP-DNA Selection and Ligation (DSL) technology. The ChIP-DSL technology has significantly improved the conventional ChIP-chip assay in terms of sensitivity and specificity. Analysis was performed under steady state conditions and after exposure to HU, which caused a three-fold increase in the levels of p53 and p73 protein. Of the ~2000 human promoters surveyed, we found 166 and 148 binding sites constitutively occupied by p53 and p73, respectively. ~50% of these binding sites are occupied by both p53 and p73. Our current results suggest that p53 and p73 constitutively co-occupy a large number of promoters in the absence of DNA damage. Following HU treatment, we found no significant change in the overall promoter occupancy profiles where ~80% of the binding sites remained bound by either p53 or p73. Functional studies by siRNA-mediated knockdown of p53 or p73 suggest that constitutive transcriptional activity of promoters co-occupied by p53 and p73 is regulated mostly by p53. Taken together, these findings support the mouse genetic studies to suggest that p53 and p73 have overlapping as well as non-redundant functions.

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Identification of a Novel p53 Target Gene Rad

B. Hsiao, T. Chang, F. Wang; Institute of Biochemistry and Molecular Biology, Yang-Ming University, Taipei City, Taiwan

The tumor suppressor p53 is a guardian of genome that elicits cell cycle arrest, apoptosis and DNA repair in stressed cells. The ability of p53 to prevent tumorigenesis is primarily mediated by many of its transcriptional target genes. To identify novel p53-regulated genes, we have established H1299 human lung cancer cells stably expressing a temperature-sensitive p53 gene. By conducting p53 ChIP DNA microarrays at permissive temperature, we have identified the Rad GTPase as a novel p53-activated gene. Rad belongs to the superfamily of Ras GTase and was first identified as a protein overexpressed in the muscle of type II diabetic patients. Expression of Rad has been reported to inhibit insulin-stimulated glucose uptake in cells and reduce plasma triglyceride levels in mice. It has been shown that Rad associates with Rho kinase ROCK and negatively influence Rho signaling. Here we show that ectopic expression of p53 increased Rad mRNA levels, elevation of Rad expression was also found in cells harboring wild type p53 exposed to DNA damaging agents, such as UV and mitomycin C. By transfection luciferase assay, we identified that nucleotides -2934 to -2904 relative to the translation start site of the Rad promoter conferred the responsiveness, this region was further shown to complex with p53 in the gel mobility shift assay. Chromatin immunoprecipitation assay (Chip) verified the binding of p53 to Rad promoter in cells overexpressing p53 or treated with adriamycin. Together our results clearly show that Rad is a direct transcriptional target of p53.

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SUMO-specific Protease SUSP4 Positively Regulates p53 by Promoting Self-Ubiquitination of Mdm2

M. H. Lee, Y. J. Jeon, J. E. Kwon, G. R. Kim, O. S. Bang, C. H. Chung; School of Biological Sciences, Seoul National University, Seoul, Republic of Korea

Tight regulation of the p53 protein level is essential for its activity in controlling cell growth, maintaining genome integrity, and responding to stresses. Mdm2 is an ubiquitin ligase that targets p53 for degradation by the proteasome. Here we isolate a new SUMO-specific protease SUSP4 that specifically removes SUMO-1 from Mdm2 and identified a novel cascade for the increase in p53 level by UV damage. Desumoylation of Mdm2 by SUSP4 has enhanced its self-ubiquitination and subsequent degradation by the proteasome, leading to stabilization of p53 and promotion of its transcriptional activity. Overexpression of SUSP4 caused cell growth inhibition, while knockdown of SUSP4 by RNA interference led to promotion of cell growth. Intriguingly, UV damage induced SUSP4 expression, leading to an increase in p53 level in parallel with a decrease in Mdm2 level. These findings establish a new mechanism for the elevation of cellular p53 level in response to UV damage.

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Nucleostemin, a Stem Cell-expressed Protein, Localizes with the TumorSuppressor Arf in the Nucleolus and Modulates p53 in the Nucleoplasm

H. Ma, T. Pederson; Biochemistry and Molecular Pharmacology, UMass Medical School, Worcester, MA

Nucleostemin (NS) is highly expressed in adult and embryo-derived stem cells, as well as in some tumor cell lines. NS is concentrated in nucleoli but dynamically exchanges with the nucleoplasm where it binds the tumor suppressor p53. Within the nucleolus, NS occupies regions distinct from those where nascent ribosomes are located (Pollitz et al., Mol. Biol. Cell 16, 3401-3410, 2005). In the present study, we found that NS is co-localized in the nucleolus with the tumor suppressor Arf. When Arf was over-expressed, the nucleolar level of NS decreased, suggesting that the two proteins may compete for common binding sites in the nucleolus. Additional results revealed that the nucleolar protein B23, already implicated in localizing Arf to the nucleolus (Korgoanaker et al., Mol. Cell. Biol. 25, 1258-1271, 2005), also plays a role in the nucleolar localization of NS. siRNA knockdown of NS resulted in an increase of p53 in the nucleoplasm and, conversely, when p53 was elevated following DNA damage, the level of NS decreased. These results suggest that NS has roles in controlling both Arf in the nucleolus and p53 in the nucleoplasm.

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Effects of GSK-3B Inhibitors on Stabilization of p53 and B-Catenin

S. R. Mudreddy, R. K. Gary; Chemistry, University of Nevada, Las Vegas, Las Vegas, NV

B-catenin is a bifunctional protein. It associates with cadherins as part of a membrane-cytoskeletal linkage in cell-cell adhesions, and it acts as a transcription factor for cell proliferation in the Wnt signaling pathway. Levels of B-catenin are controlled mainly by glycogen synthase kinase 3B (GSK-3B), a constitutively active kinase that suppresses B-catenin. There appears to be a regulatory feedback mechanism that connects GSK-3B activity and B-catenin to changes in the p53 tumor suppressor protein. Increases in B-catenin cause p53 to increase, which in turn inhibits the expression of the target gene Sestrin2 (Levina et al., Oncogene 24: 4444-4453). Here we report the use of BeS04 to investigate the relationships among these factors. In vitro, BeS04 was much more potent as a GSK-3B inhibitor than the classical inhibitor LiCl. Cells treated with BeS04 expressed increased levels of B-catenin, and showed morphological signs of defective cell-cell adhesions. BeS04-treated cells also exhibited increased levels of p53. Subcellular fractionation showed that most of the accumulated p53 was in the nucleus. Inhibition of GSK-3B causes stabilization of B-catenin by reducing the phosphorylated form of B-catenin, which is targeted for degradation. Likewise, inhibition of GSK-3B stabilizes p53 by reducing the phosphorylation of Mdm2, which needs to be phosphorylated to serve as an efficient E3 ubiquitin ligase for p53. However, these two outcomes of GSK-3B inhibition (elevated B-catenin and elevated p53) are potentially incompatible in the negative feedback model of B-catenin-p53 regulation. In BeS04-treated cells, we observed increased p53 in the absence of increased B-catenin, but never the converse, suggesting that the ability of p53 to suppress B-catenin may be dominant when both proteins are stabilized via GSK-3B inhibition. The p53 gene is frequently mutated in tumors. Such cells can be expected to lack an important check on B-catenin-mediated proliferation.
Investigating the Chromatin Regulatory Domain (CRD) of the INGI Tumor Suppressors

X. Han, K. Riabowol; Department of Biochemistry & Molecular Biology, Faculty of Medicine, University of Calgary, Calgary, AB, Canada.

Since the discovery of the INGI (Inhibitor of Growth) gene in 1996, five ING genes named ING1-5 and several different splicing forms of ING1 and ING2 have been identified. All the ING family members share a conserved plant homedomain zinc finger motif and a nuclear localization sequence. Numerous studies demonstrate that human ING proteins are involved in epigenetic regulation of gene expression through chromatin remodeling by physical interaction with protein complexes possessing histone acetyltransferase (HAT) or histone deacetylase (HDAC) activities. Through comparative sequence analyses, a novel conserved motif among all ING family members has been identified, and our hypothesis is that this unique region may play a critical role in binding HAT/HDAC complexes during chromatin remodeling and the subsequent regulation of gene expression. Our first aim was to determine whether the CRD defines a new protein motif and if it is ING-specific. By using the Basic Local Alignment Search Tool (BLAST), we got 140 matches of the query sequence, among which most were members of ING family in human and mouse, and their distant homologs in Xenopus and Drosophila melanogaster. In order to determine whether the CRD has any sequence similarity with those proteins already identified as chromatin remodeling proteins, multiple sequence alignments were performed and we found that the CRD was surprisingly unique to the ING protein family. Following the sequence searches, INGI constructs with various mutations within the CRD and multimers of this domain have been made and their interactions with HAT/HDAC complexes in cultured mammalian cells are currently being analyzed. As a conclusion, this study suggests a potential role of the CRD in ING-mediated chromatin remodeling and will help to clarify the biochemical mechanisms used by the class II tumor suppressor ING1 in proliferation, DNA repair, apoptosis, and senescence.

Loss of APC Induces Polyploidy Due to a Combination of Mitotic and Apoptotic Defects

D. Nikuradze, T. Scherthan, A. Boguski, K. Kitagawa; Molecular Pharmacology, St. Jude Children's Research Hospital, Memphis, TN, National Center for Microscopy and Imaging Research, Center for Research on Biological Structure, School of Medicine, University of California, San Diego, La Jolla, CA.

We report a novel type of mitotic cell death, caspase-independent mitotic cell death (CIMCD), which is modulated by BUB1. The spindle checkpoint prevents apoptosis by monitoring the kinetochore-microtubule attachment. When defects arise in the spindle checkpoint pathway and in the kinetochore-microtubule attachment, substantial aneuploidy results, and cell death follows. When MAD2-depleted cells had defects in the kinetochore-microtubule attachment, premature mitotic exit occurred and resulted in abnormal nuclei. Although substantial BUB1 depletion did not cause premature mitotic exit, substantial synthetic lethality occurred between BUB1 depletion and treatment with paclitaxel or 17-AAG. This finding raises the provoking question: Why does simultaneous BUB1 siRNA and paclitaxel or 17-AAG treatment cause substantial synthetic lethality, although the spindle checkpoint appeared to be active? We found that in BUB1 (partly, but not completely) deficient cells, the conditions that activate the spindle checkpoint, treatment with nocodazole, paclitaxel, or 17-AAG, or cold shock induced DNA fragmentation during early mitosis, which can explain the substantial synthetic lethality. CIMCD appeared to be independent of caspase activation or p53. Apoptosis-inducing factor (AIF) and endonuclease G (EndoG), which are effectors of the caspase-independent cell-death pathway, were released from mitochondria during the activation of CIMCD. Moreover, double depletion of EndoG and AIF abolished DNA fragmentation and suppressed the lethality. These results indicate that CIMCD is a type of programmed cell death. Many tumor cells have a diminished, but not absent, spindle checkpoint response. Further, we found that BUB1 expression levels in CIN (chromosome instability) tumors were lower than those in MIN (microsatellite instability) tumors, and that treatment with drugs (nocodazole, paclitaxel, or 17-AAG) induced CIMCD in CIN tumors but not in MIN tumors. Furthermore, ectopic expression of BUB1 suppresses CIMCD in these CIN tumors. These results suggest that CIMCD protects cells from aneuploidy by inducing the death of cells prone to substantial chromosome mis segregation.

Regulation of the Discs Large Tumour Suppressor Protein by Phosphorylation during the Cell Cycle

N. Narayan, P. Massimi, L. Banks; Tumour Virology, ICGEB, Trieste, Italy.

The Discs Large (Dlg) tumour suppressor protein along with Scribble (Scrib) and Lethal giant larva (Lgl), is known to link cell polarity and cell proliferation of epithelial cells in Drosophila. In higher eukaryotic cells less is known about its growth suppressive function, although Dlg has been shown to induce growth arrest and retard proliferation in mouse fibroblasts (Ishidate et al., 2000). A Dlg truncation mutant results in impaired morphogenesis and perinatal death during marine development (Caruana and Bernstein, 2001). Mammalian Dlg is targeted by viral oncoproteins such as the high-risk Human Papillomavirus (HPV) E6 proteins for proteasome-mediated degradation and is also sequestered by HTLV-1 Tax and Adenovirus E4 ORF1. It has been previously shown by our group that phosphorylation of Dlg is a very important post-translational mechanism of regulating the protein and that hyperphosphorylation of Dlg renders it more susceptible to degradation induced by the HPV E6 oncoprotein. Several kinases have been implicated in phosphorylation of Dlg, including the p38 MAPKs and JNK. We have now extended these studies to investigate post-translational modification of Dlg during different phases of the cell cycle. We show that Dlg is hyperphosphorylated during S phase as well as in M phase, either following Apgc6l2l5p release or Nocodazole arrest. Further, we demonstrate both in vitro and in vivo that Dlg associates with, and is phosphorylated by, the cyclinB-cdk1 complex during the M phase, and most likely by cyclinA-cdk2 during the S phase. The specificity of the kinase complex for Dlg was confirmed by treating cells with a specific inhibitor. Finally, site directed mutagenesis of the putative kinase consensus site that lies between PDZ regions 2 and 3 on Dlg also abolishes the M-phase phosphorylation of Dlg. In conclusion, we show a novel mechanism by which the Dlg tumour suppressor is regulated during normal cell proliferation in mammalian tissue.

Human DNA Topoisomerase III Alpha Serves as a Potential Anti-Cancer Block

M. Y. Hsieh, J. R. Fang, H. W. Chang, H. C. Chen, T. L. Shen, S. C. Feng, T. K. Li; 1Department and Graduate Institute of Microbiology, College of Medicine, National Taiwan University, Taipei, Taiwan. 2Department of Plan Pathology and Microbiology, National Taiwan University, Taipei, Taiwan.

Genomic instability, especially chromosome instability (CIN), and checkpoint abnormalities have been closely related to the initiation and/or progression of tumor development. Here, we showed that tumor cells are generally deficient in expression of DNA human topoisomerase III alpha (hTOP3alpha) suggesting a potential anti-tumorigenic activity for h TOP3α. Consistently, hTOP3α-deficient cell lines generated by RNA interference (siRNA-hTOP3α cells) induced/promoted tumor formation in SCID tumorigenesis model compared to parental cell lines. Conversely, tumor cells with overexpression of hTOP3α lost its ability to form tumor on SCID mice. Moreover, siRNA-hTOP3α cell lines exhibited elongated cellular morphology, less serum dependence, enriched stretch fibers, greater sensitivity to cell cycle drugs, CIN, enhanced ability in anchorage-independent growth on soft agar and greater invasiveness compared to control cell lines. Our results suggest a potential role of hTOP3α as a potential anti-cancer block functioning in maintaining genomic stability and possibly in cell cycle checkpoints. The fact that deletion of TOP3 in yeast resulted in checkpoint deficiency and hyperrecombination phenotype also support such a possibility.

Functions of ZNF217, a Gene Amplified during Neoplastic Progression

L. E. Littlepage, P. Yaswen, Z. Werb; 1Anatomy, University of California, San Francisco, San Francisco, CA, 2Lawrence Berkeley National Laboratory, Berkeley, CA.
The 20q13.1 region of the human genome is highly amplified in 20-30% of early stage human breast cancers, and this amplification correlates with poor prognosis. ZNF217, a candidate oncogene in 20q13.1, is a putative transcription factor that is a component of the co-repressor of transcription associated with the human histone deacetylase complex (CoREST-HDAC), as well as in a complex with the transcriptional co-repressor C-terminal binding protein (CBP). Overexpression of ZNF217 in human mammary epithelial cell lines leads to their immortalization. To investigate its effect on neoplastic progression in epithelial cells, we cloned mouse Zn217. We characterized the role of Znf217 as a putative transcription factor. Using Gal4-fusion constructs in transcription assays, we found that Zn217 is a strong transcriptional repressor. We then infected mouse epithelial MC2 cells with vector or mouse Zn217 in a retroviral vector. Using an antibody raised against human ZNF217 that we found cross-reacts with mouse Zn217, we found that the cells expressed the Zn217 protein when induced, as determined by western blot analysis. Cells overexpressing Zn217 had altered cell and nuclear morphology and showed increased motility in scratch assays. Taken together, our data suggest that repression of a transcriptional target by ZNF217 may lead to increased motility of epithelial cells in culture. Supported by Ruth L. Kirschstein National Research Service Award, American Cancer Society Postdoctoral Fellowship, and grant CA058207 from NCI.

2127 Interaction between the HERC1 Ubiquitin Ligase and the TSC1/TSC2 Complex

E. Casas-Terradellas, O. Hadjebi, F. Gaucin-Gonzalo, R. Bartoms, F. Ventura, J. Rosa; Ciencies Fisiològiques IL, IDIBELL-Universitat Barcelona, Hospitalitat, Spain

Tuberous Sclerosis complex is an autosomal dominant disease characterized by benign tumor formation (hamartoma) in various organs and tissues. Mutations in either of these two genes, TSC1 (or hamartin) and TSC2 (or tuberin), can cause this disease. These proteins have been shown to regulate cell growth through inhibition of the mTOR pathway. TSC1 stabilizes TSC2 forming a physical and functional complex which regulates to the Rheb small GTPase through its GAP activity. HERC1 is a giant protein containing an HECT domain in its carboxyl-terminal. Proteins containing HECT domains function as a subtype of E3 ubiquitin ligases participating in protein ubiquitination through the transfer of ubiquitin from E2 ubiquitin conjugating enzymes to specific substrates. Recently, HERC1 has been identified as a TSC2 interacting protein (Chong-Kopera et al., 2006). These initial studies, using C-terminal regions of HERC1 and overexpressed TSC1/TSC2 proteins, suggested that TSC1 stabilized TSC2 by excluding HERC1 from the TSC1/TSC2 complex. We have analyzed this interaction between endogenous proteins finding that HERC1 can interact with TSC1/TSC2 complex. This interaction is constitutive and it is not regulated by insulin or amino acids. Knock-down experiments of HERC1 seem to indicate that the stability of the TSC1/TSC2 complex is not affected. Interestingly, expression of truncation mutants of HERC1 regulated overexpressed TSC1 and TSC2 proteins. Altogether these data show the participation of HERC1 in a complex with TSC1/TSC2 and a possible role in its stability.

2128 Growth Stimulation of Prostate Cancer by Androgen Receptor Cofactor ARA70β

Y. Peng1, C. Li, F. Chen, Z. Wang, W. Gerald, L. Peng2; 1Pathology, New York University School of Medicine, New York, NY, 2Cancer Biology, The University of Texas, M. D. Anderson Cancer Center, Houston, TX

ARA70/ERl/ELE1/RFG was first identified as a gene fused to the ret oncogene in thyroid carcinoma and subsequently as an androgen receptor (AR)coactivator. Two isoforms of ARA70, full length 70 kDa ARA70α and internally spliced 35 kDa ARA70β have been identified. ARA70α can activate AR mediated transcription activation in a ligand dependent manner, possibly through its interaction with general transcription factors TFII B and pCAF. We have shown that ARA70α is decreased in prostate cancer and its overexpression inhibits prostate cancer growth in androgen dependent LNCaP cells. To elucidate the function and role of ARA70β in prostate cancer, we examined the regulatory effects of transcription, prostate cancer cell growth and invasion of ARA70β. In contrast to ARA70α function as a transcriptional coactivator of AR, ARA70β can function as an AR corepressor in androgen free condition in transient transfection assays. ARA70β promotes prostate cell growth in cell proliferation and anchorage independent growth assays. ARA70β also enhanced the ability of cell invasion in in vitro Matrigel assays. In concert to above findings, genome wide expression profiling by Affymetric DNA microarray revealed parallel changes in the upregulation of genes involved in proliferation and cell adhesion. Collectively, the above data indicates that ARA70β plays an important role in androgen dependent prostate cancer growth and invasion.

2129 Modeling Human Brain Cancer in Drosophila

R. D. Read, J. B. Thomas; Salk Institute for Biological Studies, La Jolla, CA

Gliomas, neoplasms of glial cells and their precursors, are among the most common and deadly malignant tumors of the central nervous system (CNS). These tumors diffusely infiltrate the brain and grow rapidly, properties that render them largely incurable. Formation of these tumors is a complex process involving accumulation of mutations in many genes, only some of which are known. In particular, constitutive activation of the EGFR-Ras and PI-3 kinase signaling pathways is a common feature in gliomas and is sufficient to cause glioma in mouse models. Yet, how these pathways specifically regulate glioma pathogenesis is unknown. To understand the molecular basis for this disease, we have developed a novel model in Drosophila for the purpose of carrying out large-scale genetic analyses to identify genes involved in glioma invasion and proliferation. The Drosophila CNS contains multiple glial cell types that are strikingly similar to their vertebrate counterparts in terms of function, development, and gene expression. We have developed techniques that target glia and glial precursors to allow spatial and temporal control of gene expression in the fly CNS with single cell resolution. Using these tools we found that co-activation of both the EGFR-Ras and PI-3 kinase pathways in Drosophila glia gives rise to proliferative, invasive cells that create tumor-like growths in the fly brain, mimicking the human disease. We are now performing a genetic screen for enhancers and suppressors of the EGFR-Ras-PI-3 kinase phenotype in order to identify new regulators of glial neoplasia. Furthermore, we are performing misexpression screens for additional loci that cause glioma-like phenotypes in the fly brain. The genes identified in these two screens represent excellent candidates for genes directly involved in pathogenesis.

2130 Identification of Transcripts with Altered Translational Efficiency in Human Mammary Epithelial Cells Overexpressing eIF4E

S. Li, O. Larsson, M. Peterson, S. Avdalov, P. Bitterman, V. Polansky; Pulmonary Medicine, University of Minnesota, Minneapolis, MN

Purpose: Ectopic over expression of eIF4E in human mammary epithelial cells (HMECs) harboring active telomerase (hTERT) conferred these cells with autonomy, fundamentally altering cell cycle control and apoptosis regulation. When eIF4E is aberrantly activated, it increased the translational efficiency of a subset of transcripts encoding regulators of the cell cycle and viability. Here we intend to examine the mechanism on a genome-wide scale. Methods: We combined translational efficiency estimates derived from polyribosome preparations of RNA - which stratifies RNA based on the number of bound ribosomes - with a quantitative abundance measure obtained from microarray analysis of the polyribosome associated mRNA. Briefly, HMEC/hTERT and HMEC/hTERT/eIF4E: cells were studied 18 hours after growth factor deprivation. After polyribosome fractionation, we combined those transcripts associated with the translational efficiency of transcripts governing key life cycle events.

2131 E-cadherin Promotes the Anti-proliferative Role of Caveolin-1 as Well as Down-regulation of Survivin

V. A. Torres, J. Tapia, A. Lladser, D. Rodriguez, L. Leyton, A. F. G. Quest; FONDAP Center for Molecular Studies of the Cell, ICBM, University of Chile, Santiago, Chile

Caveolin-1 is a lipid raft-enriched membrane protein that suppressor or promotes events associated with tumour progression and metastasis. These distinct roles are attributed to cell-specific variations; however, mechanistically, these differences are not understood. In HT29 colon cancer cells, caveolin-1 expression decreased survivin mRNA and protein levels via a transcriptional mechanism involving the b-catenin-Tcf/Lef pathway. Also, proliferation was reduced upon caveolin-1 expression. Surprisingly, this was not the case in metastatic HT29(US) cells. Survivin down-regulation was paralleled by co-immunoprecipitation and co-localisation of caveolin-1 and b-catenin from HT29, but not HT29(US) cells. Metastasis is often associated with loss of E-cadherin. Indeed, E-cadherin was expressed in HT29, but not HT29(US) cells and re-expression of E-cadherin in HT29(US) cells restored co-immunoprecipitation and co-localisation with b-catenin, as well as survivin down-regulation and inhibition of cell proliferation by caveolin-1. These findings provide a first mechanistic insight as to how caveolin-1 function may vary in a cell context dependent fashion and suggest that the absence of E-cadherin severely compromises caveolin-1 in this respect.
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Estrogen Mediates the Growth of Human Thyroid Carcinoma Cells via an Estrogen Receptor-ERK Pathway

Q. Zeng,1 G. G. Chen,1 A. C. Vlantis,1 C. A. van Hasselt3; 1Department of Surgery, R417, Cancer Centre, Hong Kong,2 Department of Surgery, Prince of Wales Hospital, Hong Kong There is strong evidence to show that thyroid tumors occur more frequently in females, suggesting a role of sex hormones in thyroid carcinogenesis. However, few studies have investigated the molecular mechanism responsible for this. Our previous study demonstrated that estrogen receptors (ERs) exist in thyroid papillary carcinoma (Kat5) cells. Our present study investigated how 7β-estradiol (E2) influences the proliferation of Kat5 cells. Two estrogen receptor agonists, a selective ERα agonist, PPT, and a selective ERβ agonist, DPN, were employed to selectively regulate the ER levels by MTT assay and DNA fragmentation ELISA. The response of ERK1/2 and Bcl-2 family members to E2 and ER agonists was examined by Western blot. We showed that E2 significantly promoted the proliferation of Kat5 cells. The proliferation-promoting effect of E2 was related positively to ERα but negatively to ERβ, as PPT enhanced cell proliferation whereas DPN inhibited cell proliferation. Furthermore, PPT increased Bcl-2 protein levels while DPN decreased them in Kat5 cells. DPN also elevated the expression of pro-apoptotic Bax protein. PPT markedly induced the level of phosphorylated ERK1/2, suggesting that ERK1/2 may be positively involved in the proliferation of cells induced by E2. We also demonstrated that estrogen was able to stimulate the proliferation of cancer thyroid cells and that ERα and ERβ expression had opposite effects on cell proliferation. The action of estrogen appears to be associated with an increase in Bcl-2 and a decrease in Bax levels in an ERα/ERK1/2-related pathway. Our data suggest that an imbalance between ERα and ERβ may contribute to the development of thyroid cancer.

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Structural Transitions That Regulate the Apoptotic Activity of BAX Are Dependent on Its C-terminal Alpha-9 Helix

N. Tscharmer,1 G. Zhang,1 A. Pande,1 S. Qin,1 L. L. Sechy,2 S. A. Tatulian,3 A. R. Khaled1; 1Biomolecular Science Center, University of Central Florida, Orlando, Florida, FL, 2Chemistry, University of Central Florida, Orlando, FL

A balance between processes that control life and death or homeostasis is essential for normal tissue development and the maintenance of cell population size. Activation of death proteins, such as those of the BCL-2 family, figure prominently in the homoeostatic process. Previously, we showed that loss of cytokine signaling perturbed the activation of the BCL-2 family of apoptotic mediators in lymphocytes, resulting in induction of the death activity of BAX. Hence BAX is a critical mediator of cytokine-withdrawal induced death; yet, how this lethal protein is activated is poorly understood. In the healthy cells, BAX is a cytosolic protein, with its C-terminal alpha-9 helix folded in a deep hydrophobic groove. Under apoptotic stress BAX translocates to mitochondria, causing membrane destabilization and the release of death-inducing factors. To explain this transitional behavior of BAX, we tested the hypothesis that the alpha-9 helix of BAX is an essential regulatory domain that governs its intracellular localization. Using a multi-disciplinary approach, we demonstrated that (1) the alpha-9 helix BAX is a membrane-binding domain that enables mitochondrialochondrial integration, (2) Two lysines located at the distal end of the alpha-9 helix are essential for membrane binding, and (3) Apoptotic stimuli induce a transient alkalinization that alters mitochondrial lipid dynamics, facilitating the mitochondrial integration of BAX. From these studies, we have shown that the C-terminal of BAX is responsive to apoptotic stimuli and, thereby, is an ideal molecular target for manipulation of the activity of BAX in a manner that has therapeutic application.

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Targeted Destruction of Prostate Cancer Cells with Genetically Altered Salmonella

H. Schatten,1 A. Eisenstadt,2 R. Kazmierzak,2 Z. Zhong,1 D. Newman,2 A. Fear; 1Veterinary Pathobiology, University of Missouri-Columbia, Columbia, Missouri, MO

Recently, genetically engineered non-toxic Salmonella Typhimurium strains have become attractive for cancer treatment because Salmonella preferentially infect and replicate within tumor cells and destroy solid tumors. We have engineered a Typhimurium strain (CRC1674) that is superior to previously engineered strains in the ability to attach, invade and alter cellular components in PC-3 prostate cancer cells. However, the mechanisms underlying the preferential tumor cell infection and destruction are not clear. Here we investigated the specific mechanisms that are used by the bacteria for cancer cell destruction with the goal to exploit these mechanisms for destruction of subpopulations in cancer tissue and increase therapeutic efficiency. We employed fluorescence and immunofluorescence microscopy as well as transmission electron microscopy (TEM) on S. typhimurium-infected PC-3M human prostate cancer cells fixed at 20 min, 4 hrs, and 8 hrs after inoculation. Rhodamine-phalloidin was employed to stain microfilaments, FITC-conjugated anti-tubulin antibody to stain microtubules and DAPI to stain DNA. Double and triple immunofluorescence staining was performed to determine the effects of Salmonella on the tumor cell's cytoskeleton. Our results show that the bacteria tightly attach and modify the infected PC-3M cell's microfilament system for incorporation into host cells. Ultrastructural analysis with TEM revealed Salmonella containing vacuoles (SCVs) that are formed within the host cells and connect with the host cells by tubular extensions between the vacuole and the host cell's cytoplasm. The rims of the vacuoles are stained with rhodamine-phalloidin indicating microfilament participation in the formation of the SCVs. Host cell mitochondria show deterioration and loss of cristae. These studies demonstrate that genetically modified S. typhimurium (strain CRC1674) [1] interact with PC-3 prostate cancer cells [2] and accumulate within the tumor cells in membrane-bounded SCVs. Further experiments are needed to determine the nature of the SCV-host cell interactions. Supported by Cancer Research Center.

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Essential Role of Death Domain Kinase RIP in DNA Damage-induced, p53 Independent Cell Death

G. Huh,1 M. Won,1 H. Byun,1 K. Park,1 Y. Kim,2 Z. Liu; 1Department of Pharmacology, Chungnam National University, College of Medicine, Daejeon, Republic of Korea, 2Cancer Research Institute, Daejeon, Republic of Korea

The hormonal stimulation of luteinizing hormone (LH) is an essential process in reproduction in starfish oocytes. Without fertilization, spontaneous inactivation of ERK and activation of caspase-3, which is the key enzyme to execute apoptosis, occur about 10 h after 1-MA stimulation. Also, immediately after the inactivation of MAPK, p38MAPK, which is generally considered as a death factor, is activated. When we treated eggs with p38MAPK inhibitor SB203580, apoptotic body formation was inhibited, but it did not affect activation of caspase-3. Thus, in starfish apoptosis, activation of p38MAPK and caspase-3 occur independently after ERK inactivation, and both of these cascades are needed for apoptotic body formation.

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Involvement of p38MAPK in Apoptotic Body Formation in Starfish Eggs

K. Usui,1 K. Chiba, Biology, Ochanomizu University, Tokyo, Japan

The hormonal stimulation of luteinizing hormone (LH) is an essential process in reproduction in starfish oocytes. Without fertilization, spontaneous inactivation of ERK and activation of caspase-3, which is the key enzyme to execute apoptosis, occur about 10 h after 1-MA stimulation. Also, immediately after the inactivation of MAPK, p38MAPK, which is generally considered as a death factor, is activated. When we treated eggs with p38MAPK inhibitor SB203580, apoptotic body formation was inhibited, but it did not affect activation of caspase-3. Thus, in starfish apoptosis, activation of p38MAPK and caspase-3 occur independently after ERK inactivation, and both of these cascades are needed for apoptotic body formation.

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Regulatory Role of Protein Kinase C on the Death Receptor Mediated Necrotic Cell Death

H. Byun,1 K. Park,1 M. Won,1 K. Yang,1 S. Shin,1 L. Paz,1 J. Kwak,1 J. Park,1 J. Seok,1 Z. Liu,1 G. Hur1; 1Department of Pharmacology, Chungnam National University, College of Medicine, Daejeon, Republic of Korea, 2Cell and Cellular Biology Branch, Center for Cancer Research, National Cancer Institute, NIH, Bethesda, MD, 3Cancer Research Institute, Daejeon, Republic of Korea

Protein kinase C (PKC) triggers cellular signals that regulate proliferation or death in a cell- and stimulus-specific manner. Although previous studies have demonstrated that activation of PKC with phorbol 12-myristate 13-acetate (PMA) protects cells from apoptosis induced by a number of mechanisms, including death receptor ligation, little is known about the effect or mechanism of PMA in the necrotic cell death. Here we demonstrate that PMA-mediated activation of PKC protects against TNF-induced necrosis by disrupting formation of the TNF receptor 1 (TNFR1) signaling complex. Pretreatment with PMA protected L929 cells from TNF-induced necrotic cell death in a PKC dependent manner, but did not protect against DNA damaging agents,
including adriamycin and campothecin. Analysis of the upstream signaling events affected by PMA showed that it markedly inhibited TNF-induced recruitment of TNFR1-associated death domain protein (TRADD) and receptor interacting protein (RIP) into TNFR1, subsequently inhibiting TNF-induced activation of NF-κB and JNK. However, JNK inhibitors did not significantly affect TNF-induced necrosis, suggesting that the inhibition of JNK activation by PMA is not part of anti-necrotic mechanism. In addition, PMA acted as an antagonist of TNF-induced reactive oxygen species (ROS) production, thereby suppressing activation of the ROS-mediated poly(ADP-ribose) polymerase (PARP), and thus inhibiting necrotic cell death. Furthermore, during TNF-induced necrosis, PARP was significantly activated in wild-type MEF cells but not in RIP-/- or TRAF2-/- MEF cells. Together, these results suggest that PKC activation ensures effective shutdown of the death receptor-mediated necrotic cell death pathway by modulating formation of the death receptor signaling complex. This work was supported by grant R01-2005-000-10240-0 from the Basic Research Program of the Korea Science & Engineering Foundation.

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Rottlerin Plays as a Functional Dichotomy between Apoptotic and Necrotic Modes in Response to Death Receptor Ligation by Uncoupling of Mitochondria

M. Won,1 H. Byun,2 K. Park,3 K. Yang,1 S. Shin,1 L. Piao,3 J. Park,3 J. Seok,1 G. Hur,2,3 1Department of Pharmacology, Chumgnam National University, College of Medicine, Daejeon, Republic of Korea, 2Cancer Research Institute, Daejeon, Republic of Korea, 3Department of Pathology, Chumgnam National University, College of Medicine, Daejeon, Republic of Korea.

Tumor necrosis factor (TNF)-induced cell death can be induced by apoptosis and necrosis. Our recent studies have shown that rottlerin, a potent and specific PKC-δ inhibitor, sensitizes cells from apoptosis induced by death receptor ligation, little is known about the effect or mechanisms in the necrotic cell death. To dissect the pathways of apoptosis and necrosis during death receptor ligandation in the same cell line, we used geldanamycin (GA)-induced apoptotic model in L929 murine fibroblasts, which is predominantly triggered by necrosis upon TNF treatment. Treatment with GA plus TNF caused a shift from necrosis to apoptosis characterized by caspase activation and plasma membrane blebbing, whereas TNF triggered necrosis in L929 cells. Surprisingly, whereas rottlerin sensitized apoptotic cell death induced by GA plus TNF, it protected necrotic cell death induced by TNF alone in a manner independent of its ability to inhibit PKC-δ. This protection against TNF-induced necrosis was achieved through suppressing the TNF-induced reactive oxygen species (ROS) production as well as the activation ROS-mediated poly(ADP-ribose) polymerase (PARP) leading to profound ATP depletion and subsequent necrosis. In understanding the underlying mechanisms, we found that rottlerin did not protect against H2O2-induced cell death. Furthermore, pretreatment with mitochondrial uncoupler FCCP or CCCP resulted in protection against TNF- but not H2O2-induced cell death and PARP activation. Therefore, the data from this study provide novel evidence that rottlerin regulates mitochondria-derived ROS production by uncoupling of mitochondria and thus inhibits necrotic modes of TNF-induced cell death. This work was supported by grant R01-2005-000-10240-0 from the Basic Research Program of the Korea Science & Engineering Foundation.

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Role of Protein Kinase C on the Nuclear Factor-κB signaling via Dissociation of IKK-γ and Hsp90 Complex in Human Colonic Epithelial Cells

K. Park,1 H. Byun,2 K. Yang,1 S. Shin,1 L. Piao,3 J. Kim,1 E. Junn,1 J. Park,3 J. Seok,1 G. Hur,2,1 1Department of Pharmacology, Chumgnam National University, College of Medicine, Daejeon, Republic of Korea, 2Department of Pathology, Chumgnam National University, College of Medicine, Daejeon, Republic of Korea, 3Department of Neurology, University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School, Piscataway, NJ, 4Cancer Research Institute, Daejeon, Republic of Korea.

Activation of protein kinase C (PKC) by phorbol 12-myristate 13-acetate (PMA) triggers cellular signals that lead to the activation of the transcription factor NF-kB. In various cell types. In addition to NF-κB activation by short-time PMA treatment, here we report that the prolonged exposure of human colonic cancer epithelial cells treated with PMA can also lead to a persistent inhibition of NF-κB activation. PMA selectively causes the degradation of IkB kinases (IKKs) including IKK-γ and IKK-β, and subsequent inhibition of TNF-induced IKK-γ and NF-κB activation in human colon cancer cell line HCT-116, but not in other gastrointestinal tract cells. The use of Ro31-8220 and GSK-6983, general PKC inhibitors as well as MG-132, a proteasome specific inhibitor, abrogated PMA-induced degradation of IKK-γ, and recovered the activation of IKK by TNF, suggesting that IKK complex is predominantly degraded by the proteasome pathway in PKC dependent manner. We also found that IKK-γ strongly associates with heat shock protein 90 (Hsp90) in HCT-116 cells, and that this interaction was dramatically reduced after following exposure to PMA. Furthermore, high levels of Hsp90 expression were observed in human colon cancer tissues. Taken together, these results suggest that long term activation of PKC by PMA inhibits NF-κB system in case of colon cancer cells by disrupting the interaction of IKK-γ with Hsp90 which may represent a novel regulatory mechanism of PKC-dependent cellular differentiation and limiting proliferation of colonic epithelial cells. This work was supported by grant R01-2005-000-10240-0 from the Basic Research Program of the Korea Science & Engineering Foundation.

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Mitochondrial DNA Mutations Modulate ER Stress Induced Apoptosis

J. Q. Kwong,1 A. A. Starkov,2 G. Manfredi,3 1Neuroscience, Weill Medical College of Cornell University, New York, NY, 2Neurology, Weill Medical College of Cornell University, New York, NY.

Mitochondria are responsible for aerobic respiration and ATP synthesis. They have a complex biosignature, as respiratory chain subunits are encoded by both nuclear and mitochondrial (mtDNA) genomes. Mitochondria are also a major source of reactive oxygen species and are central to apoptosis. Mutations in the mtDNA have been associated with mitochondrial diseases, cancer, and aging. Although it is has been shown that mtDNA mutations cause respiratory chain distress leading to mitochondrial dysfunction, permeability transition, and apoptosis, the exact mechanisms whereby mtDNA mutations lead to cell death have yet to be determined. We have investigated the role of mtDNA mutations in apoptotic cell death. We have studied three types of pathogenic mtDNA abnormalities, in human cybrid cells: the first affects global mitochondrial protein synthesis, the second abrogates the function of individual respiratory chain complexes, and the third impairs specifically the mitochondrial ATP synthase. Since mitochondria sequester calcium and dysregulation of calcium storages has been shown to induce apoptosis, we have studied the role of mtDNA mutations in apoptosis induced by thapsigargin, an irreversible inhibitor of the endoplasmic reticulum (ER) calcium-ATPase. We have found that, although all the mtDNA abnormalities share bioenergetic similarities resulting in reduced mitochondrial ATP synthesis, the three groups exhibit different responses to ER stress- mediated apoptosis: as compared to normal cells, the first group prevented apoptosis, the second promoted it, and the third did not change it. We have investigated the mechanisms underlying these different responses and found that they can be associated with different capacities in uptaking calcium released from the ER in mitochondria. Our findings suggest that mtDNA abnormalities either inherited or acquired, such as those accumulated in aging and cancer cells, may affect apoptosis differently, depending on their effect on mitochondrial membrane potential and mitochondrial calcium uptake in response to ER stress.

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Elucidation of the Mechanism by Which the Lim Protein Zyxin Promotes Apoptosis

M. Herry, M. C. Beckerle; Huntsman Cancer Institute, University of Utah, Salt Lake City, UT

Exposure of Ewing sarcoma cells to anti-CDD99 antibody promotes apoptosis and provides a potential targeted therapy (1). Recent evidence has implicated the LIM protein, zyxin, as a key mediator of apoptotic signaling induced by CD99 engagement (2). Zyxin is a focal adhesion protein implicated in integrin-dependent signaling and actin dynamics (3). We have determined that exposure of wildtype fibroblasts to UV-C irradiation results in apoptotic cell death, whereas cells harboring a homologous disruption of the zyxin gene display a statistically significant survival advantage. Reintroduction of a wildtype zyxin transgene restores sensitivity to UV-C irradiation illustrating that zyxin is pro-apoptotic. To explore the molecular mechanism by which zyxin contributes to apoptotic signaling, we introduced an affinity tagged version of zyxin into zyxin null fibroblasts and isolated zyxin and associated proteins from cell lysates under physiological conditions. A 130kDa protein that was co-isolated with zyxin was identified as the Cell Cycle and Apoptosis Regulator 1 (CARP-1) protein by microsequence analysis. CARP-1 was first characterized as a key protein in apoptotic signaling in breast cancer cells treated with a novel retinoid, CD437 (4). CARP-1 binds directly to zyxin, associated with its C-terminal LIM region. Zyxin that lacks the CARP-1 binding region fails to promote apoptosis in response to UV-C irradiation, thus suggesting that zyxin and CARP-1 cooperate to stimulate apoptosis. Zyxin and CARP-1 levels may be useful as predictors of responsiveness to apoptosis inducing cancer therapies. 1. Scotlandi et Al Eur J Cancer. 2006 Jan;42(1):91-6 2. Cerisano et Al Oncogene 2004 Jul 27;23(33):6064-74 3. Beckerle M.C. Cell. 1998 Dec 11;95(6):741-8 4.Rishi et Al Biol Chem. 2003 Aug 29;278(35):33422-35.

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The Effect of Crystalline Silica on Macrophages: Phagocytosis and Cell Death

G. N. Joshi, R. M. Gilberti, D. A. Knecht; Molecular and Cell Biology, University of Connecticut, Storrs, CT

Silicosis is a chronic lung disease induced by prolonged inhalation of crystalline silica dust. It is presumed that silica acts inside the cell, but there is no data showing that particles are actually phagocytosed. When macrophages are exposed to silica particles, (silica, latex, amino-latic, carboxy-latic), they extend protrusions to capture particles up to 7 μm away. Ovalbumin-opsonized silica phagocytosis is relatively slow (7% uptake at 15 minutes; 30% at 30 minutes; 71% uptake at 3 hours) compared to antibody-opsonized particles (70% uptake at 15 minutes through 3 hours).

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demonstrated significant H₂O₂ production in cancer cells but had minimal effect in non-cancer cells. The basis of this key difference is unclear at present, but the observation may have apoptosis observed upon down-regulation of CK2 is preceded by intracellular generation of H₂O₂ in various normal and cancer cells (prostatic androgen-dependent ALVA-41 and androgen-by employing antisense CK2 apoptosis in cancer cells (Ahmed et al., Trends Cell Biol 12: 226-230, 2002). Downregulation of CK2 by employing its inhibitors such as apigenin or TBB (4,5,6,7-tetrabromobenzotriazole), or CK2 (formerly casein kinase 2 or II) is a ubiquitous protein ser/thr kinase that plays important roles in cell growth and proliferation, and has also been recognized as a potent suppressor of antioxidative activity of Fenretinide, 2147 2145 P13/Akt and MAPK Signaling Pathways in DNA-damaging Drug-induced Apoptosis E. Lee, Y. Kang, J. Kim, B. Kim, H. Choi, G. Kang, Y. Oh, S. Cho; Animal Biotechnology, Konkuk University, Republic of Korea The flavonoids are a group of natural products currently receiving a great deal of attention but flavonoids became known fully explained. In the present study, we have administered several flavonoids to human HacT keratinocytes, and determined that 3,4′-dihydroxy flavone (3,4′-DHF) exerts a slight stimulatory effect on cell growth, although other flavonoids, including kaemferol, quercetin, and isohamnetin exhibited growth inhibitory properties. 3,4′-DHF was found to exert an anti-apoptotic effect on etoposide-induced cell death of HacT keratinocytes. We were also able to determine that sustained ERK activation was intimately associated with the etoposide-induced apoptosis of HacT cells, and treatment with 3,4′-DHF induced a significant suppression of etoposide-induced ERK activation, concomitant with the repression of PARP or the cleavage of pro-caspase 3. ERK overexpression significantly override the anti-apoptotic function of 3,4′-DHF, but this was not true of ERK-DN. Moreover, treatment with 3,4′-DHF resulted in the protection of cells from H₂O₂-induced cell death, and also exerted an apparent suppressive effect on the stress-induced generation of ROS. Finally, we showed that 3,4′-DHF almost completely abolished kaempferol-induced apoptosis, coupled with a concomitant suppression of both intracellular ROS generation and the activation of ERK. Taken together, our data clearly indicate that a host of psychochemicals, including etoposide and a variety of flavonoids, differentially regulate the apoptosis of human HacT keratinocytes via the differential modulation of intracellular ROS production, coupled with the concomitant activation of the ERK signaling pathway. These results underline the distinct structure-activity relationship (SAR) inherent to several flavonoids.

P13/Akt and MAPK Signaling Pathways in DNA-damaging Drug-induced Apoptosis E. Lee, J. Kim, Y. Kang, J. Kim, B. Kim, H. Choi, G. Kang, Y. Oh, M. Jeong, S. Cho; Animal Biotechnology, Konkuk University, Seoul, Republic of Korea Previous studies have shown that doxorubicin induces apoptosis at low concentrations, but induces necrosis at higher concentrations in several cancer cell variants. In this study, we have conducted an investigation into the functions of the ERK1/2, p38 MAPK, JNK and P13/Akt pathways in the context of the mechanisms underlying the apoptogenic properties of important chemotherapeutic DNA-damaging drugs, most notably doxorubicin and etoposide. Doxorubicin was determined to elicit the apoptosis of NIH3T3 cells in a dose-dependent manner. Prior to cell death, both Akt and p38 MAPK were transiently activated, and subsequently inactivated almost wholly, whereas ERK and JNK evidenced sustained activations in response to the drug treatment. The inhibition of P13/Akt and p38 MAPK both accelerated and enhanced doxorubicin-induced apoptosis and ERK inhibition apparently exerted negative effect on apoptosis. The modulation of P13/Akt activation by treatment of LY294002 or expression of Akt mutants such as Akt-DN or Myr-Akt exerted a significant effect on the activation of ERK1/2. We also observed that P13/Akt and sustained ERK activation were associated intimately with the etoposide-induced apoptosis. Interestingly, the inhibition of Akt activation resulted in a dramatic increase in ERK1/2 phosphorylation. This suggests that the activation of the ERK signaling pathway may be regulated by the P13/Akt signalling pathway in doxorubicin-induced apoptosis.

Production of Intracellular H₂O₂ Is an Upstream Event in Mediation of Apoptosis Induced by Downregulation of CK2 G. Wang, K. A. Ahmad, H. Lindvall, A. Johnson, K. Ahmed; Research Service/Lab Med Path, V.A. Medical Ctr/Univ of Minnesota, Minneapolis, MN CK2 (formerly casein kinase 2 or II) is a ubiquitous protein ser/thr kinase that plays important roles in cell growth and proliferation, and has also been recognized as a potent suppressor of apoptosis in cancer cells (Ahmed et al., Trends Cell Biol 12: 226-230, 2002). Downregulation of CK2 by employing its inhibitors such as apigenin or TBB (4,5,6,7-tetrabromobenzotriazole), or by employing antisense CK2a or siRNA results in potent induction of apoptosis in cancer cells. In an effort to define the downstream mediators of this action, here we demonstrate that cell apoptosis observed upon down-regulation of CK2 is preceded by intracellular generation of H₂O₂ in various normal and cancer cells (prostatic androgen-dependent ALVA-41 and androgen-independent PC-3 cells). In this regard, both type of prostatic cancer cells behaved similarly in response to treatment with 80 μM apigenin or TBB or with antisense CK2a ODN or siRNA. Interestingly, while chemical inhibitors of CK2 (TBB, apigenin) elicited H₂O₂ production in both the cancer and non-cancer cells, the antisense CK2a-mediated down-regulation of CK2 demonstrated significant H₂O₂ production in cancer cells but had minimal effect in non-cancer cells. The basis of this key difference is unclear at present, but the observations may have implications regarding approaches to therapeutic targeting of the CK2 signal. The intracellular H₂O₂ production induced by antisense CK2a was associated with robust caspase 3 activity, NF-kB nuclear translocation, release of cytokine c in the cytosol, and subsequent DNA fragmentation in prostate cancer cells (ALVA-41 and PC-3). These findings describe, for the first time, a relationship between CK2 and ROS such that inhibition of CK2 leads to production of intracellular H₂O₂ which may serve as a downstream mediator of apoptosis under these conditions. [Supported by grant from N.C.I., and V.A. Medical Research Fund].

N-(4-Hydroxyphenyl)retinamide, Fenretinide: An Essential Structural Component of Biological Activity and the Mechanism of its Action T. Ohba, K. Kyob, Y. Watanabe, N. Takahashi; Institute of Medicinal Chemistry, Hokush University, Tokyo, Japan Fenretinide, N-(4-hydroxyphenyl)retinamide (4-HPR) is a synthetic amide of all-trans-retinoic acid (RA), which inhibits cell growth, induces apoptosis, and is an antioxidant, and cancer chemopreventive and antiangiogenic agent. This findings led us to investigate which structural component of 4-HPR contribute to these potent activities. Our approach was to examine 4-aminophenol (4-AP), p-methylnaphylinopin (p-MAP), and p-acetaminohip (p-AAP). It was found that vitamin E, 4-AP and p-MAP scavenge α,α-diethyl-β-picyridinhydrazyl (DPPH) radicals in a 1 : 2 ratio, in contrast to 4-HP and p-AAP, where a 1 : 1 ratio and 1 : 0.5 ratio was observed relative to DPPH radicals. However, RA was inactive. Lipid peroxidation in rat liver microsomes was reduced by compounds (RA, p-MAP, 4-HP, 4-AAP) in dose-dependent manner, while p-AAP was inactive. In addition, both p-MAP and 4-HP are potent inhibitors of cell growth and inducers of apoptosis in HL60 cells. p-MAP exhibits antiangiogenic activity to the same extent as 4-HP against HL60 cells, which are a resistant clone against RA, and it inhibits growth of various cancer cell lines (MCF-7, MCF-7Adr, HepG2, and DU-145) to an extent greater than 4-AP and p-AAP, but is less potent than 4-HP. Thus, although the antioxidant activity of p-MAP is more potent than 4-HP, 4-HP is more potent than 4-HP in its anticancer activity. These results suggest that both anticancer and antioxidant activities shown by 4-HP are due to the structure of p-MAP. The retinoyl residue or long alkyl chain substituent attached to an aminophenol, may be significant for anticancer properties.
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SIRT1 Preserves DNA by Suppressing a Ubiquitin Ligase BTBD3
T. Kahyo,1 S. Sato,1 M. Setou2;3 Mitsubishi Kagaku Institution of Life Science, Machida-shi, Japan, 2National Institutes of Physiological Sciences, Okazaki-shi, Japan
SIRT proteins, including yeast Sir2, are involved in caloric restriction and lifespan extension in several species including S. cerevisiae, C. elegans and D. melanogaster. Mammalian Sir2 homologue SIRT1 is important for various cellular functions including apoptosis, differentiation and proliferation. In cells, SIRT1 functions as a NAD-dependent deacetylase and, some substrates have been reported, such as p53 and FOXO. To search for a novel SIRT1-interacting protein, we carried out yeast two-hybrid screening using SIRT1 as a bait. The C-terminal region of Broad Complex/Tramtrack/Bicaudal (BTB) domain containing protein 3 (BTBD3) was found as a SIRT1-interacting candidate. In addition to BTB domain, BTBD3 also contains PHR domain, similar to part of the PAM/Highwire/RPM-1 protein. BTBD3 formed the complex containing Cud3 and Roc1, which are known as a component of a ubiquitin ligase, and the ubiquitinated forms of BTBD3 were detected in HEK293T cells. These results indicate that BTBD3 is a component of a ubiquitin ligase. The deleted form lacking the BTB domain showed the decreased auto-ubiquitination, indicating that the BTB domain is necessary for sufficient ligase activity. Interestingly, the overexpression of SIRT1 suppressed the auto-ubiquitination of BTBD3 in HEK293T cells. The survival rate of the HEK293T cells overproducing BTBD3 was significantly decreased with the DNA-damaging agents, such as hydroxyurea and mitomycin C compared with the cells co-overproducing both BTBD3 and SIRT1 cells. The alkaline single cell gel electrophoresis (Comet) assay showed that the efficiency of DNA repair was significantly decreased in the BTBD3-overexpressing cells compared with that in the BTBD3-SIRT1-overproducing cells. These results show that SIRT1 preserves DNA by suppressing a ubiquitin ligase BTBD3.

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Dynamic O-GlcNAc Modification in Response to Glucose Deprivation
J. Kang1,2 J. Cho1,2
1Department of Biology, Yonsei University, Seoul, Republic of Korea, 2Protein Network Research Center, Seoul, Republic of Korea
About 2–5% of extra-cellular glucose is converted into uridine diphosphate-N-acetyl glucosamine (UDP-GlcNAc) through hexosamine biosynthetic pathway (HBP). UDP-GlcNAc could be used for O-GlcNAc modification of nuclear-cytoplasmic proteins by aid of O-GlcNAc transferase (OGT). Accordingly the concentration of extra-cellular glucose is tightly related not only with HBP but also with O-GlcNAc modification of proteins. decreases. However, in A549, human lung cancer cell line, O-GlcNAc modification increases in response to glucose deprivation in a time dependent manner. On the other hand, the level of OGT is not changeable at this condition. This reflects that OGT activity increases in response to starvation in A549. Moreover the activity of GFAT, the first and rate-limiting enzyme in the hexosamine biosynthesis pathway, increases in steady glucose deprivation condition. We used SWGA precipitation method and MALDI-MS to identify the proteins in which O-GlcNAc modification increased at glucose deprivation condition. In view of the results so far achieved, we could identify several cytoskeletons, heat shock protein (HSP-70), ribosomal proteins etc. HSP-70 is known not only to be O-GlcNAc modified, but also to bind to other O-GlcNAc modified proteins. Thus, we will focus on revealing how the O-GlcNAc modification protect some proteins from degradation in response to glucose deprivation.

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A Cytokine Receptor Common β Chain Box 2-associating Protein Mediates Apoptosis through Mitochondria-dependent Pathway in Hematopoietic Cells
C. Kao,1,3 K. Lin,1 P. Hwang,1 C. Li,1 J. J. Yen1; 1Institute of Biomedical Sciences, Taipei, Taiwan, 2Genome Research Center, Academia Sinica, Taipei, Taiwan, 3Stem Cell Program, Institute of Cellular & Organismic Biology, Taiwan, Taiwan
The cytokropic domain of human granulocyte-macrophage colony-stimulating factor (GM-CSF) receptor common β chain (βc) contains a region, proximal to the transmembrane domain, which is essential for induction of c-myc and pim-1 and is sufficient to mediate GS-CSF dependent mitogenic activity. This membrane proximal region has homology with other members of the cytokine receptor family and is designated as box 1 and box 2 motifs. Whereas box 1 motif is required for the recruitment and phosphorylation of JAK2 kinase, the function of box 2 motif remains largely unknown. Here we report the identification of a novel box 2-associating protein, the common beta chain associating protein (CBAP), which interacts with βc in the absence of cytokine and mediates apoptosis signal in hematopoietic cells. Yeast two-hybrid screening and GST-pull-down experiments showed that CBAP specifically associated with βc via the box 2 motif. Furthermore, co-immunoprecipitation experiments indicate that CBAP associates with βc in the absence, but not the presence, of GM-CSF in vivo via a region containing a WW-like domain on CBAP. Ectopic expression of CBAP in hematopoietic cells triggered apoptosis via mitochondria dysfunction, which could be completely abrogated by Bcl-2 overexpression. Moreover, reduced expression of endogenous CBAP by siRNA inhibited apoptosis significantly induced by GM-CSF deprivation. These findings suggest that CBAP is one of the important apoptosis regulators in hematopoietic cells. It also raises a possibility that βc may play a critical role in triggering apoptosis program in the absence of cytokine by specifically interacting with the apoptosis regulator CBAP.

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Inhibition of Poleward Flux on Mitotic Spindles Delays Entry and Progression through Anaphase
I. Matos,1 A. J. Pereira,1 L. A. Cameron,2 S. Moutinho-Pereira,1 E. D. Salmon,2 H. Maiato1; 1Institute for Molecular and Cell Biology, Porto, Portugal, 2Department of Biology, University of North Carolina, Chapel Hill, NC
Flux remains one of the most mysterious properties of metazoan mitotic spindles. Two key flux players have already been identified. CLASP promotes the input of microtubule subunits at kinetochore/microtubule attachment, whereas KLP10A couples microtubule depolymerization at the poles. Interference with the function of these proteins results in the reduction of flux rates as well as spindle abnormalities - especially short-monopolar and long spindles. As an experimental hypothesis we proposed that simultaneous depletion of CLASP and KLP10A by RNAi in S2 cells would rescue normal spindle bipolarity and cease flux. As a complementary approach we have also treated metaphase cells with taxol, which suppresses spindle microtubule dynamics. In agreement with recent results from several labs, we were also able to significantly rescue spindle bipolarity after CLASP/KLP10A RNAi. By performing fluorescent speckle microscopy we found that both treatments resulted in a significant reduction of flux rates (1.24 ± 0.32 μm/min in CLASP/KLP10A RNAi; and 0.13 ± 0.11 μm/min taxol). To understand the underlying role of flux during mitosis we followed control, CLASP/KLP10A depleted, and taxol-treated S2 cells stably expressing GFP-α-tubulin and CID-mCherry by time-lapse fluorescence microscopy and DIC. Strikingly, we found that after CLASP/KLP10A RNAi and taxol treatment, anaphase entry was often delayed or even prevented. Quantitative fluorescent analyses revealed that accumulation of BubR1 at kinetochores was proportional to the degree of flux inhibition, suggesting that the observed delay in anaphase entry was due to the inability to satisfy the spindle checkpoint. Noteworthy, those cells that were able to enter anaphase showed a 30-40% reduction in chromosome poleward movement and lagging chromosomes could frequently be observed. Taken together, these data strongly suggest that, aside from the contribution of force for chromosome segregation, flux plays fundamental roles in kinetochore-microtubule attachment, which allows a timely metaphase-anaphase transition.

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Lamin B Receptor Stimulates Nuclear Envelope Production and Targets Membrane Vesicles to Chromatin during Nuclear Envelope Assembly through Direct Interaction with Importin Beta
Y. Ma, S. Cai, Q. Jiang, Q. Zhang, S. Sodmergen, Z. Zhai, C. Zhang; The Key Laboratory of Cell Proliferation and Differentiation of Ministry of Education and The National Key Laboratory of Bio-membrane and Membrane Biotechnology, the College of Life Sciences, Peking University, Beijing, China
Lamin B receptor (LBR) is an evolutionarily conserved and developmentally essential inner nuclear membrane protein, ubiquitous in vertebrate, Drosophila, and yeast. LBR consists of a hydrophilic LaminB-binding and chromatin-binding N-terminal domain, a short hydrophobic C-terminal domain and eight predicted transmembrane segments which show a significant sequence similarity to vertebrate, yeast and plant steroid receptors. It has been shown that sea urchins LBR and an LBR like integral membrane protein are able to target membranes to the chromatin surface, which indicates an important role of LBR during nuclear assembly. The mechanism of how LBR is recruited to the chromatin surface is not clear. In the present study, based on transfection experiments, we show that overexpression of LBR in HeLa cells causes membrane overproduction, inducing NE invagination and membrane stacks formation, which is dependent on the first transmembrane domain of LBR. Biochemical analysis shows that the N-terminal domain of LBR directly interacts with importin beta in a Ran sensitive and importin α independent manner. In vitro binding assays with serials of truncated LBR shows that the region between the 45 amino acid to 90 amino acid of the N-terminal of LBR is responsible for the interaction of LBR and importin beta. Using an in vitro NE assembly assay, we also demonstrate that blocking the LBR binding sites on importin beta or competing for endogenous LBR, by addition of the LBR N-terminal domain, either reduces or seriously inhibits the recruitment of LBR containing vesicles to importin beta or Ran coated beads. Our results suggest that LBR is recruited to chromatin through direct interaction with importin beta to contribute to the fusion of membrane vesicles and formation of the NE.

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A Network Model of *C. elegans* Early Embryogenesis Identifies a Novel AT-Hook Protein That Coordinates Nuclear Envelope Function and Chromatin Maintenance

A. G. Fernandez, F. Piano; Biology, New York University, New York, NY

Using combined network analysis of large-scale functional genomics, we mapped multi-protein modules required for distinct processes during early embryogenesis (Gunsalus et al. 2005). A basic question is how these molecular modules are coordinated to ensure the proper unfolding of early developmental events. To identify proteins that could coordinate different modules we searched for proteins that bridged different modules. One such protein, MEL-28, could be placed in either the nuclear pore complex module or in the chromosome maintenance module by a network clustering algorithm M-CODE (Bader et al. 2003). Consistent with its predicted roles at the nuclear pore and in chromosome segregation, GFP fusions and anti-MEL-28 immunolocalizations show that MEL-28 shuttles between the nuclear envelope and the kinetochore during the cell cycle. Functionally, MEL-28 is required for proper nuclear envelope and chromatin integrity (Mel-28(RNAi) embryos, the embryos without nuclear pore components, are incapable of completely separating cytoplasm from nucleoplasm, failing to exclude microtubules and affecting the nuclear localization of PIE-1, a protein normally enriched in the P1 nucleus (Mello, 1996). Additionally, pronuclei fail to meet, and centrosomes do not remain attached to the paternal pronucleus and segregate prematurely. In addition metaphase spindles are not established and chromatin neither condenses, congresse, nor segregates properly. These phenotypes resemble RNAi phenotypes of genes from the Ran GTPase cycle (Asgjaer 2002). Looking for MEL-28 phenotypic neighbors using PhenoBlazt (Gunsalus et al. 2004) or phenocloning from large-scale RNAi analyses (Simonich et al. et al. 2005) we identified ~25 other genes with similar defects when analyzed by time-lapse Nomarski microscopy. Of these, genes that are part of the RanGTPase pathway (ran-1, ran-2, npp-9) were required for proper MEL-28 localization. Thus MEL-28 is critical for both mitotic and interphase cell functions and is a likely target of the Ran GTPase pathway.

**The Persistence of Lamin B from Nuclear Envelope Breakdown through Spindle Assembly**

A. E. Goldman, L. Chang, T. Dechat, H. Lipschultz, R. D. Goldman; Cell and Molecular Biology, Northwestern University, Feinberg Medical School, Chicago, IL

Mature *S. pombe* oocytes are arrested in late prophase, pre-meiosis I. The nuclei of these oocytes contain condensed chromatin which are closely associated with the lamina, the major protein of which is the nuclear lamin, L67. Upon fertilization, L67 is phosphorylated by cdk1 as nuclear envelope breakdown (NEBD) proceeds in a highly synchronous fashion. Immuno-fluorescence observations demonstrate that some L67 remains associated with chromosomes throughout NEBD, and some becomes associated with aster microtubules, spindle poles and ultimately the mitotic spindle. The remaining L67 becomes dispersed throughout the cytoplasm. Biochemical studies in support of these observations have involved the isolation of nuclei from unfertilized eggs and并通过 NEBD following fertilization. The latter resulted in nuclear remnants containing chromosomes and subsequently mitotic spindles. Immunofluorescence and immunoblotting reveal the presence of L67 in all of these isolated structures. Within the isolated spindles L67 is associated with chromosomes, spindle poles, and microtubules. Spindle-associated factors (SAFs) such as Eg2 are also present in these spindles. We have developed a method for the enrichment of spindle matrices by isolating mitotic spindles in the presence of nocodazole. These matrices are devoid of polymerized microtubules and contain L67 and chromosomes. We are presently analyzing these matrices to determine whether SAFs are associated with L67 as previously shown (Tsai et al., 2006, Science). Similar results have been obtained with mitotic spindles isolated at first cleavage. These results demonstrate that lamins are retained throughout NEBD and become incorporated into the spindle matrix. Supported by NCI and the Ellison Medical Research Foundation.

**Modeling Interactions between Rev and Microtubules**

A. Sharma, K. R. Miller, S. Bedi, D. Sweaney, S. F. Nathan, M. W. Miller; Biological Sciences, Wright State University, Dayton, OH

Watts et al. (J. Cell Biol. 150:349-360, 2000) described a novel in vitro interaction between microtubules (MTs) and the human immunodeficiency virus-1 (HIV-1) protein Rev, a 13 kDa basic protein essential for HIV infection. Highly purified Rev rapidly depolymerizes equimolar MTs and produces bilayered rings of oligomeric Rev and tubulin. Addition of purified Rev to mitotic frog egg extracts inhibits the formation of asters formed in vitro indicating that Rev can affect the polymerization state of MT in a cellular environment. A molecular explanation of Rev:MT interactions is suggested from a limited amino acid similarity shared with members of the Kinesin-13 family, proteins known to stimulate MT catastrophes. To test this hypothesis, we have modeled Rev:MT interactions using the HEX computer program to dock Rev’s arginine-rich motif onto the tubulin heterodimer believing that basic residues might create an affinity for tubulin. Of the 512 best models generated, essentially only two different models were produced. Each had a calculated binding energy of ~1300 kcal/mol with electrostatic interactions and shape compatibilities contributing equally. These models are consistent both with our efforts to dock the comparable region of MCAK (506-542) to MT as well as with structural models of Kif2c:MT interactions. We have also assessed the ability of Rev to bind, depolymerize MT and affect MT function in vitro and in vivo. Sedimentation assays show that bacterially expressed Rev, purified by FPLC using Q-sepharose and heparin-sepharose columns, bind and depolymerize purified MT. Immunoprecipitation experiments suggest that Rev:MT complexes persist in living cells when Rev is transiently and stably expressed in HeLa cells. Flow cytometric analyses and measurement of mitotic indices show that Rev expression demonstrably affects HeLa cell cycle progression. These results suggest that Rev may destabilize MTs in HIV-infected cells.

**Mammalian CLASP1 and CLASP2 Cooperate to Ensure Mitotic Fidelity by Regulating Spindle and Kinetochore Function**

A. L. Pereira, 1 A. J. Pereira, 1 A. R. R. Main, 2 K. Drabeck, 1 C. L. Sayas, 1 P. Hergett, 1 M. Lince-Faria, 1 I. Matos, 1 C. Duque, 3 T. Stepanova, 2 C. Rieder, 2 W. C. Earnshaw, 1 N. Galjart, 1 H. Maatouk, 1, 3 Institute for Molecular and Cell Biology, Porto, Portugal, 1, 3Dept. of Cell Biology and Genetics, Erasmus Medical Center, Rotterdam, The Netherlands, 1, 3New York State Dept. of Health, Division of Molecular Medicine, Wadsworth Center, New York, NY, 1Laboratory of Cell and Molecular Biology, Faculdade de Medicina, University of Porto, Porto, Portugal, 2Welcome Trust Centre for Cell Biology, Institute of Molecular Biology, University of Edinburgh, Edinburgh, United Kingdom

CLASPs are widely conserved microtubule plus-end-tracking proteins with essential roles in the local regulation of microtubule dynamics. In yeast, *Drosophila* and *Xenopus* a single CLASP orthologue is present, which is required for mitotic spindle packaging by regulating microtubule dynamics at the kinetochore. In mammals, however, only CLASP1 has been directly implicated in cell division, despite the existence of a second paralogue, CLASP2, whose mitotic roles remain unknown. Here we show that CLASP2 localization at kinetochores, centrosomes and spindle is remarkably similar to CLASP1, both showing fast microtubule-dependent turnover rates. Strikingly, primary fibrobasts from Clasp2 knockout (KO) mice show numerous spindle and chromosome segregation defects that can be partially rescued by ectopic expression of Clasp1 or Clasp2. Moreover, chromosome segregation rates during anaphase are slower in Clasp2 KO cells, which is consistent with a primary role of CLASP2 at kinetochores and spindle microtubule flux. Noteworthy, although cell viability and proliferation were not impaired in Clasp2 KO cells, the fidelity of mitosis was strongly compromised leading to severe chromosomal instability in the adult. Together, our data support that the partially redundant roles played by CLASPs during mitosis act as a possible mechanism to prevent aneuploidy in mammals.

**Spindle Assembly Is Induced by Chromatin-dependent Activation of the Aurora B Pathway**

A. E. Kelly, 1 S. C. Sampath, 2 T. A. Manier, 2 E. M. Wou, 2 B. S. Tseng, 1 H. Funabiki 2; 1Laboratory of Chromosome and Cell Biology, Rockefeller University, New York, NY, 2Laboratory of Mass Spectrometry, Rockefeller University, New York, NY

Chromatin-induced spindle assembly depends on the chromosomal passenger complex (CPC), consisting of Incenp, Survivin, Dasra (Borealin) proteins, and the kinase Aurora B. How the CPC contributes to the spatial regulation of spindle assembly remains unclear. Here we show that the Aurora B pathway is suppressed in the cytosol of *Xenopus* egg extracts, but becomes activated by chromatin via a Ran-independent mechanism. Spindle assembly requires Dasra-dependent chromatin binding of the CPC, but this function of Dasra can be bypassed simply by clustering the CPC. Dasra-dependent chromatin binding of the CPC is required for chromosome segregation and cytokinesis in eukaryotic cells. In *silico* analysis of the *Giardia* genome identified a single AK ortholog (gAK), with 61% similarity to human AK. gAK contains conserved active site residues, activation loop, and destruction box motifs.
characteristic of AKs. Similar to many other giardial gene products, gAK has a unique insert within the kinase domain. Since giardial cell division is not well understood, we tested the hypothesis that gAK plays a role in mitosis and cytokinesis through interactions with either histones or cytoskeletal elements. gAK expressed under its own promoter with a C-terminal AU1 epitope tag localized exclusively to both nuclei in interphase cells. In addition, an antibody to phosphorylated AK (pAK) reacted only with the few mitotic cells in the non-synchronized trophozoite population. Using double labeling and DAPI staining, we followed the localization of total gAK and active phosphorylated gAK throughout the cell cycle. During mitosis, gAK localized to the basal bodies/centrosomes and a spindle-like structure. Localization to the centrosomal area was confirmed through double labeling of pAK and centrin, and the spindle-like structure with pAK and alpha-tubulin. During specific stages of mitosis, gAK also localized dynamically to cytoskeletal structures unique to *Giardia*: the parflagellum dense rods of the anterior-lateral flagellum, the median body, and the parent adhesive disc. Pharmacological inhibition of gAK decreased cell proliferation and increased the percentage of cells in cytokinesis, appearing to block the progression of late stages of cell division. Treated cells also had abnormal tubulin structures. Together, these results suggest that gAK is crucial for the regulation of mitosis and cytokinesis in *Giardia lamblia*.

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**Cdk1 Acts Upstream of Aurora Kinases during G2/m Transition Revealed by Small Molecule Kinase Inhibitors and High Content Multiplex Assays**

R. D. Van Horn, Lilly Research Laboratory, Eli Lilly, Indianapolis, IN

Entry into and progression through mitosis are regulated by multiple evolutionarily conserved mitotic kinases. Mitotic kinases Cdk1, Plk1, NIMA and aurora all have essential and non-overlapping functions in mitosis. Cdk1 plays an essential role in entry into mitosis. In yeasts, activation of Cdk1 is necessary and sufficient to trigger mitosis, whereas in *Aspergillus nidulans*, activation of both Cdk1 and NIMA kinases is required. It was shown recently by RNAi that aurora-A kinase is required for mitotic commitment by recruiting Cdk1 to centrosomes where Cdk1 is initially activated to trigger mitosis. Here we developed highly selective and potent small molecule kinase inhibitors of Cdk1 and aurora kinases and high content/high throughput multiplex assays for cell cycle analysis by simultaneously measuring DNA content, cyclinB1 and phosphorylated histone H3 at the single cell level. We showed that inactivation of Cdk1 by small molecule inhibitors arrested cells in G2 with accumulated cytoplasmic cyclinB1. By contrast, inactivation of aurora A kinase arrested cells at mitosis with high levels of nuclear cyclinB1 and phosphorylated histone H3, indicating that unlike Cdk1, aurora-A kinase is not required for entry into mitosis. Indeed, using synchronized cells, we further showed that inactivation of Cdk1 prevented activation of aurora-A kinase, while inactivation of aurora kinases had no effect on the kinetics of either Cdk1 activation or entry into mitosis. Interestingly, inactivation of both aurora-A and aurora-B kinases with dual aurora kinase inhibitors promoted DNA endoreduplication in the absence of cell division. Live cell imaging revealed that the nuclear cycle of cells treated with dual aurora kinase inhibitors is uncoupled from cytokinesis and continues to oscillate in a Cdk1-dependent manner, resulting in multinucleated cells. Together, our studies with selective small molecule inhibitors and high content multiplex assays convincingly established that Cdk1 acts upstream, rather than downstream, of aurora kinases in mitosis.

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**The N- and C-terminal Domains of MCAK Spatially Control Microtubule Depolymerization Activity and Spindle Assembly in Extracts**

S. C. Enns-McClung,1 K. M. Hertzer,2 X. Zhang,3 M. W. Miller,3 C. E. Walczak2; 1Medical Sciences Program, Indiana University, Bloomington, IN, 2Anatomy and Cell Biology, Indiana University, Bloomington, IN, 3Biological Sciences, Wright State University, Dayton, OH

The proper regulation of microtubule dynamics is critical for spindle assembly and accurate chromosome segregation. MCAK, a Kinesin-13, catalytically depolymerizes microtubules, regulates microtubule dynamics in vivo and is the major catastrophe factor in egg extracts. The N-terminal domain is required for kinesin function during cell division, while the C-terminal domain regulates microtubule depolymerization in vitro. However, the degree to which the different domains contribute to or correlate with their in vitro and physiological microtubule depolymerization activity is unknown. Five domain mutants were constructed, expressed and assayed for in vitro microtubule depolymerization and physiological spindle assembly in egg extracts. The N-terminal domain truncation mutant, GM(187-731), had wild-type in vitro microtubule depolymerization activity but did not fully rescue spindle assembly due to suppressed microtubule depolymerization activity in extracts. Thus, the N-terminal domain of MCAK is not dispensable for physiological microtubule dynamics but is expendable in vitro. In contrast, the C-terminal domain truncation mutant, GM(2-592), had reduced in vitro microtubule depolymerization activity but completely rescued spindle assembly due to enhanced physiological depolymerization activity. Neither construct was as efficiently kinesin-like as Cdk1, and these results also suggested control of spindle assembly by MCAK. Our results show that high MCAK depolymerization activity in vitro does not correlate with efficient spindle assembly in extracts. We propose that MCAK activity is spatially controlled by the N-terminal domain during spindle assembly. Specifically, the N-terminal domain is essential for targeting MCAK to kinetochores, while the C-terminal domain enhances the efficiency of kinesin targeting. Additionally, the N-terminal domain is necessary for cytoplasmic MCAK activity in extracts. Surprisingly the C-terminal domain also plays an inhibitory role by limiting the extent of spindle bipolarity.

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**Mechanism of PavKLP-mediated Cortical-Spindle Interactions during Mitosis in *Drosophila* Embryos**

P. Sommi, R. Ananthakrishnan, D. K. Cheerambathur, I. Brust-Mascher, J. M. Scholey, A. Mogilner; University of California, Davis, CA

The spindle assembly checkpoint monitors microtubule-kinetochore attachments and tension across sister kinetochores to facilitate accurate division of chromosomes between daughter cells. Cortical-spindle interactions during mitosis in *Drosophila* embryos, also known as *PavKLP-mediated cortical-spindle interactions (PCSI)*, has been shown to recruit cortically localized PavKLP to kinetochores to facilitate proper spindle formation. Here we have investigated cortical-spindle interactions in *Drosophila* embryos, and observed that the kinesin-6 family member, PavKLP, influences both spindle morphogenesis and cortical dynamics. PavKLP, which behaves as a dimer in subcellular fractionation, localizes to mitotic spindles, where it is very dynamic, and to mitotic furrows, where it co-localizes with actin. The inhibition of PavKLP function causes defects in spindle dynamics, actin distribution and mitotic furrow ingression, suggesting that PavKLP is involved in stabilizing the spindle structure as well as promoting actin remodeling and membrane recruitment for furrow formation during mitosis and cellularization. Using in vivo experiments and modeling, we tested the hypothesis that PavKLP may contribute to furrow dynamics by driving membrane vesicle transport. In vivo, we observed that PavKLP interacts with Nuf1, a key component of recycling endosomes which is required for vesicle-mediated delivery and actin recruitment to the furrow, and that PavKLP inhibition interferes with Nuf1 distribution thus influencing vesicle delivery to the cortex. “Virtual Cell” modeling suggests that furrow ingression is biphasic and depends on a PavKLP-generated force during the initial slow stage, followed by PavKLP-driven vesicle transport during a subsequent fast stage. Thus PavKLP is required for proper spindle and furrow organization, and it mediates spindle-cortical interactions by transporting vesicles along astral microtubules to the cortex to promote actin remodeling and membrane recruitment.

2162

**Light Intermediate Chain 1 of Cytoskeletal Dynein Is Required for Progression through the Spindle Assembly Checkpoint**

T. L. Wadzinski, S. J. Dossey, Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, MA

The spindle assembly checkpoint monitors microtubule-kinetochore attachments and tension across sister kinetochores to facilitate accurate division of chromosomes between daughter cells. Cytoskeletal dynein function in this checkpoint, apparently by moving critical checkpoint components off kinetochores, including Mad2 and BubR1. The dynein subunit required for this function is unknown. Here we show that human cells depleted of dynein light intermediate chain 1 (LIC1) delay in metaphase. LIC1 remains intact, localized and functional in these cells implicating LIC1 directly in the metaphase delay. The kinetochore appears structurally intact as Mad2, BubR1, Mad1 and dynein are organized normally on unattached kinetochores. The prolonged metaphase requires an active spindle assembly checkpoint as depletion of Mad2 or Mad1 allows cells to progress through mitosis without delay. Moreover, Mad2 remains on some kinetochores in delayed cells suggesting that LIC1 facilitates removal of kinetochore-associated Mad2. This LIC1 function is selective as BubR1, the tension-associated protein, is normally removed from all kinetochores. The LIC1-associated metaphase delay is suppressed by re-expression of wild type LIC1 or a Cdk1 site phosphomimetic LIC1 mutant, but not by a phosphorylation-deficient mutant. We conclude that LIC1 phosphorylation at a Cdk1 site is required for removing Mad2 from kinetochores and resolution of the spindle assembly checkpoint. The presence of Mad2 on kinetochores that are under tension and lack BubR1 demonstrates that Mad2 is sufficient for checkpoint maintenance. We propose that LIC1 is an adaptor linking dynein to Mad2 either directly or indirectly, facilitating the removal of this checkpoint component off kinetochores.

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**A Novel Role for Nup98 in the Regulation of the Spindle Function**

M. Cross, M. Powers; Cell Biology, Emory University, Atlanta, GA

The nuclear pore complex is comprised of approximately 30 different nucleoporins (Nups). During interphase of the cell cycle, this complex regulates the trafficking of proteins and RNA 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. Nup98, a 98kDa nucleoporin, interacts with several proteins implicated in regulation of mitotic spindle formation. We have utilized in vitro spindle assembly in Xenopus egg extracts to determine whether Nup98 itself plays a role during mitosis. When added to spindle assembly extracts, the C-terminal domain of Nup98 stimulates excess growth of microtubules. The C-terminal domain similarly stimulates microtubule polymerization in Ran-induced asters. Added however, we have shown that excess microtubule growth does not require the known interaction between the Nup98 C-terminus and...
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 contrast, either addition of domain-specific antibodies to extract or depletion of endogenous Nup98 from extract results in dramatic inhibition of microtubule growth and formation of minimal, but stable monopolar spindles. These findings strongly suggest that Nup98 itself, through a function of the C-terminal domain, is required for assembly of a proper mitotic spindle. Similar findings were obtained using preformed mitotic spindles; the Nup98 fragment induces a dramatic increase in size and microtubule density of the spindle whereas antibody addition leads to rapid decrease in pole to pole length. We conclude that Nup98, through a previously uncharacterized activity, is acting as a regulator of microtubule dynamics during mitosis. We are using fluorescent speckle microscopy to assess the influence of Nup98 on microtubule flux within the spindle.

2165

γ-Tubulin Plays an Important Role in the Localization of Cyclin B
T. Naya, E. Sweeney, R. B. Oakley; Molecular Genetics, Ohio State University, Columbus, OH

Certain conditionally-lethal γ-tubulin alleles of Aspergillus nidulans allow normal mitotic spindle formation at restrictive temperatures, but cause dramatic failure of the regulation of late mitotic events. These data indicate that γ-tubulin has important, but incompletely defined, functions in mitotic regulation. To investigate these functions, we have GFP-tagged a number of mitotic regulators in the γ-tubulin mutant strain to determine if late mitotic events are regulated by temperature at restrictive temperatures. We find that in a temperature-sensitive delay, the spindle normally forms, but fails to progress beyond prometaphase. This reveals a requirement for γ-tubulin both in assembly and progression. Similar results were obtained for assembly and progression in a temperature-sensitive mutant of Cdc16, a different kinesin. In both cases, γ-tubulin is required for spindle pole body formation, but not for spindle pole body function. These data indicate that γ-tubulin is required for assembly and progression of the mitotic spindle, and that this requirement is not due to a temperature-sensitive defect in spindle pole body function.

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A Small-molecule Inhibitor That Disrupts Importin-beta-mediated Nuclear Import and Spindle Assembly
J. F. Soderholm, K. Weis, R. Heald; Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA

The Ran GTPase regulates nucleocytoplasmic transport and mitotic progression through its interaction with the transport receptor importin-beta. It has been hypothesized that a RanGTP gradient regulates mitotic events by releasing importin-beta-bound motif factors in a spatially-restricted manner across the chromosomes in living cells. To test this hypothesis directly we performed a high-throughput screen for small molecule inhibitors that disrupt the interaction between RanGTP and importin-beta. Our screen, which was based on fluorescence resonance energy transfer (FRET), identified about 100 compounds that interrupt the FRET signal generated by our probes. In subsequent experiments using Xenopus laevis egg extracts and cultured HeLa cells, we found that one of these compounds strongly inhibits spindle assembly and nuclear envelope assembly. The compound does not depolymerize microtubules directly in vitro. In digitonin-permeabilized tissue culture cells, this compound blocks nuclear import of GFP, and blocks nuclear localization of GFP. Importantly, the compound does not seem to affect the import of a transportin-dependent reporter in this assay, suggesting that the compound specifically targets importin-beta. We have found that this compound belongs to a larger class of easily-synthesized small molecules, many of which also abrogate spindle assembly in Xenopus extracts.

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TOG Regulates Spindle Microtubule Dynamics and Kinetochore Motility
L. Cassimeris, B. Becker; Department of Biological Sciences, Lehigh University, Bethlehem, PA

TOG, a member of the XMAP215 MAP family, is required for bipolar mitotic spindle assembly, spindle pole integrity and chromosome bi-orientation (Gergely et al. 2003; Cassimeris and Morimoto, 2004). To understand how TOG contributes to spindle assembly, we examined microtubule dynamics in Hela cells depleted of TOGp by siRNA. FRAP of GFP-tubulin expressing cells demonstrated that spindle microtubule turnover is slowed by about 2 fold in the absence of TOGp. By following microtubule regrowth after washout of the depolymerizing drug nocodazole (Tulu et al. 2006 Curr. Biol. 16:536), we find that TOGp-depleted cells have fewer microtubules associated with centrosomes, while the microtubules assembling in association with mitotic chromatin are similar to those in untreated cells. Kinetochore fibers in both untreated and TOGp-depleted cells are stable to lysis in buffer containing calcium. Taken together, our results indicate that TOGp contributes specifically to assembly of centromal microtubules and is not required for kinetochore microtubule assembly or stability. Reduced centromal microtubules should reduce ejection force on chromosome arms and reduce tension across sister kinetochore. We estimated the ejection force by measuring the pole to pole distance in monotreated-processed cells. Kinetochore fibers were located closer to the poles in the TOGp-depleted cells (2.16 µm vs 3.04 µm in untreated cells), consistent with TOGp (and centromal microtubules) contributing to the ejection force. Loss of TOGp also reduced tension across sister chromatids (1.08 µm vs 1.24 µm in untreated cells). Images of living cells expressing CENPA-GFP showed that kinetochores in TOGp-depleted cells rapidly switch between poleward and away-from-pole motility, but show little net translocation in either direction. We hypothesize that TOGp-dependent assembly of centrosomal MTs and spindle pole recruiting contribute to chromosome congression by regulating kinetochore directional instability. Supported by NIH.
Evidence That Myosin Phosphatase Antagonizes Polo-like Kinase1 during Mitosis in Mammalian Cells

S. Yamashiro, Y. Yamaoka, F. Matsumura; Molecular Biology & Biochemistry, Rutgers, Piscataway, NJ

Protein phosphatase1 (PP1) plays essential roles in various aspects of mitosis including mitotic spindle assembly, chromosome segregation and cytokinesis. While PP1 has a limited number of isoforms for its catalytic subunit (PP1C), PP1C interacts with numerous PP1C regulatory subunits, and thus generates many forms of the phosphatase with distinct substrate specificities, Protein phosphatase1 (PP1) plays essential roles in various aspects of mitosis including mitotic spindle assembly, chromosome segregation and cytokinesis. While PP1 has a limited number of isoforms for its catalytic subunit (PP1C), PP1C interacts with numerous PP1C regulatory subunits, and thus generates many forms of the phosphatase with distinct substrate specificities, enabling PP1 to antagonize a wide array of Ser/Thr kinases. Little is known, however, about which PP1C regulatory molecules are involved in mitosis and what serine/threonine kinases they antagonize. Myosin phosphatase targeting subunit1 (MYPT1) is one of the regulatory subunits of PP1C, and is believed to target PP1C to myosin II, thus controlling the contractility of smooth muscle and nonmuscle cells. Surprisingly we found that mammalian MYPT1/PP1C regulates mitosis by antagonizing polo-like kinase1 (PLK1). During mitosis, MYPT1 binds to PLK1, apparently as a consequence of mitosis-specific phosphorylation of MYPT1 by cdc2. PLK1 depletion by small interfering RNAs is known to inhibit γ-tubulin recruitment to the centrosomes in mammalian cells, blocking centrosome maturation and resulting in mitotic arrest and apoptosis. We found that co-depletion of MYPT1 and PLK1 reactivates γ-tubulin at the centrosomes, restoring the assembly of bipolar mitotic spindles. Live-cell imaging revealed that double depletion rescues mitotic arrest caused by PLK1 depletion. MYPT1 depletion, on the other hand, induced assembly of multiple (more than 3) γ-tubulin-containing foci. This MYPT1 phenotype was also suppressed by co-depletion with PLK1, indicating the dependence of the MYPT1 phenotype on normal levels of PLK1. Mutual suppression is thus likely achieved by reducing the overall levels of PLK1 to better match reduced phosphatase activity after MYPT1 knockdown. PLK1 is an essential mitotic kinase that is upregulated in many cancers. Our finding of a novel MYPT1-mediated regulatory pathway for PLK1 sheds new light on how PLK1 controls cell division in both normal and cancer cells.

Int6 and Moe1 Interact with Cdc48 to Regulate Mitotic Progression in Schizosaccharomyces pombe

J. H. Otero, 1 J. Sun, 1 E. C. Chang, 1 Cell and Molecular Biology, Baylor College of Medicine, Houston, TX, 2Breast Cancer Center, Baylor College of Medicine, Houston, TX, 3Molecular and Cellular Biology Department, Breast Cancer Center, Baylor College of Medicine, Houston, TX

INT6 is a key gene involved in the formation of breast cancer, as its frequent site of integration by the mouse mammary tumor virus. To better understand the functions of INT6, we have characterized an Int6 homolog (Yin6) in the fission yeast Schizosaccharomyces pombe. Our data support a model in which Yin6 regulates many mitotic functions, including spindle formation, chromosome segregation, and mitotic exit by controlling the localization and assembly of subunits of the proteasome. In our study, I will further characterize Yin6 by focusing on an evolutionarily conserved protein, Moe1, to which Yin6 binds directly. In two-hybrid screens, we have isolated several Moe1-binding proteins that can bind ubiquitinated proteins. Of these, we are particularly interested in Cdc48, which like Yin6 and Moe1, regulates proteasome-mediated protein degradation and spindle dynamics. In support of the data, our biochemical evidence demonstrates that Cdc48 binds Yin6 and Moe1 in S. pombe. Additionally, genetic analyses show that mutations affecting cdc48, together with mutations inactivating either yin6 or moe1, act synergistically to create a mitotic defect by affecting spindle dynamics. My data also show that human Cdc48 rescues the phenotype of yeast cdc48 null mutant. In collaboration with other members of our lab, we found that Int6 and Moe1 also binds Cdc48 in human cells. These observations support the hypothesis that the Yin6-Moe1 complex can interact with Cdc48 to regulate mitotic progression in an evolutionarily conserved fashion.

Organization of Replication Sites in HeLa Cells

A. Ligasova, 1 J. Malinsky, 1 J. Raska, 1, 4 K. Kobetina 1 Department of Cell Biology, Institute of Experimental Medicine, Prague, Czech Republic, 2Department of Microscopy, Institute of Experimental Medicine, Prague, Czech Republic, 3Department of Cell Biology, Institute of Physiology, Prague, Czech Republic, 4Institute of Cellular Biology and Pathology, 1st. Faculty of Medicine, Prague, Czech Republic

We have performed high-resolution analysis of replication sites in HeLa cells during S phase. On the light microscopy level replication sites were visualised as fluorescent foci. The number of fluorescent foci increases rapidly in early S phase from several tens to around 1000 per cell nucleus. This number gradually decreases from mid to late S phase. In late S phase, only around 100 replication foci per cell nucleus were found. They were usually located around those observed in early S phase cells. By contrast, on the electron microscopy level 2100 replication domains (RDS) exhibited DNA synthetic activity in average cell nucleus in early and mid S phase and 1400 RDS in late S phase. Thereby, we suppose that the fluorescent foci are composed of one, several or even tens of RDS. The size of the single RDS is about one tenth of the size of the fluorescent foci. According to our results, replication domains persist in cell nucleus as structural entities also after the completion of their DNA synthesis and during next cell cycle. The size of such domains is substantially reduced with respect to replication domains with ongoing DNA synthesis. It can reflect post-replicationary re-folding of the chromatin. Electron microscopy analysis of the number of replication sites in mitotic HeLa cells showed the apparent increase in the number of replication sites in mitotic cells in comparison to S phase cells. We assume that this can be attributed to the complete separation of chromosomes pairs and their reorganization during mitotic changes of chromatine organization. This work has been supported by grant from the Grant Agency of the Czech republic 304/05/0374, from AVOZ 50390512 and from the Ministry of Education, Youth and Sports MSM0021620806.
Phosphorylation of a Motif Universally Conserved within the Cdc34 Ubiquitin Conjugating Enzymes Regulates SCF Substrate Abundance

R. R. Cocklin; Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, IN

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R. R. Cocklin; Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, IN

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Substrate specificity for ubiquitin-dependent protein degradation is in large part achieved by the presence of numerous ubiquitin ligases or E3 enzymes. This family of enzymes is necessary for the final step of ubiquitination whereby ubiquitin is attached to a lysine moiety of the substrate proteins. The SCF family of ubiquitin ligases are multisubunit complexes which ubiquitylate specific cyslins and cyclin dependent kinase inhibitors in a cell cycle dependent fashion. Ubiquitylation of protein substrates by the SCF ubiquitin ligase complexes is controlled primarily through phosphorylation of the substrate proteins which generates a binding site for the recognition component of the SCF complex, the F-Box protein. We demonstrate here that an additional mechanism for regulation of these complexes is via phosphorylation of Cdc34, the ubiquitin conjugating enzyme that functions in concert with the SCFcs. Cdc34 itself is phosphorylated upon several serine residues that constitute part of a motif is highly conserved within the entire family of Cdc34 ubiquitin conjugating enzymes. It is known that these amino acid residues are completely dispensable for Cdc34 catalytic activity in certain molecular contexts and thus likely to be serving a regulatory function. Furthermore, these phosphorylation events are regulated by treatment with phenformin in yeast. Loss of phosphorylation prevents proper signaling through the yeast mating pheromone pathway and leads to gross misregulation of the SCF substrates, Sic1, Far1 and Cln1.

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Dual Roles of the Hepatitis B Virus X Protein in the ATM-dependent DNA Damage Response: Activation and Silencing

S. Kim, S. Park, W. Lim, H. Says, J. Lee, H. Cho; Biochemistry & Molecular Biology, Ajou University School of Medicine, Suwon, Republic of Korea, Department of Tumor Genetics and Biology, Graduate School of Medical Science Kumamoto University, Kumamoto, Japan

Eukaryotic cells have evolved a complex defense mechanism to maintain genomic integrity, which is often destroyed in cancer cells. During the chronic infection of the hepatitis B virus (HBV), HBx viral protein plays a multifunctional role in the development of hepatocellular carcinoma (HCC). Here, we attempted to investigate whether HBx viral protein alters genomic integrity during the pathogenesis of HCC. We found that one of the DNA double strand breakage markers, γ-H2AX foci were significantly increased in HBx expressing cells, which was accompanied with the activation of Chk2, a downstream kinase of DNA damage signaling. Consequently, the negative phosphorylation of Chk1 was increased and cell cycle progression was delayed at G1 phase. Inhibition of ataxia telangiectasia mutated (ATM) and ATM and Rad3 related (ATR) proteins, the upstream kinase of DNA damage checkpoint abrogated the HBx induced G1 delay. Intriguingly, low dose of irradiation (1 Gy) in Chang cells activated the DNA damage checkpoint pathway, evidenced by recruitment of 53BP1 to the DNA foci, increase of the phospho-Chk2 and consequent cell cycle delay at G1 phase. In contrast, the same dose of irradiation in HBx-expressing cells failed to transduce the DNA damage signaling and did not induce any delay in cell cycle progression. Taken together, we concluded that the activation of the DNA damage-induced signaling pathway by HBx viral protein weakens the ability of cells to respond extrinsic DNA damage response, which may provide a chance for increasing DNA damage accumulation.
Expression of Mesothelin Is Increased by Taxol at the G2/M Phase of Cell Cycle in Human Lung Carcinoma Cells

M. Ho, I. Bera, M. Willingham, M. Onda, R. Hassan, D. FitzGerald, I. Pastan; Laboratory of Molecular Biology, National Cancer Institute, NIH, Bethesda, MD, Department of Pathology, Wake Forest University School of Medicine, Winston-Salem, NC

Mesothelin is a glycosyl-phosphatidyl-inositol-anchored glycoprotein highly expressed in various human solid tumors including ovarian, pancreas and mesothelioma. To evaluate whether mesothelin can be a therapeutic target for the immunotherapy of lung cancer, we examined the expression of mesothelin in lung cancer tissue and cells. We also examined the effect of Taxol on the expression of mesothelin in Hela cells. The expression of mesothelin in lung cancer tissues was analyzed by immunohistochemistry and Western blotting. We observed that mesothelin expression is increased by Taxol at the G2/M phase of cell cycle in human lung adenocarcinoma cells. The expression of mesothelin is upregulated by Taxol in Hela cells and the increase is associated with the mitotic phase of cell cycle. The results suggest that Taxol interacts with the G2/M phase of cell cycle in which the canonical Wnt/beta-catenin signaling pathway is activated. This work indicates that mesothelin is a novel cell cycle-related gene. The association of mesothelin overexpression with Taxol resistance may provide a new therapeutic opportunity for treating chemotherapy-resistant lung cancer.

Regulation of the G2/M Transition by Sp1 and Plo1 Kinases
E. Lambrea, S. Lopez-Aviles, G. Mora, A. Fajardo, R. Aliguet; Cell Biology, University of Barcelona, Barcelona, Spain

The sp1 gene encodes a serine/threonine protein kinase involved in alteration of cell polarity in Schizosaccharomyces pombe. Previous studies have shown that Sp1 is required for alteration of growth polarity and actin localization after osmotic stress as well as during normal cell division cycle. The sp1 deletion causes stress sensitivity, reminiscent of defects in the stress-activated MAP kinase Sty1/Spc1. The sp1 deletion mutant exhibits monopolar actin distribution and a delay in G2/M transition (Matsusaka et al, 1995; Rupes et al, 1999). We have found that Sp1 function requires Srk1 kinase, a calmodulin-dependent protein kinase homologue in fission yeast. Srk1 kinase has a role in regulating the onset of mitosis. Deletion of srk1 gene is not essential but srk1 mutant cells divide at reduced size (9-10 μm) compared to wild type cells (14 μm). Srk1 is a nuclear kinase whose overexpression causes G2/M arrest. The G2/M arrest is dependent on Srk1 kinase and also on the Cdc25 mitotic phosphatase. Deletion of srk1 gene rescues the lethal overexpression of sp1 and it also rescues the G2/M delay of srk1 mutant cells. These results suggest that Sp1 and Srk1 are in the same pathway regulating the G2/M cell cycle transition. Moreover, recent studies have shown that Plo1 kinase is necessary for cells to undergo mitosis after DNA damage. Considering the role of Srk1 in cell cycle control, we tested a possible relation between these two kinases. Further studies describing how Srk1 phosphorylates Plo1 in vitro will be presented.

A Journey to the Centrosome for the Cdc25C Phosphatase
J. Wu, H. Jiang, W. Yang, H. Li; Children's Memorial Research Center, Feinberg School of Medicine, Northwestern University, Chicago, IL

Cdc25kinases play a central role in regulation of cell cycle progression through activating dephosphorylation of cyclin-dependent kinases. In humans three genetically distinct Cdc25 isoforms have been identified: A, B and C, with different structural and functional characteristics. Although the temporal windows of action of these phosphatases are intricate and overlapping, Cdc25B and C are most renowned for their implication in regulation of mitotic progression. It is thought that Cdc25B is responsible for triggering entry into mitosis, through initial activation of cdk1-cyclin B at centrosomes, whilst Cdc25C would act at a slightly later stage, promoting further progression through mitosis through amplification of this kinase activity in the nucleus. However the precise function of Cdc25C in mitosis, and its spatio-temporal pattern of activation still remain poorly characterized. We have found that Cdc25C localizes to centrosomes in cultured mammalian cells in G2, where it colocalizes with phosphorylated cyclin B in prophase. Moreover the first phosphorylated forms of Cdc25C are detected at the centrosome, and a fraction of Cdc25C persists at this organelle throughout mitosis and cytokinesis. We find that centrosomal localization of Cdc25C is independent of its catalytic activity and of its ability to interact with 14-3-3 and have characterized the molecular determinants involved. In addition, FRAP experiments demonstrate that like cyclin B, the centrosomal fraction of Cdc25C is mobile and rapidly exchanged with G1 cells. Intriguingly, the cyclin B levels in G2 phase of BubR1-depleted cells appear to be lower than those of control SIRNA transfected cells; however, early activation of the cyclinB/Cdk1 kinase in G2 phase was accompanied in BubR1-depleted cells. Moreover, immunofluorescence staining with antibodies against cyclin B and gamma-tubulin revealed early cyclin B localization at the centrosomes during G2 progression. This is the first indication that BubR1 regulates the G2-mitotic phase transition.

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M. Ho, T. Bera, M. Willingham, M. Onda, R. Hassan, D. FitzGerald, I. Pastan; Laboratory of Molecular Biology, National Cancer Institute, NIH, Bethesda, MD, Department of Pathology, Wake Forest University School of Medicine, Winston-Salem, NC

Mesothelin is a glycosyl-phosphatidyl-inositol-anchored glycoprotein highly expressed in various human solid tumors including ovarian, pancreas and mesothelioma. To evaluate whether mesothelin can be a therapeutic target for the immunotherapy of lung cancer, we examined the expression of mesothelin in lung cancer tissue and cells. We also examined the expression of mesothelin in normal lung tissue and cells. We observed that mesothelin is expressed on the cell surface of lung adenocarcinoma cells. In A549 cells, mesothelin expression is increased by Taxol at the G2/M phase of cell cycle in which the canonical Wnt/beta-catenin signaling pathway is activated. This work indicates that mesothelin is a novel cell cycle-related gene. The association of mesothelin overexpression with Taxol resistance may provide a new therapeutic opportunity for treating chemotherapy-resistant lung cancer and possibly other mesothelin-expressing solid tumors.

The p38 Mediated Checkpoint Delays Mitosis Under Stress in Normal Cells, but Is Progressively Lost with Tumorigenic Transformation
A. V. Mikhailov, D. Patel, D. McCance, C. L. Rieder; Molecular Medicine, Wadsworth Center of the NYS Dept of Health, Albany, NY, Biochemistry and Biophysics, University of Rochester, Rochester, NY
When growing cultures are exposed to various stresses, cells in the early stages of chromosome condensation (i.e., late G2) are rapidly delayed from entering mitosis via a cell cycle checkpoint mediated by the p38 kinase. This checkpoint protects the cells from dividing under stressful conditions which can lead to errors in chromosome segregation resulting in polyploidy or aneuploidy. We used Ansiomycin to rapidly activate this checkpoint in non-transformed, partially transformed and fully transformed cell lines. We found that when p38 is suddenly activated, entry into mitosis is rapidly and completely blocked for 4-5 hours in all primary, telomerase- or spontaneously immortalized (p53/pRb+) cells we examined. However, comparable or stronger p33 activation does not delay, or only briefly delays, entry into mitosis in tumorigenic cell lines. While there are many rapid, microplate compatible assay formats for looking generically at cell proliferation, not many are able to address mitosis specifically. A common mechanism-of-action for many anti-proliferative drugs involves disruption of cellular mitosis. While there are many ways to do this, we have developed an alternative approach optimized for small molecule or siRNA profiling and screening. This method utilizes human-derived biosensor cells that report mitotic events using positional complementation of an inactive β-galactosidase. The cells have been engineered to express two complementing fragments of β-galactosidase within different cellular compartments. When the nuclear envelope breaks down at the onset of mitosis, the two components interact in the cytoplasm, forming an active enzyme that is able to generate a detectable chemiluminescent or fluorescent signal. This unique approach provides a functional cell-based assay for screening chemical libraries for anti-mitotics, with the benefit of analysis on a simple luminometer as opposed to image analysis. Data will be shown highlighting the use of this biosensor system for both compound screening and siRNA profiling.

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A Novel, Homogeneous Biosensor Assay for Identification of siRNAs and Small Molecules That Inhibit Mitosis

M. Mathrubutham, K. R. Olson; R&D, DiscoverRx Corporation, Fremont, CA

Disruption of cell cycle progression is a key intervention point in many drug development programs focused on cancer biology. While detailed cell biology analysis is critical to determine the mechanism of action for candidate compounds, this would be used as therapeutics, there is an equally important need for assays that allow for rapid analysis of a large number of small molecule candidates that show the potential for cell cycle perturbation. A common mechanism-of-action for many anti-proliferative drugs involves disruption of cellular mitosis. While there are many rapid, microplate compatible assay formats for looking generically at cell proliferation, not many are able to address mitosis specifically. One common approach that is specific to mitosis involves high-content image analysis with DNA-specific dyes and a phospho-histoneH3 antibody labeling. While effective, this approach presents challenges with respect to profiling a large number of molecules in a timely manner. We will present an alternative approach optimized for small molecule or siRNA profiling and screening. This method utilizes human-derived biosensor cells that report mitotic events using positional complementation of an inactive β-galactosidase. The cells have been engineered to express two complementing fragments of β-galactosidase within different cellular compartments. When the nuclear envelope breaks down at the onset of mitosis, the two components interact in the cytoplasm, forming an active enzyme that is able to generate a detectable chemiluminescent or fluorescent signal. This unique approach provides a functional cell-based assay for screening chemical libraries for anti-mitotics, with the benefit of analysis on a simple luminometer as opposed to image analysis. Data will be shown highlighting the use of this biosensor system for both compound screening and siRNA profiling.

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A Subthreshold Concentration of Starfish Oocyte Maturation Hormone Causes Nuclear Entry of Cdc2/cyclin B, But Not GVBD

M. Terasaki; Cell Biology, University of California Health Center, Farmington, CT

Immature starfish oocytes are arrested in prophase of meiosis I. The maturation hormone 1-methyladenine (1-MA) at a concentration of 1 μM causes activation of Cdc2/cyclin B, resulting in GVBD after ~20 min. Using confocal imaging of cycling B-GFP in living oocytes, we previously found that Cdc2/cyclin B is present in aggregates that disperse ~7 min after 1-MA, followed a minute or two later by the beginning of Cdc2/cyclin B entry into the nucleus (MBC 14: 4685). If 1 μM 1-MA is removed when the aggregates are just beginning to disperse, the aggregates disperse completely and a significant amount of Cdc2/cyclin B enters the nucleus, but GVBD does not occur. Similarly, if oocytes are exposed to a subthreshold dose of 0.1 μM 1-MA, the aggregates disperse and Cdc2/cyclin B partially enters the nucleus without GVBD. These results indicate that the commitment to M phase occurs after the beginning of Cdc2/cyclin B entry into the nucleus. If oocytes that have been in subthreshold 1-MA for 1 hr are put into 1 μM 1-MA, GVBD occurs in ~12 min. The same speed-up also occurs if oocytes are kept in 0.1 μM 1-MA for 18 hrs, which indicates that oocytes can remain in a prolonged, intermediate state of activation.

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T56-Bcl-2 Is a Specific In Vivo Substrate of Cyclin B1/Cdk1

J. W. Jacobberger, R. M. Sramkoski, P. S. Frisa, R. K. Wetzel; Division General Medical Sciences, Case Western Reserve University, Cleveland, OH, “Cell Signaling Technology, Inc., Danvers, MA

Background: A central part of cell regulation biochemistry are specific kinase - phosphatase systems. Generally, for a specific system activity is measured by immunoprecipitation and autokinolysis of phosphorylated substrates or lyase immunoblottting with phosphorylation state specific antibodies (phospho-antibodies). An alternative approach is to quantify immunoreactivity of phospho-antibodies by cytochemistry. These assays are easier and more accurate than western blotting, but this value increases significantly when the measurement is made within the mitotic context. The object here was to obtain a measure of cyclin B1/Cdk1 activity as a function of the cell cycle in asynchronous cells.

Methods & Materials: Human cell lines and stimulated lymphocyte samples, treated with kinase inhibitors, stimulators, and specific small interfering RNAs (siRNAs), were fixed, reacted with antibodies to cyclins B1 and A, mitotic epitopes (MPM-2 and phospho-S10-histone H3), phospho-T56-Bcl-2, and stained for DNA content; then measured by flow cytometry.

Results: The intensity of phospho-T56-Bcl-2 was highest in mitotic cells that were cyclin B1 (B1) positive and pBcl2 and B1 were correlated in mitotic cells. Cyclin A positivity was not correlated. Alsterpaullone reduced pBcl2 reactivity as a function of dose and time. Specific siRNA for cyclin B1 eliminated the positive signal, but siRNA for cyclin B2 or cyclin A did not. Several other non-Cdk1 kinase inhibitors did not inhibit reactivity. Small molecule stimulators (okadaic acid; taxol) both increased reactivity that was inhibitable with alsterpaullone.

Conclusion: Phospho-T56-Bcl-2 is a specific substrate of cyclin B1/Cdk1 in mitotic cells. The period of activity is phaseprophase by late mitosis, although activity decreases significantly after metaphase.

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Xp95/Alix Is Phosphorylated within the Proline-rich Domain during Xenopus Oocyte Maturation and Mitotic Arrest of Mammalian Cells

R. Dejournet, R. Kobayashi, S. Pan, C. Wu, R. B. Clark, J. Kuang, O. Bogler; Dept. of Neurosurgery and Neuro-oncology, MD Anderson Cancer Center, Houston, TX, Dept. of Molecular Pathology, MD Anderson Cancer Center, Houston, TX, Dept. of Experimental Therapeutics, MD Anderson Cancer Center, Houston, TX, Dept. of Molecular Genetics, MD Anderson Cancer Center, Houston, TX, Dept. of Integrative Biology and Pharmacology, The University of Texas Medical School, Houston, TX

Xp95 is the Xenopus laevis ortholog of a conserved family of scaffold proteins that have in common a C-terminal proline-rich domain (PRD) and an N-terminal Brol domain. Although it is thought that the family of proteins interact with multiple partner proteins that participate in diverse cellular (endocytosis, apoptosis) and disease processes (tumor suppression and retroviral budding), their regulation is poorly understood. We previously demonstrated that Xp95 undergoes a phosphorylation-dependent gel mobility shift during progesterone-induced meiotic maturation of Xenopus oocytes, a model of M-phase induction. To further characterize phosphorylation of Xp95, we undertook two approaches. First, Xp95 was purified from immature and mature oocyte extracts and analyzed by mass spectrometry. We found a 5080 Da peptide (residues 706-756), which is phosphorylated at T745 and at least one additional site in mature oocytes. T745 is within the conserved binding site to SETA/CIN85/SHEKBP1, an adaptor protein involved in EGF receptor endocytosis. Xp95 phosphorylation at T745 was shown to inhibit SETA interaction: Xp95 from immature but mature oocytes can bind SETA in vitro, and a phospho-T745 peptide of Xp95 shows reduced binding to recombinant SETA. Xp95 phosphorylation was also characterized by identifying the minimal region (residues 705-786) which was sufficient for the gel-mobility shift during oocyte maturation. Extending these results to the mammalian ortholog, Alix, we determined that Alix also undergoes a gel-shift during mitotic arrest (MA), similar to Xp95 during Xenopus oocyte maturation. Further, deletion of residues 717-784 of Alix was sufficient to inhibit the gel-shift of Alix during MA. Lastly, transfected Xp95 in MA cells failed to bind SETA but instead could bind AMSH, a deubiquitinsase participating in vesicle sorting. Together, these findings indicate that Xp95/Alix is phosphorylated within the PRD during M phase induction and suggest that this phosphorylation regulates interaction with binding partners.

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Ypi1: A Positive Regulator of Nuclear Protein Phosphatase Type 1 during Mitosis in Saccharomyces cerevisiae

J. P. Bharucha, L. Gao, K. Tatchell; Biochemistry and Molecular Biology, Louisiana State University Health Sciences Center, Shreveport, LA

PP1 is a highly conserved phospho-serine/threonine phosphatase with roles in diverse cellular processes such as chromosome segregation, muscle contraction, and synaptic transmission. The activity of the enzyme is regulated by the association of the catalytic subunit with different regulatory subunits. In many eukaryotes, including yeast, nuclear PP1 plays an important role in cell cycle regulation, although little is known about the subunit(s) that regulates this activity. In S. cerevisiae, YPI1, an essential gene, encodes a small protein that shares homology with mammalian PP1 inhibitor-3. Ypi1 interacts with Gcl7 (yeast PP1) and inhibits its activity in vitro (Garcia-Gimeno et al., J. Biol. Chem. 278, 47444-S2). To examine its role in vivo, YPI1 was placed under the control of a galactose-inducible promoter. Ypi1 deletion causes cells to arrest in G2 of the cell cycle, due to activation of the spindle assembly checkpoint. There is a
concomitant decrease in nuclear Gli7, which localizes aberrantly as foci throughout the cells. Nuclear levels of Sds22, an essential Gli7 regulatory subunit that also interacts with Ypi1, also decrease following depletion of Ypi1. A mutant of Ypi1 whose product fails to interact normally with Gli7 also results in reduced nuclear Gli7 and Sds22, and suppresses the temperature sensitivity of a mutant in the yeast A. oryzae B ortholog (ypl1-2). The nuclear localization of Ypi1-13Myc is lost in the temperature sensitive sds22-6 mutant. These in vivo results, together with studies on other YPI1 mutants and genetic interactions between YPI1, GLC7, and SED22 alleles, suggest that Ypi1 and Sds22 act together to positively regulate nuclear Gli7 activity during the mitotic cell cycle.

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A Functional Link between Kinetochore and Spindle Assembly
A. D. McAinsh, 1 J. Winter, 1 A. Toso, 2 P. Meraldi 2; Chromosome Segregation Laboratory, Marie Curie Research Institute, Oxed, United Kingdom, 2Institute of Biochemistry, ETH Zurich, Zurich, Switzerland

Kinetochore are large multiprotein machines that perform at least two critical functions during cell division: (1) they promote the bipolar attachment of duplicated sister-chromatids by forming bridges between centromeric DNA and the plus-end of spindle microtubules; (2) they act as sensors of microtubule attachment, delaying anaphase onset via the spindle checkpoint in the presence of incorrect attachments. We have recently discovered a further function in which defective kinetochores, arising from the depletion of the kinetochore protein Mcm21R (CENP-0), can act in a dominant fashion to interfere with normal spindle assembly. Live cell imaging of double stable Histone2B-EGFP/ubrin-mRed HeLa cells reveals that Mcm21R-depleted cells fail to efficiently assemble a bipolar spindle. This phenotype is therefore distinct from that of Clasp, in which bipolar spindles form transiently, then collapse. How do kinetochores influence the assembly of the mitotic spindle? Kinetochore in Mcm21R-depleted cells remain correctly attached to microtubules, although cold treatment of cells causes the destabilization of α-fibers. This suggests that kinetochore-localized Mcm21R may act to stabilize microtubules, which in turn, is compatible with normal spindle assembly. Consistent with this idea, stabilization of microtubule plus-ends by depletion of the microtubule destabilizing kinesin MCAK or treatment with low doses of taxol can suppress the spindle assembly defect in Mcm21R-depleted cells. Future work aims to determine the precise molecular mechanism by which Mcm21R and related proteins control spindle assembly.

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Pre-Mitotic Clustering of Centromeres May Help Efficient Microtubule Search-and-Capture in Mitosis in Drosophila S2 Cells
H. Shroff, C. Gradinaru, R. Wollman, R. Vale, G. Goshima; Physiology Course, Marine Biological Laboratory, Wood's Hole, MA

Centromeres are specialized chromosome regions that build kinetochore scaffolds that capture dynamic centrosomal microtubules during prometaphase (search-and-capture model). However, a recent theoretical study revealed that the mechanism of search-and-capture alone is insufficient to account for the rapid capture of all centromeres that is observed in vivo. Here, we present experimental and computational data suggesting that centromere clustering in the prophase nucleus may enhance search-and-capture efficiency. By observing Msi2-GFP and DAPI as centromere and nuclear markers, respectively, we found that centromeres are not uniformly distributed but are rather preferentially clustered near the center of the nucleus in interphase and prophase Drosophila S2 cells. Using our experimentally measured centromere distributions, computer simulations showed a significant reduction in the microtubule search-and-capture time compared to the case where centromeres are uniformly distributed in the nucleus. Preliminary observations suggest that centromeres are clustered around nuclear RNAs, particularly the nucleolus. Our results show that centromeres are preferentially clustered near the center of Drosophila nuclei, which we suggest facilitates the rate of achieving metaphase alignment. 1. Wollman R, Cytrynbaum EN, Jones JT, Meyer T, Scholey JM, Mogilner. Curr Biol. (2005) May 10;15(9):828-32.

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The Conserved KMN Network Constitutes the Core Microtubule Binding Site of the Kinetochore
I. M. Cheeseman, A. Desai; UCSD, Ludwig Institute for Cancer Research, La Jolla, CA

The microtubule-binding interface of the kinetochore is of central importance in chromosome segregation. Although kinetochore components that stabilize, translocate on, and affect the polymerization state of microtubules have been identified, none have proven essential for kinetochore-microtubule interactions. Here, we examined the conserved KNL-1/Mis12 complex/Necl40 complex (KMN) network, which is essential for kinetochore-microtubule interactions in vivo. Previous work has suggested that these proteins function indirectly to generate kinetochore-microtubule attachments through a role in kinetochore assembly. We found that partially purified endogenous KMN network binds directly to microtubules in vitro suggesting that it is closely associated with a microtubule binding activity. Using bacterial co-expression to reconstitute these proteins, we identified two distinct microtubule binding domains within the KMN network. Future work aims to determine the precise molecular mechanism by which Mcm21R and related proteins control spindle assembly.

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Crystal Structure of a TOG Domain: Conserved Features of XMAP215/Dis1 Family Proteins and Implications for Tubulin Binding
J. Al-Bassam, 1 A. Hyman, 2 S. C. Harrison 1,2; Department of Molecular Pharmacology and Biological Chemistry, Harvard Medical School, Boston, MA, 2Max Planck Institute for Molecular Cell Biology and Genetics, Dresden, Germany, 3Howard Hughes Medical Institute, Boston, MA

Stu2p from budding yeast and XMAP215 from Xenopus belong to the conserved Dis1/XMAP215 family of microtubule-associated proteins (MAPs) that are essential for mitotic division. A common feature of MAPs in this family is the presence near the NH2 terminus of two to five, 250-residue conserved repeats termed TOG domains. Our recent work has shown that TOG domains in Stu2p associate directly with tubulin dimer and microtubule ends. When it captures a tubulin heterodimer, Stu2p homodimer undergoes a large conformational transition from an open structure to a closed one around tubulin. We have determined the structure of a single TOG domain from the C. elegans ortholog Zyg9 to 1.9 Angstrom resolution using x-ray crystallography. A TOG domain is a flat paddle-like structure composed of six HEAT repeat domains that are stacked side by side. There is a strong pattern of sequence conservation in multiple TOG domains within each family and among different members of the Dis1/XMAP215 family. The most conserved solvent-exposed residues of the TOG domain are in the turns between the first and second helices of each HEAT repeat. These conserved turns appear on a single face of the paddle-like TOG structure. We propose that this conserved face of the TOG domain contacts a tubulin heterodimer.

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The Human Spindle Checkpoint Protein Bub3 Regulates Kinetochore-Microtubule Attachments
E. Logarinho, T. Resende, C. Torres, H. Bousbaa; Molecular and Cellular Biology, ICS-N, CESPU cru, Gandra PRD, Portugal

Accurate segregation of chromosomes during mitosis is ensured by spindle checkpoint mechanisms that monitor kinetochore-microtubule (K-MT) attachments and induce a mitotic delay until full metaphase alignment is achieved. Recent data have implicated the checkpoint proteins Bub3 and BubR1 in the regulation of chromosome-to-spindle attachments. Unlike these proteins whose roles during mitosis have been well defined, Bub3 role has remained poorly studied. Here we show, using RNA interference and small molecule inhibitors, that besides its role in spindle checkpoint activity, Bub3 is also required for proper K-MT attachment. Chromosomes in Bub3-depleted cells are able to congress and organize a metaphase plate that, however, typically exhibits misaligned chromosomes. Although both aligned and misaligned kinetochores attach to microtubules in Bub3-depleted cells, these attachments are defective in terms of stability, microtubule occupancy and pulling force generation. Comparative analyses between Bub3, Bub1 and BubR1 depletion phenotypes indicate that whereas chromosome attachment and alignment are severely compromised following BubR1 repression, in the absence of Bub3 or Bub1, attachments are still established which, though defective, account for significant chromosome congression. Importantly, analysis of Bub3/Bub1, Bub3/BubR1 or Bub1/BubR1 double-depletion phenotypes strongly suggests that Bub3 acts differently from BubR1 and cooperatively with Bub1 in the regulation of K-MT attachments.

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Investigating the Spindle Checkpoint in Early C. elegans Embryos
A. W. Essex, 1 I. Cheeseman, A. Dammermann, K. Oegema, A. Desai 1; Biomedical Sciences, University of California, San Diego/Ludwig Institute for Cancer Research, San Diego, CA, 2Laboratory of Chromosome Biology, Ludwig Institute for Cancer Research, San Diego, CA

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The spindle checkpoint delays anaphase onset until all chromosomes are properly attached to the spindle. Previous studies have shown that BUB-1 and the Rod/Zwilch/Zw10 (RZZ) complex are essential for checkpoint activation and also have checkpoint-independent functions in chromosome segregation. We are utilizing the early C. elegans embroyo to investigate BUB-1 and RZZ complex functions and to analyze the relationship between checkpoint structure and checkpoint activation. To develop a robust functional assay for checkpoint activation in C. elegans, we have explored the consequences of deleting ZYG-1, a kinase required for centrosome duplication. Following ZYG-1 depletion, the first mitotic division is normal, but monopolar spindles form in both daughter cells in the second division. The presence of monopolar spindles results in a striking delay in the metaphase-anaphase transition, assessed using chromosome decondensation and onset of cortical contractility in live imaging data. Double depletion of ZYG-1, and MAD-1/MDF-2 does not affect first division timing but abolishes the delay observed in the second division. Using an integrated strain expressing GFP-MAD-2, we have found that GFP-MAD-2 localization is enriched on unattached kinetochores in monopolar spindles. By performing double depletions of ZYG-1 with other checkpoint pathway and structural kinetochore proteins, we have defined the requirements for GFP-MAD-2 localization and checkpoint activation.

We have also independently utilized quantitative assays to characterize the chromosome segregation defects observed following BUB-1 and RZZ complex inhibition. Our results indicate that both of these components contribute to the timely establishment of mechanically stable kinetochore-microtubule connections after nuclear envelope breakdown.

A Complex of Sli15-Bir1 (INCENP-Survivin) Connects Centromeres to Microtubules and Is the Likely Tension Sensor Controlling Aurora B Activation

Spindly, a Novel Regulator of Mitotic Dynein Activity

Aurora B Phosphorylation of Hec1/Ndc80 Is Required for Mitotic Spindle Assembly

Regulation of Kinetochore-Microtubules by Hec1 and Aurora B Kinase
The Multiple Roles of Mps1 Kinase Activity in Human Mitosis

N. Jelluma, R. H. Medema, G. J. P. Kops; Medical Oncology, UMC Utrecht, Utrecht, The Netherlands

The mitotic checkpoint is the primary guardian against chromosome missegregation that result in aneuploidy, a hallmark of human cancers. At the heart of the mitotic checkpoint are three kinases, Mps1, Bub1 and BubR1 that are responsible for the transduction of the checkpoint signal from unattached kinetochores. Bub1 and BubR1 have additional roles in mitosis, as both are required for chromosomes to properly attach to the spindle and Bub1 is needed for maintenance of centromeric cohesion. It is unclear whether the phospho-transfer activities of the three kinases are required for the mitotic checkpoint or for any of the additional roles in mitosis in human cells. We show here that Mps1, like BubR1 and Bub1, has multiple functions in mitosis, all of which require its kinase activity. siRNA-driven depletion of Mps1 abrogates the mitotic checkpoint and causes massive chromosome missegregations and cell death. Replacement of endogenous Mps1 with exogenous kinase-deficient protein proved that Mps1 kinase activity is indispensable for checkpoint function and viability. Like BubR1 and Mps1, Mps1 participates in the timing of mitosis, as furrow ingression follows nuclear envelope breakdown twice as fast in Mps1-depleted cells vs. control. This increased rate of mitotic progression is reverted to normal by exogenous wild-type Mps1 but not kinase-deficient Mps1. Analysis of chromosome movements in Mps1-depleted cells in real-time furthermore showed that ~80% of cells had severe chromosome alignment problems. Of these, ~75% could not reach full alignment within 2-3 hours when forced to stay in a checkpoint-independent manner. Mps1 kinase activity was also requisite for this novel mitotic function of Mps1. In short, Mps1 kinase activity is critical for mitotic checkpoint signaling, proper timing of mitosis, chromosome alignment, and viability. This places the Mps1 kinase as a central activity in controlling proper execution of mitosis.

Vor-tebrate Slugoshin 1 Is a Substrate for Aurora B Kinase and Necessary for Kinetochore Assembly

J. Pouwels, A. M. Kukkonen, W. Lan, P. T. Stuenenberg, M. J. Kalliio; VTT Medical Biotechnology, Turku, Finland, 3Department of Biochemistry and Molecular Genetics, University of Virginia Medical School, Charlottesville, VA, 4Center for Biotechnology, University of Turku, Turku, Finland

We have previously shown that Aurora B is required to generate spindle checkpoint signals and assemble kinetochores, yet the exact mechanisms of these reactions are not known. Slugoshin 1 (Sgo1) has a role in protecting the centromeric cohesion between sister chromatids, implying that Sgo1 is implicated in the tension sensing system and in regulation of kinetochore microtubule stability. We investigated the fine localization of Sgo1 in HeLa cells at the light microscope level by (Sgo1) has a role in protecting the centromeric cohesion between sister chromatids, both in somatic cells and in meiocytes. The mechanism of Sgo1 mediated protection of cohesion is unknown, but recent studies point to an essential role for dephosphorylation of cohesion by protein phosphatase 2A (PP2A). Sgo1 is functionally related to Aurora B kinase as both have been implicated in the tension sensing system and in regulation of kinetochore microtubule stability. We investigated the fine localization of Sgo1 in HeLa cells at the light microscope level by comparing localization of YFP-Sgo1 to the localization of several kinetochore marker proteins. During prophase and prometaphase YFP-Sgo1 accumulates at the inner-kinetochore between the Aurora B and Hecl1 domains. Sgo1 diminishes at each kinetochore after the chromosome arrives at the metaphase plate. Depletion experiments using siRNA in HeLa cells showed that Sgo1 binding to kinetochores depends on INCENP, Bub1, but not PIK1, Cenp-F and the spindle checkpoint protein BubR1. In a reverse experiment, we discovered that the kinetochore localization of PIK1, Bub1 and CENP-F, but not INCENP, Bub1 and Hecl1 depended on Sgo1. Xenopus Sgo1 is phosphorylated by Aurora B kinase in vitro. Moreover, depletion of the Aurora B activator INCENP by siRNA or chemical inhibition of Aurora B activity disrupts the kinetochore localization of Sgo1 and instead causes Sgo1 to accumulate on chromosome arms. These data demonstrate that Sgo1 plays a central role in proper kinetochore assembly and suggest that Aurora B kinase works through Sgo1 to regulate the spindle checkpoint and kinetochore function.

Polo-like Kinase 1 Phosphorylation and Promotes Translocation of MAD1 during G2-M Transition

Y. Chi, H. Kerstin, K. Jeang; NIAID, NIH, Bethesda, MD

The mitotic spindle assembly checkpoint (SAC) monitors attachment of duplicated chromosomes to spindle poles and ensures proper chromosome segregation in mitosis. Mitotic arrest deficient protein 1 (MAD1) is a component of the mitotic spindle assembly checkpoint. MAD1 associates with nuclear pore complex (NPC) in interphase and translocates to kinetochores as the nuclear envelope breaks down. Kinetochore-attachment by MAD1 is proposed to trigger MAD1 to depart from MAD1 and to associate with CDC20-APC/C complex leading to inhibition of premature chromosome segregation. Currently, how MAD1 is regulated during the G2-M transition is unknown. Polo-like kinase 1 (PLK1) is a Ser/Thr kinase that plays a critical role in regulating APCC/ and mitotic progression. Here we show that MAD1 is hyper-phosphorylated in M phase, and that MAD1 and PLK1 co-localize at the nuclear periphery during nuclear envelope breakdown. When the cell progresses into prometaphase, both MAD1 and PLK1 translocate to kinetochores. Knocking down PLK1 using RNAi abolished the kinetochore localization of MAD1 and erased the hyperphosphorylation of MAD1. We present evidence that PLK1 phosphates MAD1 in the latter’s N-terminus to regulate MAD1 function.

Identification of Phosphoprotein Complexes Associated with the Mitotic Apparatus


The chromosomal passenger complex (CPC) controls chromosome, cytokinesis and membrane dynamics during mitosis. By performing mass spectrometry, imaging and biochemical analyses on mitotic complexes immunoprecipitated by a phosphorylation specific antibody that cross reacts with the CPC, we identified known and potential CPC interacting proteins and a mitotic phosphorylation motif on a subset of these proteins. Proteins identified in the phosphoprotein complexes have linkages to the CPC (Aurora-B, INCENP, TD-40, cdc11, and protein phosphatase 2A), the anaphase promoting complex/cyclosome (cdh1, SMAD2), microtubule regulation (ch-TOG, stathmin-1, NudMA, cytoplasmic dynein, MAPs, tubulin), cytokinesis (Ric-8, actin, myosin, rac1, clathrin, actin-binding lim protein 1) and cell signaling pathways (SMAD2, catenins, casein kinase, cyclin B, 14-3-3 proteins, small G proteins). Phosphopeptide analysis confirmed ch-TOG and Ric-8, two proteins important for mitosis, in kinetochore assembly and cytokinesis respectively, are phosphorylated on a motif recognized by the antibody. We infer, based on co-immunoprecipitation experiments, that INCENP and cdc11 are phosphorylated during mitosis and, in the case of INCENP, this phosphorylation is associated with CPC complexes containing Aurora-B. Phospho-specific antibodies demonstrate that the TGF-beta signaling molecule, SMAD2 and Polo-like kinase 3 (Pik3) are phosphorylated on this motif during mitosis. Moreover, Pik3 phosphorylation correlates with its localization on mitotic sites that overlap with Pik1 and the CPC. These results characterize a novel receptor for the analysis of mitotic phosphoprotein complexes and identify its phosphorylation motif that may regulate the recruitment (or activation) of proteins to the mitotic apparatus and the CPC.

The Arp2/3 Complex Requires Phosphorylation of the Arp2 Subunit to Nucleate Actin Filaments


The actin-related protein (Arp) 2/3 complex is the primary nucleator of new actin filaments in most crawling cells. Nucleation promoting factors (NPFs) of the WASP/Scar family are the currently recognized activators of the Arp2/3 complex. We now report that phosphorylation of the Arp2 subunit is critical for activation of the Arp2/3 complex by NPFs. Arp2 phosphorylation increased in cells in response to growth factors and was confirmed in purified Arp2/3 complexes from Acanthamoeba, bovine, and yeast, and from baculovirus-expressed recombinant Arp2/3. We identified phosphorylation of conserved T237, T238, and Y202 and used computational modeling of bovine Arp2/3 to predict that T237 and Y238 interact with conserved R105 and R106 in the p20 subunit and pY202 interacts with a conserved R490 in the Arp3 subunit to induce a conformational change and align Arp2 and Arp3 subunits to resemble an actin dimer. Testing this prediction, mutation revealed that Arp2 must be phosphorylated on either T237/238 or Y202 to nucleate actin filaments. When dephosphorylated by either PP2Cα or YOP or a dual specificity phosphatase the Arp2/3 complex does not nucleate actin filaments. Threonine and tyrosine-dephosphorylation of Arp2/3 is able to bind the sides of actin filament and NPFs but cannot bind the pointed-ends of gelsolin-capped actin seeds. The functional significance of Arp2 phosphorylation sites was confirmed in Drosophila S2 cells, which form lamellae. S2 cells transformed with Arp2
Functional Mapping of Residues Involved in Arp2/3 Complex Activation by Nucleation-promoting Factors

A. D. Siripsa, E. D. Goleuy, E. A. Znarsers, M. D. Welch; Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA

Actin nucleation and filament branching by the Arp2/3 complex requires binding to activating factors that include nucleotide, actin, and nucleation-promoting factors (NPFs). However, the precise molecular interactions involved in complex activation remain unclear. To identify sites of interaction with binding partners and gain insight into the molecular machinery of Arp2/3 complex activation, we have performed site-directed mutagenesis on conserved Arp2/3 complex surface residues. We have identified several mutations in putative NPF binding regions of Arp2 and Arp3 that reduce the nucleating activity of the complex. Preliminary experiments suggest that mutation of these surface patches in Arp2 and Arp3 reduces the affinity of the complex for the WCA domain of the NPF WASP. Of the putative WASP-binding surfaces that we identified in Arp2 and Arp3, mutations in Arp3, but not in Arp2, also inhibit binding of the complex to the NPF cortactin, suggesting that the two NPFs share distinct binding sites, yet also differ in their interactions with the Arp2/3 complex. To identify complimentary NPF residues that are critical for Arp2/3 binding and activation, we have also generated a series of mutations in the Arp2/3-binding domain of WASP. Preliminary results suggest that WASP C-region residues that are required to promote Arp2/3 complex activation are also required to induce a conformational change in the complex, as measured by fluorescence resonance energy transfer (FRET). We will compare the effect of these mutations on NPF affinity with their effects on Arp2/3 conformation, to determine whether NPF binding, conformational change, and activation are mediated by the same residues, or whether they are separable activities. By developing a more detailed picture of the molecular interactions and conformational changes required for Arp2/3 activation, we hope to better understand the complex process by which multiple factors come together to promote regulated actin polymerization in the cell.

Distinct Roles of Myo1p and Wsp1p in Fission Yeast Actin Patch Assembly

V. A. Sirotkin, K. Macmillan, T. D. Pollard; Molecular, Cellular and Developmental Biology, Yale University, New Haven, CT

Depletion of two different subunits of the Arp2/3 complex in B35 neuroblastoma cells resulted in altered cell morphology, severe depletion of F-actin at the leading edges, inhibition of growth cone formation, and death of the cells. In addition, filopodia were absent. Filopodia are long, narrow extensions of the cell surface that are thought to protrude from the cell in a directed manner and to mediate contact guidance. The protrusion of the cell's leading edge is driven by two types of actin-based organelles, lamellipodia and filopodia, which are thought to form in a coordinated manner. Structural, kinetic and immunochemical analyses of growth cones in two neuroblastoma cell lines and two types of primary neurons revealed that filopodia are formed from lamellipodia-like veins, which have denticrystall organization and contain Arp2/3 complex and cortactin. Correlative electron microscopy demonstrated that, similar to other cells, filopodia in neuronal cells are formed by the conversion of lamellipodia to filopodia. Functional characterization of the Arp2/3 complex in the protrusive behavior of neuronal cells was tested by RNAi approach. Depletion of two different subunits of the Arp2/3 complex in B35 neuroblastoma cells resulted in altered cell morphology, severe depletion of F-actin at the leading edges, inhibition of growth cone formation, and death of the cells. In this work, we analyzed the mechanisms of protrusion in neuronal growth cones, which are thought to resemble filopodia in two neuroblastoma cell lines and two types of primary neurons revealed that filopodia are formed from lamellipodia-like veins, which have denticrystall organization and contain Arp2/3 complex and cortactin. Correlative electron microscopy demonstrated that, similar to other cells, filopodia in neuronal cells are formed by the conversion of lamellipodia to filopodia.
Traction forces impinged on the extracellular matrix (ECM) by migrating cells are dynamically regulated to create a gradient of force from the cell front to the rear. Mechanical forces are generated in the actin cytoskeleton by myosin-II motors and transmitted to the ECM via dynamic macromolecular assemblies called focal adhesions (FA). However, little is known about how dynamics of the contractile actomyosin cytoskeleton is translated into a spatiotemporally regulated pattern of traction forces exerted on the ECM. To address this question, we have developed compliant cell culture substrates compatible with both the glass trasecco microcopy and fluorescent speckle microscopy (FSM). Far red 40-nm latex spheres are co-polymerized into a polyacrylamide (PAA) gel that is covalently bound to both the glass coverslip; the gel thickness, controlled by the volume of PAA per coverslip, is varied between 5-15 microns and fibroinect is coupled to the PAA surface using established techniques. PtK1 epithelial cells are cultured overnight and we express GFP constructs of the FA protein, paxillin and micro-inject x-rhodamine labeled G-actin. Using a multi-spectral spinning disk confocal microscope, we image actin, paxillin and spheres every 10 sec. The spheres are embedded at sufficiently high density to obtain a near diffraction-limited deformation map of the substrate. Using established techniques, we reconstruct the force field generated by the cell from these substrate deformations to visualize dynamic maps of cellular traction with submicron resolution. To assess the assembly, disassembly and motion of the actin cytoskeleton with high spatiotemporal resolution, we use quantitative FSM. By correlating the speed and direction of actin, FA size and the magnitude and direction of the traction force, we hope to elucidate the biophysical mechanisms by which an actomyosin network generates traction force through substrate adhesions. This is of essential importance to further our understanding of the underlying biophysical regulation of cell migration.

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Spire and Cappuccino - Coordinated Actin Nucleators

M. E. Quinnan,1 E. Ekerhoff,2 R. D. Mullins3; 1Cell and Molecular Pharmacology, UCSF, San Francisco, CA, 2Institut für Medizinische Strahlenkunde und Zellforschung, Universität Würzburg, Würzburg, Germany

Spire (Sp) and Cappuccino (Capu) are actin nucleation factors required to establish polarity of Drosophila oocytes. Their mutant phenotypes are nearly identical, and they interact genetically. We found that Sp and Capu proteins physically interact in vitro and in vivo, and that this interaction regulates the actin nucleation and microtubule binding activities of Capu. Direct coordination between distinct actin nucleators has not been described before and begs the question of why cells require a complex that can nucleate by two mechanisms and bind microtubules.

To address this question we have studied the individual components and their interactions. Spire forms directly nucleate and elongate actin filaments through their carboxy-terminal FH (formin homology) domain and FH2 domains. Although molecular function of formin has been uncovered using actin assembly assay with pyrene-labeled actin as the standard method, the cellular roles of each formin in mammalian cells are not fully understood. To study actin polymerization in the intracellular environment, we established a simple in vitro actin polymerization assay using cytosol. Briefly, glutathione beads coated with recombinant GST-FH1-FH2 of formin were incubated with human platelet cytosol in the presence of ATP to allow actin to polymerize on beads. After incubation, beads were washed, fixed and stained with rhodamine-labeled phalloidin.

The fluorescence was observed with a fluorescence microscope and quantified with a fluorometer. The actin polymerization in the cytosol was dependent on ATP and temperature. GST or GST-FH2 alone could not polymerize actin, suggesting that some G-actin sequestering factor like profilin exists in human platelet cytosol, which is consistent with the fact that excess actin polymerization does not occur in the resting platelets. Daam1 is a member of formin family abundantly expressed in platelets and its FH2 domain consists of five regions (lasso, linker, knob, coiled-coil and post) like yeast Bni1p. Using newly established assay here, we demonstrated that Daam1 had actin polymerizing activity and the length, not specific amino acid sequence, of its linker region was significant for polymerization. This assay is simple and reproducible and could become a powerful tool to investigate the behavior of actin molecule in the intracellular environment.
PfACTI and PfACTII, which are similar to TgACT1 and also diverge from conventional actin. Molecular modeling was used to identify residues unique to apicomplexan actin that may affect polymerizes into very short filaments. Remarkably, TgACT1 has a low critical concentration, but apparently fails to fully anneal and is thus unstable.

Actin filament turnover is crucial to motility as shown by the paralyzing effects of jasplakinolide, an agent that stabilizes actin filaments. Apicomplexans contain few predicted for a Brownian ratchet. In addition, motion was saltatory with a broad distribution of step sizes that is correlated in time. These data point to a model in which thermal fluctuations of propelled microspheres using three-dimensional laser tracking. In these experiments, velocity was inversely proportional to viscosity and proportional to the amplitude of bead fluctuations as determined from videos of vectorial force transients. This result is consistent with cofilin's ability to enhance actin filament turnover and our finding that cofilin is required for EGF-induced actin filament assembly in the cell edge by supplying actin monomers for polymerization, not by directly creating new barbed ends through its severing activity. By measuring the fluorescence decay of locally photoactivated Drosha-actin in the cytoplasm, we analyzed the actin monomer population in the cytoplasm of living cells and showed that cofilin inactivation by LIM-kinase expression reduced the cytoplasmic actin monomer population and coexpression of a cofilin mutant, which has severing activity but only weak depolymerizing activity, significantly rescued it. These results suggest that the severing activity of cofilin is required for supramolecular activity in the cytoplasm. They also showed that the cytoplasmic actin monomer population correlates well with the rate of actin monomer incorporation into the tip of the lamellipodium. Cofilin inactivation suppressed the EGF-induced decrease in the rates of new Arp2/3-mediated cortical filaments not only appear to provide mechanical support but adjust the surface interactions of the vesicles as well. Our artificial cell-like system has initiated an avenue to bridge the interior of our vesicles; these structural modes are directly related to the response of the artificial cells. By analyzing images and force curves in atomic force microscopy studies, the actin filaments not only appear to provide mechanical support but adjust the surface interactions of the vesicles as well. Our artificial cell-like system has initiated an avenue to bridge the understanding of living cells with in vitro biopolymers. These results will be useful in a wide range of fields including polymer physics, cell biology, and biotechnology.

Coronin Synergizes with Cofilin In Vitro and In Vivo to Drive Rapid Turnover of Actin Filaments

M. Gandhi, B. Balcer, M. Jangi, B. Goode; Rosenthal Basic Medical Sciences Research Center, Brandeis University, Waltham, MA Dynamic remodeling of cell shape and infrastructure requires the on-going and rapid turnover of actin networks, yet the mechanisms driving actin filament disassembly have only just begun to emerge. Here, we describe a new role in promoting actin turnover for a conserved component of the actin cytoskeleton, coronin. To date, two functional activities of coronin have been described, filament bundling and direct inhibition of Arp2/3 complex, both of which require the coronin coiled coil domain. We show that budding yeast coronin (Cmr1) genetically interacts with cofilin, and that loss of Cmr1 in vivo reduces rates of actin patch and cable turnover and exacerbates the actin turnover defects of a cof1-22 mutation. A construct lacking the coronin coiled coil domain, Cmr1 (1-460), is sufficient to complement the genetic interaction between Cmr1 and cof1, indicating that the mechanism by which coronin promotes actin turnover is distinct from its previously described activities. Consistent with these genetic results, purified Cmr1 (1-460) enhances cofilin-dependent acceleration of actin filament disassembly and amplification of barbed ends in vitro. Using site-directed mutagenesis, we mapped the actin-binding surface to conserved surface residues on Cmr1 and demonstrate that actin binding is required for coronin’s function in promoting actin turnover. Further, we show that Cmr1 increases the apparent affinity of cofilin for actin filaments, suggesting a possible mechanism for the turnover function. We are now investigating how this new cellular function is integrated with coronin regulation of Arp2/3 activity and whether it is a conserved function. Coordination of the effects of coronin on Arp2/3-dependent actin assembly and cofilin-dependent disassembly may be important in a variety of species, where coronin promotes endocytosis and cell motility.

Coronin Synergizes with Cofilin In Vitro and In Vivo to Drive Rapid Turnover of Actin Filaments

Villin Severing Activity Enhances Actin Based Motility

C. Revenu, M. Coustoix, C. Sykes, D. Louvard, S. Rohme; 1UMR144, Institut CURIE, Paris, France, 2UMR168, Institut CURIE, Paris, France Villin, a protein associated with actin in microvilli, bundles, caps, macropodia and severs actin in a calcium-dependent manner in vitro. We hypothesised that villin severing activity is responsible for its reported role in enhancing cell plasticity and motility. To test our hypothesis, mutations were introduced in villin to impair this activity. By pyrene-actin assays, we demonstrate that this mutant has a strongly reduced severing activity whereas nucleation and capping remain unaffected. The bundling activity and the morphogenic effects of villin in cells are also preserved in this mutant. We thus succeeded in dissociating the severing from the other three activities of villin. To analyse the contribution of villin severing to actin dynamics, we reconstituted in an vitro actin-based motility assay in which the usual capping protein is replaced by either the WT or the severing mutant of villin. Villin severing activity enhances the velocity of beads by more than two folds and reduces the density of actin in the comets. We propose a model in which by severing actin filaments and capping their barbed-ends, villin increases the concentration of actin monomers available for polymerisation, a mechanism that might be parallelled in vivo when an enterocyte undergoes an epithelio-mesenchymal transition. We are now analysing the impact of the loss of villin severing activity on the motility of epithelial cells and on the infectious process of Shigella flexneri. The role of villin severing activity will also be tested in vivo by trying to rescue with this mutant the lack of plasticity of the villin knock-out mice.

Understanding Biological Structures through Exploring the Mechanical Response of Artificial Cell-like Systems

Y. Zhang, C. Cheng, B. Cusick, T. Kowalewski, P. LeDuc; 1Mechanical Engineering, Carnegie Mellon University, Pittsburgh, PA, 2Chemistry, Carnegie Mellon University, Pittsburgh, PA The mechanical response of a living cell is highly tuned to the structural environment that is provided by the cytoskeleton. This architectural arrangement, which has many components, provides both structural and organizational regulation that is essential for complex cellular response such as chemotaxis and mitosis. While many investigations have provided insight into determining the mechanical response of the cell from either an in vivo or in vitro perspective, a significant gap exists in determining how this response is bridged between the components such as the individual cytoskeletal elements and the higher order organism such as the living cell. To address this gap and to understand this highly efficient and hierarchical organization, we construct an intermediate model by mimicking the cell cytoskeleton in an artificial environment composed of membrane-like spherically distributed lipid bilayers. We establish an experimental model by first encapsulating G-actin into giant unilamellar vesicles using an electroformation method and then we polymerize actin filaments (F-actin) within individual liposomes. Through visualizing our system by epi-fluorescent and laser confocal microscopy, we observe that actin filaments remain inside the artificial cell membrane near the bilayer or as free filaments within the interior of our vesicles; these structural modes are directly related to the response of the artificial cells. By analyzing images and force curves in atomic force microscopy studies, the actin filaments not only appear to provide mechanical support but adjust the surface interactions of the vesicles as well. Our artificial cell-like system has initiated an avenue to bridge the understanding of living cells with in vitro biopolymers. These results will be useful in a wide range of fields including polymer physics, cell biology, and biotechnology.

Experimental Confirmation of the Brownian Ratchet Mechanism of Actin-based Motility

J. W. Shaevitz, D. A. Fletcher; 1Integrative Biology, UC Berkeley, Berkeley, CA, 2Bioengineering, UC Berkeley, Berkeley, CA A Brownian ratchet mechanism has been proposed to couple actin polymerization to cellular translocation through the rectification of thermal motions. We tested this model by following actin-propelled microspheres using three-dimensional laser tracking. In these experiments, velocity was inversely proportional to viscosity and proportional to the amplitude of bead fluctuations as predicted for a Brownian ratchet. In addition, motion was saltatory with a broad distribution of step sizes that is correlated in time. These data point to a model in which thermal fluctuations of the microsphere, and not the actin filaments, govern motility. This conclusion is supported by Monte carlo simulations of an adhesion-based Brownian ratchet.

Divergent Actin in Apicomplexan Parasites

K. M. Skillman, K. Tang, N. Sahoo, D. Sept, L. D. Sibley; 1Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, MO, 2Department 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 gliding motility mechanism that is dependent on polymerization of parasite actin. During gliding, myosin motors anchored in the inner membrane complex translocate actin filaments rearward. The actin is linked to secreted cell surface adhesins that move along the surface, resulting in forward motion of the parasite. In non-motile parasites, the majority of actin is monomeric and filaments only assemble upon initiation of gliding motility. Actin filament turnover is crucial to motility as shown by the paralyzing effects of jasplakinolide, an agent that stabilizes actin filaments. Apicomplexans contain few conventional actin binding proteins, but a conserved profilin and actin-depolymerizing factor are likely important for regulating turnover. During gliding, actin and profilin recycle from diffuse and punctate distributions in the cytoplasm, respectively, to the parasite periphery. 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. Molecular modeling was used to identify residues unique to apicomplexan actin that may affect filament stability. These studies reveal critical subdistinctions in the hydrophobic plug and in an intramolecular salt bridge in parasite actins. Consistent with these changes being responsible for
2233 Actin Filament Fragmentation and Annealing Are Responsible for Unexpectedly Large Length Diffusivities Measured In Vitro
J. Fass, J. Bamburg, A. Mogilner 1; Mathematics, University of California Davis, Davis, CA; 2; Biochemistry and Molecular Biology, Colorado State University, Fort Collins, CO
Recent studies of actin dynamics in vitro have found unexpectedly large length diffusivities that are ~30-fold greater than expected. Stochastic modeling attempting to address this discrepancy predicted that high diffusivity resulting from random exposure of the ADP-actin core should occur below but not at the critical concentration, or that the controversial assumption of vectorial hydrolysis is necessary to match observed diffusivities. However, these models disregarded fragmentation and annealing. We incorporated these two processes into multi-filament, variable volume stochastic simulations in order to evaluate their contributions to empirically observed length diffusivities. We found that annealing and fragmentation - even at the higher levels expected from the use of labeled actin - do not change the distribution of nucleotide states at the barbed end. Nevertheless, the inclusion of fragmentation and annealing yield length diffusivities comparable to those observed experimentally (~30 mon/sec). Thus we conclude that fragmentation and annealing directly increase actin filament length diffusivity, explaining recent experimental results.

2234 The Hsp90 Co-Chaperone Unc45b Targets the Myosin Motor Domain
R. Sriakulam, L. Liu, D. A. Winkelmann; Pathology and Laboratory Medicine, Robert Wood Johnson Medical School, Piscataway, NJ
Myosin folding and assembly in striated muscle is mediated by the general chaperones Hsc70 and Hsp90. This pathway may involve a myosin specific co-chaperone, UNC45. C. elegans UNC45 is a founding member of the Ucs family of proteins that all share sequence homology in a putative myosin binding domain. These genes are known to affect myosin dependent cellular processes such as muscle contraction, establishment of cell polarity, cytoskeleton, and vesicle transport. In addition to the C-terminal UCS domain, UNC45 has N-terminal TPR motifs that are common to many Hsp90/Hsc70 co-chaperones and a central domain with β-catenin homology repeats. Two UNC45 genes are found in vertebrates, including a striated muscle specific form, Unc45b/SM-UNC45, that is believed to mediate the folding and/or assembly of striated muscle myosin. We have investigated the role of Unc45b in myosin folding. Epitope tagged marine Unc45b was expressed in and isolated from muscle and non-muscle cells. Under physiological conditions, Unc45b isolates as a stable complex with the endogenous Hsp90. This interaction can be disrupted at high ionic strength, and purified Unc45b re-binding human Hsp90 in solution and rabbit Hsp90 when diluted into a reticulocyte lysate. Unc45b binds the partially folded myosin motor domain when incubated with myosin subfragments synthesized in a reticulocyte lysate. This binding is independent of the myosin rod or light chains, and Unc45b does not bind native myosin subfragments. Furthermore, Unc45b enhances the folding of the myosin motor domain when included in the lysate during synthesis. Thus, mammalian Unc45b forms a stable complex with Hsp90, binds an unfolded configuration of the myosin motor domain, and promotes motor domain folding during de novo synthesis. (Supported by NIH grant R01 AR38454)

2235 Myosin-IIA Heavy Chain Phosphorylation Regulates the Motility of Carcinoma Cells
N. G. Dullanovna, R. P. House, A. R. Bresnick; Biochemistry, Albert Einstein College of Medicine, Bronx, NY
In non-muscle cells, multiple mechanisms control the localized formation of myosin-II filaments. We demonstrated previously that the assembly of myosin-IIA is regulated by phosphorylation on the heavy chain and the binding of S100A4, a member of the calcium-regulated S100 family of proteins. Phosphorylation on the heavy chain by either protein kinase C or casein kinase 2 (CK2) significantly inhibited the assembly of myosin-IIA into filaments. In addition, CK2 phosphorylation inhibited the binding of S100A4 to myosin-IIA, thus providing an additional level of regulation for the S100A4/myosin-IIA interaction. To investigate the mechanisms controlling filament assembly and disassembly during motility and chemotaxis, we examined the EGF-dependent phosphorylation of the myosin-IIA heavy chain in human breast cancer cells. EGF stimulation of MDA-MB-231 cells resulted in transient increases in both the insolubility and phosphorylation of the myosin-IIA heavy chains. Phosphoamino acid analysis and two-dimensional tryptic phosphopeptide maps revealed that in EGF-stimulated cells, the myosin-IIA heavy chain is phosphorylated on the CK2 site (S1944). The interaction of CK2 with myosin-IIA was confirmed in a pull-down assay, utilizing His-tagged myosin-IIA rods. In vitro assays demonstrate that the assembly properties of myosin-IIA rods containing aspartic or glutamic acid substitutions at S1944 are similar to those of CK2-phosphorylated rods; however, these substitutions had no effect on S100A4 binding. Expression of full-length GFP-myosin-IIA heavy chains containing alanine, aspartic or glutamic acid substitutions at S1944 showed that S1944E and S1944D mutants were primarily cytosolic, whereas the S1944A mutant associates over assembled into stress fibers and cortical regions. Cells expressing the S1944E and S1944D mutants displayed increased migration into a wound and enhanced EGF-stimulated lamellipod extension as compared to cells expressing wild-type myosin-IIA. In contrast, cells expressing the S1944A mutant exhibited reduced migration and lamellipod extension. These observations suggest a direct role for myosin-IIA heavy chain phosphorylation in mediating motility and chemotaxis.

2236 Studies of Dictyostelium Myosin II Heavy Chain Kinase C Subdomains
M. Russ, O. Al, C. Alyssa, P. Padilla, P. A. Steinle; Biology, University of North Carolina at Greensboro, Greensboro, NC
Myosin II in Dictyostelium and nonmuscle cells exists in a dynamic equilibrium between a cytoplasmic pool of monomers and a cytoskeleton-associated assembly of bipolar filaments. Myosin II filament disassembly in Dictyostelium is driven by phosphorylation of the myosin II heavy chain (MHC) "tail" region via the activities of three structurally-related MHC kinases: MHCK-A, -B, or -C. All three of the MHCKs share homologous catalytic and WD-repeat domains. MHCK-A is the most extensively-studied of the MHC kinases. Recent studies from our lab have revealed a unique relationship between MHCK-A and F-actin, whereby F-actin is a potent activator of MHCK-A activity (50-fold); and MHCK-A, in turn, possesses the ability to organize actin filaments into bundles. In contrast to MHCK-A, there is essentially no information about the structure-function relationships defining the activities of the MHCK-B and -C enzymes. We have examined the in vivo function of the WD-repeat domain of MHCK-A by over-expressing either full-length MHCK-C (MHCK-C++) or a truncated version of MHCK-C lacking the WD-repeat domain (MHCK-CΔWD++) in Dictyostelium cells, and then assaying for myosin II-dependent activities such as cytokinesis and multicellular development. As has been reported previously (Betapudi et al., 2005, Mol Biol Cell, 16:2248), MHCK-C++ cells exhibited cytokinesis defects with reduced growth in suspension culture and increased multicellularity. Interestingly, we found that MHCK-CΔWD++ cells also do not proliferate in suspension culture; however, this growth defect is not accompanied by an increase in multicellularity. In addition, the MHCK-CΔWD++ cells, unlike their MHCK-C++ counterparts, retain the ability to undergo multicellular development. Together, these results suggest that the WD-repeat domain of MHCK-C may not serve the same myosin targeting function as the WD-repeat of MHCK-A. Moreover, our results also indicate that under certain conditions, MHCK-C may exhibit activities outside of its known MHC kinase function in the cell.

2238 The Role of C. elegans UNC-82 Serine/Threonine Kinase in Myosin Organization in Striated Muscle
A. R. Reedy, C. M. Carter, P. E. Hoppe; Biological Sciences, Western Michigan University, Kalamazoo, MI
Mutations in the unc-82 gene cause defects in thick filament organization in striated muscle (Waterston et al., 1980). UNC-82 is a serine/threonine kinase orthologous to human ARAK5 and SNARK. A rescuing UNC-82-GFP fusion localizes at or near the M-line (Chass, Flanagan, Schriefer, and Hoppe, unpublished). In current studies we are using antibody staining and FRAP analysis to define the role of UNC-82 in cytoskeletal organization. Defects in the localization of myosin and the M-line protein UNC-89/obscurin are apparent in all unc-82(e1323) null mutant embryos by the 3rd stage of embryogenesis. Abnormal aggregates of myosin are observed in addition to the organized lines of myosin signal. Integrin and vinculin localization appear normal at this stage, suggesting that UNC-82 acts in a process independent of the hypothetical-muscle-cell signaling required for early patterning of the contractile apparatus. Embryos and adults homoygous for the putative kinase-dead allele unc-82(e1220) exhibit less severe defects; however, the unc-82 catalytic domain promotes M-line or thick filament organization. To test this, we have generated constructs containing canonical kinase-dead mutations for production of transgenic worms. Our current model is that UNC-82 regulates turnover of thick filaments or M-lines during muscle cell growth and body elongation, perhaps through phosphorylation of the myosin tailpiece. The myosin defects in unc-82 mutants are similar to those observed in transgenic animals expressing truncated myosin that lacks the C-terminal phosphorylation motifs. To assess myosin filament turnover, we have introduced GFP-mycosin into mutant and wild-type worms, and monitoring FRAP experiments. Results to date suggest a faster recovery of myosin signal within organized stripes in wild-type worms in comparison to those present in unc-82 mutant animals. The abnormal myosin aggregates in unc-82 mutants show no discernible recovery within the timeframes examined to date.
Phosphorylation of Recombinant Smooth Muscle Myosin Light Chain Kinase by Various Ser/Thr Kinases

A. Nakamura, R. Ishikawa, K. Kohenm; Department of Molecular and Cellular Pharmacology, Gunma University Graduate School of Medicine, Maebashi, Japan

Smooth muscle myosin light chain (MLC) can be phosphorylated by myosin light chain kinase (MLCK) in vitro and in vivo, and it plays an important activating actomyosin-linked contractility in smooth muscle cells. However, it is not clear how MLCK is regulated by upstream signaling. We have already reported the purification of recombinant smooth muscle MLCK (rMLCK) by using an E. coli cold-shock expression system. Using this rMLCK as a substrate, we examined what kind of kinases phosphorylated rMLCK. Usually, the purified MLCK from mammalian cells may be often phosphorylated by endogenous kinase, but this rMLCK is not phosphorylated in E. coli. rMLCK was phosphorylated by MAPK, PAK1, PKA, PKC and ROCK1 in the absence of Ca/calmodulin. Among them PAK1 and PKC could not incorporate phosphate into rMLCK when calmodulin was bound to rMLCK. MLCK may be regulated by these kinases.

Additive Roles of Phosphorylation and the Extended N-terminus of the Regulatory Light Chain in Drosophila Flight Muscle Function

M. S. Miller, G. P. Farman, F. N. Soto-Adames, M. C. Reedey, J. M. Braddock, T. C. Irving, J. O. Vigoreaux, D. W. Maughan; Molecular Physiology and Biophysics, University of Vermont, Burlington, VT, 1BPCS, Illinois Institute of Technology, Chicago, IL, 2Biology, University of Vermont, Burlington, VT. 3Department of Cellular Biology, Duke University Medical Center, Durham, NC

We investigated the effects of myosin regulatory light chain (MLC2) mutations on Drosophila indirect flight muscle (IFM) function. Four MLC2 lines were examined: a rescued null control (Mlc2^-), one with a truncated N-terminal extension (Mlc2^T'), one with disrupted myosin light chain kinase phosphorylation sites (Mlc2^556/557), and one double mutant (Mlc2^556/557, 62/63). Wing beat frequency was significantly reduced in the single mutation lines compared to controls (165 ± 3 Hz, Mlc2^-; 158 ± 3 Hz, Mlc2^556/557, vs. 202 ± 4 Hz, Mlc2^-). The double mutant was unable to beat its wings. Chemically skinned fibers were compressed osmotically to in vivo resting lattice spacing, and mechanically examined in maximally Ca2+-activated (pCa 4.5) fibers using small amplitude (0.125% muscle length) sinusoidal length perturbation analysis. The frequency at which maximum power output occurs, a sensitive indicator of myosin kinetics, was significantly different between Mlc2^- (116 ± 4 Hz), Mlc2^556/557 (101 ± 3 Hz), and Mlc2^556/557, 62/63 (87 ± 3 Hz), and significantly less than control (128 ± 3 Hz). Corresponding power output was significantly reduced in both phosphorylation site mutants, with the double mutant barely able to produce power. Structural data from small angle x-ray diffraction and electron microscopy is consistent with the mechanical results, which together suggest that phosphorylation of conserved serines and the upstream (N-terminal) extension play a complementary, and in some respects additive, role in moving the myosin heads closer to actin and that the phosphorylation sites orient or pre-position the myosin heads for optimum interaction with the actin target sites. We conclude that both structural adaptations help position myosin heads for rapid binding to actin during the extremely brief wing beat cycle, thereby boosting power generation in IFM fibers and the whole fly.

Structure of Two Heads of Rabbit White Skeletal Muscle Myosin

A. Inoue; Biology, Osaka University, Toyonaka, Japan

Myosin has two heads, which react with ATP and bind with actin, however the function of two heads of myosin in muscle contraction has not yet been well clarified. We showed that two heads of myosin have different functions; Head A forms myosin-ATP complex, and head B forms myosin-P-ADP complex, respectively, as an ATPase intermediate, and only head B catalyzes actomyosin-type ATPase reaction and works as a motor, while head A helps the movement of head B, though the structure of heads A and B has not been elucidated. Here, we have cloned the cDNAs of rabbit white skeletal muscle myosin heavy chains A and B, which form heads A and B, respectively, and determined the amino acid sequences of these two heavy chains. It has shown by Elzinga that there exist two amino acids as a microheterogeneity in the amino-acid sequence of myosin heavy chain. We found that these two amino acids derive from the difference in the amino acid between heavy chains A and B. The amino-acid sequences of these two heavy chains differ by about 3 per cent. However, the nucleotides of these two cDNAs differed by almost 10 per cent. Therefore, these two chains are derived from different DNAs. The difference in the amino acid was observed not in the ATPase active site but in the actin binding site. We found that tryptic digestion pattern of 25K-50K junction are different between heads A and B. Actually, the amino-acid sequences of 25K-50K junction were different. Furthermore, there exists heterodimer-forming amino acids pairs in the neck region of myosin. We made 3 sets of heads A and B specific antibodies. These antibodies bound specifically with heads A and B. Therefore, skeletal muscle myosin is the products of these two mRNAs.

Mechanism of Myosin-IIA Regulation by S100A4 during Carcinoma Cell Motility

R. P. House, E. L. Snapp, A. R. Bresnick; 1Biochemistry, Albert Einstein College of Medicine, Bronx, NY, 2Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, NY

S100A4, a member of the Ca2+ regulated S100 family of proteins, has been shown to be a metastasis factor and is implicated in the regulation of both the motility and invasiveness of cancer cells. In vitro studies from our lab demonstrate that S100A4 regulates non-muscle myosin-IIA assembly and promotes the monomeric, unassembled state of myosin-IIA. These observations are consistent with previous reports that S100A4 may sequester myosin-IIA monomers and function as a myosin-IIA inhibitor. However, recent in vivo studies from our lab demonstrate that S100A4 enhances cell polarization and motility, which is inconsistent with S100A4 having an inhibitory function in vivo. An alternative hypothesis is that S100A4 mediates cell motility by enhancing the turnover of myosin-IIA filaments. To investigate these two models of S100A4-mediated regulation of myosin-IIA function, we are using photobleaching methods to examine the dynamics of GFP-tagged full-length non-muscle myosin-IIA heavy chain in EGF-stimulated human breast cancer cells. Our studies in MDA-MB-231 cells suggest two populations of myosin-IIA filaments with distinct patterns of movement during EGF-stimulated lamellipod extension. Studies examining myosin-IIA turnover as a function of S100A4 expression are in progress.

Development of a S100A4 Biosensor to Track Activation In Vivo

S. C. Garrett, A. Toutchkine, K. M. Hahn, A. R. Bresnick; 1Biochemistry, Albert Einstein College of Medicine, Bronx, NY, 2Pharmacology, University of North Carolina at Chapel Hill, Chapel Hill, NC

S100A4, a member of the S100 family of proteins, binds specifically to nonmuscle myosin-IIA in a calcium-dependent manner and regulates the monomer-polymer equilibrium of myosin-IIA filaments. To obtain novel information regarding the spatial and temporal activation of S100A4 during directed cell motility, we developed an S100A4 biosensor to report transient or localized calcium binding in vivo. This unique reagent will allow the activation of S100A4 to be examined directly during motility, and will permit a direct correlation between localized, transient activation of S100A4 and the regulation of myosin-IIA assembly during directed motility. The biosensor was constructed using the novel merocyanine dye ISO-IIIA, which displays sensitivity to both solvent polarity and hydrogen bonding, thus small changes in protein conformation can strongly influence the fluorescence properties of the dye. Similar to other S100 proteins, S100A4 undergoes a significant conformational change upon calcium binding; exposing a hydrophobic cleft that forms the target-binding surface. The biosensor was generated by the derivatization of a single cysteine residue (C81) proximal to the hydrophobic pocket. The resulting biosensor (Mero-S100A4) displays a 3.0-fold increase in fluorescence intensity upon calcium binding, which does not significantly change with myosin-IIA binding. A dillBAPTA calcium competition assay demonstrates that Mero-S100A4 has the same binding affinity for calcium as wild-type S100A4 (Mero: Kd=412 μM, Kd=0.85 μM; wt: Kd=2.37 mM, Kd=0.75mM). In addition, myosin-IIA cosedimentation experiments demonstrate that Mero-S100A4 has the same affinity for myosin-IIA as wild-type S100A4 (Mero: Kd=2.11 μM; wt: Kd=4.32 μM). Fluorescence calcium titration experiments demonstrate that in the presence of myosin-IIA, S100A4 exhibits a substantial increase in its binding affinity for calcium. In 3T3 fibroblasts the biosensor shows diffuse fluorescence without any sign of aggregation. In vivo studies examining S100A4 activation and its relationship to myosin-IIA are in progress.

How Might Microtubules Control Cortical Contractility during Cytokinesis?

V. E. Doe, G. von Dassow, G. M. Odell; Center for Cell Dynamics, University of Washington, Friday Harbor, WA

Using one antibody to myosin heavy chain and another to the activating phospho-ser19 of myosin regulatory light chain we show, using fixed echinoderm blastomeres, that activated myosin II (a small fraction of total myosin II) recruits to the cortex in a RhoA and cell cycle-regulated manner. During anaphase microtubule outgrowth, myosin II falls off the cortex everywhere, then recruits in active form intensely, but only to the presumptive cleavage furrow. Low nM nocodazole doses that reduce microtubule densities, block both this global anaphase dephosphorylation/deactivation and furrow-specific activation of myosin. High (20 nM) nocodazole doses that depolymerize all dynamic microtubules, reveal a population of stable microtubules.
microtubules which, starting at anaphase onset, elongates to form not only the central spindle but also an oriented fan of stable astral microtubules aimed chiefly toward the future furrow, and which disappears during abscission. Their formation schedule, the finding by Shannon et al. (2005) that taxol-stabilized microtubules can induce furrowing, and the proximity of their plus ends to areas of cortical myosin recruitment, lead us to believe these stable microtubule convey signals that activate equatorially Rhodamin-A/myosin II during furrow formation. We propose a mechanism wherein: 1) active Ect2 (RhoA GEF) binds microtubules (by complexing with MgcRacGAP/MKLP1) to the cortex than dynamic microtubules, which depolymerize out from beneath kinetosomes moving along them; 2) MgcRacGAP/MKLP1 (which transports Ect2) does not run off microtubule plus ends (Nishimura & Yonemura, 2006) but collects preferentially at tips of stabilized MTs boosting Rho/A/myosin II activation in an equatorial zone. Our computer simulations demonstrate this mechanism can produce, simultaneously, a diffusion-resistant band of Rho/A/myosin activation wherever the highest density of stable microtubule tips lie, and a general diminution of cortical Rho/A/myosin II activation elsewhere.

2245 Nonmuscle Myosin II-dependent Force Inhibits Cell Spreading and Drives F-actin Flow Y. Cai, 1 N. Bains, 1 G. Gianmone, 1 M. Tanase, 1 G. Jiang, 1 J. Hofman, 1 C. Wiggins, 2 C. Buguin, 2 A. Buguin, 3 B. Ladoux, 4 M. Sheetz, 1; 1Biological Sciences, Columbia University, New York, NY, 2Applied Physics and Mathematics, Columbia University, New York, NY, 3Physico-Chimie Curie, Institut Curie, Paris, France, 4Matière et Systèmes Complexes, Université Paris, Paris, France

Nonmuscle myosin IIA (NMIIA)-IA is involved in the formation of focal adhesions and neurite retraction. However, the role of NMIIA in these functions remains largely unknown. Using RNA interference (RNAi) as a tool to decrease NMIIA expression, we have found that NMIIA is the major myosin involved in traction force generation and retrograde F-actin flow in mouse embryonic fibroblasts (MEFs). Quantitative analyses revealed that ~60% of traction force on fibronectin-covered surfaces is contributed by NMIIA and ~36% by NMIIIB. The retrograde F-actin flow decreased dramatically in NMIIA-depleted cells, but seemed unaffected by NMIIIB deletion. In addition, we found that depletion of NMIIA caused cells to spread at a higher rate and to a greater area on fibronectin substrates during the early spreading period, whereas deletion of NMIIB appeared to have no effect on spreading. The distribution of NMIIA was concentrated on the dorsal surface and approached the ventral surface in the periphery whereas NMIIIB was primarily concentrated around the nucleus and to a lesser extent at the ventral surface in cell periphery. Our results suggest that NMIIA is involved in generating a coherent cytoplasmic contractile force from one side of the cell to the other through the croslinking and the contraction of dorsal actin filaments.

2246 Cortical Actomyosin Dynamics and the Maintenance of PAR Polarities in the C. elegans Embryo H. R. G. Clarke, C. M. Schoe, E. M. Munro; Center for Cell Dynamics, University of Washington, Friday Harbor, WA

The C. elegans egg polarizes in response to a transient cue associated with the sperm centrosomes/microtubule organizing center (MTOC). AP polarity is established during interphase when the sperm MTOC triggers an asymmetrical actomyosin contraction and cortical flows that carry the conserved polarity determinants PAR-3/PAR-6/aPKC to the anterior and allow others (PAR-1 and PAR-2) to associate with a complementary posterior domain. However, the mechanisms that maintain PAR asymmetries between the end of interphase and the onset of cytokinesis remain unclear. Here we explore how actomyosin assembly and activity are involved in the maintenance of PAR polarities. During the maintenance phase, both F-actin assembly and myosin recruitment are biased to an anterior domain defined by high levels of PAR-3/PAR-6/aPKC, and these are closely associated with persistent anterior-directed cortical flows. Myosin recruitment and cortical flow during maintenance absolutely require the small GTPase CDC-42 and its downstream effector, ROKC (Mystic dystrophy kinase-related Cdc42-binding kinase), a known activator of myosin contractility. Anterior-biased F-actin assembly during maintenance requires components of the conserved ARP-2/3 complex, which promotes the assembly of branched F-actin meshworks. ARP-2/3 is not required for the establishment of PAR polarities, but depletion of ARP-2/3 components leads to hyper-contractation and mechanical destabilization of the anterior PAR-3/PAR-6/aPKC cap during the maintenance phase, and a corresponding anterior extension of the posterior PAR-2 domain. Interestingly, mutations in the posterior PAR protein PAR-1 also lead to hyper-contraction of the anterior cap and simultaneous removal of PAR-1 and ARP-2 produce a strongly synergistic effect. We hypothesize that robust maintenance of PAR asymmetries requires two redundantly acting mechanisms: One involves conserved cross-inhibition among anterior and posterior PAR proteins; the other involves the asymmetrical contraction of actomyosin against an anterior domain that is stiffened by the assembly of a dense network of branched actin filaments.

2247 Myosin-II Activity Regulates the Development of Neuronal Polarity K. M. Collins, H. Francisco, J. Ha, G. Gallo; Drexel University College of Medicine, Philadelphia, PA

The earliest stage in the development of neuronal polarity is characterized by the extension of undifferentiated “minor processes” (MPs). Subsequently, MP growth and differentiation gives rise to structurally and functionally distinct neurites, terminated axons and dendrites. The cellular and molecular mechanisms underlying the initial outgrowth of MPs remain unclear. Through interactions with actin filaments, myosin II regulates cellular contractility and axonal extension; therefore, we investigated the role of myosin II in MP extension using cultured forebrain neurons. Both myosin IIA and IIB isoforms were expressed in developing MPs, with the IIA isoform predominating. To examine the role of myosin II in MP extension we treated neurons with the myosin II inhibitor, blebbistatin, which resulted in a 76% increase in MP length. Thus, myosin II activity restricts MP extension. Myosin II activity is determined by phosphorylation of its regulatory light chains (rMLC), which can be mediated by myosin light chain kinase (MLCK) or RhoA-kinase (ROCK). Pharmacological inhibition of MLCK or ROCK increased MP length by 22% and 43%, respectively. Combined inhibition of MLCK and ROCK resulted in an 85% increase in MP length, similar to the effect of direct inhibition of myosin II. RhoA/ROCK complex formation results in ROCK activation. Selective inhibition of RhoA signaling with cell-permeable C3 transfase produced increases in both the length (28%) and number (41%) of MPs, suggesting additional pathways for regulation of MP outgrowth. To determine whether myosin II affected the development of neuronal polarity, morphogenesis was examined in cultures chronically treated with direct or indirect myosin II inhibitors. Inhibition of myosin II, MLCK, or ROCK promoted rapid morphogenesis without altering characteristic neuronal polarity. Together, these data indicate that myosin II activity negatively regulates MP extension, and the time course for development of neuronal polarity.

2248 The B2 Alternatively Spliced Isoform of Nonmuscle Myosin II-B Lacks Enzymatic Activity and In Vitro Motility K. Y. Kim, 1 J. Baso, 1 J. R. Sellers, 2 R. S. Adelstein, 1; 1Laboratory of Molecular Cardiology, NHLBI, National Institutes of Health, Bethesda, MD, 2Laboratory of Molecular Physiology, NHLBI, National Institutes of Health, Bethesda, MD

Previous work has demonstrated that the presence of an alternatively-spliced exon in loop 1 near the ATP-binding region of the myosin II heavy chain results in an increase in both the actin-activated MgATPase activity and in vitro motility of vertebrate smooth muscle and nonmuscle heavy meromyosins (HMMs) II-B and II-C. We now report that the presence of a 21 amino acid insert in loop 2, which is near the actin-binding site of nonmuscle myosin heavy chain II-B, results in the loss of those activities. This alternative exon is normally expressed in certain neuronal cells such as the Purkinje cells in the cerebellum and has been shown to affect motor coordination in mice. Surprisingly, the F2249 max of baculovirus-expressed HMM II-B2 (inserted isoform) was less than 7% of that of the noninserted HMM II-B0 and HMM II-B2 compared to 0.15 ± 0.003 s−1 for HMM II-B0. Combined inhibition of MLCK and ROCK resulted in an 85% increase in MP length, similar to the effect of direct inhibition of myosin II. RhoA/ROCK complex formation results in ROCK activation. Selective inhibition of RhoA signaling with cell-permeable C3 transfase produced increases in both the length (28%) and number (41%) of MPs, suggesting additional pathways for regulation of MP outgrowth. To determine whether myosin II affected the development of neuronal polarity, morphogenesis was examined in cultures chronically treated with direct or indirect myosin II inhibitors. Inhibition of myosin II, MLCK, or ROCK promoted rapid morphogenesis without altering characteristic neuronal polarity. Together, these data indicate that myosin II activity negatively regulates MP extension, and the time course for development of neuronal polarity.

2249 Mechanism of Alternative Splicing of Nonmuscle Myosin Heavy Chain II-B and II-C Pre-mRNAs: Role of Fox-1 Family Proteins S. Nakahata, S. Kawamoto, NHLBI, National Institutes of Health, Bethesda, MD

The genes encoding nonmuscle myosin heavy chain (NMHC) II-B and NMHC II-C generate alternatively spliced isoforms which include or exclude a cassette of amino acids near the ATP-binding domain. Inclusion of alternative exon B2 in NMHC II-B pre-mRNAs is restricted to some neural cells. Alternative exon C1 is excluded entirely from NMHC II-C pre-mRNAs in striated muscles, but is included in nonmuscle cell mRNAs. Here, we report that the intronic RNA element UGCAUG and the RNA-binding proteins, Fox-1 family proteins, play a critical role in regulation of both B1 and C1 alternative splicing, but in a different manner. Minigene transfection analysis using a number of cell lines revealed that the UGCAUG elements located in the downstream exon of B1 was essential for B1 inclusion in cells. This RNA element was also present in both the upstream and downstream introns of C1. However, only the upstream
elements were found to be important for C1 exclusion in muscle cells. We further examined the effects of exogenous expression of Fox-1 family proteins, which can bind to the UGCAUG element, during B1 and C1 splicing. Fox-1, Fox-2 and Fox-3, which contain highly homologous RNA recognition motifs (RRMs), belong to the Fox-1 family in mammals. RT-PCR and other analyses revealed that multiple alternative promoters and alternative splicing of pre-mRNA gave rise to a number of tissue-dependent isoforms of Fox-2. We made expression constructs encoding 24 Fox-2 isoforms, which differ in their N-terminus, internal and C-terminal sequences, but share an identical RRM. Inclusion of B1 was activated by the isoforms containing a particular internal and C-terminal sequence. In contrast, exclusion of C1 could be enhanced by all isoforms. These results suggest that a different distribution of the same splicing regulatory element as well as isoform diversity of Fox-2 confer tissue-specificity on alternative splicing of B1 and C1.

2250

Using Double Knockout Nonmuscle Myosin II-B and II-C Mice to Study Cardiac Myocyte Development

X. Ma, S. Kawamoto, G. Homayounfar, R. S. Adelstein; Laboratory of Molecular Cardiology, NHLBI, National Institutes of Health, Bethesda, MD

Nonmuscle myosin IIs (NM IIs) play an important role in many basic cellular processes including cell division, cell migration and cell-cell adhesion. In addition, it has been proposed that NM II is required for sarcomere formation in cardiac myocytes. In order to study the role of nonmuscle myosins in cardiac myocyte development, we generated double knockout NM II-B and II-C (B/C/ B/C) mice. Immunofluorescence confocal microscopy of heart sections from wild-type mice shows that cardiac myocytes express NM II-A during cardiac loop development (embryonic day, E7.5-8.5) and in the arterial pole between E9.5-11.5, but not thereafter. On the other hand, both NM II-B and II-C are expressed in the developing cardiac myocytes. Ablation of II-B in mice results in partial impairment of cytokinesis in cardiac myocytes leading to binucleation, but loss of NM II-B has no effect on sarcomere formation. We attributed this to the expression of NM II-C in the B/C- ablated myocytes. Ablation of II-C alone has no obvious effect on the myocytes during embryonic heart development. Ablation of both isoforms, however, leads to abnormalities in the mitotic spindle resulting in a defect in cytokinesis, in addition to the defect in cytokinesis. More than 90% of the B/C- BC- cardiac myocytes contain abnormal nuclei. More than 90% of the B/C- BC- cardiac myocytes in mice do not affect the formation of sarcomeres in embryonic cardiac myocytes. Since there is no detectable myocyte expression of NM II-A after E9.5 (except for the outflow tract) nor is NM II-A expression increased in B/C- BC- cardiac myocytes, we suggest that NM II-A, II-B and II-C are not necessary for mouse cardiac sarcomere formation. Our results show the importance of nonmuscle myosins in cardiac myocyte cytokinesis and karyokinesis.

2251

Activated Myosin II Is Required for Cell Alignment in the Drosophila Epidermis

R. Simone, S. Dinardo; Cell and Molecular Biology, The University of Pennsylvania, Philadelphia, PA

Epithelial sheets undergo coordinated cell shape changes that are vital to organogenesis and development in general. The ventral epidermis of the Drosophila embryo is an epithelial sheet that undergoes differential cell shape changes during the parasegmentation. This epithelium is an especially promising tissue in which to study this phenomenon because much is known about the signaling pathways that underlie the establishment and maintenance of differential cell fates across the parasegment. Initially, the epithelial cells of the ventral epidermis maintain a roughly hexagonal shape and are randomly packed. Over the course of several hours, cells within this epithelium change shape: their anterior and posterior edges lengthen and their dorsal and ventral edges shorten. Our quantitative measurements showed that each row of cells in one population align their anterior and posterior edges to form parallel lines while cells in another population align their edges to a lesser degree. We observed an enrichment of Actin, Myosin II (MyoII) and MyoII related proteins along the aligning edges of these cells and suspected that MyoII-mediated actomyosin contraction might be responsible for cell alignment. To test this, we exposed embryos at the onset of cell alignment to a Rho Kinase inhibitor (Y27632), which is known to prevent MyoII activation by blocking the activation of the myosin regulatory light chain. We observed the disruption of cell alignment and order, thus implicating MyoII in the alignment of cells within the ventral epidermal sheet. Discerning the role of actomyosin contractility in cell alignment will contribute to the further description of Drosophila epibolyogenesis specifically and the understanding of epithelial morphogenesis generally.

2252

Comparison of Transcription Profiles of myo1Δ and chs2Δ Mutants in Budding Yeast Saccharomyces cerevisiae

J. F. Rodriguez-Quijones, J. R. Rodriguez-Medina; Department of Biochemistry, University of Puerto Rico Medical Sciences Campus, San Juan, Puerto Rico

Saccharomyces cerevisiae myo1Δ gene encodes the myosin type II heavy chain (Myo1p), a protein required for normal cytokinesis in budding yeast. In a myo1Δ mutant the Pck1p/Skp2p pathway is activated by cell wall stress. Delocalized chitin deposition and increased chitin synthesis by Chs3p are associated with this response. The CHS2 gene encodes chitin synthase II (Chs2p), an enzyme that catalyzes the transfer of N-acetylglucosamine (GlcNAc) to chitin and is essential for chitin synthesis in the primary septum during cytokinesis. chs2Δ strains are characterized by absence of the primary septum and share several phenotypes with myo1Δ mutants such as: abnormal cytokinesis, formation of attached cells, abnormal budding patterns, delocalized chitin deposition in the cell wall and a requirement of Chs3p expression for normal growth. The purpose of this study is to compare the transcriptional profiles of these two similar mutants in order to identify genes that could be differentially expressed in response to abnormal cytokinesis. A transcriptional profile was generated for both mutants using yeast oligonucleotide microarrays. The hybridized arrays were analyzed using statistical methods and classified by biological processes. A total of 172 genes of different categories were differentially expressed in both strains. Excluding genes of unknown function, the categories most represented in common for both profiles were: protein biosynthesis, metabolism and stress response. Notable similarities were found among down regulated genes coding for protein biosynthesis functions and RAS1. Genes involved in cell wall organization and biogenesis that were up-regulated are responsible for secretion and morphogenesis in the two strains. Our preliminary transcriptional analysis shows that ZTL transcription may not be activated in this chs2Δ mutant. Therefore these mutant strains may activate the transcriptional response by different pathways. This work was supported by a PHS grant M08224 (S06-GM08224) with partial support from NSF.

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Myosin II Activity Regulates the Shape of Three-Dimensional Epithelial Cysts

A. I. Ivanov, A. M. Hopkins, G. T. Brown, K. Gerner-Smidt, B. Babbin, C. A. Parkos, A. Nusrat; Pathology and Laboratory Medicine, Emory University, Atlanta, GA

Development of luminal organs such as gut, lungs and kidneys begins with the formation of hollow spherical cysts composed of single layers of polarized epithelial cells. This initial cystogenesis depends on reorganization of the actin cytoskeleton, however mechanisms that mediate such actin reorganization are poorly understood. This study was designed to investigate the role of the major actin motor, myosin II, in the epithelial polarization of cysts using a three-dimensional in vitro cell culture model. Caco-2 intestinal epithelial cells were embedded into Matrigel and allowed to grow for three days in the presence of either vehicle, or a myosin II inhibitor, blebbistatin. While control cells formed typical spherical cysts with a smooth surface, blebbistatin-treated cysts developed multiple radiating actin-rich surface protrusions. These protrusions contained components of the Arp2/3 complex and the neuronal Wiskott-Aldrich protein (N-WASP) and their development was inhibited by an actin-depolymerizing drug, latrunculin B or the N-WASP-inhibitor, wiscostatin. The appearance of blebbistatin-induced protrusions did not depend on the activity of Rac-1 and Cdc-42 GTPases, but was attenuated by depolymerization of microtubules with either nocodazole or vinblastin. In Caco-2 cells, blebbistatin caused disappearance of peripheral actomyosin bundles which was accompanied by reorientation of cortical microtubules and their accumulation in the vicinity of the plasma membrane. The formation of blebbistatin-induced protrusions was also blocked by pharmacological inhibition of phospholipase C (PLC). We hypothesized that inhibition of myosin II eliminates a cortical actomyosin barrier allowing cell-cell contacts to reach the plasma membrane and to activate phospholipase C. We speculated that such activation of PLC acts activating actin-severing proteins to create free actin-barbed ends and results in stimulation of N-WASP-Arp2/3 dependent act polymerization and the formation of peripheral protrusions. We conclude that myosin II controls spherical shape of epithelial cells by preventing MT-dependent actin polymerization at the cyst surface. Supported by CCF and NIH.

2254

Defective Platelet Function and Reduced Thrombosis Induced by Megakaryocyte-specific Disruption of Non Muscle Myosin IIA Gene

C. Leon,1 A. Eckly,1 B. Hecher,1 B. Aki,1 M. Freund,1 J. Cazenave,1 R. Tiedt,2 R. Skoda,2 C. Gachet1; 1Etablissement Français du Sang, INSERM U311, Strasbourg, France, 2Experimental Hematology, Basel University Hospital, Department of Research, Basel, Switzerland

Mutations in MYH9 encoding non muscle myosin heavy chain IIA (NMHC-IIA) lead to several disorders such as the May-Hegglin anomaly and the Fechtner, Sebastian or Epstein syndromes. The intracellular localization of NMHC-IIA is mainly characterized by a decrease in cortical actomyosin (enhanced thrombomodulin) associated with morphological changes with large platelets, leading to minor hemorrhagic manifestations. To understand the role of NMHC-IIA in normal platelet function and in pathology, we generated mice with disruption of MYH9 (exon1) in megakaryocytes, the precursors of platelets. MYH9A mice exhibited a selective severe deficiency in platelet NMHC-IIA, residual myosin amounting 15-20% of the normal. This decrease in myosin resulted, like in the human disease, in thrombomodulin (117±10 x 16x105 vs. 47±10 x 105 platelets/µL for wild-type and MYH9A, respectively, mean±sem). Moreover, platelets from MYH9A mice appeared...
ovoid instead of discoid, twice the normal size, with abnormally abundant rough endoplasmic reticulum and altered organelles distribution, some platelets appearing devoid of organelles. Bleeding time measurements were performed and all MYH9A mice bled for more than 600s, compared to 153±15s for wild-type mice. Clot retraction, which is important in thrombus stabilization, was totally abolished. Upon activation by soluble agonists, platelet contraction was absent in MYH9A platelets while subsequent aggregation and secretion were near normal. MYH9A platelets were still capable to adhere and extend lamellipodia on a fibrinogen-coated surface but without stress fiber formation. Thrombus growth under flow was investigated by perfusing whole blood over collagen. The MYH9A platelets adhered on collagen but thrombus were reduced in size (56% vs 22% surface coverage for wild-type and MYH9A platelet, respectively) and did not grow in height, showing the importance of platelet contraction to resist the flow. Overall, these results demonstrate the role of myosin IIA in platelet contractile phenomena which in addition to thrombocytopenia, may encounter for the haemostasis defects observed in MYH9A-disorders.
defects in arginyl-adenylylation of several human congenital heart defects is suggested that some of the underlying mechanisms of heart disease may be related to the defects of protein arginylation. Our results outline a new function of arginylation in the regulation of actin cytoskeleton in cardiac myocytes.

2260
Rho Kinase Inhibitor Y27632 Affects Initial Heart Myofibrillogenesis in Cultured Chick Blastoderm
H. Sakata, M. Skabe, H. Matsui, N. Kawada, K. Nakatani, K. Ikeda, T. Yamagishi, Y. Nakajima; 1Anatomy and Cell Biology, Osaka City University Graduate School of Medicine, Osaka, Japan, 2Department of Pathology, Osaka City University Graduate School of Medicine, Osaka, Japan, 3Anatomy, Saitama Medical School, Saitama, Japan
During early vertebrate development, Rho-associated kinases (ROCK) are involved in various developmental processes, such as gastrulation and endocardial epithelial-mesenchymal transition. Here we investigated spatiotemporal expression patterns of ROCK1 protein in early chick embryos and examined the role of ROCK during initial heart myofibrillogenesis in cultured chick blastoderm. Immunohistochemistry showed that ROCK1 protein was distributed in migrating mesendoderm cells, visceral mesoderm of the pericardial coelom (from which cardiomyocytes will later develop) and cardiomyocytes of the primitive heart tube. Pharmacological inhibition of ROCK by Y27632 in cultured posterior blastoderm did not affect the transcripts of Nkx2.5, GATA4 and sarcomeric α-actinin suggesting that ROCK inhibition did not alter the myocardial specification process. However, Y27632 disturbed the formation of striated heart myofibrils in cultured posterior blastoderm. Furthermore, Y27632 affected the formation of costamere, a vinculin/integrin-based rib-like cell adhesion site between Z-bands and the sarcolemma/extracellular matrix. In such cardiomyocytes, epithelialization was disrupted and N-cadherin was distributed in the peri-nuclear region, thereby cardiomyocytes showed a fibroblast-like phenotype. Western blot analysis showed that the expression of smooth muscle α-actin, sarcomeric integrin α6β1 and N-cadherin was downregulated in explants treated with Y27632. Pharmacological inactivation of myosin light chain kinase, a downstream of ROCK, by ML-9 perturbed the formation of striated myofibrils as well as costameres, but not myocardial epithelialization. These results suggest that ROCK may play a role in the initial heart myofibrillogenesis via actin-myosin assembly, focal adhesion/costamere and cell-cell adhesion.

2261
Krp1, a Muscle-Specific Kelch-related Protein, Is Required for Mature Myofibrill Accumulation in Primary Mouse Embryonic Cardiomyocytes
C. C. Greenberg, R. Horowits; NIAMS, National Institutes of Health, Bethesda, MD
Krp1 is a skeletal- and cardiac-muscle-specific member of the kelch repeat family of proteins. Kelch repeats form actin-binding domains, and many kelch proteins are involved in cytoskeletal organization. In transformed rat fibroblasts, Krp1 colocalizes with actin in pseudopodia and is required for pseudopod elongation. However, the physiological functions of Krp1 in muscle are unknown. Krp1 binds N-RAP, which acts as an assembly catalyst in the early stages of muscle myofibril formation; in cultured chick cardiomyocytes, Krp1 localizes to sites with laterally fusing myofibrils, indicating that Krp1 may be involved in later stages of myofibrillogenesis (Lu et al., 2003). To elucidate the role of Krp1 in myofibril assembly, endogenous Krp1 expression was reduced using RNA interference. Chemically synthesized siRNA against a Krp1 target sequence was transfected into primary mouse embryonic cardiomyocytes, and Krp1 expression was determined by quantitative PCR and immunoblotting. Krp1 transcript levels were reduced over 70% within 48 hours post-transfection compared to mock- and scrambled siRNA-transfected control cells, and Krp1 protein levels were decreased 50% by 72 hours. In contrast, levels of sarcomeric alpha-actin and muscle myosin were unaffected. Alpha-actinin organization into mature structures was used as a measure of myofibril content and assessed by confocal microscopy and morphometric analysis. Cardiomyocytes transfected with scrambled siRNA and Krp1 siRNA contained mean myofibril areas corresponding to 52% and 29% of total cell area, respectively, indicating that Krp1 is required for myofibril assembly and/or maintenance of mature myofibrils. In control cells transfected with scrambled siRNA, many broad Z-lines were observed with 1.8 to 2.2 micron periodicity. After Krp1 knockdown, broad Z-lines were often absent and alpha-actinin was organized into continuous fibers, aperiodic dots, or narrow bands or dots with 1.8 to 2.2 micron periodicity. These data are consistent with a role for Krp1 in myofibril assembly.

2262
Tropomodulin and Tropomyosin Are Required for Cardiac Myofibril Assembly and Looping Morphogenesis in the Mouse
C. R. McKewen, R. B. Nowak, V. M. Fowler; Department of Cell Biology, The Scripps Research Institute, La Jolla, CA
During heart development, the linear heart tube undergoes dramatic morphogenetic changes in order to specify chambers and become a mature, functioning organ. This process, known as cardiac looping morphogenesis, is highly dynamic and not well understood. Mechanisms that may play a role in looping morphogenesis include cell shape changes, cell proliferation, differentiation, myofibril assembly, and contractile function. We are interested in testing the contributions of cardiomyocyte myofibrillogenesis and contraction in looping morphogenesis. Mice lacking the sarcomeric proteins tropomodulin (Tmod1) or alpha-tropomyosin (αTM1) are embryonic lethal due to cardiac development defects. In both mutants, embryonic development ceases during the cardiac looping stage around day E8.5-9, suggesting that these myofibril components are also required for looping morphogenesis. In order to test the hypothesis that myofibril assembly and function are coupled to cardiac development, we have begun a comparative analysis of the αTM1 and the Tmod1 null mice. By whole-mount immunofluorescence, both αTM1 and Tmod1 null hearts display aberrant α-actinin and F-actin rod-like structures reminiscent of skeletal muscle nemaline rods. The Tmod1 null hearts do not form visible myofibrils and fail to loop. However, ex vivo imaging of cultured Tmod1 null embryos demonstrates that the hearts do have some contractile function. Together, these data indicate that myofibril assembly is required for looping but not for contractile function. Preliminary data show that the αTM1 null hearts develop slightly further than the Tmod1 nulls, initiating looping and raking nascent myofibrils.

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Myofibrillogenesis in Zebrafish Skeletal Muscle Cells
J. M. Sanger, A. Dube, B. Holloway, J. Wang, J. W. Sanger; 1Cell and Developmental Biology, SUNY Upstate Medical University, Syracuse, NY, 2Cell and Developmental Biology, University of Pennsylvania, Philadelphia, PA
The premyofibril model that we have proposed to explain myofibrillogenesis suggests that the formation of mature myofibrils is preceded by two intermediary structures: premyofibrils and nascent myofibrils. To examine myofibrillogenesis in a living animal we have begun to use the zebrafish model system where there is a temporal progression of myofibril formation that can be followed as myotomes increase in length from the youngest to the oldest. To determine if there is evidence for the premyofibril model in zebrafish, antibodies to sarcomeric alpha-actinin and muscle myosin II were used to stain embryos that were fixed at 20 - 25 hours post-fertilization. The earliest myotomes exhibited small periodic bodies of alpha-actinin (z-bodies) that were arrayed along actin fibers in a pattern that appeared identical to premyofibrils detected in avian muscle. In young myotomes, muscle myosin was present in overlapping bundles that resembled the patterns in myotomes in avian nascent myofibrils. A-bands and Z-bands were present in mature myofibrils in the elongated cells in older myotomes. However, at the ends of these cells the patterns of alpha-actinin and muscle myosin resembled the arrangement seen in younger myotomes, suggesting that as the cells elongated, premyofibrils were involved in the extension of sarcomeres that comprised cell growth. For live cell observations, zebrafish skeletal muscle alpha-actinin promoter encoding GFP-sarcomeric alpha-actinin or FATZ or skeletal actin were injected into one of the first two blastomeres. Mosaic transgenic zebrafish displayed the expected localization of alpha-actinin, FATZ and actin in the young and old myotomes even after several days. Fluorescence Recovery After Photobleaching measurements showed that the dynamics of GFP-alpha-actinin measured in the Z-bands had a recovery profile similar to that found for avian Z-bands. These observations indicate that myofibrillogenesis in zebrafish is consistent with the premyofibril model of myofibrillogenesis.

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Interaction between p94/calpain 3 and Sarcomeric α-actinin in Skeletal Muscles
K. Ojima,1,2 Y. Ono,1 N. Ooi,1 K. Yoshioka,2 Y. Kawasaki,1 H. Sorimachi1; 1Department of Enzymatic Regulation for Cell Functions, Tokyo Metropolitan Institute of Medical Science (Rinshoken), Tokyo, Japan, 2CREST, Japan Science and Technology Agency (JST), Kawaguchi, Japan, 3Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo, Japan
p94/calpain 3 is a Ca2+-regulated intracellular cytosine protease, and is predominantly expressed in skeletal muscles. A defect of its proteolytic activity leads to limb-girdle muscular dystrophy type 2A. In myofibrils, p94 is localized at Z-band, N2A, and M-line regions. At the latter two regions, p94 directly binds to connectin/titin. However, p94 binding molecule(s) of myofibrill components other than connectin/titin have not been reported. In this study, to identify a binding partner of p94 in particular in Z-band components, yeast two-hybrid screening was performed using p94 NS domain, the N-terminal region of p94, as bait. Out of the 4000 clones plated on the selection medium plates, 22 clones were identified as potential p94 binding molecule. Interaction between p94 and s-α-actinin was further investigated in mammalian cells. Both s-α-actinin and p94 were co-expressed in COS7 cells, and immunoprecipitated with specific antibodies. As a result, s-α-actinin was co-immunoprecipitated with full-length p94 as well as the p94 NS domain, indicating that the p94 NS domain directly bound to s-α-actinin. Furthermore, a specific antibody against the p94 NS domain captured p94 positive Western blot analysis showed that the expression of smooth muscle α-actin, sarcomeric α-actinin, integrin α6β1 and N-cadherin was downregulated in explants treated with Y27632. Pharmacological inactivation of myosin light chain kinase, a downstream of ROCK, by ML-9 perturbed the formation of striated myofibrils as well as costameres, but not myocardial epithelialization. These results suggest that ROCK may play a role in the initial heart myofibrillogenesis via actin-myosin assembly, focal adhesion/costamere and cell-cell adhesion.
signals in the Z-bands of cultured muscle cells, although other p94 antibodies whose epitopes were located to the C-terminal regions of p94 detected p94 in N2A and/or M-line regions, not in the Z-bands. These data suggest that the N-terminal fragments of p94 are involved in the Z-band structure by linking with s-α-actinin.

**2265**
Point Mutations in Myosin Differentially Affect ATPase and Actin Motility and Perturb Skeletal and Cardiac Muscle Structure and Physiology
A. R. Cammaroto,1,2 C. M. Dambacher,1 K. Occor,3 M. C. Reedy,3 A. F. Knowles,4 W. A. Kronert,1 R. Bodmer,5 S. I. Bernstein1; 1Biological Department, San Diego State University, San Diego, CA, 2The Burnham Institute for Medical Research, La Jolla, CA, 3Cell Biology, Duke University Medical Center, Durham, NC, 4Chemistry and Biochemistry, San Diego State University, San Diego, CA

Myosin is the molecular motor that drives muscle contraction. *Drosophila melanogaster* is a powerful model system to study the in vitro and in vivo properties of mutated myosins and their effects on different striated muscles possessing distinct contractile properties. We investigated the influence of specific mutations, from the molecular through the organ level, of the myosin heavy chain (MHC) mutants D45 (A261T), which changes a residue near β7, and Mhc1 (G200D), which alters a residue located at the base of loop I. Both regions likely regulate the rate of nucleotide exchange. D45 myosin has decreased Ca2+-ATPase activity and reduced basal and actin-stimulated Mg2+-ATPase activity as compared to the wild-type indirect flight muscle (IFM) isoform (Hill coefficient 1.6). D45 myosin shows a greater than two-fold increase in basal Mg2+-ATPase activity but a similar actin-stimulated Mg2+-ATPase rate as the IFM. The average actin sliding velocity was reduced by two-fold for D45 myosin compared to the IFM, while Mhc1 myosin increased actin filament velocity by 15%. Ultrastructural analysis of IFM expressing D45 myosin revealed white-type myofibril structure, while flies expressing Mhc1 myosin displayed severe hypercontraction. Both mutations had similar effects, however, exhibited decreased flight ability. We also investigated the effects of these mutations on cardiac structure and performance. Beating hearts of semi-intact flies were imaged using direct immersion DIC optics in conjunction with a high-speed digital camera. Movies processed to produce edge tracings allowed us to monitor heart tube movements. Compared to wild-type, D45 hearts appeared wider or dilated while Mhc1 hearts exhibited restricted, non-contractile regions. Computational analysis revealed altered systolic and diastolic function in both mutants. Ultrastructural perturbations were also found in the mutant cardiomyocytes.

Overall, our data show that distinct molecular defects in the myosin molecule differentially affect the structure of skeletal muscle and the structure and function of cardiac muscle.
charged ankryin-like repeats that are bilaterally symmetric. Supported by T32 HL 072751 (M.A.B.), by a Development Award from the Muscular Dystrophy Association (MDA; to A.K.-K.), and by the NIH (RO1 HL64304) and the MDA (to R.J.B.).

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Obscurin Interacts with Filamin C and Is Present at Costameres
A. L. Bowman, A. Kontogianni-Kstantonopoulou, A. O'Neill, R. J. Bloch; Physiology, University of Maryland, Baltimore, Baltimore, MD

Obscurin is a giant sarcomeric protein that is critical in the development of the A band and M line in skeletal muscle. Obscurin has previously been localized to structures that surround individual myofilaments at the level of Z-disks and M-bands. Using a yeast two-hybrid assay, we identified filamin C as a binding partner of the Rho-GEF/PH domains of obscurin. Amino acids 2188-2288 of filamin C contain the minimal binding region necessary for binding the Rho-GEF/PH domains in yeast. Inclusion of the dimerization domain of filamin C enhances binding in overlay assays, however, suggesting that obscurin preferentially binds dimerized filamins or that longer sequences of filamin are necessary for proper folding of the binding site. In addition to colocalizing at the periphery of Z-disks and M-bands of myofibrils, filamin C and obscurin co-localize at costameres at the sarcolemma of skeletal muscle. Both proteins are lost from the costameres that remain in muscle from mdx (dystrophin-deficient) mice, consistent with their association with the dystrophin-glycoprotein complex in healthy muscle. The identification of PC-lok (the other obscurin isoform) and the presence of costamers and its links to the underlying contractile apparatus of striated muscle. Supported by a training stipend (T32 GM08181, to ALB), grants from the NIH (RO1 HL64304 and the Muscular Dystrophy Association (to RJB) and a Development Award to AKK from the Muscular Dystrophy Association.

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Characterization of the Binding Site on Obscurin for Small Ankryin 1
B. Busby, M. A. Borzok, A. Kontogianni-Kstantonopoulou, R. J. Bloch; University of Maryland, Baltimore, MD

Obscurin is a recently discovered ~800 kDa high-mannose glycoprotein that is synthesized in striated muscle cells, where it surrounds myofilaments at the levels of Z disks and M bands. The localization of actin and tropomysin may play a role in the contractile apparatus apparatus of the sarcoplasmic reticulum (SR) of skeletal and cardiac muscles. The C-terminus of the molecule is localized near the SR and binds a small protein spanning the SR membrane known as small ankryin 1 (sAnk1). We used the yeast two-hybrid screen to identify a short sequence of amino acids in obscurin that mediate binding to sAnk1 (6324-KWVEEETEVVKK-6338) and site-directed mutagenesis to identify the amino acids involved in binding. Mutation of either K6324 or K6338 to A reduces binding strongly, whereas mutation to E inhibits binding significantly. Mutation of each of the four E's to A individually has little effect; mutation of several inhibits binding significantly; and mutation of all four completely abolishes binding. The two central residues, E6329 and E6330, contribute most to these results. R6335A has little effect on binding, whereas R6335E enhances binding. Our results suggest that this sequence and nearby amino acids, constitute a minimum binding domain for sAnk1, and that electrostatic interactions, mediated by positive charges flanking a highly negatively charged core, contribute significantly to binding. Supported by a grant to RJB from the NIH (RO1 HL64304). BB receives support from T32 GM08181.

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Tropomyosin 4 Defines Novel Filament Systems in Normal and Diseased Muscle
N. E. Vlahovich, E. Kettle, G. Schevzov, V. Nair-Shalliker, B. Iwokshi, D. Hernandez-Diveiez, R. Parton, A. Keel, K. N. North, P. W. Gunning, E. C. Harderman; 'Muscle Development Unit, Children's Medical Research Institute, Westmead, Australia, 2School of Natural Sciences, University of Western Sydney, Penrith, Australia, 3Oncology Research Unit, The Children's Hospital, Westmead, Australia, 4Neurogenetics Unit, The Children's Hospital, Westmead, Australia, 5Institute for Molecular Bioscience, University of Queensland, Brisbane, Australia

Tropomyosins (Tms), a family of filamentous proteins consisting of over forty isoforms, play an important role in defining the function of actin filaments in both muscle and non-muscle cells. Tm isoforms are derived from four genes (alpha, beta, gamma and delta) and are generated through the use of alternative promoters and alternative RNA splicing. In non-muscle cells, Tm isoforms have been shown to be functionally distinct, playing roles in diverse processes such as cell growth, differentiation, cell division and vesicle transport. We have previously shown that Tm5NM1 localizes to the Z-line associated cytoskeleton (ZLAC) in skeletal muscle fibers and disruption of this structure causes a dystrophic phenotype in transgenic mice. The current study shows that cytoskeletal Tm4 localizes to regions in muscle fibers that are distinct from those containing Tm5NM1. Using Tm5NM1 and Tm4 isoform-specific antibodies in immunofluorescence and immuno-electron microscopy, we found that Tm4 co-localizes with caldesmon at the terminal cisternae of the sarcoplasmic reticulum and Tm5NM1 co-localizes with the dihydroyphindine receptors in the T-tubules in normal muscle. In muscles undergoing remodelling and repair in response to injury, stretch and disease, Tm4 is upregulated and is present in longitudinal filaments along the length of muscle, perpendicularly to the Z-line. An elevated level of Tm4 is diagnostic of repair in dystrophies and nemaline myopathy. The discovery of these ‘nonmuscle’ filament systems in skeletal muscle demonstrates that, in parallel with all other cell systems examined, skeletal muscle contains compartmentalized cytoskeletal systems characterized by the segregation of different cytoskeletal tropomyosins. The association of distinct Tm isoforms with the internal membranes in muscle fibers suggests a role for the local actin cytoskeleton in the functions of these membranes.

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Comparison of the Localization and Dynamic Properties of Tropomyosin Isoforms: TPM1alpha and TPM1kappa in Skeletal Muscle Cells
J. Wang, J. M. Sanger, H. Thurstorn, L. Z. Abbott, D. K. Dube, J. W. Sanger; Cell and Developmental Biology, SUNY Upstate Medical University, Syracuse, NY

Tropomyosin (TM) and the troponins associate with actin filaments and regulate the interaction of the thin and thick filaments of mature myofibrils. There are four known tropomyosin genes (designated as TPM1, TPM2, TPM3, and TPM4) in vertebrates. The predominant TPM1 isoform, TPM1alpha, is specifically expressed in both skeletal and cardiac muscles. Recently a newly discovered alternatively spliced isoform, TPM1kappa, containing exon 2a instead of exon 2b contained in TPM1alpha, was found to be cardiac specific and developmentally regulated. However the role of TPM1kappa in myofibrillogenesis and contractile activity is yet to be understood. In this work, we transfected avian cardiac and skeletal muscle cells with green fluorescent proteins (GFP) coupled to Chicken TPM1alpha and Chicken TPM1kappa and compared their localizations in premyofibrils and mature myofibrils. In addition, we used the technique of fluorescence recovery after photobleaching (FRAP) to compare the dynamics of TPM1alpha and TPM1kappa in premyofibrils versus mature myofibrils. Expression of TPM1alpha and TPM1kappa did not affect muscle cell growth and myofibrillogenesis. Both TPM1alpha and TPM1kappa have similar localization and dynamic properties in skeletal and cardiac muscle cells with both isoforms exchanging more rapidly in premyofibrils than in mature myofibrils. The faster exchange properties of the TPM1 isoforms in premyofibrils associated with mature myofibrils has also been seen when the dynamics of Z-band proteins are compared in premyofibrils and mature myofibrils. These results suggest that as myofibrils assemble, proteins of the thin filament become less dynamic. This could be attributed to CapZ (binds the barbed ends of actin filaments) and nebulin (tropomyosin binding molecule) that associate with actin later in forming myofibrils than the muscle tropomyosins. These experiments are supportive of a maturing thin filament and stepwise model of myofibrillogenesis (Sanger et al., 2006). Work supported by grants from AHA, MDA and NIH.

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Expression of the Mouse e-SG Gene Having an Exon Trap Retrovirus Vector Integrated into Intron 11
M. Imamura, S. Takezaki; Department of Molecular Therapy, National Institute of Neuroscience, NCNP, Tokyo, Japan

Conventional sarcoglycans (alpha, beta, gamma and delta) comprise a subcomplex within the dystrophin-associated protein complex, which is critical for the function of the striated muscle cell membrane. A fifth broadly expressed sarcoglycan (SG), e-SG, was identified as a glycoprotein similar to a-SG that is encoded by the causative gene for autosomal recessive limb-girdle muscular dystrophy type 2D. However, unlike a-SG, loss-of-function mutations in e-SG gene cause myoclonus-dystonia syndrome (M-D) but not muscular dystrophy, indicating the importance of e-SG for the function in the central nervous system rather than in muscle cells. We found a brain-specific isoform of e-SG (e-SG2) in neuronal cells and suggested the functional importance of its C-terminal unique sequence in the brain. Therefore, to investigate the role of e-SG2, we generated a mouse lacking the C-terminal sequence by poly-A trap strategy with a removable exon trap (RET) retrovirus vector. Insertion of RET-vector into the intron 11 of the mouse e-SG gene (Sgce) resulted in making the hybrid mRNA with human bcl-2 exon 3 instead of the last two exons (exon 11b and 12) of Sgce. In the translation of the hybrid transcript, the C-terminal unique sequence of e-SG2 was replaced by the artificial 14-amino acid sequence. Reverse transcription-PCR clearly detected the expression of the hybrid transcript in a variety of mouse tissues. Analysis of heterozygous RET-vector-inserted Sgce indicated paternal expression of e-SG mRNA. Furthermore, we generated an antibody specific for the hybrid e-SG molecule and succeeded in detection of the protein product from the vector-inserted Sgce allele. M-D is a neurological disorder showing an autosomal dominant inheritance with incomplete penetrance. Our Sgce partial knockout mice would be useful for the study of inheritance mode of M-D as well as the analysis of e-SG2 function in the central nervous system.
2275 Interaction between the Muscular Dystrophy Proteins Myotilin and Telethonin
H. Suila,1 M. Moza,2 G. Faulkner,2 O. Carpen1; 1Biomedicum Helsinki, Program of Neuroscience, Department of Pathology, University of Helsinki, Helsinki, Finland, 2Muscle Molecular Biology Unit, International Centre of Genetic Engineering and Biotechnology, Trieste, Italy, 3Department of Pathology, University of Turku and Turku University Central Hospital, Turku, Finland

Myotilin and telethonin are components of the muscle sarcomere Z-disc. Missense mutations in both proteins cause muscle disorders and cardiomyopathies. Myotilin plays an important role in maintaining the structural integrity of the sarcomere and it has been shown to bind several Z-disc proteins including a-actinin and calcalcicins. It also interacts with the transcriptional regulators MrXR1 and 2 involved in sensing of mechanical load. Telethonin is a part of the muscle stretch sensor machinery and it anchors the giant muscle protein titin to the Z-disc. When analyzing myotilin knock-out mice, we noticed a consistent upregulation of telethonin mRNA and protein, suggesting a functional interplay between the proteins. The aim of this study was to analyze further the link between myotilin and telethonin. By yeast-two-hybrid and in vitro protein assays we have detected a direct interaction between the two proteins. The interaction sites are currently being mapped. We are also testing how the interaction is regulated and comparing the telethonin binding specifics of myotilin and titin.

2276 Interaction between the Myofibrillar Myopathy Proteins Myotilin and ZASP
P. von Nandanestad1, O. Carpen2; 1Department of Pathology and Neuroscience Program, University of Helsinki, Helsinki, Finland, 2Department of Pathology, University of Turku and Turku University Central Hospital, Turku, Finland

Myotilin is a component of the striated muscle sarcomeric Z-discs, which cross-link actin-containing thin filaments. Point mutations in the myotilin gene, typically replacing either a serine or threonine, causes two forms of muscle disorders, limb-girdle muscular dystrophy type 1A and myofibrillar myopathy (MFM). Functional studies on myotilin have demonstrated an association with important components of the sarcomere: α-actinin, a core structural component of the Z-disc, filamin C, and actin. Experiments using myotilin fragments with dominant negative effect have shown its critical involvement in sarcomere organization. Myotilin bundles and stabilizes actin effectively, which suggests a role for myotilin in the organization and maintenance of the Z-disc integrity. To gain further insight into myotilin’s molecular interactions, we carried out a yeast two-hybrid screen using myotilin as bait. In this screen we identified the Z-band alternatively spliced PDZ motif-containing protein (ZASP) as a novel binding partner for myotilin. Additional in vitro and in vivo studies have further verified the interaction. As both ZASP and myotilin are expressed predominantly in cardiac and skeletal muscle and their mutations can result in a similar disease phenotype characterized by disintegration of the Z-disc and by abnormal accumulation of multiple proteins, we hypothesize that the two proteins act in concert in the maintenance of the sarcomere.

2277 Nicotine Affects the Secondary Meiosis and Embryonic Development of Bovine Oocytes
G. Li, B. R. Sessions, Y. Liu, T. D. Bunch, K. L. White, L. Rickords, B. J. Pate, K. I. Astorn; Department of Animal, Dairy and Veterinary Sciences, Utah State University, Logan, UT

Effects of nicotine on the secondary meiotic spindle formation, embryo development and embryo quality were studied. The majority of the oocytes activated by ionomycin were with irregular anaphase and telophase spindles when cultured the oocytes in the presence of nicotine at 0.2 mmol concentration, the shape of spindles became worse disfiguration with the increase of nicotine concentrations. Cleavage and blastocyst rates of the embryos derived from parthenogenetical activation (PA) were not affected when the embryos were cultured in 0.2 and 0.5 nM nicotine media. With the increase of nicotine concentration to 1.0 mM the blastocyst development (9.9%) was numerically lower than the control (15.5%). Culture of the in vitro fertilized (IVF) embryos in 0.2, 0.5, and 1.0 mM nicotine media resulted in similar cleavage and blastocyst rates to the control. None of the embryos developed to beyond 16-cell stage when cultured the IVF or PA embryos in 2.0 and 4.0 mM nicotine media. Chromosomal analyses of both IVF and PA blastocysts showed that the majority (60-90%) of them were polyplody and mixoploidy. Cell number of the blastocysts derived from nicotine treatment was significantly lower than the control. Analyses of the un-cleaved 1-cell embryos after treatments with 2.0 mM and 4.0 mM nicotine showed that nicotine inhibited cytokinesis and resulted in the formation of poly-nuclei.

2278 Macromolecular Interaction of Halichondrin B Analogos E7389 and ER-076349 with Tubulin by Analytical Ultracentrifugation
J. J. Correa, P. H. Alday; Biochemistry, University of Mississippi Medical Center, Jackson, MS

Halichondrin B is an anti-mitotic drug that inhibits microtubule assembly. To understand the molecular details of its interaction with tubulin we investigated the binding of two analogs, E7389 and ER-076349, to tubulin by quantitative analytical ultracentrifugation. E7389 is currently undergoing Phase II clinical trials for cancer; ER-076349 is a closely-related analog with C.35 hydroxyl instead of C.35 primary amine (Towel MJ et al., Cancer Res. 61:1013, 2001). Below the Cc for microtubule assembly and in the presence of GDP, tubulin undergoes weak self-association into short curved oligomers. E7389 completely inhibits oligomer formation, while ER-076349 slightly stimulates oligomer formation by two fold. This is in contrast to vinblastine, which strongly stimulates large spiral polymers by 1000-fold. Colchicine binding to the intra-dimer interface has no effect on small oligomer formation, while the presence of bound colchicine also has no effect on the inhibitory activity of E7389. E7389 also inhibits vinblastine-induced spiral formation. These results suggest halichondrin B derivatives bind to the inter-dimer interface, disrupt polymer stability, and compete for vinblastine binding. Statomin is known to form a 1:2 complex with tubulin. E7389 inhibits formation of 1:2 statamin-tubulin complex, while ER-074369 dramatically weakens formation of the 1:2 complex. These results suggest E7389 is a global inhibitor of all tubulin polymer formation, disrupting tubulin-tubulin contacts at the inter-dimer interface. ER-076349 also perturbs tubulin-tubulin contacts in a polymer specific manner. This implies halichondrin B derivatives may exhibit unique cellular activities that reflect inhibition of tubulin interaction with specific regulatory, cell cycle specific factors. (Supported by Eisai Research Institute, Andover, MA.)

2279 FtsZ From Divergent Foreign Bacteria Can Function for Cell Division in E. coli
M. Osawa, H. P. Erickson; Cell Biology, Duke University Medical Center, Durham, NC

E. coli FtsZ, Mycoplasma pulmonis (MpuFtsZ) and Bacillus subtilis (BfBtsZ) are the only 46% and 52% identical in amino acid sequence to FtsZ from Escherichia coli (EcFtsZ). In the present study we show that MpuFtsZ and BfBtsZ can function for cell division in E. coli provided we make two modifications. First, we replaced their C-terminal tails with that from E. coli, giving the foreign FtsZ the binding site for E. coli FtsA and ZipA. Second, we selected for mutations in the E. coli genome that facilitated division by the foreign FtsZ’s. These suppressor strains arose at a relatively high frequency of 10^-3 - 10^-5, suggesting that they involve loss of function mutations in multi-gene pathways. These pathways may be negative regulators of FtsZ, or structural pathways that facilitate division by slightly defective FtsZ. Related suppressor strains were obtained for EcFtsZ containing certain point mutations or insertions of YFP. The ability of highly defective FtsZ to form functional Z-discs in E. coli replicates the binding specifics for EcFtsZ containing certain point mutations or insertions of YFP. The ability of highly defective FtsZ to function for division in E. coli is consistent with a two-part mechanism. FtsZ assembles the Z-ring, and perhaps generates the constrictor force, through self interactions; the downstream division proteins remodel the peptidoglycan wall by interacting with each other and the wall. The C-terminal peptide of FtsZ, which binds FtsA, provides the link between FtsZ assembly and peptidoglycan remodeling.
and TBCA. Overexpression of TBCB induces microtubule depolymerization. We found that this function is based on the ability of TBCB to form a binary complex with TBCE that greatly enhances the efficiency of this cofactor to dissociate tubulin in vivo and in vitro.

2281 Tubulin Cofactor B Expression in Developing Nervous System Suggest a Role in Neurogenesis
J. C. Villegas,1 G. Carranza,2 J. Bellido,3 D. Kortazar,3 J. C. Zabala,2 M. L. Fanaraga1; 1Anatomy & Cell Biology, University of Cantabria, Santander, Spain, 2Molecular Biology, University of Cantabria, Santander, Spain, 3Unidad de Metabolómica, CICbioGUNE, Derio, Spain Tubulin folding cofactor B (TBCB) is not apparently required for tubulin biogenesis and unnecessary for life. Here we show by Northern and Western blot that TBCB is abundantly expressed during neurogenesis. Immunostaining in neonate brain cyrossections and primary cultures demonstrate abundant TBCB levels in proliferating and migrating nestin-positive neuroblasts. In adult central nervous system TBCB immunostaining is less conspicuous being mostly localized at the ependymal cells. TBCB studies of overexpression by transitory cell transfection and expression knock down by interference RNA performed in a neuronal cell line (NG-108) suggest that TBCB participates at microtubule dynamics in neuroblasts as well as in ependymal cilia microtubule turnover.

2282 Characterization of the Colchicine Binding Site on Tubulin Isootype JVI
S. Sharma,1 B. Poliks,2 R. Ravindra,1 S. Bune1; 1Chemistry, SUNY Binghamton, Binghamton, NY, 2Physics, SUNY Binghamton, Binghamton, NY The protein tubulin exhibits seven β isotypes. All except the isotype JVI are highly homologous to each other. The sequence divergence of JVI with other β isotypes is implicated in its dissimilar in vitro assembly mechanisms. The overall aim of this project is to explore the consequences of the isotype JVI sequence divergence for ligands binding to the colchicine site. Chicken erythrocyte tubulin (CETb), which is composed solely of the αCETb, which is composed solely of the αCETb isotypes, was purified from chicken blood. Superimposition of chicken isotype JVI with the electron diffraction structure of mammalian brain tubulin-colchicine complex revealed four divergent residues within 5Å of colchicine binding site, indicating that the colchicine binding site on JVI possesses topography different from that of the other β isotypes. No evidence for colchicine binding to purified CETb was found. Taxol-induced assembly of 5 µM CETb was unaffected by up to 140 µM colchicine. No enhancement of colchicine fluorescence or quenching of intrinsic CETb fluorescence was observed at molar ratios of colchicine/tubulin up to 50:1. Podophyllotoxin inhibited taxol-induced assembly of CETb with an IC50 15-fold greater than the value for podophyllotoxin inhibition for taxol-induced assembly of bovine brain tubulin. This indicates that the colchicine A ring binding pocket on isotype JVI is structurally compatible for the trime-thoxyphenyl portion of colchicine. Surprisingly, allcolchicine, which possesses an aromatic C ring rather than the tropone ring of colchicine, binds to CETb. The kinetics of binding and fluorescence titration at 25°C revealed an apparent on rate constant and affinity constant about 23 and 28 times less than that for allcolchicine binding to bovine brain tubulin. The slow and suppressed binding of allcolchicine with iso type JVI indicates that the C ring binding pocket on JVI may either be shrunken or incompletely accessible to the colchicine C ring.

2283 The Molecular Mechanism of Taxol Function
A. Mitra,1 D. Sept2; 1Chemical Engineering, Washington University, St. Louis, MO, 2Biomedical Engineering, Washington University, St. Louis, MO Drugs such as Taxol are important antitumor agents since they stabilize microtubules and inhibit mitosis. Although we can understand this function at the cellular level, the molecular details of how these compounds work are not well understood. We have performed large-scale molecular dynamics simulations on a piece of a microtubule both with and without taxol. By comparing these two simulations, we can determine which parts of tubulin undergo conformational and/or dynamical changes upon Taxol binding. As we expected, we see differences in M-loop, H1/S2 loop interactions, however we also see long-range allosteric effects that produce significant changes in the T3, T4 and T5 loops surrounding the nucleotide in the beta monomer. These results suggest that taxol may partially counteract the effects of GTP hydrolysis and alter the interaction with the next longitudinal tubulin dimer, thereby preventing the protofilament from becoming curved as observed with GDP-tubulin.

2284 Investigation of the Paclitaxel-Binding Site in Tubulin Using Mutants of Saccharomyces cerevisiae
R. D. Winefield, T. B. Feland, R. H. Himes; Department of Molecular Biosciences, The University of Kansas, Lawrence, KS Tubulin from the budding yeast Saccharomyces cerevisiae does not bind paclitaxel. However, previously we were able to create paclitaxel binding to S. cerevisiae tubulin by mutating five residues in yeast β-tubulin (purported to play important roles in the putative taxoid-binding site in mammalian β-tubulin) to those that occur in brain β-tubulin. We also created a S. cerevisiae strain that is sensitive to paclitaxel by introducing the mutated β-tubulin gene into a yeast strain that has diminished multi-drug transporter activity. To determine the relative importance of the five residues we have now created new strains that contain different combinations of the five mutations; A19K, T23V, G26D, N227H and Y270F, and tested their sensitivities to paclitaxel. Strains containing only the N227H, Y270F, or the three N-terminal (NT) mutations showed greatly reduced sensitivity to paclitaxel. Similarly, the strains containing the N227H-Y270F or NT+N227H mutations were resistant to paclitaxel. The results indicate that one or more of the mutations in the NT region and the Y270F mutation are required to produce maximum sensitivity to the taxanes. On the other hand, the NT+Y270F strain (without the N227H mutation) was as sensitive to paclitaxel as was the strain with all five mutations. This is a surprising result since His227 has been proposed to play a significant role in paclitaxel binding. In contrast, however, the N227H mutation was necessary to produce maximum sensitivity to docetaxel. Tubulin assembly studies confirmed this result. Docetaxel was twice as effective as paclitaxel in stimulating assembly of tubulin from the strain with all five mutations, but about 60% as effective as paclitaxel when tubulin from the strain lacking the N227H mutation was used. The results indicate that His227 plays a greater role in tubulin’s affinity for docetaxel than for paclitaxel. Supported by NIH grant CA105305

2285 Vinca Alkaloids Demonstrate Differential Effects on Apoptosis
A. Aggarwal,1 A. Kruczynski,1 A. Frankfurter,1 J. Correa,1 S. Lobert1; 1Biochemistry, University of Mississippi Medical Center, Jackson, MS, 2Institute de Recherche Pierre Fabre, Castres, France, 3Biology, University of Virginia, Charlottesville, VA We compared the effects of vinblastine, vinorebine and vinflunine on mouse leukemic P388 sensitive and vinorebine-resistant cells. Interestingly, vinorebine-resistant cell lines were cross-resistant to vinblastine but retained sensitivity to vinflunine as confirmed by IC50 measurements. For example, IC50 values for vinblastine and vinorebine for resistant cell lines increased 2-16-fold relative to sensitive cell lines; while responses of the same cells to vinflunine increased less than 2-fold. Furthermore, flow cytometry demonstrated that at 10X IC50 drug concentrations, vinorebine caused earlier and more pronounced G2/M arrest compared to vinorebine and vinflunine. Apoptotic events were examined by flow cytometry using annexin-V-FITC which also showed earlier apoptosis with vinorebine. Quantitative Western blotting demonstrated that there were no changes in beta tubulin isotype levels, total tubulin or microtubule fractions in the resistant cells compared to the drug sensitive cells; however, by qRT-PCR, we found significantly increased levels of stathmin1, stathmin3 and MAP4 mRNA in the drug-resistant cell lines compared to drug-sensitive cells. Comparisons of the effects of vinblastine, vinorebine and vinflunine on mitochondrial potential change by flow cytometry and cytochrome C release by Western blotting in the drug sensitive and resistant cell lines are in progress. We conclude that differential effects of vinflunine on cell lines compared to other vinca alkaloids may due to its differential effects on apoptotic pathways.

2286 M and N Loop Mutations in Alpha-tubulin Stabilize Microtubules to Confer Dinitroaniline Resistance
L. Ganesan, C. Li, C. Ma, N. Morrisette; Molecular Biology and Biochemistry, UC Irvine, Irvine, CA Plant and protozoan microtubules are sensitive to disruption by dinitroanilines, a group of compounds used in commercial herbicide formulations. Dinitroanilines kill intracellularly Toxoplasma gondii parasites without affecting microtubule function in vertebrate host cells. We previously isolated a number of Toxoplasma lines that are resistant to dinitroanilines and harbor mutations to the single α-tubulin gene. Some of the mutations (Val41Leu, Tyr24His, Thr239Ile, Arg243Cys and Arg243Ser) map to a computationally determined binding site located beneath the N loop. Other resistance mutations (His228Glu, Phe52Ile, Phe49Cys, Phe252Leu, Phe253Thr, Met208Thr, Ala273Val, Ile275Thr, Ala295Val and Met301Thr) are localized in or near the M and N loops, domains that coordinate lateral interactions between protofilaments within the microtubule lattice. Allelic replacement of wild type tubulin with the M and N loop mutations is sufficient to
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Expression of Dinitroaniline-sensitive Tubulin for Biochemical Characterization
S. Lyons-Abbott, J. De Leon, N. Morrisette; Molecular Biology and Biochemistry, University of California—Irvine, Irvine, Irvine, CA
Microtubules in the protozoan parasite Toxoplasma gondii are sensitive to disruption by dinitroaniline compounds such as oryzalin. Computational analysis indicates that oryzalin binds as well as the α-tubulin N loop and dinitroanilines are predicted to disrupt protofilament interactions. Oryzalin resistant Toxoplasma lines contain mutations to residues in the N and M loops as well as to the predicted binding site. The M and N loops of α- and β-tubulin coordinate lateral interactions between protofilaments in the microtubule lattice and we hypothesize that point mutations in the M and N loops increase dimer-dimer affinity, compensating for dinitroaniline disruption of protofilament contacts. Similarly, we predict that binding site mutations decrease the affinity of dinitroanilines for Toxoplasma tubulin. To test these hypothesized mechanisms of resistance, we need to purify large amounts of sensitive tubulin dimers as well as heterodimers containing the individual point mutations that confer resistance. Since Toxoplasma is an obligate intracellular parasite, it would be difficult and expensive to recover sufficient amounts of parasite tubulin for biochemical characterization. We are investigating expression of sensitive α-β-tubulin dimers using E. coli, but did not co-express α- and β-tubulin or assess whether the tubulin was polymerization competent. We have developed plasmids for inducible co-expression of MBP-fusions in Toxoplasma α-tubulin and β-tubulin in E. coli. The budding yeast S. cerevisiae contains tubulin folding chaperones and we have developed an inducible co-expression construct for Toxoplasma α-tubulin and β-tubulin. Lastly the non-pathogenic ciliate Tetrahymena is an attractive expression system since it expresses abundant amounts of dinitroaniline-sensitive tubulin and has a genetically tractable system for replacement of wild type α-tubulin with tubulin mutants.

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Proteomics of the Tubulin Cytoskeletal Scaffold in Hepatic Neoplasia
L. M. Miller, 1 A. Menthen, 1 P. Verderi-Pinar, 1 P. M. Novikoff, 1 S. B. Horwitz, 1 R. H. Angeletti, 1 Developmental and Molecular Biology, Albert Einstein College of Medicine, Bronx, NY, 2Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, NY, 3Pathology, Albert Einstein College of Medicine, Bronx, NY
Understanding the changes that occur at the molecular level during the development of hepato cellular carcinoma is important in both the prevention and treatment of the disease. One characteristic of preneoplastic cells is a structural reorganization, which could be due to changes in the microtubule cytoskeleton. Using a rat liver cancer model that exhibits similar tumor progression stages as in humans, we are examining the tubulin cytoskeletal scaffold and associated proteins. Microtubules are dynamic protein polymers that play an essential role in cell division, maintenance of cell shape, transport of vesicles, and motility. They are comprised of different isotypes of α- and β-tubulin. Expression of various tubulin isotypes may not only affect the assembly of the microtubules, but may also influence the endogenous proteins that bind to microtubules. In this study, the tubulin isotypes and associated proteins present in multiple stages of liver cancer will be determined. The tubulin cytoskeletal scaffold has been purified from rat liver using a Tasel and glutamate based polymerization method. To identify the tubulin isotypes present, we measured the intact mass of the isotypes and then verified the assignment by measuring the mass of CNBr released C-terminal peptides, which contain the isoform defining region. The proteins associated with the microtubules can be released from tubulin with a high salt wash. Associated proteins were digested with trypsin, analyzed by LC-MS/MS and identifications assigned using the Mascot search engine. Initial studies indicate the presence of 3 α- and 3 β-tubulin isotypes in normal liver, along with post-translationally modified isoforms. LC-MS/MS analysis has so far identified 58 proteins that are associated with the cytoskeletal scaffold in normal liver. The methods established herein will be extended to the study of early and late stages of liver cancer.

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Versatile Yet High Specific Fluorescent Labeling of Tubulin
A. Banerjee, 1 T. D. Panosian, 1 B. Cowen, 1 O. Dilek, 1 R. Ravindra, 1 D. L. Sackett, 2 S. Bane 1; 1Chemistry, SUNY-Binghamton, Binghamton, NY, 2Laboratory of Integrative and Medical Biophysics, NICHD/NHL, Bethesda, MD
Three dimensional structures of tubulin solved to date lack the full 15-20 amino acids of the C-terminus of each subunit. These peptides are of interest because they are the primary locus of tubulin sequence heterogeneity and posttranslational modification of the protein. In polymerized tubulin, the C-terminal peptides are on the exterior of the microtubule and are implicated in the association of microtubules with other proteins in the cell. The overall goal of this project is to explore the conformational properties and protein-protein interactions of the C-termini of tubulin using fluorescence spectroscopy. The goal of this aspect of the investigation was to develop a method by which the C-terminal amino acid on one of the subunits can be covalently labeled with a fluorophore. A chemically reactive derivative of tyrosine was synthesized that was a substrate for tubulin tyrosine ligase (TTL), an enzyme that is highly specific for the C-terminus of α-tubulin. Incorporation of the modified tyrosine into α-tubulin was confirmed by immunoblotting and by mass spectrometry. Fluorophores containing a functional group that is covalently reactive with the modified tyrosine were synthesized. Covalent bond formation between the tyrosine derivative and the fluorophores was characterized. The coupling reaction can be performed under conditions that preserve the native structure of the protein (neutral pH, temperature below 37 °C, reaction time less than two hours). The fluorescein-labeled tubulin retained its ability to assemble into normal microtubules. That the fluorescent labeling was successful and specific was confirmed by SDS-PAGE of the fluorescently-labeled protein, which showed a single fluorescent band that corresponds to α-tubulin. The tyrosine derivative and the synthetic fluorophores were not cytotoxic to PC-3 cells at low micromolar concentrations, indicating that this methodology may be applicable to fluorescent labeling of tubulin in living cells.

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Behavioral and Neuropathological Abnormalities in γ-Tubulin 2 Knockout Mice
A. Kubo, 1 A. Kubo, 1 T. Miyakawa, 1 S. Tsukita, 1 S. Tsukita, 1-5 Cell Biology, Kyoto University Faculty of Medicine, Kyoto, Japan, 2Solution Oriented Research for Science and Technology, Japan Science and Technology Corporation, Kyoto, Japan, 3Dermatology, Keio University School of Medicine, Tokyo, Japan, 4Horizontal Medical Research Organization, Kyoto University Graduate School of Medicine, Kyoto, Japan, 5School of Health Sciences, Kyoto University Faculty of Medicine, Kyoto, Japan
γ-Tubulin is a universal component of microtubule organizing centers where it is believed to play an important role in microtubule nucleation. Mammalian have two γ-tubulin genes named TUBG1 and TUBG2. TUBg1 is a ubiquitously expressed γ-tubulin, and TUBg1 knockout mice showed early embryonic lethality. On the other hand, TUBg2 is a neuron specific γ-tubulin. TUBg2 knockout mice were normally developed and fertile, but they showed behavioral abnormalities. In this study, we analyzed TUBG2 -/- mice with many kinds of behavioral tests. TUBG2 -/- mice are normal on measurement of general health, neurological reflexes, sensory abilities, muscular strength, and spatial working memory, as compared to wild type littermate controls. On Morris water task, Tuba2 -/- mice do not show selective search on the probe trial. Tuba2 -/- mice can swim straight forward, but they can’t turn or search around. As a result, Tuba2 -/- mice hit the pool wall frequently. In the Barnes circular maze, another test for spatial navigation learning, Tuba2 -/- mice show normal performance on time spent around the target hole. On balance beam walking test, Tuba2 -/- mice significantly slower show walking speed and frequent slips as compared with wild type littermate mice. Tuba2 -/- mice sometimes show a sudden movement to backward. These observations could mean that Tuba2 -/- mice are normal on spatial learning, but deficient in behavioral paradigm sensitive to dysfunction of the nigrostriatal pathway. Hematoxylin and eosin staining of paraffin embedded section show many cytoplasmic eosinophilic hyaline inclusions in thalamic neural cells of TUBG2-/- brain. Shapes of the inclusions vary from small ovoid structures to longer, narrow bodies (5-10 μm in width, 10-20 μm in length). Together these findings provide the first evidence for a novel role of γ-tubulin in neural cells.

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α-Tubulin Polyglutamylation Modulates KIF1 Traffic-dependent Synaptic Vesicle Targeting
K. Ikagami, 1 M. Itarashi, 1 R. Heier, 1 H. Takagi, 1 M. Mukai, 1 S. Shimma, 1-2, S. Taira, 1 K. Hatanaka, 1-2, S. Morone, 1 M. Matsumoto, 1 Y. Yao, 1 P. Campbell, 1 S. Yuasa, 1 C. Janke, 2 G. MacGregor, 1 M. Setou, 2-4, MITLS, Machida, Japan, 2PRESTO, JST, Saitama, Japan, 3University of California, Irvine, Irvine, CA, 4NIPS, Okazaki, Japan, 5University of Tokyo, Tokyo, Japan, 6NIN, NCNP, Kodaira, Japan, 7CRBM, CNRS, Montpellier, France
Microtubules function as molecular tracks along which motor proteins transport cargo to appropriate destinations. In the nervous system, tubulins, pieces constructing microtubule, are highly polyglutamylated. Despite recent progress in the identification of tubulin polyglutamylases, the physiological significance of this post-translational modification has remained elusive. Here we demonstrate that polyglutamylation of α-tubulin is required for proper targeting of KIF1 into neurons and accounts for synaptic vesicle transport. ROSA22 mice lack functional PGCs1, a subunit of tubulin polyglutamylase. Loss of PGC1 function produced a striking and specific reduction in polyglutamyalted α-tubulin, which was normally enriched in neurons. Reduced α-tubulin polyglutamylation decreased the microtubule-binding affinity of muturotubulin-associated proteins including kinesins. In particular, KIF1A was reduced in neurons from ROSA22 mutants in

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vitro and in vivo. The density of synaptic vesicles, a cargo of KIF1A, decreased in presynaptic compartments in the CA1 region of ROSA22 mutant hippocampus. Consistent with these findings, ROSA22 mutants showed more rapid depletion of synaptic vesicles than wild-type littermates. Our results provide the first evidence for a function of tubulin polyglutamylation in vivo; modulating synaptic transmission through regulation of trafficking of KIF1 kinesin and its cargo synaptic vesicles in neurons.

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Interactions between EB1, Tubulin, and Microtubules: Implications for EB1 Function

K. K. Gupta,1 Z. Zha,2 E. S. Folker,2 S. B. Skube,1 H. V. Goodson1; 1Chemistry and Biochemistry, University of Notre Dame, Notre Dame, IN, 2Department of Anatomy and Cell Biology, Columbia University, New York, NY

EB1 and other plus-end tracking proteins (+TIPs) act at crucial interfaces between microtubules and other subcellular structures. While it seems clear that +TIPs regulate microtubule dynamics, their role in greater cell function remains obscure. EB1 is both an endogenous and conserved +TIP, and it binds to many other +TIPs. These observations suggest that EB1 is the core constituent of a +TIP network or "microtubule plus-end complex". To better understand the functions and activities of this network, we are investigating interactions between EB1, tubulin, and other +TIPs.

Two hypotheses have directed this work: 1) EB1 tracks by an end-loading mechanism driven by its having higher affinity for tubulin than for microtubules (Folker MBOC 15:256a 2004); 2) EB1 binds its own tail in an autoinhibitory conformation released by binding of the tail to partners such as CLIP-170 (Hayashi MolCell 19:449 2005). While many of our experiments are consistent with these ideas, others reveal additional complexity. First, removal of the EB1 C-terminal tail (the part involved in autoinhibition) significantly increases the apparent affinity of EB1 for microtubules. However, it does not interfere with EB1 plus-end tracking in vivo. These observations are difficult to reconcile with a straightforward end-loading mechanism. Second, the EB1 tail-minus mutant binds microtubules with surprisingly high stoichiometry (~4EB1/tubulin dimer). This suggests that EB1 has more than one binding site on tubulin, or that EB1 dimers self-associate. Moreover, CLIP-170 and the EB1 tail mutant (which lacks the CAP-GLY binding site) can bind microtubules simultaneously. We are working on models for interaction between EB1, tubulin, and other partners that are consistent with these data. In particular, we are investigating the possibility that EB1 can form higher-order structures influenced by tubulin and/or other +TIPs.

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Spatial Control of EB1 Dynamics during Neuron Polarization

D. Seetapun, D. J. Odde; Biomedical Engineering, University of Minnesota, Minneapolis, MN

Microtubule polarization requires the coordination of molecular processes to ultimately form axons and dendrites. Among these processes is the polarization of the microtubule array, since loss of dynamic microtubules (MTs) leads to polarization failure. Understanding the regulation of MT dynamics during neuron polarization is essential to understanding the control of axon specification. To investigate MT dynamics during neuron polarization, we performed time-lapse imaging of EGFP-EB1-transfected E7 embryonic chick forebrain neurons grown on polyethyleneimine-coated glass. These neurons undergo a stereotypical transition in vitro from having short, minor processes (stage 2) to having a single major process, the axon, along with a number of minor processes (stage 3), as observed for rat hippocampal neurons. Prior to axon specification (stage 2), we found that EGFP-EB1-tagged MT plus ends in minor processes (MPs) moved distally at a rate of 7.5 +/- 2.9 µm/min (N=56), similar to the rate observed in the cell body. After axon specification (stage 3), axonal Mts (AxMts) moved distally at a rate of 9.2 +/- 3.6 (N=70), again similar to the rate observed in the cell body. However, axonal growth cone MT plus ends (1.1 +/- 0.3 µm/min, N=12) and MPMT plus ends (6.8 +/- 3.41, N=47) moved significantly slower than AxMT and cell body MT plus ends. These preliminary results suggest that there is strong spatial control of MT plus end motion after axon initiation. Interestingly, axon elongation stages in 3 neurons were the same regardless of spatial location. This suggests that plus end polymerization was partially opposed by retrograde transport of Mts specifically in axonal growth cones and MPs, but not in the axon shaft or the cell body.

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The Importance of Tag Identity and Position on EB1 Behavior In Vivo

S. Skube, L. Chaverti, J. Goodson; Chemistry and Biochemistry, University of Notre Dame, Notre Dame, IN

In vivo studies are an integral part of investigating protein function and behavior. A common approach is to fuse proteins of interest to various tags that allow easier purification or detection. It is often assumed that the behavior of the tagged proteins reflects the behavior of the native protein, but this assumption can be problematic. For example, addition of a C-terminal GFP to EB1 inhibits proper binding to CLIP-170 (MBOC 16:533-2005) and interferes with CLIP-170 localization. As part of our studies of EB1 function, we have investigated the issue of EB1 tag identity and localization in more detail. We find that both N-terminal and C-terminal tags have significant effects on the behavior of EB1 and EB1 fragments. Moreover, larger tags (GFP) can have more significant effects than smaller tags (V5). For example, N-terminal tags significantly compromise the ability of expressed EB1/EB1 fragments to bind microtubules (MTs) and/or track MT plus ends. These constructs, like their C-terminally-tagged counterparts, also interfere with CLIP-170 localization, while untagged EB1 recruits CLIP-170 to MTs. As another example, N-GFP EB1-150kDa is cytosolic, while C-GFP EB1-350kDa and the untagged construct localize to mitochondria (the significance of this localization is unknown). These observations demonstrate that experiments performed using tagged EB1 constructs in vivo must be interpreted carefully. In addition to altering EB1 behavior, these tags interfere with CLIP-170 localization. Do they also interfere with localization of other EB1 binding proteins? These studies also raise concerns about the effects of tags on EB1 protein-protein interactions in vitro.

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Regulation of Dynamics of Microtubule Plus End Binding Protein through Rho GTase Activity

C. L. Kilburn, R. Uhura, S. Gupoton, C. M. Waterman-Storer; Physiology Course, Marine Biological Laboratory, Woods Hole, MA

Microtubules establish polarity necessary for cell migration. (+)TIP proteins associate with growing microtubule plus ends and may help maintain polarity by modulating microtubule plus end assembly dynamics or localizing specific signaling molecules. These putative functions of (+)TIPs could be spatiotemporally regulated by their distribution on specific microtubules and their kinetics of microtubule association. The small Rho GTases are known to modulate microtubule assembly and stability in cells. Rac promotes microtubule growth to the leading edge by decreasing catastrophes, while Rhos promotes a stable subpopulation of microtubules. We sought to test the hypothesis that the distribution and microtubule affinity of the (+)TIPs, CLASP, EB1 and APC, are regulated by Rho GTase activity. We imaged GFP conjugates of these proteins in PTK1 epithelial cells by time-lapse microscopy and analyzed the kinetics of their interactions with microtubules using FRAP on a DeltaVisionRT Imaging System. As shown previously, Rac activation promoted (+)TIP behavior by CLASP. APC exhibits three behaviors in control cells, (+)TIP clusters, cortical clusters, and small clusters that move on the microtubules. Activation of Rac caused loss of cortical and (+)TIP clusters, and increased motile microtubule-associated clusters. EB1 localization was unaffected by Rac activity state; however, Rho activation promoted EB1 association with microtubule tips and Rho inhibition increased EB1 binding to microtubule lattice. FRAP experiments revealed that EB1 and EB1 were more stably associated with microtubules in cells expressing dominant negative or constitutively active Rac alleles, suggesting that Rho GTase cycling is critical for the dynamic association of these proteins with microtubule plus ends. Also, stability of EB1 and APC binding to microtubules was increased with constitutive Rho and Rac activity, respectively. Thus, Rho family GTPases modulate (+)TIP localization and microtubule affinity which may be critical to their role in cell polarization.

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A New Role for Microtubule-plus-end Binding Protein EB1: Essential Participant in Skeletal Muscle Differentiation

T. Zhang1, K. J. M. Zaal1, G. G. Gundersen2, R. Uehara3, S. Gupton, C. M. Waterman-Storer; Physiology Course, Marine Biological Laboratory, Woods Hole, MA

Little is understood of the function or mechanism of the microtubule (MT) stabilization taking place during skeletal muscle differentiation. To investigate whether EB1 is involved, as it is in non-muscle cells, we first localized and quantified EB1 in the mouse muscle cell line C2 by immunofluorescence and immunoblotting. The typical EB1 rocket-like staining was found in both undifferentiated myoblasts and differentiated myofibers. Immunoblotting showed the expected 30 kDa band (EB1-30). An additional 20kDa band (EB1-20) appeared during differentiation and was the only other plus-end tracking protein in vivo, but was not found in fibroblasts. Overexpression of full-length EB1 had little effect but overexpression of the fragment EB1-C caused MT defects and formation of abnormally wide and short myotubes. We then silenced EB1 with different shRNA expression constructs. Compared with control cells, EB1-KD neither elongated nor fused. All indices of differentiation were suppressed or at least reduced: MT stabilization (appearance of Glu-MTs), expression of myogenin, p21, beta-catenin and cadherin, Golgi complex and centrosome reorganization. Interestingly, the inhibition of differentiation appeared preferentially linked to the disappearance of EB1-20. Reintroduction of wild-type EB1 led to partial rescue. Examination of cells that spontaneously reverted to an EB1-expressing phenotype in the EB1-KD suggested a cascade of events in which caderhin-catenin complex formation in the plasma membrane and MT stabilization in the cytoplasm happen at an early stage of muscle differentiation before myoblast elongation and fusion. We conclude that EB1 only plays a role in muscle MT stabilization but also is in fact indispensable for muscle differentiation, elongation and fusion. In addition, muscle seems to use two EB1 forms differentially.

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EB3-mediated Microtubule Capture Is Required for Myoblast Elongation and Fusion

A. Straube,1 A. Merdes2; 1University of Edinburgh, Wellcome Trust Centre for Cell Biology, Edinburgh, United Kingdom; 2CNRS - Pierre Fabre, Toulouse, France

During muscle development, myoblasts undergo a series of biochemical and morphological changes that result in elongation and fusion into syncytial myotubes. This process is characterized by a complete remodeling of the cytoskeleton, but the underlying mechanisms are unknown. In this study, we focus on the role of microtubule plus-end binding proteins related to EB1. We demonstrate that expression of EB3, but not of other EB family members, increases during early differentiation of myoblasts, and that this protein is required for the regulation of microtubule dynamics at the cell cortex in differentiating myoblasts. Our data suggest that EB3-mediated microtubule capture is necessary to provide the cytoskeletal support for cell spreading, bipolar cell elongation and establishment of fusion competence. Expression of chimeric EB1/EB3 proteins on an EB3-depletion background indicates that EB3-specific functions are mediated by its amino-terminal calponin-like domain. We identified two amino acid changes between EB1 and EB3 that are essential for the myogenic function of EB3 and are exposed at the surface of the molecule opposite to the microtubule binding site. We therefore propose that an EB3-specific interacting protein mediates microtubule capture in differentiating myoblasts and we are currently in the process of isolating this factor. Our results suggest that EB3-mediated microtubule capture at the cell cortex is a crucial step during myogenic differentiation and might be a general mechanism in polarized cell elongation.

Identification of DDA3 as a Novel Microtubule Associated Protein That Interacts with EB3 and APC2

P. Hsieh, R. Chang, F. Wang; Institute of Biochemistry and Molecular Biology, National Yang-Ming University, Taipei, Taiwan

We have previously identified DDA3 as a novel mouse p53 transcriptional target. To explore the functional role of DDA3, we screened a mouse brain cDNA library by yeast two-hybrid assay, and identified EB1, a microtubule plus-end binding protein, as a DDA3 interacting protein. The mouse EB3 was cloned and shown to share 99% sequence homology with its human counterpart. Binding of DDA3 to EB3 was confirmed by GST pull-down assay, subcellular co-localization and co-immunoprecipitation, and the results indicated that the interaction of these two proteins within cells required intact microtubules. GST pull-down assay further mapped the domains of DDA3/EB3 interaction to amino acids (a.a.) 118-241 and 242-329 of DDA3 and the N- and C-termini of EB3. Immunofluorescence analysis showed co-localization of DDA3 with microtubules in various cell phases, and regions encompassing a.a. 118-241 and 242-329 contain microtubule-interacting and bundling activities. In vitro microtubule binding assay showed that DDA3 and EB3 associated directly to microtubules. In addition, DDA3 bound to the EB3 interacting partner APC2, a homologue of the tumor suppressor adenomatous polyposis coli (APC). By ectopic expression and suppression of endogenous DDA3 and EB3 levels in cells, we showed that the two proteins countered APC2 in the Wnt/TCF-dependent transactivation. Together our results indicate that DDA3 is a novel microtubule-associated protein that binds to EB3 and APC2, and suggest a role of DDA3 in the β-catenin-mediated pathway.

Adenomatous Polyposis Coli Is Phosphorylated during Early Stages of NGF Mediated PC12 Differentiation

H. Y. Caro-Gonzalez,1 W. J. Nelson,1,2 A. I. M. Barth;1 Molecular and Cellular Physiology, Stanford School of Medicine, Stanford, CA; 2Department of Biological Sciences, Stanford University, Stanford, CA

Adenomatous polyposis coli (APC) is a multifunctional protein that binds to microtubules (MT) and localize to clusters at sites of MT-cortex interactions during cell extensions in several cell types. APC localizes to tips of neurites and is needed for differentiation of rat pheochromocytoma (PC12) cells by nerve growth factor (NGF). APC function is in part regulated by glygogen synthase kinase 3β (GSK3β) activity, which increases APC’s affinity for β-catenin and decreases MT bundling. It has been shown that, during NGF signaling in fully polarized neurons, GSK3β activity is inhibited locally at the growth cone, which allows APC cluster formation at the leading edge and rapid axon extension. In order to understand how APC mediates neurite extension in response to NGF we are analyzing APC interactions during NGF induced neurite extension in PC12 cells. Here we show that APC is phosphorylated upon stimulation of PC12 cells with NGF. This phosphorylation correlates with the rapid formation of APC cortical clusters at cell extensions. This new phosphorylation seems to be GSK3β independent and independent of PI3K and MEK pathways. We are working on the characterization of this phosphorylation and its effects on MT dynamics.

Loss of the Adenomatous Polyposis Coli Protein Alters Cytoskeletal Organisation and Leads to Decreased Cell Migration

K. Kroboth,1 I. Newton,1 D. Dikovskaia,1 K. Kata,2 C. M. Waterman-Storer,2 I. S. Nähkä1; 1Cell & Developmental Biology, University of Dundee, Dundee, United Kingdom; 2Department of Cell Biology, Scripps Research Institute, La Jolla, CA

Truncation mutations in the adenomatous polyposis coli (APC) gene are common to most colorectal cancers. The APC protein supports the formation of a protein complex that controls the availability of β-catenin in a Wnt-regulated manner. β-catenin is crucial for cadherin-mediated cell-cell adhesion and also acts as a transcriptional regulator. In addition, APC regulates cytoskeletal proteins. It binds to microtubules directly and indirectly and also interacts with proteins that regulate F-actin dynamics. Truncation mutations of APC in tumours lack binding sites for cytoskeletal components suggesting that loss of these interactions also contribute to defects in APC-mutant cells. In this study we show that loss of APC results in decreased cell migration. This effect correlates with the disappearance of cellular protrusions and a reduction in microtubule stability that is accompanied by changes in post-translationally modified microtubules. Correspondingly, overexpression of APC induces the formation of cellular protrusions. Direct binding to microtubules, but not binding of EB1, determines the effect of APC on microtubule stability and also governs the dynamics of this interaction. Surprisingly, the ability of APC to stabilise microtubules is not required for APC-induced cell shape changes, highlighting the importance of APC interaction with other cytoskeletal proteins. Our data demonstrate that cell migration and microtubule stability are linked to APC status and reveal a weakness in APC-deficient cells with potential therapeutic implications.

Aneugenic Activity of Op18/stathmin Is Potentiated by the Somatic Q18->E Mutation in Leukemic Cells

P. Holmfeldt, K. Brännström, S. Stenmark, M. Gullberg; Molecular Biology, Umea University, Umea, Sweden

Op18/stathmin (Op18) is a phosphorylation-regulated microtubule destabilizer that is frequently overexpressed in tumors. The importance of Op18 in malignancy was recently suggested by identification of a somatic Q18->E mutation of Op18 in an adencarcinoma. We addressed the functional consequences of aberrant Op18 expression in leukemias by analyzing the cell cycle of K562 cells either depleted of Op18 by expression of interfering hairpin RNA or induced to express wild-type or Q18E substituted Op18. We show here that although Op18 depletion decreases microtubule density during interphase, the density of mitotic spindles is essentially unaltered and cells divide normally. This is consistent with phosphorylation-inactivation of Op18 during mitosis. Overexpression of wild-type Op18 results in aneugenic activities, manifest as aberrant mitosis, polyploidization, and chromosome loss. One particularly significant finding was that the aneugenic activity of Op18 was dramatically altered by the Q18->E mutation. The hyperactivity of mutant Op18 is apparent in its unphosphorylated state, and this mutation also suppresses phosphorylation-inactivation of the microtubule-destabilizing activity of Op18 without any apparent effect on its phosphorylation status. Thus, although Op18 is dispensable for mitosis, the hyperactive Q18->E mutant, or overexpressed wild-type Op18, exerts aneugenic effects that are likely to contribute to chromosomal instability in tumors.

Effect of Stathmin and MAP4 Expression in Breast Cancer Cells

V. E. Cucchiarielli,1 L. Hiser,1 J. J. Correia,1 S. Lobert1; 1Department of Biochemistry, University of Mississippi Medical Center, Jackson, MS; 2School of Nursing, University of Mississippi Medical Center, Jackson, MS

The efficacy of anticancer therapy is limited by drug resistance. Stathmin and MAP4 are known to influence microtubule dynamics and may play an important role in determining the sensitivity to the antimotic agents, taxanes and vinca alkaloids. p53 is thought to regulate the expression of stathmin and MAP4. Therefore, alterations in p53 expression might lead to resistance to tubulin-binding agents. We evaluated the effects of the absence of p53 on the cellular response to these drugs. The response to taxol and vinblastine was determined by IC50 measurements in two cell lines: MCF-7 SC expressing wild type p53 and MCF-7 KD or p53 knock down cells (derived from the human breast adenocarcinoma MCF-7 cells). We found that MCF-7 KD cells are 20% more sensitive to taxol as compared with MCF-7 SC cells; while no significant difference in response to vinblastine was observed, mRNA expression of stathmin and MAP4 was measured by q-real time RT-PCR before and after treatment with taxol. Contrary to expectations, stathmin and MAP4 mRNA levels were lower in MCF-7 KD cells, suggesting that...
in this system, p53 is an activator of statmin and MAP4 genes, or that other factors that regulate statmin and MAP4 expression have been altered by p53 knockdown. The lower level of statmin mRNA in p53 knock down cells is consistent with their increased sensitivity to taxol. Upon treatment with their correspondent taxol IC50 concentrations, we found a decrease in statmin mRNA levels. These data suggest p53 regulation of statmin and MAP4 may be altered by taxol. The same experiments are being carried out with cells treated with vinblastine.

2306
Effects of Tau Pseudophosphorylation at Residues Ser262, Ser396, and Ser404 upon Microtubule Binding, Assembly, and Regulation of Dynamic Instability In Vitro

E. Kiris, M. R. Massie, M. A. Jordan, L. Wilson, S. C. Feinstein; Neuroscience Research Institute, University of California, Santa Barbara, CA

Tau is a neural specific microtubule-associated phospho-protein that is crucial for the proper development and maintenance of the nervous system. Mechanistically, it is well established that tau binds directly to microtubules, promotes microtubule assembly and regulates microtubule dynamic instability. Given that microtubules are central to many neural cell functions, it is not surprising that abnormal tau behavior has long been associated with numerous neurodegenerative diseases, including Alzheimer’s disease and related dementias; further, a large body of biochemical and genetic data implicate abnormal tau phosphorylation in pathological tau action. One technical strategy that has become widely used to overcome a variety of technical difficulties in studying protein phosphorylation is “pseudophosphorylation”, that is, the substitution of aspartic or glutamic acid residues to mimic phosphorylated serine, threonine or tyrosine. Here, we have used pseudophosphorylation to study the mechanistic effects of phosphorylation at three key phosphorylation sites in tau (ser262, ser396 and ser404). We assessed the effects of each individual pseudophosphorylation event upon the ability of tau to bind and assemble microtubules as well as to regulate microtubule dynamic instability. Consistent with a variety of cellular studies, pseudophosphorylation at ser404 has little effect on tau action in either 3-repeat or 4-repeat contexts. Alternatively, pseudophosphorylation at ser262 compromises the ability of tau to bind to microtubules at steady state by up to 2 fold in both 3-repeat and 4-repeat tau contexts. Finally, pseudophosphorylation at ser396 significantly compromises tau binding activity in 4-repeat but not 3-repeat tau. Ongoing studies of the ability of each molecule, as well as molecules harboring multiple pseudophosphorylated sites, to affect the ability of tau to assemble microtubules and to regulate microtubule dynamic instability should provide valuable insights into mechanisms of normal and pathological tau action. (Supported by NIH grants R01NS035010, R01NS13370 and R01CA57291)

2307
Tau Impacts on Src-mediated Actin Remodeling

V. Sharma; Internal Medicine, University of Iowa, Iowa City, IA

Tau, the primary component of neurofibrillary tangles in Alzheimer’s disease, is now known to have functions beyond microtubule (MT) association. We have previously shown that tau interacts with the SH3 domain of Src family kinases (SFKs), non-receptor protein tyrosine kinases that regulate several biological functions, including cell cycle and growth factor signaling. A potential consequence of SH3 interaction is the upregulation of the tyrosine kinase activity. Here we investigate the activation of Src and Fyn by tau, both in vitro and in vivo. The presence of tau increased the tyrosine phosphorylation of tubulin by Fyn in in vitro assays and in transfected COS7 cells. Tau also upregulated the activity of Src in a kinase assay. In fibroblasts, Src is known to be activated by PDGF, leading to a dramatic reorganization of the actin cytoskeleton. We found that tau appeared to prime Src for activation following PDGF stimulation, as reflected by changes in Src-mediated actin remodeling. While majority of the non-transformed cells recovered actin stress fibers by 7h following PDGF stimulation, tau-expressing cells showed a sustained actin fiber breakdown, suggesting a sustained activation of Src kinase activity. MT stabilizing function of tau appeared not to be involved in this effect. The spatial localization of activated SFKs was also altered by the presence of tau. Results obtained by inhibiting SFK activity with a pharmacologic inhibitor, or by using a tau deficient mutant in Src interaction, implicated SFKs as mediators of the effect of tau on actin organization. Our results provide evidence that the interaction of tau with SFKs upregulates the tyrosine kinase activity. This interaction would be relevant during cytoskeletal reorganization in neuronal differentiation, and also in AD where aberrant activation of cell cycle mechanisms has been implicated.

2308
Centrioles Are Essential for Ciliogenesis and Meiosis, but Not Mitosis and Fly Development

S. Blachon, J. Gopalakrishnan, A. Polyanovsky, T. Avidor-Reiss; 1Harvard Medical School, Boston, MA, 2Sechenov Institute, Russian Academy of Sciences, Petersburg, Russian Federation, 3Howard Hughes Medical Institute and University of California, San Diego, CA

The centriole has a dichotomous role in eukaryotic cell biology. During mitosis, as a component of the centrosome it radiates astral microtubules, while in interphase it becomes a basal body to the cell cilium. In Drosophila melanogaster, all cells have centrioles, however, centrioles are transformed to basal body only in sensory neurons and in male germ line. These basal bodies participate in the formation of motile and mechanosensory cilia as well as in male meiosis. To study basal body formation, we screened for the mutations that generate adult flies showing a characteristic proprioception phenotype of ciliary mutants, having immotile sperm flagellum and unable to carry out meiosis. We find three mutants in different genes that neither have basal bodies nor cilia. To study the failure to generate basal bodies, we have examined the centrioles in these mutants. Surprisingly, these mutants have either abnormal or no centrioles. These findings demonstrate that centrioles are not required for fly development but are essential for ciliogenesis. The proteins encoded by the candidate genes are localized specifically to the centriole and basal body, showing a distinct spatial and temporal localization and suggesting that they have indispensable role in different processes during centriole formation.

2309
Identification of Basal Body Proteins That Interact with Delta and Epsilon Tubulin

S. K. Dutcher, L. Li, T. H. Giddings; 1Genetics, Washington University School of Medicine, St. Louis, MO, 2MCD Biology, University of Colorado, Boulder, CO

We employed a yeast two-hybrid screen to identify novel interaction partners of Bardet Biedl Syndrome Protein, Bbs5, for β-tubulin and two genes that interact only with β-tubulin. The first group includes translation initiation factor 3, several kinases, and five novel proteins. The β-tubulin interacting class contains a AMP phosphodiesterase, a DNA I motif containing protein, and a novel protein. The β-tubulin interacting class contains a RuvB-like protein and a novel protein. The novel protein encoded by STP1, which interacts with δ-tubulin, is found in all ciliated organisms. It localizes to the basal body region by immunofluorescence. Two independent RNAi strain have morphologically normal basal bodies, but assemble very short (< 0.5 μm) flagella when the protein amount is reduced. The basal body localization of Stip1 requires the presence of δ-tubulin; it is not localized in the δ-tubulin mutant, χm3-1. However, δ-tubulin is localized properly in the Stip1 RNAi strains. RuvBII is found in all eukaryotic organisms and is involved in chromatin remodeling and transcription complexes (Bellonta et al., 2005). In Chlamydomonas, RuvBII localizes to the basal body region and to the nucleus by immunofluorescence, but localizes only to the nucleus upon deflagellation. The gene is transcriptionally upregulated by deflagellation (6x-fold) as is RuvBII or pontin (5x-fold). Its localization is dependent on fully assembled basal bodies as it is not localized in a number of mutants (bd2, bd10, bd11). RNA interference studies are underway to examine the hypomorphic phenotype.

2310
Bardet Biedl Syndrome Protein, Bbs5, for Tetrahymena Basal Body and Ciliary Function

C. G. Pearson, T. H. Giddings, M. Winey; MCDB, CU at Boulder, Boulder, CO

Human BBS5 gene mutations cause Bardet Biedl Syndrome (BBS), a disease characterized by retinal dystrophy, renal abnormalities, obesity, polydactyly, and other maladies. These phenotypes are likely associated with centriole, basal body and or/ciliary abnormalities. Loss of Bbs5 results in flagellate cells (Li et al, 2004), however, the functional role of Bbs5 in basal body and ciliary function is not understood. We have identified the Tetrahymena ortholog of the human BBS5 gene. The single BBS5 gene (TTBBS5) encodes a ~41 kDa protein with two conserved DM16 domains. Tetrahymena TTBBS5 tagged with GFP localizes at basal bodies. GFP-Bbs5 fluorescence was found along cortical rows in live cells and co-localized with the basal body marker centrin. Preliminary immuno-electron microscopy cross-section studies find GFP-Bbs5 at the lumen of the basal body, implicating this protein in early basal body duplication and assembly. We introduced into Tetrahymena several mutant alleles of GFP-bbs5 that mimic human disease mutations. In addition to elucidating human mutations that alter GFP-Bbs5 localization, we have identified TTBBS5 as a candidate for a Tetrahymena mutant that indicates that TTBBS5 is essential and protein depletion alters cellular motility and division, as expected for ciliary dysfunction. These studies further develop Tetrahymena as a model system for functional understanding of basal body and ciliary disease in human implication.
A Prefoldin-like Protein Provides a Link between the Core BBSome and the Chaperonin-like Proteins BBS6 and BBS10
A. V. Loktev,1 2 F. Bazan,3 D. Y. Nishimura,3 J. S. Beck,4 Q. Zhang,4 D. C. Slusarski,4 V. C. Sheffield,5 P. K. Jackson,3 2 M. V. Nachury3, 1 Department of Tumor Biology and Angiogenesis, Genentech, South San Francisco, CA, 2Department of Pediatrics and Howard Hughes Medical Institute, University of Iowa, Iowa City, IA, 3Department of Protein Engineering, Genentech, South San Francisco, CA, 4Department of Pathology and Stanford University School of Medicine, Stanford, CA, 5Department of Biological Sciences, University of Iowa, Iowa City, IA

Bardet-Biedl syndrome (BBS) is a pleiotropic disorder affecting retina, kidneys and body weight regulation. We have recently shown that seven of the eleven known BBS proteins are present in a highly conserved 450 kDa complex, which we named BBSome. Outside of the BBSome subunits, BBS6 and BBS10 stand out as type II chaperonin-like proteins present only in vertebrates, suggesting a link between BBS and protein folding. Yet, all evidence so far points to a role for the BBSome in membrane trafficking to and inside the primary cilium. We have now discovered stoichiometric amounts of a 10 kDa polyethylene (BBIP10) associated with the core BBSome. Bbip10 show an arrangement characteristic of prefoldins, a group of chaperones involved in transferring unfolded polypeptides to the canonical type II chaperonin complex. Like all other BBSome subunits, BBIP10 is conserved in ciliated organisms and is absent from plant genomes, thereby implicating BBIP10 in cilium function. This association is further highlighted by genome wide expression correlation data in the mouse eye showing that BBIP10 is better correlated to known BBS genes than 99.98% of all genes. Finally, we turned to the zebrafish system for functional validation. Loss of function of BBIP10 leads to malformations of the ciliated Kupffer’s vesicle, left-right asymmetry perturbations and defects in melanosome movement. The same defects were found upon depletion of BBP10, implying that BBP10 functions in the same molecular pathway as known BBS genes. In summary, we have found a novel prefoldin-like subunit of the BBSome that may provide a link to the cilia-chaperonin-like BBS proteins. A future challenge will be to identify and characterize the critical substrates of this novel folding pathway in order to uncover the missing link between BBS proteins and the variety of symptoms affecting BBS patients.

Tetrahymena thermophila Possesses Two SAS-6 Homologs That Function at Basal Bodies
B. P. Culver, T. Giddings, M. Winey; MCB, University of Colorado, Boulder, CO

Tetrahymena thermophila possesses two SAS-6 homologs that function at basal body Centrioles are a functionally and morphologically conserved organelle throughout most of the eukaryotic kingdom. SAS-6 was previously shown to be required for centriole duplication in C. elegans as well as in human cells (1,2). We have identified two SAS-6 homologs in ciliated protozoan Tetrahymena thermophila. We refer to these genes as SAS-6 like protein 1 and 2 (Sip1 and Slp2). While Tetrahymena do not possess centrosomes and associated centrioles, they do possess in abundance morphologically and compositionally similar basal bodies. Our study seeks to define a role for Slp1 and Slp2 at the basal body. We have found that both genes are expressed in dividing cells and that their GFP-tagged gene products localize to basal bodies. Slp1 and Slp2 like their homologs in other organisms have two identifiable motifs: a N-terminally located PISA domain and a centrally located coiled-coil domain (1, 2). We have shown that GFP-tagged truncations possessing the PISA domain localize to basal bodies. It therefore seems likely that the dividing cells and that their GFP-tagged gene products localize to basal bodies. Slp1 and Slp2 like their homologs in other organisms have two identifiable motifs: a N-terminally located PISA domain and a centrally located coiled-coil domain (1, 2). We have shown that GFP-tagged truncations possessing the PISA domain localize to basal bodies. It therefore seems likely that the presence of the PISA domain is sufficient for the localization of Slp1. References: 1. Dammermann, A., Müller-Reichert, T., Pelletier, L., Habermann, B., Desai, A., and Oegema, K. (2004). Science 305, 1772-1775. 2. Shi, M., Zhao, J., Stock, J. M., and Zheng, J. H. (2006). Curr. Biol. 16, 1277-1281.

A Sea Urchin Sperm Flagellar Adenylate Kinase with Triplicated Catalytic Domains
M. Kimukawa,1 2 M. Nomura, 1 V. D. Vacquier; 1 Center for Marine Biotechnology and Biomedicine, Scripps Institution of Oceanography, University of California San Diego, La Jolla, CA, 2Shimoda Marine Research Center, University of Tsukuba, Shimoda, Japan

The mitochondrion of sea urchin sperm is located at the base of the sperm head and the flagellum extends from the mitochondrion for ~40 μm. These sperm have two known flagellar, non-mitochondrial enzymatic systems to phosphorylate ADP. The first involves the phosphocreatine shuttle, where flagellar creatine kinase (Sp-CK) produces phosphocreatine to rephosphorylate ADP. The second system, studied in this paper, is adenylate kinase (Sp-AK) that uses 2 ADP to make ATP + AMP. Cloning of Sp-AK shows that, like Sp-CK, Sp-AK has three catalytic domains. Sp-AK localizes along the entire flagellum and most of it is tightly bound to the axoneme. After solubilization from the axoneme by dialysis against EDTA buffer and sedimentation by sucrose density centrifugation, the characteristics of Sp-AK were studied. The Km of Sp-AK in the direction of ATP synthesis is 0.9 mM and the Vmax 16.6 μmol ATP formed/mg protein/min. The Sp-AK activity is uniform between phi 6-9. The specific Sp-AK inhibitor Ap5A, blocks enzyme activity with an IC50 of 0.41 μM. Flagellar motility was studied using deuterated spermidine and protein. In 1 mM ADP, flagella reactive motility in 5 min; 1 μM Ap5A completely inhibits this reactivation. No inhibition of motility occurs in Ap5A when 1 mM ATP is added to the reactivation buffer. Under in vitro conditions, when both enzymes are saturated with ADP as substrate, Sp-CK contributes ~60%, and Sp-AK ~40%, of all non-mitochondrial ATP synthesis in isolated flagella. However, under in vivo conditions the role of Sp-AK in ATP regeneration to support motility is questionable. An alternative hypothesis is that Sp-AK could be involved in removal of ADP, which is a potent inhibitor of dynein ATPase.

To Maintain Motility, the Na,K-ATPase Alpha4 Isomorf Is Necessary for Extruding from Sperm Intracellular Protons Leaking from Metabolically Active Mitochondria
A. Sengupta, S. Chakraborty, P. F. James; Zoology, Miami University, Oxford, OH

The Na,K-ATPase is a major ion transport protein found in higher eukaryotic cells that establishes and maintains electrochemical gradients across the plasma membrane by transporting three Na+ out of the cell and two K+ into the cell using the energy generated from the hydrolysis of ATP. The activity of the Na,K-ATPase is essential for establishing resting membrane potential and to support the activity of other membrane transporters which move other solutes into or out of the cell. Of the four known alpha isoforms of the Na,K-ATPase, only two, the ubiquitously expressed alpha1 isoform and the sperm-specific alpha4 isoforms, are expressed in sperm. Specific inhibition of the alpha4 isoform inhibits rat sperm motility suggesting a specialized role for this Na,K-ATPase isoform in maintaining sperm motility. Here we demonstrate, by alkalining the sperm cytoplasm with NHSI, that loss of sperm motility mediated by inhibition of the Na,K-ATPase alpha4 isomorf is due to acidification of sperm cytoplasm. In addition, we identify the mitochondria as the source of protons which accumulate during Na,K-ATPase alpha4 isoform inhibition; inhibition of H+ transport by the electron transport system with KCN or Antimycin A eliminates the necessity for the Na,K-ATPase alpha4-dependant pH regulatory mechanism to maintain sperm motility. This data demonstrates that the Na,K-ATPase alpha4-dependant pH regulatory mechanism is only required to support sperm motility in presence of metabolically active mitochondria.

Characterization of the Guanylate Cyclase in Cilia of Tetrahymena thermophila
C. A. Clement,1 2 C. F. Guerra,1 A. Awu, 1 2 A. Bell,3 P. Saiti,3 S. T. Christensen3; 1 BKA, Institute of Molecular Biology and Physiology, Copenhagen, Denmark, 2Albert Einstein College of Medicine, Bronx, NY

The cGMP production in the motile cilia/flagella in Tetrahymena thermophila may play an important role in regulating cilary beat frequency (CBF) and/or function in signal transduction systems that regulate cell survival and proliferation. We have studied the mRNA up-regulation and localization of the membrane bound cGMP-generating enzyme, guanylate cyclase (GC) in T. thermophila and further investigated possible interactions with GC and the IFT raft protein, IFT139, and kin 5, which is a homodimeric kinase-2-family IFT-motor protein. With RT-PCR technique we initially conducted an analysis of whether 14 putative GC genes in the Tetrahymena database are activated after deciliation. These results showed that three of these genes were up-regulated as a result of deciliation, suggesting that these genes encode GC’s that localize to the cilium. By immunofluorescence (IF) microscopy using an antibody directed against a conserved region of GC, we show that GC strongly and preferentially localized to the cilia in T. thermophila and in a punctate pattern along the entire length of the cilium. GC quickly localized to regenerating cilia in cells after deciliation. To investigate how GC is transported into the cilium in T. thermophila, we conducted co-immunoprecipitation experiments that showed that GC colocalized with IFT139 along the cilium. They structure support the conclusion that transport of GC into the cilary membrane of T. thermophila is via IFT utilizing the homodimeric kinase kin5.

Katanin Is Required for Assembly of Motile Cilia
N. Sharma, J. Bryant, D. Wogha, J. Gaertig; Cellular Biology, University of Georgia, Athens, GA

The cilia Tetr hymena thermophila has two genes encoding a catalytic subunit of the microtubule-severing protein, katanin (p60), KAT1 and KAT2, and one gene encoding a noncatalytic subunit of katanin (p80), KAT3. Using the “knockout heterokaryon” strategy, we produced gene disruption mutants at all these loci. Cells lacking either KAT1 (p60) or KAT3 (p80) were
immotile and displayed an arrest in cytokinesis, while cells lacking KAT2 appeared normal. Cells lacking Kat1p showed an increase in the microtubule content and accumulated stable hyperacetylated microtubules in the cell body. The cytokinesis arrest appeared to be caused by abnormally long cortical microtubules, which impeded the ingressing cleavage furrow. Thus, in the cell body, the phenotype of katanin deficiency is consistent with lack of microtubule severing activity. Moreover, overproduction of Kat1p led to depolymerization of most microtubules including some ciliary doublets. Ultrastructural analyses of KAT1−/− and Kat2−/− null cells revealed severe defects in cilium biogenesis. The basal bodies formed normally, but either remained uniliated or had only an excessively short immotile cilium and only 9+0 microtubules. GFP-Kat1p localized to cortical microtubules and basal bodies. Together with an earlier demonstration that the p80 subunit is needed for the central pair assembly in Chlamydomonas (Dymek and Smith, Eukaryot. Cell, 3, 2004), these results show that the catalytic activity of katanin is required for assembly of motile cilia. We propose that katanin assists in the assembly of cilia by generating ciliary tubulin precursors via severing of preexisting MTs inside the cell body. Importantly, the phenotype of katanin mutants is identical to the phenotype of a beta-tubulin mutant lacking sites of so-called polymodifications (glutamylation and glycylation). We suggest that katanin preferentially severs polymodified tubulins.

2317
Protein Families of the Trypanosome Flagellum

N. Portman, H. Dawe, H. Farr, A. Baines, M. Ginger, K. Gull; Sir William Dunn School of Pathology, University of Oxford, Oxford, United Kingdom
We have recently produced a detergent and salt-extracted proteome for the Trypansomoa brucei flagellum. Bioinformatic investigation of the evolutionary distribution of these proteins reveals that a number of proteins encoded by a single gene in other eukaryotes exist as extended gene families in T. brucei and other kinetoplastids. Several known flagellar components such as RIB72, PACRG, PF2; Trypanin, Ectoin and MBO2 are members of small gene families in T. brucei, as well as hypothetical proteins such as DIGIT, AKR, TFI, and TAX-2 for which we have produced phenotype information. Family members are often found on different chromosomes with no conservation of flanking gene order, suggesting ancient gene duplications and subsequent divergence. We have analysed each family in terms of its domain architecture, localisation, and RNAi phenotype. In some families simultaneous RNAi ablation of multiple family members is required to produce a visible phenotype whereas ablation of a single member is sufficient in others, indicating that a requirement for functional redundancy cannot fully explain this phenomenon. We suggest that the lack of alternative splicing and introns in this extracellular parasite may favour gene duplication as a means of achieving protein diversity.

2318
Identification of Novel Ciliary Proteins Using Microarray Data of In Vitro Ciliogenesis

A. Kubo,1 A. Kubo,2 S. Tsukita,3 S. Tsukita4; 1Dermatology, Keio University School of Medicine, Tokyo, Japan, 2Solution Oriented Research for Science and Technology, Japan Science and Technology Corporation, Tokyo, Japan, 3Cell Biology, Kyoto University Faculty of Medicine, Kyoto, Japan, 4School of Health Sciences, Kyoto University Faculty of Medicine, Kyoto, Japan
To identify the factors required for the formation of ciliary epithelial cells, we analyze the gene expression patterns during ciliogenesis in the mouse tracheal epithelial cells cultured in vitro. Our previous work identified the PC1-2 protein as a component of fibrous granules appeared in the beginning of ciliogenesis. In the course of ciliogenesis, the expression of ciliary precursor proteins, including PC1-2, is elevated first followed by the expression of basal body proteins and axonemal proteins sequentially. We identified two novel proteins in this study. One shows the expression pattern of axonemal proteins and localizes to the so-called ‘lateral spokes’ at the tip of motile cilia. Its expression is restricted to the organ that has motile cilia. The other shows the expression pattern of ciliary precursor proteins and localizes at the basal bodies and the fibrous granules. We named this protein PCM-2. PCM-2 is a ubiquitously expressed coiled-coil protein with a molecular mass of ~80 kD and co-localized with the cilidional satellites protein PC1-2 and the centrosome protein γ-tubulin in U2OS cells. HA-tagged full length PCM-2 localizes to the centrosome in the interphase. Interestingly, the centrosome localization of full length PCM-2 is observed only in ~20% of transfected cells at 24h post transfection, probably via cell cycle dependent integration of PCM-2 to the centrosome. The amino-terminal domain of PCM-2 binds to PCM-1, and the carboxy-terminal domain mediates centrosome attachment. Further characterization of PCM-2 is underway to determine the function of PCM-2 in ciliogenesis and ciliogenesis.

2319
Nek1 Affects Stability of Centrioles and Cilia

M. C. White, L. M. Quarmby; Molecular Biology and Biochemistry, Simon Fraser University, Burnaby, BC, Canada
Mutations in Nek1 (NIMA-Related Expressed Kinase 1) have been identified as causal in the murine models of polycystic kidney disease katu and katu (Kidney Anemia Testis). Nek1 has proposed roles in the DNA damage response pathway and in chromosome condensation. Endogenous Nek1 is cytoplasmic and localizes to multiple spots adjacent to the centrosomes, however expression of carboxy-terminal truncations of Nek1 accumulate in the nucleus. The mechanisms and cellular significance of nuclear import and export of Nek1 remain poorly characterized. Here, we demonstrate that full-length Nek1 is cytoplasmic, but probably cycles through the nucleus. We have identified a repressive, leptomycin-B-sensitive, carboxy-terminal Nuclear Export Sequence (NES), and more than one sufficient Nuclear Localization Signal (NLS). Overexpression of truncated, eGFP-tagged mNek1 that lacks the NES region but retains the NLSs (residues 1-686) results in a depletion of γ-tubulin foci from the centrosome, and the loss of the primary cilia in the murine kidney epithelial cell line IMCD3. Expression of residues 1-686 of the putative kinase-dead mutant K33R does not deplete γ-tubulin foci or disrupt ciliogenesis. Furthermore, the truncated K33R protein localizes to the primary cilium, the centrosomes and the nucleus. The central coiled-coil domain of mNek1 is sufficient for localization to the primary cilium. To determine the domains sufficient for the disruption of γ-tubulin foci in wild-type, and primary cilium localization in the K33R mutant, we constructed eGFP-tagged truncations of mNek1 removing each of the four central coiled-coils. Coiled-coils 3 and 4 were dispensable for both these effects, whereas coiled-coils 1 and 2 were necessary. We propose that Nek1 has several kinase-depending activities related to the primary cilium and the centrosomes and that these activities are inhibited by the presence of Nek1’s carboxy-terminal domain which also inhibits nuclear accumulation of Nek1. This work is funded by a KFO6 operating grant to LMQ.

2320
Extensive Polyglycylation of Tubulin Is Not Essential for Cilia Function in Mammals

S. J. Y. Dosso, R. Hallworth, Biomedical Sciences, Creighton University, Omaha, NE, USA
Glycopolymodification is a posttranslational modification that is unique to the alpha and beta subunits of tubulin in the microtubules of cilia and flagella. It has been shown that polyglycylation of tubulin in the cilia of tetrahymena contributes to cilary function and the loss of polyglycylation in beta-tubulin may result in lethality. However, the full effects and significance of polyglycylation are still unknown. We have explored the extent of polyglycylation in functional cilia of mouse tracheal epithelial cell cultures using two antibodies, TAP 952 and ANO 49, which are specific for minimal and extensive polyglycylation, respectively. We have found that although minimal polyglycylation was present along most of the cilia length, it was excluded from the tip. In contrast, little to no extensive polyglycylation occurred in our cultures at any stage. This suggests that while some glycylation is universal in mammalian cilia, extensive polyglycylation is not required for function. Supported by RR17417-01 to Creighton University and NSF-EPSCoR EPS-0346476 to RH. We thank Marie-Helene Bre for the gift of antibodies.

2321
A Functional Component Analysis of Flagellar Motility in African Trypanosomes

P. W. Collingridge,1 E. M. Barnwell,2 N. Portman,3 M. K. Shaw,4 P. G. Mceean,1 K. Gull,4 M. L. Ginger1; 1Sir William Dunn School of Pathology, University of Oxford, Oxford, United Kingdom, 2Department of Biological Sciences, Lancaster University, Lancaster, United Kingdom, 3Department of Genetics, University of Cambridge, Cambridge, United Kingdom, 4Department of Biological Sciences, University of Oxford, Oxford, United Kingdom
We have used RNA interference (RNAi) and epitope-tagging strategies to interrogate the regulation of motility in Trypanosoma brucei. Based upon likely carbon source availability in the environments occupied by trypanosomes we have argued that intraflagellar energy homeostasis is maintained by flagellar adenylate kinase activity (J.Biol.Chem. 280:11781-9). Here, we show the presence of an axonemal trypanosome-specific glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-like protein. The protein is unlikely to be catalytically active and we speculate that its presence in the trypanosomal structural flagellum is a reminder of when glycolysis could have been an intraflagellar pathway in the free-living flagellate ancestor from which the parasitic trypanosome lineage diverged. We argue that an important role for this gene is likely to play an important role regulating trypanosome motility, and provide new insight into the complexity and control of flagellar motility through the molecular characterisation of two novel trypanosome axonemal adenylate kinase-related (AKR) proteins. We show these proteins are important for motility and, moreover, form part of a flagellate-specific super-family containing unusually structured adenylate kinases and several classes of AKR protein. We suggest the trypanosome AKR proteins evolved following fusion of an adenylate kinase and a nucleoside diphosphate sugar epimerase. Although homologs are present in evolutionarily diverse taxa, no homology of the trypanosome AKR proteins is present in Chlamydomonas, but intriguingly other members of the AKR super-family are present and important for motility. In general, the phylogenetic distribution of AKR proteins supports a hypothesis that the trypanosome AKR proteins (and homologs in other flagellates) play a role in central pair dependent regulation of dynein arm activity. Using the ultrastructural phenotypes that arise following RNAi against various flagellar proteins (including radial spoke and outer arm docking complex components) for comparison, we provide arguments for both structural (passive) or regulatory (direct) roles for trypanosome AKR proteins in the control of flagellar motility.
2322
Comparison of Motility in Primate Sperm Using Optical Tweezers
J. Nascimento, L. Shi, E. Botvinick, S. Meyers, P. Gagneux, M. Berns, 1, 2 Bioengineering, University of California, San Diego, La Jolla, CA, 3Anatomy, Physiology, and Cell Biology, University of California, Davis, CA, 4Glycobiology Research and Training Center, University of California, San Diego, La Jolla, CA, 5Beckman Laser Institute and Medical Clinic, University of California, Irvine, Irvine, CA

Laser tweezers have been used to trap sperm cells, determine their swimming forces, and relate these forces to sperm curvilinear swimming velocity, VCL (swimming force increases with VCL). After trapping a sperm, laser power is reduced using a polarizer until the sperm is capable of escape (escape power, Pesc, and VCL are recorded). Sperm from Rhesus macaques, whose mating system is multimale-multifemale, are found to be on average both the fastest (86.1 µm/sec) and strongest (93.2 µmN). Sperm from western lowland gorillas, known to be strictly monogamous, are found to be on average the slowest (23.5 µm/sec) and weakest (4.6 µN). Sperm from human males, considered from an evolutionary viewpoint to be at neither extreme, lie between the two other species (52.5 µm/sec, 16.5 µN). The VCL and Pesc distributions for each species are statistically different (P < 0.005). It is also found that the linear regression applied to the scatter plot of Pesc vs. VCL for human sperm is statistically the same as that for gorilla (t > 1.96). Both of these regressions are statistically different than that for Rhesus macaque (t > 1.96). These findings support the theory that primary sperm from multimale-multifemale mating systems compete within the female tract. Such high evolutionary pressures would lead to sperm that swim stronger and faster than sperm from monogamous primates.

2323
Empirical Model of the Ciliary Waveform of Human Airway Epithelium Developed Using Video Microscopy
P. R. Sears, K. Thompson, C. Davis, Cystic Fibrosis Center, University of North Carolina, Chapel Hill, Chapel Hill, NC

Mechanical models of ciliary motion are validated by their ability to reproduce true ciliary waveforms. However, there is currently no empirical model of the ciliary waveform. Of particular interest are cilia beating in epithelial sheets where they are difficult to visualize. An empirical model was developed that parameterizes the ciliary shapes using two Gaussian functions to define the curvature. The waveform is constructed by visualizing the model superimposed on slowed videos of beating cilia and using 15 smoothed parameter sets that span one cycle. To acquire videos of beating cilia, cultures of human bronchial and nasal epithelial cells were wrapped around a #1 coverglass, placed between two coverglasses, and viewed in profile using a 60x oil objective (NA 1.4) fitted with a Bioptechs objective heater. The waveform was recorded using a Megaplus ES-310T camera at 250 frames per second. The ciliary waveforms were ranked according to their parameter values and the median waveform was used to calculate internal displacements. The displacement of the dyneins along B-tubules was determined for all dyneins over the entire cycle. Dynein displacement at the tip is 18 nm across tubelet sets 7-8 at the end effective stroke when the integrated curvature is greatest. But the central portion of the cilium, where the displacement relative to the base is similar to that at the tip, has a displacement of 36 nm relative to its position at the end of the recovery stroke. This region is also the only one to remain relatively straight for much of the cycle. This suggests that the central portion of the cilium has a critical role during the effective stroke and that processivity may be important since the net displacement of dyneins in this region is greater than four times the expected step size.

2324
An Investigation of the Mechanical Properties of Passive Sea Urchin Sperm Flagella
D. W. Pelle, K. A. Lesich, C. B. Lindemann; Biological Sciences, Oakland University, Rochester, MI

Recently, we lab reported that passive, vanadate-inhibited (50 µM + 0.1 mM ATP), rat sperm flagella exhibit a phenomenon described as counter-bend formation (Lindemann et al. 2005 Biophys. J. 89:1165-1174). When the basal portion of the flagellum is bent with a glass microprobe, the portion of the flagellum distal to the probe contact point develops a bend in the opposite direction to the imposed bend. In this study, we confirm that a similar phenomenon is observed in sea urchin flagella. Therefore, the counter-bend phenomenon is integral to the basic 9 + 2 microtubular axoneme and is not derived from the special features of mammalian sperm morphology. We further determined that removing the end flagellum of the by micro-dissection eliminates the response, as does mechanocally splitting the flagellum longitudinally. If the axoneme is immobilized in the middle of the flagellum, bends imposed on the basal section result in a counterbend in the distal section, but manipulation of the distal portion does not produce a counterbend in the basal portion. All of these results confirm that the phenomenon depends on the generation of inter-doublet shear. Based on measurements under our experimental conditions the flagellum has a total stiffness of ~ 6.0 x 10¹⁰ Nm⁻². We used this value to calculate the torque produced in the counterbend and find a shear resistance of ~ 7.4 pNnm for the flagellate microtubules. On this basis, the shear resistance of the sea urchin flagellum would account for ~ 1/3 of the total stiffness of the passive axoneme. Supported by N.S.F. grant #MCB-0516181.

2325
Disruption of Hydin in Tetrahymena Causes Defects in Swimming Speed and Ciliary Beat Frequency
A. Kabli, J. Zhao, M. L. Robinson; Zoology, Miami University, Oxford, OH

Conagential hydremas are a common birth defect with an estimated occurrence of 1 in 1000 live births in the United States. Hydin (Hydrocephalus-inducing) is a very large gene that contains 167 exons, spanning 400 kb, and is expressed throughout the nervous system in all organisms. Hyduperturbated cilia beat at a frequency of 33 beats/sec in wild-type cells. Although disruption of Hydin affects ciliary motility, mutants can alter ciliary activity in response to specific external stimuli. Both wild-type cells and Hydin mutants increase swimming speed when exposed to hyperpolarizing conditions and decrease swimming speed when exposed to mild depolarizing stimuli. Like wild-type cells, mutants also exhibit a transient reversal of ciliary beat direction when exposed to strong depolarizing stimuli. Our data suggest that Hydin is required for normal ciliary motility but is not required for at least some alterations in ciliary activity. Our data also suggest that mammalian Hydin mutants develop hydrocephalus because ciliary beating is affected. This work was supported by NIH AREA grant R15GM098553-3 to DGP.

2326
The Rate of Microtubule Sliding: Its Stability and Load Dependency
S. Ishijima, Bioengineering, Tokyo Institute of Technology, Tokyo, Japan

Flagellar beat of Suncus, golden hamster, and monkey spermatozoa before and after hyperpolarization were analyzed using high-speed video microscopy and digital image processing in order to understand the sliding mechanism of the flagellar beating and the function of outer dense fibers of mammalian spermatozoa. Although these spermatozoa have a different morphology and movement characteristics, change in the flagellar beating during the hyperpolarization was almost identical; namely, sharp bends at the base of the flagellum and a low beat frequency. Flagellar bends in other regions of the flagellum appear to have a limited involvement in the hyperpolarization. The nonhyperpolarized (activated) spermatozoa exhibited nearly constant-curvature beating, whereas the hyperpolarized spermatozoa displayed nearly constant-frequency beating, suggesting that the hyperpolarization is a mode conversion from constant-curvature beating to constant-frequency beating. To examine the microtubule sliding of sperm flagella, shear angle, which is the angle of the tangent to the flagellar shaft with reference to the sperm head axis, was measured because the degree and the rate of the shear angle are proportional to the amount of microtubule sliding and sliding velocity, respectively. The sharp bends at the flagellar base were generated by the increase in the total length of the microtubule sliding. The sliding velocity of each species was statistically different (P < 0.005). A comparison of the sliding velocity of the flagellar beating of Suncus, golden hamster, and monkey spermatozoa with the moment of inertia of the cross section at the flagellae base revealed that relationship between these two parameters was expressed by an exponential equation, similar to the characteristic equation of the muscle contraction. Data from sea urchin sperm also reveal the same extrapolated values from the values of the mammalian spermatozoa, suggests that the same sliding microtubule system functions in both the mammalian and echinoderm spermatozoa.
Fiber Diffraction Study on Flagellar Axonemes from a Wild Type and Mutant Strains of Chlamydomonas

S. Toba,1 H. Ivamoto,2 H. Sakakibara,1 K. Oiwa,1 Kobe Advanced ICT Research Center, National Institute of Information and Communications Technology, Kobe, Japan, 3Japan Synchrotron Radiation Research Institute, SPring-8, Harima, Hyogo, Japan

We have carried out X-ray diffraction studies on whole flagella isolated from Chlamydomonas and have obtained structural information of the axoneme both with and without nucleotides. We used several types of flagella prepared each from strains of wild type, and mutants lacking the whole outer arm (oda1), spoke (pf14), central apparatus (pf18) and the α chain of the outer arm dynein (oda11). We aligned axonemes in a flow cell by continuous shear flow, and then obtained diffraction patterns with the synchrotron radiation x-ray of 0.9 Å wavelength at SPring-8, BL45XU. The diffraction pattern showed several distinct meridional and equatorial reflections. Compared under the same nucleotide conditions, the 24-nm meridional reflection of the wild type was stronger than that of oda1, suggesting that this reflection originates mainly from the axial repeat of the outer dynein arms. The weaker 32-nm meridional reflection of pf14 compared with the wild type suggests that this reflection mainly comes from the repeat of the spoke. The 19-nm equatorial and 16-nm meridional reflections of pf18 were weaker than in the wild type, and these changes were presumably caused by the lack of the central sheath in pf18. Diffraction patterns were also obtained in different nucleotide states: no nucleotide, AMP-PNP, ADP/V, and ADP. In the ADP/V state, the 24-nm meridional reflection of the wild type became over twice as strong as that in the no nucleotide state. Although the origin of the equatorial reflections is yet to be determined, the addition of ATP/V or ADP caused the 27-nm equatorial reflection to shift towards the meridian and the 21-nm one away from it, indicating some nucleotide-dependent axial / azimuthal motion of axonomal structures. These changes are not simply due to uniform shrinkage/expansion of the axoneme. Acknowledgement: We thank Dr. S. Kamimura for the use of his flow cell.

Analysis of Structure and Functions of Flagellar Microtubule Tektin/Tubulin Protifilament Ribbons

P. W. Setter,1 E. Malvezy-Dorn,2 W. Steffen,1 R. E. Stephens,1 R. W. Linck3; 1Genetics, Cell Biology & Development, University of Minnesota, Minneapolis, MN, 2All About Children Pediatrics, Eden Prairie, MN, 3Molecular and Cell Physiology, Medical School, Hannover, Hannover, Germany, 4Physiology and Biophysics, Boston University School of Medicine, Boston, MA

Flagellar microtubule protifilament and tektin filaments were analyzed to elucidate their polypeptide composition and structural interactions. Tripneustes gratilla seaurchin sperm flagellar axonemes were fractionated with 0.5% Sarkosyl into protifilament ribbons, and these in turn were fractionated with 3-3.5 M urea to obtain tektin filaments. These entities were analyzed by quantitative SDS-PAGE immunoblotting with monospecific, polyclonal antibodies to L. pictus echinoderm tektin, and with antiserum to protein phosphatase 2A. Results indicated that there are two equimolar isomers of tektin A (A1, A2) and that in 3 M urea-stable filaments the tektins were present in equimolar ratios: (A1+ A2)/B=C. Tektin filaments were then fractionated with 4.5 M urea to yield highly stable AB filaments and pure soluble tektin C. These fractions were crosslinked with bis(sulfosuccinimidy) suberate, demonstrating that tektins A and B were nearest neighbor heterodimers and higher oligomers in the extended polymer; whereas soluble tektin C exists as homodimers, trimers and tetratomers. Finally, by extracting axonemes with 1 M NaSCN, filamentous remnants were obtained that contained tektins in a unitary ratio plus tightly-bound tubulins equivalent to the tektins, i.e., n=(A1+ A2)/B=C, and residual dynein heavy chains. The tightly-bound tubulin and dynein could be extracted from tektins with Sarkosyl. By negative stain EM, the NaSCN remnants appeared as tektin filaments with semi-periodic globular elements that disappeared after Sarkosyl extraction. We conclude that NHE1 activity contributes to the generation of (i) a defined cell surface pH needed for adhesion and spreading and (ii) a longitudinal pH gradient needed for migration. The proton gradient in moving direction. The proton concentration was higher at the leading edge than at the rear end. This longitudinal gradient is not present when the Na+/H+ exchanger is inhibited. Stimulation of the Na+/H+ exchanger leads to the formation of numerous protrusions firmly attached to the extracellular matrix. The proton gradient is much lower than at the surface of the cell body (ΔpH 0.25 in the glycolaxis). We conclude that the longitudinal pH gradient at the outer surface of the plasma membrane of melanoma cells contributes to the dynamics of cell-matrix contacts and thereby to cell migration. The more acidic pH at the front favors the formation, and the more alkaline pH at the rear end of the cell matrix contacts.

Protons at the Cell Surface as Glue for Cell-Matrix Contacts in Migrating Melanoma Cells

A. Schwab, M. Mueller, S. Mally, C. Stock; Institute of Physiology II, University of Muenster, Muenster, Germany

Migration of melanoma (MVM3) cells (i) is based on a coordinated formation and release of focal adhesion contacts mediated by integrins, (ii) depends on extracellular pH and (iii) requires the activity of the Na+/H+ exchanger NHE1 in the lamellipodium. The Na+/H+ exchanger is part of focal adhesion complexes. We suggest, that protons extruded by the Na+/H+ exchanger create an extracellular pH nanoenvironment at sites of cell-matrix contacts. The local extracellular pH may then influence the strength of cell adhesion and thereby modulate cell migration. In order to test this hypothesis we measured the extracellular pH (pHeμ) within approximately the first 50 nm from the outer surface of the plasma membrane. We labeled the plasma membrane and the glycolaxis with pH-sensitive dyes (phosphoethanolamine and wheat germ agglutinin, respectively) and determined pHeμ by means of ratiometric video imaging techniques. pHeμ decreased or increased as soon as pH of the bulk solution was lowered or elevated. Cells establish a proton gradient in the direction of movement, with the extracellular proton concentration being higher at the leading edge than at the rear edge (ΔpHe 0.2 at the cell membrane and ΔpHe 0.1 in the glycolaxis). This longitudinal gradient is not present when the Na+/H+ exchanger is inhibited. Stimulation of the Na+/H+ exchanger leads to the formation of numerous protrusions firmly attached to the extracellular matrix. The proton gradient is much lower than at the surface of the cell body (ΔpH 0.25 in the glycolaxis). We conclude that the longitudinal pH gradient at the outer surface of the plasma membrane of melanoma cells contributes to the dynamics of cell-matrix contacts and thereby to cell migration. The more acidic pH at the front favors the formation, and the more alkaline pH at the rear end of the cell matrix contacts.
Increased numbers of stellate cells (PSC) around pancreas carcinoma (PAC) might be due to two factors: (i) increased proliferation of PSC (recently demonstrated by our group) and (ii) recruitment of PSC from surrounding areas. Here we show whether carcinoma cells stimulate motility of PSC and attract PSC towards tumor cells. In addition we investigated the molecular mechanisms of stimulated PSC motility. The effect of tumor cell (MiaPaCa2, Panc1 and SW580) supernatant on PSC motility was demonstrated by time-lapse microscopy using the wound-assay and an agarose-chemotactant assay. The molecular mechanisms of stimulated PSC motility were studied by immunofluorescence microscopy (F-actin, paxillin, phosphorylated focal adhesion kinase (p-F-AK), and total tyrosine-phosphorylation) and Western analysis (total tyrosine-phosphorylation, p-PDG-F-R, p-F-AK, P-ERK, P-Src). In addition the compounds SB203580 (p38MAPK), PD98059 (MEK) and Y27630 (Rock), AG1295 (PDGF-R), anti-PDG-F-bb, and LY294002 (PI3K) were used to inhibit PSC motility and to determine the signal transduction pathways. In the presence of 0.1, 1.0, 5.0 and 10 % fetal calf serum, respectively, TC-SN (25%, 50%, 75% final concentration) dose dependently stimulated PSC motility and chemotaxis. Within 5 min after addition of TC-SN to cultured PSC the formation of focal adhesions complexes and phosphorylation of PDGF-F-R, FAK, ERK and Src was observed. Preincubation of TC-SN with anti-PDG-F-bb or preincubation of cultured PSC's with AG1295, SB203580, PD98059, Y27630 and LY294002 reduced stimulated PSC motility. Strongest inhibition was obtained by the ERK-inhibitor PD98059. The PI3K inhibitor LY294002 almost completely blocked basal and stimulated PSC motility. Our data indicate that pancreas carcinoma cells release PDGF and stimulate hereby random motility and chemotaxis of PSC towards a PDGF-gradient. In vivo these mechanisms result in an increased number of PSCs around pancreas carcinomas. PI3K, ERK, and P38MAPK and seem to play a role in the PDGF-induced signal transduction pathway leading to stimulated motility of PSCs.

Does Cdk6 Induce Cell Migration through Integrin Pathways?

T. Baker, M. J. Grossel; Biology, Connecticut College, New London, CT

Previously thought to function solely by regulating the G1 phase of the cell cycle through phosphorylating retinoblastoma protein (pRb), evidence now implicates cyclin-dependent kinase 6 (cdk6) in the process of differentiation. Over-expression of cdk6 in cultured mouse astrocytes was recently shown to induce changes in motility, gene expression, and actin dynamics - processes known to be associated with differentiation. Interestingly, wound assays demonstrated that cdk6-expressing astrocytes showed enhanced motility on fibronectin- and collagen-coated plates, but not on laminin- or vitronectin-coated plates. This ligand-specific motile response may indicate that cdk6 is involved in an integrin-mediated pathway. These data support a model of cdk6 interaction with integrin pathways, which may be a factor in the increased migratory ability characteristic of glioblastoma multiforme.

PDEF Mediated Cytoskeleton Re-organization Inhibits Invasive Properties of Breast Cancer Cells

D. P. Tumelty; Pathology and Laboratory Medicine, Medical University of South Carolina, Charleston, SC

Breast cancer mortality is attributable to metastasis as cells migrate from the primary tumor and invade into the circulatory system to establish at alternative sites. In order to acquire the polarized morphology required for directed movement, metastatic cells must dissociate from normal cell-to-cell contacts, reorganize their cytoskeleton and transiently re-localize cell-matrix interactions. These processes require extensive alterations in gene expression profiles. Our work has demonstrated that the ETS transcription factor PDEF (prostate derived ETS factor) is a possible negative-regulator of metastatic potential through the transcriptional control of metastasis-associated genes. PDEF expression is reduced in human invasive breast cancer and is absent in invasive breast cancer cell lines. Constitutive and inducible PDEF expression in invasive cells results in the inhibition of migration, invasion and cellular growth. Correlated with these phenotypic changes is the altered expression of metastasis-associated genes including the serine protease aPAP (arokinase-type plasminogen activator) and the metastasis suppressor maspin. Morphological differences observed between control cells and cells expressing PDEF led us to examine possible changes in cytoskeleton organization. Cells not expressing PDEF display polarized morphology associated with a migratory, including a defined single leading lamellipodia, a distinct trailing edge and polarized focal adhesions. In contrast, cells expressing PDEF, no longer show polarized morphology; but displayed characteristics of a more stationary cell. Specifically, they were rounded in shape, displayed altered G-F actin ratios and had randomly localized focal adhesions. Gene ontology analysis confirmed the differential expression of known effectors and modulators of cell-cell and cell-matrix interaction pathways. Collectively, these data indicate that PDEF expression in invasive cancer cells alters the ability of motile cells to form the temporal structures required for efficient motility. These results provide new insights into the mechanisms whereby PDEF functions as a potential metastasis suppressor.

Using Mammalian and Yeast Systems to StudyMemo, a Novel ErbB2 Effector Protein.

M. Meira, R. Masson, N. Hynes; Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland

The Neu/ErbB2 receptor tyrosine kinase plays an important role in cancer cell motility and metastases formation. ErbB2 is often overexpressed in human breast and ovarian tumors. Our studies focused on signaling molecules that interact with autophosphorylated tyrosine residues of ErbB2. We have discovered that two autophosphorylation sites, Tyr 1201(YC) and Tyr1227(YF) are crucial to restore the migratory phenotype and promote cell motility to breast carcinoma cells upon Heregulin (HRG) stimulation, whereas the receptor lacking the 5 major autophosphorylation sites is impaired in stimulating migration. A novel protein has been identified and named Mmo for Mediator of ErbB2-driven cell Motility. Mmo interacts with the phosphorylated Tyr1227(YD) and is essential for HRG induced motility (1). Based upon the fact that Mmo knock-down (KD) does not block the ability of cells expressing the Tyr 1201 add-back mutant to migrate, we hypothesized that two parallel pathways stimulate migration in the context of a wild type ErbB2 receptor. We have found that PLCγ1 binds to Tyr 1201, and KD of PLCγ1 blocks HRG-induced cell migration. Furthermore, PLCγ1 KD cells, like Mmo KD cells, show decreased mitochondria outgrowth and strengthened actin stress fibers. We are focusing on the mechanisms which mediate the effects of Mmo and PLCγ1 on motility. Mmo has been conserved protein and has an orthologue with 40% sequence identity in S. cerevisiae. Strains lacking the Mmo gene (YJR080W) are viable. Accordingly, we have performed a synthetic lethal screen (in collaboration with the group of Dr. Matthias Peter in Zurich) by mating a Mmo deleted strain to an ordered array of 4700 yeast strains containing non essential gene deletions. This has led to the identification of a few genes that give a synthetic phenotype. (1) Marone, R. et al. 2004. Nature Cell Biol. 6: 515 - 522

Investigating Cytoplasmic Functions of Cdk6 in Differentiation

Z. E. De la Cruz, H. Stanish, E. Elliot, M. J. Grossel; Biology, Connecticut College, New London, CT

Recent evidence implicates the cyclin-cyclop cellcycle cycle, cyclin-dependent kinase (cdk6) in the processes of differentiation. Over expression of cdk6 in cultured mouse astrocytes was recently shown to induce changes in motility, gene expression, and actin dynamics. To better understand the role of cdk6 in changes in these processes, the sub-cellular localization of cdk6 is under study in these cells. Indirect immunofluorescence (IF) performed in our lab has localized cdk6 to the nucleus and the cytoplasm of cdk6-expressing mouse astrocytes. Our studies show that astrocytes infected with cdk6 stained more heavily in the cytoplasm and perinuclear regions, as opposed to control AP cells, which stained primarily in the nucleus. Together these results suggest that cdk6 may be involved in cytoplasmic functions. Indeed, published studies have demonstrated a role for cdk6 in the αβ, integrin regulatory pathway (Fahraeus, 1999). Also, another cyclin-dependent kinase, cdck, has been shown to modulate cell migration together with αβ integrin (Manes, 2003). Cdk6 coexpressed cells plated on fibronectin and collagen showed an unusual change in morphology. These ECM proteins resulted in increased nodules in cytoplasmic extensions. To further investigate this, immunofluorescence was performed on astrocytes over-expressing cdk6 on fibronectin. After being seeded on fibronectin, cdk6 was localized to the cytoplasm of the astrocytes including cytoplasmic nodules. A differential response by cdk6 to ECM proteins in both migration and morphological changes implies an involvement by cdk6 in integrin-mediated pathways. Localization of cdk6 to the cytoplasm further supports this possibility. The mechanism of this involvement, however, is unknown. These studies are intended to determine the mechanism of cdk6 function in the process of differentiation.

Interaction of Non-receptor Tyrosine Kinase Jak3 with Cytoskeletal Protein Villin Regulates Intestinal Epithelial Restitution

N. Kumar, J. Mishra, C. M. Waters; 1Physiology, University of Tennessee, Memphis, TN, 2College of Pharmacy, University of Tennessee, Memphis, TN

Intestinal epithelial restitution is a process by which epithelial continuity is rapidly reestablished following various forms of injury. The integrity of these epithelial barriers is compromised during the course of various intestinal disorders including inflammatory bowel disease (IBD). Enhancement of reparative processes through different interleukins can be a viable therapeutic alternative for different intestinal disorders. Although involvement of various (i.e. cytoketamine) and hormonal (i.e. growth factors and cytokines) components has recently been documented the mechanism of humoral signaling regulating mucosal recovery are not understood completely. To understand the significance of humoral IL-2 activated tyrosine kinase in the regulation of intestinal epithelial restitution, post-confluent differentiated intestinal epithelial cell lines were used to study in-vitro wound healing in response to recombiant human IL-2. Application of exogenous IL-2 enhances the restitution of both Caco-2 and HT-29 CL-19A cells in a dose dependent manner. Activation by IL-2 led to redistribution and tyrosine phosphorylation of the non-receptor tyrosine kinase Jak3. In these cell-culture model Jak3 interacts with villin in an IL-2 dependent manner. The time course of Jak3 activation coincided with
the tyrosine phosphorylation of intestinal and renal epithelial cell-specific cytoskeletal protein villin. Inhibition of Jak3 activation resulted in loss of tyrosine phosphorylation of villin and a significant decrease in wound repair of the intestinal epithelial cells. Activation of with IL-2 also led to co-localization of Jak3 and villin to cell margin in the migrating cells. We have earlier reported that villin can regulate cytoskeletal dynamics through its binding to different ligands or tyrosine phosphorylation. Present studies show that Jak3 plays a significant role in the signal transduction pathways involved in intestinal epithelial restitution through its interaction with interleukin receptors and interactions with cytoskeletal component villin. *This study was supported by a Research Fellowship Award (RFA) from the Coshen’s Colitis Foundation of America to NK.*

**2338 Regulation of Epithin by TGFβ and Its Role in Epithelial-Mesenchymal Transition**

H. Lee, S. Kim, Y. Cho, M. Kim, D. Park; Seoul National University, Seoul, Republic of Korea, 2Cho In University, Incheon, Republic of Korea

Epithin (matriptase, MT-SP1 in human) is a type II transmembrane serine protease, expressed in epithelium-derived tumors and epithelial components of most normal tissues. Epithin is consistently overexpressed in epithelium-derived tumors, and it has been demonstrated that Epithin could promote cancer metastasis in several reports. From these reports, it is expected that epithin has important roles in tumorigenesis, tumor growth, and metastasis. But the regulation of Epithin expression and its roles in cancer progression remain unknown. We found that TGFβ upregulated Epithin by transcriptional activation. This is mediated by Smad-independent, p38 MAPK-dependent pathway. In addition, TGFβ can facilitate the shedding as well as activation of Epithin. Although TGFβ is a well-known potent growth inhibitor, it has also been shown to promote metastasis, especially through induction of epithelial-mesenchymal transition (EMT). We investigated whether Epithin is implicated in TGFβ-mediated EMT. To test this possibility, we produced the Epithin-knockdown stable cell line (Epi-KD-17) using small interference RNA for Epithin. The expression of EMT markers were reduced in Epithin-knockdown cell line, and TGFβ-induced cell migration was also inhibited by Epithin knockdown. These results suggest that Epithin plays a crucial role in TGFβ-mediated EMT.

**2339 Abl and Its Interaction Partners Regulate Membrane Dynamics and Rate of Cell Spreading during Attachment**

S. Antoku, K. Sakoela, B. Mayer; University of Connecticut Health Center, Farmington, CT, 1Haartman Institute, University of Helsinki, Helsinki, Finland

During attachment, the cell dynamically changes its membrane structure to adapt to the new environment. Cell attachment and spreading involve two actin-based protrusive membrane structures, filopodia and lamellipodia. The Abi family of non-receptor tyrosine kinases, Abi and Arg, induces filopodia during attachment to fibronectin-coated surfaces. However, how Abi regulates this filopodium formation and its significance remain elusive. To determine the mechanism whereby Abi enhances filopodium formation, we introduced mutations in various domains and motifs of Abi, and re-expressed the mutants in mouse embryonic fibroblasts lacking Abi and Arg. We found that Abi carrying mutations in the cluster of proline-rich (PxxP) SH3 binding motifs significantly reduced filopodium formation upon attachment. We used a phage display library containing all known SH3 domains to identify potential binding partners for the Abi PxxP motifs. To determine which of these were involved in Abi-mediated filopodium formation, we overexpressed each candidate protein in spreading cells. We found that Nck family adaptor proteins enhanced filopodium formation, while Crk family adaptor proteins reduced filopodium formation and enhanced lamellipodium formation. All other candidates had no effect in this assay. These data suggest that binding of Nck and Crk to Abi through its PxxP motifs regulates filopodium formation. We performed further experiments to up- and down-regulate the expression levels of Abi, Nck, and Crk in spreading cells to manipulate the dynamics of filopodia and lamellipodium formation. We consistently observed that the average cell area during attachment was increased under conditions where lamellipodium formation was enhanced, and decreased where filopodium formation was enhanced. This implies that the balance between filopodium and lamellipodium formation controls the spreading speed of the cell. Our data indicate that Abi plays a critical role in attachment by regulating the dynamics of these actin structures through its interactions with Nck and Crk.

**2340 The Biological Function of iNOS in Cell Migration by RNAi-mediated Gene Silencing**

S. Liu, S. Chung, P. J. Chu; Institute of Biomedical Sciences, National Chung Hsing University, Taichung, Taiwan

We have described a tumor-associated NADH oxidase (iNOS) and its constitutive activation in transformed culture cells. Certain anticancer drugs have been shown to inhibit preferentially the iNOS activity in transformed cells and the inhibition of activity is well correlated with the poorer survival of transformed cells and subsequently results in the induction of apoptosis. However, the precise function of iNOS in transformed cells has not been fully studied. To further identify the biological function of iNOS, we utilize RNA interference (RNAi) approach and generate a stable iNOS RNAi cell line in HeLa cells. The stable cell line is, therefore, used to study the loss-of-function phenotypes. The expression of iNOS protein is reduced in iNOS-RNAi cells when compared with wild type HeLa cells and the protein expression associates with the lower survival in RNAi-transfected HeLa cells. In addition, the iNOS-RNAi cells exhibits less ability to migrate as suggested in wound-healing assay. To further investigate the effect of iNOS knock down in cell migration, we examine the key regulators involved in the pathway such as Rac protein. Surprisingly, the Rac protein level is significantly decreased in iNOS-RNAi cells. The detailed mechanisms by which iNOS regulates cell migration is investigated. (Supported by grants from National Healthy Research Institute NIHRI-EX95-941 IBC and Taichung Veterans General Hospital and National Chung Hsing University TCVGH-NCHU-957615).

**2341 α6β4 Integrin-mediated AKT Signaling Regulates Slingshot and Cofilin Activity in Human Keratinocytes**

K. R. Klipp, P. DeBiasi, J. C. Jones; Cell and Molecular Biology, Northwestern University, Chicago, IL

The motility of keratinocytes is essential for wound healing and metastasis of skin tumor cells. In vitro, the specific motile behavior of keratinocytes is dictated by the assembly of laminin-332 tracks. The assembly of these tracks is dependent upon α6β4 integrin signaling to the actin cytoskeleton, namely to the actin severing protein cofilin. Here we have analyzed how cofilin phosphorylation is regulated by mediators downstream of α6β4 integrin signaling in human keratinocytes. In human skin cells, expression of either dominant negative Rac1 or dominant inactive Slingshot-1 (SSH-1) induces phosphorylation of cofilin and leads to assembly of aberrant arrays of laminin-332. Like cofilin, SSH-1 is inactivated by phosphorylation on serine residues at the C-terminus. One kinase that may regulate SSH-1 in keratinocytes is AKT. To assess whether AKT phosphorylates SSH-1 in skin cells downstream of Rac 1 activity, we first determined the level of active AKT in keratinocytes induced to express dominant negative Rac1. Expression of dominant negative Rac1 not only increases levels of phosphorylated cofilin, but also leads to an increase in phospho-AKT levels. Conversely, expression of constitutively active Rac1 leads to decreased levels of phosphorylated cofilin and phospho-AKT. Moreover, treatment of human keratinocytes with the PI3K inhibitor LY294002 leads to a decrease in phospho-cofilin levels, providing additional support that SSH-1 is a target of AKT. Taken together, these data define a molecular pathway mediating keratinocyte motility. In the pathway, the regulation of AKT by Rac1 is a key determinant of SSH-1 and cofilin activity. SSH-1 and cofilin govern laminin-332 matrix assembly and ultimately regulate precise keratinocyte motility behavior.

**2342 Regulation of TRIP6 Function in Cell Motility by PTP-L1 Phosphatase**

Y. Lai, F. Lin; Cell Biology, Univ. of Alabama at Birmingham, Birmingham, AL

TRIP6 is a zyxin family member that has been implicated in actin dynamics and signal transduction important for cell adhesion and migration. Previously we have shown that LPA stimulation induces the recruitment of TRIP6 to the activated LPA 2 receptor and promotes the association of TRIP6 with several components of focal complexes, including p130 cas, FAK and Src. TRIP6 is a zyxin family member that has been implicated in actin dynamics and signal transduction important for cell adhesion and migration. Here we further identify PTPL1 as a tyrosine phosphatase responsible for dephosphorylating TRIP6 in vitro and in cells. Overexpression of PTP-L1 inhibits LPA-induced tyrosine phosphorylation of wild-type TRIP6, but not a TRIP6 mutant lacking the C-terminal PTP-L1 binding domain. Furthermore, LPA-induced morphological changes and the turnover of TRIP6 at focal adhesion sites are retarded in cells overexpressing PTP-L1. Consistently, PTP-L1 reduces LPA-induced cell migration. Taken together, these results suggest that a coordinate regulation of TRIP6 phosphorylation and dephosphorylation by c-Src kinase and PTP-L1 phosphatase is important for TRIP6 function in LPA-induced cell migration.

**2343 Regulation of Sperm Motility by Unique C-Complex-encoded Inhibitory Subunits of PPE172**

J. Lu, L. Chen, C. Myers, D. Solar, R. Wadlow, S. Vijayaraghavan, S. Pilder; Anatomy and Cell Biology, Temple University School of Medicine, Philadelphia, PA, 2Biological Sciences, Kent State University, Kent, OH
Ppp1r11 encodes a heat-stable inhibitor of PPI1cy2, a protein phosphatase 1 catalytic subunit whose activity is inversely related to sperm motility. Mouse Ppp1r11 localizes to the c complex, a naturally occurring polymorphism of the proximal third of chromosome 17 represented by a family of closely related haplotypes that carry similar mutant alleles of genes implicated in sperm dysfunction. The mutated t-allele of Ppp1r11 is thought to code for one of several proteins whose expression in sperm from t/t males coincides with an aberrant motility phenotype strongly associated with sterility. The initial purpose of this study was to compare the pattern of PPP1R11 expression in sperm using protein purification, western blotting, pull-down assays, immunoprecipitation, and immunofluorescence. Results indicate that PPP1R11+ is highly expressed in testis and spermatozoa as multiple isoforms, including a 27-kDa isoform identified in numerous tissues, and a testis- and sperm-specific 55-kDa isoform. The wild-type 55-kDa polypeptide does not bind PPI1cy2 in the testis, but binds it quantitatively in motility-activated sperm. Its t-isoform is extremely volatile in both newly activated sperm and sperm capacitated in vitro. Interestingly, microcin pull-down experiments demonstrate that a large, N-terminally unique isoform of another inhibitory subunit of PPI1cy2, PPI1R2, mapping to the c complex, is abundant in motility-activated wild-type sperm, but absent from sperm produced by t/t males. Based on our findings, we hypothesize that interplay between PPI1cy2 and the t-isoforms of these two c complex-derived regulatory subunits promotes the onset and maintenance of sperm motility and hyperactivation, while the poor motility of t-sperm derives from the instability or absence of the t-isoforms of these PPI1cy2 regulators. Efforts are currently underway to determine the complete molecular identities of these sperm-specific, c complex-encoded isoforms of inhibitors of PPI1cy2.

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Regulation of Actin Dynamics and Cell Motility by Chromophin, a Cofilin Phosphatase, during EGFR Stimulation
C. DerMardirossian, V. Delorme, T. Huang, G. Bokoch; Immunology, The Scripps Research Institute, La Jolla, CA
As a crucial mediator of actin dynamics and cell motility, cofilin is regulated by cycling between an inactive, phosphorylated form, mediated by LIM kinase, and an active unphosphorylated form that depolymerizes and/or severs actin. Dephosphorylation of cofilin is mediated by slingshot (SSH) and a previously uncharacterized phosphatase, chromophin (CIN). We investigated the contributions of CIN to growth factor-induced actin dynamics and cell motility. We show that in serum-starved MTLn3 carcinoma cells, overexpressed CIN is mostly diffusely distributed in the cytosol. In response to epidermal growth factor (EGF) stimulation, marked transient localization of overexpressed CIN at the leading edge of the lamellipodium occurs. Interestingly, neither inactive CIN (D25N) or SSH translocate to the cell edge. As a consequence of the translocation, CIN becomes active and decreases by 25% the P-cofilin level at the cell edge. We find that the EGF-induced re-localization of CIN to actin-rich ruffles and membrane protrusions is dependent on PI3-kinase and Rac activity. To assess the function of CIN, we measured actin dynamics in vivo using Fluorescence Speckle Microscopy (FSM). We show that CIN expression induces the formation of a unique region at the cell edge in which the F-actin undergoes a fast retrograde flow. Kinetic analysis indicated that CIN increases the rate of F-actin treadmilling in vivo and widens the region of F-actin polymerization at the leading edge, consistent with the increase of free barbed ends induced by CIN at 3 min post EGF stimulation. Finally, analysis of cell speed using phase-contrast microscopy revealed that MTLn3 injected with CIN migrated nearly twice as fast as cell controls. The effect of chromophin knock-down on P-cofilin levels, actin polymerization, and cell motility in EGF stimulated cells will be evaluated.

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Hepatocyte Growth Factor-induced Motility through Transactivation of the Epidermal Growth Factor Receptor
J. K. Klarlund, J. K. Spis, E. R. Block, E. Y. Clay; Visual Sciences Research Center, University of Pittsburgh, Pittsburgh, PA
Signaling by HGF (hepatocyte growth factor or scatter factor) through its receptor c-MET has been implicated in epithelial wound healing. HGF/MET has also been found to play important roles in numerous forms of cancer. HGF was found to induce robust scattering of colonies of an immortalized line of human corneal limbal epithelial (HCLE) cells, and it accelerated healing of wounded areas in confluent sheets of the cells. Unexpectedly, HGF was also found to induce activation of the epidermal growth factor (EGF) receptor by immunoblotting with an antibody against the receptor phosphorylated on tyrosine 1173. Studies with neutralizing antibodies and inhibitors demonstrated that HGF-induced EGF receptor activation proceeds via the triple membrane-passing signal mode of activation. Activation of the EGF receptor was necessary for HGF induced scattering and healing of wounds in sheets of corneal epithelial cells. However, activation of the EGF receptor was not necessary for activation of ERK1/2 or for internalization of E-cadherin. Activation was inhibited by the src inhibitor pp2, suggesting a role for a src-family kinase in transactivation, but protein kinase C or phospholipase D did not seem to be involved. Importantly, the EGF receptor is also activated by HGF in epidermoid carcinoma A431 and in MDCK cells, suggesting a general role for transactivation of this receptor in HGF action. We suggest that the EGFR receptor may be a relevant therapeutic target for at least some cancers driven by aberrant HGF/MET signaling.

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ARF6-dependent Activation of ERK and Rac1 Is Regulates Cytoskeletal Remodeling during Epithelial Tube Development
J. Tushir, C. D’Souza-Schorey; Biological Sciences, University of Notre Dame, Notre Dame, IN
Tubes are the building blocks of epithelial organs and form in response to cues derived from morphogens such as hepatocyte growth factor (HGF). Investigating the mechanisms and regulation of tube development or tubulogenesis, is important not only to define fundamental aspects of development but also to understand the cellular basis of a variety of disease states. Recently little is known about signaling pathways that orchestrate the cellular behaviors that constitute tubulogenesis. Tube ‘initiation’ is characterized by a partial EMT, which would allow ‘pioneer’ cells from a cyst to become invasive and project long basolateral membrane extensions into the extracellular matrix. Therefore, cytoskeletal remodeling events are likely vital to tubule extension. Here, using three dimensional cell cultures of MDCK cells we show that the ARF6 GTPase is a critical determinant of tubule initiation in response to HGF. ARF6 is transiently activated during tubulogenesis and perturbing the ARF6/GTP/GDP cycle by inducible expression of ARF6 mutants defective in GTP binding or hydrolysis, inhibits the development of mature tubules. The effect of ARF6 on tube initiation is twofold. We show that not only does ARF6 regulate the subcellular distribution of the Rac1 GTPase at tubule extensions, but that ARF6-induced ERK activation is required for Rac1 activation and consequently for cytoskeleton remodeling during tubule initiation. We also demonstrate that uPAR signaling downstream of the ARF6/ERK pathway is critical for Rac1 activation during tubule outgrowth. Importantly, we have identified a cellular apparatus downstream of HGF signaling which regulates membrane and cytoskeleton remodeling necessary for the early stages of epithelial tube development.

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IRSp53 is Required for CSF-1 and Rac Dependent Membrane Ruffling and Cell Migration, but Not for Podosomes and Cdc42-mediated Filopodia Formation in Macrophages
W. G. Abou-Kheir, B. Isaac, H. Yamaguchi, D. Cox; Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, NY
WAVE (Wiskott-Aldrich syndrome protein [ WASP] family g-actin homologous) proteins play a major role in Rac-induced actin dynamics, but Rac does not bind directly to WAVE proteins. While it was initially demonstrated that IRSp53 (Insulin receptor substrate protein 53) linked WAVE to Rac, it has recently been shown that Rac can also bind to a complex of proteins containing Abelin interaction protein 1 (Abi1). We have previously shown that WAVE2 is crucial for actin cytoskeleton remodeling and migration of macrophages in response to colony-stimulating factor 1 (CSF-1) and that Abi1 is required for WAVE2 protein stabilization and function. A role for IRSp53 in CSF-1-induced actin cytoskeletal reorganization has not been reported in macrophages. We found that IRSp53, WAVE2 and Abi1 were co-localized in CSF-1-induced F-actin rich membrane protrusions in primary macrophages and in Rac-induced (RacQ61L) protrusions in a murine monocyte/macrophage cell line (RAW/LR5). Moreover, immunoprecipitation experiments demonstrated that IRSp53, WAVE2 and Abi1 existed in a complex in RAW/LR5 cells over-expressing constitutively active RacQ61L. Depletion of endogenous IRSp53 by RNA-mediated interference (RNAi) in RAW/LR5 cells resulted in a significant reduction of CSF-1 or RacQ61L-induced membrane ruffles, although formation of CSF42L (activated)-induced filopodia was not affected by depletion of IRSp53. Consistent with previous results observed in RAW/LR5 cells with reduced WAVE2 expression, cell migration in response to CSF-1 in RAW/LR5 cells with reduced IRSp53 expression was also significantly impaired. However, IRSp53 and WAVE2 were not involved in the formation of podosomes (actin rich, dot-like adhesion structures) suggesting that IRSp53 is not required for all actin dependent events in macrophages. Altogether, these data suggest that IRSp53 links Rac to WAVE2 and its function is crucial for CSF-1-induced F-actin rich protrusions and cell migration in macrophages.

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Cortactin Is Required for Lamellipodia Retraction and Substrate Adhesion in MTLn3 Breast Cancer Cells
A. Gatesam Ammer,1 L. C. Kelley,2 S. A. Weed;1 Dept of Neurobiology and Anatomy, West Virginia University, Morgantown, WV, 3West Virginia University, Morgantown, WV
Changes in the actin cytoskeleton are mediated by a variety of actin-binding proteins and are essential for many cellular processes, including migration, invasion, and adhesion. Cortactin is an F-actin binding protein and Src family kinase substrate that is enriched in lamellipodia and is required for tumor cell motility and invasion. Cortactin functions to stimulate Arp2/3-mediated actin polymerization necessary for lamellipodia formation and extension, and serves to stabilize Arp2/3-F-actin networks. In this study we have investigated the role of tyrosine and serine phosphorylation in regulating motility and cortactin function using MTLn3 breast adenocarcinoma cells, a model system for studying lamellipodia. Lamellipodia formation in MTLn3 cells stimulated with EGF was monitored using time-lapse video microscopy. MTLn3 cells expressing control siRNA displayed normal lamellipodial dynamics, with maximal extension at about 3-4 minutes, and retraction by 10 minutes. In contrast, MTLn3 cells transfected with cortactin siRNA exhibited normal lamellipodia extension, but were dramatically impaired in their ability to
Retract, resulting in increased lamellipod persistence. Rescue of cortactin-silenced cells by expression of GFP-tagged human cortactin partially restored the ability of these cells to retract the lamellae. Our data suggest that cortactin serves to regulate lamellipodia retraction, an essential requirement for productive cell motility. Additionally, the role of cortactin in substrate adhesion and cell migration was examined using Electric Cell-substrate Impedance Sensing (ECIS). Our results suggest that cortactin is necessary for both cell-substrate adhesion and cell migration, as a loss of cortactin resulted in a delay in MTA2 adhesion and a decrease in adhesion strength, as well as impaired cell migration in response to EGF stimulation. We are currently examining the function of several mutants of cortactin, including tyrosine (Y421F, Y470F, Y486F, and Y498F) and serine (S405A and S418A) mutations, to dissect the requirements of cortactin in lamellipodia extension and retraction, substrate adhesion, and migration.

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Fascin Promotes Colon Carcinoma Cell Migration by Alterations to Leading Edge Protrusions
Y. Hashimoto,1 M. Parsons,2 A. A. Smith,1 J. C. Adams1; 1Cell Biology, Lerner Research Institute, Cleveland Clinic, Cleveland, OH, 2Randall Division of Cell and Molecular Biophysics, King's College London, Guy's Campus, London, United Kingdom

Despite major improvements in diagnosis and treatment, tumor invasion and metastasis remain major causes of cancer morbidity and mortality. The actin-bundling protein, fascin, is not expressed by most normal epithelia but is up-regulated in many carcinomas. With our clinical colleagues we have found that high fascin expression correlates retrospectively with poor survival in patients with colorectal carcinoma. To test the hypothesis that up-regulation of fascin contributes to tumor cell migration and invasiveness, inducible shRNA for fascin knockdown (KD) was introduced into SW480 human colon cancer cells, that have a high level of fascin, and cloned cell lines were derived. Fascin-KD cells were unchanged in their proliferation rate, content and localisation of E-cadherin and beta-catenin, surface expression of beta integrins and initial attachment to extracellular matrix components. Distinct changes in phenotype were apparent in migrating cells. Parental cells contained fascin in cell protrusions and in the cell body. In both parental and fascin-KD cells, the major adhesions were at the protrusive tips. On laminin matrices, fascin-KD cells were increased in maximum length and had thinner and fewer actin-containing protrusions. By timelapse microscopy, the velocity of fascin-KD cells was significantly decreased (p<0.0001), as was polarised cell migration and the turnover of cell protrusions. This correlated with an altered distribution of focal adhesion components and decreased sensitivity to inhibition of endogenous Rac function. Phosphorylation of fascin by protein kinase C is known to regulate its actin-bundling activity, and FRET/FLIM microscopy of living cells migrating on laminin revealed strong, trans and Rac-dependent interaction between GFP-fascin and mEFP-protein kinase C gamma, localised at leading edge protrusions. Our working model is that fascin contributes to the cellular aggressiveness of CRC by promoting the turnover of cell protrusions necessary for directional cell migration. Fascin could represent a novel therapeutic target in strategies to arrest carcinoma cell invasion.

2350
Neural Crest Migration and Dorsal Root Ganglion Formation in Zebrafish erbB3 Mutants
Y. Honjo, J. S. Eisen; Institute of Neuroscience, University of Oregon, Eugene, OR

ErbB3 is a receptor-type tyrosine kinase that has been shown to have important roles in glial development and in sympathetic ganglion formation. Mice with a targeted mutation of ErbB3 lack sympathetogenic brain and dorsal root ganglion (DRG) development. However, raising the possibility that normal neural crest migration is defective. To learn whether erbB3 is required for normal neural crest migration, we have taken advantage of the transparency of zebrafish embryos and studied neural crest migration in erbB3 zebrafish mutants. Previous studies showed that in zebrafish, as in mice, erbB3 mutants have a defect in gla development (Lyons et al., 2005, Current Biology, 15:513). We found that erbB3 mutants also have a defect in DRG formation, as revealed by absence of neurogenin1-positive nascent DRG cells at 30 hours postfertilization and by absence of Hac/D-positive DRG neurons at 4 days postfertilization. However, markers that label migrating neural crest cells revealed no obvious defects in the pattern of neural crest migration in erbB3 mutants. To learn whether other aspects of neural crest migration are affected in these mutants, we followed neural crest migration in live transgenic embryos in which GFP expression is driven by the zebrafish soxx10 promoter (Wada et al., 2005, Development, 132:3977). Treating embryos with the ErbB receptor inhibitor, AG1478, did not appear to affect overall neural crest motility, but did appear to affect the ability of some migrating neural crest cells to stop near the position where the DRG normally forms. These results suggest that erbB3 may be involved in the ability of DRG progenitors to recognize their target position during migration.

2351
A Special Function for Nckα Adapter: Maintaining Paxillin Level During Neuronal Differentiation
S. Guan; University of Southern California, Los Angeles, CA

Nck adapter has an evolutionarily conserved function in neuronal differentiation, in which it links cell surface cues to the actin cytoskeleton-orchestrated cellular events. However, the mechanism of Nck action remained little beyond speculation. We investigated the roles of Nckα (Nck/Nck1) versus Nckβ (Grb4/Nck2) in nerve growth factor (NGF)-induced differentiation of the rat adrenal pheochromocytoma cell line, PC12, and in fully differentiated rat primary neurons. Here we show that genetic silencing Nckα completely blocks NGF-induced neurite outgrowth. In contrast, down-regulation of Nckβ slightly enhances the effect of NGF. We provide evidence that the role of Nckβ is to maintain the steady-state levels of paxillin, a critical focal adhesion molecule. Down-regulation of Nckα causes subsequent down-regulation of paxillin at the post-translational level. Furthermore, the differentiation defects of Nckα PC12 cells can be duplicated by genetic silencing paxillin in PC12 cells and rescued by re-expressing a siRNA-resistant Nckβ, but not Nckα, in the Nckβ cells. Thus, this study unveiled a novel Nckβ-paxillin mechanism by which Nckβ is the Mother Nature selection to maintain cellular paxillin level during neuronal differentiation.

2352
A “Traffic Control” Role for TGFβ3: Orchestrating Dermal and Epidermal Cell Motility during Wound Healing
W. Li; University of Southern California, Los Angeles, CA

Cell migration is a rate-limiting event in skin wound healing. In un-wounded skin, cells are nourished by plasma. When skin is wounded, resident cells encounter serum for the first time. As the wound heals, the cells experience a transition of serum back to plasma. We report here that human serum selectively promote epithelial cell migration and halts dermal cell migration. In

2353
Extracellular Hsp90α Mediates Hypoxia Signaling to Promote Human Skin Cell Migration
W. Li; University of Southern California, Los Angeles, CA

Hypoxia is a microenvironmental stress in skin wounds, where it causes increased motility of epidermal keratinocytes and dermal fibroblasts. The mechanism was unclear. Here, we report that hypoxia promotes human dermal fibroblast (HDF) migration by inducing hypoxia-inducible factor-1alpha (HIF-1α), which in turn causes secretion of heat shock protein-90alpha (hsp90α) into the extracellular environment. Expression of a constitutively activated HIF-1α replaced hypoxia for promoting hsp90α secretion and HDF migration. Permanent down regulation or functional inhibition of several mutants of cortactin, including tyrosine (Y421F, Y470F, Y486F, and Y498F) and serine (S405A and S418A) mutations, completely blocks hypoxia- and HIF-1α-enhanced HDF migration. In contrast, PDGF-βBB-stimulated migration of the same cells was unaffected. Finally, recombinant hsp90α alone promoted HDF migration under normoxia as potently as hypoxia, even in the absence of its co-factors or ATP. Thus, this study demonstrates a novel mechanism of hypoxia > HIF-1 > extracellular hsp90α > cell migration. Considering to HDF migration into skin wounds to help wound healing and remodeling, identification of this novel function for extracellular hsp90α provides a potential therapeutic target for better management of human skin wounds.

2354
Distribution and Fate of YFP-transfected Donor Cell Mitochondria in Cloned Porcine Embryos
Z. Zhong, M. Katayama, R. Li, L. Lai, Y. Hao, D. Wax, L. Spate, K. Whitworth, Q. Sun, R. S. Prather, H. Schatten; 1Veterinary Pathobiology, University of Missouri-Columbia, Columbia, MO, 2Division of Animal Science, University of Missouri-Columbia, Columbia, MO, 3State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing, China
Production of live offspring from cloned embryos is still low in mammalian species (ca. 1-5%) compared to in-vitro produced embryos which has been linked to a variety of cellular incompatibilities between the donor cell nucleus and the enucleated oocyte. Here we investigated whether donor cell mitochondria may exert adverse effects on embryo development. We have previously shown that perinuclear mitochondria aggregation indicates low developmental potential in vivo and in vitro fertilized pig eggs while abnormal mitochondria distribution is associated with developmental failures in in vitro fertilized oocytes. Here we investigated whether abnormal mitochondria distribution may be a contributing factor to the low cloning efficiencies and whether donor cell mitochondria are integrated into the entire mitochondria population in reconstructed pig embryos. In cloned embryos, two mitochondria populations are typically present, one contributed by the oocyte and one contributed by the donor cell. During normal fertilization, the oocyte contributes all mitochondria while the sperm’s mitochondria are destroyed by ubiquitination. We used either CMXRos-preloaded pig fibroblast cells or YFP-mitochondria transfected 3T3 mouse fibroblasts as donor cells to reconstruct interspecies and intraspecies cloned embryos, respectively, and follow donor cell mitochondria distribution. Our results revealed that donor cell mitochondria always aggregated near the donor cell nucleus but shortly after nuclear transfer (NT) dispersed into the ooplasm of the reconstructed embryo. In early stage embryos, there are no obvious differences in mitochondria distribution between IVF and nuclear transfer (NT) embryos. In later stage NT embryos, donor cell mitochondria displayed decreased labeling intensity which may indicate degradation or compromised mitochondrial function. Further studies are underway to investigate whether donor cell mitochondria exert negative effects on development, perhaps by inducing abnormal patterns of apoptosis. Funded by NIH grant R03-HD43829-02.

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An Intact Actin Cytoskeleton Is Important for CCN2 Expression in Chondrocytes

D. Pala, 1 K. M. Lyons, 1,2 F. Beier, 1 A. Leask 1; 1 CIHR Group in Skeletal Development and Remodeling, University of Western Ontario, London, ON, Canada, 2 Department of Biological Chemistry, UCLA, Los Angeles, CA

Connective tissue growth factor (CTGF, CCN2) is expressed by mesenchymal cells during mammalian development. Mice genetically deficient in CCN2 die immediately after birth, displaying severe bone defects, including an expansion of hypertrophic cartilage and a decrease in ossification. The molecular basis of this defect is not known. An examination of the mechanisms underlying the control of CCN2 expression in primary chondrocytes was undertaken. CCN2 is expressed in chondrocytes, correlating with their differentiation stage. Using a combination of promoter analysis involving deletion series and point mutations, inhibitors and further functional assays we have identified cis acting sequences and trans-acting factors involved with CTGF expression. Remodeling of the actin cytoskeleton is essential for the early stages of chondrogenesis; and it has been found that expression of CCN2 in primary chondrocytes is blocked by the actin depolymerising agent cytochalasin D. Our results have identified a key sequence in the promoter that is responsive to the actin cytoskeleton. These results suggest that CCN2 is required for early chondrogenic events, downstream of remodeling of the actin cytoskeleton.

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The Hereditary Neural Amyotrophy Causing Sept9 Mutations Are Associated with Altered Interaction with Other Septins, and Resistance against Rho/Rhotekin-dependent Septin Filament Disruption

K. Nagata, H. Ito, I. Iwamoto, R. Morishita, T. Asano; Molecular Neurobiology, Institute for Developmental Research, Aichi Human Service Center, Kasugai, Japan

Single point-mutations in Sept9 were shown to cause hereditary neural amyotrophy (HNA) but the cellular consequences of the mutations in HNA have not been determined. Since Sept9 is thought to exert its functions through interaction with other septins and small GTPase Rho-mediated signaling, we analyzed biochemical and cellular biological properties of HNA-associated Sept9 missense mutants, S93F/Sept9-v3 (Sept9F) and R88W-Sept9-v3 (Sept9W). We show that 1) Sept9F, but not Sept9W, affects Triton-X100 solubility of Sept7 and Sept11 when co-expressed in COS7 cells. 2) Sept9W, but not Sept9F, has reduced affinity to the interactive partner Sept4 in the cells, 3) both mutants, but not the wild-type, coordinately forms filamentous structures with Sept7 in NMuMG cells, and 4) the septin filaments comprising the mutants are not affected by the Rho/Rhotekin signals in the cells. Taken together, HNA-causing mutations in Sept9 are likely to alter the biochemistry of properties of Sept7 and modes of interaction with its interactive partners in different manners, and consequently might contribute to the pathogenesis of HNA.

2357

The Alteration of Cell Shape, Cytoskeleton, and the Adhesive Structures Induced by Antioxidants

L. V. Domina, O. Y. Ivanova; Laboratory of Mathematical Methods in Biology, A.N.Belozersky Institute of Phys.-Chem. Biology, Moscow State University, Moscow, Russian Federation

The aim of the work is investigation of the effect of antioxidant Mito Q (mitochondrial targeted antioxidant) which in very low concentration protects cell from H2O2-induced apoptosis. Human skin fibroblasts (HSF), rat fibroblasts (Rat 1), mouse normal and Ras-transformed fibroblasts, human epithelial carcinoma cells (HeLa) and rat epithelial cells (IAR 2) were used. The cells were incubated with mitochondria targeted antioxidant- mito Q for 7 days, 50-100 μM H2O2 was added for last 24 hr. The confocal microscope in combination with immunofluorescence were used to study cytoskeleton. and in combination with tetera metil rodamine to study mitochondria. Treatment of cells with the antioxidant mito Q led to significant increase in cell areas and number of focal contacts. In spite of cell area growing up - cell elongation decreased. These changes in Ras-transformed fibroblasts made them morphologically indistinguishable from the normal ones. Cytoskeleton alteration consisted of actin filament increase in number and size. O-Smooth actin appeared in 26-30% of the treated fibroblasts, that could be considered as the differentiation step. Consequently wound healing by these fibroblasts was 2 times more intensive than in the control cultures. Number of mitochondria increased in the cells treated with mito Q. Mito Q led to formation of mitochondrial network from separated mitochondria and protect the cells from H2O2-apoptosis.

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Cdc42 Is Required in Glucose-induced Insulin Secretion and Rac1 Activation in Pancreatic Beta-cells

Z. Wang, E. Eh, D. C. Thurmond; Biochemistry & Molecular Biology, Indiana University School of Medicine, Indianapolis, IN

Cdc42 and Rac1, members of the Rho subfamily of small GTPases, have each independently been found to play important roles in glucose-stimulated insulin secretion from pancreatic islet β-cells. Although Cdc42 and Rac1 are presumed to function in actin remodeling and insulin granule mobilization to the plasma membrane, it is unknown whether they are linked in a common signaling pathway. Using the GST-Pak1-PBD binding assay, we demonstrated that glucose stimulation Cdc42 is rapidly activated at the plasma membrane (PM) within 3 min, while Rac1 is not activated until a later time point of ~20 min. Both Cdc42 and Rac1 are activated only in response to D-glucose, otherwise unresponsive to non-metabolizable analogs 2-deoxyglucose and 3-orthoethyl-glucose or KCl, suggesting a requirement for glucose metabolism or a metabolite therefrom for activation. Interestingly, depletion of Cdc42 resulted in impaired glucose-induced activation of Rac1 at 20-30 min, supporting the notion that Rac1 exists downstream of Cdc42 in a common pathway. Furthermore, the p21-activated-kinase 1 (Pak1) became phosphorylated within 5–10 min of glucose stimulation, and thus may mediate the link between Cdc42 and Rac1 in this pathway. Functionally, Cdc42 depletion specifically impaired the 2nd phase of glucose-stimulated insulin secretion from isolated mouse islets, consistent with the notion that 2nd phase secretion requires actin remodeling for granule mobilization. Together, these data support a new mechanism whereby glucose initiates the mobilization of insulin granules early in stimulus-secretion coupling via first activating Cdc42 which then leads to Rac1 activation, and may involve Pak1 as an intermediary protein to coaggle Cdc42 and Rac1. This mechanism is only responsive to metabolism of D-glucose, consistent with the known requirement for stimulation of 2nd phase secretion. Further experiments are needed to elucidate the mechanisms leading to activation of Cdc42 and linking to Rac1 to facilitate second phase insulin secretion.

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Phosphopeptide Analogues of HSP20 Modulate TGFβ-1 Stimulation of Human Keratin Fibroblasts

L. B. Lopes, 1 P. Komalavilas, 1,2 C. R. Flynn, 1 A. Panitch, 3 E. Furnish, 1 C. M. Brophy 1,2; 1 Harrington Department of Bioengineering, Arizona State University, Tempe, AZ, 2 Carl T. Hayden Veterans Affairs Medical Center, Phoenix, AZ, 3 Weldon School of Biomedical Engineering, University of Purdue, West Lafayette, IN

Transforming growth factor β-1 (TGFβ-1) stimulation leads to stress fiber formation, myofibroblast phenotype, and extracellular matrix production in fibroblasts. Phosphorylation of heat shock related protein (HSP) 27 and 40 is associated with actin cytoskeleton stabilization and stress fiber formation. Treatment with a phosphopeptide analogue of HSP20 conjugated to a protein transduction domain (PTD-pHSP20) induces loss of stress fibers and coloifin dephosphorylation. We investigated whether TGFβ-1 induces the phosphorylation of HSP27 and coloifin in human dermal keratin fibroblasts, and whether fibroblasts were growth 80% confluenve, serum-starved for 48 hr, and subsequently stimulated for 24 hr with TGFβ-1 (2.5 ng/mL), in the presence or absence of PTD-pHSP20 (0.05 mM). Untreated cells were used as controls. Immunoreactive phosphorylated HSP27 and coloifin were increased 5 and 2.2 times (over control), respectively. TGFβ-1 stimulation, PTD-pHSP20 decreased the TGFβ-1-induced phosphorylation of HSP27 by 52.4 ± 6.8% (n=3) and of coloifin by 31.5 ± 5.9% (n=3). PTD-pHSP20 treatment also led to loss of actin stress fibers with only cortical actin remaining. In addition, the expression of collagen in TGFβ-1-stimulated cells was decreased by 44.8 ± 13.2% (n=4) after PTD-pHSP20 treatment. The results suggest that phosphorylation of HSP27 and coloifin might mediate stress fiber formation induced...
Hyperosmotic stress increases phosphoinositide levels, reorganizes the actin cytoskeleton and induces multiple acute and adaptive physiological responses. We have found that phosphatidylinositol 4,5-bisphosphate (PIP2) levels increases rapidly in HeLa and Cos cells during hypertonic treatment. Depletion of the human type 1 phosphatidylinositol 4-phosphate 5-kinase (isoform PIP5KIβ) by RNA interference (RNAi) impairs both the PIP2 and actin cytoskeletal responses. PIP5KIβ is recruited to membranes and activated by hypertonic stress through ser/thr dephosphorylation. Cycalcin A (a caly A, a protein phosphatase 1 inhibitor, blocks the hypertonicity-induced PIP5KIβ dephosphorylation/activation as well as PIP2 increases in cells. Urea, which causes a rise in osmolarity without inducing cell shrinkage, neither promotes dephosphorylation nor increases PIP2 levels. Disruption or stabilization of the actin cytoskeleton, or inhibition of the Rho kinase, does not block the PIP2 increase or PIP5KIβ dephosphorylation. Therefore, PIP5KIβ is dephosphorylated in a volume-dependent manner by a caly A-sensitive protein phosphatase, which is activated upstream of actin remodeling and independently of Rho kinase activation. Our results establish a cause-and-effect relation between PIP5KIβ dephosphorylation, lipid kinase activation and PIP2 increase in cells. This PIP2 increase orchestrates multiple downstream responses, including the reorganization of the actin cytoskeleton.

Cytoskeletal Reorganization in a Kidney Cell Line (LLC-PK1) under Hypertonic Stress

A. S. Khanna, S. Murphy, M. Kasschau; Biological Sciences, University of the Sciences in Philadelphia, Philadelphia, PA

Mammalian kidney cells are normally exposed to high extracellular salt concentrations as a consequence of their participation in the urinary concentration mechanism (Natalia et al., Fundamental and Molecular Mechanisms of Mutagenesis, 2005). Cell structure and volume dependent remodeling of cytoskeleton is known to be influenced as an adaptive response to withstand this hypertonic stress (Di Ciano et al., Am J Cell Physiol, 2002). In diuresis and anti-diuresis conditions in mammals, the kidney cells are exposed to fluctuating levels of NaCl, urea and ammonia (Bustamante et al., Am J Physiol Renal Physiol, 2003). In our laboratory the effect of NaCl induced hypertonicity on cytoskeletal rearrangement in kidney cells was assessed by fluorescence microscopy. In LLC-PK1 cells, the distal tubular epithelial cell line from the cortical region of Sus scrofa (pig) kidney, actin and tubulin reorganized with exposure to 1 hour of hypertonic medium (1530 mOsm) as compared to cells in isotonic medium (330 mOsm). More than 95% of the LLC-PK1 cells grown in isotonic DMEM displayed colocalized actin and tubulin cortical rings. In approximately 80% of the cells, the actin and tubulin cortical rings were disrupted when cells were treated with a hypertonic medium for 1 hour. The cytoskeletal rings were reorganized, within 3 hours as cells recovered from the hypertonic treatment. One possible reason for this cytoskeletal reorganization could be due to the cell shrinkage as a result of hypertonicity. Comparative studies with mIMCD-3 cells, the inner medullary collecting duct cell line from Mus musculus (mouse), will enable us to understand whether cells from different regions of the kidney respond to hypertonic stress in a similar fashion.

Role of HSP70 and the Cytoskeleton in Podial Formation in a Human Hematopoietic Cell Line under Hypertonic Stress

M. D. Dutt, M. Kasschau, S. Murphy; Biological Sciences, University of the Sciences in Philadelphia, Philadelphia, PA

We study podia formation under hypertonic conditions in a human hemopoietic cell line KG1. KG1a cells are derived from the bone marrow of a patient with acute myelogenous leukemia (AML). KG1a cells are known to be highly sensitive to osmotic stress and undergo acute and adaptive physiological responses. Focal adhesion kinase (FAK) and its downstream targets, p38 MAPK and JNK, are involved in the regulation of podia formation in KG1a cells. FAK activation in KG1a cells under hypotonic conditions is mediated by Src.

The Rho-specific GEF Net1 Interacts with Scaffolding Proteins of the MAGUK Family

R. Garcia-Mata,1 A. Dubash,1 L. Sharek,1 K. Burridge2,3; 1Cell and Developmental Biology, UNC-Chapel Hill, Chapel Hill, NC; 2Lineberger Comprehensive Cancer Center, UNC-Chapel Hill, Chapel Hill, NC

Neuropilin remodeling factor 1 (NET1) is a RhoA specific guanine nucleotide exchange factor which was originally identified in a screen for oncogenic genes. Unlike the majority of Rho-GEFs which reside in the cytosol, Net1 localizes into the nucleus at steady state. A deletion in its N-terminus redistributes the protein to the cytosol, where it activates RhoA and can promote cell transformation. Net1 contains a conserved PDZ binding motif at the C-terminus which is essential for its transformation properties. In this study we found the Net1 interacts through its PDZ binding motif with the tumor suppressor protein SAP97/DLG1, as well as with other members of the same family, including SAP102 and SAP97. Interestingly, interaction between Net1 and its PDZ partners promotes the translocation of the PDZ protein to the nucleus specifically to nuclear subdomains associated with PML bodies. Deletion of the PDZ binding tail in Net1 prevents the Net1-mediated translocation of the PDZ protein which remain in the cytosol. The oncogenic mutant of NET1, which lacks the nuclear localization signals, is unable to shuttle the PDZ proteins to the nucleus, although the proteins still associate as clusters in the cytosol. Our recent studies suggest that the interaction of the NET1 mutant to transform cells may be in part related to its ability to sequester tumor suppressor proteins like SAP97/DLG1 in the cytosol preventing them to perform their normal cellular function. In agreement with this, we observe that the transformation potential of oncogenic Net1 is reduced when it is co-expressed in NIH3T3 cells together with SAP97/DLG1 or SAP102. Taken together our results suggest that the interaction between Net1 and SAP97/DLG1 could play a role in the mechanism of Net1 mediated transformation. Supported by a Susan Komen Foundation postdoctoral Fellowship (RGM) and by NIH GM25860 (KB).

Stretched to the Limit: Actin Dynamics at Extreme Cell Deformations

K. L. van Beek,1 M. G. Coppolino,1 M. Skalski,2 M. J. Kean,2 D. S. Fudge1; 1Integrative Biology, University of Guelph, Guelph, ON, Canada, 2Molecular and Cellular Biology, University of Guelph, Guelph, ON, Canada

Recent work on the intermediate filament (IF) network in cells suggests that IFs are highly extensible, and that the network can passively withstand cell strains up to 140%. In contrast, actin filaments are known to be at least two orders of magnitude stiffer than IFs and not nearly as extensible. This suggests that the actin cytoskeleton cannot survive large scale cell deformations and likely undergoes dramatic reorganization at large strains. To test these hypotheses we seeded Madin-Darby Canine Kidney cells onto rectangular silicone rubber strips coated with collagen IV, and stretched them with a custom uniaxial stretching device. Using this device we achieved cell strains in excess of 100%, which is several fold higher than the vast majority of cell stretching studies, which typically have maximum uniaxial strains of 25%. Rhodamine-phalloidin staining of control and stretched cells revealed that large uniaxial strains lead to considerable disruption of stress fibers (SFs). Similarly, large strain followed by relaxation of the rubber substrate also led to disruption of SFs. Cells stretched and held at large strains for 30 minutes exhibited SF buckling when the rubber substrate was relaxed. When viewed under phase contrast, these cells exhibited dramatic "stretch marks" perpendicular to the direction of stretch, indicating a partial loss of adhesion during the time they were held in the stretched state.

Analyzing the Role of Tension in Focal Adhesion Dynamics and Maturation

A. M. Pasapera-Limon,1 A. Kirby,2 W. D. Shim,1 A. N. Murray,1 C. M. Waterman-Storer1; 1Cell Biology, The Scripps Research Institute, La Jolla, CA; 2Biology, Duke University, Durham, NC
Focal adhesions (FAs) play a central role in cell migration. They link the actin cytoskeleton to the extracellular matrix through transmembrane integrin receptors which in turn recruit a complex of anchoring proteins leading to the formation of Focal complexes which mature into FAs. RhGTPase-regulated myosin II contraction of the cytoskeleton linked to adhesion induces integrin clustering and FA “maturation.” We hypothesize that tension-induced maturation of FAs results in changes in protein composition and dynamics that may influence adhesion morphology, strength, and signaling, thus regulating cell migration. In order to understand the relationship between adhesion morphology, maturation and protein binding/dissociation in FAs, we perform FRAP analysis of GFP-conjugated FA proteins while manipulating cellular tension across cell substrates.

To analyze the array of structural and signaling of FA components, we plated MEFs on 5 and 10 μg/ml of fibronectin and transfected then with GFP-conjugates of integrin α5, FAK, paxillin, zyxin, talin, or vinculin that were plated. FRAP analysis of these proteins showed remarkable diversity in their dissociation kinetics; 1/2 of fluorescence recovery was fast for paxillin, FAK, zyxin, and talin (~25s) while vinculin and α5 integrin were more stably bound in FAs (1/2 >100s) and only recovered partially. To manipulate cell contractility we inhibited myosin II activity by using the MLCK inhibitor ML-7 (20 μM). This revealed that 1/2 for α5 integrin, vinculin and zyxin were reduced, indicating that contractility promotes tighter binding of these proteins to FAs.

2366 Evolution of Actin Filament and Focal Adhesion Organization in Micro-patterned Cells
Department of Cell and Molecular Biology, Northwestern University Medical School, Chicago, IL, and Department of Chemical and Biological Engineering, Northwestern University, Evanston, IL, Marine Biological Laboratory, Woods Hole, MA

Heterogeneity of cell morphology and behavior precludes clear-cut quantitative analysis of cytoskeleton organization and dynamics across cell populations. Here, we show that control of cell geometry allows effective analysis of structural and functional characteristics of one component of cell motility, namely focal adhesion actin reorganization. Cell shape was controlled by patterning cell-adhesive islands separated by non-adhesive regions with micro-contact printing (μCP) of self-assembled monolayers of alkanethiolates onto gold. We used geometrically-defined cells to analyze the evolution and dynamics of the actin bundle and focal adhesion organization. B16F1 mouse melanoma cells were fixed with 30-minute time intervals from 2 to 12 hours after plating on fibronectin-coated patterned islands of circular and triangular shapes (area 1,250 micron square). Actin and focal adhesion structures were visualized with fluorescent phallolidin staining and immunofluorescence staining for paxillin. The images obtained from 25 single cells for each time point were combined to yield intensity maps of average distribution of cytoskeleton in the cell population. In circular cells, actin filaments re-organized from initial uniform distribution across entire cell area to preferential enrichment at the cell periphery with concordant increase of actin polymer mass. In triangular cells, actin filaments radiating out of the vertices as internal bundles progressively aligned with triangle’s edges. The re-organization of actin filaments was correlated with increased accumulation of focal adhesions at the rim of circular cells and at the vertices of triangular cells. Modeling of experimental data will be used to understand the cooperation between re-organization of actin bundles and focal adhesions. Our approach provides a powerful framework for quantitatively analyzing complex dynamic cellular processes from simple read-out of time series of averaged static images. Supported by NIH Grant 1 U54 CA119341 (GBB), Camille and Henry Dreyfus New Faculty Award (BG), and DOD Breast Cancer Research Program Postdoctoral Fellowship W81XWH-05-1-0312 (KKG).

2367 Cytosplastic Dynamics of Ovarian Cancer and the Role of RhGTPases
M. S. Thompson, P. Panorchan, J. S. H. Lee, A. Shrestha, D. Wirtz; Chemical and Biomolecular Engineering, Johns Hopkins University, Baltimore, MD

Ovarian cancer is the most deadly gynecological disease and progresses through two distinct pathways, complicating the search for effective diagnostics and treatments. Using multiple particle tracking microscopy, we have discovered that the cells corresponding to the two pathways of ovarian cancer progression vary distinctly in their cytoplasmic viscoelasticity. Benign ovarian surface epithelium and high-grade carcinomas share similar viscoelastic profiles, while the low-grade carcinomas exhibit a much softer cytoplasm. Upon wounding, the cytoskeleton of all three cell lines become stiffer, with the low-grade carcinomas showing the greatest change in viscoelasticity with a profile nearly identical to the benign and high-grade forms. These differences are correlated with vastly different migration rates in which the low-grade carcinomas migrate at twice the speed of the benign cells. However, the high-grade carcinomas, which are the most lethal via intraperitoneal dissemination and invasion, migrated the least. Immunofluorescence revealed vastly different cytoskeletal structures among the benign epithelium, low-grade carcinomas and high-grade carcinomas, suggesting that mediators of cytoskeleton organization may be essential to regulating ovarian cancer migration. We found that the RhGTPases were differentially expressed in the benign, low-grade and high-grade forms of ovarian cancer. Inhibition of Rac1 decreased the highly motile nature of the low-grade malignant and abolished migration of the high-grade carcinomas.

2368 Active Stiffening and Contractility of Cytoskeletal Networks Driven by Molecular Motors
G. H. Koenderink, Z. Dogic, M. P. Hansen, C. P. Brangwynne, D. A. Weitz
Experimental Soft Condensed Matter, Harvard University, Cambridge, MA, Complex Fluids Group, Rowland Institute at Harvard, Cambridge, MA

The cytoskeleton is a highly dynamic network of interconnected protein filaments that lends cells their mechanical integrity, but also actively generates forces. The most important contribution to shape changes and cellular movements stems from the actin cortex underlying the cell membrane. This cortex is contractile due to embedded myosin motor assemblies, which slide actin filaments past one another and thereby build up tension in the cross-linked actin cortex. In this talk I will present recent experiments on simplified model networks of purified actin and myosin II, which are aimed at a quantitative, microscopic understanding of the contractile properties of active actin/myosin networks. We probe the network mechanics on different length scales ranging from macroscopic scales (using macrotaxis) to micron scales (using embedded fluorescent microtubules as microscopic probes). The networks macroscopically stiffen due to internal stresses generated by contractile myosin activity, provided that cross-linking proteins are present to anchor the filaments. On small length scales the motors generate contractile fluctuations that show up in large transient shape deformations of embedded microtubules. From a materials perspective, our model system is a unique, active soft material. At the same time, the system allows to quantitatively explore molecular mechanisms of active cellular processes.

2369 Mechanical Properties of a Prokaryotic Actin-like Filament
B. Tseng, S. Sen, D. Mullins
Physics Course, Marine Biological Laboratory, Woods Hole, MA, Chromosome and Cell Biology, Rockefeller University, New York, NY, Control and Dynamical Systems, California Institute of Technology, Pasadena, CA, Cellular and Molecular Pharmacology, UCSF, San Francisco, CA

Recent work has identified homologs of actin and tubulin in bacteria. As in eukaryotes, these protein forms polymerize to maintain cell shape; transport cargo through the cytoplasm; and organize intracellular compartments. Understanding the mechanical properties of the eukaryotic polymers has helped explain the molecular mechanisms underlying their cellular functions. At present, almost nothing is known about the mechanical properties of prokaryotic cytoskeletal polymers. Using fluorescence microscopy of labeled proteins, we measured the flexural rigidity of actin-like filaments in Caulobacter crescentus, leveraging the phage-encoded protein ParM. Assembly of ParM filaments pushes plasmids to opposite poles of rod-shaped cells in a process analogous to the chromosome segregation function of microtubules. We determined a persistence length of approximately 10 μm for freely fluctuating filaments formed from a non-hydrolyzing ParM mutant.

In addition, we determined a persistence length of 20 μm for ParM filament bundles formed in the presence of parC and ParR. The persistence length of an individual ParM filament is similar to that of a eukaryotic actin filament - approximately 4-5-fold larger than the length of a bacterium. This result suggests that individual filaments may be stiff enough to segregate pairs of plasmid subunits. To analyze the array of structural and signaling of FA components, we plated MEFs on 5 and 10 μg/ml of fibronectin and transfected then with GFP-conjugates of integrin α5, FAK, paxillin, zyxin, talin, or vinculin that were plated. FRAP analysis of these proteins showed remarkable diversity in their dissociation kinetics; 1/2 of fluorescence recovery was fast for paxillin, FAK, zyxin, and talin (~25s) while vinculin and α5 integrin were more stably bound in FAs (1/2 >100s) and only recovered partially. To manipulate cell contractility we inhibited myosin II activity by using the MLCK inhibitor ML-7 (20 μM). This revealed that 1/2 for α5 integrin, vinculin and zyxin were reduced, indicating that contractility promotes tighter binding of these proteins to FAs.

2370 Quantitative Morphological Cytometry for Measuring Shear Stress in Cellular Systems
D. Lenz, B. Bayraktar, S. Leavesley, J. Robinson, B. Rajwa; Bindley Bioscience Center, Purdue University, West Lafayette, IN

Shear stress is known to have a significant effect on the state of cellular differentiation. It also induces morphologic responses including changes to cytoskeletal organization subsequently leading to changes in cell shape. In fact, fluid shear stress caused by blood flow is a major determinant of vascular remodeling and can lead to development of atherosclerosis. The morphological changes are usually evaluated using boundary-based shape descriptors (e.g. form factor) or binary geometrical moments on manually segmented cells. Although any one of the many automated segmentation method could be employed, these techniques are known to be complex, time consuming, and often require user input to operate properly, which is especially problematic for high-content screening (HCS) systems processing thousands of images during a single run. Therefore, development of robust, quantitative morphological measurements that are not dependent on precision and reproducibility of segmentation is extremely important for a substantial improvement of shear-stress analysis. The goals of this study were to find simple
morphological descriptors that could be applied to cells isolated by tessellation in order to enable a high-throughput screening of morphological shear-stress response, and to determine the amount of fluid shear stress to which endothelial cells were exposed on the basis of changes in their morphology. The proposed technique is based on the monitoring of changes in cytoskeleton organization using texture descriptors, rather than on quantifying cell-boundary modifications. We showed that objects identified by Voronoi tessellation carried enough information about cytoskeleton texture of individual cells to create a robust classifier. Our approach provided higher discriminant and predictive powers, and better classification capability, than traditional boundary-based methods. The robustness of classification in the presence of segmentation difficulties makes the proposed approach particularly suitable for automated HCS systems.

2371
Interaction between XB130 and Fish/Tks5 and Involvement in Podosome Formation
H. Xiao, M. Liu; Thoracic Surgery Research Laboratory, Toronto General Hospital, Department of Physiology, University of Toronto, Toronto, ON, Canada
Src family protein tyrosine kinases play an important role in tumor progression and metastasis. We recently cloned a new adaptor protein, XB130, from a human lung adenocarcinoma cell line, and found that XB130 can activate c-Src, and leads to Akt-P-1,SRE transactivation. Using yeast-two-hybrid system, we found 64 positive clones that potentially interact with XB130, of which five clones encode partial sequence of Fish (Five SH3 domain-containing protein), which is called Tks5 and is required for podosome formation in cancer cells. Endogenous XB130 and Fish expression was found in several thyroid cancer cell lines by RT-PCR and western blotting. Interactions among Src and XB130 and Fish in these cells were found by co-immunoprecipitation and western blotting. Cell lysates from XB130-transfected COS7 cells were incubated with GST fusion proteins containing each of the five SH3 domains of Fish. The last SH3 domain at the C-terminus of Fish was identified as the major binding site to XB130. This was further confirmed by using a single amino acid mutant (W1056A) of this SH3 domain. A group of constructs were generated to partially truncate or delete functional domains in XB130. The N-terminus of XB130 containing putative SH3 and SH2 domain binding sites was responsible for the binding to Fish. When GFP XB130 construct was transfected into v-Src transformed NIH3T3 cells, XB130 was clustered in the podosomes. With prolonged incubation podosomes in these cells became smaller and disappeared. The morphology of these GFP XB130 transfected cells also changed. We speculate that the adaptor protein XB130 may interact with Fish to participate in the regulation of podosome formation and other Src related signaling and functions. Keyword: Fish/Tks5, Src, podosome, adaptor protein, signal transduction

2372
Involvement of MLCK in Actin Stabilization and Podosome Development in the Contracting A7r5 Smooth Muscle Cell
S. E. Thatcher, 1 M. E. Fultz, 1 H. Tanaka, 1 K. Kohama, 2 G. L. Wright 1; Physiology, Pharmacology, and Toxicology, Joan C. Edwards School of Medicine, Huntington, WV, 1Biology, Morehead State University, Morehead, KY, 2Molecular and Cellular Pharmacology, Gummer University, Maebashi, Japan
Myosin light chain kinase (MLCK) is a serine/threonine protein kinase that is responsible for phosphorylating the serine-19 position on the regulatory light chains of myosin. MLCK also contains actin-binding sites located within its N-term which provides binding and bundling of actin filaments. Colocalization imaging and fluorescence resonance energy transfer (FRET) analysis indicated n-actin/MLCK association in resting cells and in podosomes of phorbol 12,13-dibutyrate (PDBu)-stimulated A7r5 smooth muscle cells. By comparison, β-actin/MLCK association was observed in stress fibers and in diffuse distribution in the perinuclear region of both control and PDBu-treated cells. Downregulation of MLCK by siRNA transfection resulted in variable patterns of actin isoform reorganization in control cells. n-Actin formed a dense system of filaments at the cell periphery leaving the central region of the cells devoid of structure. In contrast, β-actin stress fibers disassembled with this isoform diffusely distributed in the cell. In PDBu-treated cells, transfection with MLCK-siRNA resulted in an approximate 70% reduction in the formation of podosomes. The introduction of a peptide containing the I-41 N-terminal amino acid sequence of MLCK by peptide-mediated uptake or microinjection resulted in loss of α-actin stress fibers from the central region of the cell. The results indicate that MLCK plays an important role in maintaining n- and β-actin stress fibers and in the phorbol ester-induced reorganization of these isoforms. Furthermore, this role appears to be related to the N-terminal actin binding properties of the kinase.

2373
Large Scale Cytoskeletal Structures Support Grasshopper Oogenesis
K. A. Edwards, E. Murray, D. Whitman, K. Vadali; Biological Sciences, Illinois State University, Normal, IL
Much of our knowledge concerning the cellular and molecular mechanisms of insect oogenesis is derived from Drosophila genetic studies. However, Drosophila ovaries are rather small and do not encounter the extreme mechanical challenges faced by large insects such as the Lubber grasshopper Romalea microptera. We are studying the differences in ovariore structure and the behavior of the follicular epithelium in these two species, to better understand the evolution of oogenesis in response to size variation. We find at least five structures that are heavily reinforced by either actin or microtubules, and differ dramatically from the homologous structures in Drosophila; these include the cellular sheath surrounding the ovariole, the terminal filament, the interfollicular stalks, the basal cytoskeleton of the oocyte follicle cells, and the follicle cells that remain after egg laying. The observed cytoskeletal structures all require the coordination of filament orientation across multiple cells. In two cases (the sheath and terminal filament) the cytoskeletal structures appear to reside in syncytia: the filaments pass continuously alongside multiple nuclei. In the remaining cases, actin filaments are coordinated from cell to cell, presumably through cadherin complexes or focal adhesions. These observations indicate that supracellular actin networks provide mechanical stability during insect oogenesis, and suggest that similar structures may arise in other developing tissues.

2374
Cytoskeletal Regulation of Cell Shape Changes during Xenopus Gastrulation
J. Lee, R. M. Harland; Department of Molecular and Cell Biology, UC Berkeley, Berkeley, CA
Cell shape changes, such as apical constriction, are essential to diverse processes during development and cancer, ranging from morphogenesis to epithelial-to-mesenchymal transitions. Despite its importance and apical, surprisingly little is known regarding the cytoskeletal mechanisms that regulate apical constriction. To study apical constriction in a developmental context, we are examining the role of the cytoskeleton during Xenopus laevis bottle cell formation. Bottle cells undergo shape changes typical of apically constricting cells, transforming from cuboidal-shaped to flask-shaped while simultaneously undergoing cell elongation. In Xenopus, these cell shape changes create a crenicle to direct the first steps of mesodermal involution. It has been suggested, but never shown, that actin and myosin drive bottle cell apical constriction, whereas microtubules may be involved in elongation but not for contraction. To test these hypotheses, we first examined the localization and function of actin, myosin, and microtubules. Significant levels of F-actin and activated myosin accumulate at the apical surface of bottle cells. When actin or myosin function was inhibited with pharmacological agents, bottle cells were either completely absent (actin inhibitors) or exhibited less constricted apices (myosin inhibitor). Microtubules also localize to the apical side, though in an apicobasally-directed filamentous array. Intriguingly, microtubule depolymerization did not interfere with cell elongation; instead, it inhibited full constriction of the apical surface, presenting a novel role for microtubules during apical constriction. Together, our results show that, and to a lesser extent myosin and microtubules, are required for discrete aspects of the cell shape changes associated with apical constriction. We are currently examining the role of additional actin-dependent mechanisms, investigating the regulation of myosin activity, and determining the mechanism by which microtubules contribute to apical constriction. Using Xenopus bottle cells as a model, we hope to gain broader insight into the cytoskeletal mechanisms controlling cell shape changes.

2375
Co-ordination of Actin and Microtubules during Drosophila Dorsal Closure
T. Millard, B. Stramer, P. Martin; University of Bristol, Bristol, United Kingdom
Dorsal closure is a process occurring during Drosophila embryogenesis whereby two epithelial sheets sweep towards one another over dorsal surface of the embryo and fuse when they meet at the midline. Dorsal closure is a well-studied model of epithelial morphogenesis. Previous work has elucidated the dynamics and function of the actin cytoskeleton during dorsal closure as well as signals that regulate actin during the process. A contractile actin-myosin cable is observed along leading edge of the two epithelial sheets and filopodia and lamellipodia protrude beyond this cable, which are involved in ‘rappelling’ of the two epithelial sheets. We have performed experiments designed to observe the dynamics and function of the microtubule cytoskeleton during dorsal closure. We have used two-colour live imaging to simultaneously observe actin filaments and microtubules during dorsal closure. We find that actin and microtubules display clearly distinct arrangements during dorsal closure. Microtubules are highly dynamic and are arranged in parallel bundles perpendicular to the leading edge actin cable. Dynamic microtubules frequently make contact with the leading edge actin cable and protrusions. Depolymerisation of microtubules disrupts dorsal closure, resulting in a dorsal hole. Live imaging suggests that ‘rappelling’ of leading edge cells fails following microtubule depolymerisation, while amnioserosa contraction proceeds as normal. Our results suggest that the microtubule and actin cytoskeletons of epithelial cells co-operate to achieve dorsal closure.
Role of the Yeast RGS Protein Sst2p in Cell Morphogenesis

T. Yi,1 H. Tanaka,1 H. Kitano2; 1Developmental and Cell Biology, UCI, Irvine, CA, 2The Systems Biology Institute, Tokyo, Japan

Yeast (2). Here, we investigated the role of Sst2p in forming proper shmoo. The Sst2D cells exhibited morphology defects during late polarization. The hRGS4 cells also exhibited similar morphology defects, even though hRGS4 could rescue the pheromone supersensitivity phenotype in sst2D cells. To investigate the differences between Sst2p and hRGS4, we observed the localization of marker proteins including Spa2p, Ste18p (Gg), Fus3p, Ste20p and Cdc12p (septin). We noted interesting spatial dynamics in the hRGS4 cells. Finally, in contrast to the late polarization defects, mating and early polarization in hRGS4 cells were similar to wild-type cells. (1) Billingmaier S and Snyder M, JCB 164:207-218, 2004 (2) Spinavisa SP, Bernstein LS, Blumer KJ and Linder ME, PNAS 95: 5584-5589, 1998

2378 Alanine Scanning Mutagenesis of the Yeast Cdc11p Septin

M. S. Longtine, A. Rajendran; Cell Biology and Physiology, Washington University in St. Louis, St. Louis, MO

role of septins to localize, and sometimes activate, septin-associated proteins. Recent data suggest that septin GDP binding is important in the formation of septin complexes and, thus, in septin filament formation and localization. Cdc11p and Cdc12p interact directly and stoichiometrically and this interaction requires both proteins to be bound to nucleotide. To better understand septin function, we have carried out a systematic alanine-scanning mutagenesis of Cdc11p with the aim of identifying regions involved in the interactions of Cdc11p with Cdc12p and with septin-associated proteins. Using this approach, we have identified four mutations that disrupt Cdc11p function. First, we identified two dominant-negative mutations in Cdc11p which do not prevent the interaction with Cdc12p or localization to the cell cortex. However, these mutations result in dominant defects in septin localization to the neck of the mutated Cdc11p and other septins and in septin function. We are currently testing the idea that these mutations result in the formation of abnormal septin filaments. Second, we have identified two mutations that disrupt the interaction of Cdc11p with Cdc12p. These mutations may identify surface regions of Cdc11p involved in the interaction with Cdc12p.

2379 Structural Analysis of Septin 2, 6, and 7 Complexes

C. Low,1 M. Macara1; 1Biochemistry, University of Virginia, Charlottesville, VA

Complexes. Yeast septins localize to the yeast mother-bud neck, the site of cytokinesis and mutations in CDC3, CDC10, CDC11 or CDC12 result in defects in cytokinesis, cell-cycle progression, chitin deposition, and other processes, resulting in inviability. Septin function appears to involve the formation of a membrane-associated septin filament serving as a barrier at the neck that has required for cell differentiation and cytokinesis. Septin function also involves the ability of septins to localize, and sometimes activate, septin-associated proteins. Recent data suggest that septin GDP binding is important in the formation of septin complexes and, thus, in septin filament formation and localization. Cdc11p and Cdc12p interact directly and stoichiometrically and this interaction requires both proteins to be bound to nucleotide. To better understand septin function, we have carried out a systematic alanine-scanning mutagenesis of Cdc11p with the aim of identifying regions involved in the interactions of Cdc11p with Cdc12p and with septin-associated proteins. Using this approach, we have identified four mutations that disrupt Cdc11p function. First, we identified two dominant-negative mutations in Cdc11p which do not prevent the interaction with Cdc12p or localization to the cell cortex. However, these mutations result in dominant defects in septin localization to the neck of the mutated Cdc11p and other septins and in septin function. We are currently testing the idea that these mutations result in the formation of abnormal septin filaments. Second, we have identified two mutations that disrupt the interaction of Cdc11p with Cdc12p. These mutations may identify surface regions of Cdc11p involved in the interaction with Cdc12p.

2380 p115-RhoGEF Controls RhoA Activity downstream of Fibronectin Adhesion

A. D. Dubash,1 K. Wennerberg,2 R. Garcia-Mata,3 M. Menold,1 K. Burridge1; 1Cell and Developmental Biology, University of North Carolina at Chapel Hill, Chapel Hill, NC, 2Southern Research Institute, Birmingham, AL

In order to understand how Rho proteins control actin reorganization, there is little knowledge about which specific GEFs are involved in different RhoA activation pathways, and the mechanisms via which these GEFs are activated. The current study was therefore focused on identifying the specific GEFs involved in activation of RhoA downstream of the FN adhesion signal. Using the nucleotide-free mutant of RhoA (which has high affinity for activated GEFs), we demonstrate that adhesion of fibroblasts to FN causes an increase in activity of p115-RhoGEF (p115RG), a RhoA exchange factor. Importantly, knockdown of Lsc (the murine homolog of p115RG) greatly reduces the ability of FN to activate RhoA in fibroblasts. Further, fibroblasts overexpressing a catalytically inactive mutant of p115RG demonstrate spreading defects, an inability to form stress fibers and focal adhesions, and no increase in RhoA activity when presented with a FN stimulus. Our studies therefore support an important role for p115RG function in modulating RhoA activity downstream of FN stimulation. Supported by DOD BCRP 2005 Predoctoral Traineeship Award BC051092 and NIH grant HL45100.

2381 Role of the Yeast RGS Protein Sst2p in Cell Morphogenesis

T. Yi,1 H. Tanaka,1 H. Kitano2; 1Developmental and Cell Biology, UCI, Irvine, CA, 2The Systems Biology Institute, Tokyo, Japan

2382 Cytoskeletal Changes during Nuclear and Cell Divisions in a Freshwater Alga Zygnema cruciatum (Chlorophyta, Zygnematales)

M. Yoon1, J. W. Han1; 1Biological and Environmental Research, Korea Institute of Science and Technology, Republic of Korea

Cytoskeletal changes were observed during cell division of the green alga Zygnema cruciatum using FITC-conjugated Phallacidin for F-actin staining and FITC-anti-a-tubulin for microtubule staining, and time-lapse videography. The f-actin ring appeared as a cleavage furrow. During the metaphase, FITC Phallacidin staining appeared on the chromosomes and nuclear surface, but the F-actin ring reduced and then disappeared. At the telophase, FITC Phallacidin staining did not appear at all. FITC-anti-a-tubulin staining revealed that microtubules were arranged beneath the protoplasm during the interphase and then localized on the nuclear region at the prophase, and the mitotic spindle was formed at the metaphase. The microtubules appeared between dividing chloroplasts. To our knowledge, this is the first record on coordinate involvement of both actin and microtubules in nuclear division. Our results showed that the F-actin began to appear on the chromosome and nuclear surface at prophase and then disappeared at metaphase and, therefore, F-actin and microtubules play an important role during cell division in this species.

2383 Effectives of Rho-family GTPases and Ras on the Cell Contact-dependent Regulation of the Smooth Muscle Actin Promoter during Epithelial-Mesenchymal Transition

A. Sebe1,2 Z. Peteri1, L. Fan2, A. Thirone3, L. Rosiwall2, I. Musci1, A. Kapus1; 1Dept. of Pathophysiology, Semmelweis University, Budapest, Hungary, 2Dept. of Surgery, St. Michael's Hospital, University of Toronto, Toronto, ON, Canada

Epithelial-mesenchymal transition (EMT) of tubular cells into o-smooth muscle actin (SMA)-expressing myofibroblasts is a central pathomechanism in kidney fibrosis. Our recent studies have shown that an initial injury of intercellular contacts in LLC-PK1 tubular cells is a prerequisite for the transforming growth factor- (TGFβ)-induced EMT. We found that intercellular contact disassembly facilitates Rho activation and Rho kinase-mediated myosin light chain (MLC) phosphorylation, which in turn contributes to the translocation of serum response factor (SRF) and its cofactor, myocardin-related transcription factor (MRTF) into the nucleus, where they induce the activation of the SMA promoter. Dominant negative (DN) Rho, DN-MLC and DN-MRTF inhibited the contact disruption- and TGFβ-induced SMA promoter activation. The present study demonstrates that other Rho-family GTPases, Rac and Cdc42 also participate in the contact- and contactility-dependent regulation of the SMA promoter. Specifically, overexpression of constitutively active Rac1, Cdc42 and their downstream effector p21-activated kinase (PAK) led to MLC phosphorylation, nuclear accumulation of SRF and MRTF, and activation of the SMA promoter. Contact disassembly induced by Ca2+ removal enhanced PAK phosphorylation. Importantly, DN-Rac1, DN-Cdc42 or DN-PAK1 inhibited the contact disruption-induced nuclear translocation of MRTF and the activation of the SMA promoter. These data suggest that various Rho GTPases promote contact injury-provoked EMT, partially through actin/phosphomycin-dependent nuclear translocation of MRTF. In sharp contrast to Rho GTPases, wild type...
Ras inhibited the Ca<sup>2+</sup>-removal-triggered SMA promoter activation, whereas DN-Ras markedly activated the promoter. These results suggest that Rho GTPases and Ras are both involved in the contact-dependent regulation of the SMA promoter, exerting an opposite effect: Rho GTPases promote whereas Ras GAPase mitigates the process.

**2383 Regulation of PKL/GIT2 Complexes by Protein Kinase A**

A. K. Howe, L. C. Baldor, S. Campbell, P. B. Deming; Pharmacology and the Vermont Cancer Center, University of Vermont, Burlington, VT

The interplay between integrin and growth factor receptor signaling is critically important for regulating both anchorage-dependent growth and chemotactic cell migration. Previously, we have shown that the cAMP-dependent protein kinase (PKA) plays an important role in anchorage-dependent growth factor signaling and in chemotaxis. The p21-activated kinase-1 (PAK1) is an important regulator of cell migration and is a direct target for PKA in the regulation of anchorage-dependent growth factor signaling. To further investigate the ability of PKA to regulate PAK-associated functions, PAK-containing complexes were immunoprecipitated and incubated with purified PKA to identify other PAK-associated substrates for PKA. Among the substrates identified were previously described targets - including PKA itself, the adaptor protein Nck, and the Rac/Cdc42 exchange factor beta-PIX - as well as a prominent 95 kDa protein. This protein was identified as PAKL/GIT2, a multifaceted linker protein that couples PAK1 and beta-PK to the focal adhesion protein paxillin, binds G-protein coupled receptor kinases (GRKs), and exhibits Arf/GAP activity. Further analyses confirm that PKA phosphorylates PAKL both in vitro and in vivo and can mediate PAKL signaling events. Specifically, inhibition of PKA activity blocks the PDGF-stimulated interaction of PAKL with beta-PX, and activation of PKA directly induces PAKL-paxillin and PKL-GRK2 interaction, and is required for the induction of these complexes following PDGF stimulation. These biochemical data correlate with PKA-dependent regulation of GFP-PKL localization in growth factor-stimulated cells. Together, these data implicate PKL/GIT2-containing complexes as major targets for PKA in events coordinately regulated by integrins and growth factors.

**2384 The Effects of Ginseng Fibroblasts Carried SOS1 Mutation on 3-D Collagen Matrices**

S. Jang, E. Lee, D. Pallos, T. C. Hart; 1NIDCR-CRC, NIH, Bethesda, MD, 2NIAMS, NIH, Bethesda, MD. 3University of Tahoe T, Tahoe, Brazil

Mutation of the son of sevenless-1 gene (SOS1) is etiologic for hereditary gingival fibromatosis (HGF) (Hart et al., Am. J. Hum. Genet. 70:943-954, 2002), a rare autosomal dominant disorder characterized by benign, nonhemorrhagic, fibrous enlargement of gingival tissues. Previous studies of HGF fibroblasts grown in monolayer culture under starving conditions showed constitutive MAP kinase signaling activation compared to control fibroblasts. To determine how the mutant SOS1 affects the extracellular matrix and leads to gingival enlargement we used a physiologically relevant 3-D collagen matrix model to study cell-matrix interactions. When equal densities of normal control and HGF fibroblasts were embedded in 3-D, regardless of the matrix shape (rectangular or circular), the 3-D HGF fibroblasts contracted to half the size of the 3-D culture control after 4 days incubation. The degree of contraction correlated with cell density in both cell types but HGF samples contracted 8-times faster than normal controls. To determine if contraction was associated with the higher proliferation rate of HGF fibroblasts, SOS1 was depleted through siRNA. No contraction was observed in the HGF 3-D mutant SOS1 knock-down. Morphological differences were observed as early as 2 hr after seeding in 3-D and maintained under starving medium (DMEM-HEPES): while normal fibroblasts appeared dendritic in shape with long, slender extensions, HGF fibroblasts showed a stellate/bipolar morphology. These data suggest that the induction of HGF fibroblasts to higher proliferation of gingival fibroblasts through the rearrangement and result in global remodeling of the collagen matrix.

**2385 Galectin-8 Induces Phosphatidic Acid-dependent Decrease of PKA Activity Leading to Sustained ERK Activation and Apoptosis in Jurkat T Cells in Suspension**

C. Metz, A. Norambuena, L. Vicuña, C. Cárcamo, E. Pardo, C. Oyanadel, A. González, A. Soza; 1Reumatología, Fac. Medicina, and Centro de Regulación Celular y Patología, Fac. Ciencias Biológicas, Pontificia Universidad Católica de Chile, and MIFAB, Santiago, Chile, 2Neurología, Fac. Medicina, Pontificia Universidad Católica de Chile, Santiago, Chile

Homeostatic regulation of immune response includes apoptosis induced by repeated T cell activation. This apoptotic process is mediated by prolonged activation of ERK. In several adherent cells, the Ras/Raf/Mek/MAPK pathway induced by growth factors depends on integrin-mediated cell attachment and is not activated in cells in suspension. In T cells, MAPK activation has been observed during cell adhesion to either integrin or TCR ligands. In Jurkat T cells, activation of β1 integrins in suspension failed to increase the activity of ERK. Galectin-8 are a family of carbohydrate-binding proteins secreted by non-canonical genes and recently implicated in a variety of cellular processes, including T cell homeostasis. Galectin function is exerted by binding to cell surface glycoproteins, specially integrins. In Jurkat T cells, we found that galectin-8 (Gal-8) binds to specific β1 integrins and constitutes a strong stimulus of ERK, even in cells in suspension. Gal-8 provokes a maintained ERK activation for at least 1 hour. A pathway from integrins to ERK involves activation of Rac-1 GTPase. Gal-8 as matrix induced activation of Rac1, but not in suspension. Primary alcohol known to abrogate phospholipase D-derived phosphatidic acid (PA) inhibited Gal-8 induction of ERK. PA has been described to activate specific cAMP phosphodiesterases leading to decreased cAMP levels and PKA activity. Accordingly, Gal-8 inhibited the activity of PKA, which in adherent cells has been described to allow anchorage-independent activation of MAPK. As expected for a prolonged activation of ERK, Gal-8 provoked apoptosis within 6 h of stimulation. These results suggest that Gal-8 constitutes a novel extracellular stimulus for T cells, which induces ERK activation in cells in suspension by involving a nexus between PA and cAMP/PKA pathways, with modulating potential upon the immune response (Financiado in part by FONDECYT grant #1050715, FONDAP grant # 13980001 and Millenium Project, Ministerio de Planificación).

**2386 Autophagy-mediated Survival of Prostate Epithelial Cells on Laminin Requires Integrin-mediated Activation of the EGFR/Erk and Src Signaling Pathways to Maintain Bcl-ExL Expression**

M. Edick, L. Lamb, B. Knudsen, C. Miranti; 1Van Andel Institute, Grand Rapids, MI, 2Fred Hutchinson Cancer Research Center, Seattle, WA

In vivo the prostate gland basal epithelial cells adhere to laminin 5 (L5M) via α3β1 and α6β4 integrins. When placed in culture primary basal prostate epithelial cells (PECs) secrete and adhere to their own L5M-rich matrix. In this study we have elucidated the mechanism whereby integrin-mediated signaling regulates the survival of PECs on L5M-rich matrices. Survival of PECs on L5M requires α3β1, but not α6β4, and is dependent on integrin-mediated, ligand-independent activation of the epidermal growth factor receptor (EGFR) and the cytoplasmic tyrosine kinase Src, but not PI-3K. Integrin-mediated EGFR activation supports cell survival by signaling downstream to Erk, but not Akt. Both Erk and Src act to maintain elevated levels of anti-apoptotic regulator Bcl-XL. Down regulation of Bcl-XL by siRNA in L5M-adherent PECs is sufficient to induce cell death. Despite the dependence on Bcl-XL for survival, death induced by inhibition of EGFR or Src is not mediated through cytochrome C release or caspase activation. Blocking autophagy in L5M-adherent cells also induces cell death and caspase activation. However, blocking autophagy did not prevent cell death induced by blocking EGFR signaling, and cell death under these conditions was compromised by an increase in caspase activity. Together these data indicate that survival signals by integrins on L5M is regulated by at least two pathways, signaling through EGFR/Erk and Src to target Bcl-XL and through an autophagy survival pathway. In the presence of an intact autophagy survival pathway, death induced by blocking EGFR/Erk signaling is caspase-independent. However, in the absence of autophagy, inhibition of signaling through this pathway leads to caspase-dependent death.

**2387 Regulation of Raf-MEK-ERK Signalling Pathway in Primary Mouse Mammary Epithelial Cells Forming 3D-acinar Structures**

M. K. Gajewska, E. McCadle, F. Martin; Conway Institute, University College Dublin, Dublin, Ireland

Primary mouse mammary epithelial cells supported on a laminin-rich ECM form 3D-acinar structures that mature to form polarised monolayers surrounding a lumen. Using this three-dimensional culture system, we have previously shown that JNK activity is necessary for normal mammary acinus development. Inhibition of JNK activity with SP600125 results in failure of cell polarisation and lumen clearance leading to formation of "disorganised" spheres filled with surviving cells. We show that during early acinus formation the Raf-MEK-ERK signalling axis is active, but the activity falls in time. In addition, focal adhesion complex components, paxillin, Src and FAK, which are recruited downstream of ECM-integrin interactions, are activated/phosphorylated. In spheres treated with the JNK inhibitor, acinus maturation failed and phosphorylation of the focal adhesion complex components was decreased. Raf-1 and MEK1/2 phosphorylation was unaffected, however ERK1/2 exhibited sustained high levels of phosphorylation. This sustained phosphorylation of ERK1/2 is crucial to the effect of JNK inhibition as normal acinus formation could be rescued by its inhibition using PD98059. We hypothesized that control of ERK1/2 phosphorylation/activation may also be regulated, to a degree, downstream of the ERK-MAPK pathway. Indeed normal acinus formation is associated with JNK-dependent Erk-specific phosphatase (MKP-2/-3) expression. By regulating the extent of MAP kinase phosphorylation, MKP-2 and MKP-3 are expressed (MKP-2 particularly in nuclei), whereas JNK inhibitor treatment abolishes the expression of both phosphatases. In addition, broad inhibition of cellular phosphatases by pervanadate induced cell illisation, sustained high level of ERK1/2 phosphorylation and formation of disorganised acini. This could be partially rescued with the MEK1/2

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inhibition by PD98059. Our data suggest that necessary control of ERK1/2 phosphorylation/activation during acinus formation may indeed be contributed to by the action of ERK-specific phosphatases.

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Mechanical Stimuli Activation of Calpain Is Required for Myoblast Differentiation and Occurs via an ERK/MAP Kinase Signaling Pathway
A. Grossi, A. H. Karlsson, M. A. Lawson; Department of Food Science, The Royal Veterinary and Agricultural University - KVL, Frederiksberg C, Denmark
Myogenesis is a complex sequence of events, including the irreversible transition from the proliferation-competent myoblast stage into fused, multinucleated myotubes. During embryonic development, myogenic differentiation is regulated by positive and negative signals from surrounding tissues. Stimulation due to stretch- or load-induced signaling is now beginning to be understood as a factor which affects various signal transduction pathways, gene sequences and protein synthesis. Evidence of the involvement of mitogen-activated protein kinase (MAPK) cascade activation in myoblast fusion, cell membrane and cytoskeleton component reorganization due to the activity of ubiquitous proteolytic enzymes known as calpains has been reported. Whether there is a link between stretch- or load induced signals, the MAPK pathway and calpain expression and activation is not known. Using a magnetic bead stimulation assay and C2C12 mouse myoblasts cell population, we have shown that mechanical signals transmitted through the C2C12 cells interaction with laminin cause an increase in cellular differentiation. This signaling results in an increase in the number of myotubes formed in the cultures, with each individual myotube containing a reduced number of nuclei. Mechanical stimulation increases not only the expression of m-calpain but also the overall activity of calpain in the cells through the MAPK signaling cascade. Our findings underline that the mechanical modulation of MAPK signaling cascade enhances the expression and activity of m-calpain, which may play a pivotal role during myoblast fusion, strengthening the idea of its implication during the initial events of muscle development.

2389
JNK-dependent Regulation of Polarization in Primary Mouse Mammary Cells
J. A. Whyte, L. Thornton, E. McAdze, F. Martin; School of Biomolecular and Biomedical Sciences, Conway Institute, University College Dublin, Dublin, Ireland
Primary mouse mammary epithelial cells organize as spherical acini when grown on laminin-rich ECM. Acini formation begins with the apico basal 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 to form a hollow lumen. Polarization in an epithelial sheet is initially established by the generation of an active ternary signaling complex consisting of the atypical PKCζ, Par3 and Par6. We have previously shown that JNK activity is necessary for functional acinus formation and we have recently demonstrated that PKCζ is phosphorylated (at Tyr140) and activated in the early stages of acinus formation in a JNK-dependent fashion. In addition, inhibition of PKCζ activity is sufficient to disrupt the assembly of polarized acini. We now investigate other downstream systems that are regulated by JNK and contribute to cell polarization in the acini. We observe an increase in expression of the basolateral polarity protein scribble during the early stages of acinus formation and this increase is significantly reduced in JNK inhibited spheres. The reduction in scribble protein expression is most likely though the repression of c-jun-mediated transcription. In JNK inhibited spheres the low levels of scribble protein remaining is mislocalized. Inhibition of JNK also ablated expression of the scribble binding protein, discs large (dlg) and significantly reduced the expression of the apical polarity complex protein pahl1. These findings highlight a role for JNK signaling in the regulation of multiple polarity proteins. We therefore propose that JNK regulates key molecular events leading to the activation of PKCζ and the expression of key polarity proteins, which are necessary for early events in cell polarization.

2390
The Activity of the JNK-c-Jun Axis during Mammary Epithelial Acinus Formation
J. Stack, E. Gilligan, F. Martin; UCD School of Biomolecular and Biomedical Sciences, Conway Institute, University College Dublin, Dublin, Ireland
2391
Low Substratum Rigidity Induces Ubiquitin Ligase Culll Mediated-c-Jun Degradation in Nucleus of Epithelial Cells
Y. Hu, Y. Wang, M. Tang; Physiology, National Cheng-Kong University, Tainan, Taiwan, 2Basic Medical Sciences, National Cheng-Kong University, Tainan, Taiwan
The proto-oncprotein c-Jun is a component of the transcription factor AP-1 (activator protein-1) involved in cellular proliferation, differentiation and death. Maintenance of c-Jun protein levels plays an important role in proliferation and survival of epithelial cells. Previous studies in our lab showed that epithelial cells cultured on collagen gel developed apoptosis due to low substratum rigidity. Low rigidity-induced cell apoptosis was mediated by degradation of c-Jun, which was observed only in epithelial, but not transformed cells. The purpose of my study was to delineate the underlying mechanism whereby low substratum rigidity induced degradation of c-Jun. The low rigidity-induced degradation of c-Jun could be reversed by 26S proteasome specific inhibitors. Here we showed that Cul1, a ring-domain ubiquitin ligase, had a specific physiological role in low rigidity-induced c-Jun degradation. Low substratum rigidity induced Cul1 neddylation and enhanced Cull1 mediated polyubiquitination. Under low substratum rigidity condition, Cull1 physically interacted with c-Jun. Immunofluorescence study showed that low rigidity induced the accumulation of Cul1 in the nucleus, which was associated with degradation of c-Jun. In addition, low rigidity also triggered translocation of 26S proteasome into the nucleus. Taken together, we demonstrate that low substratum rigidity induces Cul1 neddylation which triggers c-Jun polyubiquitination and results in c-Jun degradation through ubiquitin-proteasome proteolysis in epithelial cells.

2392
Abl Tyrosine Kinase Inhibits Cell Spreading on Fibronectin
H. Jin, J. Wang; Division of Biological Sciences, University of California, San Diego, La Jolla, CA
Cell adhesion and spreading on extra cellular matrix (ECM) is an important process for cell growth, migration and differentiation. The non-receptor tyrosine kinases Abl is activated upon cell adhesion to fibronectin, which is a component of ECM. To determine the effect of Abl kinase on fibronectin-induced cell spreading, we prepared Abl-FKBP fusion proteins, which were activated by a cell-permeable dimerizer. Dimerization of Abl-FKBP in serum-starved and suspended 3T3 cells led to tyrosine phosphorylation and activation of Abl. When plated on fibronectin, cells with activated Abl-FKBP exhibited a significant reduction in the rate of spreading, and the degree of inhibition could be correlated with the level of Abl activation. We also found inhibition of endogenous Abl kinase with imatinib could induce an increase in the rate of cell spreading, and this effect was not seen in an imatinib-resistant Abl (AblT315I) stable expressing cells, as well as Abl and Abl related gene, Arg, double knock out cells. Lastly, we found Rac activation was not impaired in spreading cells by Abl. Taken together, these results suggest that consistent with its role in cell spreading, Abl exerts a negative effect on cell spreading through a pathway that probably lies downstream of Rac activation.

2393
FAK FERM Domain-enhanced p53 Degradation Controls a p21-dependent Block in Cell Proliferation
There is an undefined role for the p53 tumor suppressor (p53) in promoting cell cycle arrest when integrin-matrix signals are disrupted. Here, we demonstrate a novel link between p53 and integrin-activated focal adhesion kinase (FAK) during development and in the control of primary mouse and human fibroblast proliferation. Genetic deletion of FAK results in mesodermal growth arrest by E8.5, p53 accumulation, and the inability of FAK−/−p53−/− embryonic cells to proliferate ex vivo. Loss of FAK was not associated with increased cell apoptosis. However, FAK−/− mesodermal cell proliferation block was reversed by genetic deletion of either p53 or the p53-responsive target, p21Cip/Waf1 (p21). Interestingly, p53 protein stability and nuclear localization levels are elevated in FAK−/−p21−/− compared to FAK−/−p21−/− primary fibroblasts. By re-expressing FAK or various FAK mutants in FAK−/−p21−/− fibroblasts, we find that the FAK N-terminal domain is required for p53 nuclear localization. The data further suggest that cytoplasmic localization of p53 is required for FAK to promote increased p53 transcriptional activity. FAK-FERM-mediated p53 degradation is blocked by MG132 and increased p53 ubiquitination in the absence of FAK-FERM is dependent on MDM2. Biochemical fractionation and fluorescent microscopy showed that FAK-FERM is strongly localized to the nucleus and G2/F-FAK is nuclear-localized upon leptotignin B addition. A series of point mutations within the FAK-FERM domain identified a functional bipartite nuclear localization sequence (NLS) for FAK-FERM, which is associated with p53 and promote p53 degradation. To determine whether FAK expression regulates p53 turnover and cell proliferation.

2394

Role of Gelsolin in Cyclosporin-induced Inhibition of Collagen Phagocytosis

M. W. C. Chan, P. D. Arora, C. A. McCulloch; CIHR Group in Matrix Dynamics, University of Toronto, Toronto, ON, Canada

Background: Gelsolin (Gsn) inhibits collagen degradation by interfering with the ligand binding step of the phagocytic pathway. In rapidly remodeling connective tissues such as human periodontium this manifests as marked tissue overgrowth and loss of function. Previous data have shown that CsA inhibits the release of Ca2+ from internal stores, which is required for the binding step of collagen phagocytosis. As gelsolin is a Ca2+-dependent actin severing protein that is required for collagen phagocytosis, we determined if gelsolin is a CsA target. Objectives: To examine the role of gelsolin in collagen phagocytosis following CsA treatment. Methods: Gingival tissues from wild-type and gelsolin-null mice treated with CsA were analyzed by histological sections. Collagen-coated beads were used to study collagen phagocytosis and calcium signaling in fibroblasts following CsA treatment. Results: Compared to vehicle controls, CsA treatment of wild-type mice markedly increased collagen accumulation in periodontal tissues; equivalent increases of accumulation were seen in vehicle-treated gelsolin-null mice. Similarly, collagen degradation by phagocytosis in vitro was blocked by CsA or absence of gelsolin. This inhibition was attributable to the collagen binding step but not the internalization step. CsA blocked actin assembly, gelsolin recruitment to sites of collagen binding and collagen-induced Ca2+ fluxes subjacent to bound collagen beads. CsA inhibited gelsolin-dependent severing in wild-type and gelsolin-null cells showing low to into gelsolin-null cells, collagen binding was similar to that of cells treated with CsA. In cells loaded with a peptide that interferes with the PI(4,5)P2 regulatory site of gelsolin and that inhibits collagen internalization, CsA did not further reduce collagen internalization. Conclusions: One mechanism of CsA-induced accumulation of collagen in the extracellular matrix involves disruption of actin severing by gelsolin, thereby inhibiting the binding step of collagen phagocytosis.

2395

Effect of Basement Membrane on Hox A5 Transcriptional Activity


Hox genes are transcriptional regulators which play key roles during embryonic development and differentiation, and in adults contribute to pathological tissue remodeling. We previously showed that HoxA5 blocks angiogenesis by down regulating pro-angiogenic genes including VEGFR2 and Ephrin A1 and up regulating anti-angiogenic genes including Thrombospondin-2. Moreover, in endothelial cells cultured in the presence of a three dimensional reconstituted basement membranes (3DhBM), HoxA5 promotes formation of stable capillary networks as siRNA against endogenous HoxA5 blocks tube formation and overexpression of HoxA5 leads to increased branching and formation of extensive adherens junctions. The HoxA5 mediated increases in capillary networks and adherens junctions is associated with increased expression and activity of Akt1. Interestingly, the increase in Akt1 mRNA in HoxA5 expressing endothelial cells is observed only when cells are plated in 3DhBM but not in conventional 2D tissue culture plastic, suggesting that signaling through the BM facilitates HoxA5 mediated transcriptional activation. Indeed, pre-incubation of HoxA5 expressing endothelial cells with function blocking antibodies against β4 integrin impaired the HoxA5 dependent increases in Akt1 mRNA expression. To determine whether culturing on 3DhBM impacts chromatin organization and/or accessibility of Hox A5 to target genes, we pre-treated cells cultured in the absence of 3DhBM with an inhibitor of histone deacetylases. Incubation with Trichostatin-A restored the ability of HoxA5 to activate Akt1 mRNA in the absence of 3DhBM. Together our results suggest that signaling through the BM cooperates with HoxA5 to promote accessibility to downstream target genes and the formation of stable capillary networks.

2396

Interaction of Alpha Actinin 4 with NFkB in Human Keratocyes Depends on Extracellular Matrix Proteins

D. Tentler,1 L. Turoverova,1 V. Babakov,1 O. Petukhova,1 A. Bolshakova,1 K. Magnusson,2 G. Pinacv; 1Dept. of Cell Cultures, Institute of Cytology, Russian Academy of Sciences, St. Petersburg, Russian Federation, 2Division of Medical Microbiology, Linkoping University, Linkoping, Sweden

The NFkB protein complex is a second messenger, which activates transcription of a number of genes in various tissues. The most abundant form of NFkB protein complex is a heterodimer of the p55 DNA-binding subunit and the weak DNA-binding subunit p65. The p50/p65 complex also interacts with a number of other proteins regulating its activity. The inactive form of the NFkB complex translocates from the cytoplasm into the nucleus. The exact mechanism for this remains unclear. We have earlier demonstrated that p65 subunit can interact with the actin-binding protein alpha actinin 4 (actn4), which proposes an important role of the cytoskeleton in the NFkB translocation. In order to elucidate a possible role of actn4 in the NFkB function, we investigated its distribution in nuclear keratocytes complexes. The complexes were purified by the immuno-precipitation with p65 antibodies from normal and transformed (epidermoid carcinoma cell line A431) human keratocyes cultured on different substrates such as plastic, collagen 1, collagen IV, laminin 2/4 and fibronectin. Western blot analysis of the actn4 in the purified complexes showed that not all of the complexes containing p65 had actn4. The distribution of the nuclear complexes with both p65 and actn4 depended on the substrate, and changed after the EGF treatment. Moreover, the amount of complexes with p65 and actn4 was different in the normal and transformed cells. The data provide evidence that the extracellular matrix may regulate NFkB activity through the actin cytoskeleton. The differences in distribution of p65/actn4 complexes between normal and transformed keratocytes may also signify that there is a mechanism of NFkB pathway regulation specific for cancer cells.

2397

Does Discoidin Domain Receptor 2 Recognize the Fibrillar State of Collagen?

D. Garbellini, C. Mihal, G. Agarwal; Dorothy Davis Heart and Lung Research Institute, The Ohio State University Medical Center, Columbus, OH

Discoidin domain receptors (DDRs) are widely-expressed cell surface receptors belonging to the tyrosine kinase family. Earlier studies have shown that collagen are their ligand and their activation leads to up-regulation of matrix-metalloproteases (MMPs). It has also been noted in earlier observations that the activation of the DDR kinases by collagen follows a delayed time course relative to conventional growth factor receptors. This delay could be due to that time it takes for collagen to achieve a particular fibrillar state. What remains to be explored is if DDR-activation leads to up-regulation of matrix-metalloproteases (MMPs). It has also been noted in earlier observations that the activation of the DDR kinases by collagen follows a delayed time course relative to conventional growth factor receptors. This delay could be due to that time it takes for collagen to achieve a particular fibrillar state. What remains to be explored is if DDR-activation leads to up-regulation of matrix-metalloproteases (MMPs). In this study we employed cell-based assays and stimulated DDR2-transfected cells with collagen in fibrillar or non-fibrillar form. Our results show that activation of DDRs was enhanced when collagen was in a fibrillar state as compared to a monomeric state. Further, our in-vitro binding assays confirm that DDR2 binding is enhanced when collagen is in a fibrillar state. These observations together with our earlier results [1-2] may provide new insights on how DDR2 influences the deposition of collagen and the process of fibrosis. Ref: [1] Agarwal G. et al "Binding of DDR2 to Collagen type 1", Biochemistry, 2002 Sep 17;41(37):11091-8 [2] Mihal C. et al "DDR2 inhibits fibrillogenesis of collagen type 1" JMB
proteins. After the screening of 4 × 10^7 colonies, 133 positive clones were selected. While NSP is an intracellular protein, 52% of the clones encoded genes for extracellular matrix proteins, including Collagen type 1 alpha2 (Col1a2), 30%; Col1a1, 5%; Fibulin 2 (Fbn2), 4%; Laminin receptor 1 (Rpsa), 4%. Coimmunoprecipitation experiments using in vitro translated proteins confirmed binding of NSP-N to the fibrillar collagens C-terminal domain (COL2A1). Although the physiological significance of the interactions is currently under investigation, these results revealed that NSP interacts with fragments of extracellular matrix proteins at least under the tested condition.

2399 An α-Dystroglycan Binding Peptide Derived from the Laminin α2 Chain
M. Nomizu, T. Yoshimura, N. Suzuki, S. Urasuhita, Y. Kikawa; Tokyo Univ. of Pharm. and Life Sci., Hachioji, Japan
Laminins, consisting of α, β, and γ chains, are major components of basement membranes and have diverse biological activities. The laminin α2 chain, which is a component of laminin-2/4/12, is mainly expressed in skeletal muscles or peripheral nerves and interacts with dystroglycan and heparan sulfate proteoglycans. Here, we focused on the laminin α2 chain LG4-5 module and to identify their heparin and dystroglycan binding activities to better understand the biological function of the molecule. We prepared a recombinant laminin α2 chain LG4-5 module (res-α2LG4-5) using 293T cells. The rec-α2LG4-5 showed heparin and α-dystroglycan binding activities. To identify the binding sites in the laminin α2 chain LG4-5 module, we synthesized 42 overlapping peptides covering the entire molecule. First, we examined the effect of the peptides on the heparin binding of rec-α2LG4-5. The A2G78 peptide (GLIFYMARINHA, mouse laminin α2 chain 2796-2807) inhibited the heparin binding of rec-α2LG4-5. A2G78 showed heparin binding activity in a solid phase binding assay. The minimal active sequence of A2G78 for heparin binding was found to be LLYFMAR1. Next, we tested the α-dystroglycan binding activity of the A2G78 sequence. The A2G78 peptide showed inhibitory effect on α-dystroglycan binding to the rec-α2LG4-5 protein. These results suggest that the A2G78 site plays an important role in the interaction of rec-α2LG4-5 with heparin/heparan sulfate and α-dystroglycan and the peptide is useful to investigate the α-dystroglycan mediated biological functions.

2400 Receptor for Advanced Glycation End Products (RAGE) Signaling Stimulated by S100A4 Increases Chondrocyte Matrix Metalloproteinase-13 Production
R. Yammani; Molecular Medicine, Internal Medicine, Wake Forest School of Medicine, Winston-Salem, NC
S100A4 belongs to a family of 100 calcium binding proteins which function both intracellularly and extracellularly. In the current study, treatment of chondrocytes with exogenous S100A4 was found to increase production of MMP-13. Soluble RAGE and AGE-BSA blocked the production of MMP-13 suggesting a role for RAGE. A pull down assay found that RAGE co-precipitates with S100A4, confirming S100A4-RAGE association. Increased production of MMP-13 was found associated with increased phosphorylation of MAP kinases and NF-κB. Pretreatment with MetTAP, an antioxidant, followed by S100A4 stimulation blocked the production of MMP-13, suggesting the role for ROS in S100A4 stimulation of MMP-13. BAPTA-AM, an intracellular calcium scavenger also inhibited MMP-13 production upon stimulation with S100A4. However, nifedipine, an L-type calcium channel blocker had little inhibition suggesting a role for intracellular calcium levels. Pyk-2, a non-receptor calcium dependent tyrosine kinase was also found phosphorylated upon S100A4 stimulation. Inhibition of phosphorylation of Pyk-2 by either BAPTA-AM or triflupin A9 blocked the downstream activation of MAP kinases especially JNK and the transcription factor NF-κB with subsequent inhibition of MMP-13 production. This suggests a major role for Pyk-2 along with ROS in S100A4-RAGE mediated production of MMP-13. Our study also found that chondrocytes express S100A4 and its expression was up regulated in osteoarthritic (OA). Taken together, we conclude that in chondrocytes, S100A4 serves as a RAGE ligand and activates a signaling cascade leading to increased production of MMP-13. Since both S100A4 and RAGE are up regulated in OA cartilage, this signaling pathway could contribute to cartilage degradation in OA.

2401 Undersulfation of Chondroitin Sulfate Proteoglycans Results in Reduced Chondrocyte Proliferation via the Indian Hedgehog Signaling Pathway
M. Cortes,1 A. Baria,2 J. Henry,2 N. B. Schwartz;1 1Biochemistry and Molecular Biology, University of Chicago, Chicago, IL, 2Pediatrics, University of Chicago, Chicago, IL
The functional role of heparan sulfate proteoglycans (HSPGs) in regulating signaling pathways such hedgehog (Hh), Wingless-type (Wnt) and fibroblast growth factor (FGF) has been previously described. In contrast, the role of chondroitin sulfate proteoglycans (CSPGs) in these signaling pathways remains elusive. PAPS synthetase 2 (PAPSS2) is one of the two isoforms responsible for the synthesis of PAPS, the universal sulfur donor. The brachymorphic (bm) mouse is a PAPSS2 kinase mutant characterized by preferential undersulfation of CS chains and 50% reduction in limb length. In situ hybridization analysis of the bm mouse growth plate with various markers, performed to determine whether signaling pathways regulating chondrocyte proliferation and differentiation are affected by undersulfation, revealed reduced mRNA levels of Indian hedgehog (Ihh), and Patched (Ptc). Immunostaining with Ihh and Parathyroid hormone related peptide (PTHrP) antibodies showed decreased extracellular diffusion of both signaling molecules in the bm mouse growth plate compared to wildtype. Consistent with reduced Ihh signaling, BrDU labeling experiments of post-natal day 6 growth plates showed a significant decrease in chondrocyte proliferation, specifically in the proliferative zone. In order to determine if CSPGs directly interact with Ihh, binding experiments with N-Ihh AP-fusion protein and various forms of chondroitin sulfate (CS) and heparan sulfate (HS) chains revealed that HS-C, CS-4, CS-6, and CS-0 interact with Ihh with decreasing binding affinities, via hedgehog’s proteoglycan binding domain. Mutations of the Indian hedgehog proteoglycan binding domain result in complete loss of binding to both HS and CS chains. In sum, undersulfation of CSPGs in the brachymorphic mouse results in reduced chondrocyte proliferation due to diminished Ihh signaling, and sulfation of CSPGs is required for Ihh interaction via its conserved proteoglycan binding domain.

2402 C-Src Signaling Promotes Phagocytosis of αvβ5 Integrin-bound Photoreceptor Particles
S. C. Finneman,1 Y. Chang,1 M. Anand1; 1Dyson Vision Research Institute, Weill Medical College-Cornell University, New York, NY, 2Dyson Vision Research Institute, Graduate Program in Physiology, Weill Medical College-Cornell University, New York, NY
Retinal pigment epithelial (RPE) cells are among the most actively phagocytic cell types. Every morning, each RPE cell in the retina engulfed and digests numerous outer segment fragments (OS) shed in a circadian rhythm by adjacent photoreceptor neurons. Daily, synchronized OS clearance by RPE cells is critical for vision. Mechanistically, phagocytosis of OS by RPE cells is similar to engulfment of apoptotic cells by other phagocytes. OS recognition by apical (OS) shed in a circadian rhythm by adjacent photoreceptor neurons. Daily, synchronized OS clearance by RPE cells is critical for vision. Mechanistically, phagocytosis of OS by RPE cells is similar to engulfment of apoptotic cells by other phagocytes. OS recognition by apical OS shedding by photoreceptor neurons is essential to trigger particle engulfment. Here, we test whether and which Src family kinases (SFK) link OS-phagocytosis to MerTK activation and retinal phagocytosis. Furthermore, they show that FAK activation requires c-Src activation to promote phagocytosis. In contrast, active c-Src is sufficient to stimulate phagocytosis independently of FAK.

2403 14-3-3ζ Up-regulates MMP-2 (type IV Collagenase) Expression
E. Lee, S. Park; Pathology, Inha University, Incheon, Republic of Korea
14-3-3ζ is a highly conserved acidic protein family, composed of seven isoforms in mammals and involved in cellular processes like signal transduction, cell cycle arrest, and apoptosis. In this study, we demonstrate that the expression of 14-3-3ζ gene up-regulates the expression of MMP-2 gene independent of TIMP-1 levels. MMP-2, 72kDa enzyme, degrades multiple ECM components, including gelatins, type IV and V collagens. Overexpression of 14-3-3ζ protein induced MMP-2 mRNA and its protein expression in both NIH3T3 fibroblast and human keratinocyte cells. In addition, expression of 14-3-3ζepilson increased the secretion of MMP-2 and MMP-9. These up-regulations of MMP-2 by 14-3-3ζepilson were partially blocked by p38 MAPK inhibitor SB203580. These results strongly suggest that 14-3-3ζ induces MMP-2 expression via p38 MAPK signaling pathway.
Adhesion-induced Actin 1 and Actin 4 Redistribution in A431 Cells Spread on Fibronectin and Laminin 2/4
A. Bohlakova,1 Q. Petukhova,1 L. Turoverova,1 V. Babakov,2 D. Tenler,1 K. I. Magnusson,2 G. Pinacev;1 1Cell Cultures, Institute of Cytology, St. Petersburg, Russian Federation, 2Medical Microbiology, Ludwig Institute, Cape Town, South Africa
2404
Alpha-actinin 1 and 4 belong to family, originally described as actin-binding proteins in non-muscle cells. They share about 80 % of homology but play different roles in cell motility and adhesion. Actinin 4 is more abundant in highly motile cells especially in the dorsal ruffle. In contrast to actin 1, actin 4 is prone to the nuclear translocation and interaction with transcription factors. To delineate functional differences between the actinins, we assessed actinin redistribution after EGF stimulation in A431 cells, conventionally cultivated on plastic or spread on either fibronectin or laminin 2/4, using confocal microscopy and Western-blot. Both actinin 1 and 4 colocalized with cytoplasmic F-actin in spread cells and reorganized with F-actin after EGF stimulation. Moreover, actinin 1 accumulated near the nuclear membrane, and actinin 4 in the nucleus. A truncated form of actinin 4 resided in the cytoplasm of conventionally grown cells and truncated actinin 1 appeared after cell spreading, which was diminished with cytochalasin D. In nuclear extracts the antibody to actinin 4 precipitated full-size actinin 4, a truncated actinin 4 different from the cytoplasmic one, and an unknown, high molecular weight protein. These were found both in the nuclear protein fraction with transcription factors, and in the chromatin fraction. The redistribution between these nuclear fractions differed between normally cultivated cells and cells spread on fibronectin or laminin 2/4. This suggests that differences in the sub-cellular distribution of actinins can be explained by the appearance of truncated actinins regulated by the cell adhesion. Thus, the function(s) of actinin 4 in the nucleus could be regulated by specific cell adhesions, also affecting its partitioning between the chromatin- and transcription factor- containing protein pools. Acknowledgements: The work was supported by grants from the Leading Scientific School, Russia (97852.2006.4) and the Visby Programme of the Swedish Institute (#1361/2006).

Beta-1 Integrin Regulates Epithelial Polarity through Rho GTPases
W. Yu, M. Zegers, K. Mostov;1 1Anatomy, University of California, San Francisco, San Francisco, CA, 2Surgery, University of Cincinnati, Cincinnati, OH
2405
The kidney is primarily comprised of epithelial cells. Epithelial cells are highly polarized and their plasma membrane is divided into discrete domains. The apical surface faces the lumen and the basolateral surface interacts with other cells and underlying extracellular matrix. Establishment and maintenance of polarity is critical to the functioning of epithelial cells. Most work on epithelial polarization has used cells grown on artificial filter support. We have used a three dimensional culture system in which is closer in vivo to study how β1 integrin and Rho GTPases coordinate to control the epithelial polarization. Individual Madin-Darby canine kidney (MDCK) cells grown in collagen gel from cyst spherical cysts comprising a monolayer of cells surrounding a hollow lumen. The cells are polarized. The β1 integrin function-blocking antibody A7B2 gives rise to cysts with inverted polarity. We showed that normal polarity is restored by either expression of constitutively active Rac or expression of exogenous laminin. We also found that inhibition of ROCK, a major effector of RhoA, or inhibition of myosin not only revert β1 integrin blocking induced phenotype, but also polarize the expression induced by expression of dominant-negative Rac. These findings indicate that β1 integrin orients polarity through Rho GTPases and laminin assembly.

Epithelial-Mesenchymal Transition via Actin Organization in Integrin α5-Expressing Cells
M. Oh, J. Lee;1,2 1Department of Tumor Biology, Cancer Research Institute, College of Medicine, Seoul National University, Seoul, Republic of Korea, 2Department of Molecular and Cell Oncology, Cancer Research Institute, College of Medicine, Seoul National University, Seoul, Republic of Korea
2406
Cell adhesion receptor integrins are well-known to transduce signaling activities leading to actin reorganization on integrin-extracellular matrix (ECM) protein engagement. This integrin-mediated actin reorganization is prerequisites of diverse cellular functions including epithelial-mesenchymal transition (EMT) and cell migration. In this study, the significance of integrin α5β1 signal transduction in actin reorganization leading to regulation of EMT and cell migration was explored by using normal rat intestinal epithelial cells. Whereas ectopic expression of integrin α5β1 wildtype responded to an actin-disturbing reagent by showing an EMT and severe retraction in morphology depending on c-Src and PI3K activities, cytoplasmic tailless mutant α5β1 caused no such changes and retarded migration. Expression of tailless α5β1 resulted in more focal adhesions and actin fibers along the cell-cell contacts, irrelevant to c-Src and PI3K inhibition. PKCδ inactivation via rotlinn or dominant negative adeno-PKCδ infection of wildtype α5β1-expressing cells abolished the actin disruptor-mediated EMT. Adeno-PKCδ wildtype infection to tailless α5β1 cells rendered the sensitivity to actin disruptor for EMT. Altogether, these observations indicate that integrin α5β1, but not tailless mutant α5β1, regulates dynamically actin organization especially along the cell-cell contacts sites leading to efficient regulation of migration.

Tension Induces a Conformational Change in α5β1 Integrin-Fibronectin Bonds That Controls Downstream Signals
D. Boettiger; Microbiology, University of Pennsylvania, Philadelphia, PA, USA
2407
Integrin β3 Integrin Ligand Binding Is Regulated by Serine Phosphorylation of the β3 Cytoskeletal Tail
A. M. Gonzalez, J. C. R. Jones; Cell and Molecular Biology, The Feinberg School of Medicine at Northwestern University, Chicago, IL
2408
Previously we demonstrated that integrin α5β1 antagonists negatively regulated α5β3 integrin-ligand binding in endothelial cells. To determine the mechanism regulating α5β3 integrin activation, we performed vitronectin binding assays on endothelial cells in suspension. Vitronectin binding was significantly inhibited upon β1 integrin clustering using antibody 656, indicating that 656-modulated α5β3 integrin affinity. Since talin is known to regulate integrin affinity, we assessed whether over-expression of talin would abrogate the impact of β1 integrin clustering on α5β3 integrin-ligand binding. It did not. Rather, β1 integrin clustering resulted in a two-fold increase in protein kinase A (PKA) activity and serine phosphorylation of β3 integrin. Inhibition of PKA activity rescued cell adhesion in the presence of β1 integrin antibodies and blocked β3 integrin serine but not tyrosine phosphorylation. These results indicated that affinity modulation of α5β3 integrin involves β3 integrin serine phosphorylation. β3 integrin contains a serine residue at position 752. To confirm the importance of this residue, we mutated it to alanine (β3S752A) or aspartic acid (β3S752D), expressed wild-type and mutated proteins in CHO cells and assessed cell adhesion to α5β3 integrin ligand. Cells expressing wild-type or β3S752A integrin attached robustly to ligand. Cells expressing β3S752D integrin did not. Since the β3 cytoskeletal tail lacks a PKA consensus site, it was unlikely that PKA is acting on β3 integrin. Rather, we tested the hypothesis that PKA inhibits the calcineurin/inhibitor-1 pathway, blocking the activation of protein phosphatase 1 (PP1) that, in turn, regulates β3 phosphorylation. In support of this, blocking PP1 activity in the presence of β1 integrin and PKA antagonists inhibited α5β3 integrin adhesion and resulted in β3 integrin serine phosphorylation. These results indicate a novel mechanism via which clustered β1 integrin negatively modulates α5β3 integrin-ligand binding. In this mechanism, PKA and PP1 play antagonistic roles in regulating 4 residue of α5β3 integrin.

Integrin α6β4 Chelation
M. C. Demetriou, K. A. Kwok;1 R. B. Nagle,2 G. T. Bowden,3 A. E. Cress;1 1Biological Sciences, University of Cyprus, Nicosia, Cyprus, 2Stanford University, Stanford, CA, 3University of Arizona, Tucson, AZ
2409
We have previously identified a structural variant of the α6 integrin (Laminin receptor) called α6p. The α6p variant is a 70 kDa form of the full-length α6 integrin (140 kDa) that is paired with either the β1 or β4 subunit on the cell surface. α6p is produced by removal of the extracellular β-barrel domain while the receptor is on the cell surface. Urokinase-type plasminogen activating
(uPA) cleaves the α6 integrin directly without the involvement of plasmin. Using site directed mutagenesis we have determined the site of cleavage on the α6 integrin. In order to determine whether α6p occurs in tissue, we have found that α6p is present in mouse papillomas and squamous cell carcinomas induced by DMBA, TPA and MNNG treatments. Subcutaneous injection into athymic nude mice of a malignant mouse keratinocyte derived cell line (6M90) that is α6p negative, results in the development of tumors that contain α6p integrin. Taken together, these data suggest that the cell surface clipping of the α6 integrin occurs in tissue and may be an important step during skin tissue remodeling and during carcinogenesis. We are currently investigating the distribution of α6p in human and mouse tissues, using immunohistochemistry approaches.

2410 Oscillation of GPIIb/IIIa Surface Expression on Platelets
Y. Ma, K. Wong; Research and Development, Canadian Blood Services, Edmonton, AB, Canada

Background. It was observed that GPIIb/IIIa expression on platelets fluctuated to varying extents between samples or platelet rich plasma preparations. An objective of the present study was to show that such fluctuations were due to more than assay noise. Method. The expression of GPIIb/IIIa on platelets was studied by flow cytometric analysis of platelet rich plasma (PRP) sampled periodically, fixed and tagged with fluorochrome-conjugated, anti-GPIIb mAb. Results show that GPIIb/IIIa fluctuations of live platelets were significantly greater than that of prefixed cells. Periodicity in GPIIb/IIIa time series was not discerned but fluctuations in GPIIb/IIIa and forward scatter were positively correlated. Surface GPIIb/IIIa amplitudes subsided with storage within 140 min but increased with vigorous agitation of PRP. The former correlated with transient activation of platelets in PRP induced by processing. PAC-1 mAb binding shows that GPIIb/IIIa oscillated concomitantly at a low level between active and inoperative conformations. The average amplitude of such oscillations declined similarly after 140 min. Compared with room temperature results, PAC-1 binding oscillated with greater amplitudes at 4°C, an activating milieu for platelets. Surface GPIIb/IIIa increased slowly in washed platelets after > 6 h storage at room temperature. Cytochalasin B inhibited this rise in GPIIb/IIIa while exposing an underlying oscillation. Conclusions. GPIIb/IIIa surface expression and activation oscillated at a low level in nominally resting platelets. Present results suggest that GPIIb/IIIa oscillated between an inner and outer compartment independently of actin filament polymerization. It is proposed that subacute activation of platelets reduced cytoskeletal restraints on integrin movements, and GPIIb/IIIa interconversion between active and inoperative conformations.

2411 The Role of Calcium on the Reassociation of the Glycoprotein IIa-IIIb Complex in EDTA-treated Platelets
Y. Ma, K. Wong; Research and Development, Canadian Blood Services, Edmonton, AB, Canada

Incubation of platelets with EDTA at 37°C at alkaline pH causes GPIIb-IIIa (integrin, dIb/III) to dissociate into monomeric subunits. The objective of this study was to demonstrate that this dissociation and consequent loss of function were reversible. GPIIb-IIIa was measured by flow cytometric analysis of platelets labeled with fluorophore-conjugated mAbs specific for the glycoprotein complex or a monomer. In agreement with previous studies, EDTA chelation of extracellular calcium, [Ca^2+]_, caused the heterodimer to dissociate in a time and temperature dependent manner. Resuspension and incubation of platelets in nM [Ca^2+]_ allowed reassociation of GPIIb-IIIa in a manner dependent on [Ca^2+]_. While complete GPIIb-IIIa reassociation was induced by thrombin independently of [Ca^2+]_, Suppression of rises in cytosolic calcium concentration by BAPTA blocked the effect of all three agonists. Thus, internal Ca^2+ release was necessary and sufficient for the thrombin effect while Ca^2+ influx was necessary for the collagen and ADP effects. The aggregatory response of EDTA-treated platelets was inhibited differently depending on the agonist and was not correlated with the extent of GPIIb-IIIa reassociation. In conclusion, EDTA-induced changes in platelets were variably reversed depending on the agonist used and the cellular parameter measured.

2412 MyH9 (Non-muscle Myosin Heavy Chain-IIA) Provides a Key Mechanical Link between Integrin LFA-1 and the Cytoskeleton during T Cell Migration
N. A. Morin,* Y. Hyan,* P. W. Oakes,* J. X. Tang, E. Y. Chin,* J. S. Rechiner,* M. Kim; Surgery, Brown Medical School, Rhode Island Hospital, Providence, RI, *Physics, Brown University, Providence, RI

The process of cell migration involves at least three interdependent events including attachment of active integrin at the leading edge, cell contraction, and integrin detachment at the trailing edge. To identify key cytoskeletal molecules that allow LFA-1 de-adhesion from ICAM-1 during efficient T cell migration, LFA-1 was immunoprecipitated selectively from adherent human primary T cells on ICAM-1 substrate. Mass spectrometry analysis revealed that MyH9 is recruited to LFA-1 together with αL, β2 integrin, and the cytoskeleton during T cell migration. MyH9 transfected T cells showed the distinct distribution of MyH9 at the uropod of migrating T cells. Blebbistatin, a highly specific inhibitor of class II myosin ATPase activity, blocked MyH9 association with LFA-1 and resulted in extreme uropod elongation, defective tail detachment, and reduction in overall migration on ICAM-1 substrate. Dynamic LFA-1 activation by SDF-1, distribution of active LFA-1 at the leading edge, and periodic contraction of lamellipodia of migrating T cell were not affected by blebbistatin, suggesting MyH9 recruitment to LFA-1 is necessary for the collagen and ADP effects. The aggregatory response of EDTA-treated platelets was inhibited differently depending on the agonist and was not correlated with the extent of GPIIb-IIIa reassociation. In conclusion, EDTA-induced changes in platelets were variably reversed depending on the agonist used and the cellular parameter measured.

2413 EHD1 Regulates Beta-1 Integrin Endosomal Transport: Effects on Focal Adhesions, Cell Spreading, and Migration
M. Jovic,* N. Naslavsky,* D. Rapaport,* M. Horowitz,* S. Caplan; *University of Nebraska Medical Center, Dept. of Biochemistry and Molecular Biology, University of Nebraska Medical Center, Omaha, NE, *Dept. of Cell Research and Immunology, Tel Aviv University, Ramat Aviv, Israel

Beta-1 integrins bind to the extracellular matrix and stimulate signaling pathways that lead to a variety of critical cellular functions, including proliferation, apoptosis, cell spreading and migration. Consequently, control of beta-1 integrin function depends upon its subcellular localization, and current research aims at unravelling the complex regulatory mechanisms involved in beta-1 trafficking. Recent studies have identified the C-terminal EH domain-containing protein, EHD1, as a regulator of endocytic recycling for receptors internalized either through clathrin-coated pits or in a clathrin-independent manner. Furthermore, it has been shown that EHD proteins functionally interact with ARF6 and coordinate recycling events with Rab6 and Rab11, both of which are known regulators of integrin trafficking. Our current objective is to further elucidate the role EHD1 plays in regulating beta-1 integrin transport. Recently we have shown that the RNAi knockdown of EHD1 results in impaired recycling of beta-1 integrins and their accumulation in a transferrin-containing endocytic recycling compartment. The role of EHD1 in beta-1 integrin recycling was further assessed by using embryonic fibroblasts derived from EHD1 knockout mice (MEF-/-). These cells exhibited decreased levels of beta-1 integrins on the plasma membrane and longer, more prominent focal adhesions, resulting from slower kinetics of focal adhesion disassembly. The delay in beta-1 integrin recycling and the decreased rate of focal adhesion turnover impaired the ability of MEF-/- cells to migrate and spread on fibronectin. These defects could be similarly induced by EHD1-RNAi treatment of normal MEF +/- cells. They could also be rescued by transfection of wild-type EHD1 into MEF-/- cells. Our data supports a role for EHD1 in the recycling of beta-1 integrins, and demonstrates that loss of EHD1 impairs integrin-mediated downstream functions.

2414 Stimulation of Clearance Phagocytosis by the Tetrapsin CD81 Requires αvβ5 Integrin
Y. Chang,1 S. C. Finnemann 2,3; 1Dyson Vision Research Institute, Graduate Program in Physiology, Biophysics and Systems Biology, Weill Medical College of Cornell University, New York, NY, 2Dyson Vision Research Institute, Department of Ophthalmology, Weill Medical College of Cornell University, New York, NY, 3Graduate Programs in Cell and Developmental Biology, and Physiology, Biophysics and Systems Biology, Weill Medical College of Cornell University, New York, NY

Retinal pigment epithelial (RPE) cells are among the most active phagocytes in the body. Every morning, circadian shedding of photoreceptor outer segment fragments (POS) activates a prompt phagocytic response by RPE cells that is critical for vision. RPE cells employ αvβ5 integrin to recognize POS and to stimulate Mer tyrosine kinase (MerTK) activation that is essential to trigger POS engulfment. RPE cells lacking MerTK bind POS via αvβ5 but fail to internalize bound POS. In contrast, RPE cells lacking αvβ5 show greatly decreased POS binding. Like αvβ5, the tetrapsin CD81 is abundantly expressed at the apical, phagocytic surface of the RPE. Here, we test whether CD81 contributes to POS uptake. CD81 overexpression increased function of CD81 protein down-regulation by RNA silencing decreased binding but not internalization of isolated POS by RPE cells. Conversely, CD81 overexpression by transient transfection increased POS binding. These effects were specific for CD81 as similar experiments expressing or inhibiting function of CD9, another tetrapsin at the phagocytic surface of RPE cells, had no effect on POS uptake. Wild-type RPE cells and RPE cells lacking the engulfment receptor MerTK equally reduced and increased POS binding in response to CD81

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inhibition and CD81 overexpression, respectively. In striking contrast, neither CD81 inhibition nor CD81 overexpression had any effect on POS binding by RPE cells lacking αvβ5 integrin. Furthermore, CD81 and αvβ5 formed a complex at the apical surface of wild-type RPE cells and shared similar detergent resistance. Taken together, our results identify a novel role for CD81 in POS binding by the RPE. They demonstrate that CD81 does not function as phagocytic receptor by itself but increases particle binding through functional and possibly physical interaction with αvβ5 integrin. To our knowledge, this is the first report of transsialin function in integrin-dependent phagocytosis by any cell type.

2415

Cell Surface Proteoglycans in αβ1 Integrin-mediated Cell Adhesion to Collagen

K. Vuoriluoto, J. Ivaska; VTT Medical Biotechnology, University of Turku, Turku, Finland

We have investigated whether cell-surface proteoglycans play a role in cell-collagen interactions. Collagen receptor null Chinese hamster ovary (CHO) cells transfected to express αβ1 integrin became able to bind to and spread on type I collagen. Interestingly, proteoglycans are also required for αβ1 mediated adhesion to collage, since mutated CHO cell lines lacking HS and chondroitin sulfate (CS) glycosaminoglycan chains show impaired αβ1 mediated adhesion to collage. Importantly, this requirement applies to other cell types as well since enzymatic disruption of HS and CS from MDA-MB-231 human mammary carcinoma cells results in defects in αβ1-mediated adhesion to collagen. Impaired adhesion to type I collagen in cells lacking glycosaminoglycan chains is reflected in their inability to assemble integrin containing focal adhesion and in dramatic changes in the actin cytoskeleton. The requirement for proteoglycans applies only to adhesion to monomeric collagen since HS and CS disruption influence adhesion to fibronectin and fibrillar collagen only moderately. Furthermore, adhesion to the specific αβ1 recognition sequence, FGOGER, is not affected by the absence of HS and CS glycosaminoglycan chains, suggesting that proteoglycan co-receptors are required only for adhesion to full-length collagen. Integrin αβ1 is a known regulator of matrix metalloproteinase-1 (MMP-1) gene expression. Using a luciferase reporter construct, we show that αβ1 mediated activation of MMP-1 is also dependent on proteoglycans since MMP-1 activation in response to collagen is impaired in CHOαβ1 cells lacking HS. Finally, wound healing experiments have shown that CHOαβ1 cells lacking HS have a decreased migratory ability when plated on type I collagen. In summary, our results suggest that proteoglycans function as co-receptors with integrins in collagen adhesion, migration and cell signalling.

2416

Dominant-Negative β1 Integrin Mice Have Region-specific Myelin Defects Accompanied by Alterations in MAPK Activity and Oligodendrocyte Behavior

K. K. Lee,1,2 Y. De Repentigny,1 R. Saulnier,1 P. Rippstein,3 W. B. Macklin,4 R. Kothary1,2,5; 1Molecular Medicine, Ottawa Health Research Institute, Ottawa, ON, Canada, 2Cellular and Molecular Medicine, University of Ottawa, Ottawa, ON, Canada, 3Department of Neurosciences, The Lerner Research Institute, Cleveland, OH, 4Department of Medicine, University of Ottawa, Ottawa, ON, Canada

Myelination is a process by which oligodendrocyte precursor cells proliferate, migrate and differentiate into mature oligodendrocytes within the CNS, and eventually form a myelin sheath. Recent studies have demonstrated the importance of β1 integrin in oligodendrocyte maturation in vitro. Here, we demonstrate the importance of β1 integrin in myelination in vivo. We have generated a transgenic mouse line that expresses a dominant-negative β1 integrin ΔC transgene under the control of the protocoll protein (PLP) promoter that drives expression of the transgene specifically to CNS tissues. The dominant-negative β1 integrin ΔC transgenic mice have hypomyelinated and increased numbers of unmyelinated axons in the spinal cord and the optic nerve, however the corpus callosum remains unaffected. The dominant-negative β1 integrin ΔC transgenic mice, under a demyelination and remyelination model, demonstrated significantly reduced number of remyelinated axons within the corpus callosum when compared with wild type mice. The defects in myelination and remyelination in the dominant-negative β1 integrin ΔC mice were attributed to the disruption of the MAPK signaling pathway. Histological and immunohistochemical analysis on sections of tissues from the central nervous system is allowing us to determine whether the numbers of proliferating oligodendrocyte progenitors and/or mature oligodendrocytes are affected in the dominant-negative β1 integrin ΔC mice. Our work highlights the importance of β1 integrin-mediated signalling in CNS myelination and remyelination in vivo.

2417

NGF as a Ligand for α9β1 Integrin Initiates Progression of Glioblastoma

I. Staniszewska,1 M. Higgins,1 P. Lazarowicz,2 C. Marcinkiewicz2; 1Department of Neuroscience, Temple University, Philadelphia, PA, 2Department of Pharmacology, Hebrew University, Jerusalem, Israel

The α9β1 integrin is characterized as receptor for ligands including VCAM-1, tenasin-C, osteopontin, TSP-1 and certain ADAMs family members and two growth factors, VEGF-C and VEGF-D. In presented studies we found that α9β1 integrin is a receptor for nerve growth factor (NGF). Adhesion and ELISA assays revealed that this integrin is a high affinity receptor for NGF, similar to previously characterized trKA. NGF also induced signal transduction in cells expressing α9β1 integrin, including phosphorylation of MAPK Erk1/2 and paxillin. Using adhesion, flow cytometry, immunocyto staining and immunoprecipitation assays we found α9β1 integrin expressed on the surface of grade IV astrocytoma cell line, LN229, whereas normal human astrocytes and grade III astrocytoma cell lines showed no presence of this integrin. This finding indicates that expression of α9β1 integrin may be related to malignancy of glioblastoma multiformes (GBM), although another grade IV glioma cell line, LN18, does not express this integrin. We found that NGF induced proliferation of LN229 cells, whereas it inhibited proliferation of LN18 cells. This phenomenon could be explained that NGF-promoted proliferation is related to its binding to α9β1 integrin, but in the absence of this integrin NGF binds to low affinity receptor p75NTR. We found this receptor on both cells, however, in the absence of high affinity pro-survival receptor, α9β1 integrin, p75NTR, dominates and following interaction with NGF leads to induction of apoptosis, which blocks of proliferation of LN18 cells. NGF was also potent chemotactant for LN1229 cells in the Boyden chamber, in the contrast to LN18 cells. The pro-attractive and pro-migratory ability of LN229 cells induced by NGF was abolished following silencing of α9 integrin subunit using siRNA method. Moreover, specific inhibitors of α9β1 integrin such as disintegrin, VLO5 and monoclonal antibody V9A2, blocked NGF interaction with α9β1 integrin in adhesion, proliferation and migration assays.

2418

The Integrin Alpha9Beta1 Contributes to Granulopoiesis by Modulating G-CSF Receptor Signaling

C. Chen, X. Huang, D. Sheppard; Lung Biology Center, UCSF, San Francisco, CA

The integrin α9 subunit forms a single heterodimer, α9β1, and is widely expressed on neutrophils, smooth muscle, hepatocytes, endothelia and some epithelia. We found that integrin α9-deficient mice had a dramatic defect in neutrophil development, with decreased numbers of circulating neutrophils, decreased numbers of granulocyte precursors in bone marrow, and impaired differentiation of bone marrow cells into granulocytes. In response to the neutrophil growth and differentiation factor, granulocyte colony stimulating factor (G-CSF), α9-deficient bone marrow cells or human bone marrow cells incubated with α9β1 blocking antibody demonstrated decreased phosphorylation of STAT5, an early target of G-CSF receptor signaling, and decreased phosphorylation of the G-CSF receptor. Ligation of integrin α9β1 in the hematopoietic cell line, BaF3, enhanced G-CSF-induced phosphorylation of Erk1/2 and STAT3 but had no effects on activation of STAT5 or Akt. These effects both depend on the presence of an intact α9β1 subunit cytoplasmic domain and the distal region of G-CSF receptor cytoplasmic domain. We conclude that integrin α9β1 is required for granulopoiesis and that integrin ligation enhances activation of a specific subset of signaling intermediates downstream of the G-CSF receptor.

2419

Fatty Acid Binding Proteins (FABPs) as Modulators of Integrin Function in Breast Cancer Cells

J. B. Nero, J. Ivaska; Centre for Biotechnology, University of Turku, Turku, Finland

The cells need to connect to each other and to the surrounding matrix to form tissues. Integrins are major cell surface receptors mediating cell-cell and cell-matrix interactions and play a critical role in many vital cellular functions like cell migration, adhesion, tissue organization, and the regulation of cell survival, differentiation and proliferation. The role of integrin β subunit in cell signalling has been studied in detail, whereas very little is known about the signaling functions mediated by the integrin α cytoplasmic tails. I have been involved in screening for protein-protein interactions with the yeast-two hybrid method using the α cytoplasmic tails of the collagen binding integrins α11, α21, α31 and α111 as baits. Members of fatty acid-binding protein (FABP) multilamily have emerged from three independent screenings. FABPs are known to be small cytosolic proteins that mainly facilitate the solubility and intracellular transport of free fatty acids. I have studied in detail the biological effects of FABPs on integrin function. Interestingly, FABP3, also known as MDGI (mammary derived growth inhibitor), is found to down-regulate integrin mediated cell adhesion to type I collagen in several breast cancer cell lines. Most breast cancer cell lines have lost FABP3 expression, and our data demonstrates that one of the functions of this breast cancer tumor suppressor protein is to influence cell proliferation possibly via attenuated cell matrix interactions and integrin signalling.

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Regulation of Inflammatory IL-6 and INOS by Substratum and Cell-Cell Interaction in Mammary Epithelial Cell Lines
S. W. Maalouf,1 D. C. Borger,1 R. S. Talhouk,2 F. L. Schanbacher1; 1Animal Sciences, The Ohio State University, OARDC, Wooster, OH, 2Biology, The American University of Beirut, Beirut, Lebanon
Recent studies linking chronic inflammation to the risk of cell transformation and cancer likely would apply to the mammary gland and breast cancer. Mammary epithelium is comprised of secretory and myoepithelial cells in close contact, represented by SCp2 and SCg6 cell lines respectively; both derived from the CID-9 mouse mammary cell line. From our previously described response of SCp2 cells to inflammation induced by bacterial endotoxin (ET), we further investigated the effect of exogenous extracellular matrix (ECM) or co-culture of secretory SCp2 with myoepithelial SCg6 mammary cells (mimicking cell organization in the mammary epithelium) on inflammatory responders interleukin-6 (IL-6) and nitric oxide (NO) in response to ET. SCp2 cells were cultured on plastic +/- extracellular matrix (ECM) or on a monolayer of SCg6 cells. ET-induced IL-6 secretion and NO production were monitored by ELISA immunoassay and Griess reaction assay respectively. Endotoxin induced a 4-7 fold increase in IL-6 secretion in SCp2 cells independent of ECM addition. However, in the absence of ET the co-culture of SCp2 with SCg6 cells induced a surprising 10 fold increase in secreted IL-6 compared to that of cells on plastic +/- ECM. ET treatment of the co-cultured cells further doubled IL-6 secretion. Unlike IL-6, NO production was dependent on ET treatment regardless of substratum. However, co-cultured SCp2 and SCg6 cells gave only 1/3 the NO production as SCp2 cells alone after ET treatment. These results suggest that NO induction is dependent on ET-induced inflammation in mammary epithelial cells. However, IL-6 secretion by mammary cells is drastically increased not only by ET but also by cell-cell interaction in the absence of ET, suggesting a potential role of IL-6 in mammary cell growth or differentiation independent of its pro-inflammatory role.

tg-beta Effects on Desmosomal Components in MCF10A Cells
S. A. Keim, Genetics, Cell Biology and Anatomy, University of Nebraska Medical Center, Omaha, NE
Epithelial to mesenchymal transition (EMT) is a normal part of embryonic development, and is a potential mechanism in carcinoma progression. During EMT, epithelial cells decrease cell-cell interactions and acquire an invasive phenotype. Epithelial cells interact with one another via several junctional complexes including the adherens junction and the desmosome. Changes in the adherens junction have been characterized during EMT, but desmosomes have received only limited attention. We have previously characterized the desmosomal components in MCF10A cells when they undergo TGF-beta induce EMT. Immunofluorescence and western blot analysis showed that the general trend is a decrease in the desmosomal components at the protein level by days three and four of TGF-beta treatment. We are now interested in the mechanism that causes the decrease in the desmosomal components. Quantitative Real Time PCR showed that mRNA levels of the desmosomal components are not significantly decreased with TGF-beta treatment suggesting that the decrease is due to a posttranscriptional mechanism. Since the plakophilins are greatly reduced at the protein level after TGF-beta treatment, we sought to determine if the proteins would be stabilized by the proteasome inhibitor MG-132. When MCF10A cells were treated with both TGF-beta and MG-132, the fates of plakophilins 2 and 3 differed from one another. Plakophilin 3 levels did not decrease with treatment, while plakophilin 2 levels where similar to those cells treated with only TGF-beta. These results suggest that the decrease in plakophilins during TGF-beta induced EMT contribute to the disassembly of desmosomes.

down-regulation of ras/erks cascade in scattered cells renders the egfr phosphorylation-dependent formation of cell-cell contacts
E. Kang,1 J. Lee2; 1Department of Molecular and Cellular Oncology, Cancer Research Institute, College of Medicine, Seoul National University, Seoul, Republic of Korea
Epithelial-mesenchymal transition (EMT) plays important roles in the development and dissemination of cancer cells from a primary tumor body. The reversal of EMT, mesenchymal-epithelial transition (MET) can occur at metastatic sites, although its regulation mechanisms are unclear. In this study, we observed MET as determined by cell-cell contact formation between scattered rat intestinal epithelial cells after treatment with a specific MEK inhibitor (U0126). U0126 treatment-mediated MET correlated with increased EGFR phosphorylation, in a ligand-independent manner, since the phosphorylation still occurred in the absence of serum or in cells preincubated with neutralizing anti-EGF antibody. Moreover, pretreatment with EGFR kinase inhibitor abolished U0126 treatment-mediated EGFR phosphorylation and MET, and the expression of H-Ras N17 dominant negative mutant allowed EGFR phosphorylation and MET even without U0126 treatment. Furthermore, the expression of a nonphosphorylatable EGFR Y5F mutant abolished U0126-mediated MET. U0126 treatment also caused less efficient wound healing by keeping monolayer intact, compared to control untreated cells. This U0126-mediated reduction in wound healing was further altered either by pretreatment of EGFR kinase inhibitor or expression of H-Ras N17 or EGFR Y5F. Taken together, this study supports a unique mechanism of cell-cell contact formation through MEK/Erks inhibition-mediated EGFR phosphorylation.

Subcellular Localization of RhoA/Rho Kinase Signaling in Endothelial Cells
G. P. van Nieuw Amerongen, J. Hodzic, C. M. L. Beckers, R. J. P. Musters, M. A. Engelse, V. W. M. Van Hinsbergh; Faculty of Medicine, Department of Physiology, VU Medical Center, Amsterdam, The Netherlands
We have previously shown that thomrin is a potent activator of RhoA. Thomrin is vascular hyperpermeability-inducer. It disturbs endothelial barrier integrity by a contractile mechanism, involving activation of RhoA and its downstream target Rho kinase. Rho kinase on its turn inactivates the Myosin Phosphatase (MP). Here, we investigated the subcellular localization of RhoA-mediated signaling. To visualize Rho kinase activity in endothelial cells, a phosphospecific antibody against the myosin phosphatase targeting subunit (Thr^422/Thr^423/Thr^425) was used as a surrogate marker for Rho kinase activity. Upon stimulation with thomrin, enhanced Rho kinase activity at contractile F-actin stress fiber was observed by 3D deconvolution fluorescence microscopy. In addition, live cell imaging using a fluorescence resonance energy transfer (FRET)-based probe for RhoA activity, confirmed an increase in RhoA activity in cytosolic areas upon thomrin stimulation Unexpectedly, at sites of thomrin-induced gap formation RhoA activity decreased, as was evidenced by a FRET probe that localized mostly at the plasma membrane by means of a K-Ras4B carboxy-terminal region. Furthermore, a basal activity of both RhoA and Rho kinase was observed in junctional areas of nonstimulated cells, suggesting a contribution of basal RhoA/Rho kinase activity to maintenance of cell-cell contacts. Indeed, inhibition of basal Rho kinase activity attenuated endothelial barrier integrity, opposing previously observed protection from the thomrin response. In conclusion, we provide evidence for dual role of RhoA/Rho kinase signaling in regulation of endothelial barrier function: 1) a basal activity injurious areas that contributes to maintenance of cell-cell adhesion, and 2) an enhanced RhoA/Rho kinase activity in cytosolic regions upon thomrin stimulation, resulting in barrier disruption.
Cerebral cavernous malformations type 1 (CCM-1), a disease associated with defective endothelial junctions, results from autosomal dominant CCM-1 mutations that cause loss of KRIT-1 protein. KRIT-1 (Krill Interaction Trapped-1) was discovered through its interaction with Rap1A in a yeast two-hybrid screen. We now demonstrate that KRIT-1 protein binds tightly to GTP-loaded Rap1A but not to the nucleotide free form to nor to activated H-Ras, establishing that it is an authentic Rap-specific effector. Furthermore, we developed anti-KRIT-1 monoclonal antibodies and used them to establish that Rap1 can regulate the conformation of KRIT-1 thus unmasking its FERM domain. The KRIT-1 FERM domain was masked through an interaction with a sequence in the KRIT-1 N-terminus. FERM domains often mediate interactions with the cytoplasmic tails of transmembrane proteins; we find that integrin β tails are such a membrane protein ligand for the KRIT-1 FERM domain and that Rap1 regulates the KRIT-1-integrin interaction. In addition, KRIT-1 protein is expressed in cultured arterial and venous endothelial cells and is present in both the nucleus and cell-cell junctions. We now find that the junctional localization of KRIT-1 is mediated by the FERM domain and that activation of Rap1 enhances this localization. Thus, these studies establish that KRIT-1 is a Rap1 effector located at cell-cell junctions, and provide a molecular explanation for aspects of the CCM phenotype.

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Connexin 43 May Be Involved in IL-1β-mediated Cell Survival in Human Tenocytes
J. Qi,1 L. Chi,1 D. Bynum,2 A. J. Banes1; 1Flexcell International Corporation, Hillsborough, NC, 2Joint Department of Biomedical Engineering, North Carolina State University, Raleigh, NC, Orthopaedics, University of North Carolina at Chapel Hill, Chapel Hill, NC. Joint Department of Biomedical Engineering, University of North Carolina at Chapel Hill, Chapel Hill, NC

Introduction - Interleukin-1β (IL-1β) is a proinflammatory cytokine that is up-regulated by mechanical loading. IL-1β treatment increased cell survival in mechanically loaded human tenocytes by relaxing the cytoskeleton and decreasing the Young’s modulus. Overexpression of connexin 43 (Cx 43) increased cell survival in neuron cells. Cx 43 is the major connexin expressed in human tenocytes. To further investigate the mechanism of cell survival under mechanical loading, the effects of IL-1β on the Cx 43 expression were studied. Method - Primary tenocytes (HTFPs) were isolated from discarded human tendon tissue as described previously. HTFPs from passages 2 to 4 were used in this study. HTFPs were grown in three-dimensional type I collagen gels in the presence of 2% fetal bovine serum with or without 100 pM IL-1β. Cells were collected at 1, 8 and 24 h post-addition of IL-1β and total RNA was extracted with an RNaseasy mini kit (Qiagen), CDNA was synthesized with SuperScriptII (Invitrogen) and real time PCR for Cx 43 was carried out using a SYBR premix Ex Taq kit from Takara. For a dose-localization study, cells in type I collagen gels were treated with 1, 10 or 100 pM IL-1β for 24 h. Results - IL-1β up-regulated the expression of Cx 43 in human tenocytes in a dose-dependent manner by 24 h post-addition of 100 pM IL-1β. Conclusion - These results indicate that up-regulation of Cx 43 to increase cell-cell communication may be a potential mechanism by which IL-1β increases cell survival after mechanical loading in connective tissues. The latter action would be important in the compliant wound tissue during convalescence post-injury.

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Exosomes Released by Activated T Cells May Contribute to Atherogenesis by Promoting Monocyte Cholesterol Accumulation and TNF-α Production
L. Zakharova, M. Svetlova, A. F. Fomina; Physiology and Membrane Biology, University of California, Davis, Davis, CA

Activated T lymphocytes release vesicles, termed exosomes, enriched in cholesterol and exposing phosphatidylserine (PS) at their surface. Although CD4+ activated T lymphocytes infiltrate an atherosclerotic plaque, the effects of T cell exosomes on the atheroma-associated cells have not been studied. Transformation of monocytes and macrophages into cholesterol-loaded foam cells is a hallmark of atherosclerosis. Therefore, we investigated the effects of T cell exosomes on cholesterol accumulation and cytokine production by human monocytes and PMA-activated THP-1 cells. Resting CD4+ T lymphocytes were purified from the peripheral blood of healthy volunteers and were activated in vitro with phytohaemagglutinin P to stimulate exosome production. Exosomes were isolated from T cell culture supernatants using the high speed centrifugation method. Purified exosome preparations were added to the cultured human monocytes or PMA-activated THP-1 cells. After overnight incubation, the total cholesterol content in monocyte whole-cell lysates was determined with an enzymatic cholesterol-detection assay. We found that exosomes strongly stimulated cholesterol accumulation in both cultured human monocytes and THP-1 cells. This effect was abolished by anti-PS receptor antibodies, indicating that this receptor may be involved in exosome internalization. Staining with filamin and Nile Red, the specific markers for free and esterified cholesterol respectively, revealed a number of lipid droplets composed of esterified cholesterol or cholesterol ester and free cholesterol in the cytosol of exosome-treated monocytes. The enhanced production of proinflammatory cytokine TNF-α in monocyte cell cultures was observed in parallel with cholesterol accumulation. We conclude that within an atherosclerotic plaque, the effects of T cell exosomes on rupture. Events tagged KIR molecules are being used to monitor KIR interactions during clustering and upon zinc binding. Preliminary results indicate that FRET decreases upon chelation of zinc. Analysis of NK Cell Inhibitory Receptor Clustering by FRET
P. Srinivasan, E. O. Long; National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, MD

Natural killer (NK) cells defend the body against intracellular pathogens via cytokine production and destruction of infected cells. NK cell activation is regulated by inhibitory receptors such as KIR (killer cell immunoglobulin-like receptor) that recognize MHC Class I molecules on target cells. KIR2DL1, an inhibitory receptor specific for the class I molecule HLA-C, accumulates at the interface of NK cells and resistant, HLA-C-expressing target cells. The divalent metal zinc contributes to KIR clustering at NK cell immune synapses, and to the inhibitory signal delivered by KIR2DL1. FRET (fluorescence resonance energy transfer) is being used to analyze dyeimerization and clustering of KIR, in order to define the role of zinc in the inhibitory function of KIR. KIR2DL1 has been tagged at the cytoplasmic tail with CFP and YFP. In addition, CFP and YFP have been fused to the N-terminus of KIR, adjacent to the putative zinc binding site. These tagged KIR molecules are being used to monitor KIR interactions during clustering and upon zinc binding. Preliminary results indicate that FRET decreases upon chelation of zinc. Events recorded on live cells will provide further insights into the role played by zinc in KIR2DL1 signaling.

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Killer Cell Ig-like Receptor Promotes Clustering of Activation Receptors at Inhibitory NK Cell Immune Synapses
M. E. March,1 N. Schleiniz; 1Molecular Cellular Immunology Section, NIH/NIAD, Rockville, MD, 2Centre d’Immunologie, Institut National de la Santé et de la Recherche Médicale/Centre National de la Recherche Scientifique de Marseille Luminy, Marseille, France

Activation of NK cells by contact with target cells is controlled by inhibitory receptors for MHC class I, which include the human killer cell Ig-like receptors (KIR) specific for HLA-C. Inhibitory KIR accumulate at the site of contact with HLA-C-expressing target cells, and prevents phosphorylation of activation receptor 2B4 and its recruitment to detergent-resistant membrane domains. The hypothesis that KIR blocks activation receptor signaling by preventing anti-non-written receptor accumulation at immune synapses was tested here. Using primary human NK cells in contact with target cells expressing defined combinations of ligands for NK cell receptors, we show that accumulation of KIR correlated with exclusion of LFA-1 from the NK-target cell interface. In contrast to the exclusion of LFA-1, activation receptors CD2 and 2B4 were not excluded from inhibitory NK cell immune synapses, but accumulated and colocalized with KIR. In fact, KIR promoted the clustering of CD2 and 2B4 at inhibitory synapses by lifting the requirement for actin polymerization in CD2 and 2B4 accumulation. Therefore, inhibitory KIR does not prevent CD2 and 2B4 signaling by blocking actin-dependent accumulation at NK cell immune synapses, but by blocking the ability of activating receptors to signal within inhibitory synapses.

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Regulation of Erzin Localization by Rac1 and Pipkin in Human Epithelial Cells
E. Auvinen,1 N. Kivi,1,2 A. Vaheri1; 1Department of Virology, HUSLAB Clinical Microbiology, HUS Helsinki, Finland, 2Department of Virology, Haartman Institute, University of Helsinki, Helsinki, Finland

Regulation of erzin and other ERM proteins is not completely understood, but the involvement of Rho GTPases seems crucial. In this work, expression plasmids encoding full-length, deleted or truncated ezrin were constructed and coexpressed with Rac1 GTPase in HeLa human epithelial cells in order to elucidate the mechanisms of ezrin activation and function. We observed induction of actin stress fiber formation by ezrin constructs harboring the F-actin binding site but devoid of sequences required for intra- or intermolecular binding. Stress fiber-inducing ezrin mutants were localized at cell-cell adhesions and also colocalized with F-actin in stress fibers. This localization required the activity of Rac1 and phosphorylidyinositol-4-phosphate 5-kinase and involved RhoA. We suggest that Rac regulates ezrin localization at cell-cell adhesions.
Thrombomodulin Cytoplasmic Domain Interacts with α-Melanocyte and Ezrin

G. Shi, G. C. Huang, C. Kuo, F. Lin, M. Chen, H. Wu
Department of Biochemistry and Molecular Biology, National Cheng Kung University, Tainan, Taiwan, 2Institute of Medical Sciences, National Taiwan University, Tainan, Taiwan

Thrombomodulin (TM), a type I integral membrane glycoprotein, is a well-studied potent anticoagulant factor. However, expression of TM in nonvascular cells such as epithelial squamous keratinocytes highly suggests that TM may possess other functions beyond its anticoagulant activity. Previously we have reported that TM functions as a novel cell-cell adhesion molecule which mediates cell aggregation. To be an effective adhesion molecule, cytoplasmic domain of TM needs to associate with cytoskeleton either directly or indirectly through linkage proteins. To investigate molecules involved in this interaction, by employing pMACS magnetic isolation we identified two candidates, ezrin and α-actinin, potentially associated with TM cytoplasmic domain. Co-immunoprecipitation analysis further proved their interaction with TM. In vitro binding assay provided evidence that both the ezrin N-terminal and α-actinin spectra domain bound to the TM cytoplasmic domain directly without the requirement of other proteins. We also studied the regions in the TM cytoplasmic domain implicated in the binding to ezrin and α-actinin. By truncating and mutating TM cytoplasmic domain we showed that the ezrin- and α-actinin-binding sequence was located in the positively charged juxtamembrane region KRRKQGAARAR. Immunohistological data in cultured cells revealed that TM colocalized with ezrin in actin-rich microvilli and membrane ruffles while α-actinin overlaid TM at the leading edges of lamellipodia and cell-cell contacts. Moreover, mutation of the positively charged binding sites for ezrin and α-actinin on TM prevented surface protrusions and cell aggregation phenotype. We therefore conclude that the positively charged residues in juxtamembrane region among TM cytoplasmic domain directly interact with ezrin and α-actinin which link membrane protein to actin filaments.

Thrombomodulin-mediated Cell Adhesion, Cytoskeletal Rearrangement, and Morphological Conversion

C. W. Huang, G. Y. Shi, H. Y. Yang, H. C. Huang, H. P. Chen, H. L. Wu
Institute of Basic Medical Sciences, National Cheng Kung University, Tainan, Taiwan, 2Department of Biochemistry and Molecular Biology, National Cheng Kung University, Tainan, Taiwan

Podocytes are highly differentiated kidney epithelial cells responsible for maintaining the selective permeability barrier of the glomerulus. They form specialized modified adherens junctions that are important for maintaining the selective permeability barrier of the glomerulus. They form specialized modified adherens junctions that are important for maintaining the selective permeability barrier of the glomerulus. They form specialized modified adherens junctions that are important for maintaining the selective permeability barrier of the glomerulus. They form specialized modified adherens junctions that are important for maintaining the selective permeability barrier of the glomerulus.

Effects of Changing Endogenous Alpha-Catenin Localization on Cell Adhesion and Migration

J. Nelson, Biological Sciences and Molec. Cell. Physiology, Stanford University, Stanford, CA

α-Catenin regulates cell-cell adhesion and migration in vertebrates and invertebrates. It is generally accepted that strong cell-cell adhesion is mediated through cadherin binding to cytoplasmic α-Catenin is a protein essential for cell migration. In addition, we found that α-Catenin localization to F-actin cell-cell contacts and cell migration has been extensively investigated, but whether and how cadherin function is directly affected, as well as the role of the underlying actin cytoskeleton, remain less well understood issues. Ep8, a substrate of receptor tyrosine kinases, in a complex with Ab1 and Sos-1 induces Rac activation leading to actin cytoskeleton remodeling. Recent studies have shown that Ep8 regulates the growth of actin filaments by capping their barbed ends, an activity essential for cell motility. Here, we report that Ep8 localizes to cell-cell junctions, potentially regulating the interaction with the N-terminus of p120 catenin. Through the use of RNAi and the reconstitution with mutants selectively defective for Ep8 capping activity, the latter is shown to be required for normal cadherin-dependent adhesion, cortical actin organization, epithelial cell migration and HGF-driven cell scattering. This study provides novel functional information on Ep8 as a cell-cell adhesion regulator and increases our understanding of the roles of cell cing proteins in cell adhesion.
Beta-catenin Directly Regulates Bacteria-induced Inflammation In Vivo

Y. Duan, A. Liao, S. Kupipreddi, M. J. Ciancio, J. Sun
Pathology, The University of Chicago, Chicago, IL, The Inflammatory Bowel Disease Research Center, Department of Medicine, The University of Chicago, Chicago, IL
Wild-type (WT) Salmonella typhimurium cause acute intestinal inflammation by activating the nuclear factor kappa B (NF-kappaB) pathway. Interestingly, WT Salmonella typhimurium infection also causes degradation of beta-catenin, the regulator of cell proliferation and differentiation. Regulation of beta-catenin and the inhibitor of NF-kappaB, IkappaBalpha, are strikingly similar, involving phosphorylation at the same N-terminal serine sequence sites, ubiquitination by the same E3 ligase complex, and subsequent proteasomal degradation. However, how beta-catenin directly regulates the NF-kappaB pathway during bacteria-induced inflammation in vivo is unknown. The purpose of this study, therefore, was to investigate the role of beta-catenin in modulating the pro-inflammatory response mediated by NF-kappaB subsequent to Salmonella infection in vivo. Using streptomycin-pre treated mice challenged with WT Salmonella typhimurium entericenter, we demonstrated that WT Salmonella stimulated beta-catenin degradation and decreased the physical association between NF-kappaB alpha and beta-catenin (NF- kappaB-alpha/beta-catenin complex) in intestinal epithelial cells by Western blot and immunoprecipitation assays. Accordingly, WT Salmonella infection decreased the expression of c-myc, a beta-catenin-regulated target gene and increased the levels of IL-6 and TNF-alpha, the NF-kappaB-regulated target genes. Bacterial infection directly stimulated phosphorylation of beta-catenin, both in vivo and in vitro. In conclusion, we demonstrated that the transcription factor c-myc, a beta-catenin-regulated gene, is important in the regulation of the pro-inflammatory response in intestinal epithelial cells by WT Salmonella infection.

The Formin Homology Protein Dia1 Is Required for the Integrity of Cell-Cell Junctions in Epithelial Cells

L. Carramusa, A. Bershadsky; Molecular Cell Biology, Weizmann Institute of Science, Rehovot, Israel
Recent research demonstrated that Zipcode Binding Protein 1 (ZBP1) inhibits translation of beta-actin mRNA, until the mRNA reaches its destination at the leading edge of fibroblasts. To investigate the physiological significance of this process in living cells, we utilized a live-cell imaging assay to determine the spatial distribution of beta-actin mRNA. Experiments with GFP-fused Dia1 constructs demonstrated that junctional localization of Dia1 depends on the N-terminal domain of the Dia1 molecule, which contains the Rho-binding domain. Thus, constitutively active GFP-Dia1 mutants truncated at the N- terminus did not demonstrate junctional localization, while a mutant preserving the N-terminal domain but lacking the C-terminal autoinhibitory domain (GFP-Dia1-deltaDAD) was enriched at the junctions. As expected, constitutively active RhoA mutant, RhoA-V14, did not rescue formation of adherens junctions in the cells with down regulated Dia1, but enhanced recruitment of beta-catenin at the cell-cell contact area in GFP-Dia1-deltaDAD expressing cells. Moreover, coexpression of RhoA-V14 with full length GFP-Dia1 strongly promoted localization of GFP-Dia1 to the cell-cell junction, which was accompanied by greatly increased recruitment of E-cadherin to the junctional area. Thus, we propose that Dia1 localizes to adherens junctions in a RhoA-dependent manner, and is required for the proper junctional localization of beta-catenin and associated proteins, for which further studies are needed.

The Recruitment of Adherens Junction Components Requires Zipcode Mediated Local Translation of beta-Actin

A. J. Rodriguez, S. Burke, A. L. Wells, S. M. Shenoy, J. Condeelis, R. H. Singer; Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, NY
Recent research demonstrated that Zipcode Binding Protein 1 (ZBP1) inhibits translation of beta-actin mRNA, until the mRNA reaches its destination at the leading edge of fibroblasts. To investigate the physiological significance of this process in living cells, we utilized a live-cell imaging assay to determine the spatial distribution of beta-actin mRNA. Experiments with GFP-fused Dia1 constructs demonstrated that junctional localization of Dia1 depends on the N-terminal domain of the Dia1 molecule, which contains the Rho-binding domain. Thus, constitutively active GFP-Dia1 mutants truncated at the N-terminus did not demonstrate junctional localization, while a mutant preserving the N-terminal domain but lacking the C-terminal autoinhibitory domain (GFP-Dia1-deltaDAD) was enriched at the junctions. As expected, constitutively active RhoA mutant, RhoA-V14, did not rescue formation of adherens junctions in the cells with down regulated Dia1, but enhanced recruitment of beta-catenin at the cell-cell contact area in GFP-Dia1-deltaDAD expressing cells. Moreover, coexpression of RhoA-V14 with full length GFP-Dia1 strongly promoted localization of GFP-Dia1 to the cell-cell junction, which was accompanied by greatly increased recruitment of E-cadherin to the junctional area. Thus, we propose that Dia1 localizes to adherens junctions in a RhoA-dependent manner, and is required for the proper junctional localization of beta-catenin and associated proteins, for which further studies are needed.

The Role of the Beta-1 Subunit of the Na,K-ATPase and Its Glycosylation in Cell-Cell Adhesion

O. Vagin, E. Tolkhtseva, G. Sachs; Physiology, UCLA, Los Angeles, CA
OBJECTIVE. The goal of this study is to identify the role of the Na,K-ATPase beta1 subunit in cell-cell adhesion. METHODS. YFP-linked fusion proteins of the Na,K-ATPase beta1 subunit and its glycosylation-deficient mutants were stably expressed in MDCK cells. Distribution and endogenous subunits of the Na,K-ATPase and adherens junction proteins was studied by immunohistochemistry, confocal microscopy and surface-selective biotinylation followed by Western blot analysis. RESULTS. In polarized monolayers of MDCK cells, the Na,K-ATPase alpha1 and beta1 subunits were detected exclusively in the sites of cell-cell contact and were resistant to Triton X-100 extraction suggesting association with the cytoskeleton. The Na,K-ATPase was co-localized with the markers of adherens junctions, beta1 Na,K-ATPase, directly underneath the tightest adhesion. Co-localization with the adherens junctions was retained before the tightest adhesion. The association of the Na,K-ATPase with the adherens junction proteins and cytoskeleton occurred when cell-cell contact initiated. Upon disruption of intercellular contacts by incubation of cells in a Ca++-free buffer, the Na,K-ATPase was internalized along with beta1-catenin. Mutagenic removal of N-glycosylation sites of the Na,K-ATPase beta1 subunit dissociated the pump from the adherens junctions and cytoskeleton as inferred from a significant removal of the mutated beta1 subunit and the endogenous Na,K-ATPase alpha1 subunit from the lateral membrane and loss of co-localization with beta1-catenin after treatment of cells expressing the mutant with Triton X-100. Incubation of attached MDCK cells with an antibody against the extracellular domain of the Na,K-ATPase beta1 subunit inhibited cell-cell adhesion. CONCLUSIONS. The Na,K-ATPase beta1 subunit is involved in intercellular adhesion and is responsible for association of the heterodimeric alpha1,beta1 Na,K-ATPase with the adherens junctions. N-glycosylation of the Na,K-ATPase beta1 subunit is essential for the stable association of the pump with the adherens junctions and hence the cytoskeleton.

A Possible Role for Cno/AF-6 in Linking Actin to Adherens Junctions during Morphogenesis

J. K. Sawyer, C. Beattie, M. Peifer; Biology, UNC-Chapel Hill, Chapel Hill, NC, Laboratory of Development Neurogenetics, Rockefeller University, New York, NY
Cell-cell adhesion is critical for development. Adherens junctions (AJs) were thought to form mechanical attachments between cells by linking actin in neighboring cells together through the cadherin-catenin complex. However, recent work from the Nelson and Weis labs questions whether this linkage is direct. Additional proteins are recruited to AJs whose function is less well understood. We hypothesize that these may act partially redundantly to regulate junctional plasticity by regulating connections to actin and to signal transduction machinery. To test this hypothesis we are defining the function of Cno (Cno) in AJs. Cno is a scaffolding protein that is thought to bring together multi-protein complexes to modulate adhesion. In mammals, AF-6 and the cell adhesion molecule nectin form a multi-protein complex that exists in adherens junctions. Our model predicts that these complexes regulate the formation and function of AJs by providing a link between AJs and actomyosin contractile ring.
A Role for Rap1 in *Drosophila* Morphogenesis

N. J. Harris, J. K. Sawyer, M. Feifer; University of North Carolina at Chapel Hill, Chapel Hill, NC

Animal development is dependent on proper cell-cell interactions, which are mediated in part by adherens junctions (AJs). These junctions mediate cadherin-based adhesion and also help organize the cortical actin cytoskeleton within cells. In addition to the cadherins and catenins at these junctions, other proteins are recruited to AJs which are thought to have additional functions. Our lab has been investigating the function of canon (cno), the *Drosophila* homolog of mammalian Afadin/AF-6, and its role in morphogenesis. Cno has a number of binding partners, among which is the small GFP-tagged Rap1. Mammalian Rap1 has been recently proposed to regulate integrin adhesion, while *Drosophila* Rap1 is suggested to regulate cadherin localization in imaginal discs. Rap1 has functions in early morphogenesis, but how it functions with cno to regulate adhesion or other intercellular events is not well understood. Currently, we are investigating Rap1’s role in cell-cell adhesion by analyzing maternal/zygotic mutants, and looking for possible genetic interactions with other proteins that localize to the AJ or are predicted to bind Rap1 based on work in other organisms. In addition, we are examining a GFP fusion of Rap1 from Nick Brown to learn more about its localization inside the cell during embryo morphogenesis.

Long-term Effects of Limited p120-ablation in the Mouse Intestine

W. G. Smalley, 1 P. E. Burnett, 1 M. A. Davis, 1 M. K. Washington, 2 S. Robine, 4 D. L. Guumcu, 2 R. J. Coffey, 2 A. B. Reynolds, 1 Cancer Biology, Vanderbilt University, Nashville, TN, 2Dermatology, Vanderbilt University, Nashville, TN, 3Pathology, Vanderbilt University, Nashville, TN, 4Institut Curie-CNRS, Paris, France, 5University of Michigan Medical School, Ann Arbor, MI, 6Gastroenterology, Vanderbilt University, Nashville, TN

We have established a novel mouse model for determining the long-term effects of p120-ablation in the small intestine and colon. In our first generation model, p120 ablation was induced by crossing p120 floxed mice to villin-Cre transgenic mice provided by D. Guumcu. A mosaic p120 knockout was observed in approximately half of epithelial cells from the stomach to the anus, with no other tissues affected. The damage, however, was severe, and animals bled to death by 3 weeks of age due to widespread epithelial shedding and inflammation. To examine long-term effects of p120 ablation, we have crossed our animals to a new villin-Cre-Ert2 transgenic mouse from S. Robine’s laboratory, and established tamoxifen dosing conditions to limit p120 loss to less than 10% of epithelial cells in the intestine and colon. Interestingly, morphologic and adhesive defects are similar to those characterized previously but are limited to fields of cells derived from individual p120-null stem cells. E-cadherin is significantly downregulated in p120-null regions, and cell morphology and adhesion is perturbed, but animals do not suffer the early health problems associated with the full knockout. By two months of age, however, all animals develop numerous cyst-like villi within villus structures in the intestines. This new model will allow detailed analysis of the long-term fate of these unusual structures.

Dissecting the Role of ALP-1 in *C. elegans* Epidermal Morphogenesis

H. Han, 1,2 C. R. McKeown, 2 M. C. Beckerle 1,2; 1Oncological Sciences Department, University of Utah, Salt Lake City, UT, 2Huntsman Cancer Institute, Salt Lake City, UT

The epidermis is a multilayered epithelial tissue that provides mechanical support and regulates drug transport. Despite its importance, very little is known about the molecular mechanisms that control epidermal morphogenesis. In this study, we have investigated the role of ALP-1, a protein that binds to the cadherin-catenin complex and cooperates with the adherens junction to regulate cell adhesion and morphogenesis. ALP-1 is an adhesion molecule that interacts with multiple proteins, including cadherins, catenins, and integrins. In *C. elegans*, ALP-1 cooperates with proteins in the catenin-cadherin complex to regulate cell adhesion and morphogenesis. Our data suggest that ALP-1 plays a role in cell adhesion and morphogenesis by interacting with the cadherin-catenin complex. ALP-1 also interacts with integrins, suggesting that it may function as a scaffold for protein assembly at cell-cell contacts. Our results provide new insights into the molecular mechanisms that control epidermal morphogenesis and suggest that ALP-1 may have a conserved role in cell adhesion and morphogenesis across multiple species.
Regulation of Adhesion by the Intracellular Trafficking of DE-cadherin during *Drosophila* Morphogenesis

J. F. Roeth, M. Peifer; Biology, University of North Carolina at Chapel Hill, Chapel Hill, NC

Adhesions junctions (AJ)s are critical to normal cell adhesion (mediated by intercellular homophilic interactions between Cadherin molecules on adjacent cells). It is generally thought that AJs must maintain a high level of plasticity to accommodate cell shape changes during morphogenesis and cell division. Studies in cultured mammalian cells suggest that the intracellular trafficking of E-Cadherin is an essential determinant of adhesion. Rab proteins form a family of small GTPases that act as key regulators to orchestrate multiple stages of protein transport. Rab5 mediates fusion of internalized vesicles with early endosomes, and may also play a role in clathrin-mediated endocytosis. Rab11 is thought to regulate vesicle transport at the sorting/recycling endosome, and can help to target proteins to the plasma membrane from either the endocytic or exocytic pathway. We hypothesize that the balance between endocytosis and recycling of AJ proteins is a potential mechanism to rapidly modulate epithelial cell adhesion, and that AJ proteins are actively transported via Rab5- and Rab11-dependent endosomal pathways. Using the GAL4-UAS system in *Drosophila*, we have expressed dominant Rab5 and Rab11 mutants in specific regions at various developmental stages during *Drosophila* embryogenesis and examined the consequences. In epidermal cells, constitutively active Rab5 induced the formation of enlarged endosomes that accumulate DE-Cadherin and recruit F-actin. Both constitutively active and dominant negative Rab5 mutants inhibited apical constriction and cell shape changes necessary for proper epithelial cell migration during dorsal closure. Finally, expression of a dominant negative Rab11 mutant resulted in the breakdown of apical AJs, and eventually led to major defects in epithelial integrity. Thus, the endosomal trafficking of AJ proteins in epithelial cells is important for normal embryonic development. We currently are investigating how these mutants affect the transport of other membrane proteins and how protein trafficking via these pathways is regulated during development.

### The PDZ Binding Domain of WTI1 Is Important for Assembly of Podocyte Cell-Cell Contacts

A. Mukherjee, J. H. Kim, A. Padiyar, S. H. Schechtmann, M. Konieczkowski, J. R. Schelling, J. R. Sedor; Medicine, Case Western Reserve University, Cleveland, OH

Podocyte differentiation is critical for glomerular filtration barrier function and regulated by WTI1, a zinc finger transcription factor. We have identified a novel, WTI1 interacting protein (WTIP) that maps to human chromosome 19q13.1, a region with familial focal segmental glomerulosclerosis genes. We hypothesized that WTIP is important for assembly of cell-cell contacts in cultured podocytes. In vivo WTIP is expressed in glomeruli in a podocyte pattern and co-localizes with synaptopodin. After density gradient ultracentrifugation fractionation using rat glomerular lysates, WTIP co-fractionates with nephrin and caveolin, suggesting association with nephrin in podocyte lipid rafts. In kinetic studies in cultured podocytes, GFP-WTIP co-localized with focal adhesion protein vinculin at the tips of actin stress fibers before cell-cell contacts are established and co-precipitated with the focal adhesion protein paxillin. In contacting cells, GFP-WTIP is targeted to the adherens-based cell-cell junctions and co-localized with ZO-1, -catenin and pan-cadherin. When cell-cell contacts are formed, GFP-WTIP co-precipitated with -catenin, -catenin, and cadherin, suggesting it is a component of the adherens junction complex. WTIP membrane targeting was calcium-dependent, consistent with cadherin association. The C-terminal, PDZ-binding domain is critical for cell-cell junction assembly. When GFP-WTIP knockdown is expressed, cell junctions are disordered; -catenin and ZO-1 fail to target to the plasma membrane, WTIP and podocyte adherens components no longer co-precipitate and cell-cell contacts fail to seal. In summary WTIP is a member of the adherens junction signaling and is important for formation and maintenance of adherens junctions in cultured cells and for normal glomerular slit diaphragm assembly in vivo.

### Helicobacter Pylori Significantly Decreases the Transepithelial Resistance of a Confluent Gastric Epithelial Monolayer

V. S. Conlin, R. I. Allan, D. Menard, E. D. Moore, B. B. Finlay, N. Ng, A. M. J. Buchan; 1Biological Chemistry, University of Michigan, Ann Arbor, MI, 2Molecular and Integrated Physiology, University of Michigan, Ann Arbor, MI, 3Internal Medicine, University of Michigan, Ann Arbor, MI

Objective To investigate the effects of *H. pylori* infection on the integrity of a gastric epithelial monolayer, with respect to transepithelial resistance, paracellular permeability and the distribution of paracellular junction proteins, occludin, ZO-1 and E-cadherin. Methods Transepithelial Resistance (RT) Measurements-The gastric epithelial cell line, HGE-17 was grown to confluence on snapwell filters. Cells were infected with wild type, G27 *H. pylori* and two isogenic mutants, Cap- [lacking cag pathogenicity island (PAI)] and VacA- [lacking vacuolating toxin A]. Resistances were monitored over 24 hours. Cells were then either fixed or processed for flux experiments (see below). Immunocytochemistry-Snapwell filters were fixed and labeled with monoclonal antibodies directed against occludin, ZO-1 and E-cadherin. Paracellular flux- 10microM 10,000KDa (HMW) dextran-conjugated to Texas Red was added to the apical chambers of control and infected monolayers. Samples from the basal chambers were removed and fluorescent levels measured. Results All *H. pylori* capPAI+ strains decreased the RT of *H. pylori* monolayers (G27 42% decrease, Cap- 8% decrease, VacA- 57% decrease p<0.001 *p=NS, n=6). Paracellular flux experiments (at 24 hours post-infection) revealed increased permeability across monolayers with a decreased RT, compared to control monolayers (control = 1 (normalized), G27 = 1.48, Cap- = 1.06*, VacA- = 1.25 p<0.05, *p=NS, n=6). ICC analysis revealed punctate labeling at tight junctions and a redistribution of proteins away from the lateral membranes into the cytoplasm. Conclusion Our research suggests that chronic *H. pylori* capPAI+ infection alters the barrier properties of an intact gastric epithelial monolayer. The presence of the capPAI results in redistribution of both tight junction and adherens junction proteins from the lateral membrane into the cytoplasm. In vivo, this has the potential to compromise the integrity of the gastric epithelial barrier.

### Low-dose Aspirin and Helicobacter Pylori Infection Significantly Decrease the Transepithelial Resistance of a Confluent Gastric Epithelial Monolayer

V. S. Conlin, R. I. Allan, D. Menard, E. D. Moore, B. B. Finlay, N. Ng, A. M. J. Buchan; 1Biological Chemistry, University of Michigan, Ann Arbor, MI, 2Molecular and Integrated Physiology, University of Michigan, Ann Arbor, MI, 3Internal Medicine, University of Michigan, Ann Arbor, MI

Objective To investigate the effects of aspirin and *H. pylori* on the integrity of a gastric epithelial monolayer, with respect to transepithelial resistance and the distribution of paracellular junction proteins, occludin, ZO-1 and E-cadherin. Methods Transepithelial Resistance (RT) Measurements-The gastric epithelial cell line, HGE-17 was grown to confluence on snapwell filters. Cells were treated with 5mM aspirin and/or wild type (G27) *H. pylori*. Resistances were monitored over 24 hours. Cells were then fixed at various time points or processed for paracellular flux experiments. Immunocytochemistry-Snapwell filters were fixed and labeled with monoclonal antibodies directed against occludin, ZO-1 and E-cadherin. Paracellular flux- 10microM 10,000KDa (HMW) dextran-conjugated to Texas Red was added to the apical chambers of monolayers. Samples from the basal chambers were removed and fluorescent levels measured. Results The addition of *H. pylori* to confluent HGE-17 monolayers resulted in a dramatic decrease within the initial 4 hours (aspirin 65%, aspirin & G27 68% p<0.001, n=6) and a gradual recovery over 24 hours; however, the presence of *H. pylori* impaired the RT recovery (aspirin 14%, aspirin and G27 49% p<0.05, n=6). Infection with G27 alone revealed no statistical difference in RT at 4 hours (G27 11% increase p=NS, n=6). After 24 hours a 55% decrease in monolayer RT was observed in G27 infected monolayers (p<0.05, n=6). Paracellular flux experiments revealed increased permeability across monolayers (control = 1 (normalized), G27 = 1.64, aspirin = 1.34, aspirin & G27 = 1.81 p<0.05, n=6). ICC revealed punctate labeling at tight junctions and a redistribution of proteins away from the lateral membranes into the cytoplasm. Conclusion Aspirin is thought to interfere with bicarbonate secretion, weakening the mucosal protection above the gastric epithelium. Here we provide evidence for aspirin-induced disruption of an intact gastric epithelium and how an existing *H. pylori* infection may exacerbate the situation.

### PALS1 Regulates Tight Junction Formation and E-Cadherin Trafficking in Mammalian Epithelial Cells

Q. Wang, X. Chen, B. Margolis; 1Biological Chemistry, University of Michigan, Ann Arbor, MI, 2Molecular and Integrated Physiology, University of Michigan, Ann Arbor, MI, 3Internal Medicine, University of Michigan, Ann Arbor, MI

PALS1 is an evolutionarily conserved scaffold protein that targets to the tight junction in mammalian epithelia. Prior work in our laboratory demonstrated that the knockdown of PALS1 in MDCK cells lead to tight junction and polarity defects. We have created new PALS1 stable knockdown cell lines with more profound reduction of PALS1 expression, and a more severe defect in tight junction formation was observed. Unexpectedly, we also observed severe adherens junction defect, and both defects were corrected when PALS1 wild type and certain PALS1 mutants were expressed in the knockdown cells. We found that the adherens junction structural component E-cadherin was not effectively delivered to the cell surface in the PALS1 knockdown cells, and E-cadherin vesicles accumulated in cell periphery. The ezocyst complex was also found to be mislocalized in PALS1 knockdown cells, potentially explaining why E-cadherin trafficking is disrupted. Our results suggest a broad and evolutionarily conserved role for the tight junction protein PALS1 in the biogenesis of adherens junction.
During spermatogenesis, inter-Sertoli tight junctions at the blood-testis-barrier must be assembled and disassembled in a timely regulated manner so that developing spermatocytes can translocate from the basal to the adluminal compartment of seminiferous epithelium for further development. Recently, a novel tight junction transmembrane protein, CLMP, was found to be expressed in mouse testis. However, the functional importance and its regulation in testis are entirely unknown. By transient transfection assay performed in a mouse Sertoli cell cell line, TM4 cells, we found that the minimal CLMP promoter was located between nucleotides -550 and -288 (relative to translation start site). By the use of site-directed mutagenesis, three motifs namely GATA, KLF4 and SRY within the minimal promoter region were found to be functionally cooperated with one another to regulate CLMP gene transcription. By EMSAs, a ternary protein complex consisting of GATA-1/GATA-6/KLF4 was found to be present in all the three identified motifs, suggesting that a looping mechanism might involve in regulating CLMP gene transcription.

Interestingly, apart from the identification of three transcription factors in ternary protein complex, a ubiquitously expressed transcription factor Sp1 was also found in this ternary complex over the KLF4 motif. Overexpression of KLF4 significantly increased the promoter activity whilst overexpression of Sp1 has an opposite effect. Co-transfection studies showed that Sp1 could significantly abolish KLF4 transactivation of the CLMP promoter, suggesting that KLF4 and Sp1 might compete for the same binding site on the CLMP promoter. Taken together, the differential interaction of transcription factors, GATA-1, GATA-6, KLF4 and Sp1, might provide a precise machinery in regulating CLMP gene expression in Sertoli cells. This work was supported by Hong Kong Research Grant Council grants (HKU7536/05M and HKU 7599/06M).

2452
Identification of a New Protein That Interacts with the Tight Junction Protein ZO-2
S. Lechuga, J. Solano, L. Gonzalez-Marial; Physiology, Biophysics and Neuroscience, Center for Research and Advanced Studies (Cinvestav), Mexico, D.F., Mexico
With the purpose of identifying proteins that interact with the middle portion of the tight junction (TJ) protein ZO-2, we have used a yeast two-hybrid system with the 3PSG segment of ZO-2 as bait. 3PSG includes the third PDZ domain, the SH3 region and the GK module (1555-1621 m) of ZO-2 canine cDNA. The sequence of the isolated protein, named protein binding 3PSG (pb3PSG), is matched to human chromosome 7q22, has an open reading frame of 737 bp and exhibits polyadenylation sites at its 3’ end. The sequence of pb3PSG predicts a protein containing a NES, a WW domain, two SH3 binding motifs PXSP, and a PDZ binding motif located 5 residues upstream of the carboxyl terminal end. Gene specific primers for pb3PSG allowed the RT-PCR amplification of the corresponding cDNA in epithelial MDCK cells. Down pulls assays confirm the interaction of a pb3PSG-GST fusion protein with ZO-2 present in an extract of MDCK cells. When the PDZ binding motif of pb3PSG-GST is altered by a point mutation (V163A), the association to ZO-2 is lost, while the mutation of the PXXP motifs (P112Q, P113S) exerts no effect. Transfected pb3PSG, co-immunoprecipitates with ZO-2 in MDCK cells, indicating an in vivo interaction between these two proteins. In epithelial cells the transfected protein pb3PSG concentrates at the nucleus and the cellular borders. The overexpression of pb3PSG diminishes the cell border presence of ZO-2.

2453
The Tight Junction Protein ZO-2 Has Four Functional Nuclear Export Signals
L. Alarcon, A. Ponce, B. E. Jaramillo, L. Gonzalez-Marial; Physiology, Biophysics and Neuroscience, Center for Research and Advanced Studies (Cinvestav), Mexico, D.F., Mexico
The tight junction (TJ) protein ZO-2 changes its subcellular distribution according to the state of confluency of the culture. Thus in confluent monolayers it localizes at the TJ region whereas in sparse cultures it concentrates at the nucleus. The canonical sequence of ZO-2 displays four putative nuclear export signals (NESes), two at the second PDZ domain (NES-0 and NES-1) and the rest at the GK region (NES-2 and NES-3). The functionality of NES-0 and NES-3 was unknown; hence we have explored it with a nuclear export assay, injecting into the nucleus of MDCK cells peptides corresponding to the ZO-2 NES sequences chemically coupled to ovalbumin. We show that both NES-0 and NES-3 are functional and sensitive to leptomycin B. We also demonstrate that NES-1, previously characterized as a non-functional NES, is rendered capable of nuclear export upon the acquisition of a negative charge at its Ser148 residue. Experiments performed injecting at the nuclear WT and mutated ZO-2 GST fusion proteins revealed the need of both NES-0 and NES-1, and NES-2 and NES-3 for attaining an efficient nuclear exit of the respective amino and middle segments of ZO-2. Moreover, the transfection of MDCK cells with full length ZO-2 revealed that the mutation of any of the NESes present in the molecule is sufficient to induce nuclear accumulation of the protein.

2454
Deciliation Is Associated with Dramatic Remodeling of Polarized Epithelial Cells
C. E. Overgaard, A. Sandra, C. Yeaman; Anatomie & Cell Biology, University of Iowa, Iowa City, IA
Primary cilia mediate various chemo- and mechanosensory functions, and ciliary defects are associated with diseases affecting kidney and liver epithelia. To test the hypothesis that ciliary signaling regulates epithelial polarity, we have pharmacologically deciliated cells with chloroquine (CQ) or dibucaine. Deciliation of polarized MDCKII monolayers promotes tight junction remodeling resulting in significant, but reversible increase in trans-epithelial resistance. This reflects coordinated reduction in tight junction proteins associated with "leaky" epithelia (eg. Claudin-2) and increase in proteins associated with "tight" epithelia (eg. Claudins-4 and -7). Deciliation also affects junction remodeling in other ways. In control cells, calcium chelation inhibits cadherin-mediated adhesion, and constituents of intercellular junctions are internalized. However, deciliated cells maintain tight intercellular contacts in the absence of extracellular calcium and fail to internalize junctional complexes. Deciliation also promotes redistribution of non-cadherin-associated apical proteins. Specifically, gp114 is redistributed into a novel apical pattern and ultimately misoriented to the basolateral surface. In addition, gp80/clusterin, a protein that is normally secreted apically, is randomly secreted when cilia are removed. Dramatically, expression of gp135/podocalyxin, a protein that serves to organize apical subdomains, is significantly reduced. Deciliated cells in 3-D collagen/matrik gel cultures do not form organized cysts with well-defined apical and basolateral domains. Furthermore, polarity reversal characteristic of untreated cells upon transfer of cysts from agasone to matrigel is impaired following deciliation. Insight into mechanisms responsible for these effects is provided by analysis of clonal MDCK lines impaired in ciliogenesis, showing that cilia resorption does not, by itself, result in junction remodeling and that presence of a mature primary cilia is not required for the deciliation-triggered effects. Also, LLC-PK1 cells neither lose primary cilia nor remodel junctions following CH treatment. We conclude that CH targets a mechanism that functions to promote deciliation, tight junction remodeling, altering post-Golgi sorting and cell polarity.

2455
Mechanism of NC-1059 Induced Increase in Paracellular Permeability of Epithelial Monolayers
S. Somasekharan, J. M. Tomich, D. Schuler; 1Biochemistry, Kansas State University, Manhattan, KS, S. Anatomy and Physiology, Kansas State University, Manhattan, KS
The goal of this study is to identify the mechanisms by which NC-1059 increases epithelial paracellular permeability. NC-1059 is a synthetic channel forming peptide that increases short circuit current (Isc) and decreases transepithelial resistance (Rte) in a variety of epithelia. Exposure of MDCK monolayers to 100 and 200 micromolar NC-1059 results in a concentration-dependent change in both Lsc and Rte. NC-1059 enhances the permeation rate of large molecular weight dextrans across monolayers, which documents a peptide induced opening of the paracellular route suggesting a reorganization of tight junction proteins. The effects on immunolabeling of occludin, ZO-1 and actin as viewed with confocal microscopy reveal substantial redistribution or loss of these proteins upon exposure to both 100 and 200 micromolar NC-1059 in MDCK monolayers. The effect of NC-1059 on the tight junctions and actin occurs in a time and concentration dependant pattern with a more rapid change occurring at 200 micromolar as compared to 100 micromolar. 100 micromolar NC-1059 causes the Rte of MDCK monolayers to drop by at least 90%. The epithelia show an average recovery in Rte of approximately 34 % at 24 hours which reaches pretreatment levels after 48 hours. The modulation of the epithelial barrier in a reversible manner has therapeutic potential to increase the efficiency of drug delivery across barrier membranes. (Supported by GM 074096 to JMT)

2456
Urinary Epithelial Growth Factor; Plausible Physiological Regulator of Tight Junction Permeability along the Nephron
D. Flores-Benitez, A. Ruiz-Cabrera, C. Flores-Maldonado, M. Cereijido, R. G. Contreras; 1Fisiologia, Biofisica y Neurociencias, CINVESTAV, D.F., Mexico, 2Laboratorio de Medicina, FES-Iztacala, UNAM, D.F., Mexico
Epithelia can adjust the permeability of their paracellular permeation route to physiological requirements, pathological conditions, and pharmacological challenges. This is reflected by a substrate dependent change in both Lsc and Rte. NC-1059 enhances the permeation rate of large molecular weight dextrans across monolayers, which documents a peptide induced opening of the paracellular route suggesting a reorganization of tight junction proteins. The effects on immunolabeling of occludin, ZO-1 and actin as viewed with confocal microscopy reveal substantial redistribution or loss of these proteins upon exposure to both 100 and 200 micromolar NC-1059 in MDCK monolayers. The effect of NC-1059 on the tight junctions and actin occurs in a time and concentration dependant pattern with a more rapid change occurring at 200 micromolar as compared to 100 micromolar. 100 micromolar NC-1059 causes the Rte of MDCK monolayers to drop by at least 90%. The epithelia show an average recovery in Rte of approximately 34 % at 24 hours which reaches pretreatment levels after 48 hours. The modulation of the epithelial barrier in a reversible manner has therapeutic potential to increase the efficiency of drug delivery across barrier membranes. (Supported by GM 074096 to JMT)
2457 IL-1β Modulation of Epithelial Tight Junction Permeability
R. Al-Sadi, 1 T. Ma 1; 1 Internal Medicine, University of New Mexico, Albuquerque, NM; 2 VA Medical Center, Albuquerque, NM

IL-1β is a prototypical pro-inflammatory cytokine that plays a central role in the regulation of epithelial barrier function. Recent studies have shown that IL-1β affects the expression of tight junction proteins in various cell types, including enterocytes, intestinal epithelial cells, and Sertoli cells. In this study, we investigated the effects of IL-1β on tight junction permeability in Caco-2 cells, a cell line that has been widely used as a model for intestinal epithelial cells.

We found that IL-1β induced a progressive time-dependent increase in transepithelial permeability in Caco-2 cells. This effect was mediated in part by the activation of NF-κB pathways but not apoptosis. Further, the IL-1β-induced increase in TJ permeability was mediated in part by the activation of NF-kappaB pathways but not apoptosis.

2458 Differential Modulation of Claudins in Skin Tumours
K. Turkson, A. Arabzadeh, T. Troy; Ottawa Health Research Institute, Ottawa, ON, Canada

It is widely recognized that the Claudin (Cldn) family of tetraspan transmembrane proteins is crucial for skin barrier function. Recent studies indicate that Cldn overexpression and Cldn deletion mutants are associated with cell permeability defects in vivo, and that the level of Cldn expression appears to be crucial in skin barrier integrity and function. Knowledge of the overall structure-function of the Cldn family of proteins is limited, however they are thought to have three distinct and characteristic functional domains: (i) four transmembrane-spanning regions, (ii) two extracellular loops responsible for barrier formation within the paracellular space and specific ion selectivity residing within the first extracellular loop; and (iii) a cytoplasmic C-terminus that functions as a transmembrane protein.

2459 Dual Transcriptional Control of Claudin-11 via an Overlapping GATA/NF-Y Motif: Positive Regulation through the Interaction of GATA, NF-Y, and CREB, and Negative Regulation through the Interaction of SMAD, HDAC1, and mSIN3A
L. Wang, W. Lee, Zoology, The University of Hong Kong, Hong Kong

The expression of claudin-11, a key tight junction protein, is tightly regulated to ensure that the integrity of the semipermeable epithelium can be maintained during the translocation of spermatozoa at the blood-testis barrier at stages VIII-IX. In this study, we elucidate how the overlapping GATA/NF-Y motif within the core promoter of claudin-11 gene is modulated by differential binding of various transcription factors, resulting in dual transcriptional control. We have found that GATA, NF-Y, and CREB form a complex in vivo and bind to the GATA/NF-Y region to promote claudin-11 gene transcription. GATA and CREB transactivation could be further modulated by the presence of Smad3 and Smad4 proteins. Binding of Smad proteins at the GATA/NF-Y motif could repress the GATA and CREB transactivation of claudin-11 gene.

2460 Occludin and Claudin-11 Participate in the Formation of Epidermal Tight Junction and the Barrier Function in Human Keratinocytes
Y. Ito, 1 M. Kasurawa, 2 S. Kuroda, 1 Y. Yamamoto, 1 H. Sasaki 1; 1 POLE Chemical Industries, Inc., Yokohama, Japan; 2 Department of Molecular Cell Biology, Institute of DNA Medicine, Jikei University School of Medicine, Tokyo, Japan

The tight junctions (TJs) of the epidermis are the most apical component of the junctional complex and play a vital role in cell-cell adhesion in epithelial and endothelial cells. TJs create a primary paracellular barrier that regulates the movement of water and solutes, and also keep cell polarity to divide the apical and the basolateral membrane domains. In the epidermis of the skin, the evidence suggests that the epidermal barrier should be contributed not only by the stratum corneum but also by TJs in the granular layer of the epidermis are accumulated. The detailed mechanism of the epidermal barrier, however, is not clearly understood. The roles of TJs and the barrier function in human skin epidermal keratinocytes, we investigated the interactions of TJ molecules using the differentiated keratinocytes induced by calcium switching (3.3 mM) and RNA interference of TJs molecules. After the calcium switching, TJs molecules that characterised claudin-11, occludin and claudin-12 were localized to cell membrane and the transepithelial electrical resistance (TER) was elevated in accordance with keratinocyte differentiation.

2461 TNFα Induces Degradation of CLMP mRNA in Sertoli Cells through a JNK-dependent Pathway
K. Sze, W. Lee, W. Lui; Zoology, The University of Hong Kong, Hong Kong

During spermatogenesis, extensive restructuring of blood-testis barrier takes place to facilitate the migration of preleptotene /leptotene spermatocytes from the basal to the adluminal compartment in the seminiferous epithelium. However, the biochemical mechanisms that regulate this event remain largely unknown. Recent studies have shown that proinflammatory cytokine tumor necrosis factor-alpha (TNFα) plays a crucial role in this event by inhibiting the expression of tight junction transmembrane proteins in Sertoli cells. In this study, we sought to examine the effect of TNFα on the expression of CLMP (cox-5a-like membrane protein), a novel identified tight junction transmembrane protein, in the testis. Addition of TNFα (10 ng/ml) to Sertoli cell culture (TM4 cells) significantly reduced the steady-state CLMP mRNA level as shown in quantitative RT-PCR. In the presence of actinomycin D, the TNFα-induced decrease in CLMP mRNA expression was not affected. On the other hand, occludin expression did not influence the localization of claudin-1, suggesting that occludin plays a more important role in the formation of epidermal barrier than claudin-1 itself, and claudin-1 is rather important for recruiting occludin to cell membrane to form epidermal permeability barrier. These data provided us the new insight into not only the TJ localization and formation during keratinocyte differentiation but also the roles of TJs on epidermal barrier function.

2462 Cldn12 and Cldn18 in different stages of mouse skin carcinogenesis. The expression of Cldns may also be involved in the initiation and progression of carcinogenesis. Here, we present data on the expression of Cldn1, Cldn6, Cldn11, Cldn12 and Cldn18 in different stages of mouse skin carcinogenesis. The comparison of Cldn expression on the keratinocyte membranes of papilloma cells clearly revealed expression and localization changes correlating with the level of tumour differentiation. This work is supported by the Canadian Institutes of Health Research.

2463 Regeneration through the Interaction of SMAD, HDAC1, and mSIN3A
W. Lui, E. Wong, W. Lee, Zoology, The University of Hong Kong, Hong Kong

The expression of claudin-11, a key tight junction protein, is tightly regulated to ensure that the integrity of the semipermeable epithelium can be maintained during the translocation of spermatozoa at the blood-testis barrier at stages VIII-IX. In this study, we elucidate how the overlapping GATA/NF-Y motif within the core promoter of claudin-11 gene is modulated by differential binding of various transcription factors, resulting in dual transcriptional control. We have found that GATA, NF-Y, and CREB form a complex in vivo and bind to the GATA/NF-Y region to promote claudin-11 gene transcription. GATA and CREB transactivation could be further modulated by the presence of Smad3 and Smad4 proteins. Binding of Smad proteins at the GATA/NF-Y motif could repress the GATA and CREB transactivation of claudin-11 gene. Such repression required the recruitment and physical interactions of histone deacetylase 1 and its co-repressor, mSin3A with Smad proteins. It is believed that cyclic changes in the ratio of positive regulators (GATA, NF-Y and CREB) to negative regulators (Smads) in the semipermeable epithelium during the spermatogenic cycle might provide a precise control in claudin-11 gene transcription. This work was supported by the Hong Kong Research Grant Council grant (HKU7536/05M), HKUCRCG Small Project Funding and HKUCRCG Seed Funding.

2464 Ocludin and Claudin-11 Participate in the Formation of Epidermal Tight Junction and the Barrier Function in Human Keratinocytes
Y. Ito, 1 M. Kasurawa, 2 S. Kuroda, 1 Y. Yamamoto, 1 H. Sasaki 1; 1 POLE Chemical Industries, Inc., Yokohama, Japan; 2 Department of Molecular Cell Biology, Institute of DNA Medicine, Jikei University School of Medicine, Tokyo, Japan

The tight junctions (TJs) are the most apical component of the junctional complex and are responsible for barrier formation within the paracellular space and specific ion selectivity residing within the first extracellular loop; and (iii) a cytoplasmic C-terminus that functions as a transmembrane protein.

2465 Dual Transcriptional Control of Claudin-11 via an Overlapping GATA/NF-Y Motif: Positive Regulation through the Interaction of GATA, NF-Y, and CREB, and Negative Regulation through the Interaction of SMAD, HDAC1, and mSIN3A
W. Lui, E. Wong, W. Lee, Zoology, The University of Hong Kong, Hong Kong

The expression of claudin-11, a key tight junction protein, is tightly regulated to ensure that the integrity of the semipermeable epithelium can be maintained during the translocation of spermatozoa at the blood-testis barrier at stages VIII-IX. In this study, we elucidate how the overlapping GATA/NF-Y motif within the core promoter of claudin-11 gene is modulated by differential binding of various transcription factors, resulting in dual transcriptional control. We have found that GATA, NF-Y, and CREB form a complex in vivo and bind to the GATA/NF-Y region to promote claudin-11 gene transcription. GATA and CREB transactivation could be further modulated by the presence of Smad3 and Smad4 proteins. Binding of Smad proteins at the GATA/NF-Y motif could repress the GATA and CREB transactivation of claudin-11 gene. Such repression required the recruitment and physical interactions of histone deacetylase 1 and its co-repressor, mSin3A with Smad proteins. It is believed that cyclic changes in the ratio of positive regulators (GATA, NF-Y and CREB) to negative regulators (Smads) in the semipermeable epithelium during the spermatogenic cycle might provide a precise control in claudin-11 gene transcription. This work was supported by the Hong Kong Research Grant Council grant (HKU7536/05M), HKUCRCG Small Project Funding and HKUCRCG Seed Funding.

2466 Occludin and Claudin-11 Participate in the Formation of Epidermal Tight Junction and the Barrier Function in Human Keratinocytes
Y. Ito, 1 M. Kasurawa, 2 S. Kuroda, 1 Y. Yamamoto, 1 H. Sasaki 1; 1 POLE Chemical Industries, Inc., Yokohama, Japan; 2 Department of Molecular Cell Biology, Institute of DNA Medicine, Jikei University School of Medicine, Tokyo, Japan

The tight junctions (TJs) are the most apical component of the junctional complex and are responsible for barrier formation within the paracellular space and specific ion selectivity residing within the first extracellular loop; and (iii) a cytoplasmic C-terminus that functions as a transmembrane protein.
Taken together these results, we conclude that ZO-2 downregulates cyclin D1 transcription, probably by interacting with the c-Myc/E box element and recruiting HDAC1. We use ChIP and reporter gene assays employing HDACs inhibitors. Our results demonstrated that HDACs are necessary for ZO-2 repression and also that HDAC1 is recruited to the E box.

To understand how ZO-2 represses cyclin D1 promoter activity, we used deletion analyses, finding that ZO-2 negatively regulates cyclin D1 transcription via a distal E box present in cyclin D1 promoter. To establish the identity of transcription factors bound to this E box in MDCK, we employed EMSA and ChIP assays that pointed to the transcription factor c-Myc. Over-expression of c-Myc and ZO-2 resulted in a clear negative effect over transcriptional promoter activity, where c-Myc is acting as a repressor, cooperating additively with ZO-2. We also noticed that ZO-2 localizes at the E box region by ChIP assays in MDCK cells. Finally, we explored HDACs participation in cyclin D1 downregulation by the c-Myc/ZO-2 combination. To address this question, we use ChIP and reporter gene assays employing HDACs inhibitors. Our results demonstrated that HDACs are necessary for ZO-2 repression and also that HDAC1 is recruited to the E box. Taken together these results, we conclude that ZO-2 downregulates cyclin D1 transcription, probably by interacting with the c-Myc/E box element and recruiting HDAC1.

Rho-ROCK II Signaling Regulates Disassembly of Epithelial Apical Junctions
S. N. Samarin, A. I. Ivanov, C. A. Parkos, A. Nusrat; Pathology, Emory University, Atlanta, GA

The apical junctional complex (AJC) encompassing tight junction (TJ) and adherens junction (AJ) regulates epithelial barrier function. Diverse physiologic and pathologic stimuli have been observed to induce reversible disassembly of AJC associated with disruption of epithelial barrier. Nevertheless, signaling pathways that regulate A JC disassembly are poorly understood. Using a calcium depletion model of rapid junctional disassembly, we have previously shown that disintegration of the AJC is driven by myosin II-mediated contraction of the peripheral acto-myoelin ring. This study was designed to dissect signaling pathways upstream of myosin II that control A JC disassembly. Depletion of extracellular calcium in TS4 and SK-C015 model intestinal epithelial cell lines induced reorganization of the perijunctional F-actin into contractile rings, which was accompanied by disruption and internalization of apical junctions. Pharmacological inhibition and protein localization studies revealed no role for myosin light chain kinase (MLCK) in disruption of the A JC. In contrast, inhibition of Rho-associated kinase (ROCK) with Y-27632 and H-112 prevented disassembly of the AJC and loss of epithelial barrier function in calcium-depleted cells. Moreover, both isoforms of ROCK, ROCK I and ROCK II were recruited to contractile F-actin rings. However, only siRNA knock-down of ROCK II attenuated A JC disassembly. Additionally, Rho GTPase, an upstream activator of ROCK, was transiently activated and co-localized with internalized junctional proteins in calcium-depleted cells. Pharmacological inhibition of Rho with a Rho specific inhibitor DCBII completely prevented A JC disassembly. GEF-H1, a guanine nucleotide exchange factor for Rho, also accumulated in contractile F-actin rings in calcium-depleted cells. Furthermore, si-RNA-mediated down-regulation of GEF-H1 expression significantly attenuated disassembly of A JC. These results suggest that GEF-H1/Rho/ROCK II signaling pathway is a major regulatory mechanism responsible for disassembly of epithelial A JC.

Negative Curvature Contributed by Specific Lipids Is an Essential Component of Ca2+-triggered Native Membrane Fusion
M. A. Churchward, T. Rogashevskia, R. C. Taylor, J. R. Coorsen; 1Physiology and Biophysics, University of Calgary, Calgary, AB, Canada, 2Hotchkiss Brain Institute, University of Calgary, Calgary, AB, Canada

Fast Ca2+-triggered membrane merger is the defining step of regulated exocytosis. In native secretory vesicles, cholesterol (CHOL) functions in the fundamental fusion (e.g. bilayer merger) mechanism (1), and CHOL sphingomyelin - enriched microdomains define the efficiency (Ca2+ sensitivity and kinetics) of fusion (2). The contribution of cholesterol to the process of membrane fusion itself is mimicked by structurally dissimilar lipidic membrane components having spontaneous negative curvatures similar to or greater than that of CHOL. Dioleoylphosphatidylethanolamine with a single mol% of cholesterol, together triggered bilayer merger of CHOL-depleted vesicles, correlating quantitatively with the negative curvature each contributes to the membrane. Unable to substitute for CHOL in stabilizing microdomains, these lipids do not rescue fusion efficiency. Lipids of less spontaneous negative curvature than CHOL, including dioleoylphosphatic acid, are unable to effectively support fusion. This quantitative relationship between negative curvature and membrane fusion appears most consistent with the stalk-pore model (3), demonstrating that negative curvature itself is an essential component of the fundamental native fusion mechanism. The data also suggest that different fusion sites, vesicles, or secretory cell types could use other lipidic components, in addition to CHOL or similar endogenous sterols, to provide optimal local negative curvature and even to modulate the fusion process.


Hemifusion Arrest by Complex Is Relieved by Ca2+-Synaptotagmin I
B. Doneske, 1 J. R. Schaub, 2 K. Lu, 2 Y. Shin, 1 J. A. McNew; 1Department of Biochemistry and Cell Biology, Rice University, Houston, TX, 2Department of Biochemistry, Biophysics, and Molecular Biology, Iowa State University, Ames, IA

Synaptic transmission relies on an exquisitely orchestrated series of protein-protein interactions that ultimately results in neurotransmitter release into the synaptic cleft. The final step in this reaction is membrane fusion of the synaptic vesicle with the presynaptic plasma membrane catalyzed by SNARE proteins. Several extrinsic protein factors impinge on the cycle of SNARE complex assembly to regulate vesicle release in space and time. These include general regulators such as the Rab family of GTPases and the Munc18/Sec1 (SM) proteins, as well as specific regulators of synaptic transmission including complexin and synaptotagmin. The interplay of these regulatory factors and their precise mechanisms of action remain an area of intense investigation. Here we show that fusion driven by a t-SNARE complex of Syntaxin/I/SNAP25 and the v-SNARE VAMP2, but not their yeast equivalents, is inhibited by complexin. Furthermore, inner leaflet mixing is strongly impaired related to total lipid mixing indicating that inhibition by complexin arrests fusion at a hemifusion intermediate. When the calcium sensor synaptotagmin is added in the absence of calcium to the complex-inhibited reaction, the arrest persists. However, when calcium is introduced, complexin inhibition is relieved and full fusion rapidly proceeds as evidenced by restoration of inner leaflet mixing. Our results suggest that the combination of complexin and synaptotagmin provides a strong calcium-dependent clamp to inhibit full fusion at hemifusion until released by calcium influx.

Sphingosines Interfere with Antigen-stimulated, Calcium-dependent Fusion of Recycling Endosomes with the Mast Cell Plasma Membrane by Perturbing its Inner Leaflet
N. L. Smith, D. Holowka, B. Baird; Chemistry and Chemical Biology, Cornell University, Ithaca, NY

Proteins and lipids undergo trafficking to and from the plasma membrane via a spatially organized pool of intracellular membranes termed recycling endosomes. FITC-labeled cholera toxin B (FITC-CtxB) bound to the ganglioside GM1 is used to monitor IgG receptor stimulated trafficking of these endosomes to the plasma membrane in RBL mast cells (Naal et al., Traffic 4, 190, 2003). This process is refractory to most inhibitors of stimulated degranulation in these cells. We find that certain sphingosine derivatives, including D- or L-sphingosine and N,N-dimethylsphingosine (DMS), effectively inhibit this endosomal trafficking response stimulated by multivalent antigen or calcium ionophore. These amphiphiles cause a transient relief of self-quenching of FITC-CtxB at the cell surface that is restored as these compounds flip to the inner leaflet. In contrast to sphingosine and DMS, trimethyl sphingosine (TMS) stably inserts into the outer leaflet of the plasma membrane where it causes sustained relief of FITC-CtxB self-quenching and does not inhibit stimulated trafficking. Unlike their effect on recycling endosome trafficking, sphingosine and DMS only modestly inhibit ionophore-stimulated exocytosis of secretory lysosomes, suggesting different mechanisms for exocytosis of these two intracellular organelles. Our results are consistent with a model in which sphingosines alter intracellular signaling processes by physical perturbation of the cytoplasmic leaflet of cell membranes.

Thiol-Reactivity as a Route to Identifying Proteins Involved in Ca2+-Triggered Membrane Fusion
K. L. Ferber, 1 D. M. Brandman, 2 A. L. Yergey, 1 J. R. Coorsen; 1Physiology & Biophysics, Hotchkiss Brain Institute, Faculty of Medicine, University of Calgary, Calgary, AB, Canada, 2Cellular & Molecular Biophysics, NICHD, National Institutes of Health, Bethesda, MD

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Ca2+-triggered membrane fusion is an essential and conserved process for the rapid release of cellular components. Despite substantial progress in identifying proteins involved in exocytosis, the mechanisms underlying regulated membrane merger remain poorly understood. Here we use isolated sea urchin egg cortical vesicles (CV), a minimal, stage-specific preparation of Ca2+-sensitive, release-ready secretory vesicles, to investigate the proteins involved in the native fusion mechanism. Thiol-reactive reagents, which alkylate free sulphydryl groups, have been consistently shown to dose-dependently inhibit the extent, Ca2+-sensitivity, and kinetics of triggered fusion. However, in this study, iodocadaverine (IA) was found to selectively enhance the Ca2+-triggered steps of membrane fusion. Treatments of 10mM-100mM IA caused an increase in Ca2+-sensitivity indicated by a maximal leftward shift in EC50 of 14.4 ± 1.5µM [Ca2+]o, with treatments in the range of 20mM-80mM IA also potentiating fusion kinetics. If Sr2+; a weak Ca2+ mimetic, was used to trigger fusion, the increase in Sr2+-sensitivity was approximately 2-fold compared to the increase in Ca2+-sensitivity. Further investigation established that IA did not increase the extent or quality of CV attachment / docking either to the plasma membrane or to other CV. Comparison of IA to other thiol-reactive reagents indicates that the differential effects of these reagents can be attributed to lipophilicity. Ongoing studies show that the same enhancement of fusion can be elicited by treatment with Lucifer yellow IA (LYIA); therefore, this fluorescent IA can be employed to simultaneously potentiate fusion and label the critical protein(s) involved. By coupling the functional assays with two-dimensional gel electrophoresis (2DE), proteins that showed a dose-dependent increase in LYIA labeling, corresponding to an increased efficiency of fusion, were isolated. These are currently being sequenced by mass spectrometric techniques. This work identifies potential candidate proteins that may act at or near the fast Ca2+-triggered steps of native membrane fusion.

Quantitative Analysis of Recycling Endosomal Trafficking in RBL Mast Cells Revealed by Real-Time Fluorescence Microscopy

S. Hammond, N. L. Smith, D. Holowka, B. Baird; Chemistry and Chemical Biology, Cornell University, Ithaca, NY

Recycling endosomes are spatially organized pools of intracellular membranes that dynamically recycle endocytosed lipids and proteins back to the plasma membrane. This membrane pool can be labeled by cholera toxin subunit B (CTxB), which binds to the ganglioside GM-1, and fluorescently tagged CTxB serves as a marker to monitor trafficking from this organelle in mast cells. Stimulation via IgE receptors, FceRI, results in a net outward trafficking response, as monitored by an increase in FITC-CTxB fluorescence (Naal et al., Traffic, 4:190, 2003). To further characterize this process, we have developed a real-time assay to monitor outward trafficking of recycling endosomes to the plasma membrane in individual cells using three-color fluorescence confocal microscopy. In this approach, surface GM1 is first blocked by unlabeled CTxB; upon stimulation, newly delivered GM1 from recycling endosomes can then bind Alexa488-CTxB, resulting in increased fluorescence at the plasma membrane. On average, stimulation by antigen or calcium ionophore increases the rate of net outward trafficking of recycling endosomes 2-3-fold. In addition, D-sphingosine inhibits the rate of calcium ionophore-stimulated outward trafficking of recycling endosomes, consistent with the results from our fluorimetric assay. Spatial proximity of newly-trafficked CTxB/GM1 to FceRI and other cell surface components can also be monitored by this method. The development of a microscopy method to study this process will be useful in characterizing the functional roles and mechanism of recycling endosomal trafficking in these and other cells.

Regulation of Syntaxin 7 Expression and Function in Macrophages by CSF-1

K. Subraamnian; Biochemistry Department, University of Osnabrueck, Osnabrueck, Germany

Protein palmitoylation is a reversible lipid modification by fatty acids, usually palmitic acid, to cysteine thiols. It stably anchors specific proteins to membranes, but may also have a direct effect on the function of a protein. The yeast protein Vac8 is required for efficient vacuole fusion, inheritance and cytosol-to-vacuole trafficking. It is anchored to vacuoles by an N-terminal myristoylation site and three palmitoylation sites, also known as the SH4 domain. Here, we address the role of Vac8 palmitoylation and show that the position and number of substrate cysteines within the SH4 domain determine the vacuole localization of Vac8: stable vacuole binding of Vac8 requires two cysteines within the N-terminus, regardless of the combination. Importantly, our data suggest that palmitoylation adds functionality to Vac8 beyond simple localization. A mutant Vac8 protein, in which the palmitoylation sites were replaced by a stretch of basic residues, still localizes to vacuole membranes and functions in cytosol-to-vacuole transport, but can only complement the function of Vac8 in morphology and inheritance if it also contains a single cysteine within the SH4 domain. Our data suggest that palmitoylation is not a mere hydrophobic anchor required solely for localization, but influences the protein function(s).

Interaction of SNAREs with ArfGAPs Precedes Recruitment of Sec18p/NSF

C. Schindler, A. Spang; Biozentrum, University of Basel, Basel, Switzerland

SNAREs are key components of the fusion machinery in vesicular transport and in homotypic membrane fusion. The ArfGAP Gsc1p accelerated v-t-SNARE complex formation in vitro, indicating that ArfGAPs may act as folding chaperones. These SNARE complexes were resolved in the presence of ATP by the yeast homologues of α-SNAP and NSF, Sec17p and Sec18p, respectively. Furthermore, the ArfGAPs Gsc1p and Glp3p, but not Age2p, induced conformational changes in v- and t-SNAREs that promoted recruitment of the small GTPase Arf1p in stoichiometric amounts. Yet, not Arf1p bound to the ‘activated’ SNAREs: Sec1p and Sec1p also recognized the SNAREs even when they were not engaged in v-t-SNARE complexes. Here again the induction of a conformational change by ArfGAPs was essential. Surprisingly, the mere recruitment of Sec1p to SNAREs did not require Sec17p or ATP hydrolysis. Moreover, Sec1p displaced prebound Arf1p from SNAREs, indicating that Sec1p may have more than one function: one to ensure that all vesicle coat proteins are removed from the SNAREs before the engagement in a trans-SNARE complex and a second one to resolve cis-SNARE complexes after fusion has occurred.

Neuronal Sec1 Binds the t-SNARE Complex and Stimulates Fusion

T. L. Rodkey, J. A. McNew; Biochemistry & Cell Biology, Rice University, Houston, TX

Neuronal Sec1 binds the t-SNARE complex and stimulates fusion driven by neuronal SNAREs in a concentration and time dependent manner. However, stimulation is specific for Syntaxin1a/SNAP25 and VAMP2 since their yeast counterparts are unaffected by the addition of n-Sec1.
Membrane fusion was blocked. Experiments are underway to determine if FusMp and Fus1p interact with each other. Blast analysis shows that fusion. Bioassays demonstrated that gametes transformed with HA-tagged FusMp showed that FusMp is localized on the male mating structure and preliminary results indicate that FusMp levels were reduced immediately after undergoing normal flagellar adhesion with female gametes, but failed to fuse. Transformation with the wild-type gene rescued fusion in cells and we have systematically studied their topology and assembly pathway. Our results suggest that both topology and assembly pathway of trans-SNARE complexes differ from the ones suggested based on earlier studies with purified proteins. These discrepancies probably reflect the control of SNARE conformation and association imposed by accessory factors in the physiological membrane.

The formation of complexes between v- and t-SNAREs is believed to be crucial for many intracellular fusion reactions. Studies with purified SNARE proteins have suggested that the t-SNARE subunits assemble on one fusion partner and that the v-SNARE is donated by the other fusion partner. We have now used a physiological membrane system, the cell-free fusion of yeast vacuoles to detect trans-SNARE complexes and we have used purified recombinant proteins, we demonstrate that yeast Sec1p binds directly and specifically to ternary SNARE complexes in vitro. Our data are consistent with models in which Sec1p binding is sensitive to the assembly state of the target membrane SNAREs. We have also initiated trials for obtaining crystals of Sec1-SNARE complexes to understand the molecular details of this interaction.

The Conserved Oligomeric Golgi (COG) complex is a widely conserved eight-subunit tethering complex required for retrograde Golgi transport, and hence for proper maintenance of Golgi protein localization. Defects in several subunits, including Cog7, have been identified as causes of congenital disorders of glycosylation in humans. The complex is thought to act upstream of SNAP Receptor (SNARE) involvement by tethering the incoming vesicle to its target membrane at a distance. Supporting this, the complex has been shown to interact with SNAREs, and several of the mammalian complex, including Cog5, have been shown to interact with Golgi-localized Rab proteins which regulate vesicle tethering. Existing partial structures of S. cerevisiae Cog2p and of subunits of the weakly homologous Exocyst complex display a common theme of bundles of α-helices, but the crystal structures of the majority of the COG subunits, and of any subunit in its context in the complex, have yet to be determined. To the end of obtaining the crystal structure of a greater number of subunits while preserving their interactions, we have purified from E. coli a recombinant subcomplex of S. cerevisiae COG consisting of the strongly interacting Cog5p and Cog7p. Proteolytic fragments of these proteins in complex, suitable for crystallographic trials, have been identified and purified, and indicate that the N-terminal 80 residues of Cog5p appear to contain its interaction domain with Cog5p.

The molecular mechanisms of gamete fusion in fertilization are unknown. The aim of this work is to study the forces that drive cell fusion from local intermediates to syncytium formation. The event of fusion is a critical step in development and in enveloped virus infection. While a lot is known about structures of some fusogenic proteins and early fusion intermediates, later fusion stages that involve the expansion of the initial fusion pore to fully merge the volumes of the fusing cells and form a new cellular entity, remain elusive. To investigate the interplay between cell fusion and rearrangements of cytoskeleton, we have used marine fibroblasts (NIH 3T3 cells) that stably express hemagglutinin, the fus fusion protein. After short-term applications of trypsin followed by acidic medium, these cells fused with each other yielding multinucleated cells in about 3 hours. Time lapse fluorescence microscopy experiments have been performed to dissect the process of syncytium formation. We found that incubation on ice blocks transition from local fusion (assayed as redistribution of fluorescent cell trackers) to syncytia formation. The actin and microtubule cytoskeleton have been labeled with phallolidin and antibodies to better understand their involvement. In addition, the rate of syncytium formation has been quantified using drugs affecting either actin cytoskeleton (latrunculin B) or microtubule cytoskeleton (taxol, nocodazole). Our data suggest that while the microtubule cytoskeleton is not significantly involved in syncytia formation, the actin cytoskeleton appears to restrain the expansion of the fusion pores. These data on syncytium formation by viral fusion protein disagree with the results published for syncytia obtained with electroporation suggesting that mechanisms of the syncytium formation may depend on numbers and/or properties of initial fusion pores. Further elucidation of the mechanisms of syncytium formation will hopefully bring important insights into the mechanisms by which living cell controls its shape and responds to its changes.

The formation of complexes between v- and t-SNAREs is believed to be crucial for many intracellular fusion reactions. Studies with purified SNARE proteins have suggested that the t-SNARE subunits assemble on one fusion partner and that the v-SNARE is donated by the other fusion partner. We have now used a physiological membrane system, the cell-free fusion of yeast vacuoles to detect trans-SNARE complexes and we have used purified recombinant proteins, we demonstrate that yeast Sec1p binds directly and specifically to ternary SNARE complexes in vitro. Our data are consistent with models in which Sec1p binding is sensitive to the assembly state of the target membrane SNAREs. We have also initiated trials for obtaining crystals of Sec1-SNARE complexes to understand the molecular details of this interaction.
Role of Cholesterol in Organization of the Neuronal Fusion Core

A. Jeremic, W. Cho, B. Jena; Physiology, School of Medicine, Wayne State University, Detroit, MI

Neurotransmitter release depends on the regulated release of neurotransmitter vesicles in the synaptic cleft and subsequent activation of post-synaptic sites. Neuronal fusion pore or poreosome, a supramolecular assembly localized at the presynaptic membrane, has been identified as the molecular structure for neurotransmitter release. To further determine the composition, organization and function of this molecular machinery at the nerve terminal, we carried out the present study. In this study we show using biochemical and high-resolution imaging approaches, that membrane cholesterol is an essential component of the neuronal presynaptic. Cholesterol, together with pre-synaptic proteins: syntaxin-1, SNAP-25 and synaptotagmin-1, co-clusters and separates out as a high-molecular weight supramolecular complex (~700 kD) estimated using gel filtration and sucrose gradient density centrifugation, respectively. Depletion of synaptic membrane cholesterol by saponin treatment abrogated binding between t-SNAREs (syntaxin-1 and SNAP-25) and N-type Ca2+-channels, leading to disassembly of the poreosome complex. In contrast to the effect of cholesterol on the organization of SNAREs in synapsosomal membrane, interactions and organization of recombinant t-SNAREs in artificial lipid membranes were not influenced by cholesterol. This study demonstrates that in the native state, cholesterol is part of the poreosome complex, and is required to maintain its integrity. It also suggests that in the native state, cholesterol regulates SNAREs in such a way that cholesterol modifies the interaction of the poreosome complex. In summary, our studies demonstrate that cholesterol controls the synaptic membrane interacts with t-SNAREs, N-type Ca2+-channels and other proteins to organize functional fusion machinery at the nerve terminal. Supported by NIH grants (BPH).

Alcohol-induced Pathological Ectopic Exocytosis in Cholinergic-stimulated Rat Pancreatic Acini

L. I. Cosen-Binker, P. Lam, M. G. Binker, H. Y. Gaisano; University of Toronto, Toronto, ON, Canada

Chronic alcoholic consumption is a major factor associated to pancreatitis. However, the susceptible mechanism of alcohol-induced pancreatitis remains unknown. Using a combination of conventional epifluorescence and confocal microscopy, electron microscopy, subcellular fractionation, immunoprecipitation and biochemical techniques, we show that acute low-dose alcohol in vitro exposure or chronic alcoholic diet reduces postprandial cholinergic-stimulated amylase secretion from rat pancreatic acinar cells by redirecting normal apical exocytosis to the less efficient basolateral plasma membrane. The molecular mechanism involves the displacement of Munc18c from the basolateral plasma membrane, which becomes receptive to pathological ectopic exocytosis, leading to interstitial pancreatitis.

Akt Regulates Insulin-mediated Recruitment/Docking of GLUT4 Vesicles to the Plasma Membrane of Adipocytes

E. Gonzalez, T. McGraw; Biochemistry, Weill Medical College of Cornell University, New York, NY

Insulin modulates glucose disposal in muscle and adipose tissue by regulating the cellular redistribution of the GLUT4 glucose transporter. Insulin promotes the translocation of GLUT4 from intracellular sites to the plasma membrane by accelerating GLUT4 exocytosis and inhibiting GLUT4 internalization. Protein kinase Akt/PKB is a central mediator of insulin-regulated translocation of GLUT4; however, the GLUT4 trafficking step(s) regulated by Akt is not known. We used acute pharmacologic: Akt inhibition and shRNA-mediated Akt knockdown to dissect the role of Akt signaling on GLUT4 trafficking in 3T3-L1 adipocytes. We observed that acute inhibition of Akt activity strongly impaired insulin-induced GLUT4 redistribution to the plasma membrane and glucose transport in adipocytes. Insulin-induced GLUT4 translocation was impaired following downregulation of Akt2 but not Akt1. Using a HA-GLUT4-GFP reporter and single cell microscopy analyses we found that activation of Akt is required for insulin-induced GLUT4 exocytosis whereas regulation of GLUT4 internalization is independent of Akt activity. To further explore the steps on GLUT4 exocytosis regulated by Akt we developed a novel total internal reflection microscopy assay and show that along the exocytotic pathway, Akt activity is specifically required for an insulin-mediated pre-fusion step involving the recruitment and/or docking of GLUT4 vesicles to within 250 nm of the plasma membrane. By contrast, the insulin-stimulated fusion of GLUT4 vesicles with the plasma membrane can occur independent of Akt activity, although based on inhibition with wortmannin, it is dependent on PI3-kinase activity. These studies propose Akt as a central regulator of insulin-induced GLUT4 vesicle recruitment/docking at the plasma membrane and show that to achieve full redistribution of GLUT4 into the plasma membrane, insulin signaling bifurcates to independently regulate both fusion and a pre-fusion step(s).

Glut4 Vesicle Mobility Is Independent of Insulin Stimulation in 3T3-L1 Adipocytes

C. A. Eyster, A. Olson; Biochemistry, University of Oklahoma Health Sciences Center, Oklahoma City, OK

GLUT4 is an integral membrane glucose transporter protein responsible for insulin-mediated glucose uptake in adipose and muscle tissue. Insulin mediated GLUT4 redistribution to the cell surface is required to maintain glucose homeostasis, and is defective in insulin resistance. It has previously been suggested the insulin treatment increases GLUT4 vesicle movement to and fusion with plasma membrane. To directly test the effect of insulin on GLUT4 vesicle movement, we carried out Fluorescence Recovery After Photobleaching (FRAP) studies of GLUT4-GFP in live 3T3-L1 adipocytes. Surprisingly, we found that GLUT4 has highly mobile in the basal state at the cell surface, in the cytosol, and in the perinuclear compartment. In addition, we determined that insulin stimulation does not alter GLUT4 mobility in any of the three compartments tested. It had also been hypothesized that insulin signals to the microtubule network in order to increase the rate of GLUT4 vesicle mobility. Indeed, pharmacological inhibition of the microtubule network has been shown to block insulin mediated GLUT4 redistribution. However, recent work from our lab suggests that the microtubule requirement lies upstream of Akt activation in formation of the insulin signaling complex. Using a constitutively active Akt (myr-Akt), we tested if the requirement for the microtubule network was bypassed. As previously reported, insulin-dependent GLUT4 redistribution was blocked by Nocodazole, a microtubule inhibitor. However, Nocodazole did not inhibit GLUT4 redistribution signaled by expression of myr-Akt. This suggests that the microtubule network functions upstream of Akt activation. Indeed, in Nocodazole treated adipocytes, insulin-dependent Akt activation is blocked. These data suggest that insulin does not alter GLUT4 vesicle mobility. GLUT4 vesicles are not tethered in the basal state, and the microtubule requirement lies upstream of Akt activation.

Insulin Modulates Perinuclear GLUT4 Redistribution Independently of Toxin-sensitive VAMP2

V. K. Randhawa,1 A. W. P. Cheng,2 A. Klip 1; Programme in Cell Biology, Hospital for Sick Children, Toronto, ON, Canada; 1Department of Biochemistry, University of Toronto, Toronto, ON, Canada

Skeletal muscle and adipose are the main insulin-responsive tissues with regards to glucose uptake via the GLUT4 glucose transporter. GLUT4 resides intracellularly in compartments sequestered about the perinucleus and dispersed within the cytosol. Insulin increases surface GLUT4 levels by reducing its total internal complement. However, little is known of the nature of insulin-sensitive pools that furnish surface GLUT4. To this end, we made use of an L6 skeletal muscle cell line stably expressing exocytosis myr-tagged GLUT4 that allows high spatial resolution of these internal domains. Confocal microscopy revealed a predominantly conical perinuclear distribution of fluorescent-tagged GLUT4mGFP in the basal steady state. Insulin elicited a redistribution of perinuclear GLUT4 into a concentric morphology. As well, insulin increased the GLUT4 found with VAMP2 but not VAMP3 in both punctate cytoplasmic elements and the insulin-induced actin mesh. Insulin also increased GLUT4 recycling as measured by uptake of Cy3-anti-HA antibody by HA-tagged VAMP2. While insulin signals may directly modulate these intracellular sites, it was recently proposed that insulin regulation lies nearer to the plasma membrane. To determine if the insulin-induced perinuclear remodeling could arise from queuing of GLUT4 when vesicle fusion is prevented, we examined perinuclear GLUT4 distribution in cells transfected with tetanus toxin (TeTx). While TeTx abolishes insulin-dependent GLUT4 externalization in L6 myoblasts due to cleavage of VAMP2 (Randhawa et al, Mol Biol Cell 11:2403, 2000), we show here that TeTx does not alter the remodeling of perinuclear GLUT4 by insulin. Taken altogether, these results suggest that this remodeling is independent of GLUT4 fusion with the plasma membrane. Rather, we propose a model whereby GLUT4-containing compartments are in dynamic equilibrium and insulin may regulate the acquisition of VAMP2-positive GLUT4 vesicles en route to furnishing transporters the cell surface.

α-Actinin-4 Binds to Glut4 and Participates in Insulin-dependent GLUT4 Mobilization Towards the Plasma Membrane

I. Talon,1 V. K. Randhawa,1,2 A. Klip 1; Cell Biology, Hospital for Sick Children, Toronto, ON, Canada; 1Biochemistry Department, University of Toronto, Toronto, ON, Canada

Glucose transporter-4 (GLUT4) translocates from intracellular pools to the plasma membrane in response to insulin acidic secretion from rat pancreatic acinar cells by redirecting normal apical exocytosis to the less efficient basolateral plasma membrane. The molecular mechanism involves the displacement of Munc18c from the basolateral plasma membrane, which becomes receptive to pathological ectopic exocytosis, leading to interstitial pancreatitis. In contrast to the effect of cholesterol on the organization of SNAREs in synapsosomal membrane, interactions and organization of recombinant t-SNAREs in artificial lipid membranes were not influenced by cholesterol. This study demonstrates that in the native state, cholesterol is part of the poreosome complex, and is required to maintain its integrity. It also suggests that in the native state, cholesterol regulates SNAREs in such a way that cholesterol modifies the interaction of the poreosome complex. In summary, our studies demonstrate that cholesterol controls the synaptic membrane interacts with t-SNAREs, N-type Ca2+-channels and other proteins to organize functional fusion machinery at the nerve terminal. Supported by NIH grants (BPH).
domestic actin remodelling is essential for insulin-induced GLUT4 translocation, we hypothesized that α-actinin-4 may link GLUT4 to actin filaments in the course of its mobilization to the cell surface. Here we report increased co-precipitation of α-actinin-4 with GLUT4myc (but not GLUT1) from L6 muscle cells in response to insulin (but not PDGF), by immunoblotting with isoform-specific α-actinin-4 antibodies. Co-precipitation was significantly decreased by Latrunculin B, an inhibitor of actin filament reassembly. Interestingly, GLUT4myc co-localized with α-actinin-4 in regions within the insulin-remodelled actin mesh in L6 myotubes. Knockdown of α-actinin-4 (60%) via specific siRNA reduced the insulin-stimulated glucose uptake and gain in surface GLUT4myc, compared to unrelated siRNA. Notably, α-actinin-4 knockdown prevented GLUT4myc arrival at the membrane, scored by the lack of a submembrane perinuclear rim of myc immunofluorescence in permeabilized, rounded-up myotoblasts. Consequently, there was also no exocytotic myc rim in intact cells. Conversely, blocking PDK or AS160 allowed significant GLUT4myc arrival but prevented fusion with the membrane. α-actinin-4 siRNA did not perturb GLUT4 or α-actinin-1 expression, perinuclear GLUT4 distribution, insulin-stimulated actin remodelling or Akt activation. Collectively, this work suggests that the interaction of α-actinin-4 with GLUT4myc contributes to GLUT4 mobilization to the cell surface through its association with the actin cytoskeleton, and that GLUT4 mobilization is likely an insulin-regulated step. Supported by CIHR, BBDC and Sickkids.

**2485**
Dominant Negative Rab11-FIP2 Trafficking Mutants Reveal Novel Protein Interactions Regulating Recycling
N. A. Ducharme,1 J. R. Goldenring2; 1Cell and Developmental Biology, Vanderbilt University, Nashville, TN, 2Surgery, Vanderbilt University, Nashville, TN
Rab11-FIP2 binds Rab11a and myosin Vb and regulates trafficking through the apical recycling system in polarized cells. We isolated a mutant EGFPRab11-FIP2 construct, Rab11-FIP2(S229A/R413G), which demonstrated a dominant negative phenotype for the Rab11a-containing recycling system. We assessed the effects of each single mutant and the double mutant in stable MDCK cell lines. EGFPRab11-FIP2(S229A) has no discernable effect on the morphology of the recycling system. EGFPRab11-FIP2(R413G) was a less severe dominant negative than EGFPRab11-FIP2(S229A/R413G), which caused a marked collapse and tubulation of the recycling system. The collapsed EGFPRab11-FIP2(S229A/R413G) tubular cisternae were often located eccentrically towards one corner of the cell. This was in marked contrast to our previously characterized dominant negative Rab11-FIP2 mutant, EGFPRab11-FIP2(129-512), a Rab11-FIP2 construct lacking the C2 domain which elicited generally a round, more regular shaped cisternal structure. Like EGFPRab11-FIP2(129-512), Rab11-FIP2(S229A/R413G) caused an inhibition in plgA trafficking in MDCKs cells. Rab11-FIP2(R413G) mutant effects on the recycling system that are distinctly separate from the EGFPRab11-FIP2(129-512) mutant-induced collapse of early endosomal system towards the centrosome. However, this alteration is not seen in Rab11-FIP2(S229A/R413G) cells. In contrast, Rab11-FIP2(S229A/R413G) expressing cells showed a partial collapse of GP135 to the tubular cisternae that is not seen in Rab11-FIP2(129-512) cells. The availability of multiple mutants in the Rab11a pathway will allow a more rigorous analysis of the regulation of plasma membrane recycling.

**2486**
From Sorting Endosome to Exocytosis: Imaging the Recycling Pathway Using Novel Multifocal Plane Microscopy
P. Prabhath,1 Z. Gao,1 J. Chao,2 S. Ram,1 C. Vaccaro,1 S. Gibbons,1 R. J. Ober,2 E. S. Ward1; 1Center for Immunology, University of Texas Southwestern Medical Center, Dallas, TX, 2Department of Electrical Engineering, University of Texas at Dallas, Richardson, TX, Joint Biomedical Engineering Graduate Program, University of Texas at Arlington/University of Texas Southwestern Medical Center, Arlington/Dallas, TX
Elucidation of intracellular events on the recycling pathway from sorting endosomes to exocytosis on the plasma membrane is of fundamental importance. Epifluorescence microscopy is typically used to study recycling events inside the cell, whilst Total Internal Reflection Fluorescence (TIRF) microscopy provides valuable insight into the nature of exocytic events on the plasma membrane. However, events on the recycling pathway preceding exocytosis are poorly characterized. This is primarily due to the fact that the imaging of fast moving transport containers (TCs) on the recycling pathway poses significant technical challenges. To overcome this limitation, we recently developed a microscopy technique [1] that enables the simultaneous imaging of distinct focal planes in a specimen. Using this technique, events on the plasma membrane of a cell can be imaged with TIRF excitation whilst simultaneously imaging additional focal planes inside the cell using widefield excitation. This has enabled us to track events preceding exocytosis on the recycling pathway of the MHC Class I related receptor, FcRn, in human endothelial cells. We observe both direct and indirect delivery of TCs from sorting endosomes to exocytic sites at the plasma membrane. In the direct delivery pathway, vesicular and tubular TCs that are in close proximity to sorting endosomes undergo fusion. In some cases, TCs that extend from sorting endosomes to the plasma membrane as tubular extensions can be seen. In contrast, for the indirect pathway, exocytosis involving TCs which are not in close proximity to sorting endosomes is observed. These TCs can move large distances within the cell prior to arresting in ‘holding zones’ above the plasma membrane. TCs can also interact with multiple sorting endosomes prior to exocytosis. 1. Prashant Prabhath, et. al., IEEE Transaction on Nanobioscience, 3(4), 257-242, (2004) Supported by grants from the NIH (R01 AI39167, GM071048).

**2487**
Interaction between SCAMPs and Complexins
S. A. Shestopal, D. Castle, Cell Biology, University of Virginia, Charlottesville, VA
Secretory carrier membrane proteins (SCAMPs) comprise a family of tetraspan transmembrane proteins, implicated in membrane trafficking. Complexins are proteins that are able to bind assembled SNARE complexes. Mammalian complexins 1 and 2 are soluble and expressed in neuronal and neuroendocrine cells where they are involved in regulation of exocytosis, but their exact role is not yet established. Using a pull-down assay, we have demonstrated interaction between recombinant complexins 1 and 2 and SCAMPs 1, 2, 3 and 5 from brain and PC12 cell lysates. We made a deletion mutant of complexin 2 (residues 456-497) which eliminates the interaction with complexin and map within a localized groove of the primary sequence, all map in a localized groove of the high resolution nsec1 structure. Thus, phylogenetic analysis provides a powerful tool for probing the precise function of Sec1p in membrane trafficking.

**2488**
A Sec8/Sec6/Sec5 Sub-complex of the Mammalian Exocyst Mediates Polarized Endocytic Recycling via Rab GTPase during Myoblast Migration
X. Chen,1 Q. Wang,1 S. C. Hsu,2 A. R. Saitiel,3 University of Michigan, Ann Arbor, MI, 3Rutgers University, Piscataway, NJ
Spatial regulation of endocytic recycling during polarized cellular processes remains a central question to be understood. The protein complex exocyst has been suggested to provide spatial control of endocytic recycling. Here we report that the mammalian exocyst subunits Sec8, Sec6, and Sec5 form a stable sub-complex. Although the assembly of this subcomplex is independent of RabA; RaA activity is important for recruiting these exocyst proteins to the exocytic vesicles. Sec8 regulates the stability of Sec6 and Sec5, which in turn bridges RabA-localized Rab GTPase to the plasma membrane. Unlike Sec6 and Sec5 accumulate to the membrane ruffle region of myoblast in response to HGF signaling, while knockdown of RaA or Sec5 inhibits HGF-induced myoblast migration. Our data provided insights into the molecular architecture of the mammalian exocyst and suggested that RaA specifies the targeting of RE vesicles via recruitment of the Sec6/Sec8/Sec5 sub-complex.

**2489**
Exploiting Phylogenetic Analyses to Explore the Function of Sec1p in Saccharomyces cerevisiae
J. L. Hutton,1 C. Chiu,1 C. M. Carroll1; 1Pathology, UMDNJ-RWJMS, Piscataway, NJ, 2Life Sciences, Rutgers, Piscataway, NJ
Sec1p/Munc 18 (SM) proteins are essential for SNARE-mediated vesicular trafficking and fusion in eukaryotes and, in S. cerevisiae, loss of function mutations can result in a lethal phenotype. However, despite extensive work in various eukaryotic species, the precise role of SM proteins is unclear. Here, aligning over 150 SM proteins from 30 diverse organisms including S. cerevisiae, C. elegans and H. sapiens followed by phylogenetic analyses, yields insights into their evolution and functionally important residues. Our findings confirm that SM proteins represent a superfamily comprised of four paralogous families (Sec1, Sty1, Vps33 and Vps45) that function at different points of protein trafficking/ secretary pathways. These subfamilies arose from ancient gene duplication events, since all eukaryotic organisms examined contained orthologous proteins. Interestingly, we have been able to place previously un-annotated genes into SM subfamilies. Furthermore, our data suggests that the Vps33 subfamily was the last to appear and this subfamily of proteins contains the largest amount of diversity between orthologues in different species and between paralogues within the same species. Finally, several highly conserved amino acids, spread throughout the primary sequence, all map in a localized groove of the high resolution nsec1 structure. Thus, phylogenetic analysis provides a powerful tool for probing the precise function of Sec1p in membrane trafficking.

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Specifity of Yeast Sec1p for the Exocytic SNARE Complex Defines a New Binding Mode for Sec1/Munc-18 Proteins

J. Togneri; UMDNJ, Piscataway, NJ

The Sec1/Munc-18 (SM) family of proteins is required for vesicle fusion in eukaryotic cells and has been linked to the membrane-fusion proteins known as SNAREs. SM proteins may activate the target-membrane SNARE, syntaxin, in support of the fusion event, and SM proteins bind directly to their cognate syntaxins. An exception is the yeast Sec1p, which does not bind the yeast plasma-membrane syntaxin, Sso1p. This exception could be explained if the SM interaction motif was blocked by the highly stable conformation of Sso1p. We tested the possibility of a latent binding motif using Sso1p mutants in yeast and reconstituted the Sec1p binding specificity observed in vivo with purified proteins in vitro. Our results indicate that there is no latent binding motif in Sec1p. Instead, Sec1p binds specifically to the ternary SNARE complex, with no detectable binding to the binary S-N/P complex or any of the three individual SNAREs in their uncomplexed forms. We propose that vesicle fusion requires a specific interaction between the SM protein and the ternary SNARE complex.

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Overexpression of Synaptotagmin 1 in the Brain of Down Syndrome Mouse Models Causes Defects in PtdIns(4,5)P2 Metabolism and Learning Deficits

S. V. Voronov1, S. G. Fere1, S. Giovedi1, E. A. Pollina1, C. Schmidt1, L. Cimasoni1, C. Borel1, D. M. Wenk2, E. S. Antonarikas1, K. Gardner3, M. T. Davison4, O. Arancio5, P. De Camilli1, G. D. Paoli1, 1Pathology, Columbia University, New York, NY, 2Cell Biology, Yale University, New Haven, CT, 3The Jackson Laboratory, Bar Harbor, ME, 4Genetic Medicine and Development, University of Geneva, Geneva, Switzerland, 5Biochemistry, National University of Singapore, Singapore, 1Eleanor Roosevelt Institute at the University of Denver, Denver, CO

Down syndrome (DS) is the most common cause of mental retardation. In DS, integrated gene expression is altered due to the presence of a third copy of human chromosome 21 (H21C). Although mental retardation has been linked to non-overlapping regions of H21C, indicating the multicogenicity of its etiology, the relative contribution of single genes to this phenotype is unknown. Among all the HC21 genes, SYNJ1 is a strong candidate for contributions to mental retardation in DS. SYNJ1 encodes synaptotagmin 1, a brain-enriched lipid phosphatase that dephosphorylates phosphatidylinositol-4,5-bisphosphate [PtdIns(4,5)P2]. This lipid regulates multiple biological functions, including signal transduction and membrane trafficking. Our previous work has shown that PtdIns(4,5)P2 regulates synaptic vesicle trafficking and thereby, neurotransmission. This property is largely due to its ability to recruit to the plasma membrane key components of the endocytic machinery, which mediates the recycling of synaptic vesicles via a clathrin-dependent pathway. Here we show that SYNJ1 is overexpressed in the brain of a mouse model for segmental trisomy 21 (Ts65Dn). Furthermore, gene dosage imbalance for SYNJ1 in Ts65Dn brains correlates with an increase in PtdIns(4,5)P2 phosphorylation and a corresponding reduction in the mass of PtdIns(4,5)P2. These phenotypes are recapitulated by transgenic mice overexpressing SYNJ1 [Ts(Synj1)] at levels comparable to those found in Ts65Dn mice and rescued by genetically restoring a normal Synj1 copy number in the segmentally trisomic mice. Finally, Ts(Synj1) mice exhibit learning deficits in the Morris water maze paradigm, suggesting that normal PtdIns(4,5)P2 balance is critical for proper cognitive functions. Altogether, our study indicates that the mild overexpression of a single gene, SYNJ1, causes PtdIns(4,5)P2 dyshomeostasis in the brain of DS mouse model and that this biochemical defect results in discernable behavioral deficits, likely as a result of altered synaptic vesicle trafficking and suboptimal neurotransmission.

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Temporal Analysis of Synaptojanin 1 Recruitment at Clathrin Coated Pits

R. M. Perera1, R. Zoucs1, L. Lucast1, P. De Camilli1, D. Toomre1, 1Cell Biology, Yale University School of Medicine, New Haven, CT, 2Cell Biology/HHMI, Yale University School of Medicine, New Haven, CT, 3Department of Cellular and Molecular Medicine, University of California, San Diego School of Medicine, Howard Hughes Medical Institute, La Jolla, CA, 4 Ludwig Cancer Research Institute, La Jolla, CA

Synaptojanin (SYNJ1) is a non-synaptic polyphosphoinositide phosphatase that dephosphorylates phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P2) with endocytic clathrin adaptors, while functional studies using cell-free systems or intact cells have demonstrated the importance of PtdIns(4,5)P2 synthesis and dephosphorylation in clathrin coating and uncoating, respectively. Furthermore, genetic manipulations of kinases and phosphatases involved in PtdIns(4,5)P2 metabolism result in major defects in synaptic vesicle recycling and other forms of exo-endocytosis. However, a dynamic visualization of these enzymes at clathrin coated pits has yet to be conducted. We have used live TIRF imaging of cells transfected with fluorescently tagged proteins to determine the spatial and temporal recruitment of the polyphosphoinositide phosphatase synaptojanin 1 at endocytic clathrin coated pits. The 145 KDa isoform of synaptotagmin 1 (i.e. the isoform that by far predominates at synapses), was recruited together with endophilin at a late stage and closely correlated with a burst of dynamin recruitment. This is consistent with a role of synaptotagmin 145 during late stages of vesicle formation and uncoating. In contrast, the 70 KDa isoform of synaptotagmin, which has a ubiquitously tissue distribution and predominates in brain before synaptogenesis, was recruited to clathrin-coated pits at an early stage and persisted on the coat throughout its lifetime. Recruitment of synaptotagmin-170 was dependent on the presence of clathrin and AP2 binding sites contained in this isoform. These results suggest that normal PtdIns(4,5)P2 balance is critical for proper cognitive functions. Altogether, our study indicates that the mild overexpression of a single gene, SYNJ1, causes PtdIns(4,5)P2 dyshomeostasis in the brain of DS mouse model and that this biochemical defect results in discernable behavioral deficits, likely as a result of altered synaptic vesicle trafficking and suboptimal neurotransmission.

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Regulation of Phosphoinositide Kinase Signaling at the Plasma Membrane

C. Stefan1, D. Baird1, Y. Ling1, A. Audhya1, S. Emr1, 1Department of Cellular and Molecular Medicine, University of California, San Diego School of Medicine, Howard Hughes Medical Institute, La Jolla, CA

Phosphoinositides (PIs) are thought to play an important role in clathrin coated pit dynamics. Biochemical and structural studies have shown that a direct interaction of phosphatidylinositol-4,5-bisphosphate (PI(4,5)P2) with endocytic clathrin adaptors, while functional studies using cell-free systems or intact cells have demonstrated the importance of PI(4,5)P2 synthesis and dephosphorylation in clathrin coating and uncoating, respectively. Furthermore, genetic manipulations of kinases and phosphatases involved in PI(4,5)P2 metabolism result in major defects in synaptic vesicle recycling and other forms of exo-endocytosis. However, a dynamic visualization of these enzymes at clathrin coated pits has yet to be conducted. We have used live TIRF imaging of cells transfected with fluorescently tagged proteins to determine the spatial and temporal recruitment of the polyphosphoinositide phosphatase synaptojanin 1 at endocytic clathrin coated pits. The 145 KDa isoform of synaptotagmin 1 (i.e. the isoform that by far predominates at synapses), was recruited together with endophilin at a late stage and closely correlated with a burst of dynamin recruitment. This is consistent with a role of synaptotagmin 145 during late stages of vesicle formation and uncoating. In contrast, the 70 KDa isoform of synaptotagmin, which has a ubiquitously tissue distribution and predominates in brain before synaptogenesis, was recruited to clathrin-coated pits at an early stage and persisted on the coat throughout its lifetime. Recruitment of synaptotagmin-170 was dependent on the presence of clathrin and AP2 binding sites contained in this isoform. These results suggest that normal PtdIns(4,5)P2 balance is critical for proper cognitive functions. Altogether, our study indicates that the mild overexpression of a single gene, SYNJ1, causes PtdIns(4,5)P2 dyshomeostasis in the brain of DS mouse model and that this biochemical defect results in discernable behavioral deficits, likely as a result of altered synaptic vesicle trafficking and suboptimal neurotransmission.

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Essential and Unique Roles of PIP5K1a and γ in FcγR-mediated Phagocytosis

M. Yamagua1, Y. S. Mats1, H. Q. Sun1, M. Yun2, P. De Camilli1, M. Muller3, C. Y. Lu1, H. L. Yin1, 1Physiology, University of Texas Southwestern Medical Center, Dallas, TX, 2Cell Biology, Yale University Medical School, New Haven, CT, 3Internal Medicine, University of Texas Southwestern Medical Center, Dallas, TX

Fc receptor (FcγR)-mediated phagocytosis is a spatially and temporally regulated process that includes three morphologically defined phases: the attachment of the IgG-opsonized particle, the extension of pseudopodia to form a phagocytic cup, and the engulfment of the particle to form a phagosome. The actin cytoskeleton is extensively restructured during these steps; actin assembles at the nascent phagocytic cup and at pseudopodia during the ingestion phase, and actin disassembles during the completion phase. Previous studies have shown that actin remodeling during phagocytosis is regulated by localizations of phosphatidylinositol-4,5-bisphosphate, and a type 4 phosphatidylinositol 5 kinase (PIP5K4) that generates PIP2 is recruited to the phagocytic cup. Here we investigated the roles of the PIP5KIs on phagocytosis by depleting PIP5K1a isoforms individually by RNA interference in FcγRI receptor overexpressing CHO cells (CHO-IA) and, in one case, by gene knockout in mice. We found that PIP5K1a (human nomenclature) or γ depletion inhibited different steps in phagocytosis by CHO-IA cells. PIP5K1a depletion prevented particle binding and decreased the macroscopic clustering of FcγR by IgG immune complexes. Unlike control cells, the PIP5K1a-depleted CHO-IA cells did not dismantle their actin stress fibers on exposure to the IgG-opsonized particles, suggesting that the primary defect may be due to an inability to initiate the actin disassembly process that is required for stable particle attachment and FcγR clustering. Likewise, bone marrow-derived macrophages isolated from PIP5K1a knockout mice also did not bind the IgG-opsonized particles normally. In contrast, the PIP5K4-depleted CHO-IA cells bound the particles, but were unable to engulf them. Actin assembly under the phagocytic cup was decreased, suggesting that the defect is due to an inability to polymerize actin at the site of particle contact. Our results establish that PIP5K1a and γ have essential and unique roles in the different stages of FcγR-mediated phagocytosis.

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Differential Regulation of PIP5Ks by the Syk Protein Tyrosine Kinase during Fc Receptor-mediated Phagocytosis

Y. J. Wei, B. Liu, M. Yamaga, H. Q. Sun, H. L. Yin, Physiology, UT Southwestern Medical Center, Dallas, TX

During FcR-mediated phagocytosis, type I phosphatidylinositol 5-kinases (PIP5Ks) are recruited to the nascent phagocytic cup to increase phosphatidylinositol 4,5-bisphosphate (PIP2) generation in situ. We now report that PIP5Kγ (human nomenclature) and PIP5Kβ, but not PIP5Kα, are also tyrosine phosphorylated during phagocytosis. PIP5Kγ and β tyrosine phosphorylation increases with a time course that parallels the activation the Syk tyrosine kinase, which has an essential apical role in the FcγR signaling cascade. PIP5K phosphorylation is decreased by the Syk inhibitor, piceatannol, and Syk overexpression promotes PIP5K phosphorylation in CHO-IA cells, which has much less Syk than the professional phagocytes, during phagocytosis. Using an in vitro protein kinase assay, we found that Syk phosphorylates the two differentially spliced PIP5Kγ variants (90 and 87 kDa), as well as PIP5Kβ. Furthermore, Syk immunoprecipitation pulls down endogenous PIP5Kγ in the RAW macrophage-like cells, and also overexpressed PIP5Kγ/87 and 90 in non-phagocytic COS cells. Taken together, our results establish that these PIP5Ks are bona fide Syk substrates, and suggest that they are likely to bind Syk directly. In vitro lipid kinase assay shows that tyrosine phosphorylation increases the activity of PIP5Kγ/87 and 980, but not of PIP5Kα.

We conclude the PIP5K isoforms are differentially regulated by Syk during phagocytosis, and that PIP2 is increased at the phagocytic cup by recruiting PIP5Ks into the membranes. The recruitment of select PIP5Ks by tyrosine phosphorylation for functional roles in phagocytosis is regulated by Syk has broader significance beyond PIP2 regulation of phagocytosis, because Syk has crucial roles in multiple leukocyte signaling pathways, and Syk has been implicated in cytokinesis and tumorigenesis in non-hematopoietic cells as well.

Effects of SHIP1 on the Dynamics of 3' Phosphoinositides during Fc Receptor-mediated Phagocytosis

L. A. Kamen, J. Levinsohn, J. A. Swanson, Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, MI

Lipid kinases and phosphatases regulate receptor-mediated phagocytosis in macrophages. PI 3-kinase, which generates PI(3,4,5)P3 from PI(4,5)P2, is necessary for later stages of the phagocytosis of particles larger than 3 µm in diameter. Overexpression of the phosphatase PTEN, which catalyzes the reverse reaction, inhibits phagocytosis after formation of phagocytic cups, much like inhibitors of PI 3-kinase. SHIP1, a lipid phosphatase that hydrolyses PI(3,4,5)P3, to PI(3,4)P2 also inhibits phagocytosis when overexpressed in macrophages. We hypothesize that the inhibitory activities of SHIP1 contribute to phagosome formation by modifying the distribution or PI(3,4,5)P3,5-PIP2 in the phagocytic cup. To test this, we quantitatively analyzed the distributions of SHIP1, PTEN, YFP, or phosphatase PLT (the regulatory subunit of PI3K), and YFP chimeras of phosphoinositide-binding domains that recognize PI(3,4,5)P3 (BikPH), PI(3,4,5)P2/PI(3,4)P2 (PiclPH), PI(3,4)P2 (Tapp1PH), and PI3P (2xFYVE) during phagocytosis. PTEN-YFP never localized to phagosomes, but SHIP1-YFP and PI(3,4)P2-YFP localized to the phagosomes with similar rates of association. Coexpression of SHIP1-YFP and PI(3,4)P2-CFP revealed that SHIP1-YFP associated from phagocytic cups earlier than did PI(3,4)P2-CFP, indicating that SHIP1 inhibitory activity may be relieved before phagocytosis is completed. Although the phospholipid profile of the phagocytic cups was distinct from that of the plasma membrane, no significant gradients of phosphatidylserine were detected within the membranes of the cup. Rather, PI(3,4,5)P3 and PI(3,4)P2 increased and decreased similarly over the forming cup. Using an IRES vector system, we examined the effects on 3' phosphoinositide dynamics of overexpressing SHIP1 or inhibitory SHIP1-ΔSH2 domains. All IRES-based manipulations of SHIP1 function decreased levels of PI(3,4,5)P3 and PI(3,4)P2 on the phagosome. These results support a model in which transient recruitment of SHIP1 to forming phagosomes permits early PI 3-kinase-independent activities and delays the later PI 3-kinase-dependent activities necessary for completion of phagocytosis.

Cooperation of Phosphoinositides and Bar Domain Proteins in Endosomal Tubulation

N. Shinozaki-Narikawa,1,2 T. Kodama,1 Y. Shibasaki1; 1Laboratory for Systems Biology and Medicine, RCAST, University of Tokyo, Tokyo, Japan, 2Yokogawa Electric Corporation (NEDO fellow), Tokyo, Japan

Phosphorylated derivatives of phosphatidylinositol (PtdIns) regulate many intracellular events, including vesicular trafficking and actin remodeling, by recruiting proteins to their sites of function. PtdIns(4,5)P2 (PIP2) is recruited to the nascent endocytic tubules in COX-7 cells. ADP-ribosylation factor (Arf) has been shown to act upstream of PIP5K and regulate endocytic transport and tubulation. ACAP1 has GTPase activating protein (GAP) activity for Arf6. While there were few tubules induced by the expression of ACAP1 alone, numerous endosomal tubules were induced by coexpression of PIP5K and ACAP1. ACAP1 has a pleckstrin homology (PH) domain known to bind phosphoinositide, and a Bin/amphiphysin/Rvs (BAR) domain that has been reported to detect membrane curvature. All of these three sub-domains, the hydrophobic ridge, the helix 0 and the rigid crescent main body, are necessary to induce plasma membrane tubulation in cells over-expressing the endophilin-A1 BAR domain. Our results support the putative liposome binding activity. These results suggest that the rigid crescent dimer shape of the BAR domain is crucial for the tubulation. All of these three sub-domains, the hydrophobic ridge, the helix 0 and the rigid crescent main body, are necessary to induce plasma membrane tubulation in cells over-expressing the endophilin-A1 BAR domain. Our results support the putative mechanism that the BAR domain dimer imparts its intrinsic curvature on membrane to tubulate it. We have propose that the BAR domain drives membrane curvature by coordinate action of the crescent’s scaffold mechanism and the ridge’s membrane insertion in addition to membrane binding via the helix 0.

Endophilin-A1 BAR Domain Uses Two Mechanisms to Drive Membrane Curvature

M. Masuda,1 S. Takeda,2 M. Sone,3 T. Ohki,4 H. Mori,2 Y. Kamioka,1 N. Mochizuki1; 1Structural Analysis, National Cardiovascular Center Research Institute, Suita, Japan, 2Cardiac Physiology, National Cardiovascular Center Research Institute, Suita, Japan, 3Laboratory of Structural Biochemistry, RIKEN Harima Institute at SPring-8, Mikazuki-cho, Sayo, Japan

Membrane dynamics in a cell, such as membrane budding, tubulation, fission and fusion, is associated with vigorous changes in membrane curvature. The crescent-shaped BAR domain of human endophilin-A1 consists of a pair of helix-loop appendages sprouting out from the crescent-shaped main body. The short helices of the appendages form a hydrophobic ridge, which runs across the concave surface of the main body at its centre. Liposome binding and tubulation assays of the recombinant BAR domain and its mutants indicate that the ridge penetrates into the membrane bilayer and enhances liposome tubulation. The N-terminal amphipathic helix preceding the BAR domain (helix 0) is crucial for liposome binding. The crystal structure of a designed mutant solved at 2.4 Å shows flexibility in the helix kinks that make the curved molecular shape. This flexible-arm mutant loses liposome tubulation activity yet retaining intact liposome binding activity. These results suggest that the rigid crescent dimer shape of the BAR domain is crucial for the tubulation. All of these three sub-domains, the hydrophobic ridge, the helix 0 and the rigid crescent main body, are necessary to induce plasma membrane tubulation in cells over-expressing the endophilin-A1 BAR domain. Our results support the putative mechanism that the BAR domain dimer imparts its intrinsic curvature on membrane to tubulate it. We have propose that the BAR domain drives membrane curvature by coordinate action of the crescent’s scaffold mechanism and the ridge’s membrane insertion in addition to membrane binding via the helix 0.

Core Protein Machinery for PtdIns 3,5-P2 Synthesis and Turnover Controls Mammalian Endosome Carrier Vesicle Formation/Detachment: Novel Sac3 Phosphatase Joins the ArPKfyve-Pikfyve Complex

D. Shroira, G. Demel, T. Iain, T. Takenawa, A. Shisheva; Physiology, Wayne State University, Detroit, MI

PtdIns 3,5-P2, plays an essential role in regulating diverse endosome-related events. This role is conserved in evolution as evidenced by the similar aberrant morphology of endocytic organelles, associated with inactivation of structurally related PtdIns 3,5-P2-producing enzymes, Fab1, in yeast, and PIKfyve, in mammalian cells. The conservation of the PtdIns 3,5-P2 pathway is reinforced by the structural and functional homology between yeast Vac14 and mammalian ArPKfyve, which activate Fab1 and PIKfyve, respectively. Regulation of PtdIns 3,5-P2 levels occurs by ArPKfyve and ArPKfyve turnover, and a Sac3, 35-kDa, PtdIns 3,5-P2-specific phosphatase. Fig. 8, in interaction with Vac14, turns over PtdIns 3,5-P2, in yeast. Whether such mechanism operates in mammalian cells is unknown. We identified and partially characterized mammalian Sac3, a 90 kDa Sac domain phosphatase and putative ortholog of Sac3, Sac3, ArPKfyve or ArPKfyve antibodies coinmunoprecipitated the other two proteins in several mammalian cell types, consistent with the presence of endogenous PIKfyve/Sac3 ternary complexes. In this heterologous system, Sac3 and ArPKfyve, but not PIKfyve, could self-assemble as revealed by coimmunoprecipitation in COS7 cells coexpressing HA- and Myc-tagged forms of either protein. Individual siRNA-mediated depletion of Sac3 or ArPKfyve in 3T3-L1 fibroblasts resulted in their coincidental loss, consistent with a mutual dependence of the two proteins for stability. Sac3 cofractionated with PIKfyve by density gradient sedimentation in HEK293 cells. Fluorescence microscopy in cotransfected COS7 cells visualized Myc-Sac3 diffuse or on vesicles colocalizing in part with PIKfyve- or ArPKfyve-positive signals. Depletion of either protein altered the in vitro formation of endosome carrier vesicle intermediates, consistent with the role of PtdIns 3,5-P2 in this process. Physical assembly of core protein machinery for regulated PtdIns 3,5-P2 synthesis and turnover ensures a tight coupling between these two processes and fulfills the crucial need for a firm spatial and temporal control of PtdIns 3,5-P2 on endosome membranes.
Phosphatidylinositol 3-kinase C2α Links Clathrin to Microtubule-dependent Movement

Y. Zhao, I. Gaidarov, J. H. Keen; Dept. of Biochemistry and Molecular Biology, Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA

Phosphatidylinositol 3-kinase C2α (PI3K-C2α) has been implicated in several important membrane transport and signaling processes. We previously showed that overexpression of PI3K-C2α inhibits clathrin-mediated membrane trafficking and induces proliferation of clathrin coated structures within the cytoplasm. Using fluorescently tagged fusions of PI3K-C2α and clathrin, we find that these structures contain both proteins. Further, they exhibit localized, extremely rapid mobility such that they can be reliably tracked for only several frames captured at video rate. The movement is microtubule-based as revealed by use of inhibitors, and PI3K-C2α rapidly and reversibly accumulates on microtubules upon cytoplasmic acidification, which also blocks movement. The microtubule-based movement is mediated by dynamin, as its p50 subunit both immunoprecipitates with PI3K-C2α and, upon overexpression, inhibits movement. Based on these findings, we have more closely evaluated the dynamics of GFP-clathrin coated structures in the absence of exogenous PI3K-C2α expression. In addition to the relatively stationary distribution of cell surface coated pits and established vectorial movement of putative coated endosomes/vesicles, a proportion of transient GFP-clathrin spots exhibited localized and rapid movements similar to those widespread in PI3K-C2α-expressing cells. Importantly, these hyper-motile clathrin coated structures repeatedly interact with microtubules. Collectively, these results reveal a novel population of intracellular clathrin coated structures characterized by localized fast mobility and suggest that PI3K-C2α is a functional linker of these and potentially other clathrin coated membranes to the microtubule motor machinery.

Domain-mediated Oligomerization, Phosphoinositide Binding, and Membrane Targeting by Human DIP13/APPL Rab5 Effector Proteins

H. J. Chiai,1 P. Lenart,2 R. Wu,3 L. C. McPhail,4 Y. Q. Chen;1 Dept. of Neurology and Neurological Sciences and the Neuroscience Institute at Stanford, Stanford University School of Medicine, Stanford, CA,4 Stanford Research Institute of Molecular Pathology (IMP), Vienna, Austria,5 Dept. of Cancer Biology, Wake Forest University School of Medicine, Winston-Salem, NC,6 Dept. of Biochemistry, Wake Forest University School of Medicine, Winston-Salem, NC

α-DIP13 and α-DIP13β are a pair of highly homologous proteins that contain three predicted domains: a N-terminal Bin/Amphiphysin/Rvs (BAR) domain, a central pleckstrin homology (PH) domain, and a C-terminal phosphorysytline binding (PTB) domain. DIP13α interacts with a diverse set of receptors and signaling proteins, including DCC (Liu et al., 2002), FSHR (Nechamen et al., 2004), AdipoR1 and AdipoR2 (Mao et al., 2006), AKT proteins, and PI3K subunits (Mitsuuchi et al., 1999). DIP13α and DIP13β also interact with GTP-bound Rab5, and are proposed to function in an endosome-mediated signaling pathway linking the cell surface to the nucleus (Miaczynska et al., 2004). Here, we investigated the function of DIP13 BAR, PH, and PTB domains. BAR domains form crescent-shaped dimers capable of sensing and inducing membrane curvature, whereas PH and PTB domains are similar in structure and interact with both phosphoinositides and protein targets. Our co-immunoprecipitation and yeast two-hybrid studies demonstrate that full-length DIP13 proteins form homo-oligomers (DIP13α-DIP13α and DIP13β-DIP13β) and hetero-oligomers (DIP13α-DIP13β), and that DIP13 BAR domains are necessary and sufficient for mediating these interactions. We also found that DIP13 isolated BAR domains are targeted to novel cell membrane structures that overlap with regions of altered cellular morphology. We developed a dual-mode confocal microscopy-based approach for fluorescence resonance energy transfer (FRET) studies in which a single cell is analyzed using four independent FRET methods, and observed strong FRET signal for DIP13α-DIP13α, DIP13α-DIP13β, and DIP13β-DIP13β isolated BAR domain FRET pairs. Finally, we found that full-length DIP13 proteins bind membrane-immobilized phosphoinositides, and that DIP13 isolated PH or PTB domains are sufficient for phosphoinositide binding in vitro and membrane targeting in vivo. Taken together, these findings suggest that DIP13 proteins may function as protein scaffolds that organize Rab5 subdomains on signaling endosomes via their underg00 do segment-mediated oligomerization, phosphoinositide binding, and membrane targeting.

PI4P Promotes the Recruitment of GGA Adaptor Proteins to the trans-Golgi Network and Regulates Their Recognition of the Ubiquitin Sorting Signal

J. Wang, H. Yin, H. Sun; Physiology, University of Texas Southwestern Medical Center at Dallas, Dallas, TX

Phosphatidylinositol 4-phosphate (PI4P) is highly enriched in the trans-Golgi network (TGN). Here we establish that PI4P is a key regulator of the recruitment of the GGA clathrin adaptor proteins (GGAs) to the TGN and show that PI4P has a novel role in promoting their recognition of the ubiquitin (Ub) sorting signal. Knockdown of PI4KIIe by RNA interference (RNAi), which depletes the TGN’s PI4P, impaired the recruitment of the GGAs to the TGN. The GGAs bind PI4P through their GAT domain, in a region called C-GAT which also binds Ub but not Arf. We identified two basic residues that are essential for PI4P binding in vitro and for the recruitment of the full-length GGAs to the TGN. These GGA mutants failed to rescue the normal TGN phenotype of the GGA RNAi-depleted cells. These residues partially overlap with those that bind Ub, and PI4P increased the binding affinity of the GAT domain for Ub. Since the recruitment of clathrin adaptors and their cargoes to the TGN is mediated through a web of low affinity interactions, our results show that the dual roles of PI4P can promote specific GGA targeting and cargo recognition.

Membrane Targeting of Phosphatidylinositol 4 Kinase Type II beta

G. Jung, B. Barylo, J. Abanesi; Department of Pharmacology, UT Southwestern Medical Center, Dallas, TX

Phosphorylation of phosphatidylinositol (PI) at the D-4 hydroxyl yields PI(4,5)P2, the principal precursor in the synthesis of two signaling lipids, P(4,5)IP2 and P(3,4,5)IP3. Mammalian cells express four enzymes that catalyze this reaction, the PI4-kinases known as PI4KIIα, PI4KIIβ, PI4KIIIα, and PI4KIIIβ. This study is concerned specifically with PI4KIIα, a 55kDa protein that is almost evenly distributed between cytosol and membrane. The cytosolic pool is unmodified and catalytically inactive, whereas the membrane-associat ed pool is both phosphorylated and palmitoylated and expresses a catalytic activity of about 100 min⁻¹. Palmitoylation, but not phosphorylation, is essential for activity. A portion of cytosolic PI4KIIα redistributes to the plasma membrane particularly to cell-cell junctions, in response to Rac activation. The N-terminal 90 amino acids are required for this rec localization but are not involved in overall binding of the kinase to membranes. Instead, its relative distribution between membranes and cytosol is determined by the C-terminal catalytic domain (residues 91-481). A candidate binding partner for this catalytic domain is Hsp90, which protects PI4KIIα from proteolytic degradation, but also appears to sequester it to the cytoplasm.

Phosphoinositide-mediated ACAP4 Recruitment to the Plasma Membranes of Endothelial Cells Stimulated by EGF

F. Wang,1,2 Z. Fang,3 F. Wu,4 L. Ge,5 X. Cao,2 Y. Liu,2 X. Ding,2 X. Yao,1,6 M. Powell1; 1Cellular Proteomics, Morehouse School of Medicine, Atlanta, GA, 2Univ. of Sci. & Tech. of China, Hefei, China, 3Cellular Dynamics, Univ. of Sci. & Tech. of China, Hefei, China, 4Physiology, Morehouse School of Medicine, Atlanta, GA, 5Medicine, Beijing Univ. of Chinese Med., Beijing, China, 6Hefei National Laboratory, Hefei, China

ARF6 is a conserved regulator of membrane trafficking and actin cytoskeleton dynamics at the leading edge of migrating cells. A key determinant of ARF6 function is the lifetime of the GTP-bound active state, which is orchestrated by GTPase-activating protein and GTP-GDP exchange factor. However, very little is known about the mechanisms underlying ARF6-mediated cell migration. To systematically analyze proteins that regulate ARF6 activity, we performed a proteomic discovery of proteins selectively bound to active ARF6 using mass spectrometry and identified a novel ARF6-specific GTPase-activating protein ACAP4 (Mol. & Cell Proteomics. 2006. 5, 1437-1449). ACAP4 co-distributes with ARF6 in the cytoplasm but relocates the plasma membrane particularly to cell-cell junctions, in response to Rac activation. The N-terminal 90 amino acids are required for this relocalization but are not involved in overall binding of the kinase to membranes. Instead, its relative distribution between membranes and cytosol is determined by the C-terminal catalytic domain (residues 91-481). A candidate binding partner for this catalytic domain is Hsp90, which protects PI4KIIα from proteolytic degradation, but also appears to sequester it to the cytoplasm.

Phosphoinositide-mediated ACAP4 Recruitment to the Plasma Membranes of Endothelial Cells Stimulated by EGF

F. Wang,1,2 Z. Fang,1 F. Wu,4 L. Ge,5 X. Cao,2 Y. Liu,2 X. Ding,2 X. Yao,1,6 M. Powell1; 1Cellular Proteomics, Morehouse School of Medicine, Atlanta, GA, 2Univ. of Sci. & Tech. of China, Hefei, China, 3Cellular Dynamics, Univ. of Sci. & Tech. of China, Hefei, China, 4Physiology, Morehouse School of Medicine, Atlanta, GA, 5Medicine, Beijing Univ. of Chinese Med., Beijing, China, 6Hefei National Laboratory, Hefei, China

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ARF6 is a conserved regulator of membrane trafficking and actin cytokinesis dynamics at the leading edge of migrating cells. A key determinant of ARF6 function is the lifetime of the GTP-bound active state, which is orchestrated by GTPase-activating protein and GDP-GTP exchanging factor. However, very little is known about the mechanisms underlying ARF6-mediated cell migration. To systematically analyze proteins that regulate ARF6 activity, we performed a proteomic discovery of proteins selectively bound to active ARF6 using mass spectrometry and identified a novel ARF6-specific GTPase-activating protein (ACAP4; Mol. Cell Proteomics, 2006; 5, 1437-1449). ACAP4 co-localizes with ARF6 in the cytoplasm but relocates the plasma membrane ruffles upon the EGF stimulation. Our computational analysis reveals that ACAP4 contains one PH domain, one GAP motif, and two ankyrin repeats. Within the PH domain, ACAP4 contains four PI(2)P-binding sites. To illustrate the functional relevance of the PI(2)P-binding sites in the recruitment of ACAP4 to the plasma membrane, we carried out systematic mutational analyses to change lysine residue individually or jointly and assessed the ability of mutant proteins to locate to the plasma membrane in response to EGF stimulation. Our analyses revealed that the four PI(2)P-binding sites cooperate to provide an adaptor for the recruitment of ACAP4 to the plasma membrane. Significantly, overexpression of the PI(2)P-binding deficient mutant abolished the recruitment of ACAP4 to the plasma membrane, which results in accumulations of ACAP4 and ARF6 in the cytoplasm and an inhibition in membrane ruffling. In addition, overexpression of the PI(2)P-binding deficient ACAP4 mutant suppresses ARF6-dependent cell migration in wound-healing. These results suggest that phosphoinositide-mediated recruitment of ACAP4 is essential for EGF-stimulated membrane dynamics underlying cell migration. Using fluorescence resonance energy transfer measurements in living cells, we are evaluating how PI2P determines the segregation of proteins in the membrane ruffle induced by EGF.

Actin Comet Tails Rocketing EGF-containing Endosomes Are Induced by Phosphatidic Acid
A. Norambuena, E. Pardo, A. González; Reumatología, Fac. Medicina, and Centro de Regulación Celular y Patología, Fac. Ciencias Biológicas, Pontificia Universidad Católica de Chile, and MIFAB, Santiago, Chile
Endocytosis of EGF can be induced in the absence of ligand and independently of tyrosine phosphorylation and ubiquitilation of the receptor by inhibiting basic PKA activity (Salazar and González, Mol Cell Biol 13; 1677-1693. 2002). This indicates that the permeability of inactive EGF at the cell surface can be transmodulated. Here, we explored the role of phosphatidic acid (PA) as a signaling lipid involved in the regulation of clathrin-mediated endocytosis, actin cytoskeletal function and phosphodiesterase(PDE)-dependent cAMP levels. In several cell lines, inhibition of phosphatidic acid phosphohydrolase by propranolol treatment increased the membrane levels of PA, leading to induction of clathrin-mediated endocytosis of inactive EGF. This PA-induced endocytosis negative regulation of PKA, through activation of rolipram-sensitive cAMP-PDEs, as well as activation of RhoA and Rac-1. Strikingly, in NIH3T3 cells permanently transfected with human EGF, propranolol treatment both in the absence or presence of EGF, induced actin tails emerging from EGF-containing endosomes in a process involving Cdc42 and N-WASP activity. Generation of these actin tails requires the expression of the EGFFR. Although actin comets have been widely described as propelling forces for certain bacterial and viral pathogens as well as for endocytic vesicles and other membranous organelles, to our knowledge, this is the first time that detection of actin tails associated with endosomes containing a signaling receptor. We also showed a novel function for a await known mechanism in this paper and PA pathways, namely, control of the endocytic trafficking of EGF. The mechanism involves an actin role at different steps. Because PA levels is regulated by a variety of stimulus, such a control system provides wide possibilities of transmodulating both the accessibility of empty EGF to extracellular stimuli and the spatial determinants of receptor signaling (Financed in part by FONDAP grant# 1398001 and Millennium project from Ministerio de Planificación).

Sorting Nexin 9 Links Assembly Dynamics to Membrane Remodeling
D. Yarar, C. Waterman-Storer, S. L. Schmid; The Scripps Research Institute, La Jolla, CA
Remodeling of the plasma membrane is essential for multiple types of endocytosis. However, the factors that contribute to these distinct pathways are not clearly understood. We previously demonstrated that Sorting Nexin 9 (SNX9) interacts with dynamin and functions in clathrin-mediated endocytosis. Here, we demonstrate by siRNA-mediated knockdown that SNX9 is also critical for other clathrin-independent, but nonetheless actin-dependent, fluid phase internalization pathways. Moreover, we report that SNX9 localizes to circular dorsal ruffles and peripheral ruffles, actin-rich structures implicated in fluid-phase uptake. To identify the mechanism by which SNX9 functions at these sites, we investigated the connection of SNX9 with established components of the actin cytoskeleton. We find that SNX9 binds directly to the N-WASP, an Arp2/3 complex nucleation promoting factor, and stimulates N-WASP/Arp2/3-mediated actin nucleation. At low, physiological concentrations, the ability of SNX9 to stimulate WASP/Arp2/3-mediated actin polymerization is greatly enhanced by the presence of PI(2)P-containing liposomes, which is in part due to PI2P-induced SNX9 oligomerization. Isolated SNX9 oligomers have significantly higher N-WASP stimulatory activity than unassembled forms of SNX9. These results define a novel mechanism for the regulation of N-WASP-dependent actin assembly and implicate SNX9 in the coupling of actin dynamics to membrane remodeling.

Dynamic Actin Facilitates Endosomal Traffic
D. A. Schafer; Biology, University of Virginia, Charlottesville, VA
Actin filaments participate in several steps of endocytosis and trafficking: to internalize ligands at the plasma membrane, to propel endosomes about the cytoplasm and to promote vesicle fusion. To investigate how actin filaments function during intracellular endosomal trafficking, we observed dynamic actin and endocytic tracers in live cells. Several pools of dynamic actin were visualized in Ptk1 fibroblasts stably expressing GFP-capping protein (GFP-CP), a convenient marker for actin filaments undergoing rapid turnover. One pool of dynamic actin exists as small foci that move in a salutatory manner about the periphery of perinuclear vesicular compartments but do not power movement of the compartments. Arp2/3 complex, cortactin, capping protein, and N-WASP contribute to this dynamic actin pool. The vesicular compartments containing actin foci loaded with TexasRed-transferrin (TR-Tf) within 5-10 minutes. In fixed cells, the vesicular compartments labeled with antibodies to the early endosome marker, EEA-1, however, its distribution and that GFP-CP at the periphery of these compartments was not controlled. In contrast, the distribution of one of the ubiquitously expressed secretory carrier membrane proteins, SCAMP3, was coincident with actin foci. Thus, early endosomes assemble actin foci within discrete domains on their surfaces. Inhibition of actin dynamics with Latrunculin B stopped the movement of endosome-associated actin foci and blocked the trafficking of TR-Tf. Two observations point to a connection between actin dynamics and the formation of tubules that might sequester sorted proteins during trafficking. First, endosomes in LatB-treated cells became enlarged and more "tubular" compared to those in untreated cells. Second, a phospholipaseA2 antagonist that inhibits tubule formation on endosomes and blocks recycling traffic also blocks actin dynamics on endosomes. These findings are consistent with a role for dynamic actin filaments in selective sorting via tubulovesicular compartments early in the endocytic pathway.

Transmembrane Ser/Thr Kinase KPI-2 Is a Myosin VI-Binding Protein Involved in Endocytic Membrane Trafficking
T. Inoue,1 T. Kon,1 R. Okaura,2 H. Yamakawa,2 O. Ohara,1 J. Yokota,1 K. Sutoh1; 1Department of Life Sciences, Graduate School of Arts and Sciences, University of Tokyo, Tokyo, Japan, 2Kazusa DNA Research Institute, Chiba, Japan, 3Biology Division, National Cancer Center Research Institute, Tokyo, Japan
Endocytic membrane traffic in eukaryotic cells is a fundamental process in sorting and delivering membrane components to various intracellular compartments. Interactions of endocytic components with the cytoskeleton and associated motor proteins have important roles in this pathway. Several studies have shown that the cellular functions of myosin VI are involved in clathrin-mediated endocytosis, in intracellular transport of clathrin-coated and uncoated vesicles, in the maintenance of Golgi-distributed morphology and in secretion in mammals. The diverse functions and cellular localizations of myosin VI are considered to be mediated by interacting with distinct cargos proteins. In this study, using a yeast two-hybrid screen, we have identified and characterized a novel myosin VI-binding partner, KPI-2 (Kinesin/Phosphatase/Inhibitor-2), a transmembrane Ser/Thr kinase. Several binding experiments confirmed the interaction of KPI-2 with myosin VI in vivo in vitro. Immunocytochemical analyses showed that KPI-2 localizes to perinuclear recycling endosomes (REs), where transferrin receptor or transfected GFP-Rab11 resides. Overexpression, the cells whose KPI-2 was depleted by siRNA were still able to internalize transferrin molecules and transport them to early endosomes (EE), but could not further transport them to perinuclear REs. EAE-based analysis also revealed that the rate of transferrin uptake was reduced in KPI-2-depleted cells. These results support the notion that KPI-2 is essential for transition of endocytosed vesicles from EEs to perinuclear REs, also suggesting a possible involvement of myosin VI in this pathway.

Role of the Rho Family GTP-binding Protein Rac1 and Its Guanine Nucleotide Exchange Factor Ost in Negative Regulation of Receptor Endocytosis
K. Ieguchi, S. Ueda, T. Katoaka, T. Satoh; Division of Molecular Biology, Department of Molecular and Cellular Biology, Kobe University Graduate School of Medicine, Kobe, Japan
Clathrin-mediated endocytosis is the main pathway for internalization of cell surface receptors. In this study, we examined the involvement of the Rho family GTP-binding protein Rac1 and the Dbl family guanine nucleotide exchange factor Ost in receptor endocytosis. The GTP-bound active form of Rac1, but not other Rho family members including RhoA and Cdc42, negatively inhibits internalization of epidermal growth factor receptor (EGFR) by re-localizing it to the plasma membrane and promoting ligand-induced receptor tyrosine phosphorylation.
regulated endocytosis of the transferrin receptor (TfR) in HeLa cells. Downregulation of Rac1 expression by siRNA treatment significantly increased TfR endocytosis. The Ost splice variant Ost-III, which contains a unique C-terminal region including an SH3 domain, potently inhibited TfR endocytosis like activated Rac1, whereas expression of other splice variants Ost-I and Ost-II had virtually no effect. Downregulation of Rac1 expression by siRNA treatment, in fact, canceled the inhibitory effect of Ost-III. Furthermore, we identified γ-amino butyric acid type A receptor-associated protein (GABARAP) as a protein that binds to the C-terminal region of Ost-III. When ectopically co-expressed, GABARAP potently suppressed Ost-III inhibition of TfR endocytosis, suggesting that GABARAP modulates Rac1 activity by binding to Ost-III. Indeed, Ost-III-dependent Rac1-GTP formation in transfected cells as assessed by pull-down assays was significantly reduced upon co-expression of GABARAP. Ost-III inhibited epidermal growth factor receptor endocytosis as well, implicating Ost-III/Rac1-dependent inhibition as a general regulatory mechanism for clathrin-mediated receptor endocytosis.

A Rhoa Guanine Exchange Factor Associates with the Early Endosome and Regulates Clathrin-dependent Uptake

M. Liu, A. Horowitz; Medicine, Angiogenesis Research Center, Dartmouth Medical School, Lebanon, NH

We have recently reported (MBC, 17:1880-7, 2006) that the targeting and activity of the novel Rhоa guanine exchange factor (GEF) Syx depends on its interaction with the PDZ adaptor protein synectin, a member of the immunoglobulin superfamily. Syx accounted for a major portion of the total RhoA activity in rat microvascular endothelial cells and was essential for the maintenance of cell migration. Synectin is involved in vesicle traffic and in the recruitment of the molecular motor myosin VI to uncoated vesicles (MBC, 14: 2728-43, 2003; PNAS, in press, 2006). We asked, therefore, if the activity of Syx, the ensuing activation of Rhoa, and the remodeling of the actin cytoskeleton are involved in the clathrin-dependent uptake of transferrin. We found that Syx colocalized with the early endosome markers Rab5 and EEA1. These findings are in agreement with the known participation of synectin in the traffic of uncoated vesicles between the plasma membrane and the early endosome and with its colocalization with Rab5. Furthermore, Syx co-immunoprecipitated with both EEA1 and Rab5, suggesting these proteins participate in the same complex. In order to examine the possible involvement of Syx in vesicle traffic we tracked the uptake of fluorescently-labeled EGF, a known clathrin-dependent process. We compared the rate of EGF uptake in endothelial cells transfected with Syx-specific siRNA to that of control cells. In pulse-chase experiments EGF reached perinuclear late endosomes in 15 min in close to a 100% of control siRNA-transfected cells. In the same time interval EGF reached perinuclear late endosomes in less than 20% of the Syx-specific siRNA-treated cells. These results suggest that the Rhоa GEF Syx modulates vesicle traffic, most likely that of uncoated vesicles up to their fusion with early endosomes. Consequently, Syx is an attractive target to probe actin dynamics accompanying vesicle movement in the cytoplasm.

Rac1 Regulates the Endosomal Localization and Function of the Rab5 Guanine Nucleotide Exchange Factor ALS2/asin via Rac1-activated Macropinocytosis

R. Kunita,1,2 A. Otomo,1 H. Mizumura,7 K. Suzuki-Utsunomiya,7 S. Hadano,1,2 J. Keddi1,2; 1Department of Molecular Life Sciences, Tokai University School of Medicine, Isehara, Kanagawa, Japan, 2Solution Oriented Research for Science, Japan Science and Technology Agency, Kagawuchi, Saitama, Japan

ALS2/asin is a member of guanine nucleotide exchange factors for Rab5 (Rab5GGEF) and a modulator in endosome dynamics. It has been demonstrated that, in cultured cells, ALS2 is mostly sequestered in cytoplasm with a rare distribution onto early endosome autoantigen 1 (EEA1)-positive endosomes, while a constitutively active form of ALS2 lacking the N-terminal RCC1-like domain is exclusively recruited to endosomes, promoting their enlargement. Thus, the intracellular localization and function of ALS2 can be regulated by certain upstream molecular signalings as well as its own domain structures. However, such regulatory mechanisms remain unknown. Here, we show that the subcellular localization of ALS2 is controlled by Rac1 signaling. ALS2 preferentially interacts with activated Rac1 in the cells and in vitro. Further, cytoplasmic ALS2 was exclusively recruited to membrane ruffles by activated Rac1 and then relocated to nascent macropinosomes via Rac1-activated macropinocytosis. At later endocytic stages, macropinosomal ALS2 enhanced fusion of the ALS2-associated macropinosomes with the transferrin-positive endosomes, depending on the ALS2-associated Rab5GGEF activity. These results suggest that ALS2 mediates fusion between distinct endosomal compartments under the control of Rac1 signaling. To our knowledge, ALS2 is the first Rab5GGEF that acts as a Rac1 effector and is involved in Rac1-activated macropinocytosis. ALS2 is a novel clue to delineate a molecular basis of the Rac1-Rab5 signaling.

Interaction of the μ Subunit of the Adaptor Protein Complex AP-4 with the Small GTPase RhoB

C. Rondanino, L. Leiva-Vega, J. Kunikle, G. Apodaca; Medicine/Cell Biology, University of Pittsburgh, Pittsburgh, PA

RhoB, a member of the Rho family of GTPases that localizes in part to endosomes, was recently shown to modulate basolateral traffic in polarized Madin-Darby Canine Kidney (MDCK) cells. However, the mechanism by which RhoB mediates this process is unknown. We hypothesize that RhoB acts through downstream effectors. To identify novel RhoB binding partners, we performed a yeast-two hybrid screen of a human kidney library. Both dominant active RhoB (RhoBV14) and dominant negative RhoB (RhoBN19) were found to interact with the μ subunit of the adaptor protein complex AP-4 (μ4). AP-4 is a heterotetrameric complex shown to be also present on endosomes and to regulate basolateral sorting in MDCK cells. We mapped the RhoB interaction site to the C-terminal domain of μ4. Among the small GTPases, the switch I and switch II regions have been shown to be the sites of interaction with effector molecules. The T37Y mutation in the switch I domain of RhoB, known to impair the binding to both GDP and GTP, abolishes the interaction with μ4. However, other mutations in the switch II domain, such as F39A, F170L, F197L, and C42Y, were found to be inactive. RhoA, which is 92% homologous to RhoB, also bound the C-terminus of μ4 whereas other GTPases such as Cdc42 and Rac1 did not. Myc-tagged RhoBV14 and RhoBN19 were overexpressed in MDCK cells together with full length μ4 bearing an internal HA tag. Both proteins were found to co-localize in vesicular structures. Moreover, full length μ4 co-immunoprecipitated with RhoBV14. Taken together, these data show that μ4 is a novel RhoB binding protein. RhoB may modulate endocytic traffic through its interaction with μ4.

Gus Regulates Endosome Maturation

A. Beas, C. Lavoie, I. R. Niesman, M. G. Farquhar; Cellular and Molecular Biology, University of California San Diego, La Jolla, CA

 Trafficking from early endocytic vesicles to lysosomes involves endosomal growth by fusion, multivesicular body (MVB) sorting, and endosome-lysosome fusion. This pathway is utilized to downregulate many cell surface receptors including the EGFR receptor (EGFR). Previously, we found that the heterotrimeric G-protein stimulatory n-subunit (Gαs) and its GAP, RGS-PX1, may regulate EGFR sorting (Zheng, B. et al., 2001, Science 294: 1939; Zheng, et al., 2004, MBC 15:5538). In the current study, we report a possible role for Gus in regulating endosome size. HeLa cells overexpressing GusGFP contain smaller EEA1-labeled endosomes compared to control cells; conversely, Gus depleted cells have larger endosomes. EM analysis demonstrates that Gus depleted cells accumulate larger multivesicular endosomes (MVB) than control cells. Also, Gus depletion shifts EEA1 to membrane fractions, while Gus overexpression shifts EEA1 to cytosolic fractions. Overall, our data suggests Gus regulates endosome size and/or maturation and in turn, affects trafficking from endosomes to lysosomes.

The Role of Newly Identified Protein, Melanoregulin in Phagocytic Processes

D. Mamek-Poprawa, K. Boese-Battaglia; Biochemistry, University of Pennsylvania, School of Dental Medicine, Philadelphia, PA

Introduction: Deletion mutation of melanoregulin (Mreg) (a.k.a. dilute suppressor protein dsu), a recently identified 26 kDa product of the Wnd2 gene (a.k.a. dou, Gm074, MGC32980), affects phagocytosis, but not the inhibition by activated Rac1, suggesting that melanoregulin functions downstream of Rac1. Melanoregulin containing protein-complexes were analyzed using co-immunoprecipitation with mAb 165 and anti-Merk antibodies. Results: Human retinal pigment epithelium (ARPE-19) cells express high level of melanoregulin mRNA comparing to lymphocytes and Jurkat cells. Confocal analysis was performed by confocal microscopy using fluorescent probes to detect Mreg and a membrane raft associated marker GM1. Dark- and light-adapted cells were analyzed in order to take into account membrane rearrangement during the rhytmic phagocytic activity. Melanoregulin co-precipitated with phagocytic receptor c-mer tyrosine kinase (Merk) from ARPE-19 cell lysate. Mreg level was further validated and confirmed in vitro on primary photoreceptor cells by temporary transfection and phagocytosis processes, including phagocytosis. Our results suggest that melanoregulin is localized to lipid rafts in response to light. Moreover, melanoregulin interacts with phagocytic receptor Merk. Therefore, we propose that the light-dependent dynamic association of melanoregulin with rafts is necessary for normal phagocytosis in RPE cells and consequently for continuing photoreceptor renewal process.
Rab5, a member of the Rab-family GTPases, plays important roles in the endocytosis and homotypic early endosome fusion. However, the function of Rab5 has been examined mainly by cell biological experiments using constitutively-active (CA) and dominant-negative (DN) mutants, or in vitro biochemical analyses. Thus, it is largely unknown when and where Rab5 is activated and inactivated during endocytosis. In order to address this issue, we developed a fluorescence resonance energy transfer (FRET)-based probe, designated Raichu-Rab5, for visualizing Rab5 activity in living cells. Raichu-Rab5 consists of YFP, the Rab5 binding domain of EEA1, CFP, and Rab5a. Raichu probes for wild-type, CA, and DN mutants of Rab5 were expressed in 293F cells and their emission profiles were examined. Compared with the wild-type Rab5 probe, Raichu-Rab5-CA had a markedly increased FRET efficiency, while Raichu-Rab5-DN had a decreased FRET efficiency. The dynamic range of Raichu-Rab5 was over 90%, proving that Raichu-Rab5 was a useful tool for monitoring the change of Rab5 activity. The GTP loading of the Rab5 probes correlated well with that of the authentic Rab5 proteins. Next, we confirmed the increased and decrease in the FRET level of Raichu-Rab5 by the expression of GEFs and GAPs acting on Rab5, respectively. The subcellular distribution of Raichu-Rab5 was similar to that of mRFP-Rab5 and the localization of the probe to early endosomes was ascertained using markers for PI(3)P. We also confirmed that Raichu-Rab5 bound to RabGDI, which controls the shuttling of Rab5. In Swiss3T3 cells, we observed a higher activity of Rab5 in the endosomal structures than in the cytosol. In order to elucidate the precise role of Rab5 in endocytic processes, we are currently trying to obtain informative images of Rab5 activation during endocytosis.

Interaction of Drosophila Rab GDI with Gint3, a UBX-domain Containing Protein

P. Amin, M. Lawson, A. Chen, B. Richardson, K. Ayres, A. Moore, N. Sangi, M. Keese, C. M. Cheney; Biology, Pomona College, Claremont, CA
Rab GTPases play multiple roles in vesicle transport. Rab GDP dissociation inhibitor (GDI) regulates Rab polymerization by retrieving Rab proteins from the cytoplasm to donor membranes. GDP plays a critical role in ensuring that the correct Rab is placed in the correct membrane. To identify proteins that interact with GDI, we have carried out screens for GDI interactors, using Drosophila as the model system, since the fly genome has a single GDI, but multiple Rab5a. In a yeast two-hybrid (Y2H) screen, a protein with a ubiquitin-like UBX domain was identified as a GDI inhibitor. This protein was named Gint3, for GDI interacting protein 3. Gint3 has a PUG-N-glycanase-associated domain and a UBX ubiquitin-like domain, but no obvious transmembrane domain. Gint3 did not interact with L319 mutant GDI, which has a mutation that alters the lipid-binding pocket of Domain II of GDI (Ricard et al, 2001, Genesis 31:17). Gint3 also did not interact with AK037 GDI, which has E349K mutation. Phosphoryl analysis of Gint3 and UBX domain proteins shows that Gint3 forms a distinct subfamily of UBX domain proteins with the highest bootstrap value. The region between the PUG and UBX domains also shows a high degree of conservation. Supported by NSF-RUI grant 0212730 to CMG.
mediated by different vSNAREs (VAMP3, VAMP4, VAMP7 and VAMP8). VAMP3 and VAMP8 are v-SNAREs of the early and late endosomes respectively. TI-VAMP has been proposed to mediate vesicular transport from endosomes to lysosomes. Here we have investigated the role of these different v-SNAREs in the trafficking of the EGF/EGFR complex by silencing their expression level by RNA interference. Our results indicate that silencing TI-VAMP expression results in increased EGF compared with control and VAMP3, VAMP4 or VAMP8 silenced cells. The precise membrane trafficking steps involving TI-VAMP in EGF degradation are under investigation.

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Regulation of EGF Receptor Degradation by SCAMP3

Q. Aoh, D. Castle; Cell Biology, University of Virginia, Charlottesville, VA

Downregulation of cell surface receptors by endocytosis and degradation in the lysosome is vital to regulating cell signaling events and maintaining homeostasis. Ubiquitylation plays a key role in recognition of downregulated receptors such as the epidermal growth factor receptor (EGFR) and in regulating and mediating interactions with accessory and adapter proteins, e.g. epsins, Hrs and ESCRT complexes, that act along this pathway. Here we report that SCAMP3, a member of the Secretory Carrier Membrane Protein (SCAMP) family is multi-monoubiquitylated and interacts with both Hrs and Nedd4 family ubiquitin ligases. A portion of SCAMP3 localizes to compartments that traffic EGF. Additionally, SCAMP3 associates with endocytic cargos through a carboxy-terminal proline-rich (PR) motif. Our results demonstrate that SCAMP3 plays an important role in ubiquitinating EGFR and regulate the sorting efficiency of ubiquitinated EGFR degradation. These results suggest SCAMP3 has important roles in organizing the early endosomal compartment and in regulating sorting between degradative and recycling pathways.

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The Role of Oligo-ubiquitin and Endocytic Adaptors in Misfolded CFTR Degradation from the Cell Surface

H. Barriere, C. Nemes, G. L. Lukacs; Cell Biology, Hospital for Sick Children, Toronto, ON, Canada

Recently we have demonstrated that ubiquitination plays a pivotal role in the rapid elimination of non-native CFTRs from the plasma membrane by rerouting the mutant channel from constitutive recycling towards lysosomal degradation. This process is conceivably mediated by the ubiquitin-dependent endosomal sorting machinery, including components of ESCRT-I, II and III (J. Cell. Biol. 2004, 164-923-933). To gain insight into the molecular basis of ubiquitinated transmembrane protein, including CFTR, internalization and endosomal sorting, we used type-I and type-II membrane proteins harbouring ubiquitin (Ub) as their exclusive sorting signal. Relying on CD4 and invariant-chain Ub-chimeras in combination with functional, biochemical and genetic techniques, we obtained evidences indicating that poly- and monoubiquitin, but not monomeric Ub can be recognized as an endocytic sorting signal by a subset of UIM containing clathrin adaptors (eps15/15R and epsin) in higher eukaryotes. The preferential binding of poly-Ub by eps15 was also verified in vitro. Combination of morphological and genetic techniques also showed that Ub chimeras were targeted for clathrin-dependent and caveolin-independent internalisation. Similar internalisation route and adaptor requirement were identified for CD4 ubiquitinated by MARCKS, a highly cationic receptor as well as ubiquitinated mutant CFTR. Measuring CFTR internalization rates in clathrin-adaptor-depleted cells demonstrated that CFTR ubiquitination provoked the switch from an AP-2-dependent internalization, driven by Tyr- and di-Leu based endocytic motifs of native channel to eps15/epsin-dependent endocytosis of misfolded CFTR. Ongoing experiments aim at identifying the ubiquitin chain topology and coat requirement for lysosomal sorting of ubiquitinated cargo at early endosomes.

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The Pathways of Protein Cellular Delivery with Transportan

M. Pooga,1 2 K. Padar,3 P. Säälä,2 A. Niiwatt4,2 M. Hamsten4,2 U. Langel4; 1Estonian Biocentre, Tartu, Estonia, 2Institute of Zoology and Hydrobiology, University of Tartu, Tartu, Estonia, 3Institute of Cell and Molecular Biology, University of Tartu, Tartu, Estonia, 4Department of Biochemistry and Genetics, Tartu University, Tartu, Estonia, Sweden

Cell penetrating peptides (CPP) consisting of up to 30 amino acids are capable of translocation through cell membranes and delivering different cargoes. Despite numerous efforts the internalization mechanisms of CPPs have not been fully understood. The uptake of CPPs is dependent on the properties and concentration of peptide, cellular energy and lipid rafts of plasma membrane. Binding to the cell surface proteoglycans, followed by endocytic uptake is considered as the main mechanism of highly cationic CPPs’ cell-entry. However, the type of endocytosis induced by CPPs, further intracellular targeting, and liberation from vesicular structures are still under debate. We have focused on the characterization of the interaction sites with cells and intracellular trafficking of transportans (TP). The results of fluorescence and transmission electron microscopy show that TP and its constructs with proteins associate both with transferrin and transferrin receptor-positive vesicles and flat areas of cell surface. Interaction of TP-protein constructs with the plasma membrane induces formation of invaginations and vesicles pinch off into cells. The size and shape of forming vesicles is dependent primarily on the concentration of peptide and properties of cargo protein. Big non-homogenous vesicles with irregular shape, reminiscent macropinososomes, form at high TP concentration, while at lower peptide concentration small vesicles are induced suggesting the raft- or caveolae mediated uptake. At the initial steps of uptake, the cargoes coupled to transportans show moderate colocalization with proteins characteristic for main endocytic pathways - transferrin, caveolin or flotillin. Later cargoes translocated into cells concentrate in LAMP-positive vesicles in perinuclear area. A small fraction of protein targeted into cells is not confined to vesicular structures but localizes in cytosol, both in cortical and central area. Our results suggest the involvement of different endocytic pathways in the transportans-mediated cellular delivery of proteins and several routes may be used in parallel.

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Multiple Factors Associate with the SH3 Domain of Hse1 to Mediate Cargo Sorting into Multivesicular Bodies (MVB)

J. Ren,1 Y. Kee,2 J. Hubbrecht,3 R. Piper1; 1Physiology and Biophysics, University of Iowa, Iowa City, IA, 2Institute for Cellular and Molecular Biology, University of Texas at Austin, Austin, TX, 3Institute for Cellular and Molecular Biology, University of Texas at Austin, Texas, USA

Ubiquitinated integral membrane proteins are delivered to the interior of the lysosome/vacuole for degradation. This process relies on specific ubiquitination of potential cargo and the recognition of ubiquitin by sorting receptors at multiple compartments. The protein complex Hse1-Vps27 functions as a sorting receptor on yeast endosomes. Hse1 is composed of a N-terminal VHS domain, an UIM domain and an SH3 domain, which are critical for Hse1 function. The SH3 domain is required for MVB formation and proper sorting of vascular hydrolases. Thus, we set out to identify factors that could interact with Hse1 SH3 domain and determine how those interactions contribute to MVB biogenesis. We found the novel protein Hua1 associates with SH3 domain of Hse1. Hua1 also interacts with an E3 ubiquitin ligase, Rsp5, and links Hse1 to the Rsp5-Rsp1-Ubp5 complex. The SH3 domain of Hse1 also binds to the ubiquitin-peptidase complex that regulates the sorting efficiency of proteins into the MVB. The SH3 domain of Hse1 also binds Doa1/Ufd1. We find that Doa1 binds ubiquitin through multiple domains and cells lacking Doa1 have impaired MVB sorting defect. Doa1 is known to interact with multiple factors that are involved in proteasome-mediated ubiquitinated protein degradation. Our data imply that these factors may play additional role in MVB biogenesis.

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MVB Sorting: The Assembly of ESCRT-III at the Endosome

S. Saksena, D. Teis, J. Sun, S. D. Emr; CMM, UCSD, HMI, La Jolla, CA

The endosomal system is required for many key cellular processes including down-regulation of activated cell-surface receptors, antigen presentation, and the sorting of biosynthetic cargoes to the lysosome. The Endosomal Sorting Complex Required for Transport (ESCRT) is essential for the sorting of transmembrane receptors/proteins into Multi-Vesicular Bodies (MVB) and into lysosomes. Thus the ESCRT complex contributes critically to the regulation of cell surface receptor signalling during development and disease. Here we address the molecular mechanism that drives assembly and dynamics of the ESCRT-III core complex, Vps20p, Snf7p, Vps24 and Vps30p on endosomes. ESCRT-III function is required for the final protein-sorting step and membrane invagination during MVB vesicle formation. Unlike ESCRT-I and -II, which are recruited to endosomes as preformed complexes, ESCRT-III subunits remain monomeric in the cytoplasm, driven by the cytosolic conformation of the individual ESCRT-III subunits. What are the signals that initiate the assembly of ESCRT-III on endosomes? To address these issues, we initiated a genetic screen for ESCRT-III mutations that trigger premature activation and assembly in the cytosol. We will report on the characterization of certain “constitutively active” mutants. Our results strongly indicate that the assembly of ESCRT-III on endosomes requires an activating conformational switch from an inactive cytoplasmic monomer. This activation is mediated by ESCRT-II. Once activated, ESCRT-III assemblies on endosomal membranes in a tightly regulated cascade-like manner. Disassembly of the membrane complex requires the ESCRT-II into the individual cytosolic monomers is driven by the AAA-protein Vps4. Based on our findings we propose that ESCRT-II triggers a conformational change in Vps20p, which subsequently drives the assembly of ESCRT-III on endosomes to regulate the final steps in the MVB sorting process.
The multivesicular body (MVB) pathway of eukaryotic cells is an endosomal protein sorting system that delivers transmembrane proteins to the lumen of lysosomes/vacuoles for degradation. The MVB pathway functions in the degradation of cell surface proteins and therefore plays an important role in many cellular functions, such as cell signaling, nutrient uptake and immune response. The sorting of MVB cargo is executed by the protein complexes ESCRT-I, ESCRT-II, ESCRT-III which are recruited to the endosomal membrane and assemble into a large protein network. When sorting is completed the AAA-type ATPase Vps4 disassembles the protein network and recycles the ESCRT machinery. Vps4 is the only known enzyme of the ESCRT machinery and therefore is ideally positioned to play a key role in regulating the MVB pathway. We identified Nrv1 as a negative regulator of Vps4 function. For example, an increase in proteasomal activity would result in decreased levels of Nrv1, which consequently would cause a more efficient delivery of cargo through the MVB pathway. This regulatory connection formed by Nrv1 might allow the cell to regulate simultaneously both major protein degradation systems, the proteasome and the MVB pathway.

Cargo Ubiquitination Along the Endocytic Pathway: Regulation by SKD1/Vps4
K. Umebayashi,1 H. Stemman,2 T. Yoshimori1; 1Research Institute for Microbial Diseases, Osaka University, Osaka, Japan, 2Department of Biochemistry, The Norwegian Radium Hospital, Oslo, Norway

Ubiquitin conjugation to plasma membrane proteins, such as the epidermal growth factor receptor (EGFR), triggers their downregulation. Ubiquitin acts as a sorting signal in internalization from the plasma membrane and multivesicular body (MVB) sorting in endosomes, thereby directing cargoes to lysosomes. EGFR undergoes autophosphorylation upon ligand-binding, and the protooncogene product c-Cbl, which turns out to be a ubiquitin ligase, is recruited to the receptor via the resultant phosphotyrosine residues. However, it remains an open question where and how the c-Cbl-dependent receptor ubiquitination takes place in the endocytic pathway. To address this issue, we devised the following events after EGFR stimulation: localization of c-Cbl, binding of c-Cbl to EGFR and ubiquitinated status of EGFR. Strikingly, after 20 min of EGFR addition, c-Cbl was clearly relocated to endosomes. It was colocalized with EGF in endosomal subdomains marked by Hrs, which sorts ubiquitinated cargoes to the MVB lumen. Up to this chase period, coimmunoprecipitation of c-Cbl with EGFR was increased. Ubiquitination of EGFR occurred immediately after the EGFR stimulation, but it proceeded afterwards, as more c-Cbl was communoprecipitated with the receptor. These results strongly suggest that EGFR is progressively ubiquitinated during its transport to endosomes. This may ensure enough ubiquitin to be recognized by the endosomal sorting machinery. At a longer chase period, c-Cbl was not colocalized with EGFR, indicating that it was dissociated from EGFR prior to the MVB sorting. The dissociation of the EGFR/c-Cbl complex was dependent on the function of the AAA ATPase SKD1/Vps4: overexpression of the ATPase-deficient SKD1(E235Q) resulted in prolonged association of c-Cbl with EGFR in aberrant endosomes. Concomitantly, ubiquitinated status of EGFR was not attenuated for a long chase period. We propose that SKD1/Vps4 dissociates c-Cbl from EGFR and thereby terminates receptor ubiquitination in endosomes.

Functional Characterization of the Chorea-acanthocytosis Gene VPS13A in Saccharomyces cerevisiae
L. I. Grad, E. Combear; Medical Genetics, University of British Columbia, Vancouver, BC, Canada

Mutations in the gene VPS13A are responsible for chorea-acanthocytosis, a neurodegenerative disorder that closely resembles Huntington’s disease. VPS13A is one of four human genes that encode proteins homologous to the yeast protein, Vps13. The function of Vps13-like proteins has yet to be determined. However, yeast Vps13 appears to regulate protein sorting between the endosome and Golgi compartments. In addition, previous studies have demonstrated that Vps13 behaves as part of a large proteinaceous complex that is peripherally associated with intracellular compartments. Since vesicle traffic is highly conserved in eukaryotes and Vps13-like proteins are ubiquitously expressed, they are likely to play analogous roles in vesicle-mediated protein trafficking in humans. We have therefore utilized the highly amenable yeast model system to further investigate the role of Vps13 in the regulation of endosomal transport processes. Results from our genome-wide phenotypic analysis of yeast knockout collections support a role for Vps13 in the regulation of multiple endosomal sorting pathways. We predict that Vps13 is a multifunctional protein that associates with different adaptor proteins to carry out cargo sorting or vesicle formation. Therefore, we have undertaken genetic and biochemical approaches to identify interacting proteins. We have identified a Vps13-interacting protein that co-immunoprecipitates from cell lysates. This protein represents the first known binding partner for Vps13. We have also identified proteins required for the intracellular localization of Vps13. Further characterization of these relationships and the identification of other interacting proteins will allow us to characterize the pathways regulated by Vps13 and contribute to our understanding of the molecular mechanisms of neurological disease pathogenesis caused by defective Vps13-like proteins.

New Roles for an Old Player: Daxx as a Mitotic Regulator That Determines Taxane Sensitivity
C. R. Lindsay, A. Scholz, A. M. Ishov; Anatomy & Cell Biology, University of Florida, Gainesville, FL

Daxx, originally identified as a pro-apoptotic Fas-interacting partner and later demonstrated to have anti-apoptotic activity, is a ubiquitously expressed and highly conserved nuclear protein that accumulates in PML bodies and at condensed heterochromatin during interphase. Daxx knock out mouse embryos are lethal by E11.5 and are characterized by extensive apoptosis. Increased prometaphase index in Daxx deficient embryos, aneuploidy of Daxx knockout cells and an elevated level of Daxx in mitotic cells suggest a potential function of Daxx in mitotic progression. During prometaphase and metaphase, Daxx is released from PML bodies and is associated with mitotic spindles where it co-localizes and interacts with tumor suppressor Rassf1. This mitotic Daxx/Rassf1 interaction disrupts Rassf1/Cdc20 binding thus releasing Cdc20, which activates APC to catalyze the degradation of cyclins A and B and promotes the prometaphase/metaphase transition. Increased stability of mitotic cyclins in anti-Daxx siRNA cell lines confirms this model. Dereguulation of most mitotic checkpoint proteins leads to altered response to microtubule affecting drugs, usually enhancing sensitivity to these compounds. Daxx knockout and Daxx-depleted cells have increased resistance to treatment by the taxane paclitaxel, an anti-cancer chemotherapeutic agent that affects mitosis progression by hyper-stabilizing microtubules. While Daxx positive cells commit mitotic catastrophe and micronucleation upon paclitaxel treatment, Daxx deficient cells accumulate in prometaphase and survive after drug withdrawal. Thus, Daxx is a mitotic checkpoint control protein that determines sensitivity to paclitaxel chemotherapy, and is a potential predictive marker for taxane response in cancer patients.
2532
Nucleolar Dynamics Revealed by Monoclonal Antibodies Against Specific Structural Regions of the Nucleus
Y. Hirai,1 M. Kumaeta,1 Y. Hirano,1 S. H. Yoshimura,1 K. Takeyasu,1 Kyoto Univ., Faculty of Integrated Human Studies, Kyoto, Japan, 2Kyoto Univ., Graduate School of Biostudies, Kyoto, Japan
[Background] Recent studies have revealed that a nucleolus is a dynamic nuclear structure which plays important roles in ribosome syntheses, formation of the nuclear speckles and transcriptional regulations. We have constructed a series of monoclonal antibodies against nucleolar proteins and 14 of them recognized nucleolus in immunofluorescence microscopy. So far, several antigens have been identified by mass spectrometrical analysis and cDNA library screening. [Results] (i) One of them, the Junmoni C (JmcC) domain-containing protein (potential histone de-methylase), bound to DNA and localized in the granular compartment (GC) region of the nucleolus. This protein dispersed into the cytoplasm at the onset of prophase and gradually assembled into the PNs (pre-nuclear body) on the mitotic chromosomes. A comparison of the cell-cycle dynamics of this protein with those of well-characterized nucleolar proteins (Fibrillarin (DFC) and B23 (GC)) revealed that the JmcC domain-containing protein may be one of the leading proteins in the reorganization process of the nucleolus at telophase. (ii) The antigen of 2-30C antibody, the human homologues of Suppressor-of-Hairy-Wing localized to the fibrillar compartment (FC) region of the nucleolus. Co-immunostaining of this protein with UBF (upstream binding factor) suggested that this protein localized to rDNA regions on the chromosomes throughout the mitosis. (iii) ObgH2, a human homologue of Bacillus Subtilis GTPase, localized in the dense fibrillar compartment (DFC) region of the nucleolus. Co-staining with DFC markers revealed that its reassembly to the nucleolar region at telophase was completely merged with Fibrillarin, but slightly earlier than Ki67 antigen.

2533
Human Small G Proteins, ObgH1 And ObgH2, Participate in the Maintenance of Mitochondria and Nucleolar Architectures
K. Kimura,1 Y. Hirano,1 R. L. Ohtniwa,2 C. Wada,1 S. H. Yoshimura,1 K. Takeyasu,1 Faculty of Integrated Human Studies, Kyoto University, Kyoto, Japan, 2Graduate School of Biostudies, Kyoto University, Kyoto, Japan
The small G proteins act as molecular switches and play crucial roles in the regulation of fundamental cellular processes, such as protein synthesis, nuclear transport, membrane trafficking and signal transduction. The Obg sub-family proteins belong to the P-loop GTPase super class and their primary structures are highly conserved in many organisms from bacteria to human. The Obg homolog in Escherichia coli, ObgF, is essential for chromosome partitioning as well as cell growth. Our previous study revealed that ObgE coexists with 23S and 16S rRNA in vitro in the present of GTP and bind to 30S and 50S subunits, but not 70S. These evidences suggested that ObgE functions in ribosome maturation. Although all species in other kingdoms have only one sub-family protein, exploring the genomic database revealed that animals and plants have multiple obg homologs as well. In fungi Aspergillus nidulans, the obg gene is an essential gene for cell proliferation. A knockdown of ObgH1 by RNAi induced a mitochondria elongation and abnormal nuclear morphology, whereas a knockdown of ObgH2 resulted in a disorganization of the nucleolar architecture. These results implicated that ObgH2 may go through the specific evolutionary process and has an important role for the maintenance of nucleolar structure.

2534
Nucleolar Proteins of Aspergillus nidulans Are Segregated via a Cytoplasmic Nucleolar Remnant
L. Ueki,1 C. De Souza,1 H. Liu,2 S. A. Osman1; Molecular Genetics, The Ohio State University, Columbus, OH
The nucleolus is a prominent nuclear structure whose mitotic segregation is poorly understood. During yeast mitosis the nucleolus segregates intact with rDNA. In contrast, during open mitosis the nucleolus is disassembled then reassembled. In Aspergillus nidulans, nuclei synchronously complete mitosis in a common cytoplasm. This model filamentous fungus also undergoes partially open mitosis, which is an evolutionary intermediate between open and closed mitosis. We therefore determined how A. nidulans nucleoli are segregated during mitosis. Unlike Saccharomyces cerevisiae nuclear proteins, few A. nidulans nucleolar proteins segregate with DNA. Instead we have defined three patterns by which different nucleolar proteins segregate during mitosis: (1) Dispersal into the cytoplasm at the onset of mitosis and re-accumulation into daughter nucleoli during G1, (2) Same as pattern 1 but with some protein remaining bound to DNA, (3) A novel pattern in which nucleolar proteins remain surrounded by nuclear envelope in a nucleolar remnant, distinct from daughter nuclei, before re-accumulating into daughter nucleoli during G1. Dual labeling of nucleolar proteins and nuclear envelope markers reveal the nucleolar remnant in segregation pattern 3 is generated as a result of a double nuclear envelope fission event. This double fission occurs around a nucleolar protein mass during telophase. This mechanism generates two transport competent daughter nuclei and a very transient nucleolar remnant containing class 3 nucleolar proteins. This study indicates A. nidulans undergoes mitotic disassembly then reassembly of its nucleolus, as do higher eukaryotes, and that generation of daughter nuclei occurs via a double fission mechanism, not a single fission as occurs in yeasts. We suggest the novel mitotic nucleolar remnant we have defined may serve as a sink for unwanted cytoplasmic proteins or RNAs that gain access to nuclei during A. nidulans mitosis and can also serve as a positional cue for double nuclear envelope fusion.

2535
Initial Characterization of Mugsy, a Drosophila Nucleostemin-like GTPase
R. O. Rosby,1 P. DiMario; Biological Sciences, Louisiana State University, Baton Rouge, LA
Nucleostemin is a nucleolar GTPase implicated in stem cell and cancer proliferation (Tsai and McKay, 2002, 2005; Misteli, 2005). We have identified a nucleostemin-like protein in Drosophila. It is encoded by CG3983 in cytological region 89E11 on chromosome 3R. We refer to the encoded protein as Mugsy. It is 581 amino acids in length, and it displays 33.7% identity and 64.2% similarity to mammalian nucleostemin. Immuno-staining localized the endogenous protein to nuclei of larval Malpighian tubule cells, and to nuclei of nested imaginal (stem) cells within the larval midgut. Other than yet unidentifed cells also contained Mugsy. Preliminary RNAi observations include larval and pupal lethality, delayed development, and melanotic tumor formation in the hemocoel of third instar larvae. The few adults that managed to eclose displayed bristle, eye, and wing deformities. Some of these phenotypes fall within the Minute syndrome. RNAi knockdown of Mugsy also caused an apparent reduction in the number of midgut imaginal cells in larvae. A p-element disruption of CG3983 displayed a similar loss of midgut imaginal cells and a more penetrant larval lethality.

2536
A Test of NuMA as a Structural Element of Nuclei and Spindle Poles
A. D. Silk, D. W. Cleveland; CMM, UCSD, La Jolla, CA
NuMA (Nuclear Mitotic Apparatus protein) is a large, abundant, coiled-coil protein component of the nucleus in vertebrates, which localizes to the spindle poles during mitosis. A role at spindle poles has long been implicated, especially from NuMA’s requirement as a microtubule crosslinker that in vertebrates acts, in concert with its partner cytoplasmic dynein, to tether spindle microtubules into a focused pole. Building on its abundant, intranuclear localization and likely assembly into coiled-coil nuclear filaments, a role as an essential element in structuring the interphase nucleus has also been proposed. To test NuMA’s functional roles in both of these contexts, two alleles of the NuMA gene have been generated through homologous recombination in embryonic stem cells. A constitutive disruption was created by the insertion of a transcription unit into a downstream intron so as to preclude normal NuMA RNA splicing, thereby producing a hyposmorphic, nearly null allele. As anticipated, NuMA is essential, as embryos homozygous for this allele die between E3.5 and E10.5. A conditional allele has also been generated in which the exon encoding the nucleolar localization sequence (NLS) is flanked by loxP recombination sites. After Cre recombinase action in primary fibroblasts homozygous for this allele, NuMA is relocalized to the cytoplasm. This produces normal mitoses in vitro and nuclei deficient in NuMA. A definitive test for a functional role for NuMA in nuclear organization and structure, including effects on long term gene expression, has been posed by following the consequences of in vivo deletion of the NuMA NLS through activity of Cre within the nuclei of long lived cells, including motor or Purkinje cell neurons.

2537
Lamin A-dependent Nuclear Defects in Human Aging
P. Scalff, T. Mischel; NICI, NIH, Bethesda, MD
Mutations in the nuclear envelope structural protein lamin A are responsible for the childhood premature aging disease Hutchinson-Gilford Progeria Syndrome (HGPS). The molecular basis of HGPS is the generation of a truncated, dominant gain-of-function lamin A protein. Although HGPS individuals show several typical aging symptoms, it has been difficult to envision a role of lamin A in the normal aging process since the dominant gain-of-function protein was thought to be exclusively present in HGPS patients. We find that the same molecular mechanism responsible for HGPS is active at a low level in healthy cells. Cell nuclei from old individuals acquire similar defects as HGPS patient cells including downregulation of specific nuclear
2538 Molecular Characterization of the Ran-binding Zinc Finger Domain of Nup153
M. M. Higa; Oncological Sciences, Huntsman Cancer Institute/University of Utah, Salt Lake City, UT
The nuclear pore complex is the gateway for selective traffic between the nucleus and cytoplasm. To learn how building blocks of the pore create specific docking sites for transport receptors and regulatory factors, we have studied a zinc finger module present in multiple copies within the nuclear pores of higher eukaryotes. Biophysical studies indicate that all four zinc fingers found in human Nup153 can bind the small GTPase Ran, with dissociation constants ranging between 5 and 40 μM. Nuclear Magnetic Resonance (NMR) structural studies reveal that a representative Nup153 zinc finger adopts a zinc finger ribbon structure previously characterized as the Npl4 zinc finger (NZF) module. Chemical shift analysis was used to map the amino acids involved in Ran binding and a three amino acid motif (Lx3/Vx4/Ax3) located within the two zinc coordination loops was found to be key to Ran recognition. Mutational analysis confirmed the importance of these residues within the context of the zinc finger scaffold. Although this zinc finger domain has been previously reported to interact preferentially with RanGDP, we found, surprisingly, that a Nup153 zinc finger binds GDP- and GTP- forms of Ran with similar affinities, indicating that this interaction is not dependent on conformational determinants in Ran that are influenced by the nucleotide bound. Taken together, these studies elucidate the Ran-binding interface on Nup153 and, more broadly, provide insight into the versatility of this zinc finger binding module.

2540 X- Chromosome Inactivation in the Tetraploid Rodent Tympanoctomys barareae
C. N. O. Bacquet1, T. Neidez1, A. Paldi1, M. Gallardo2, Instituto de Ecología y Evolución, Universidad Austral de Chile, Valdivia, Chile, 1Génétique, Evry, France
To balance the unequal X-chromosome dosage between the XX female and XY male, mammals have adopted a unique form of dosage compensation in which one of the two X chromosomes is inactivated in the female. Initiation of X-chromosome inactivation (XCI) involves ‘counting’ of the X chromosomes to ensure that only one X remains active per autosome set, and a ‘choice’ as to which chromosome to inactivate. In addition, the inactivated X chromosome acquires particular heterochromatic characteristics. In the present study we have investigated for the first time the XCI in a naturally-occurring tetraploid mammal. To detect inactive X chromosome(s) we used immunofluorescence on interphase nuclei with specific antisera raised against MeSH2K7 and macroH2A1. We detect one inactivated X in female cells, indicating that this species presents a diploid-like XCI process, most probably due to the presence of a single pair of sex chromosomes. To further understand nuclear organization in T. barareae, the distribution of H3K4, and the acetylated variants of H3 and H4 was examined. The overall intensity of antibody signal in these proteins was not significantly different in T. barareae compared with the diploid controls. However, the spatial distribution of fluorescence was strikingly different when tetraploid and diploid cells were compared. We conclude that T. barareae behaves like a diploid species in the process of XCI, but it keeps important differences with diploids in terms of chromatin organization of its duplicated genome.

2541 Is ADP Unblocking in the Hexameric Structure of Human RuvBL1 the Key to its Functional Activity?
S. Gorynia,1,2 P. Matias,3 P. Donner,1 M. Carrondo2; 1Proteinchemistry/ET, Schering AG, Berlin, Germany, 2Protein Crystallography, ITQB, Oeiras, Portugal
RuvBL1 is an evolutionarily highly conserved eukaryotic protein related to the AAA+ family of ATPases (ATPase associated with diverse cellular activities) and plays important roles in essential signalling pathways like e-Myc and Wnt, in chromatin acetylation and remodelling, in transcriptional and developmental regulation, in DNA repair and apoptosis. Although its closest homolog is the bacterial helicase RuvB, no helicase activity could be detected from purified human RuvBL1. Tests of DNA/RNA binding and ATPase activity of RuvBL1 show that it binds ssDNA/RNA and dsDNA, but has a marginal ATPase activity. We have solved the three-dimensional structure of human RuvBL1, refined using diffraction data to 2.2 Å resolution. The crystal structure of the hexamer bound to ADP reveals that human RuvBL1 consists of three domains, of which the first and the third are involved in ATP binding and hydrolysis. Structural homology suggests that the second domain, which is weakly associated to AAA+ proteins and not present in RuvB, is a novel DNA/RNA binding domain. The structure of the RuvBL1/ADP complex, combined with our biochemical results, suggest that, while RuvBL1 has all the structural characteristics of a molecular motor, even of an ATP-driven helicase, one or more of yet undetermined co-factors are essential to its activation.

2542 Barrier-to-Autointegration Factor (BAF) Regulates Histone Acetylation In Vitro and In Vivo
R. Montes de Oca1, K. L. Wilson2; Cell Biology, Johns Hopkins Medical School, Baltimore, MD
To balance the unequal X-chromosome dosage between the XX female and XY male, mammals have adopted a unique form of dosage compensation in which one of the two X chromosomes is inactivated in the female. Initiation of X-chromosome inactivation (XCI) involves ‘counting’ of the X chromosomes to ensure that only one X remains active per autosome set, and a ‘choice’ as to which chromosome to inactivate. In addition, the inactivated X chromosome acquires particular heterochromatic characteristics. In the present study we have investigated for the first time the XCI in a naturally-occurring tetraploid mammal. To detect inactive X chromosome(s) we used immunofluorescence on interphase nuclei with specific antisera raised against MeSH2K7 and macroH2A1. We detect one inactivated X in female cells, indicating that this species presents a diploid-like XCI process, most probably due to the presence of a single pair of sex chromosomes. To further understand nuclear organization in T. barareae, the distribution of H3K4, and the acetylated variants of H3 and H4 was examined. The overall intensity of antibody signal in these proteins was not significantly different in T. barareae compared with the diploid controls. However, the spatial distribution of fluorescence was strikingly different when tetraploid and diploid cells were compared. We conclude that T. barareae behaves like a diploid species in the process of XCI, but it keeps important differences with diploids in terms of chromatin organization of its duplicated genome.

2543 The Physical Plasticity of the Mammalian Nucleus Is Related to the Epigenetic State
I. Pajerowski,2,3 D. E. Discher; Bioengineering, University of Pennsylvania, Philadelphia, PA
Molecular mechanisms of nuclear remodeling include changes in gene expression during differentiation and refers, in particular, to the ability of epigenetic modifications to influence ultrastructural reorganization. We hypothesized that such changes would parallel changes in nuclear rigidity, and have used biophysical methods to demonstrate that naïve human hematopoietic stem cells are on average 2.2 times softer than differentiated human skin fibroblasts. We explore the mechanism behind this observation in an epithelial cell line ideally suited to such measurements. The histone deacetylase inhibitor Trichostatin A is used to model chromatin decondensation that is typical of undifferentiated cells, and excess divalent salts are used to hyper-condense nuclei to varying degrees perhaps mimicking drug treated nuclei. Several different mechanisms of reorganization or relaxation were assessed by Fluorescence Recovery After Photobleaching (FRAP) coupled to Fluorescence Imaged Microdeformation (FIMD). FRAP-FIMD of GFP-Histone-H2B during micropipette aspiration demonstrate stable association of H2B during chromatin extension and relaxation in situ. However, between Trichostatin A decondensation that softens the nucleus and salt-induced condensation that stiffens the nucleus, a ten-fold change is seen in the susceptibility to conformational remodeling, with decondensed states remodeling fastest. Nuclear distortions that persist longer than ~8 seconds lead to irreversible reorganization of chromatin and nucleoli. Comparisons between the remodeling kinetics non-linearly suggest that slow post-translational modifications are often rate-limiting for the ultrastructural changes associated with nuclear plasticity.

2544 Programmed DNA Elimination in the Ciliate Stylonychia lemnac Is Accompanied by DNA Methylation
C. K. Maercker,1,2 C. Rutenberg,1,3 C. Schmitt,3 J. Marhold1; 1Biotechnology, University of Applied Sciences, Mannheim, Germany, 2RZPD German Research Center for Genome Research, Heidelberg, Germany, 3Epigenetics, German Cancer Research Center (DKFZ), Heidelberg, Germany
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Ciliates contain micronuclei which are degraded and transcriptionally inactive, whereas the small DNA molecules of the DNA-rich macronuclei are transcribed during vegetative life cycle. After conjugation, mitotic products of the micronuclei are exchanged between conjugating partners and fuse with the stationary haploid micronuclei, resulting in a diploid zygote nucleus. Following mitotic division, one of the daughter nuclei differentiates into a new macronucleus, and the other differentiates into new micronuclei. The development of the somatic macronucleus from the germ-line micronucleus is accompanied by genome-wide DNA elimination. Several rounds of DNA replication to form polyploid chromosomes. These chromosomes are degraded again, and most of the DNA is eliminated. The remaining DNA is fragmented into the small DNA molecules of the macronucleus. We asked if in addition to small RNAs, H3K9 methylation, and chromodomain proteins, DNA methylation also is involved in the epigenetic mechanism of DNA elimination in electrophoresis method detected a cytosine methylation grade of 1.38 % in genomic DNA from fractionated macronuclei. For experiments on the sub-cellular level, we used an antibody specifically recognizing 5-methylcytosine (5mC) in order to investigate cells after conjugation and starvation. We found cytosine methylation in macronuclei of both types of cells. Interestingly, the antibody also stained polyene chromosomes bands, which are eliminated during macronuclear differentiation. This indicates that specific DNA sequences are cytosine-methylated before elimination.

2545  Cell Rounding Induced Global Histone Decacytation in Mammary Epithelial Cells
R. Xu,1 J. Lehbeyec,2 S. Moonee,2 C. M. Nelson,1 A. Richk,1 M. J. Bissell1; 1Life Science Division, Lawrence Berkeley National Laboratory, Berkeley, CA, 2Centre des Cordeliers, Unite mixte 505 INSERM / U1096, Paris, France
Extracellular matrix (ECM) regulates cell morphology, gene expression in vivo; these relationships are maintained in three-dimensional (3D) cultures of mammalian epithelial cells. In the presence of laminin-rich ECM (lECM), mammary epithelial cells become less adhesive to the substratum and undergo global histone deacetylation, which we previously showed was critical for their phenotypic differentiation. Here, using 3D culture models as well as nonadherent and micropatterned substrata, we showed that the cell rounding caused by ECM was sufficient to induce global deacetylation of histones H3 and H4 in human mammary epithelial cells. Microarray analysis demonstrated that this deacetylation correlated with a global reduction in gene expression. Whereas cells cultured on plastic substrata formed prominent stress fibers, those rounded in 3D lECM or on micropatterns lacked these cytoskeletal structures. These results indicate a tight link between ECM-controlled cell morphology and chromatin structure mediated by the actin cytoskeleton, supporting the concept of a tissue matrix in the control of gene expression.  

2546  Sumoylation of Tip60 Forms a Link between UV Irradiation Signaling to DNA Lesion Repair
Z. Cheng,1 X. Ding,1 H. Wang,2 Z. Liu,1 P. Wang,1 Y. Ke,2 Q. Wu,1 Y. Xu,1 H. Huang,1 X. Yao1; 1Laboratory of Cellular Dynamics, University of Sci. & Tech. of China, Hefei, China, 2Beijing University of Chinese Medicine, Beijing, China
Sumoylation-dependent acetylation of Tip60 regulates DNA damage response following genotoxic stress by acetylyzing histone and remodeling chromatin, the molecular mechanisms underlying the Tip60-dependent response to UV-induced DNA damage remain poorly understood. To systematically analyze proteins that regulate Tip60 activity during UV irradiation, we perform a proteomic analysis of Tip60-containing complexes. Our results show that Tip60 forms part of a stress-specific complex with KAP1. This complex contains proteins such as CIZ, belonging to the YAB subfamily, and ZC3H7B. Modulation of Tip60 acetylation by KAP1 is essential forTip60-dependent DNA damage response. Our biochemical characterization demonstrates that Tip60 is sumoylated by SUMO1 at its C-terminal in response to UV irradiation. This sumoylation targets Tip60 to the PML nuclear body. Interestingly, the sumoylation recruits p53 to the nuclear body and facilitates the trans-activation of its target genes. As a result, p21 protein expression is elevated in a Tip60-sumoylation-dependent manner, which induces a G1 arrest in response to UV irradiation. Inhibition of Tip60 sumoylation abrogates the p53-dependent DNA damage response while sumoylation of Tip60 augments its acetytransferase activity in vitro and in vivo. Significantly, this sumoylation-amplified HAT activity of Tip60 induces ATR activation cascade. This mechanism provides an explanation for how Tip60 links the UV-irradiated DNA damage response signaling to DNA lesion repair.  

2547  Using Multiplex ELISA to Obtain an Acetylation Profile of Three Core Histones and p53
C. R. Lyman,1 T. Lam,1 B. K. Baumgartner2; 1Quansys Biosciences, Logan, UT, 2Cell Signaling Technology, Danvers, MA
Acetylation of core histone lysine has been associated with increased gene transcription. CBP, CREB-binding protein, is a transcriptional co-activator endowed with histone acetytransferase (HAT) activity, transferring an acetyl-group to the ε-amine group of a lysine residue. The acetylation level of histones in chromatin has been established to be a key mechanism in regulating gene transcription can be controlled by both transcription factors and chromatin remodeling proteins. Multiple studies have suggested that chromatin remodeling proteins can manipulate the binding affinity between histones and through modification of core histone tails. Histone covalent modifications include acetylation, methylation, phosphorylation and ubiquitination. Acetylation of core histone lysine has been associated with increased gene transcription. CBP, CREB-binding protein, is a transcriptional co-activator endowed with histone acetytransferase (HAT) activity, transferring an acetyl-group to the ε-amine group of a lysine residue. The acetylation level of histones in chromatin has been established to be a key mechanism in regulating gene transcription. Recently, many studies have indicated that Ezrin, a member of the Rho GTPase family, binds to actin filaments and to the membrane skeleton and regulates cell migration and invasion. Ezrin expression is upregulated in the metastases of mammary and pancreatic adenocarcinoma, osteosarcoma, melanoma and rhabdomyosarcoma. Little is known about the regulation of Ezrin gene expression. We performed a series of experiments to determine the effect of Tip60 on Ezrin gene expression. We used a Tip60 knockdown HeLa cell line and a Tip60 overexpression stable cell line. We found that Tip60 knockdown resulted in a decrease in Ezrin expression, while Tip60 overexpression resulted in an increase in Ezrin expression. These results indicate a tight link between ECM-controlled cell morphology and chromatin structure mediated by the actin cytoskeleton, supporting the concept of a tissue matrix in the control of gene expression.

2548  The CREB-binding Protein Has a Dual Effect on Ezrin Gene Transcription
Y. Yu, G. Merlino; National Cancer Institute, Bethesda, MD
Gene transcription can be controlled by both transcription factors and chromatin remodeling proteins. Multiple studies have suggested that chromatin remodeling proteins can manipulate the binding affinity between histones and through modification of core histone tails. Histone covalent modifications include acetylation, methylation, phosphorylation and ubiquitination. Acetylation of core histone lysine has been associated with increased gene transcription. CBP, CREB-binding protein, is a transcriptional co-activator endowed with histone acetytransferase (HAT) activity, transferring an acetyl-group to the ε-amine group of a lysine residue. The acetylation level of histones in chromatin has been established to be a key mechanism in regulating gene transcription. Recently, many studies have indicated that Ezrin, a member of the Rho GTPase family, binds to actin filaments and to the membrane skeleton and regulates cell migration and invasion. Ezrin expression is upregulated in the metastases of mammary and pancreatic adenocarcinoma, osteosarcoma, melanoma and rhabdomyosarcoma. Little is known about the regulation of Ezrin gene expression. We performed a series of experiments to determine the effect of Tip60 on Ezrin gene expression. We used a Tip60 knockdown HeLa cell line and a Tip60 overexpression stable cell line. We found that Tip60 knockdown resulted in a decrease in Ezrin expression, while Tip60 overexpression resulted in an increase in Ezrin expression. These results indicate a tight link between ECM-controlled cell morphology and chromatin structure mediated by the actin cytoskeleton, supporting the concept of a tissue matrix in the control of gene expression.

2549  Transcriptional Reprogramming of Gene Expression in Bovine Somatic Cell Nuclear Transfer Embryos
N. Rodriguez-Osorio,1 Z. Wang,2 S. Kasinathan,2 G. Page,1 E. Memilli1; 1Animal and Dairy Sciences, Mississippi State University, Starkville, MS, 2Hematech LLC, Sioux Falls, SD, 3Public Health, University of Alabama-Birmingham, Birmingham, AL
Successful reprogramming of a somatic genome to produce a healthy clone by somatic nuclear transfer (SCNT) is a rare event and the mechanism(s) involved in this process are poorly defined. We took a unique approach by performing multiple rounds of cloning to produce bovine clones up to four generations (NT4), and then using cDNA microarray (Affymetrix), to compare the transcriptomes of blastocysts derived from NT1 and NT4 to those derived from IVF blastocysts. In parallel, we analyzed gene expression of the donor cells used to produce the NT1 and NT4 clones (DC1 and DC4, respectively). Since calving rate declines with the increased rounds of cloning, such comparisons would reveal epigenetic errors accumulated through each round of cloning, shedding lights on the reprogramming process. We found that there were 270 differentially expressed transcripts between IVF and NT1 (147 upregulated in IVF), and 411 differentially expressed between NT4 and NT1 (218 upregulated in IVF). We also found that there were 193 differentially expressed transcripts between NT1 and NT4. A large number of differentially expressed transcripts were found between NT1 and DC1, and between NT4 and DC4 (2459 and 2276, respectively). Of the 83 differentially expressed transcripts between both donor cells, 49 were at higher level in DC1 and 34 in DC4. The majority of genes upregulated in both NT groups compared with IVF embryos included those involved in protein folding and signal transduction such as LEC2 and DNAJC5. Genes with upregulated expression in IVF compared to both NT groups included those with receptor and catalytic activities such as PLAUR, PTGER4 and PDE1A. There were 23 differentially expressed genes in NT1 vs. NT4. Such study provides a unique data set for identifying the epigenetic errors in somatic cloning and may allow us to better understand the epigenetic reprogramming process in SCNT.
2550
Trophoblastic Invasion In Vitro and In Vivo: A Comparison
C. Helige, A. Hammer, G. A. Dohr; Institute of Cell Biology, Histology and Embryology, Medical University of Graz, Graz, Austria

The basic mechanisms of trophoblast invasion are far from being completely understood. This may be due to the lack of suitable in vitro models, which contain several cell types considered to be involved in trophoblast invasion and which enable an experimental modulation of this complex process. The study aims to compare two factors considered to be implicated in trophoblast invasion, the expression of HLA-G by trophoblastic cells and apoptosis, in vitro and in vivo. A three-dimensional tissue culture model, which closely resembles the in vivo conditions during placentation was used. For that purpose, tissue fragments of human 1st trimester decidua parietalis (800 µm in diameter) were put in close contact with multicellular spheroids of AC-1:MW9 trophoblast/choriocarcinoma hybrid cells (300 µm in diameter) as a model of the invasive trophoblast. Cryostat sections from these co-cultures were immunohistochemically stained and compared with 1st trimester decidua basalis. The trophoblastic cells on the invasion front as well as the cells, which had already invaded deeply into the decidual tissue showed an intensive staining for HLA-G, whereas the cells on the periphery of the confrontation culture exhibited only a weak staining. A similar staining pattern was found in vivo. Invasive extravillous trophoblast cells were highly HLA-G positive, whereas the trophoblast cells of the cell columns were only weakly stained. Both in vitro and in vivo CD45+ apoptotic leukocytes were frequently detected in close proximity to the invasive trophoblastic cells. Only a few of the trophoblastic cells were apoptotic and most of them were not in close contact with the decidual leukocytes. In conclusion, in this three-dimensional tissue culture model key factors considered to be implicated in trophoblast invasion in vivo can also be demonstrated in vitro. Therefore, it can be used to study cellular interactions and factors regulating trophoblast invasion.

2551
Effect of Melatonin on Development of Porcine Preimplantation Embryos In Vitro
N. Rodriguez-Osorio,1 I. Kim,1 S. Jung,1 A. Kaya,2 E. Memili,1 1Animal and Dairy Sciences, Mississippi State University, Starkville, MS, 2Department of Animal Sciences, University of Wisconsin-Madison, Madison, WI

Melatonin, a reactive oxygen species scavenger, plays a role in prevention of apoptosis and has been used to promote in vitro development of mouse, sheep and cow embryos. The aim of this study was to determine the effects of melatonin on in vitro porcine embryo development, particularly on cleavage, blastocyst rates, and blastocyst cell number. Porcine abattoir-derived oocytes were subjected to standard in vitro maturation and fertilization procedures. Fertilized oocytes were randomly allocated to groups in PZM-3 medium supplemented with Melatonin at concentrations of 10^{-3}, 10^{-6}, 10^{-9} and 10^{-12} M, starting 5 hours after insemination (hpi), and cultured in a humidified incubator with 5% CO₂ at 39ºC. Cleavage rates were assessed 48 hpi, and blastocyst rates were determined at day 6 post fertilization. Number of cells per embryo was assessed after epifluorescent staining with DAPI. The experiments were repeated 8 times and the results were analyzed using t-test. The study showed that Melatonin at 10^{-9} M concentration had a positive effect on cleavage rates with 45%±1.94 (mean±SEM) compared to 39%±2.01 in the control group (p<0.05). There were no differences in embryo development from the groups with Melatonin at concentrations 10^{-12} M and 10^{-6} M. Blastocyst cell numbers were higher in the Melatonin 10^{-9} M group, with 65±3.22 (cell number±SEM), compared to 50±2.07 for the control group (p<0.01). Our data indicate that melatonin (10^{-9} M) supports development of porcine preimplantation embryos and increases total number of cells in the blastocysts.

2552
Individual and Collective Neural Crest Cell Migration Requires Cdc42 and RhoA
P. A. Rupp, P. M. Kulesa; Stowers Institute for Medical Research, Kansas City, MO

Programmed cell invasion is a key aspect of many developmental and adult processes, yet despite knowing many of the extrinsic guidance molecules involve in shaping migratory patterns, the function of cell extensions in accurate pathfinding remains unclear. The neural crest is a major migratory cell population in the vertebrate embryo that sorts into complex patterns of discrete migratory streams. Previously, we have shown that filopodial extensions may influence a trailing neural crest cell’s choice of direction through contact with other neural crest cells. We analyze the in vivo role of small Rho GTPases in neural crest cell dynamics and migratory patterning. We show that when the ability of a neural crest cell to correctly form filopodia and make meaningful cell-cell contacts is perturbed by altering Cdc42 or RhoA function, individual cell trajectories are less directional and cells are delayed in reaching the peripheral targets. Disrupted cell filopodial dynamics lead to a breakdown or delay of the follow-the-leader chain migratory behavior observed in post-otic neural crest. This altering of Cdc42 or RhoA signaling at the individual cell level perturbs the normal two-phase process of neural crest cell migratory patterning. We conclude that Cdc42 and RhoA are required for the accurate pathfinding and timely migration of avian neural crest cells.

2553
Characterization of the Role of Planar Cell Polarity during Axial Elongation Movements in PTK7-null Mouse Embryos
W. Yen,1 A. Periasamy,2 C. Burdsal,3 X. Lu,2 A. E. Sutherland 1; 1Dept. of Cell Biology, University of Virginia, Charlottesville, VA, 2W. M. Keck Center for Cellular Imaging, University of Virginia, Charlottesville, VA, 3Dept. of Cell and Molecular Biology, Tulane University, New Orleans, LA

In Drosophila melanogaster, a ‘core’ group of planar cell polarity (PCP) genes including frizzled (ft), flamingo (fln), disheveled (dsh), Van Gogh (vang)/strabismus and prickle, function through a non-canonical Wnt-like pathway to regulate the orientation of specialized cells, such as wing hairs, bristles and ommatidia, within the plane of epithelial tissues. This PCP pathway is conserved in vertebrates and functions in regulating the planar polarization of cell protrusive activity and intercalation that drives the convergent extension movements of Xenopus gastrulation. It also plays a major role in mouse neural tube closure, although the mechanism is unknown. Recent studies have identified protein tyrosine kinase 7 (PTK7) as a novel regulator of PCP in mouse sensory hair cells and in Xenopus neural convergent extension and tube closure. We have used both static and live (time-lapse) confocal microscopy and three-dimensional reconstruction software to investigate the role of PTK7 in the PCP pathway in the anteroposterior elongation movements of developing mouse embryos. Embryos from heterozygous crosses mutant for PTK7 were dissected at 8.5 days of gestation and labeled with phalloidin, to examine actin organization and cell shape, and with antibodies to fibronectin to examine tissue organization. We found that there are major morphological differences between homozygous mutants for PTK7 and their wild-type counterparts that are consistent with a defect in anterior-posterior elongation. These include the lack of a visible node and notochord, a shorter and thicker primitive streak, somites that are wide mediolaterally and narrow anteroposteriorly, and an open neural tube that is convoluted in both the dorsoventral and mediolateral axes. There is also a difference in localized deposition of fibronectin fibrils at tissue boundaries. We propose three models for how PTK7 may regulate axial elongation in the mouse: through regulating cell division planes, by directing cell motility or by polarizing fibronectin fibril deposition.

2554
Functional Disruption of the p23 Gene in a Mouse Leads to Perinatal Death
I. Grad,1 C. A. Miller,2 T. A. McKee,3 S. M. Ludwig,2 G. W. Hoyle,2 P. Ruiz,4 W. Wurst,4 D. Picard 1; 1Cell Biology, University of Geneva, Geneva, Switzerland, 2Environmental Health Sciences, Tulane Univ. School of Public Health and Tropical Medicine, New Orleans, LA, 3Service de Pathologie Clinique, University of Geneva, Geneva, Switzerland, 4GSF-Forschungszentrum fur Umwelt und Gesundheit, Neuherberg, Germany

The p23, a component of the Hsp90 chaperone complex, is thought to stabilize the client-interacting form of Hsp90. Among its various activities, it is crucial for assembly of mature steroid receptor complexes and in vitro it exhibits prostaglandin E2 synthase activity. To address the question of the in vivo function of p23, we have generated a mouse model using gene-trap approach, where p23 is functionally disrupted. Two independent mouse lines have shown that the p23 null is not crucial for overall prenatal development and morphogenesis, however p23 null neonates exhibit premature infant phenotype. The transition into air environment requires extensive changes in lung and skin of the embryo, which normally appear in the last phase of intrauterine development. The p23 null neonates die upon birth, their lungs are atelectatic with reduced expression of surfactant genes, but unaltered proportions of surfactant-composing lipids and their skin barrier is compromised. Correlating with the known function of glucocorticoids in promoting lung and skin maturation and the role of p23 in the assembly of a hormone-responsive glucocorticoid receptor-Hsp90 complex, p23 null fibroblast cells have a defective glucocorticoid response. Thus, p23 contributes a non-redundant, temporally restricted, and tissue-specific function during mouse development which, at least in part, is connected with its role in maturation of glucocorticoid receptor.
Identification of Genes Differentially Expressed in Endometrium from Women with Unexplained Recurrent Spontaneous Abortion

J. Lee, C. Cho; Life Science, Gwangju Institute of Science and Technology, Gwangju, Republic of Korea

Recurrence spontaneous abortion (RSA), defined as three or more clinically consecutive miscarriages during the first trimester, occurs in approximately 1% of pregnant women. The etiology in approximately 40% of RSA is unexplained, but it has been suggested that a proportion of unexplained RSA is due to endometrial factors that cause alteration of essential components for implantation and pregnancy. In this study, to identify the endometrial factors in unexplained RSA, we selected 31 genes of which expression levels are altered during the implantation period (between proliferative and secretory phases) from various microarray data sets previously reported. We compared the expression levels of these implantation-related, 31 genes in the endometrium between normal controls and patients with unexplained RSA, using a real time PCR assay. We found for the first time that four genes were differentially expressed between the controls and unexplained RSA patients with statistical significance. They were cellular retinoic acid binding protein 2 (CRABP2), neuronal olefimothionin related ER localized protein (Olf1), B-cell CLL/Jymphoma 6 (BCL6) and complement component 4-binding protein alpha (C4BPs).

Regulation of Muscle Plasticity by the Homeobox Factor Barx2

K. Gonzalez, H. Makarenkova, C. Wen Tsau, R. Meech; 1Dept. Neurobiology, The Scripps Research Institute, La Jolla, CA, 2The Neurosciences Institute, San Diego, CA

Muscle development is a dramatic example of terminal differentiation: myoblasts differentiate into myocytes that exit the cell cycle and fuse to form multinuclear myotubes. An intriguing question in skeletal muscle biology is the extent to which differentiated myofibers in adult animals retain developmental plasticity. Studies in different vertebrate phyla suggest that two different mechanisms for muscle repair exist: 1) recruitment and differentiation of satellite cells; and 2) dedifferentiation of existing myofibers to produce a pool of proliferative cells that can redifferentiate into new fibers. Satellite cell-mediated repair has been extensively studied; however the ability of muscle fibers to dedifferentiate remains controversial. Dedifferentiation and/or transdifferentiation of muscle have been reported in various vertebrates and low phyla vertebrates. For example, in jellyfish striated muscle undergoes transdifferentiation and S-phase re-entry and in urodile amphibians transacted myofibers dedifferentiate and contribute to multiple tissues during limb regeneration. Mammalian myotubes also retain developmental plasticity as indicated by observations that treatment of cultured myotubes with extract from regenerating newt limbs, or ectopic expression of the homeodomain transcription factor Msx1, can induce their dedifferentiation. However, whether muscle dedifferentiation occurs endogenously in mammals is presently unclear. Here we present evidence that another homeodomain factor that is expressed in skeletal muscle, Barx2, may regulate muscle plasticity. Ectopic expression of Barx2 in C2C12 myotubes and in MyoD-induced C1H10T1/2 myotubes causes cleavage and de-differentiation concomitant with down-regulation of muscle differentiation markers. Moreover, BrdU labeling suggested that Barx2 induced entry of muscle cells into S-phase. Among several homeodomain proteins studied, only Barx2 and Msx1 could induce myobrade breakage and downregulation of muscle specific markers, indicating that this phenomenon is specifically regulated by these proteins.

Changes in Muscle Fiber Properties of Murine Digastric Muscle Before and after Weaning

K. Ohkubo, K. Sakiyama, K. Kurokawa, S. Abe; 1Anatomy, Tokyo Dental College, Chiba, Japan, 2Sports Dentistry, Tokyo Dental College, Chiba, Japan

The importance of muscle plasticity is a major topic of research during several stages of an individual's life. Several studies have focused on changes in muscle fiber properties before and after weaning. We studied the digastric muscle before and after weaning to understand the changes in muscle fiber properties and to analyze the potential mechanisms involved.

Expression of the Regulatory Gene Pax-6 Precedes the Initial Stages of Corneal Epithelial Cell Differentiation

R. Garcia-Villegas, M. Hernandez-Quintero, E. Sanchez-Guzman, F. Castro-Munozzolo; 1Dept. of Physiology, Biophysics and Neurosciences, CINVESTAV-IPN, Mexico City, Mexico, 2Dept. of Cell Biology, CINVESTAV-IPN, Mexico City, Mexico

Pax6 is a regulatory gene, which plays a major role during development ofvisual and nervous systems in Metazoans. Although Pax-6 expression was described in several eye tissues, its involvement in corneal epithelial development is not understood. In order to analyse Pax-6 function during corneal epithelial differentiation, we used the RCE1 rabbit corneal epithelial cell line, previously described as a model that mimicks corneal differentiation. After RT-PCR with specific oligos, we cloned the full-length messengers encoding two Pax-6 isoforms. Both isoforms were found in vitro in confluent stratified epithelia formed by corneal keratinocytes, but not in proliferating cells. Northern blot and RT-PCR experiments showed that Pax-6 is not expressed in early cell cultures; afterwards, the transcription factor shows low levels in proliferating colonies. These results were supported by immunostaining of proliferating colonies with antibodies specific for Pax-6. In such colonies, the transcription factor was only found in the cells that were already positive for K3 keratin and therefore suggest that Pax-6 is expressed in cells that begin the differentiation process. After confluence, Pax-6 mRNA’s levels raised up to 5-fold. The authors conclude that the expression of this transcription factor preceded the increase in those messengers encoding for LDH isozymes H and M, previously shown as early markers of corneal epithelial cell differentiation. Similarly, the expression of the mRNA’s encoding these isozymes preceded by 2.5 days the expression of the differentiation-linked K3/K12 keratin pair. Finally, the ectopic expression of Pax-6 in corneal epithelial keratinocytes led to a decrease in the proliferative ability of these cells. Together, the results suggest that Pax-6 expression switches on the differentiation of corneal keratinocytes, leading to a knock down of proliferation. Supported in part by CONACyT grants 39690-Q and 1314P-N9507 (to FCML) and 43128-Q (to MRGV).

Elucidating the Activation Function of Msx Homeodomain Proteins

F. Zhang, C. Shuler, Y. H. Lee; Center for Craniofacial Molecular Biology, University of Southern California, Los Angeles, CA

The importance of Msx genes in regulating development of oro-craniofacial structures has been well established. It was shown that null mutations in the Msx1 gene can result in facial and palatal clefting, adontia, palatal bone synostosis and loss-of-function mutations in the Msx2 gene could lead to delayed closure of palatine foramina. Overexpression and ectopic expression of the Msx2 gene in transgenic animals can accelerate membrane bone formation and cause microphthalmia. Previous studies have shown that Msx proteins regulate gene transcription.
predominantly through repression by forming transcriptionally inactive heteromeric complexes. In contrast to their known suppressor activities, gene expression studies using either the gain-of-function or the loss-of-function mutants revealed many gene targets whose expression relies on functional Msx proteins. In order to study the activator function of Msx proteins, we established transcriptional assay system based on the mouse Hspa1b promoter. Mxs proteins activate Hspa1b promoter through a mechanism that relies on their conserved C-termini and Heat Shock Factors. DNA binding by Mxs proteins is not essential although the homeodomain does contribute to the over all activation activity.

2561 Smos2, a Sphingomyelin Synthase Gene Expressed in Calculifying Osteoblasts and Osteocytes

N. Ishikura, C. Shinotsuka, H. Akizuki, K. Ikeda, K. Watanabe; Dept. Bone & Joint Disease, National Center for Geriatrics & Gerontology, Obu, Japan

Osteocytes are thought to play crucial roles in bone metabolism, although limited information has been available on their specific products and physiological function. To isolate genes expressed in osteocytes, we employed subtractive PCR method and microarray techniques, and identified 5 novel genes expressed in osteocyte-enriched bone fraction. One of the genes encodes a novel enzyme involved in phospholipid metabolism, which has recently been found as a sphingomyelin synthase (Smos2: Huitema et al. (2004); Yamaoka et al. (2004). Expression of Smos2 was detected in long-term cultured osteoblasts, but not in bone marrow stromal or embryonic carcinoma cell lines. To determine the expression of Smos2 in vivo, LacZ gene was introduced into mouse Smos2 locus so its expression was driven by the endogenous Smos2 promoter. At E11.5, the expression, as monitored by X-gal staining, was detected only in skeletal elements, especially in the areas enriched in osteoblasts. The expression was observed not only in osteoblasts but also in osteocytes of E16.5 embryo. Smos2 was expressed in some differentiated chondrocytes of long bones, but not in notochord or cartilage elements which remain uncalcified. It has been reported that sphingomyelinase activity was detected in matrix vesicles, which play a pivotal role in matrix mineralization, and that sphingomyelin was hydrolyzed upon mineralization, which prompted us to hypothesize that sphingomyelin may have some inhibitory effects on mineralization. In fact, when Smos2 gene was overexpressed in osteoblasts, mineralization was significantly suppressed. Also, a sphingomyelinase inhibitor, Bn09, reduced mineralization when added to osteoblast cultures. It is suggested that sphingomyelin metabolism is tightly regulated in matrix mineralization and that Smos2, specifically expressed in calcifying osteoblasts and chondrocytes, may be involved in the regulation of mineralization.

2562 Phospholipase C-delta1 and -delta3 Are Required for Normal Development of Placenta

Y. Nakamura,1 T. Takenawa,2 K. Fukami; 1Tokyo University of Pharmacy and Life Sciences, Tokyo, Japan, 2Department of Biochemistry, Institute of Medical Science, University of Tokyo, Tokyo, Japan

Phosphoinositide-specific phospholipase C (PLC) is a key enzyme in phosphoinositide turnover and is involved in a variety of physiological functions. PLC can be categorized into six types, beta, gamma, delta, epsilon, zeta, and eta on the basis of sequence homology and activation mechanism. PLC-delta types are evolutionarily conserved from lower to higher eukaryotes and these isoforms are thought to be the primary forms expressed in mammals. To elucidate physiological functions of PLC-delta types, we generated PLC-delta1 and PLC-delta3 mice lacking both PLC-delta1 and PLC-delta3 died at embryonic day 11.5 (E11.5) to E13.5. PLC-delta1/PLC-delta3 double-knockout mice exhibited severe disruption of the normal placental architecture in the placenta and decreased placental vascularization, as well as abnormal proliferation and apoptosis of trophoblasts in the labyrinth area. Furthermore, PLC-delta1/PLC-delta3 double-knockout embryos supplied with a normal placenta by the tetraploid aggregation method survived beyond E14.5, clearly indicating that the embryonic lethality is caused by a defect in trophoblasts. On the basis of these results, we conclude that PLC-delta1 and PLC-delta3 are essential in trophoblasts for placental development.

2563 Conditional Functional Deletion of Rb within Chondrocytes Results in Dwarfed Phenotype

D. S. Vale-Cruz, P. A. LoValle; Anatomy and Cell Biology, University of Florida, Gainesville, FL

Longitudinal bone growth occurs through the process of endochondral ossification. Cartilage cells i.e. chondrocytes, undergo proliferation and terminal differentiation in order to provide the cartilage template for ossification into calcified bone. Our lab has previously described the expression profiles of the cell cycle regulatory proteins retinoblastoma (Rb), p107 and p130 in developing bone in response to wild-type or mutant activating transcription factor-2 (ATF-2) protein. ATF-2 has also been shown to regulate Rb promoter activity. ATF-2 regulation of the Rb promoter within chondrocytes was verified by chromatin immunoprecipitation. In order to assess the role Rb plays in normal skeletal development, a conditional knockout mice were generated. Rbloxp mice were crossed with Cre recombinase expressing mice under the control of the collagen type 2 promoter (Col2Cre) specific to chondrocytes. Heterozygous animals developing bone in response to wild-type or mutant activating transcription factor-2 (ATF-2) protein. ATF-2 has also been shown to regulate Rb promoter activity. ATF-2 regulation of the Rb promoter within chondrocytes was verified by chromatin immunoprecipitation. In order to assess the role Rb plays in normal skeletal development, a conditional knockout mice were generated. Rbloxp mice were crossed with Cre recombinase expressing mice under the control of the collagen type 2 promoter (Col2Cre) specific to chondrocytes. Heterozygous animals (Rbloxp+/−; Col2Cre+/-) were crossed with Rbloxp homozygotes to maximize the number of knockout animals. Resulting offspring were genotyped and assessed for skeletal abnormalities. Rb conditional knockout (Rbloxp−/−) animals exhibited reduced size and some skeletal abnormalities. Immunohistochanism analysis showed disruption of normal growth plate architecture. There was an increase in extracellular matrix combined with a decrease in overall cell number. Primary chondrocytes from Rbloxp mice were infected with an adenovirus expressing Cre to determine changes in cell cycle progression in response to Rb deletion. Cell cycle progression was altered in chondrocytes following Rb deletion. Rb expression is required for normal skeletal development. Disruption of Rb expression yields a dwarfed skeletal phenotype as a result of changes in chondrocyte proliferation and differentiation.

2564 Improper Notochord Detachment Due to Abrupt Bmp Signaling in the Pathogenesis of Esophageal Atresia

Y. Li,1 Y. Litingtung,2 P. T. Djike,2 C. Chiang; 1Cell and Developmental Biology, Vanderbilt University, Nashville, TN, 2Department of Molecular Biology, Leiden University Medical Center, Leiden, The Netherlands

Human foregut malformation known as esophageal atresia with tracheoesophageal fistula (EA/TEF) occurs in 1 in 4,000 live births with unknown etiology. We found that mice lacking PTEN activity, a tumor suppressor gene normally expressed in the notochord, had a phenotype similar to human EA/TEF. Our results showed that loss of PTEN expression is not essential for proper development of the notochord, because Noggin expression was reduced in the EA/TEF notochord. Indeed, normal notochord branches were evident in Noggin−/− embryos, and cellular and molecular examinations indicated delayed detachment of the notochord from the underlying dorsal foregut endoderm. Consistently, non-nothochordal, likely endodermal, cells were found in Noggin−/− notochord, suggesting Noggin function is required in the notochordal plate for its proper delamination from the dorsal foregut. Notably, ablating Bmp7 in Noggin−/− embryos rescued EA/TEF and notochordal branching defects, thus establishing a critical role of Noggin-mediated Bmp7 antagonism in EA/TEF pathogenesis.

2565 Alteration of Intestinal Epithelial Architecture and Proliferation in Mice with Impaired PTEN Expression

M. J. Langlois, B. A. Auclair, S. Roy, S. Turgeon, N. Rivard, N. Perreault; Anatomy and Cell Biology, University of Sherbrooke, Sherbrooke, PQ, Canada

PTEN is a tumor suppressor gene localized in a chromosomal region frequently altered in human tumors (10q23). PTEN dephosphorylates phosphatidylinositol 3-kinase (PI3K) activation. Germlinal mutations of PTEN have been identified in juvenile polyposis syndrome, a disease characterized by the development of hamartomatous polyps in the digestive tract and associated with an increased risk of cancer. Objective. To investigate the role of PTEN in the development, regulation of cell function and maintenance of the intestinal epithelium. Methods and Results. With the use of the Cre/loxP system, we have generated a mouse with a deletion of PTEN exclusively in the intestinal epithelium by crossing floxed PTEN mice with the Villin-Cre line. Macroscopically, we observed in mice with loss of intestinal epithelial PTEN expression, a dramatic lengthening of the digestive tract in addition to an hypertrophy and dilatation of its circumference. A disorganization of the intestinal epithelium architecture and an important thickening of the muscular layer are also observed in the mutants. Villin-Cre/LoxP (-90 min) and Cre/LoxP (90 min) mice develop similar phenotypes. Immunohistochemical analysis of fixed tissue revealed a delay in the appearance of villin staining, consistent with a delay in villin expression. ALDH activity was decreased in the villus tips of the mutants. The decrease in the ALDH activity may indicate decreased bile salt, which are known to be required to promote proliferation. Furthermore, a delay in the appearance of proliferating cell nuclei may indicate that the expression of cell cycle regulatory proteins may be delayed in the villus tips of the mutants. In summary, our data indicate that PTEN expression is required for normal development of the intestinal epithelium.
Localizing CDC-42 Activity in the Early C. elegans Embryo

K. Kumfer, J. G. White; Laboratory of Molecular Biology, University of Wisconsin, Madison, Madison, WI

Attaining a defined axis of polarity is necessary for a cell to execute diverse functions, such as directed motility, the generation of daughter cells with disparate fates and for the morphogenesis of ensembles of cells into organs. In C. elegans, the site of sperm entry defines the posterior end of the major axis of the fertilized oocyte. The paternally provided centrosome triggers actinomyosin-driven cortical flows toward the anterior, leading to the segregation of cytoplasmic determinants that maintain polarity and signal asymmetric cell division. The establishment and transition of a polarized microtubule cytoskeleton directs the localization of Cdc-42, which is required for anterior localization of several polarity factors and for the cytoskeletal reorganization that occurs during cell division.

2571

Localizing CDC-42 Activity in the Early C. elegans Embryo

K. Kumfer, J. G. White; Laboratory of Molecular Biology, University of Wisconsin, Madison, Madison, WI

Attaining a defined axis of polarity is necessary for a cell to execute diverse functions, such as directed motility, the generation of daughter cells with disparate fates and for the morphogenesis of ensembles of cells into organs. In C. elegans, the site of sperm entry defines the posterior end of the major axis of the fertilized oocyte. The paternally provided centrosome triggers actinomyosin-driven cortical flows toward the anterior, leading to the segregation of cytoplasmic determinants that maintain polarity and signal asymmetric cell division. The establishment and
Correlation between β-catenin and APC Inhibits Nuclear Localization of β-catenin during Asymmetric Division in C. elegans

H. Sawa; Center for Developmental Biology, Riken, Kobe, Japan

Asymmetric division is fundamental for generation of cellular diversity. In C. elegans, asymmetry of most cell divisions is regulated by the Wnt signaling pathway. In other organisms, β-catenin has two separate functions in cell adhesion at cell cortex and in Wnt signaling in the cytoplasm and nucleus. In C. elegans, we previously showed that WRM-1/β-catenin that does not function in cell adhesion localized asymmetrically on the anterior cell cortex before and during divisions and to the posterior nucleus at telophase and after division. To know the function of cortical β-catenin, we expressed membrane target form of β-catenin to find that nuclear localization of β-catenin was inhibited in both nuclei. In contrast, in mutants of APC, β-catenin localized in both daughter nuclei, even if cortical WRM-1 was still asymmetric. Furthermore, we found that APC and Axin localized to the anterior cortex as β-catenin and that the APC localization was regulated by β-catenin. These results suggest that asymmetric cortical β-catenin recruits APC to the anterior cortex which inhibits nuclear localization of β-catenin. Our results indicate that β-catenin has antagonistic functions at the cortex and in the nucleus during asymmetric cell division in C. elegans.

The Planar Cell Polarity Protein Vangl2 Signals via Scribble - βPIX Complex to Modulate Epithelial Cell Migration

A. Gangar, S. Audebert, M. Montcouquiol, J. Borg; INSERM UMR599, Marseille, France, Institut F. Magendie des Neurosciences, INSERM, Bordeaux, France

Vangl2, a core planar cell polarity (PCP) protein is required for the uniform orientation of cells within the plane of an epithelium and convergent extension movements. The development of animal tissues requires dynamic remodeling of epithelial structure. For this remodeling, cells must balance two opposing but essential influences on cell-cell junctions: the active maintenance of contacts between all cells in an epithelial sheet. This balance maintains widespread epithelial integrity during morphogenesis but its molecular bases have been unclear. Here, we identify a novel role for the polarity regulator atypical Protein Kinase C (aPKC) as a key regulator of adherens junction (AJ) symmetry during Drosophila gastrulation. aPKC regulates apical microtubule organization to counterbalance planar polarized AJ assembly required for cell intercalation events. Without aPKC, centrosomal microtubules become abnormally dominant cues for planar polarized AJ clustering--AJs form discrete puncta associated with microtubule asters at the dorsal and ventral sides of each epithelial cell, sites where new contacts form as the cells intercalate. With aPKC, AJ symmetry and epithelial structure are maintained. aPKC appears to disrupt microtubule-cadherens associations in the apical domain, thereby inhibiting the AJ-microtubule interactions that otherwise break symmetry. In apparent contrast to microtubules, the actin cytoskeleton maintains AJ symmetry around the apical domain. In other tissue types as well, actin and microtubules appear to compete for AJ interactions and have distinct effects on AJ assembly and maintenance around the apical domain. This work reveals that specific molecular mechanisms regulate AJ symmetry during morphogenesis. Interplay between actin and microtubules, regulated by cell polarity regulators such as aPKC, functions in orchestrating the balanced remodeling of AJs during epithelial development.

E-cadherin Mediated Cell-Cell Adhesion Assembles a Targeting Patch That Specifies Basal-Lateral Vesicle Delivery to Initial Cell-Cell Contacts

L. N. Nejum, L. Edgington, W. J. Nelson; Molecular and Cellular Physiology, Stanford University Medical School, Stanford, CA

Formation of apical and basal-lateral membrane domains in polarized epithelial membranes requires cell adhesion to the extracellular matrix and to other cells, but the mechanisms involved are unknown. We tested whether assembly of the basal-lateral cell polarity complex is required for initial targeting of E-cadherin mediated cell-cell contacts in MDCK cells. Using aquaporin-3 (AQP3) and AQP5 as homologous marker proteins of kidney epithelial cell (basal-) lateral and apical membrane domain respectively, we show that AQP3, but not AQP5, was delivered in post-Golgi structures directly to cell-cell contacts where it co-accumulated precisely with E-cadherin during initial assembly of cell-cell adhesion. Components of the Exocyst and the t-SNARE complex also localized precisely with E-cadherin upon cell-cell adhesion, and selective disruption of microtubules, the Exocyst or the t-SNARE complex inhibited delivery of AQP3 to forming cell-cell contacts. These results show that E-cadherin mediated cell-cell adhesion initiates the rapid, localized assembly of a targeting patch for selective delivery of basal-lateral vesicles thereby initiating the specification of that site as the forming (basal-) lateral membrane domain.

The Exocyst Complex Regulates Multiple Post-Endocytic Trafficking Pathways in Polarized MDCK Cells

A. Oztan, L. Leiva-Vega, M. Sivis, N. Bradbury, S. Hsu, J. Goldenberg, C. Yeaman, G. Apodaca; Department of Medicine, University of Pittsburgh, Pittsburgh, PA, Department of Pharmacology, University of Pittsburgh, Pittsburgh, PA, Department of Cell Biology and Physiology, University of Pittsburgh, Pittsburgh, PA, Department of Physiology and Biophysics, Rosalind Franklin University, North Chicago, IL, Department of Cell Biology and Neurosciences, Rutgers University, Piscataway, NJ, Department of Surgery and Cell & Developmental Biology, Vanderbilt University, Nashville, TN, Department of Anatomy and Cell Biology, University of Iowa, Iowa City, IA

The exocyst is an octomeric complex that regulates delivery of the newly synthesized trans-Golgi network (TGN) proteins to the basolateral plasma membrane domain of polarized Madin-Darby canine kidney (MDCK) cells. In these cells, the exocyst has been variously localized to the lateral plasma membrane near the junctional complex, the trans-Golgi network, and transferrin-positive recycling endosomes. At present, it is unknown whether the exocyst is localized to additional endocytic compartments or whether it regulates transport within the endosomal system of polarized epithelial cells. Analysis of the distribution of the exocyst subunits Sec6, Sec8, and Exo70 in polarized MDCK cells by confocal microscopy confirmed that in addition to the lateral membrane, these proteins were partially localized to vesicles that are positive for the basolateral recycling marker transferrin. There was also a significant subapical pool of Sec6/Sec8/Exo70 that showed extensive colocalization with transcytosing IgA and the apical recycling endosome marker Rab11. Immunolocalization experiments further confirmed that Sec8 subunit was found on Rab11-positive endosomes. Streptolysin-O (SLO)-permeabilized MDCK cells were used to analyze the involvement of the exocyst complex in endocytic trafficking pathways. Recycling of transferrin in this assay was inhibited 46% by the addition of function blocking Sec8 antibodies, but was not affected by the addition of a non-specific control antibody. Furthermore, Sec8 antibodies inhibited IgA transcytosis 57% and IgA recycling 50%. Previously Sec15 subunit of the exocyst complex was shown to interact with Rab11. In order to test the role of this interaction in IgA trafficking, Rab11 binding domain of Sec15 (Sec15CT) fused to EGFP was expressed in MDCK cells. Rab11 and IgA receptor pGFP co-localized with Sec15CT and expression of this domain inhibited IgA trafficking. These results indicate that the exocyst complex is localized to endocytic compartments and regulates multiple post-endocytic pathways in polarized epithelial cells.

Control of Adherens Junction Symmetry during Embryogenesis

T. J. Harris, M. Peifer; Dept. of Cell and Systems Biology, University of Toronto, Toronto, ON, Canada, Dept. of Biology/Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, NC

The development of animal tissues requires dynamic remodeling of epithelial structure. For this remodeling, cells must balance two opposing but essential influences on cell-cell junctions; the disassembly and assembly of individual cell-cell contacts and the active maintenance of contacts between all cells in an epithelial sheet. This balance maintains widespread epithelial integrity during morphogenesis but its molecular bases have been unclear. Here, we identify a novel role for the polarity regulator atypical Protein Kinase C (aPKC) as a key regulator of adherens junction (AJ) symmetry during Drosophila gastrulation. aPKC regulates apical microtubule organization to counterbalance planar polarized AJ assembly required for cell intercalation events. Without aPKC, centrosomal microtubules become abnormally dominant cues for planar polarized AJ clustering--AJs form discrete puncta associated with microtubule asters at the dorsal and ventral sides of each epithelial cell, sites where new contacts form as the cells intercalate. With aPKC, AJ symmetry and epithelial structure are maintained. aPKC appears to disrupt microtubule-cadherens associations in the apical domain, thereby inhibiting the AJ-microtubule interactions that otherwise break symmetry. In apparent contrast to microtubules, the actin cytoskeleton maintains AJ symmetry around the apical domain. In other tissue types as well, actin and microtubules appear to compete for AJ interactions and have distinct effects on AJ assembly and maintenance around the apical domain. This work reveals that specific molecular mechanisms regulate AJ symmetry during morphogenesis. Interplay between actin and microtubules, regulated by cell polarity regulators such as aPKC, functions in orchestrating the balanced remodeling of AJs during epithelial development.
Roles of the Discs Large SH3-GK Intramolecular Interaction in Asymmetric Cell Division
R. A. Newman, K. E. Prehoda, University of Oregon, Institute of Molecular Biology, Eugene, OR
Members of the membrane-associated guanylate kinase (MAGUK) superfamily are involved in organizing molecular assemblies that regulate the formation and function of specialized regions of the membrane. Allosteric regulation of an intramolecular association of MAGUK SH3 and GK domains is thought to play a central role in regulating MAGUK function. For example, all genetically identified mutations in the SH3 and GK domains from the tumor suppressor Discs large (Dlg) have been shown to disrupt the SH3-GK intramolecular interaction. One such allele, dlg⁶⁵⁴, encodes a form of Dlg that disrupts the intramolecular association while leaving the SH3 and GK domains intact, providing an excellent model system for assessing the role of this interaction in MAGUK function. To determine the functional role of the SH3-GK intramolecular interaction in asymmetric cell division of Drosophila neuroblasts we assessed localization of cortical polarity markers in Drosophila neuroblasts. While the intramolecular interaction plays no role in Dlg localization, as the sw form of Dlg localizes like wild-type Dlg, it is required for restriction of the protein Miranda to the basal cortex. This similarity to the Dlg null phenotype suggests that while the dlg⁶⁵⁴ is properly localized it is unable to associate with protein partners that allow for restriction of cell fate determinants to the basal cortex. This hypothesis is currently being addressed by comparison of Dlg-associated proteins in dlg and dlg⁶⁵⁴ cell extracts by gel filtration of clarified cell extracts followed by co-immunoprecipitation and mass spectrometry. These studies will contribute to our understanding of the molecular mechanism by which Dlg regulates asymmetric cell division.

PAR-3 and PAR-1 Cooperate to Generate the LET-99 Band Pattern during Asymmetric Cell Division
J. Wu, R. Leslie; MCB, UC Davis, Davis, CA
During asymmetric cell divisions in early worm embryos, cell polarity is regulated by PAR proteins. PAR proteins also act as a through G protein signaling pathway to generate forces that position nuclei and spindles. Our lab has previously shown that the LET-99 protein acts downstream of the proteins and negatively regulates G protein signaling. Unlike several PAR proteins that localize to either the anterior or posterior cortex, LET-99 localizes in a distinct pattern as a posterior cortical band. We previously showed that cortical LET-99 asymmetry is dependent on the anterior PAR-3/PAR-6/PKC-3 complex. To further elucidate how the LET-99 band pattern is generated, we examined LET-99 localization in other polarity and spindle positioning mutants. We found that LET-99 localization is dependent on cortically localized proteins of the PAR pathway, but not on several cytoplasmically localized components. In the absence of low LET-99 levels at the anterior cortex in par-1 mutants shows that PAR-3 inhibits LET-99 localization independently of PAR-1. Further, quantification of staining intensities revealed a gap between the anterior LET-99 band and the posterior PAR-1 domain that correlates with the region of highest cortical LET-99 intensity. In a par-1 kinase mutant, LET-99 is also mislocalized to the entire posterior cortex, which suggests that LET-99 localization is regulated by phosphorylation. We propose that the anterior PAR-3/PAR-6/PKC-3 complex and posterior PAR-1 both inhibit LET-99 localization through their kinase activities, and the resulting cortical LET-99 band is required for asymmetric spindle positioning events. We are currently examining if LET-99 is phosphorylated by PAR-1 and PAR-3/PAR-6/PKC complex using 2D gel analyses.

Lentiviral Transduction of Bone Marrow Hemopoietic Progenitor Cells Ex Vivo
Methods of gene transfer into mammalian cells has been significantly progressed over recent years. Gene transfer by means of lentiviruses-based vectors permits to introduce a foreign gene into non-dividing cells, including stem cells. This work was performed with the aim to optimize the method of transducing hematopoietic progenitor cells with such a genetic construct ex vivo. The differentiation potential of transduced cells was studied by analysing spleen colonies. Cell subpopulation enriched with Lin- c-kit- hemopoietic progenitor cells (LK) was isolated from murine bone marrow by magnetic separation. This population was characterized by FACS-analysis of cells pre-treated with MAb against c-Kit, Sca-1 and CD34 antigens (BD). LK-cells were incubated in vitro for 24 h with HIV-based pseudoviral particles harboring genes of red fluorescent protein (dsRed) under the control of MSCV promoter. Transduced cells were cultured for 1 week, and dsRed expression was then determined using FACS-analysis. Spleen colonies were generated in lethally irradiated female CBA/C57Bl6 mice after administration of 3000 transduced LK-cells from syngenic males. In 10 days spleens from chimeric mice were used for morphological investigation and for isolation of single colonies, FACS-analysis of dsRed expression, and PCR-detection of Y-chromosomal marker and a fragment of integrated provirus genome. In 1 week post transduction 21% of cultured cells were shown to express dsRed. Spleen colonies were non-differentiated, and most of them cells expressed dsRed. In isolated colonies, percent of fluorescent cells varied from 0.5 to 78%. Y-chromosomal marker was detected in all colonies, while a fragment of integrated provirus genome was detected in most of them. Thus, HIV-based lentiviral vector can stably and effectively transduce LK-cells in vitro. LK-cells containing transgene are capable of giving rise to fully viable spleen colonies of megakaryocyte differentiated type in vivo. Supported by grant of RF President NS-7675.2006.7 and CRDF grant RUB1-576-MO-04.

Characterization of Cytoplasts from Embryonic Stem Cells
A. Kijima,1,2 S. Tone,1 Y. Nagahara,1 M. Ikekita,1 T. Shinomiya2; 1Tokyo University of Science, Tokyo, Japan, 2National Research Institute for Child Health and Development, Tokyo, Japan,
Methods of gene transfer into mammalian cells has been significantly progressed over recent years. Gene transfer by means of lentiviruses-based vectors permits to introduce a foreign gene into non-dividing cells, including stem cells. This work was performed with the aim to optimize the method of transducing hematopoietic progenitor cells with such a genetic construct ex vivo. The differentiation potential of transduced cells was studied by analysing spleen colonies. Cell subpopulation enriched with Lin- c-kit- hemopoietic progenitor cells (LK) was isolated from murine bone marrow by magnetic separation. This population was characterized by FACS-analysis of cells pre-treated with MAB against c-Kit, Sca-1 and CD34 antigens (BD). LK-cells were incubated in vitro for 24 h with HIV-based pseudoviral particles harboring genes of red fluorescent protein (dsRed) under the control of MSCV promoter. Transduced cells were cultured for 1 week, and dsRed expression was then determined using FACS-analysis. Spleen colonies were generated in lethally irradiated female CBA/C57Bl6 mice after administration of 3000 transduced LK-cells from syngenic males. In 10 days spleens from chimeric mice were used for morphological investigation and for isolation of single colonies, FACS-analysis of dsRed expression, and PCR-detection of Y-chromosomal marker and a fragment of integrated provirus genome. In 1 week post transduction 21% of cultured cells were shown to express dsRed. Spleen colonies were non-differentiated, and most of them cells expressed dsRed. In isolated colonies, percent of fluorescent cells varied from 0.5 to 78%. Y-chromosomal marker was detected in all colonies, while a fragment of integrated provirus genome was detected in most of them. Thus, HIV-based lentiviral vector can stably and effectively transduce LK-cells in vitro. LK-cells containing transgene are capable of giving rise to fully viable spleen colonies of megakaryocyte differentiated type in vivo. Supported by grant of RF President NS-7675.2006.7 and CRDF grant RUB1-576-MO-04.

Cellular Characteristics of the Distal Tip Cell, a Somatic Cell Required for Maintenance of Germline Stem Cells in C. elegans
D. T. Byrd,1 S. L. Crittenden,2 K. J. Schmidt,1 K. A. Leonhard,1 J. Kimble2; 1Department of Biochemistry, University of Wisconsin-Madison, Madison, WI, 2Howard Hughes Medical Institute, Madison, WI
Germ cells in adult C. elegans hermaphrodites are spatially organized with mitotically dividing cells at the distal end adjacent to the somatic distal tip cell (DTC), which provides a niche for germ cell proliferation. As cells move away from the DTC, they enter the meiotic cell cycle and undergo oogenesis. The region of mitotically dividing germ cells (the mitotic region) includes germline stem cells since it is self-renewing and produces cells that can differentiate into gametes. To investigate how germline stem cells are maintained, we examined the self-renewal and...
division patterns of germ cells in the mitotic region. We found that all germ cells within the mitotic region are actively cycling, as assayed by BrdU labeling, and that there is no consistent orientation of the mitotic divisions with respect to the DTC. Therefore, germline stem cells are likely maintained by proximity to the DTC rather than by programmed oriented cell divisions. To further investigate what other cellular characteristics of the DTC are important for its function in providing a niche for the germline stem cells, we made a panel of fluorescent protein markers to examine the cellular structure of the DTC in vivo. Using a membrane marker, we found that contact between the DTC and germ cells in the mitotic region is extensive and dynamic. The DTC extends membrane processes that surround adjacent germ cells. 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 provide more localized signaling to maintain the germline stem cells.

2583
Cellular Basis for Autologus, Adult Stem Cell Mediated Regeneration of Damaged Myocardium in a Canine Model for Myocardial Infarct
C. Culshaw,1 G. Wisenberg,2 F. Prato,3 H. Kong,2 K. Leks,1 J. Sykes,1 R. Mann,4 P. Merrifield3; 1Anatomy & Cell Biology, University of Western Ontario, London, ON, Canada, 2Cardiology, London Health Sciences Complex, London, ON, Canada, 3Imaging, Lawson Health Research Institute, London, ON, Canada

Heart failure, as a consequence of myocardial infarction, is the leading cause of death in North America. The use of autologous, adult stem cell therapy to sustain and/or regenerate damaged myocardium has been successfully used in rodents, but clinical trials are in development. In this study, we report a canine model of myocardial infarct. BMSCs were isolated from adult dogs, expanded in vitro, labeled with the fluorescent marker PKH26 and injected into myocardium damaged by 3 hrs of ischemia. Animals were imaged using MRI at weekly intervals to determine infarct size and left ventricular ejection fraction, sacrificed at 12 weeks post-injection and the hearts dissected and frozen for immunohistochemical analysis using a panel of vascular, myogenic and cardiomyogenic specific antibodies. MRI revealed that, compared to 5 control dogs (which received an infarct but no stem cells), dogs receiving BMSCs (5) experienced a greater recovery of damaged myocardium (ie reduction in infarct size over time). Immunohistochemical analysis with Mab CD31 demonstrated more neovascularization of infarcted regions in dogs receiving BMSCs compared to control dogs. Similar results have been reported by others for MSCs. The extent of neovascularization is currently being analysed. These studies will provide insight into the relative contributions of neovascularization and neocardiogenesis in improving cardiac function following stem cell therapy. 1 Ye et al (2006). Exp. Biol. Med 231, 8-19. 2 Oshima et al, 2005 Mol. Ther. 12, 1130-1141. (Supported by grants from HSFO and CCHR).

2584
The W-57 Allele of the Mouse Kit Oncogene Confers Advantages for HSC Function
Y. Sharma, C. Astle, D. Harrison; Hematology and Immunology, The Jackson Laboratory, Bar Harbor, ME

Throughout the life of an animal, cells for the blood and immune systems are descended from hematopoietic stem cells (HSC). The best-defined adult stem cell. HSC development organizes as a hierarchy, from the unipotent stem cells to the specialized blood cells. One essential regulator of HSC function is the Kit oncogene (c-Kit), previously identified as the White-spotting locus. Our objective is to define the effects of 3 Kit alleles on HSC and progenitor function. Several mutant Kit alleles have been identified with varying degrees of defects on hematopoiesis. This study focuses on 3 mutations - Kit-W-57 (W-57), Kit-W-41 (W-41), and Kit-W-42 (W-42). The W-57 mutation deletes 2.5 Kb of the minimal promoter, leaving the coding region intact, while W-41 and W-42 are single residue changes in the Kit intracellular phosphotransfer domain. Interestingly, the W-57 mutation increases HSC function. On the other hand, the defect caused by the W-42 mutation is so severe that homozygous die before birth; effects of W-42/+ on HSC differentiation are comparable to W-41/W-41. These 2 deleterious Kit mutations have normal numbers of progenitor cells in the HSC pathway, so they reduce HSC and progenitor functions rather than numbers. With the competitive repopulation assay, HSC from W-42/+ and W-41/W-41 mice have similar degrees of repopulating defects, which are significantly less functional than +/- W-57/W-57 BMC repopulate about twice as well as +/- controls. Progenitor cell function is also tested with colony spleen assays. We looked at allelic interactions between W-57 and each mutation to determine if the W-57 mutation confers advantages to HSC and progenitor cell function. Each mutation has a unique pattern of effects on specific stages of precursor cell differentiation when placed with W-57. These studies will improve understanding of KIT receptor in HSC function and could suggest clinical therapies for hematopoietic stem cell disorders.

2585
Enrichment of Muscle Satellite and Stem Cells with Chemotherapeutic Drugs: A Paradigm for Enhanced Stem Cell Transplantation
E. C. Hardeman1, P. Kahatapiyita1, B. Kramer1,1 E. J. Joya1, R. Liu1, J. Hook1, G. Schevova2, F. Lemnicker3, T. Borovina1, I. Alexander3, G. McCowage4, P. W. Gunning5; 1Muscle Development Unit, Children's Medical Research Institute, Westmead NSW, Australia, 2Oncology Research Unit, Children's Hospital at Westmead, Westmead NSW, Australia, 3Department of Oncology, Children’s Hospital at Westmead, Westmead NSW, Australia, 4Gene Therapy Research Unit, Children’s Hospital at Westmead, Westmead NSW, Australia

We describe a novel application of a selective enrichment strategy, initially established for hematopoietic cells, in a skeletal muscle system. Cells expressing a mutant form of the drug MGMT-P140K were selected and transplanted into regenerating muscle beds. These studies will provide insight into the relative contributions of neovascularization and neocardiogenesis in improving cardiac function following stem cell therapy. 1 Ye et al (2006). Exp. Biol. Med 231, 8-19. 2 Oshima et al, 2005 Mol. Ther. 12, 1130-1141. (Supported by grants from HSFO and CCHR).

2586
Regulatory Mechanism for Reprogramming
M. Nakagawa, K. Takahashi, K. Okita, T. Ichisaka, S. Yamanaka; Stem Cell Biology, Institute for Frontier Medical Sciences, Kyoto University, Kyoto, Japan

Embryonic stem (ES) cells derived from inner cell mass of mammalian blastocysts grow rapidly and infinitely while maintaining pluripotency. Although human ES cells could be used in cell transplantation therapy, their clinical application faces ethical objections against utilizing human embryos. One solution is to generate pluripotent cells directly from somatic cells. As candidates for factors inducing pluripotency, we selected 24 factors, which are specifically expressed in ES cells and/or play important roles in the maintenance of pluripotency. These include transcription factors Oct3/4, Sox2, and Nanog, as well as several (proto) oncogenes. We have also developed an assay system in which nuclear reprogramming can be detected as marker gene expression. By using this system, we have recently shown that retrovirus-mediated transfection of four factors, including Sox2, Oct3/4, c-Myc, and Klf4, could induce pluripotent cells, which we designated induced pluripotent stem (iPS) cells, directly from mouse embryonic and adult fibroblast cultures. However, the molecular mechanisms for this phenomenon remain to be resolved. One open question is whether sustained expression of the four factors is required for the generation and maintenance of IPS cells. To answer this question, we are introducing some of the factors transiently by adenoviral vectors, with the remaining factor(s) by retroviral vectors. We are also trying to express some of the four genes conditionally by the tetracycline-controlled system. Another important question is whether the four factors can be replaced by their mutants, their family genes, or other (proto) oncogenes. In this meeting, we will present progress in these experiments.

2587
Temporal-Spatial Emergence of HSC in the Fetal Mouse Liver
M. L. Edwards, M. Ferkowicz, S. Khan, W. C. Shelley, M. C. Yoder; Pediatrics, IU School of Medicine, Indianapolis, IN

Published data supports the origin of definitive hematopoietic stem cells (HSC) in the yolk sac, primitive spleenoplerue and aorto- gonado-mesonephros. The molecular signals that promote engraftment and expansion of definitive HSC in the newly developing fetal liver remain undefined. The object of this work was to define the earliest time and place that HSC arrived at the developing fetal liver to provide an optimal frame of reference for subsequent molecular studies. The nascent liver is formed as the vitelline veins invade the septum transversum and foregut endoderm differentiates into hepatoblasts. Definitive progenitor assays and transplantation data have been used to indirectly support the presence of HSC in the fetal liver as early as 28 somite stage (post conception day 19.5). Groups of ES 8.5-11 embryo mice were fixed and dissected at post conception day 19.5 to optimally demonstrate the developing abdominal structures and then incubated with fluorescein-labeled antibodies for a variety of cell lineage specific antigens including FLK1 and VE Cadherin (vascular endothelium), Proxl and HNF3B (hepatoblasts), and CD150 and CD41 (HSC). The tissues were imaged using a Biorad single photon confocal microscope system. The raw images were processed with Metamorph and Vox software and composite images compiled with Adobe Photoshop. Results support the hypothesis that small numbers of HSC arrive at the
nascent liver hematogenously and cross the vascular endothelium to engraft the extra-vascular hepatic parenchyma 3-4 somite pairs earlier than the 28 somite stage. Three-dimensional images in separate color channels support a direct interaction of individual HSC with vascular endothelial cells and hepatoblasts. We conclude that signals in 24-28 somite stage fetal murine liver may promote diapedesis of HSC to the extra-vascular region and that the hepatic microenvironment during this period should be the focus of future molecular studies of engraftment signaling.

2588
Optimizing Conditions for Low Toxicity, High Efficiency Transfection of Difficult-to-Transfect Cells Such as Primary Fibroblasts and Stem Cells Using Lipofectamine™ LTX
Transfection is an essential tool for numerous in vitro applications including studies of gene expression, promoter analysis, intracellular signaling pathways, epigenetics, and for therapeutic strategies such as tissue engineering and gene therapy. Common methods used in delivery of DNA into cells include calcium phosphate precipitation, liposome mediated transfer, electroporation, and virus-based delivery. Of these, cationic liposomes consistently provide the simplest and most effective method without requirements for additional equipment, cloning, or production of recombinant virus. Lipofectamine™ LTX is a new and novel reagent that provides high transfection efficiency and high levels of transgene expression in a range of mammalian cell types in vitro using a simple protocol. Optimal transfection efficiency and subsequent cell viability depend on a number of variables such as cell culture conditions, liposome and DNA concentrations, liposome-DNA complexing time and type of media in which the complexation occurs. Based on these general conditions, optimized protocols for transfection of a variety of cell lines have been established, including cell types often regarded as hard to transfect. The importance of these factors in Lipofectamine™ LTX mediated transfection is evaluated and used in the development of specific applications for transfection of several primary cell types, stem cells, and disease relevant (NCI60) cell lines.

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Self-Tolerance and Vascularization of Tissue Mediated by “Non-Coding” RNA Bioaptamers
J. H. Wissler, J. E. Wissler, E. Legermann; ARCONS Institute for Applied Research & Didactics, Bad Nauheim, Germany
OBJECTIVE: Tolerance concerns development and immune self-nomelf recognition and discrimination processes with spatiotemporal, basic and clinical problems associated with tissue vascularization alike. As evident from paradigmatic Le Dorain phenomena and peptideiton complexity, involved mechanisms are not fully understood [Burnet, Medawar: http://nobelprize.org/nobel_prizes/medicine/laureates/1960/index.html; Coutinho, Int.J.Dev.Biol. 4:131-136,2005; Yewdell, Science 301:1334-1335,2003]. Therefore, RNA/RNP functions in tolerance and associated epigenetic [non-Mendelian] phenotype alterations were investigated. METHODS were developed for isolation of endogenous mammalian [oligo+] RNA, extracellular DNA and RNP, guided by self-tolerance bioassays of cellular differentiation and tissue morphogenesis [Wissler et al. Proteins Biol.Fluids 34:517-536,1996; Arch.Surg. 124:693-698,1999; Materialwiss.Werkstofftech. 32:994-1008,2001; Ann.N.Y.Acad.Sci. 961:292-297,2002; TET 213:163-184,2004; FEBIS J. 27:1-116,4-5P,2005; Mol.Biol.Cell Suppl. 12:149a150a,2001; FASEB J. 20:A30-A31 & A930-A931,2006]. RESULTS: By fluid shear stress, Fenton-type redox reactions, hypoxia [reactive oxygen species], metal [Ca,Cu,Zn] ions and mitogens, cells form many endogenous bioactive redox- and metalloregulated, edited and modified "non-coding" small hairpin shRNA bioaptamers [200 bases] and extracellular RNP cytokines [ribokines]. shRNA that feedback protein structure/folding to translation and transcription were sequenced. By copper ion-structured UCUG-hairpin loops, they address binding domains in epigenetic regulator protein conformers, termed KRX3H [KRXxXH], i.e. t/sx/Rg/x/RxxRx/y/n/q/dx/x-hh/y,x-x/Rx-x/KRx/q/eh with accessory basic [RK], R/K-zipper, SR/K/Rx and/or Hhx/h/Hyy/q segments. shRNA apparently represent cryptic codes for epigenetic alteration: By sequence edition, base modification [e.g. to isoguanine], redox- and metalloregulation, i.e. code extension/alteration, they are not directly retranslatable to inheritable genome codes. CONCLUSIONS: The results suggest that such shRNA are specific clonal recognition addresses for epigenetic phenotype alterations in RNA-, redox- and metalloregulated conformation phase pathway-locked loops. In estimated repertoires of recognition entities, RNA bioaptamers are effective and superior in diversity/specificity [~1017] than blood group type [~104], antibody [~108], T-cell receptor [~1010], and MHC [~1012] protein systems. For tolerance, all attributes make such RNA bioaptamers efficient and competent to recognize and integrate information flow on all possible molecular facets back to genomic mechanisms.

2590
Membrane Bound Cardiomyocyte Differentiation from Embryonic Stem Cells
S. Sheridan, M. Wilgo, S. Gil; Cell Based Assay Development, Millipore Corporation, Danvers, MA
Cardiomyocytes are an important model system to evaluate potential drug interactions that may affect the development and/or activity of heart tissue. However, cardiomyocytes from primary sources are limited, particularly when human cardiomyocytes are required. Embryonic stem cells (ESCs) have the capacity to differentiate into all three somatic germ layers and are a potential source for tissues for a variety of uses. The ability to differentiate these cells into specific tissue types has extensive implications in the areas of regenerative medicine, tissue engineering and drug discovery. Several studies have demonstrated that ESCs can be differentiated into cardiomyocytes, expressing the same markers and showing equivalent electrophysiology as isolated cardiomyocytes. These results support the idea that engineered cardiac tissue from embryonic stem cells can be used as a model for studies of tissue development and function in vitro and may provide a cell source for cell therapy and drug discovery screening. We have evaluated the use of porous synthetic polymer substrates in facilitating the tissue-specific differentiation of embryonic stem cells into cardiomyocytes. Cardiomyocyte differentiation was initiated via embryoid body formation followed by adherent growth to a porous lum polymer (PET) membrane surface. Phenotype was verified over time by visual observation of beating cell clusters, immunohistochemistry and flow cytometry. Typically, differentiation studies have been carried out on 2D solid bottom plastic growth substrates. However, such cultures have limitations, such as lack of nutrient and gas exchange in the interior of cell clusters. Membrane-based growth and differentiation overcomes these limitations by providing a more in vivo like 3D surface topography conducive to adherence while the pores allow for both nutrient and gas exchange from multiple directions. In addition, membrane-based differentiation culture allows for co-culture experiments to examine indirect cell-cell interactions.

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Expansion of Embryonic Stem Cell Clones by Porous Membrane Based Co-Culture
S. Sheridan, S. Gil, M. Wilgo; Cell Based Assay Development, Millipore Corporation, Danvers, MA
Embryonic stem cells (ESCs) are essential tools in the developmental genetics of modern genetically modified mouse models. The requirement that the ESCs are maintained in the undifferentiated state is a general challenge during microwell plate based clone isolation and expansion. Current methods typically accomplish this by a labor-intensive multi-step direct co-culture of ESCs on a mouse embryonic fibroblast (MEF) feeder layer in 96 well tissue culture treated plates. We have investigated the ability to expand undifferentiated clonal colonies in a 96 well membrane based cell culture plate. Porous membranes allow for the co-culture of cells from different origins in order to study how cells interact through indirect signaling or by providing a conditioned niche for the proper growth and identity of cell type. In this study, we examine the ability of MEFs to condition the media for the growth and undifferentiated clonal isolation of ESCs in a porous membrane based indirect co-culture system. The configuration of this co-culture setup involves growing the MEFs in a feeder tray below wells containing ESCs separated by a porous membrane filter. This arrangement allows for a physical separation between the two cell types eliminating the need for mitotically inactivating the MEFs while continuing to allow the MEFs to condition the media for maintenance of ESCs pluripotence. Since the ESC containing wells share a larger volume of media, media change frequency can be reduced. In addition to improving the culturing efficiency/specificity of embryonic stem cell clones, the separation of the cell types during this filter based co-culture eliminates the requirement for removing the MEFs before blastocyst injection. Variations in media for maintenance of ESCs pluripotence. Since the ESC containing wells share a larger volume of media, media change frequency can be reduced. In addition to improving the culturing efficiency/specificity of embryonic stem cell clones, the separation of the cell types during this filter based co-culture eliminates the requirement for removing the MEFs before blastocyst injection. Variations in media for maintenance of ESCs pluripotence. Since the ESC containing wells share a larger volume of media, media change frequency can be reduced. In addition to improving the culturing efficiency/specificity of embryonic stem cell clones, the separation of the cell types during this filter based co-culture eliminates the requirement for removing the MEFs before blastocyst injection. Variations in media for maintenance of ESCs pluripotence. Since the ESC containing wells share a larger volume of media, media change frequency can be reduced. In addition to improving the culturing efficiency/specificity of embryonic stem cell clones, the separation of the cell types during this filter based co-culture eliminates the requirement for removing the MEFs before blastocyst injection. Variations in media for maintenance of ESCs pluripotence. Since the ESC containing wells share a larger volume of media, media change frequency can be reduced. In addition to improving the culturing efficiency/specificity of embryonic stem cell clones, the separation of the cell types during this filter based co-culture eliminates the requirement for removing the MEFs before blastocyst injection. Variations in media for maintenance of ESCs pluripotence.
expression of liprotein lipase and peroxisome proliferator activated receptor γ mRNA, however, by 30 PDLs, calcium deposits were also apparent. In summary, proliferative and differentiation capacity of adipose derived hMSCs is relatively unchanged for 25 PDLs following isolation. These data suggest large numbers of multipotent hMSCs can be derived from a single isolation. Potential applications include tissue engineered autologous bone grafts, where a patient’s own cells could be used in a custom designed implant to repair a specific defect or injury.

2593
Selection of Smooth Muscle Cells from Differentiating Mouse Embryonic Stem Cells
A. Kumar, J. Szucz, J. L. Lessard; Developmental Biology, Cincinnati Children’s Hosp Med Ctr, Cincinnati, OH
Disorders of smooth muscle affect both vascular and visceral organ systems. The goal of this study was to develop a strategy to select smooth muscle cells from cultures of differentiating mouse embryonic stem cells. Toward this end, we have exploited a 13.7-kb DNA fragment containing the promoter, upstream regions and all sequences up to the translational start codon in the Smooth Muscle Gamma-Actin (SMGA) gene (Qian et al., Dev. Dynamics 207:135-144, 1996). This promoter drives expression of reporter genes in a tissue specific and developmentally-regulated manner that is similar, if not identical, to that of the endogenous SMGA gene which is expressed almost exclusively in smooth muscle. Moreover, unlike Smooth Muscle Alpha-Actin (SMAA), SMGA is not expressed in early cardiomyocytes, developing skeletal myocyte, or other non-smooth muscle cell types. SMGA expression occurs subsequent to SMAA expression during smooth muscle development; thus, SMGA expression is a hallmark of a mature and/or highly differentiated smooth muscle phenotype. We have generated a construct containing a selectable puromycin marker and the 13.7-kb SMGA promoter to drive the expression of neomycin phosphotransferase. Puromycin resistant, undifferentiated ES cell clones were selected after electroporation of this construct into feeder-independent E14TG2E ES cells. Following differentiation of these ES cells and selection with neomycin, populations of cells were obtained in which most, if not all, cells stained for both SMGA and SMAA. Furthermore, these cells displayed a characteristic morphology of cultured smooth muscle cells and Northern analysis and microarray analysis confirmed the expression of numerous smooth muscle-related mRNAs in these cells. These cells provide an important resource for the study of smooth muscle differentiation that may lead to insights into the smooth muscle myopathies and form the basis for novel cell-based therapies.

2594
Nanoscale Topography Modulates Human Embryonic Stem Cell Self-Renewal
D. R. McFarlin, K. J. Finn, P. F. Nealey, C. J. Murphy; 1School of Veterinary Medicine, University of Wisconsin-Madison, Madison, WI, 2Chemical and Biological Engineering, University of Wisconsin-Madison, Madison, WI
Significant challenges in human embryonic stem cell biology include the maintenance of an undifferentiated lineage as well as devising optimal strategies to coax large populations of cells down pathways of preferred differentiation (e.g. neurons). Numerous environmental factors influencing stem cell behavior have been investigated including surface chemistry, growth factors, and mechanical forces. Our laboratory has previously reported, for a number of cell types, that topographic features, independent of surface chemistry, profoundly modulate fundamental cell behaviors. We hypothesized that topographic cues, in the nano to micron scale range, would play a role in modulating stem cell behaviors. Soft lithography was used to stamp polyurethane substrates containing nanoscale through micron scale grooves and ridges, ranging from 4000 nm down to 400 nm pitch (pitch = ridge width + groove width). H1 HES cells were cultured for five days on flat and topographically patterned substrates. Cells were fixed and scored for the presence of alkaline phosphatase, a known marker of undifferentiated stem cells. The provision of topographically patterned substrates (in the nanoscale through microscale range) improved maintenance of the self-renewing phenotype (p ≤ 0.0009). To our knowledge these are the first experiments documenting that the physical topography of cell culture surfaces influences HES cell differentiation and self-renewal. Nanoscale topographic cues should be considered a fundamental environmental factor that has relevance to emerging strategies of stem cell engineering.

2595
Microfluidic Platform for Live Cell Screening
P. J. Lee, P. J. Hung; CellASIC Corporation, Richmond, CA
We have developed an innovative microfluidic chip for multiplexed cell culture and high content analysis. The current chip enables a researcher to control 16 independent perfusion experiments on adherent cells over time periods of days to weeks. The design of the microfluidic culture regions and nutrient flow paths resemble the physiological microcirculation, and ensure uniform culture conditions while eliminating cross-contamination. The “tubing free” construction of the system overcomes many of the current limitations of microfluidic screening. Each experimental unit contains a volume in the nanoliter range with flow rates on the order of nanoliters per minute. The cells can be directly imaged through the #1 thickness borosilicate glass bottom, enabling high magnification light and fluorescence microscopy. Our microfabrication process enables reliable production of features down to 1 micron, with a cost per data point over 10X lower than current flow cell systems. This chip has been used to demonstrate cytotoxicity of anti-cancer drugs on cultured cell lines. An alternate geometry has also been used to create and maintain well defined multicellular tumor models in a multiplexed microfluidic format. Due to the small volume requirements for the microfluidic chip, multiplexed screening of primary cell cancer models can be readily implemented. The continued development of microfluidic tissue culture technologies will enable more accurate in vitro results for primary cell screening experiments.

2596
Microfabricated Arrays for Separating Adherent Cells
C. E. Sims, Y. Wang, M. Bachman, G. Li, G. Young, G. Salazar, N. Albritton; 1Physiology and Biophysics, University of California-Irvine, Irvine, CA, 2Integrated Nanosystems Research Facility, University of California-Irvine, Irvine, CA, 3Biomedical Engineering, University of California-Irvine, Irvine, CA, 4Physiology and Biophysics, Biomedical Engineering, University of California-Irvine, Irvine, CA
A fundamental need in almost all areas of biomedical research is the ability to separate single or small groups of cells from within a heterogeneous population. In order to obtain a living cell possessing a desired characteristic, individual or homogeneous groups of cells within the population must be analyzed followed by identification and isolation of the target cell(s). Most live-cell separation methods require that cells be dispersed into a single-cell suspension, but removal of adherent cells from their growth surface may at times be undesirable. A flexible cell array platform composed of releasable microfabricated “pallets” and an integrated laser microscope system has been developed for analyzing, sorting and collecting viable cells from a mixed population while the cells remain adherent to their growth surface. Individual pallets containing single cells or colonies can be released and collected with minimal perturbation. Preliminary studies have shown that adherent cells cultured on the array can be analyzed and selected using standard imaging methods. Furthermore, target cells can then be collected with high viability and efficiently cloned. Benefits of this new approach include improved cell viability, smaller sample size requirements, and broader alternatives for cell selection. Mating of the technique with image cytometry can be expected to provide a valuable tool for selection of adherent cells for cloning or further analysis.

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Islet on a Chip: A Novel Array of Living Beta Cells Reveals Functional Subclasses
M. Hibo, J. Wikstrom, B. Corkey, S. Katz, B. Israel, O. Shirihai; 1Pharmacology, Tufts University, Boston, MA, 2Obesity Research Center, Boston University, Boston, MA
Pancreatic beta cells form a functionally heterogeneous population within the islets of Langerhans. Currently, the understanding of beta cell population dynamics in response to glucose and other physiologically relevant agents is limited. This limitation is caused by the lack of a suitable tool with which to study a large population of primary cells over time at the single cell resolution. Here we present the first such study of beta cell heterogeneity using a novel imaging approach that enables real-time, ongoing observation of cellular events from thousands of individual living cells, followed by structural and post-fixative measurements on the same cells. This technology is based on a densely packed array of transparent micron-sized wells. Each well is designed to contain an individual cell, facilitating visual observation and manipulation of the individual cells. Using this slide-based imaging technology, we have demonstrated that beta cells can be sorted into distinct subpopulations based on their individual responses to glucose stimulation. Mitochondrial membrane potential was used as an indication of beta cell function and was correlated to distinct non-responding, weak responding, and strong responding sub-populations. This methodology facilitates the characterization of the different metabolic phenotypes of beta cell populations and potentially establishes predictive parameters for beta cell function and susceptibility to glucolipotoxicity.
X-ray Tomography: New Tool for Imaging Whole, Hydrated Cells

W. Gu, M. A. Le Gros, L. D. Ekin, C. A. Larabell; Department of Anatomy, University of California, San Francisco, CA, 2Physical Biosciences Division, Lawrence Berkeley National Lab, Berkeley, CA, 3Department of Molecular and Cell Biology, University of California, Berkeley, CA, 4Department of Anatomy, University of California, San Francisco, CA.

X-ray microscopy is a new imaging technique that bridges the gap between light and electron microscopy. Whole, hydrated cells are examined using photons with energies in the so-called 'water window,' where organic material absorbs approximately ten times more than water. This generates high contrast images of cellular structures in their native state, without the need for contrast enhancement reagents. Immunolocalizations are conducted in hydrated cells using metal, rather than fluorescent, probes. Unlike fluorescence microscopy, however, information about the location of the protein is superimposed upon the cellular structures. Using tomographic techniques it is possible to acquire 3D images of whole cells and, since we use the x-ray absorption coefficient, the information obtained is quantitative. We report here the use of soft X-ray tomography to examine cell division in the fission yeast, Schizosaccharomyces pombe. The cells were rapidly frozen and maintained at cryogenic temperature during imaging. No fixatives or contrast enhancement reagents were used, so the cell structures observed in these images closely approximate the natural state. During S. pombe mitosis, F-actin reorganizes at the cell equator to form a contractile actomyosin ring. X-ray tomography reveals the filaments that form the actomyosin ring using the natural contrast mechanism, revealing structural differences between the early and late stages of cell division. The unlabeled actomyosin ring-like structure forms a circumferential ring just beneath the cell membrane in early cell division, and forms a solid planar sheet bisecting the two cells at the culmination of cell division. Using X-ray tomography, we can now localize the macromolecules involved in cell division to the division plane throughout the cell cycle.

Development of a 'Trap and FRAP' Microscopy System for Cell Migration Studies

W. D. Shin, S. Ross; C. M. Waterman-Storey; Cell Biology, The Scripps Research Institute, La Jolla, CA, 2Nikon Instruments Inc., Melville, NY

Cell migration is dependent on forces generated by the actin cytoskeleton to the extracellular environment through focal adhesions. We are interested in understanding how the pulling force on focal adhesions between the extracellular environment and the actin cytoskeleton is regulated by how tightly the molecules make up the focal adhesions bind to one another. In order to address this question, we have developed a microscopy system that allows simultaneous measurement of the forces exerted by the actin cytoskeleton and focal adhesions on an extracellular structure and at the same time allows measurement of the tightness of binding of focal adhesion molecules to one another. The microscope system combines optical gradient trapping and Fluorescence Recovery After Photobleaching (FRAP) capabilities using a Nikon TE2000-E2 motorized inverted microscope. A 1 W 1064 Nd:YAG laser provides the optical gradient trapping force, while FRAPing is accomplished with a 65 mW Ar laser and/or a 10 mW 561 nm solid state laser. Laser light, which is focused to a diffraction limit spot at the specimen plane, is used to move and position objects with precision. FRAPing is accomplished with a 65 mW Ar laser and/or a 10 mW 561 nm solid state laser. Laser light, which is focused to a diffraction limit spot at the specimen plane, is used to move and position objects with precision. FRAPing is accomplished with a 65 mW Ar laser and/or a 10 mW 561 nm solid state laser. Laser light, which is focused to a diffraction limit spot at the specimen plane, is used to move and position objects with precision. FRAPing is accomplished with a 65 mW Ar laser and/or a 10 mW 561 nm solid state laser. Laser light, which is focused to a diffraction limit spot at the specimen plane, is used to move and position objects with precision. FRAPing is accomplished with a 65 mW Ar laser and/or a 10 mW 561 nm solid state laser. Laser light, which is focused to a diffraction limit spot at the specimen plane, is used to move and position objects with precision. FRAPing is accomplished with a 65 mW Ar laser and/or a 10 mW 561 nm solid state laser. Laser light, which is focused to a diffraction limit spot at the specimen plane, is used to move and position objects with precision. FRAPing is accomplished with a 65 mW Ar laser and/or a 10 mW 561 nm solid state laser. Laser light, which is focused to a diffraction limit spot at the specimen plane, is used to move and position objects with precision.
Time-resolved microscopy is the ultimate tool for investigating dynamic events in cells and sub-cellular structures. Its applications previously have been limited to wavelengths longer than 370 nm because microscope optics does not transmit in the deep UV. We have introduces a filter-based confocal system (the DynaMic™) to measure fluorescence lifetime and intensity directly under the microscope in the deep UV and visible wavelength range of 240-850 nm. The system features TCSPC single photom sensitivity and fast acquisition for any fluorescence lifetimes of 100ps-100 ns. An Oxford 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. A pinhole turret is placed in the emission light path with variable pinholes sizes from 0.1 up to 10mm for different spatial resolutions. With a laser diode as excitation light source, confocal measurements can be achieved with this system. This system can also be used for mapping of fluorescence lifetimes and intensity with variable spatial resolution (<1μm) with an automated stage. The paper will present fluorescence lifetime measurement from a single protein crystal with diameter of ~70 μm, and lifetime mapping obtained from stained mouse kidney section. The potential application for detection of single protein molecules without fluorescence label will be discussed.

2604

**Structured Illumination Microscopy on Meiotic Chromosomes**

P. M. Carlton, R. Wang, W. Z. Cande, J. W. Sedat; 1Department of Biochemistry and Biophysics, University of California, San Francisco, San Francisco, CA, 2Department of Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA

In order to bridge the gap between the high resolution of electron microscopy and the high labeling specificity of conventional optical microscopy, we have developed a new optical microscope that utilizes structured illumination. Structured illumination uses a sine wave pattern of light to excite fluorophores, rather than a uniform field of light. The light emitted from the specimen contains high-frequency spatial information in the form of interference fringes, which can be reconstructed into an image that reveals features below the Rayleigh limit. To demonstrate the high resolution and specificity of this technique, we took advantage of the well-established dimensions of the synaptonemal complex (SC), a proteinaceous, ribbon-like structure that holds homologous chromosomes together at a distance of 200nm during prophase I of meiosis. Maize meiotic cells were immunostained for two components of the SC (AFD1, a maize Rec8 homolog; and ASY1, a Hop1 homolog) and imaged in three dimensions. While the two axial elements of the SC appear as a single fiber in conventional optical microscopy, SI microscopy reveals their dual nature and 200nm spacing, as well as their tendency to twist around each other. The exceptional quality of this 3D image data allows the full path of each of the ten maize chromosomes to be traced and analyzed. Additionally, the morphology of DNA, visualized by DAPI staining, can be directly correlated to the pairing state of each chromosome. This demonstrates that structured illumination microscopy is a powerful new tool for exploring basic cell biological questions, and should be readily adaptable toward a variety of systems.

2605

**Fluorescence Correlation Imaging for Biophysics and Cell Biology Applications**

D. J. Needelman, T. J. Mitchison; 1Department of Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA

We have developed a novel imaging technique, Fluorescence Correlation Imaging (FCI), which will allow the dynamics of soluble molecules to be studied in detail. FCI is a radical extension of Fluorescence Correlation Spectroscopy (FCS), transforming this technique from a spectroscopy, which measures dynamics at one point, to a microscopy. FCS can be used to determine binding constants and to measure diffusion in a fluid. Fluorescence Correlation Imaging offers two advantages over conventional FCS: it can be used to image molecules on substrates, and it can be used to image a single point in a sample due to movement of labeled molecules relative to a small, fixed illumination volume. However, FCS is only applied to a single point at a time, so no spatial detail is obtained. FCI performs FCS type measurements simultaneously on ~10,000 points, essentially creating an “image” of FCS measurements which provides quantitative temporal and spatial data. In addition to enabling multiple FCS measurements, which can be used to characterize heterogeneous structures like cells, entirely new information can be obtained from FCI by analyzing the correlations in the fluctuations at different points, including component resolution and large length scale, collective motions. We have used FCI to study the dynamics of colloids, globular proteins, networks of cytoskeletal filaments, and soluble molecules inside cells.

2606

**Live Cell Imaging of Activated Human Umbilical Cord Blood Derived Cultured Mast Cells**

J. Malakal, T. Dvori, D. Kempuraj, M. Tagen, I. Biran; 1Molecular Cytomics Inc., Boston, MA, 2Tufts University School of Medicine, Boston, MA

mast cells are target effector cells in the onset of IgE-mediated allergic and inflammatory response. When the mast cells are activated they undergo regulated release of intracellular granules by exocytosis. This includes inflammatory cytokines such as IL-6, TNF-α and other cytokines and chemokines. In this study we examined mast cell activation using a microscope slide based pico-well array at a single cell resolution. The CD34 positive cells were isolated from human umbilical cord blood and cultured with Stem Cell Factor (SCF) to obtain mature mast cells. The mast cells were sensitized with human IgE for 48 hours, counted and 2.5μl of cell suspension were added to the pico-wells of the array (LCA, Molecular Cytomics, Inc. Boston, MA). The cells in the array were covered with a sliding cover slip allowing each cell to settle into an individual well. The cover slip also allowed for repeated treatment and washing of reagents via capillary flow. The cells were treated with anti-human IgE to induce activation and were labeled with anti-CD63 PE, a fluorescently tagged marker of mast cell activation. During the entire washing and labeling procedure the cells remained in their individual wells. Time-lapse and single time-point live cell imaging showed activated mast cells and varying degree of activation as indicated by levels of fluorescence. Following activation, the cells fluoresced nonspecifically under various wavelength filters; however, when tagged with anti-CD63 PE, only activated mast cells exhibited fluorescence. Among the activated mast cells, significant heterogeneity was observed and only less than 50 percent of the cells exhibited activation. The ability to culture, treat and image non-adherent mast cells in the pico-well array allowed us to study single cell response in a mast cell population.

2607

**High Efficiency Peptide and Antibody Delivery for Cell Functional Study Using Flow Cytometric Analysis**

N. Quan, J. Wu, S. Huang, J. Li, G. McMasters, F. Witney, F. Heitz; 1Molecular Detection, Panomics, Fremont, CA, 2Molecular Biophysics and Therapeutics, CRBM-CNRS, Montpellier, France, 3CRBM-CNRS, Montpellier, France, 4Cellular Analysis Business, Beckman Coulter, Inc., Fullerton, CA

Cell deliveries of peptides and proteins are critical to the study of cellular function of proteins. However, conventional lipid-based or electroporation technologies are not always capable of efficiently delivering these macromolecules into many cell types such as Jurkat and K562. Here we demonstrate that the viral derived amphiphatic delivery peptide “MPG” efficiently imports both peptides and proteins into cells. MPG that consists of hydrophobic and hydrophilic domains interacts non-covalently with its cargo forming nano size particles capable of passive diffusion through the cell plasma membrane and avoiding the endosomal pathway. Peptides and proteins were labeled with FITC or TAMRA and delivered to cells using MPG. Delivery efficiencies were determined using the CellLab Quanta SA flow cytometer. Viability of transfected and non-transfected cells was quantified by both staining with propidium iodide (PI) or 7-amino-actinomycin D and determining the electronic volume and side scattering of the cells. We delivered peptide inhibitors to Cdk2 and G-protein βγ, subunit with MPG and carried out cell functional studies. The effect of the Cdk2 peptide inhibitor on cell cycle was analyzed by staining the cells with Hoechst 33342 and PI and determining Cyclin A2 expression using an anti-cyclin A2 antibody conjugated to FITC. Inhibition of the G-protein βγ resulted in activation of Erk1/2 as measured by phospho-specific antibodies. We conclude that the combination of MPG delivery and flow cytometry analysis provides a powerful tool to study cell function of proteins. Note: For research use only. Not for use in diagnostic procedures.

2608

**Three-dimensional Visualization of Cell and Tissue Architecture Using Dual Beam Electron Microscopy**

J. Heymann, S. Subramaniam; Laboratory of Cell Biology, National Cancer Institute, NIH, Bethesda, MD

Current approaches to 3D imaging at subcellular resolution using confocal microscopy and electron tomography, while powerful, are limited to relatively thin and transparent specimens. We have recently reported on the use of a new generation of dual beam electron microscopes capable of site-specific imaging of the interior of cellular and tissue specimens at spatial resolutions about an order of magnitude better than those currently achieved with optical microscopy [1]. The principle of imaging is based on using a focused ion beam to create a cut at a designated site in the specimen, followed by viewing the newly generated surface with a scanning electron beam. Iteration of these two steps several times results in the generation of a series of surfaces maps of the specimen at regularly spaced intervals, which can be converted into a three-dimensional map of the specimen. We demonstrate here that this strategy can be successfully used for site-specific 3D imaging of entire individual cells or of one or more tissues within a tissue. We show that such images can be obtained at resolutions adequate to detect molecular machines such as the nuclear pore and ribosomes. Imaging of melanocytes and melanoma cell lines in 3D reveal the distribution of melanosomes and other organelles providing a new base to investigate localization and organization of cellular components in diseased and healthy cells or tissues. Such information might provide unprecedented opportunities for understanding the structural mechanisms underlying the onset of diseases such as malignant melanoma especially when combined with molecular labeling techniques. [1] Heymann JA, Hayles M, Gestmann I, Giannuzzi LA, Lich B, Subramaniam S., J. Struct. Biol. 155: 63-73, 2006. Site-specific 3D imaging of cells and tissues with a dual beam microscope.
Thin Sectioning Improves Visualization Quality in Imaging Mass Spectrometry

Y. Sugura, 1 S. Simma, 2 M. Setou 3 1Department of Bioscience and Biotechnology, Tokyo Institute of Technology, Tokyo, Machida, Japan, 2Okazaki Institute for Integrative Biosciences, National Institutes of Natural Sciences, Okazaki, Aichi, Japan, 3The Graduate University for Advanced Studies, School of Life Science, Okazaki, Aichi, Japan

The direct molecular imaging of biological tissue using mass spectrometry (MS) is the new technique to image distribution of biomolecules, such as proteins, peptides, and lipids in the tissue section. This method has already been shown to provide useful biological information for biological applications, pathological applications and drug discovery. To carry out successful profiling and mapping of biomolecules, though, techniques for correct sample preparation are required. Here we focus on the influences of tissue thickness on mass spectra. We obtained the mass spectra and imaging results of proteins as a function of thickness from 2 to 40 µm, then evaluated the spectrum quality. The peak intensity and number of observed peaks drastically increased as the sections became thinner (< 10 µm). Significant images of high molecular weight proteins were acquired only from the thinner slices. We thus concluded that the thickness of biological tissue slices was an important factor in obtaining high quality mass spectra. There could be two reasons for inefficient analyte ionization of thicker slices: electrical nonconductive properties and impurities in thick tissue sections. Furthermore, we applied this technique to the direct analysis of lipid species. Improved ionization efficiency permitted not only MS but also MS/MS of neutral lipid and phospholipid species. Enriched neutral lipid and phospholipid species were used to obtain high-quality MS imaging results and structural information of these molecules. In conclusion, this investigation extends the capability of imaging mass spectrometry by optimizing the thickness of the tissue samples.

Modification and Conjugation of Proteins, Peptides, Oligonucleotides, and Carbohydrates to Cell Surfaces

D. Schwartz; Solulink Biosciences, San Diego, CA

Methods to transport small molecule drugs, especially antiviral and anti-cancer drugs, antibodies, siRNA and other polynucleotides across cell membranes is an area of intense research. TAT-like cationic peptides have been shown to be able to mediate transport of both proteins and oligonucleotides across membranes. As is well recognized with lipid-based plasmid transfection reagents transfection efficiency can be reagent dependent. Solulink has engineered a series of chemically activated cationic peptides containing cleavable disulfide bonds for release of the payload in the endosome or cytoplasm. These peptides are readily conjugated to any protein or oligonucleotide of interest. A series of linkable cationic peptides will be used to compare transfection efficiency across various cell lines using both antibodies and labeled siRNA.

A Transgenic Mouse System with Bioluminescent and Color-Switching Dual Fluorescent Cell Tracers

L. Zheng, C. Njauw, M. Martins-Green; Cell Biology and Neurosciences, University of California, Riverside, CA

Interleukin-8 (IL-8) is a prototype chemokine that plays multiple roles in the wound healing process. Human IL-8 has two receptors, CXCR1 and CXCR2, which mediate its different functions. In order to establish an animal model system to decipher the functions of IL-8 in vivo, we have made a transgenic mouse model system that: (i) can express CXCR1 in specific tissues in a Cre/loxP controlled manner; (ii) incorporates bioluminescence and color-switching dual fluorescence as tracer systems that allows us to distinguish the transgene-activated tissues/cells (labeled with eGFP/luciferase) from the wild type tissue (labeled with mRFP). A transgenic mouse line with this vector carrying CXCR1 has been generated and here we show validation of various features of this transgenic system. The F1 founder line expresses mRFP whereas no CXCR1 or eGFP/luciferase activity has been detected. The offspring of F1 founder crossed with different lines of Cre mice enabled us to examine the Cre-dependent color-switching and transgene expression in the designated tissues. In the tissues/cells in which the transgene CXCR1 expression has been activated by Cre, the original mRFP gene is genetically deleted and the protein lost within 8 days and these cells have been relabeled with another multifunctional marker which is a membrane-localizing eGFP/luciferase fusion protein. The mRFP/eGFP conversion has been examined in most of the tissues of the transgenic mice. The luciferase activity is also being examined to turn on along with the transgene expression and colorswitching. This transgenic model system is a versatile and flexible system that allows temporal and spatial control of the transgene expression. It also allows using different imaging techniques to localize, quantify and even manipulate the cells of interest in vivo.

Image Informatic Tools for Multidimensional Light Microscopy

C. T. Rueden, M. Linkert, F. Wong, C. Peterson, P. Huettl, M. Hvalenka, E. Hathaway, K. W. Eliceiri; Molecular Biology, University of Wisconsin-Madison, Madison, WI

With the increasing use of digital image capture microscopy in the biomedical sciences, it has become a major challenge to locate, view and interpret large numbers of images collected in a diversity of formats. Many biological research laboratories have a pressing need to archive and annotate vast numbers of images collected by video, laser-scanning microscopy and other photonic-based imaging techniques. Multidimensional images, such as four-dimensional images from multi-focal plane time-lapse recordings, or images from spectral and lifetime microscopy, make the challenge even greater. Without careful organization, important research data can be difficult or impossible to find, much less visualize and analyze effectively. We are developing a complete, open system for handling biomedical images, including image acquisition, data storage, metadata (experimental data associated with an image), visualization, analysis, annotation, and database interconnectivity. The software makes extensive use of several established open source systems for handling images: the Open Microscopy Environment (OME; image database), VisAD (image visualization), and ImageJ (image processing). We have also developed an open file format called OME-TIFF: fully compatible multi-page TIFF with rich metadata embedded in OME-XML format, the OME standard. Other major components of the software include: Bio-Formats, a Java library for reading and writing dozens of common microscopy file formats, including processing and conversion of metadata into OME/TIFF format; VisBio, a biological visualization tool for facilitating visualization and analysis of complex multidimensional image data; an OME plugin for ImageJ for transferring images between ImageJ and an OME database; and a multidimensional Data Browser plugin for ImageJ to enable more effective visualization of 3D image data within ImageJ. We are working to integrate these packages into a cohesive whole to provide microscopists with a complete toolkit for overcoming the challenges of working with digital multidimensional images.

Automated Image Analysis of Protein Translocations and DNA Content for Cell- and Tissue-based High Content Screening Assays

N. L. Prigoshina, L. Zheng, E. A. Hunter, J. Mikie, D. R. Roop, M. A. Mancin, D. A. Zacharias, J. T. Price, P. M. McDonough; 1The Burnham Institute, La Jolla, CA, 2Baylor College of Medicine, Houston, TX, 3Hunter Miki & Callaway, San Diego, CA, 4The Whitney Laboratory for Marine Bioscience, St. Augustine, FL, 5Vala Sciences, La Jolla, CA

High throughput (HT) image cytometers analyze individual cells in digital photomicrographs by first assigning pixels within each image to plasma membrane (PM), cytoplasm, nucleus or other regions. In this study, we tested a novel algorithm that identifies PM regions to measure changes in PM-associated proteins (protein kinase C alpha, N-cadherin, E-cadherin, VE-cadherin, and pan-cadherin) that regulate cell division, migration, and adhesion. Validation assays were performed for these proteins on cells cultured in 96-well plates and also for tissue sections obtained from transgenic and chemical carcinogenic models of skin cancer. The algorithm successfully quantified phospho-PM (PMA) induced PM localization of protein kinase C (PKCalpha) in HeLa cells (Z′ of 0.88). Additionally, PMA activated translocation to the PM of N-cadherin (in HeLa cells), E-cadherin (in A431 cells), and VE-cadherin (in human dermal microvascular endothelial cells (HDMECs)), suggesting a relationship between PKCα activity and cadherin localization. For VE-cadherin, a Z′ of 0.52 was observed between serum-free media, which increased VE-cadherin, and EGFα, which diminished VE-cadherin at the PM. For sections obtained from the transgenic skin cancer model, analysis of images with the PM algorithm revealed that tumor cells exhibited cadherin expression that was just 34% of that expressed by surrounding normal tissue; furthermore, tumor cells expressed elevated DNA content, consistent with development of aneuploidy. In contrast, increased DNA content did not occur for tumor cells produced by chemical carcinogenesis. The results demonstrate that this new algorithm for PM image cytometry enables analyses in a variety of applications in both cultured cells and tissue sections.

Manual Tracking: A Semi-Automated Tool to Keep a Track of Movements in 2 and 3D

F. P. Cordelières; Plateforme d’Imagerie Cellulaire et Tissulaire, Institut Curie - Centre de recherche/CNRS UMR 146, Orsay, France

Over the past 5 years, development of 2D and 3D video-microscopes has made it possible to record the dynamic behavior of objects within the full volume of samples. While the acquisition techniques are now widely available, as part of imaging workflows, a new pole of interest has emerged, aiming to get dynamics’ quantization out of the images. Two main approaches may be used to characterize object’s motion: 1- fully automated approaches based on isolation and recognition of structures over time series; 2- manual identification and tracking. The first kind

VisBio, a biological visualization tool for facilitating visualization and analysis of complex multidimensional image data; an OME plugin for ImageJ for transferring images between ImageJ and an OME database; and a multidimensional Data Browser plugin for ImageJ to enable more effective visualization of 3D image data within ImageJ. We are working to integrate these packages into a cohesive whole to provide microscopists with a complete toolkit for overcoming the challenges of working with digital multidimensional images.
of methods requires a good segmentation of the objects, which may be tricky in highly noisy corrupted situations. The second approach is more widely applicable, while being somewhat slower as it requires user’s input. We here introduce the “Manual Tracking” plugin to the ImageJ software as a new tool to keep a track of objects’ motion in 2 and 3D. It offers a way to record the coordinates of objects on two-dimensional time series’ images, together with their velocity and intensity. “Manual Tracking” may also be used to generate graphical representation of tracks as overlays to the original images or synthetic views. In the particular case of tracking recovered from 2D+time projections of 3D time series, “Manual Tracking” proposes a semi-automated retrieval of the third coordinate (z coordinate). Parameters such as velocity and volumetric intensity are calculated on a sub-volume which boundaries are user defined. Finally, “Manual Tracking” offers a 3D representation of tracks and object movements as VRML files which may be opened with freely available plugins to web browsers.

2615 Co-localisation Studies in Cell Biology: Specificity and Limits Highlighted by JACOp
F. P. Cordélies,1 S. Bolté,2 Plateforme d’Imagerie Cellulaire et Tissulaire, Institut Curie - Centre de recherche/CNRS UMR 146, Orsay, France, 2Plateforme d’Imagerie et de Biologie Cellulaire, IFR 87-CNRS, Gif-sur-Yvette, France

In optical microscopy applied to cell biology, it is commonly admitted that co-localisation is achieved when two proteins are found at the same location, provided that they are seen on overlapping pixels, using the most appropriate sampling rate and magnification for image acquisition. However, two observers might not draw the same conclusions from this coincidental event. Cell biologist would conclude that two proteins co-localise while physicist would rather say that under current conditions, it can not be excluded that the two proteins are at the same location! These simplistic interpretations are already a first step of controversy. The situation is even more complicated when the localisation pattern of two proteins is mixed (partial co-localisation). The use of appropriate coefficients and/or representation methods is therefore crucial to estimate more accurately the co-localisation event. A plethora of methods exists and is used despite the absence of appropriate guidelines on how and when to use these methods. As a consequence, the most well known co-localisation indexes (such as correlation coefficients) are most frequently used beyond their domain of application and quite often resulting in mis-interpretation, over- or underestimation of the cellular reality. We provide an overview of the three levels of co-localisation evaluation: 1-visualisation, 2-quantification by considering the image as a collection of “intensities”, 3-quantification by considering the image as a collection of “objects”. For each level, we provide insights into the methods that might be employed, the coefficients that might be calculated, their limits and the conclusions that might be drawn. We furthermore introduce a new tool encompassing all reviewed methods in a single toolbox, named JACOp (Just Another Colocalisation Plugin, http://rsb.info.nih.gov/ij/plugins/track/jacop.html) as plug-in to the public domain ImageJ software (Rasband, W.S., 1997-2006).

2616 Rab6 Acts Upstream of ZW10 and COG in the Maintenance of Golgi Apparatus Structure in HeLa Cells
Y. Sun, A. Shestakova, V. Lupashin, B. Storrie; Physiology and Biophysics, University of Arkansas for Medical Sciences, Little Rock, AR

The maintenance of Golgi apparatus structure is a balance between proteins involved in anterograde and retrograde trafficking. We have taken an epistatic approach to order the role of Rab6 relative to ZW10 or COG (Conserved Oligomeric Golgi), all proteins implicated in Golgi trafficking and organization. Depletion of ZW10 resulted in inhibition of Golgi enzyme recycling to the endoplasmic reticulum (ER) during a 5 h ER-exit block. siRNA transfection to silence ZW10 or COG results in disruption of Golgi organization. With ZW10 silencing, we found that the Golgi apparatus was broken down into a perinuclear cluster of small fragments while with COG silencing scattered vesicles were observed in addition to larger Golgi fragments in agreement with previous results. In contrast, silencing of Rab6 using an siRNA directed against a sequence common to both Rab6a and Rab6b resulted in, if anything, a more continuous juxtanuclear Golgi apparatus as revealed by the distribution of Golgi glycosyltransferases. Co-depletion of Rab6 selectively inhibited the disruptive effects of both ZW10 and COG inactivation on Golgi organization. The effects of Rab6 loss-of-function were specific with no cross-talk observed between either Rab6 and endosomal Rab5 or Rab7 and the p115 Golgi-associated anterograde tether. ZW10 depletion induced disruption of Golgi structure was also inhibited when the dominant negative protein, GDP-Rab6a, was overexpressed. Overexpression of the C-terminal fragment of Binacudal D, a protein that bridges between Rab6 and dynamin, inhibited ZW10 depletion induced disruption of Golgi organization suggesting that the upstream effect of Rab6 is likely mediated by a dynin motor as effector. We conclude that Rab6 acts upstream of ZW10 and COG in the maintenance of Golgi apparatus organization. Supported by grants from the NSF (MCB-0549001 to B.S. and MCB-0234822 to V.L.).

2617 Role of Rab33b in the Cycling of Proteins through the Golgi Apparatus
T. Starr,1 Y. Sun, K. Forest-Williams,2 B. Storrie,3 Physiology and Biophysics, University of Arkansas for Medical Sciences, Little Rock, AR, 2Chemical Engineering, Virginia Tech, Blacksburg, VA

We tested the role of a medially located Golgi apparatus Rab GTPase Rab33b, in the cycling of proteins through the Golgi apparatus. We examined the transport of three proteins: GPP130 (a cis Golgi protein that cycles to endosomes), Shiga-like toxin B subunit (SLTB, the targeting subunit of Shiga toxin that moves from endosomes to the Golgi and then ER), and GaNAcT2-GFP (a stably expressed Golgi glycosyltransferase chimeric protein that cycles to the ER). In the case of GPP130, the protein was accumulated in endosomes by treatment of HeLa cells with monensin and then transported to the Golgi initiated by drug washout. SLTB fed to HeLa cells was also accumulated in endosomes by a 19.5°C temperature block and then entered the Golgi via temperature shift. Upon shift to permissive conditions, GPP130 resumed a steady-state-like cis Golgi distribution with a half-time of 30 min. By confocal microscopy, cis and trans Golgi markers could be resolved and the Rab6-positive trans-Golgi apparatus appeared to be an intermediate step in GPP130 re-equilibration to the cis Golgi apparatus. For GalNAcT2, RNAi silencing of Rab33b, as indicated by a pronounced reduction in protein levels by immunoblotting, appeared to inhibit GTP-Rab6a induced redistribution of GaNAcT2-GFP. Further experiments are in progress to test in a detailed manner if Rab33b and the medial Golgi apparatus act as intermediaries in Golgi protein cycling from the trans to cis Golgi. Initiated with support from NIH grant GM65233 and continued with support from NSF grant MCB 05.

2618 TRAPP-specific Subunits Are Required for the Specificity Switch of a Ypt/Rab Nucleotide Exchanger
N. Morozova,1 Y. Liang,2 A. A. Tokarev,2 S. H. Chen,3 V. Secora,2 S. Ernö; N. Seges1; 1Biological Sciences, University of Illinois, Chicago, IL, 2Cellular and Molecular Medicine, University of California at San Diego, San Diego, CA

Intracellular trafficking in eukaryotes is regulated by GTPase Rab and Rab GTAPases. These GTAPases are activated by guanine-nucleotide exchange factors (GEFs). When in the GTP-bound state, Ypt/Rabs interact with effectors that mediate individual steps of the protein trafficking pathways. An attractive possibility is that Ypt/Rabs and their accessory factors not only regulate the separate steps of protein transport pathways, but also coordinate these separate steps. Here, we propose a novel mechanism for such coordination in the Golgi. The modular multi-protein Golgi-associated complex, TRAPP, acts as a GEF for Ypt1 and Ypt31/32, the GTAPases that regulate entry into and exit from the yeast Golgi, respectively. TRAPP comes in two configurations: the seven-subunit TRAPP is required for ER-to-Golgi transport, whereas the ten-subunit TRAPP functions in late Golgi. The two essential TRAPP-specific subunits, Tns130 and Tns120, are conserved from yeast to man, and are candidates for several human disorders. We show that Tns120/130 determine the specificity of the dual-GEF complex; they are required for switching the GEF activity of TRAPP from a Ypt1-GEF to a Ypt31/32-GEF. Moreover, a tns120/130 mutation confers opposite effects on the intra-cellular localization of Ypt1 and Ypt31/32. We propose that TRAPP acts as a Ypt1 GEF in early Golgi, whereas in late Golgi the Tns120/130 sub-complex is required for switching this activity off, and turning the Ypt31/32 GEF activity on. Such a switchable dual-specificity GEF would ensure activation of Golgi Ypts at the correct cisterna, thereby coordinating entry into and exit from the Golgi apparatus.

2619 The GARP (Vps52-Vps3-Vps4) Complex Is Essential for Transport from Endosomes to the TGN in Mammalian Cells
F. P. Perez-Victoria, J. S. Bonifacino; Cell Biology and Metabolism Branch, NICHD, National Institutes of Health, Bethesda, MD

Tethering factors are required in every major transport step within the secretory, endocytic and lysosomal trafficking pathways. A multi-protein complex known as GARP associated retrograde protein (GARP; also known as VFT) has been shown to function as a tethering factor for transport from endosomes to the late Golgi in yeast. A similar complex consisting of at least three subunits, Vps52, Vps53 and Vps54, exists in mammalian cells. GARP is an effector of the Rab6 GTAPase, and co-immunoprecipitation studies have shown that it also interacts with the t-SNARE, syntaxin 10. We have studied the function of this complex by examining the trafficking phenotypes of cells depleted of GARP complex subunits using siRNA oligonucleotides. We find that depletion of GARP subunits from the TGN to the periphery GARP mRNA and GARP protein is reduced. In contrast to their transport to the TGN in control cells. The steady-state levels of mature cathepsin D are decreased by more than 10-fold in GARP-depleted cells, most likely due to the inability
of the CI-MPR to return to the TGN. Likely as a consequence, lysosomes are swollen and not fully functional, as assessed by a delay in the degradation of EGF-R. These observations indicate that the GARP complex is involved in retrograde transport from endosomes to the TGN.

2620

The TGN Accessory Protein p56 is Necessary for Vesicular Transport to the Endosomal-Lysosomal System

G. Mardones, P. Burgos, J. Bonifacino; NICHD, Cell Biology and Metabolism Branch, Bethesda, MD

The paradigm of TGN-endosome trafficking is the mannose 6-phosphate (M6P) receptor system. In the TGN two M6P receptors bind M6P-containing soluble acid hydrolases (such as cathepsin D), and transport them to endosomes. This process involves binding of the hydrolases to the receptor, packaging of the ligand-receptor complexes into vesicular carriers, transport to endosomes and recycling of the receptors back to the TGN. The formation of these vesicular carriers starts with the association of cytosolic adapter proteins that link the cytosolic tail of M6P receptors to clathrin coats. Clathrin adapters functioning at the TGN are the heterotetrameric AP-1, and the monomeric GGA proteins (GGAs-1-3), all of which have been implicated in M6P receptor trafficking. A number of accessory proteins interact with these adapters, but little is known about their functions. One of these accessory proteins is p56, which was identified through its ability to interact in vitro with the γ-adaptin subunit of AP-1 and the GGAs. To get insight on p56 function, we performed a combination of microscopic imaging and RNA interference (RNAi) experiments. Using fluorescence microscopy we observed that p56 is recruited to TGN membranes by GGAs overexpression, suggesting that p56 interacts with GGAs in vivo, an observation confirmed by Fluorescence Resonance Energy Transfer. RNAi of GGA-3, but not of γ-adaptin, resulted in reduced levels and decreased TGN association of p56. RNAi of p56 restricted the movement of GGA1-containing vesicles and the processing of cathepsin D. Our results indicate that p56 is necessary for normal vesicular transport from the TGN to the endosomal-lysosomal system.

2621

Comprehensive Protein Analysis of Naked2-associated Exocytic Vesicles by LC/MS-MS

Z. Cao, C. Li, R. Graves-DeAl, J. Franklin, J. Higginbotham, A. Ham, R. Coffey; Gastroentontology, Vanderbilt University, Nashville, TN, Pediatric Infectious Diseases, Vanderbilt University, Nashville, TN, The Proteomics Core of the Mass Spectrometry Research Center, Vanderbilt University, Nashville, TN

Polarized epithelial cells have developed specialized mechanisms for targeting transGolgi network (TGN)-derived vesicles to the apical or basolateral membrane. We have reported that Naked2, but not Naked1, interacts with Golgi-processed form of TGFβ and escorts TGFβ-containing exocytic vesicles from the TGN to the basolateral surface of polarized MDCK cells, where the vesicles dock and fuse in a Naked2 myristoylation-dependent manner. In myristoylation-deficient (G2A) Naked2-expressing MDCK cells, Naked2-associated TGFβ-containing vesicles are trapped in the cytoplasm and TGFβ is unable to reach the plasma membrane. To determine the protein compositions of these Naked2-associated vesicles, we performed a biochemical enrichment followed by flow cytometric purification. Using a 10-40% iodixanol gradient centrifugation, we first isolated G2A Naked2-EGFP-containing vesicle fractions de-enriched in plasma membrane markers as measured by western blotting for Naked2 and markers of subcellular compartments. This pool of vesicles was then subjected to dual flow sorting using GFP and DiD enrichment followed by flow cytometric purification. Using a 10-40% iodixanol gradient centrifugation, we first isolated G2A Naked2-EGFP-containing vesicle fractions de-enriched in plasma membrane markers as measured by western blotting for Naked2 and markers of subcellular compartments. This pool of vesicles was then subjected to dual flow sorting using GFP and DiD enrichment followed by flow cytometric purification.

2622

Regulation of Beta-1AR Delivery to the Cell Surface by Golgin-160

D. M. Zuckerman, D. Sagaram, C. E. Machamer; Cell Biology, Johns Hopkins University School of Medicine, Baltimore, MD

Golgins are a family of peripherally associated Golgi residents that have been implicated in vesicle tethering and maintenance of Golgi structure. Golgin-160 is a cis- and medial-Golgi resident involved in transducing certain apoptotic signals. Unlike several golgins, knockdown of golgin-160 by RNAi did not lead to an overall trafficking phenotype, nor a change in Golgi morphology. However, the trafficking of specific cargos, for example, beta-1-adrenergic receptor (beta-1AR), was perturbed. Beta-1AR is a seven transmembrane G-protein coupled receptor, and stimulation by epinephrine or norepinephrine leads to activation of adenylyl cyclase and increased cAMP production. We previously found that overexpression of golgin-160 lead to an increase of surface levels of beta-1AR, and knockdown of golgin-160 by RNAi lead to a decrease, though total beta-1AR levels remained consistent. We reason that since golgin-160 is not on the trans-Golgi, it likely is involved in a step prior to Golgi exit. While much research has focused on signaling and post-signaling events related to adrenergic receptors, very little is known about the mechanisms of surface delivery, including post-translational modifications. To characterize the contribution of golgin-160 to beta-1AR biogenesis, we are analyzing the trafficking of GFP-tagged beta-1AR in live cells in the presence or absence of golgin-160. To investigate the interaction of beta-1AR and golgin-160, we are performing in vitro binding experiments. A small region of the soluble N-terminal head domain of golgin-160 binds to beta-1AR, and mapping the region of beta-1AR recognized by golgin-160 is in progress. We are also examining post-translational modifications of beta-1AR, including dimerization and palmitoylation, addressing where in the secretory pathway these modifications take place, and the role of golgin-160. These studies will expand our understanding of golgins beyond vesicle tethers and Golgi structural proteins, and give insight into the biogenesis of beta-1AR, including the modifications early in the secretory pathway.

2623

Study of the Suborganellar Organization of the Golgi Apparatus by Using a Novel In Vivo Biotinylation Approach for Purification Coupled with the Selection of Recombinant Antibodies

O. Vielmeoyer, S. Motel, B. Goud, F. Perez; Cnr/c im144, Institut Curie Research Section, Paris, France

In mammalian cells the Golgi apparatus maintains a distinct ribbon-like structure throughout interphase despite a large bidirectional flux of membranes and cargo. To maintain this distinct architecture it contains several structural and regulatory proteins. We are particularly interested in golgins and Golgi associated Rab proteins. The former are stably associated with membranes and function as structural elements/vesicle tethers while the latter bind to target membranes in a highly regulated manner via nucleotide exchange. Traditionally, rat livers have been used for purification of Golgi stacks and only immunopurification would lead to separation of suborganellar fractions. Animal tissues, however, are not easily amenable to drug/siRNA treatment prior to purification. In order to better understand how golgins and Golgi associated Rab-proteins organize Golgi subdomains, we have developed a new approach, which allows for rapid purification of suborganellar fractions from cultured cells. Golgins of interest, fused with a biotinylation tag, are overexpressed and efficiently and specifically biotinylated inside mammalian cells. Stable clones expressing such in vivo biotinylated proteins are used as starting material for rapid streptavidin-based affinity purification. After washing on a magnet, such enriched and purified fractions are specifically eluted using an integral TEV recognition site. In this fashion GI130, Rab6A, Rab8A and Rab1A positive suborganellar membrane fractions are characterized by comparative mass spectrometry analysis and Western blotting. In addition, novel Rab effectors are being sought after addition of detergent. Lastly, purified protein complexes are used as starting material for in vitro extension of highly specific recombinant antibodies using the phage display technology. In summary, purification of subcellular fractions after in vivo biotinylation of target proteins is a powerful novel approach to study the suborganellar organization of the Golgi apparatus. This approach can easily be adopted for any organelle of interest and can be coupled with gene silencing techniques.

2624

Novel Regulation of Golgi Complex Structure and Function by a Transmembrane Lysophosphatidic Acid Acyltransferase

J. A. Schmidt, W. J. Brown; Molecular Biology and Genetics, Cornell University, Ithaca, NY

Phospholipase (PLaG) and lysophospholipid acyltransferase (LPAT) activities appear to have antagonistic roles in the formation of Golgi membrane tubules and retrograde trafficking to the Endoplasmic Reticulum (ER) Golgi terminal. A Golgi tube formation is promoted by PLaG activity and inhibited by LPAT activity. These enzymes may alter the shape of membrane bilayers to regulate the formation of trafficking intermediates. The human genome encodes six transmembrane (TM) PLaGs, however, the function of only one is known. Here we report the identification of a specific TM LPAT (LPATγ) that regulates Golgi membrane tubule formation. When transfected into HeLa cells, LPATγ-GFP was localized primarily to the Golgi complex and also to ER membranes. Interestingly, over-expression of LPATγ significantly inhibited the rapid formation of Golgi membrane tubules that are induced by brefeldin A (BFA). Additionally, retrograde trafficking of Golgi membranes to the ER in BFA-treated cells was significantly slower. This phenotype was also observed when tubules were induced by CI-976, an LPAT inhibitor. LPATγ with mutations in the catalytic region did not show this phenotype. Cells with knocked-down expression of LPATγ that are hyper-sensitive to BFA. We also show that Golgi membranes from LPATγ over-expressing cells have elevated lysophosphatidic acid acyltransferase activity. LPATγ may control Golgi membrane dynamics and trafficking either by aiding in the fission of Golgi-derived vesicles that consume membrane tubules, or by re-acylating tube-forming lysophospholipids that were generated by PLaGs. These data are the first to show a direct role for a TM LPAT in regulating Golgi membrane trafficking.
Identification of Effector Proteins That Mediate Signaling between Golgi Vesicles and the Cytoskeleton

W. Xu, M. Starmer; Department of Physiology & Biophysics, University of Iowa, Iowa City, IA

Efficient intracellular protein trafficking requires regulation of cytoskeleton-mediated motility. For example, the Golgi-localized GTP-binding protein Cdc42 coordinates Golgi vesicle assembly with the recruitment of the motor protein dynein. Cdc42 in a complex with the COPI coat protein, coatimer, regulates both actin polymerization and dynein recruitment. Cdc42 regulates actin polymerization through the WASP and the Arp2/3 complex. However, the effectors that mediate the effects of Cdc42 on microtubule motors at the Golgi complex remain undefined. Here we present evidence that the actin binding protein, mAbs, is a candidate effector for Cdc42 at the Golgi complex. Like Cdc42, mAb binding is regulated by the GTP-binding protein ARF1 and is sensitive to the presence of the putative cargo receptor p23. Interestingly these properties of mAb are conferred by the actin-binding domains. Also, we show that ARF1-dependent recruitment of dynein can be reconstituted on liposomal membranes. As we found previously with Golgi membranes, the actin-plus-end-binding toxin cytochalasin D causes an increase in ARF1-dependent dynein recruitment to liposomes. This suggests that a specific cytochalasin D-dependent mAb-bound actin pool may regulate dynein. This reconstitution system has been exploited to identify and characterize effectors that function during dynein recruitment. Finally, we provide evidence that phosphorylation is involved in regulating these effectors. Together, our data indicate that the Cdc42 complex may act through a cascade of proteins to ensure that dynein recruitment occurs with correct spatial and temporal regulation.

The Conserved Oligomeric Golgi (COG) Complex Regulates Formation of the Intra-Golgi SNARE Complex

A. Sheshakova, E. Suvorova, O. Pavlyuk, G. Khaidakova, V. Lupashin; Physiology and Biophysics, UAMS, Little Rock, AR

Vesicle tethering factors mediate the initial loose tethering of transport vesicles to their target membranes. This loose interaction is followed by the more stable docking and consequent fusion of membranes involving the SNARE molecules. We used both yeasts and mammalian cells to demonstrate that the formation of the intra-Golgi SNAREs complex is regulated by the putative vesicle tethering COG complex. In yeast, the COG complex was specifically co-immunoprecipitated (co-IP) with intra-Golgi SNAREs Sed5 (yeast homolog Syntaxin5), Gos1 and Yk6. The highest recovery of the t-SNARE Sed5 was observed with anti-Cog4 IP. Purified COG complex in vitro preferentially interacted with the pre-formed intra-Golgi SNARE complex and the SNARE domain of Sed5. In mammalian cells the COG complex was co-IP with the Golgi SNAREs Syntaxin5 and Gs28. Yeast two-hybrid assay identified the Cos4 and Cog6 as the potential direct partners of the Syntaxin 5. Immunofluorescence microscopy showed that cells depleted of the Cog7 or Cog3p accumulated uncompled form of Syntaxin5. Furthermore, in Cog3 depleted cells intra-Golg SNAREs Gs28 and Gs15 were re-localized into the non-tethered vesicles. Native IP recovered decreased amount of the intra-Golgi SNARE complexes from the Cos3 siRNA treated cells. In vivo formation of the intra-Golgi SNARE complex was determined by RET in cells co-expressing GFP-GS15 and DoRed-Syntaxin5. We concluded that the COG complex acted to promote formation and/or stabilization of the intra-Golgi SNARE complex. Supported by grants from the NSF (MCB-0234822) and Mizutani Foundation for Glycoscience.

Dysfunction of Golgi Tethers, SNAREs and SNAPs in Monocrotaline-induced Pulmonary Hypertension

S. Mukhopadhyay; 1P. B. Sehgal; 1Cell Biology and Anatomy, New York Medical College, Valhalla, NY, 2Cell Biology and Anatomy and Medicine, New York Medical College, Valhalla, NY

Monocrotaline (MCT) is widely used to produce pulmonary hypertension in experimental animals. At the pulmonary arterial endothelial cell (PAEC) level, MCT produces loss of caveolin-1 (cav-1) in plasma membrane rafts and consequent hyperactivation of promitogenic STAT3 and ERK1/2 signaling. We have investigated the initiating mechanism involved. PAEC in culture showed trapping of cav-1 in Golgi membranes as early as 6 hr after exposure to MCT pyrrole (MCTP). Phenotypic megaloctysis and a reduction in interorganelle trafficking through the Golgi was analyzed in a gene transfer-based assay of horseradish peroxidase secretion was evident within 12 hr. Cell function and immunofluorescence techniques revealed the marked trapping of cav-1, eNOS, bone morphogenetic factor type 2 receptor (BMPR2), and of Golgi tethers, SNAREs and SNAPs which regulate vesicular trafficking (GM130, p115, giantin, golgin 84, clathrin heavy chain; syntagmin-4, -6, -Vit-1a, Vit1b, Gs15, Gs28, Gs28; SNAP23 and α-β-SNAP) in the enlarged/circumnuclear Golgi organelle in megalocytic PAEC and A549 cells (a “Golgi blockade”). Live-cell imaging of caveolar and intracellular NO production was carried out using the membrane-permeant reporter DAF2-DA. While control PAEC showed both caveolar and cell-centric NO production, MCTP-treated endothelial cells showed loss of caveolar but increased cell centric NO consistent with the trapping of eNOS in perinuclear vesicular elements. Immunofluorescence studies of lung tissue from MCT-treated rats confirmed enlargement of perinuclear Golgi elements in target lung cells as early as 4 days after MCT, thus preceding the development of symptomatic PH. These data provide the novel insight that pulmonary hypertension represents a disease state involving dysfunction of Golgi tethers, SNAREs and SNAPs and, thus, reduced trafficking of diverse vaso-relevant proteins (such as cav-1, eNOS, BMPR2, Tie-2, PEMAC-1) to the vascular cell surface.

Role of a Golgin-160 Fragment in Transducing Golgi Stress Signals

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

Golgin-160 is a coiled-coil peripheral Golgi membrane protein that is cleaved early during apoptosis by caspases. Previous studies from our lab have shown that a potential cleavage product of golgin-160 by caspase-2/caspase-3 (60-311) is targeted to the nucleus when exogenously expressed, potentially suggesting a role for golgin-160 in a Golgi stress sensing pathway. We hypothesize that the nuclear translocated golgin-160(60-311) fragment directly or indirectly modulates gene expression during stress repair. To this end, we performed yeast-2-hybrid assay with HeLa cDNA library using golgin-160(60-311) as bait and identified a few candidate proteins whose functions have been implicated in cell cycle and gene regulation. Confirmation of interaction of these candidates with golgin-160(60-311), and identification of their possible role(s) in Golgi stress sensing are in progress. Our preliminary results taken together with other data from our lab suggest that the golgin-160(60-311) fragment is translocated to the nucleus in response to Golgi stress where it regulates gene expression indirectly via other nuclear proteins and transcription factors.

GCP60 Preferentially Interacts with a Caspase-generated Golgin-160 Fragment

J. L. Shioda, C. Machamer; Cell Biology, Johns Hopkins University School of Medicine, Baltimore, MD

Golgin-160, ubiquitously expressed in vertebrates, localizes to the cytoplasmic face of the Golgi complex. Golgin-160 has a large coiled-coil C-terminal domain and a non-coiled-coil N-terminal (head) domain. The head domain contains important motifs, including a nuclear localization signal, a Golgi targeting domain, and three aspartates that are recognized by caspases during apoptosis. Some of the caspase cleavage products accumulate in the nucleus when overexpressed. Expression of a non-cleavable form of golgin-160 impairs apoptosis induced by some pro-apoptotic stimuli, thus cleavage of golgin-160 appears to play a role in apoptotic signaling. To screen for interactors of the golgin-160 head, we used a yeast two-hybrid assay, and hypothesize that the nuclear translocated golgin-160(60-311) fragment directly or indirectly modulates gene expression during stress repair. To this end, we performed yeast-2-hybrid assay with HeLa cDNA library using golgin-160(60-311) as bait and identified a few candidate proteins whose functions have been implicated in cell cycle and gene regulation. Confirmation of interaction of these candidates with golgin-160(60-311), and identification of their possible role(s) in Golgi stress sensing are in progress. Our preliminary results taken together with other data from our lab suggest that the golgin-160(60-311) fragment is translocated to the nucleus in response to Golgi stress where it regulates gene expression indirectly via other nuclear proteins and transcription factors.

Disrupted Golgi Complex Reorganization during Myogenesis by GSK3-β Inhibitors Leaves VSV-G Trafficking Intact

K. J. Zaal, E. R. Reid, A. Mehta, E. Ralston; Light Imaging Section, NIH, NIAMS, Bethesda, MD

During muscle differentiation, the classic compact juxtanuclear Golgi complex is dispersed into numerous smaller stacks found around the myotube nuclei and throughout the cytoplasm. GSK3-β is central to signaling pathways involved in microtubule stabilization, which is assumed to play a role in Golgi complex reorganization. We have found that the GSK3-β inhibitors lithium chloride and organometallic compound 1-OH disrupt Golgi complex reorganization during differentiation. In treated C2C12 mouse muscle cells, unusual morphologies were observed,
Pulse-chase Experiments with Photoactivatable Proteins Reveal Stress-dependent Turnover Kinetics of Autophagosomes
D. W. Hailey, J. Lippincott-Schwartz; NICHD, National Institutes of Health, Bethesda, MD
Macroautophagy is an inducible process by which proteins and whole organelles are sequestered in vesicles and delivered to lysosomes. Autophagosomes can accumulate in cells via two mechanisms: by increased rates of formation; or by decreased rates of turnover through lysosomal fusion. To discriminate between these mechanisms we used photoactivatable proteins (PAPFs) to monitor autophagosome lifetime. We then asked whether particular stresses result in autophagosomes with distinct lifetime characteristics. LC3 is a marker for maturing autophagosomes. The protein is covalently bound to phosphatidylethanolamine in autophagosomal membranes and persists on those membranes through their fusion with lysosomes. We constructed stable cell lines that express PAPF-LC3 and characterized cellular stresses that accumulate autophagosomes in these lines. We then analyzed the lifetimes of the induced structures using targeted laser illumination to activate PAPF-LC3 and bleach PAPF-LC3 proteins not incorporated in membranes. "Highlighted" autophagosomes were then monitored with live-cell confocal microscopy. Amino acid deprivation, proteasomal inhibition, disruption of ER calcium levels, and lysosomal inhibition all dramatically increased the number of observable PAPF-LC3-labeled structures. Whereas lysosomal inhibition results in an accumulation of LC3-labeled structures that do not turn over, autophagosomes formed in response to starvation are rapidly degraded. In contrast, structure labeled under conditions of amino acid deprivation and calcium disruption have dramatically increased lifetimes. Our data suggest a model in which the autophagic pathway serves different roles under different stress conditions: under conditions of amino acid deprivation, autophagosomes rapidly turn over to recover amino acids and energy; under conditions that induce sudden accumulations of misfolded proteins (proteasomal inhibition or calcium disruption) the pathway serves to sequester deleterious proteins from the functional cellular environment.
Identification and Analysis of a Novel Atg Protein, Atg29
T. Kawamata, Y. Kamada, Y. Ohsumi; Department of Cell Biology, National Institute for Basic Biology, Okazaki, Japan

Autophagy is the primary intracellular catabolic process whereby cytoplasmic components are sequestered for bulk degradation in the vacuole/lysosome. Nutrient starvation induces a high level of autophagy to provide materials required for survival. In the yeast S. cerevisiae, 17 Atg (Autophagy-related) proteins are essential for autophagosome generation. Of these, Atg1 kinase plays a pivotal role in regulation of autophagy, but the substrate of Atg1 remains to be identified. We recently identified Atg29 as an additional protein required for autophagy. Atg29 is phosphorylated under starvation conditions and this phosphorylation does not occur in atg1-delta cells. In vitro kinase assays demonstrate that Atg1 directly phosphorylates Atg29, indicating that Atg29 is a physiological substrate for Atg1 kinase. In atg29-delta cells, induction of autophagy was delayed, and the autophagic activity was severely impaired. We also found that in atg29-delta cells, the Cvt (Cytoplasm to vacuole transport) pathway, which shares mechanistic components with autophagy, proceeds normally. Thus, Atg29 is specifically required for autophagy. Atg29 localizes to the PAS, similar to most of the Atg proteins involved in the Cvt and autophagy pathways. We hypothesize that Atg29 might function as a molecular trigger situated downstream of Atg1 kinase to initiate switching from the Cvt pathway to autophagy. We will also discuss about interactions of Atg29 with known Atg proteins and its role on autophagosome formation.

Dynamic Organization of LAMP-2A, the Receptor for Chaperone Mediated Autophagy at the Lysosomal Membrane
U. Bandopadhayay, A. Cuervo, Anatomy & Structural Biology, Albert Einstein College of Medicine, Bronx, NY

Chaperone-mediated autophagy (CMA) is responsible for the degradation of selective cytosolic proteins inside the lysosomes. This type of autophagy is maximally activated in conditions such as nutrient deprivation or mild-oxidative stress. CMA substrates bind to a constitutive cytosolic chaperone (hsc70), which directs them to the lysosomal membrane. There the chaperone/substrate complex binds to the lysosomal membrane protein type 2-A, LAMP-2A, a receptor for the substrates of this pathway. Substrates are then translocated through the lysosomal membrane into the lumen, assisted by a resident lysosomal chaperone. Binding of substrates to the receptor is limiting for this pathway, but the relationship of the receptor with the translocation complex remains unknown. In this work, we characterize the organization of LAMP-2A in different molecular weight complexes at the lysosomal membrane and the changes in this organization with changes in CMA activity. Using isolated rat liver lysosomes in conjunction with techniques such as native electrophoresis, gel filtration chromatography and sucrose density gradient centrifugation, we have found that LAMP-2A is present in 6-7 different molecular weight complexes at the lysosomal membrane. Conditions that upregulate CMA (starvation, oxidation) stabilize LAMP-2A in a particular high molecular weight complex. Addition of hsc70 favors the presence of monomeric forms of LAMP-2A, pointing toward an active role of this chaperone in the disassembly of the LAMP-2A complexes. Assembly/disassembly of the LAMP-2A complexes is also modulated by GTP. We found that GTP exerts opposite effects on binding and uptake of substrates suggesting the possible involvement of a GTP-binding protein in this process. Our findings support that the organization of LAMP-2A in different size complexes at the lysosomal membrane is a dynamic process and that the step-wise assembly/disassembly of these complexes determines CMA activity.

New Insights on the Mechanism and Function of Sortilin
M. Camel,1 J. Hassan,2 A. Balbiss,3 K. Kommu,4 S. Kiernan,1 C. R. Morales1; 1Anatomy and Cell Biology, McGill University, Montreal, PQ, Canada, 2Polypeptide Hormone Laboratory, McGill University, Montreal, PQ, Canada

Background: Delivery of soluble lysosomal proteins to the lysosomes is dependent primarily on the mannose 6-phosphate receptor (M6P-Rc). However, in 1-cell disease (ICD) fibroblasts, where the M6P-Rc pathway is non-functional, some soluble lysosomal proteins continue to traffic to the lysosomes. Hypothesis: In this investigation, we have tested the hypothesis that cathepsins D and H, two soluble proteases that exhibit M6P-Rc independent trafficking, are targeted to the lysosomes by sortilin. In order to identify differences in trafficking via sortilin and the M6P-Rc, we have investigated that sortilin and its ligands are associated with sphingolipid detergent-resistant membranes (DRMs). Results: Using a dominant-negative sortilin construct and small interfering RNA (siRNA) we have demonstrated that while cathepsin D transport is dependent upon sortilin and the M6P-Rc, cathepsin H requires only sortilin for its transport to the lysosomes. Analysis of DRMs fractions has shown the presence of sortilin, sortilin, cathepsins H and D. On the other hand, the M6P-Rc and its ligand cathepsin B were found to be excluded from DRM fractions. Conclusions: These results suggest that sortilin functions as an alternative sorting receptor to the M6P-Rc for some soluble lysosomal proteins and that ligand-sortilin complexes exit the Golgi apparatus within vesicles containing lipid rafts. Supported by CIHR.

AP-1 and Retromer Play Opposite Roles in the Trafficking of Sortilin
M. Camel,1 S. Lefrançois,1 J. Zeng,2 C. R. Morales2; 1Anatomy and Cell Biology, McGill University, Montreal, PQ, Canada, 2Cell Biology, NIH, Bethesda, MD

Background: Sortilin has been implicated in the sorting and lysosomal targeting of certain soluble hydrolases and sphingolipid activator proteins. The cytoplasmic tail of sortilin contains an acidic cluster-dileucine motif structurally similar to that of the mannose 6-phosphate receptor. Recent studies have indicated that the cytoplasmic tail of sortilin interacts with both AP-1 and retromer complexes to mediate sorting of ligands to the lysosomes. In this investigation, we have tested the hypothesis that retromer is responsible for the retrograde transport of sortilin and also demonstrate a role for AP-1 in the endosomal trafficking of this novel receptor. Depletion of retromer with RNA-interference results in sortilin's accumulation and retention in the lysosomal compartment as well as in the secretion of the sortilin cargo protein, prosaposin, into the extracellular space. These results indicate that GAK plays a critical role in lysosomal enzyme sorting in conjunction with AP-1.

Determinants of the Interaction of GAK/Auxillin-2 with the AP-1 Complex
S. Kametaka,1 S. Koriyama,2 P. Burgess,3 L. Greene,4 E. Eisenberg,4 J. Bonifacino2; 1NICHD, NIH, Bethesda, MD, 2New England Inflammation and Tissue Protection Institute, Boston, MA, 3NIDDK, NIH, Bethesda, MD

Proper sorting of proteins to their corresponding destinations within cells is prerequisite to maintain the homeostasis of living organisms. Many proteins that function at endosomes and lysosomes are sorted out from the secretory pathway at the trans-Golgi network (TGN) by incorporation into clathrin-coated vesicles (CCV). Adapter protein complex-1 (AP-1) is a heterotrimeric complex that recruits clathrin and other factors from cytoplasm to the TGN membrane to facilitate the CCV formation and cargo selection. A large subunit of AP-1, γ-adaptin, possesses a C-terminal globular domain referred to as “ear” that functions as a platform for the recruitment of a variety of accessory proteins. Here, we report that GAK, a known CCV-associated kinase, directly interacts with ear domain of AP-1-γ-adaptin through a canonical tetrapartite motif, G(P/D/E)(Φ/L/M), in the clathrin-binding domain of GAK. Mutation of this motif or RNA-mediated depletion of AP-1 causes decreased association of GAK with the TGN. Depletion of GAK by RNAi impairs the proteolytic maturation of the lysosomal protease, cathepsin D, in cells. Re-expression of RNAi-resistant, wild-type GAK suppresses the defect in cathepsin D maturation caused by GAK depletion, whereas the AP-1-binding mutant cannot reverse the defect. These results indicate that GAK plays a critical role in lysosomal enzyme sorting in conjunction with AP-1.

Sortilin and Mannose 6-Phosphate Receptor Mediate the Trafficking of Acid Sphingomyelinase to the Lysosomes
X. Ni, C. R. Morales; McGill University, Montreal, PQ, Canada

Background: Acid sphingomyelinase (ASM), a member of the saposin-like protein (SAPLIP) family, is a lysosomal enzyme that hydrolyze sphingomyelin to ceramide. Deficiency of ASM causes a variant form of Niemann-Pick disease. The mechanism of targeting of ASM to the lysosomes is poorly known. Previous studies suggest that ASM could use in part the mannose 6-phosphate receptor (M6P-Rc). Sortilin, a type I transmembrane glycoprotein that belongs to a novel family of receptor proteins, presents structural features of receptors involved in lysosomal targeting. Results: We examined the hypothesis that sortilin may be implicated in the trafficking of ASM to the lysosomes. Hypothesis: Using a dominant-negative sortilin construct lacking the cytoplasmic tail, essential to recruit adaptor proteins and clathrin, we have demonstrated that sortilin is also involved in the lysosomal targeting of ASM.
revealed that truncated sortilin partially inhibited the lysosomal trafficking of ASM in COS-7 cells and abolished the lysosomal targeting of ASM in I-cells. Pulse-chase experiments corroborated that sortilin is involved in normal sorting of newly synthesized ASM. Furthermore, over-expression of truncated sortilin accelerated and enhanced the secretion of ASM from COS-7 cells and I-cells. Co-immunoprecipitation assays confirmed the interaction between sortilin and ASM. Conclusion: ASM uses sortilin as an alternative receptor to M6P-Re to be targeted to the lysosomes. Supported by CHRI.

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Snf7-mediated Protein Interactions at Multivesicular Bodies

M. Wehmer, G. Odorozzi, MCD Biology, University of Colorado, Boulder, Boulder, CO

Multivesicular bodies (MVBs) are late endosomal organelles that function in sorting integral membrane protein cargos to the vacuole to be degraded. The sorting of monoubiquitinated cargo proteins through MVBs is a tightly regulated and highly conserved process. Snf7, a component of the Endosomal Sorting Complex Required for Transport (ESCRT) III in Saccharomyces cerevisiae, is required for the correct sorting of cargo proteins through MVBs. However, the mechanism by which Snf7 regulates sorting is unclear. Using a yeast two-hybrid system, we have discovered that the predicted third coiled coil of Snf7 is required for recognition by Bro1 to the MVB. Disruption of this region abolishes recognition of Bro1 and causes defects in cargo sorting. Additionally, we have mapped the region of interaction between Vps20 and Snf7. These observations provide a deeper understanding of protein recognition and complex assembly that mediate cargo sorting at the multivesicular body.

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Reading and Writing the Ubiquitin Code for Gap1p Trafficking

A. L. Risinger, C. A. Kaiser; Biology, Massachusetts Institute of Technology (MIT), Cambridge, MA

The expression, localization, and activity of the high capacity general amino acid permease in S. cerevisiae, Gap1p, is regulated by amino acids such that it transports available amino acids from the surrounding medium into the cell only when internal amino acid levels are low. This dynamic regulation of Gap1p allows the cell to rapidly upregulate amino acid import when internal amino acid levels become limiting while avoiding excess amino acid import that can be lethal to cells expressing a non-sortable form of Gap1p. To completely redistribute Gap1p from the plasma membrane to intracellular compartments, Gap1p present at the plasma membrane is endocytosed while newly synthesized Gap1p is redirected to the vacuole. Both sorting steps require ubiquitination as the non-ubiquitinated Gap1pK9R,K16R mutant is found exclusively at the plasma membrane even in the presence of high levels of amino acids. It has been previously suggested that identical cis and trans acting factors are involved in ubiquitin-mediated delivery of both newly synthesized and endocytosed Gap1p to the vacuole. Intriguingly, upon further dissection, we clearly defined two unique ubiquitin-mediated pathways by which Gap1p can be delivered to the vacuole: 1) a direct path from the trans-Golgi to the endosome via Bul1/2p-dependent internal polyubiquitination of Gap1p on either lysine 9 or 16; 2) endocytosis via Bul1/2p-dependent ubiquitination of Gap1p on either lysine 9 or 16; and 3) endocytosis via Bul1/2p-independent monoubiquitination of Gap1p on lysine 16. Our finding that distinct ubiquitin-mediated sorting steps employ unique trans-acting factors, ubiquitination sites on Gap1p, and types of ubiquitination demonstrates a previously unrecognized level of specificity in ubiquitin-mediated protein sorting. Table of Contents

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The Leading Role of Microtubules in Endothelial Barrier Dysfunction - Disassembly of Peripheral Microtubules Leaves Behind the Cytoskeletal Reorganization

I. B. Aliev,1,2 K. M. Smarova,3 A. A. Birukova,1 A. D. Verrit;1 Department of Medicine, University of Chicago, Chicago, IL; 3A.N. Belozersky Institute, Moscow University, Moscow, Russian Federation

Pulmonary endothelial cell (EC) barrier dysfunction is often associated with dramatic cytoskeletal reorganization, activation of actomyosin contraction and finally gap formation. The role of microtubules (MTs) in EC barrier regulation is not fully understood, but clinical observations suggest that intravenous administration of anti-cancer drugs and MT inhibitors can lead to the sudden development of pulmonary edema in breast cancer patients. Our observations have been forced us to examine the role of MT reorganization in EC barrier regulation. Substantial studies, including our own, have previously demonstrated that endogenous agent, thrombin, induced rapid and reversible increase of EC barrier permeability. In quiescent EC MT density is highest in the centrosome region and decreases exponentially from the cell center to the cell margin, while actin network consists of thin bundles located mainly at the cell periphery. Thrombin (25 nM)-induced human EC barrier dysfunction associated with rapid (within 5 min) decreasing of peripheral MT density, which preceded formation of actin-stress-fibers and further MT reorganization in the internal cytoplasm. Both effects were reversible (within 1 h cytoskeleton returned to the normal state). From the other side, MTs disassembly by nocodazole (1 μM) itself induced rapid increases in EC permeability, actin cytoskeletal remodeling, activation of focal adhesions and formation of paracellular gaps, indicating EC barrier dysfunction. Collectively, our data demonstrate the leading role of MT disassembly in pulmonary EC barrier compromise - peripheral MT disruption points the way for the following cytoskeleton reorganization. Supported by NIH grants HL 067307, HL 58064 and RBFR grant 06-04-49233.

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Characterization of Aortic Endothelial Cells Cultured on Amniotic Membrane

S. H. Tsai,1 Z. W. Zhou,1 Y. W. Liu,2 W. S. Ton,1 C. Y. Huang,1 J. K. Chen,2 V. C. Yang 1; 1Department of Life Science, Tunghai University, Taichung, Taiwan, 2Graduated Institute of Basic Medical Sciences, Chang Gung University, Taoyuan, Taiwan

Arterial occlusive disease remains a leading cause of death in western countries and in Taiwan. The limited supply of suitable vascular graft has led to the development of tissue engineered arteriovenous (TVE) grafts. These grafts rely on the culture of autologous vascular endothelial cells (ECs) for viability, and a major problem has been the loss of EC barrier function. Endothelial permeability increases with age and hypertension, and the use of tissue culture medium may not accurately reproduce in vivo conditions. We investigated the expression, localization, and activity of the high capacity general amino acid permease in S. cerevisiae, Gap1p, regulated by amino acids such that it transports available amino acids from the surrounding medium into the cell only when internal amino acid levels are low. This dynamic regulation of Gap1p allows the cell to rapidly upregulate amino acid import when internal amino acid levels become limiting while avoiding excess amino acid import that can be lethal to cells expressing a non-sortable form of Gap1p. To completely redistribute Gap1p from the plasma membrane to intracellular compartments, Gap1p present at the plasma membrane is endocytosed while newly synthesized Gap1p is redirected to the vacuole. Both sorting steps require ubiquitination as the non-ubiquitinated Gap1pK9R,K16R mutant is found exclusively at the plasma membrane even in the presence of high levels of amino acids. It has been previously suggested that identical cis and trans acting factors are involved in ubiquitin-mediated delivery of both newly synthesized and endocytosed Gap1p to the vacuole. Intriguingly, upon further dissection, we clearly defined two unique ubiquitin-mediated pathways by which Gap1p can be delivered to the vacuole: 1) a direct path from the trans-Golgi to the endosome via Bul1/2p-dependent internal polyubiquitination of Gap1p on either lysine 9 or 16; 2) endocytosis via Bul1/2p-dependent ubiquitination of Gap1p on either lysine 9 or 16; and 3) endocytosis via Bul1/2p-independent monoubiquitination of Gap1p on lysine 16. Our finding that distinct ubiquitin-mediated sorting steps employ unique trans-acting factors, ubiquitination sites on Gap1p, and types of ubiquitination demonstrates a previously unrecognized level of specificity in ubiquitin-mediated protein sorting. Table of Contents

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Regulation of Actin Dynamics by ANP-modulated Cyclic-GMP Signal Transduction in Endothelial Cells

A. J. Lin,1 H. Chen,2 T. Michel,2 D. E. Golian;1 Biological Chemistry & Molecular Pharmacology, Harvard Medical School, Boston, MA; 2Cardiovascular Division, Brigham and Women's Hospital, Boston, MA

Cyclic-GMP (cGMP) signaling cascades initiated by nitric oxide (NO) and atrial natriuretic peptide (ANP) play an important role in the maintenance of cardiovascular homeostasis. The molecular signaling mechanisms downstream of cGMP are not well understood, however. We here use siRNA technology to specifically knock down a series of signaling proteins in bovine aortic endothelial cells (BAEC), and combine biochemical analyses with laser confocal imaging techniques to investigate cGMP-regulated cytoskeletal dynamics. ANP activation of particulate guanylate cyclase (GC-A) leads to a substantial, dose-dependent, rapid, and sustained increase in intracellular cGMP, whereas NO activation of soluble guanylate cyclase (sGC) yields only a weak and transient response. Furthermore, ANP greatly enhances the phosphorylation at Ser239 of the vasoconstrictor-stimulated phosphorylase (VAP), a protein of the Ena/VASP family that is a major substrate of cGMP-dependent protein kinase (PKG) and that intimately influences actin dynamics. As observed by confocal imaging, enhanced Ser239 phosphorylation is accompanied by an increase in the formation of cellular actin stress fibers. Transfection of BAEC with duplex siRNAs constructs that specifically target GC-A, VASP, or PKG leads to an 80-95% decrease in target protein abundance compared to cells transfected with control siRNA. Knock down of PKG or GC-A completely abolishes VASP Ser239 phosphorylation and ANP-induced enhancement of stress fiber formation. siRNA targeting of PKG also blocks endothelial tube formation on a Matrigel substrate, while control siRNA exhibits no effect. Together with a recent in vivo study reporting that ablation of endothelial GC-A abrogates the ANP-induced endothelial permeability increase in mice, our findings suggest that ANP-mediated cGMP signal transduction regulates actin dynamics through VASP Ser239 phosphorylation, which in turn plays an important role in the regulation of endothelial permeability and angiogenesis.
Amelioration of Acute EA of a Selective Inhibitor of Vascular Endothelial Growth Factor Receptor 2 (VEGFR2)
M. E. Welsh, M. Feng, S. J. Karfik; Physiology and Pharmacology, The University of Western Ontario, London, ON, Canada
Experimental allergic encephalomyelitis (EAE) is a T cell-mediated inflammatory disorder of the central nervous system, frequently used as an animal model for multiple sclerosis (MS). An increased expression of vascular endothelial growth factor (VEGF) by astrocytes and vascular endothelial cells is associated with demyelinated lesions in both EAE and MS. In order to investigate the contribution of VEGF to lesion development in acute EA, we treated immunized mice with SU5416 (Semaxinin), a potent and selective inhibitor of VEGF receptor 2 (VEGFR2). EAE was induced in C57 BL/6J mice by immunization with myelin oligodendrocyte glycoprotein (MOG35-55) and animals were assessed daily by a blinded observer for clinical manifestations of disease. Animals received seven daily injections of SU5416 (50 mg/kg) or vehicle beginning on the day of disease onset. Brain and spinal cord were collected on the day of sacrifice for histology/immunohistochemistry and quantitative microscopy was used to determine demyelination, cell infiltration and VEGF expression. SU5416 treatment produced a significant clinical improvement versus control mice (p<0.001), with less demyelination (37%) and cellular infiltration (23%) in the spinal cord (p<0.05). The amount of cellular infiltration was very strongly correlated with demyelinated lesion size in both control (r=0.943) and SU5416-treated (r=0.913) animals. Total VEGF immunoreactivity was the same in both groups. As well, VEGF expression correlated with lesion size for both control (r=0.723) and treated (r=0.681) animals. VEGFR2 blockade partially decreased the clinical and pathological changes in acute EA without effecting the extent or distribution of VEGF expression. SU5416 treatment produced smaller lesions without altering lesion composition. Therefore, VEGFR2 signaling represents an important component of lesion development in acute disease.

Methylalthoxone Inhibits Opiate and VEGF-induced Angiogenesis: Role of Receptor Transactivation
P. A. Singleton, J. Moss, J. G. N. Garcia; Medicine, University of Chicago, Chicago, IL
Angiogenesis or the formation of new blood vessels is important in the growth and metastatic potential of various cancers. Therefore, agents that inhibit angiogenesis have important therapeutic implications in numerous malignancies. We examined the effects of methylalthoxone (MNTX), a peripheral mu opioid receptor antagonist, on agonist-induced human EC proliferation and migration, two key components in angiogenesis. We observed that MNTX inhibits opioid and VEGF-induced migration and proliferation in a dose-dependent manner using human pulmonary microvascular EC. On a mechanistic level, morphine sulfate (M) and DAMGO induced Src activation which was required for VEGF receptor transactivation and opioid-induced EC proliferation and migration. MNTX inhibited M, DAMGO and VEGF induced tyrosine phosphorylation (transactivation) of VEGF receptors 1 and 2. Further, MS, DAMGO and VEGF induced RhA activation which was inhibited by MNTX or VEGF receptor tyrosine kinase inhibition. Finally, MNTX or silencing RhA expression (siRNA) blocked MS, DAMGO and VEGF-induced EC proliferation and migration. Taken together, these results indicate that MNTX inhibits opioid-induced EC proliferation and migration via inhibition of VEGF receptor phosphorylation/transactivation with subsequent inhibition of RhA activation. These results suggest that MNTX inhibition of angiogenesis can be an useful therapeutic intervention for cancer treatment.

Optimizing Transfection Conditions to Facilitate Cell Migration and Angiogenesis in HUVEC Cells
S. Vasa, L. Vozza-Brown, J. Fan, X. Yu, H. C. Chou; Invitrogen Corp, Carlsbad, CA
HUVEC and other primary endothelial cells represent important tools in studies of cancer, angiogenesis, and cell differentiation. The success of much of this research rests on the ability to manipulate gene expression and/or to deliver specific marker or reporter constructs while maintaining a cell's ability to progress normally through downstream developmental programs. Transfection, the method of choice for gene delivery, can involve tradeoffs since high transfection efficiency and expression often correlate with high levels of cytotoxicity, which can impair cell physiological responses and compromise performance in downstream assays. During development of LipofectamineTM LTX, which is specifically designed as a low toxicity transfection reagent, we observed that different reagents and transfection conditions can exert a significant impact on the cell’s ability to proceed through normal physiological programs. Here we describe optimized conditions for transfection of HUVEC cells using LipofectaminTM LTX that achieves high transfection activity without interfering with normal functioning of the cells, as indicated by cell differentiation indices, cell migration and the ability to undergo angiogenesis as measured in an endothelial tube formation assay.

Inhibition of Angiogenesis by Xanthohumol
M. Negrao, I. Azvedo, R. Soares; Department of Biochemistry, Faculty of Medicine, University of Porto (U38-FCT), Porto, Portugal
Angiogenesis, the formation of new blood vessels from pre-existing ones, is a process that occurs in many physiological conditions, being also essential to the development and progression of several pathologies, such as cancer and cardiovascular disease. Recent Western diet is believed to contribute to an increased lifetime risk of cancer and cardiovascular disorders. On the other hand, diets high in plant-derived foods offer a protective effect. Recent studies indicate that the development of these pathologies is inversely associated with the consumption of natural polyphenolic compounds, which are known to affect angiogenesis. Therefore, an important strategy for prevention of cancer and cardiovascular disease is the identification and characterization of dietary phytochemicals that are able to block, slow, or reverse pathological stages. One of these compounds is xanthohumol, a prenylated polyphenol obtained from the Humulus lupulus and, thus, present in beer. The purpose of this study was to investigate the effect of xanthohumol in angiogenesis, namely evaluating its effects on cell viability, apoptosis, migration, invasion and formation of capillary-like structures in human umbilical vein endothelial cells (HUVEC). Incubation of HUVEC with 10 µM xanthohumol resulted in a significant decrease in cell viability assessed by MTT assay (23.28±7.77%), as well as in an increase in the number of apoptotic cells by TUNEL assay (436.67±52.03%). Xanthohumol treatment also led to a reduction in cell migration to injured areas (injury assay), a strong decrease in the invasive capacity using double-chamber assays (27.85±7.89%) and in the formation of capillary-like structures (53.27±13.25%). Our findings indicate that 10 µM xanthohumol, exerts inhibitory effects on endothelial cell’s proliferation, migration and invasion, processes that are required for the development of physiological and pathological angiogenesis.

Therapeutic Potential of CD31 Negative Dental Pulp SP Cells for Vasculogenesis in Dentin-Pulp Regeneration
M. Nakashima, K. Iohara, L. Zheng, T. Into, K. Matsushita; Laboratory of Oral Disease Research, National Institute for Longevity Sciences, Obu, Japan
Dentin-pulp complex was demonstrated in this study. SP cells contained CD31--;CD146-, CD31++;CD146- and CD31++;CD146+ population, at the concentration of 45%, 48% and 7%, respectively. Only CD31--;CD146- SP cells formed capillary-like structures when cultured with VEGF on Matrigel and expressed activated endothelial cell markers, CEACAM1 and vWF, and mature endothelial cell markers, VE-cadherin and occludin, after seven days. VEGF induced a much stronger chemotactic response in CD31++;CD146+ SP cells compared with CD31- SP cells. VEGF also enhanced the proliferation of CD31++;CD146 SP cells. In addition to vasculogenic potential, CD31-;CD146 SP cells demonstrated neurogenic and odontogenic potential. In conclusion, the potential utility of CD31--;CD146 SP cells for regeneration of dentin and pulp complex was demonstrated in this study.

Human Blood Brain Barrier Disruption by Retrovirus-infected T Lymphocytes: Mechanism of Endothelial Tight-Junctions Disorganization by Proinflammatory Cytokines
P. Afonso; Institut Pasteur, Paris, France
The blood-brain barrier (BBB), which constitutes the interface between blood and brain parenchyma, has been shown to be disrupted during retroviral associated neuroinflammatory diseases. During Human T cell Lymphotropic Virus (HTLV) Associated Myelopathy/Tropical Spastic Paraparesis (HAM/TSP), a slowly progressive neurodegenerative disease associated with HTLV-1 infection, evidence of BBB disruption has been brought by demonstrating the presence of lymphocytic infiltrates and plasma protein leakage through brain endothelium. We have developed a human cellular model to study the mechanisms involved in endothelial permeability changes induced by HTLV-1-infected lymphocytes. We demonstrated that IL-1α and TNFα secreted by infected lymphocytes are responsible for BBB disruption, associated with the disorganization of tight junctions (TJ) between endothelial cells, and changes in expression patterns of tight junction...
proteins such as Zonula Occludens 1. Such a disruption could be confirmed in sections of spinal cord from HAM/TSP patient. In vitro, these changes were associated with an enhancement in lymphocyte migration across the barrier. Such BBB disruption could be prevented by inhibiting the NFκB pathway or MLCK activity. Our results provide, in a human in vitro model, evidence of the central role of proinflammatory cytokines in BBB endothelial TJ disorganization during HAM/TSP, with involvement of NFκB and MLCK. We propose that such a mechanism could be a general initial step in pathogenesis of retroviral associated neurodegenerative diseases.

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Non-clathrin Non-caveolin Vascular Cells Involved in Transcytosis
D. N. Predescu, S. Predescu, I. Knezevic, R. Neamu, N. Knezevic, A. Malik; Pharmacology, University of Illinois, Chicago, IL
Transcytosis is an active process by which, in fluid-phase or receptor mediated, some toxins, viruses, certain growth factors, secreted and circulating proteins are transported across different cell types in a polarized mode within a membrane bound carrier. For a long period of time it was believed that only caveolin and clathrin coated vesicles are selectively moving material between two environments without affecting the cellular milieu. Taken into account the importance and the uniqueness of endothelial transcytosis and of its corresponding carriers the endothelial caveolae, we decided to investigate what happens in a model from which caveolin1 was removed genetically. Here we present data showing that in caveolin 1 knockout mice: i) the transendothelial transport is not shifted toward clathrin-mediated transcytosis, ii) non-clathrin non-caveolin (double NC carriers) are present in different vascular segments, and iii) the double NC carriers are involved in transcytosis. Using an in situ perfusion system we were able to find out that the double NC carriers: i) have an average dimension of 100nm (90-110nm), fact that place them between the classical caveolae (60-80 nm) and clathrin coated vesicles (120-150 nm), ii) are less numerous even than the clathrin coated pits that occupy < than 0.2% of endothelial surface and have a small or very limited contribution to the ability of the cells to internalize fluid volumes, iii) depend on cholesterol for their function and subsistence and iv) seem to use the same basic targeting and fusion machinery for their movement across the endothelial cells. In the same in situ perfusion system it appears that the double NC carriers are different from the vesicular carriers used by SV40 viruses to enter the cells. The reality of double NC vesicles has been in time controversial, but our data demonstrate their existence and more than that prove their functionality.

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Thrombin Peptide, TP508, Upregulates Expression of eNOS and Prevents Hypoxia-induced eNOS Downregulation in Human Endothelial Cells
B. Olszews-PAzdrak, A. R. Hart-Van Tasse, D. H. Carney; Biochemistry & Molecular Biology, UTMB, Galveston, TX
TP508 (Chrysin®) is a nonpeptidic peptide representing a portion of human thrombin involved in receptor binding with cellular effects distinct from those of proteolytically active thrombin. TP508 is an angiogenic factor currently undergoing human clinical trial testing for healing of bone fractures and diabetic foot ulcers, and preclinical testing for myocardial revascularization. In animal models, TP508 accelerates repair and revascularization of dermal and muscoskeletal tissue, and increases the number and density of blood vessels in ischemic myocardium. We therefore studied the effects of TP508 on endothelial cells and signals related to angiogenesis. Impaired nitric oxide (NO) production reduces the responsiveness of endothelial cells to angiogenic factors and causes loss of endothelial function in ischemic and inflamed blood vessels contributing to a number of chronic diseases. We hypothesized that TP508 may produce angiogenic and other tissue repair effects by activating or upregulating nitric oxide synthase (NOS) in endothelial cells, and if so, that it may have potential therapeutic value in tissues and diseases exhibiting endothelial dysfunction. In our studies, TP508 increased phosphorylation of eNOS at Ser 1177, relative to controls, in human coronary artery endothelial cells cultured under normoxic and 1% hypoxic (1% O2) conditions (1.8 ± 0.2-fold and 2.5 ± 0.2-fold, respectively). TP508 also upregulated eNOS mRNA and protein expression in normoxic and hypoxic conditions as determined by real time PCR and Western blots. Moreover, TP508 prevented hypoxia-induced decreases in eNOS protein expression. In contrast, TP508 stimulation had no significant effect on TNFα-induced downregulation of eNOS mRNA and protein expression, but increased phosphorylation of eNOS when compared with TNFα alone. These studies demonstrate that TP508 initiates signals that increase eNOS phosphorylation, upregulate eNOS expression, and selectively inhibit effects of hypoxia. Therefore, TP508 may exert its effects in a number of tissues by modulating endothelial cell eNOS and NO production.

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Laser Capture Analysis of Endothelial Tip Cells, a Vascular Counterpart to Axonal Growth Cones
G. A. Strasser; Genentech, South San Francisco, CA
The vascular and nervous systems form extensive and often parallel networks that control, monitor, and nourish all the tissues of the body. Recently, a number of secreted and membrane bound factors known to be required for the proper wiring of the nervous system have been found to be involved in the development of the vasculature as well. Like the guidance of a neuronal axon to its synaptic partner, the growing endothelial stalk appears to be guided to its target through the activity of a specialized cell located at its tip. This tip cell, like an axonal growth cone, is highly motile and characterized by the presence of dynamic filopodia. However, little is known about how these specialized cells detect guidance cues and translate this information into motility and, subsequently, vascularization of a tissue. To analyze the gene expression patterns of tip cells, we have developed a method to isolate them using laser capture microdissection, permitting subsequent expression analysis. We have found that tip cells express genes indicating they are highly motile and characterized by the presence of dynamic filopodia. We therefore studied the function of heparanase in controlling arterial remodeling. Under normal physiological conditions endothelial cells produce soluble factors that inhibit the growth of vascular smooth muscle cells (vSMCs). This process is fundamental to vascular and nervous systems form extensive and often parallel networks that control, monitor, and nourish all the tissues of the body. Recently, a number of secreted and membrane bound factors known to be required for the proper wiring of the nervous system have been found to be involved in the development of the vasculature as well. Like the guidance of a neuronal axon to its synaptic partner, the growing endothelial stalk appears to be guided to its target through the activity of a specialized cell located at its tip. This tip cell, like an axonal growth cone, is highly motile and characterized by the presence of dynamic filopodia. However, little is known about how these specialized cells detect guidance cues and translate this information into motility and, subsequently, vascularization of a tissue. To analyze the gene expression patterns of tip cells, we have developed a method to isolate them using laser capture microdissection, permitting subsequent expression analysis. We have found that tip cells express genes indicating they are highly motile and characterized by the presence of dynamic filopodia. We therefore studied the function of heparanase in controlling arterial remodeling. Under normal physiological conditions endothelial cells produce soluble factors that inhibit the growth of vascular smooth muscle cells (vSMCs). This process is fundamental to vascular remodeling. Under normal physiological conditions endothelial cells produce soluble factors that inhibit the growth of vascular smooth muscle cells (vSMCs). This process is fundamental to vascular remodeling.
Translational Regulation of VEGF Expression by Akt in Tumor Derived Endothelial Cells in Hepatocellular Cancer

F. Meng, R. Henson, H. Webbe, T. Patel, Internal Medicine, Scott & White Hospital, Temple, TX

Angiogenesis is essential for the growth and metastasis of human hepatocellular carcinoma (HCC). Tumor derived endothelial cells are phenotypically distinct and genetically unstable compared to normal endothelial cells. Targeting tumor derived endothelial cells may be a promising new strategy for HCC. Akt / PKB can regulate the expression of key mediators of angiogenesis and cell survival but the role of Akt in liver cancer is unknown. Our aims were to determine the role of Akt kinase regulation in VEGF-mediated angiogenesis and tumor growth in HCC. Liver tumors were experimentally induced in male mice by i.p. injections of N-diethylnitrosamine (Den). Microvascular endothelial cells (EC) were isolated from HCC tumor as well as normal liver tissues using a magnetic bead immunoaffinity technique. Angiogenesis was quantitated using an in vitro assay, and the expression of Akt and VEGF were analyzed by Western blot. Transfection with NF-κB increased Akt phosphorylation and VEGF expression in both normal and tumor-derived EC. However, co-transfection of dominant negative Akt with NF-κB blocked VEGF up-regulation only in the tumor derived EC. Transfection of Akt directly increased VEGF expression as well as the angiogenesis index only in tumor derived ECs. Moreover, overexpression of Akt conferred resistance to gemcitabine induced apoptosis in tumor derived MVEC, but not in non tumor derived MVEC. Thus, there are differences in the role of Akt in VEGF dependent angiogenesis and cell survival in tumor and normal liver EC. Next, we examined the effect of the translational inhibitor cycloheximide. Pre-incubation with 50 μg/mL cycloheximide prevented the increase in VEGF expression by Akt, suggesting the need for new mRNA translation. These results highlight a novel function of Akt kinase: direct involvement VEGF dependent HCC tumor angiogenesis. It is suggested that Akt can contribute to angiogenesis in hepatocellular carcinoma via translational regulation of VEGF mRNA expression.

The Anti-cancer Effect of Combination Therapy with Conditionally Replicating Adenovirus and Adenovirus-expressing P33/β or P53 in Cervical and Ovarian Cancer Cell Lines

S. Kim, J. Lee, S. Park, D. H. Lee, J. Park, J. Lee, Cancer Research Institute, Seoul National University College of Medicine, Seoul, Republic of Korea, 2Department of Internal Medicine, Seoul National University College of Medicine, Seoul, Republic of Korea

Gene therapy is most often described as a technique for introducing the foreign genetic material into cells with a correction of a dysfunctional gene as its final goal. Consequently, we need to develop new therapeutic strategies. Conditionally replicating adenoviruses (CRAds) enhance the tumor specificity and thus avoid damage to other tissues. We were exploring the combination therapy effect of CRAds and non-replicating adenovirus in cervical cancer cell and ovarian cancer cell. Therefore, we reconstitute adenoviruses expressing the β-isoform of P33/β, a p53 homologue, by CMV promoter (Ad/CMVp73β) and the p53 by CMV promoter (Ad/CMVp53). Expression of protein level and induction of target genes was analyzed by Western blot. Infection of human ovarian cancer cell (SKOV3) and human cervical cancer cell (C33A) with Ad/CMVp73β or Ad/CMVp53 resulted in several folds increase of p73β and p53 levels. And so p21WAF1/CIP1 and BAX protein, the apoptosis and growth arrest related proteins which are responsive to p73β and p53, increased in SKOV3 and C33A. We then evaluated the inhibitory effect of Ad/CMVp73β or Ad/CMVp53 in SKOV3 and C33A using MTT assay and crystal violet staining. Also, we monitored the induced expression of p73β, p53, p21WAF1/CIP1 and BAX upon Ad/CMVp73β or Ad/CMVp53 infection. Ad/CMVp73β-infected cells showed several times higher amounts of p73β, p53, p21WAF1/CIP1 and BAX in comparison to Ad/CMVp73β, Ad/CMVp53 or CRAds alone. Further study of cell death with combination gene therapy will be discussed by cell cycle analysis. The effect by combination therapy of CRAds and non-replicating adenovirus was more effective than by non-replicating adenovirus in human ovarian cancer cell (SKOV3) and human cervical cancer cell (C33A). These results suggest that CRAds + non-replicating adenoviruses containing a therapeutic gene can improve gene transfer.

Over-expression of Rab25 Causes Transformation of Intestinal Epithelial Cells

L. A. Lapierre,1,2 C. M. Caldwell,1 R. J. Coffey,1 R. D. Beauchamp,1 J. R. Goldering1,2,1 Surgery/Epithelial Biology, Vanderbilt Univ. Medical Center, Nashville, TN, 2Vanderbilt-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 up-regulated in a number of epithelial cancers and over-expression may increase the aggressive phenotype of 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 GFP-Rab25 in RIE cells leads to transformation including 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. This induced morphological transformation was not observed in an RIE line that inducibly overexpressed GFP-Rab11b. We do not see any increased phosphorylation of either MAPK (p44/42) or AKT (Ser473) in the GFP-Rab25 overexpressing cells. In addition, we have found that over-expression of Rab25 does not alter phospho-Smad2 levels, indicating that altered TGFβ-signaling is not responsible for transformation. We also did not observe any changes in EGFR receptor phosphorylation and addition of EGFR tyrosine kinase inhibitors did not alter transformation. These results suggest that over-expression of GFP-Rab25 can transform intestinal epithelial cell using mechanism separable from classical EGFR receptor mediated signal transduction.

mDia1 Knockout Mice Phenocopy Human Pre-Leukemic/Myelodysplastic Syndromes


While acting as effectors for Rho GTPases, mammalian Diaphanous-related (mDia) form families participate in cytoskeletal remodeling events triggered by migratory and/or proliferative signaling. Using mDia1 deficient mice, we have found that over-expression of Rab25 does not alter phospho-Smad2 levels, indicating that altered TGFβ-signaling is not responsible for transformation. We also did not observe any changes in EGFR receptor phosphorylation and addition of EGFR tyrosine kinase inhibitors did not alter transformation. These results suggest that over-expression of GFP-Rab25 can transform intestinal epithelial cell using mechanism separable from classical EGFR receptor mediated signal transduction.

Characterization of Heparanase from Malignant Breast Cancer Cells

M. Yu,1 Y. Wang,2 R. S. Beek,2 M. A. Kosin2,2 Surgery, Wayne State University, Detroit, MI, 1Immunoology, Wayne State University, Detroit, MI, 2Surgical Service, John D. Dingell VA Medical Center, Detroit, MI, 3Breast Program, Karmanos Cancer Institute, Detroit, MI

Heparanase is an endo-beta-D-glucuronidase that degrades glycosaminoglycan chains of heparin sulfate proteoglycans (HSPG) at specific sites. Elevated levels of mammalian heparanase (known as HPSE) are associated with the tumor cell proliferation, invasion, angiogenesis, and metastasis. Extracellular heparanase activity that is not HPSE has been identified as an 80-kDa heparanase (and 97-kDa precursor) from malignant breast cancer cells (MCF-10CA1A11 cells) that shares homology to the CXC chemokines of the platelet basic protein (PBP) family (also known as CXCL17). To identify and characterize the extracellular heparanase activity in malignant breast cancer cells several approaches were used: 1) Heparanase activity chromatography of conditioned medium (CM) followed by Western blot antibody to the homologous C-terminal domain and verification of heparanase activity. Finally, 2-D gel to separate isoforms. 2) Immunoprecipitation of cell lysates from malignant MCF-10 CA1C11 cells by anti-human CXCL7/NAP2 monoclonal antibody. RESULTS: Cell lysate and CM of MCF-10 CA1C11 cells demonstrated 80-kDa PBP-like protein by immunoblot. In the CM, an 80-kDa PBP-like protein was identified as responsible for heparanase activity. Furthermore, separation of molecular weight of heparanase was observed by immunoblotting CM from MCF-10 CA1C11 cells with anti-human CXCL7/NAP2 monoclonal antibody. This suggested that the 80-kDa heparanase is not NAP2 like protein but a PBP-like protein. In conclusion, malignant breast cancer cells show another heparanase activity in CM and cell lysates with homology to the
chemokine CXCL7. The 80-kDa heparanase, a PBP-like molecule rather than HPSE 1 is likely responsible for the heparanase activity in the CM of MCF-10 CA1A.C11 cells. This may be specific to malignant human breast cancer, or result from additional mechanisms in malignant cells that are yet to be elucidated.

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Identifying Desmoplakin's Contribution to Prostate Cancer

C. M. MacCarthy; Biochem. and Molecular & Cellular Biology, Georgetown University, Washington, DC

We have found a relationship between desmoplakin and prostate cancer cell phenotype in vitro (i.e. increased cell division, decreased contact inhibition). Desmoplakin is known primarily for its crucial role in cell-cell adhesion complexes, desmosomes. Preliminary data reveal an additional function for desmoplakin as a cell signaling intermediate in the Rho signaling pathway, which is shown to regulate cell growth, cell motility and cytokinesis. We hypothesize that overexpression of desmoplakin in prostate cancer cells hyperactivates the Rho pathway which leads to excessive cell proliferation and potential metastasis. To determine if desmoplakin contributes to the prostate cell cancer phenotype, we have begun to use small interfering RNA (siRNA) against desmoplakin mRNA to knockdown protein translation in PC3 prostate cell cultures. Knocking down desmoplakin levels in PC3 cells resulted in decreased cell division and the recurrence of contact inhibition. Additionally, a Constitutively active Dsp-GFP vector was transfectioned into a less aggressive cancer cell line, CA-HPV-10, and reverse characteristics were observed. Morphological changes as well as protein distribution were determined by a combination of immunofluorescence staining and microscopy. Cells stained with fluorescein labeled molecules specific for desmoplakin, E-cadherin and F-actin provided an image of protein distribution as well as relative proximity of mentioned proteins. Confocal microscopy images revealed desmoplakin and active Rho proximally localized in the cytoplasm. It also showed disorganized F-actin networks. A Rho specific activity assay was used on the same cell types under the same or similar conditions to establish a link with Rho activity. Elevated levels of active Rho corresponded to elevated levels desmoplakin. The combination of these results further indicates that desmoplakin may have a non-canonical function as a signaling intermediate in prostate cancer. There is potential for this research to uncover a novel signaling mechanism of oncogenic transformation possibly leading to improved targeted cancer therapy and prevention.

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Coronin-1C Regulates Actin Dynamics during Metastasis

D. W. Roadcap, J. E. Bear; Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC

Coronins are highly conserved F-actin binding, WD repeat proteins that have been implicated in cell motility in model organisms. Research from our laboratory suggests that the type 1 family of Coronins (1A, 1B, and 1C) coordinate Arp2/3 and Cofilin activity at the leading edge. Coronin 1C is transcriptionally regulated, and microarray analysis suggests that it is overexpressed in multiple forms of cancer. We examined Coronin 1C expression in both melanoma and astrocytoma by immunohistochemistry, and found a strong correlation between Coronin 1C expression and cancer progression, with particularly high levels in metastatic cancers. Furthermore, we have found that Coronin 1C expression correlates with phospho-ERK levels in melanoma, which indicates that Coronin 1C expression may be linked to the mutual activation of the upstream regulators of ERK activity, N-Ras and B-Raf, which are commonly found in melanoma. Taken together, these data support a role for Coronin 1C in regulating migration and invasion during metastasis. To further examine Coronin 1C's in vivo significance, we have generated a Coronin 1C gene-trap mouse. Analysis of this mouse is currently underway, and embryonic fibroblasts (MEFs) have been isolated from gene-trap embryos. We interrogated these cells using a panel of motility and invasion assays to determine exactly how loss of Coronin 1C alters cell behavior. These studies reveal a critical role for Coronin 1C regulation of the actin cytoskeleton during migration, and demonstrate that Coronin 1C plays an important role in promoting the changes in cytoskeletal regulation that drive metastasis.

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Expression and Function of Pyk2 in Pancreatic Cancer

A. Galloway, J. Hubbard, Y. Yan, D. J. Cole; Surgery, MUSC, Charleston, SC; Alexion Pharmaceuticals, Cheshire, CT

Pancreatic cancer (PC) is the fourth leading cause of cancer related deaths in the US. However, the mechanisms underlying the highly invasive and metastatic phenotype of PC have not been fully elucidated. The focus of our study is the non-receptor tyrosine kinase Pyk2, which has been implicated in several cancers, but has not been fully explored in PC. Overexpression of Pyk2 mRNA in PC was demonstrated by real time RT-PCR analysis of patient-matched PC and normal pancreatic tissue. Increased Pyk2 activation in PC was also observed by immunohistochemical analysis of a tissue core array stained for Pyk2 phosphorylation on tyrosine 402. As the epidermal growth factor (EGF) and lysosphosphatic acid (LPA) pathways have been implicated in PC proliferation and metastasis and have been shown to signal through Pyk2 in other cell contexts, their activation of Pyk2 in PC cell lines was analyzed. Pyk2 was found to be phosphorylated at tyrosine 402 in a time and dose dependent manner following stimulation of both the human AsPC1 and murine Panc02 PC cell lines with EGF or LPA. Further, infection of PC cell lines with a dominant negative Pyk2 adenoviral construct, AdPy2 Y402F, reduced proliferation and induced an accumulation of cells in G2 phase. Expression of this Pyk2 dominant negative mutant also significantly reduced wound healing. Taken together, these results suggest that Pyk2 may contribute to the aggressive phenotype of this disease and may represent a potential therapeutic target.

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A Role for the Lactate Transporter MCT4 in Regulating Cell Surface Expression of CD147 in the Metastatic Breast Cancer Cell Line MDA-MB-231

S. M. Gallagher, D. Wang, Z. Jiang, N. J. Philip; Pathology, Anatomy and Cell Biology, Thomas Jefferson University, Philadelphia, PA

The matrix metalloproteinase inducer, CD147, plays an important role during tumor invasion. In metastatic cancer cells, CD147 is expressed at high levels and stimulates the production of matrix metalloproteinases in the stroma, thereby enhancing tumor cell migration and facilitating angiogenesis. CD147 has also been shown to form heteromeric complexes with members of the monocarboxylate transporter family (MCT) and is required for the trafficking of these transporters to the plasma membrane. While MCT1 is widely expressed, MCT4 is primarily detected in tissues dependent on glycolysis for the production of ATP. Since a switch from oxidative to glycolytic metabolism is a key feature of metastatic cells, we investigated whether the increase in CD147 in cancer cells is linked to the expression of MCT4. Using the metastatic breast cancer cell line MDA-MB-231, we find that MCT4 expression is increased at both the message and protein level compared to non-metastatic cancer cell lines and cells derived from normal mammary tissue. Immunofluorescence microscopy and co-immunoprecipitation studies reveal that the MCT4/CD147 heteromeric complex co-localizes to the plasma membrane and membrane blebs that are shed from the cell surface. Silencing of MCT4 with siRNA impairs the maturation and trafficking of CD147 to the cell surface, resulting in accumulation of CD147 in the endoplasmic reticulum. Likewise, silencing CD147 causes a reduced expression of MCT4 which leads to a decrease in lactate efflux and cellular levels of ATP. Also, when cells are treated with CD147 siRNA, MCT4 is not trafficked to the plasma membrane, but is targeted instead to endosomes. These studies establish that the increase in CD147 observed in metastatic cancer cells is mediated through its association with MCT4. Therefore, our results suggest that upregulation of MCT4 may be critical for cancer progression and metastasis making it a potential target for anti-cancer therapies.

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The Selectivity of Ras Signaling in Tumor Formation

C. Cheng, B. Onken, M. R. Philip, E. C. Chang; Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX; Cell Biology and Pharmacology, New York University School of Medicine, New York, NY

The Ras G-protein signaling pathways are very complex in humans. There are four Ras proteins. The N-termini of these Ras proteins are over 90% identical in primary sequences, which include the effector-binding domains, and in vitro they interact with many effectors with nearly the same efficiency. However, how a given Ras protein selectively activates a particular effector is poorly understood. One leading model suggests that the C-termini of Ras proteins undergo specific post-translational modification, e.g., lipidation, and that such modifications allow Ras to localize and signal from at least two distinct compartments: the plasma membrane and the endomembrane. To unambiguously test this model, we have turned to the fission yeast Schizosaccharomyces pombe, which has only one Ras protein, but it is still capable of controlling two highly conserved pathways. Our data show that J. pombe Ras selectively activates a MAP kinase pathway to mediate periodic signaling at the plasma membrane, while it activates Cdc42 at the endomembrane to control cell polarity and mitosis. Ras also appears to act via Cdc42 to transform mammalian cells, and Cdc42 can be found at the endomembrane. We have transformed fibroblasts with endomembrane-restricted Ras and showed dominant-negative Cdc42 can block endomembrane-restrictive Ras induced transformation. Together, these results suggest that Ras in mammalian cells can interact selectively with Cdc42 in the endomembrane. In the current study, we plan to test this idea and to determine if such interaction between Ras and Cdc42 may be important for tumour formation.
2668 Tumor Suppression by Inhibition of p130Cas/BCAR1 Signaling in Tamoxifen Resistant Breast Cancer Cells
S. Soni; Biochemistry, Boston University School of Medicine, Boston, MA

Breast cancer cell lines and primary breast carcinomas express high levels of p130Cas/BCAR1 (breast cancer anti-estrogen resistance-1) in the cytoplasm. Elevated protein expression in mammary tumors is a marker of poor prognosis and poor overall survival. Moreover, p130Cas (CAS) signaling has been associated with anti-estrogen resistance. The central objective of our research is to investigate the effects of inhibition of CAS on breast tumor formation and resistance to Tamoxifen. We employed the human breast cancer cell lines MCF7 and its Tamoxifen resistant derivative TAM-R. For our studies we established cells that were stably infected with a dominant negative CAS - blocking signal transduction downstream of the CAS substrate domain ([CAS]D). Kirsch et al., 2002, BMC Cell Biol 3:18 - as well as CAS knockdown cell populations established by siRNA. These cells were monitored for effects on proliferation, morphology, migration, survival and sensitivity to growth inhibition or apoptosis upon treatment with Tamoxifen. The expression of the dominant negative CAS as well as CAS siRNA induced morphological changes, which were consistent with a more normal epithelial-like phenotype. This reversion was accompanied by reduced cell proliferation and migration. Interestingly, we observed cytostatic effect using the dominant negative CAS, whilst the expression of CAS siRNA had a cytotoxic effect. The signaling pathways affected by inhibition of CAS in this system are currently under investigation. In summary, our findings suggest that targeting the product of the BCAR1 gene may provide a new molecular avenue for treatment of anti-estrogen resistant breast cancers. This work has been supported by the NIH CA106468 to S.H. Kirsch. Reference: Kirsch K.H., M. Kensinger, H. Hanafusa and A. August . 2002. A p130Cas tyrosine phosphorylated substrate domain

2669 Regulation of Proteasome Dynamics and Assembly by Int6/yin6
Z. Sha,1 H. Yen,2 E. Chang1; 1The Breast Center, Baylor College of Medicine, Houston, TX, 2Harvard University, Houston, MA

INT6 is a target of MMTV, whose insertion generates truncated proteins that induce tumor. Our lab demonstrates an evolutionarily conserved function of Int6 in the model system Schizosaccharomyces pombe: regulation of proteasome transport and assembly. The overall objective of this study is to better understand how Int6 regulates this process. By FRAP/FLIP assays, we found that the proteasome submit Rpn11-GFP is highly dynamic, and is actively entering and exiting the nucleus. The dynamics increases when deleting int6 or rpn11, a proteasome subunit regulated by int6. These results are consistent with our previous findings that proteasome become disassembled when int6 or rpn11 is deleted. Furthermore, the dynamics of Rpn11-GFP appears to increase when treated with MIB, a microtubule-depolymerizing drug, but decrease when treated with canavanine, an arginine analog, which generates proteasome stress. These results raise interesting questions whether proteasome assembles into larger complexes when they are required for protein turn-over, and whether microtubules play a role in proteasome assembly. I am also carrying out a structure-function analysis focusing on the fact that many proteasome subunits and Int6 contain a PCI domain, whose truncation induces mouse breast tumors. I have characterized a gene encoding a novel PCI proteasome subunit, Rpn7, which was isolated as a high copy suppressor to rescue growth defects in int6 mutants. I showed that Rpn7 is essential for proper chromosome segregation, and PCI subunits could preferentially interact. For example, both Rpn5 and Rpn7 bind Rpn9 and Int6 interacts with this complex via binding to Rpn9. Through protein sequence analyses and mutagenesis, I identified a conserved leucine residue in Rpn7, which is critical for its localization and binding with Rpn9. The importance of this residue has been further verified in Int6. These data imply that PCI domain facilitates protein-protein interaction that is pivotal for the assembly of the proteasome.

2670 Appropriate Localization of Retinoblastoma Protein (pRB) in the Nucleus Correlates with Tumor Suppressor Activity
L. A. Collins,1 F. K. Kornelak,1 University of Washington, Seattle, WA

Past studies have shown that the retinoblastoma protein (pRB) exists in the nucleus as a mobile pool, associating with different nuclear subcompartments, such as the nucleolus and the soluble nucleoplasm. pRB transiently localizes to the nucleolus during passage through G1, which can be disrupted by the presence of viral oncoproteins. In order to gain insight into whether nuclear localization is necessary for pRB’s tumor suppressor activity, we have screened various point mutants including low penetrance tumor alleles, C712R and R661W, for their localization patterns compared with wild-type pRB in cells lacking endogenous pRB. We find that C712R cannot attain nuclear localization, whereas another point domain mutants, such as R661W and K713A, can reside at the nucleolus but at lower frequencies than wild type pRB. Importantly, pRB’s localization at nucleoli coincides with induction of senescence in SAOS2 cells, and persists during the formation of large condensed nucleoli as determined using simultaneous GFP and senescence-associated ß-gal labeling. One exciting possibility is that pRB localization to the nucleolus induces large-scale chromatin condensation of rDNA resulting in the morphologically distinct single nucleolus observed in senescent cells. Possible causes for different localization of RB alleles are being examined, and include altered phosphorylation status and protein stability. The substitution of different residues at C712 results in different frequencies of nucleolar residence, suggesting that strongly destabilizing substitutions are more likely to abrogate localization. The failure of mutants to accumulate at sites of activity is being explored in light of a proposal by Otterson et al. 1999 that low penetrance tumor-derived alleles of pRB fail in their function as tumor suppressors due to fact that they are less stable.

2671 In Silico Modeling of ERK2 Docking Domains and Development of Substrate Selective Inhibitors
F. Chen, A. Macias, A. D. MacKerell, P. Shapiro; Pharmaceutical Science, University of Maryland School of Pharmacy, Baltimore, MD

Extracellular signal regulated kinase proteins (ERK1 and ERK2) are important in regulating cell growth, proliferation, and differentiation. Over-activation of the ERK pathway by mutations in upstream components is thought to play a significant role in the development and progression of cancer. However, ERK substrates are not only involved in cancer cell proliferation and survival, but also participate in physiological functions of non-cancerous cells. Therefore, selective inhibition of ERK regulation of substrates involved in cancer cell proliferation may be a potential approach to improve existing therapies for treating cancers with elevated ERK pathway activity and reduce drug toxicity in normal cells. In these studies, we used computer aided drug design (CADD) with the 3D structure of active ERK2 to identify novel compounds that have the potential to selectively disrupt ERK2 interactions with substrate proteins. CADD identified small molecular weight compounds that potentially targeted a groove between the CD domain (Aspartate 316 and 319) and ED domain (Threonine 157 and 158) on ERK2. These compounds were analyzed in immunoblotting, cell proliferation and ERK2 binding assays. The results revealed that several compound can effectively inhibit phosphorylation of the ERK substrate, ribosomal S6 kinase-1 (RSK-1), which interacts with the CD domain, and proliferation of cultured cancer cells. Importantly, none of the test compounds inhibited phosphorylation of ERK by the upstream MKK1/2 or p38 MAP kinase mediated phosphorylation of the transcription factor, ATF2. In addition, active test compounds showed ERK2-specific binding using fluorescence quenching assays. These studies demonstrate the utility of using CADD in silico modeling and biological assays to rapidly identify small molecular weight compounds that are non-competitive for ATP binding and show selective inhibition of ERK interactions with substrate proteins.

2675 Mono and Dual Phosphorylated Erk1 and Erk2 Measured in Breast Cancer Cell Lines
K. Hacker,1 U. Nguyen,1 E. Gentlen,1 J. Knittle,1 N. Parker,1 K. Voss,1 R. Neve2; 1Cell Biosciences, Palo Alto, CA, 2Lawrence Berkeley National Laboratory, Berkeley, CA

Multiple techniques are being used to discover biomarkers to stratify cancer biopsies and predict response to drug therapies. Erk1 and Erk2 are key kinases in the MAP kinase cascade that play a roll in cell growth. Erk1 and Erk2 are activated by phosphorylation of a threonine and a tyrosine. Here we use a capillary system to separate singly and doubly phosphorylated forms of Erk1 and Erk2. We then measure the percent phosphorylation without phospho specific antibodies. Western blotting, which separates proteins based on size, is unable to resolve these different phosphorylated forms. In our initial studies, three breast cancer cell lines were compared with a normal tissue culture line, MCF10A. We observed significant differences in the ratios and levels of mono and dual phosphorylation forms of Erk1 and Erk2 across the breast cancer cell lines. We are now expanding our studies to look at Erk and other protein phosphorylations in a panel of 50 breast cancer cell lines.

2676 Fast Dissociation of Cancer Cell Colonies by Lysophosphatidic Acid (LPA) Causes ß-Catenin Dependent Gene Expression Changes by a Novel Perinuclear Accumulation Mechanism
Y. Kann, H. Yamashita, V. Quaranta; Department of Cancer Biology, Vanderbilt University, Nashville, TN

Lysophosphatidic acid (LPA) has been suggested as a potent modulator of motility and proliferation of epithelial cancer cells through its specific G-protein coupled receptors. We previously reported that LPA induces rapid colony dispersal in A431 carcinoma cells (Jouquin et al. (2006) J. Cell Physiol.). The colony dispersal is accompanied by rapid dissociation of cell-cell adherens junctions (AJ) and robust cell migration. Here we report that AJ components including E-cadherin, α-catenin and β-catenin were internalized within minutes and accumulated at the perinuclear region upon LPA stimulation. Even before dissociation of cell-cell adhesion became obvious, β-catenin rapidly reached a perinuclear region that appeared to overlap with early
endosome distribution. E-cadherin was colocalized in the same region. Perinuclear β-catenin was translocated into nucleus when GSK-3β was inactivated by LiCl, or proteasome functions were impaired by MG132. However, LiCl or MG132 alone could not effectively induce β-catenin nuclear translocation. After 12-24 hours from LPA stimulation, changes in cell motility-associated gene expression were detectable by real-time PCR. Nuclear β-catenin has been directly implicated in cancer cell phenotypic changes (e.g., epithelial-mesenchymal transition) that prelude to invasion. Our results unveil a novel intracellular transport mechanisms whereby β-catenin can reach the nucleus, via a previously unknown perinuclear sorting compartment. More generally, our results suggest that LPA, released in the tumor microenvironment (e.g., by platelets, due to the frequent occurrence of inflammatory or remodeling processes in tumor tissue), may play an immediate and long-lasting role in inducing invasive behavior in cancer cells.

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Mig-7 Expression Allows Carcinoma Cells to Sense a 3D Environment, to In invade and to Form Vessel Structures
A. P. Petty,1 K. L. Garman,1 J. S. Lindsey2; 1School of Molecular Biosciences, Washington State University, Pullman, WA, 2Pharmaceutical Sciences, Washington State University, Pullman, WA
Cancer cell interactions with the tumor microenvironment are critical for disease progression. However, the molecular mechanisms are not fully understood. We hypothesize that an atypical gene expression, Mig-7, which is induced by receptor tyrosine kinase and src55 integrin signaling, plays a role in this process because cancer cell dissemination in vivo requires these signal transduction. Specifically, we hypothesize that Mig-7 expression causes tumor cells to masquerade as endothelial cells, a process called vasculogenic mimicry thought to be important for cancer progression. We base this hypothesis on our previous data that suggest Mig-7 expression is limited to embryonic cytrophoblast cells (CTB) that invade and undergo pseudovascularization during placenta formation and to invasive carcinoma cells from multiple tissue types. Our results show that tumor microenvironment growth factors (HGF, IGF-1 and EGF) that regulate PI3K, a signaling pathway required for vasculogenic mimicry, induced Mig-7 expression in src55-positive carcinoma cells. In 3D cultures, stably transfected colon carcinoma cells expressing Mig-7 formed vessel-like structures in contrast to empty vector transfected cells that formed colonies. Mig-7 expression significantly diminished adhesion to laminin, an extracellular matrix heterotrimer that has a y chain cleaved during vasculogenic mimicry and invasion. We also found that melanoma cells previously characterized to invade aggressively and undergo vasculogenic mimicry expressed Mig-7 while noninvasive melanoma cells lacked Mig-7 expression. In our nude-mouse model of metastasis, immunohistochemistry revealed Mig-7 protein predominantly co-localized with Factor VIII-associated antigen, a plasma protein and endothelial cell marker, to vessel-like structures of metastasized lymph node. Given these findings, we speculate that Mig-7 expression allows cancer cells to sense their environment, to invade and to undergo vasculogenic mimicry. Therefore, Mig-7 may serve as a molecular target for future therapies aimed to modulate tumor progression.

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ROCK II Modulates Tumor Cell Invasion in Colon Cancer via Invadopodia
S. C. Glover,1 J. Huq,1 R. Vishnubhota,2 G. Guzman,3 M. Bulic,4 S. Sun,4 M. Cho5; 1Medicine, University of Illinois at Chicago, Chicago, IL, 2Pathology, University of Illinois at Chicago, Chicago, IL, 3Bioengineering, University of Illinois at Chicago, Chicago, IL
MET in the colon follows a strict 2-D axis as stem cells differentiate into enterocytes and migrate up the crypt-villus axis. In colon cancer, this axis is disrupted and tumor cells undergo migratory reversal allowing them to invade into the submucosa and beyond. One major pathway that has been shown to be critically influenced by the tumor microenvironment is the Rho/ROCK pathway. Evaluation of these proteins in resected human colon cancers revealed up-regulation of ROCK II, a downstream effector of Rho, at the invasion front. ROCK II has been linked with other greater invasion and metastasis in other solid tumors but its role in colon cancer invasion has yet to be fully defined. The goal of this study was to evaluate the behavior of ROCK II in non-malignant (NCM 460) and malignant (Caco-2 EP90 and SW 620) colon epithelial cell lines grown in a 3-D type 1 collagen scaffolds. Scaffolds were evaluated at 1, 3, and 5 days post invasion for phenotypic appearance of cells, depth of invasion, ROCK II expression and localization, proliferation, collagenase activity, and overall ROCK activity. Multiphoton images revealed that both NCM 460 and Caco-2 EP90 cells invaded the scaffolds and formed "crypt-like" structures. SW 620 cells penetrated deeply into the scaffold, degraded collagen two-fold more, but had significantly lower proliferation. When probed with antibodies specific for ROCK II, structures resembling invadopodia were noted in both Caco-2 EP90 and SW620 cells but not in NCM 460 cells. Finally, ROCK activity was three fold higher in the SW 620 cells 24 hours post invasion as compared with the other cell lines evaluated. This data suggests that ROCK II may play a direct role in colon cancer invasion through its action at the site of invadopodia.

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Midkine, a Potential Tumor Biomarker, Is Enhanced in Huh7 and HepG2 Human Hepatoma Cells
Y. Zhou, H. Xu; R & D, AbboMax Inc, San Jose, CA
Midkine (MK) is currently discovered as a new member of a unique heparin-binding neurotrophic factor or cytokine family which is composed of MK and PTN (Pleiotrophin). MK plays important roles in development, neuronal survival and differentiation, tissue remodeling and carcinogenesis. The augmented expression of midkine has been revealed in many advanced tumors at very high frequency in non-tissue specific manner. The serum midkine level is increased in many cancer patients, making the detection level of MK a potential tumor marker in clinical diagnosis. We have developed immunoassays to detect midkine based on a panel of high affinity and specificity antibodies against human midkine. The Huh7 and HepG2 hepatoma cells were grown in high-glucose DMEM with essential supplements. The culture mediums were collected at 4th passage with 80% confluence for midkine ELISA detection. The protein extracts were prepared from 10 7 cells/ml by adding lysis buffer, half of the samples were used for midkine ELISA detection, and the other half were fractionated by sodium deoxycholate-sulfate-polyacrylamide gel electrophoresis (10% SDS-PAGE). We found that the antibodies can recognize ~14 kDa protein from the hepatoma cell lysates, these immunoreactive bands can be blocked by recombinant protein midkine. Further more, MK levels are detectable in the hepatoma cell culture mediums (12.6ng/ml, n=20, CV=9.8%) as well as the cell lysate preparations (15.1 ng/10 6 cells, n=10, CV=14%) compared to the non-detectable MK levels from the negative controls. In conclusion, the fast and sensitive ELISA method for midkine detection can be a potential tool for clinical diagnosis. The attempt of targeting midkine might be a plausible approach to illustrate the midkine in anti-apoptotic, angiogenic and other carcinogenesis-related activities.

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Activation of Rapid Signaling Pathways and the Subsequent Transcriptional Regulation for the Proliferation of Breast Cancer MCF-7 Cells by the Treatment with the Extract of Glycyrrhiza glabra Root
M. Tanji1, S. Dong2, A. Iroue3, Y. Zhou,4 R. Kiyama1,2; 1InfoGenes Co., Ltd., Tsukuba, Ibaraki, Japan, 2Signaling Molecules Res. Lab., AIST, Tsukuba, Japan
Midkine (MK) is currently discovered as a new member of a unique heparin-binding neurotrophic factor or cytokine family which is composed of MK and PTN (Pleiotrophin). MK plays important roles in development, neuronal survival and differentiation, tissue remodeling and carcinogenesis. The augmented expression of midkine has been revealed in many advanced tumors at very high frequency in non-tissue specific manner. The serum midkine level is increased in many cancer patients, making the detection level of MK a potential tumor marker in clinical diagnosis. We have developed immunoassays to detect midkine based on a panel of high affinity and specificity antibodies against human midkine. The Huh7 and HepG2 hepatoma cells were grown in high-glucose DMEM with essential supplements. The culture mediums were collected at 4th passage with 80% confluence for midkine ELISA detection. The protein extracts were prepared from 10 7 cells/ml by adding lysis buffer, half of the samples were used for midkine ELISA detection, and the other half were fractionated by sodium deoxycholate-sulfate-polyacrylamide gel electrophoresis (10% SDS-PAGE). We found that the antibodies can recognize ~14 kDa protein from the hepatoma cell lysates, these immunoreactive bands can be blocked by recombinant protein midkine. Further more, MK levels are detectable in the hepatoma cell culture mediums (12.6ng/ml, n=20, CV=9.8%) as well as the cell lysate preparations (15.1 ng/10 6 cells, n=10, CV=14%) compared to the non-detectable MK levels from the negative controls. In conclusion, the fast and sensitive ELISA method for midkine detection can be a potential tool for clinical diagnosis. The attempt of targeting midkine might be a plausible approach to illustrate the midkine in anti-apoptotic, angiogenic and other carcinogenesis-related activities.

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Ubiquitination of Mitochondrial Proteins Triggers Apototic Cell Death in Cell Culture Models of Parkinson's Disease: A Novel Regulatory Function of Lysine 48 and -63 Ubiquitin Sites
F. Sun, V. Anantharam, A. Kanthasamy, A. G. Kanthasamy; Biomedical Sciences, Iowa State University, Ames, IA
Ubiquitin proteasome system (UPS) dysfunction and mitochondrial impairment have been linked to neurodegeneration of Parkinson's disease (PD). Recently, we demonstrated that environmental neurotoxic, estrogen, dieldrin, and Mg-2+ enhanced UPS function in dopaminergic cells and that the proteasomal inhibitor MG-132 promotes nigral dopaminergic degeneration in both in vitro and in vivo models. To further determine the inter-relationship between UPS dysfunction and mitochondrial impairment, we systematically examined the sequence of cellular events in dopaminergic neuronal (N27) cells following exposure to dieldrin and MG-132. Subcellular analysis of ubiquitinated protein revealed a rapid and profound accumulation of polyubiquintinated proteins in mitochondria. The magnitude of mitochondrial ubiquitinated accumulation was much more than that of cytosolic accumulation, indicating that mitochondria are

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early sensors of ubiquitinated protein stress. Time course studies revealed that accumulation of ubiquitinated proteins in mitochondria precedes activation of mitochondrial apoptotic cascade. To determine whether the accumulation of polyubiquitinated proteins in the mitochondria is involved in the mitochondria apoptosis events, we established cell lines stably expressing wild type or two ubiquitin site mutants lysine 48 (K48R) and lysine 63 (K63R). Results showed that K48R mutant ubiquitin significantly suppresses dieldrin and MG-132 induced caspase-9, and -3 activation, suggesting that accumulation of K48R polyubiquitin tagged proteins plays a critical role in activating mitochondrial apoptosis. Interest in A53T alpha-synuclein mutation at lysine 63 (K63R), which is not required for dieldrin polyubiquitination, is increasing for its potential for UPS degradation, greatly sensitized N27 cells to dieldrin or MG-132 induced apoptosis, indicating that lysine 63 may have some protective function during ubiquitinated protein stress. Collectively, these novel findings demonstrate an intrinsic link between mitochondrial ubiquitination, UPS dysfunction and apoptotic cell death in dopaminergic neuronal cells and also suggest that polyubiquitination sites may determine the fate of cell survival by regulating the mitochondrial dependent apoptotic cascade (supported by NIH grant NS45133).

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Budding Yeast Model for Alpha-Synuclein: Evaluation of E46K, Catalase Deletion, and Multi-Vesicular Body (MVB) Disruption
M. D. White, M. Vahedi, M. Zorniak, K. Brandis, S. Herrera, T. Saylawala, J. Price, S. DebBurman; Biology, Lake Forest College, Lake Forest, IL
Parkinson’s disease (PD) is a common neurodegenerative disorder that results from the selective loss of midbrain dopaminergic neurons. Misfolding and aggregation of the protein α-synuclein, oxidative damage, and degradation impairment are all hypotheses for the molecular basis of this selective neurotoxicity. Here, we extend our Saccharomyces cerevisiae model to evaluate α-synuclein misfolding, aggregation, and toxicity (Sharma et al. 2006, J Mol. Neurosci. 28, 161-178). First, we describe the toxicity-inducing ability of the recently discovered E46K α-synuclein mutant and familial combination mutants (A30P/E46K, A53T and A30P/E46K/A53T). Unlike wildtype, A30P and A53T α-synuclein, the E46K mutants were strongly toxic to several, but not all, strains. They retained plasma membrane localization of α-synuclein with cytoplasmic aggregates developing after 24 hours of induction. When yeasts were challenged with an oxidant (hydrogen peroxide), α-synuclein was extremely lethal to cells that lacked manganese superoxide dismutase Mn-SOD (sod2Δ), but not to cells that lacked copper, zinc superoxide dismutase Cu,Zn-SOD (sod1Δ). Despite the toxicity, sod2Δ cells never displayed intracellular aggregates of α-synuclein. We suggest that the toxic α-synuclein species in yeast are in smaller aggregates and may involve α-synuclein membrane association and is particularly sensitive to mitochondrial oxidative stress.
Lastly, to assess lysosomal involvement in α-synuclein degradation, we assessed if α-synuclein was toxic to yeast that were deficient in the Vps family of proteins critical to the endosome-based multivesicular body pathway and thus far shown that α-synuclein accumulated and caused toxicity in only one Vps deletion strain. Thus, yeasts have emerged effective organisms for characterizing factors and mechanisms that regulate α-synuclein toxicity in PD. (Supported by NSF-MRI, NSF-CCLI & NIH R15)

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Functional Validation of the Neuroprotective Capacity of Small Molecule Effectors of Alpha-Synuclein Misfolding
A. M. K. Clark, 1 C. Makowsky, 1 R. Thomas, 1 X. Meng, 3 M. Zhu, 1 J. Li, 1 A. L. Fink, 2 G. A. Caldwell, 1 K. A. Caldwell 1; 1Biological Sciences, The University of Alabama, Tuscaloosa, AL, 2Department of Chemistry and Biochemistry, University of California-Santa Cruz, Santa Cruz, CA
Parkinson’s disease (PD) involves the progressive loss of dopamine (DA) neurons over the course of aging. Mutations or multiplication of wildtype gene encoding human α-synuclein are among the most common causes of familial Parkinson’s disease. Mitochondrial dysfunction, oxidative stress, and misfolded α-synuclein contribute to the cell death in PD. This model for α-synuclein-induced DA neurodegeneration. Worms overexpressing wildtype human α-synuclein undergo a reproducible DA neuron decline that worsens with age. This degeneration can be arrested by the addition of transgene expressing either human torsinA, a protein that exhibits molecular chaperone activity, or mammalian Rub1, a guanosine triphosphatase (GTPase). Importantly, select compounds identified from the in vitro α-synuclein misfolding assays are also effective in significantly reducing the degeneration of DA neurons in vivo. This screening strategy facilitates our ability to prioritize specific candidate molecules in determining their suitability as potential disease modifying agents in mammalian models of PD.

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Fission Yeast Model for Alpha-Synuclein: Evaluating Concentration Threshold to Induce Aggregation in Living Cells
L. Kukreja, M. Zorniak, K. Brandis, S. DebBurman; Biology, Lake Forest College, Lake Forest, IL
Despite fission yeast's history of modeling salient cellular processes, it has not been extensively used to model human neurodegeneration-related protein misfolding. Since α-synuclein misfolding and aggregation are linked to Parkinson's disease (PD), we recently reported a fission yeast model that evaluated alpha-synuclein misfolding, aggregation, and toxicity (Brandis et al. 2006, J Mol. Neurosci 28, 179-191). Using thiamine repressible promoters (pNMT1, pNMT4, pNMT1), wild-type alpha-synuclein and familial mutants were expressed in increasing concentrations to directly test in living cells the nucleation polymerization hypothesis for alpha-synuclein misfolding and aggregation. In support of this hypothesis, both wild-type and A53T alpha-synuclein formed cyttoplasmic aggregates within fission yeast cells in a concentration and time-dependent manner. A53T alpha-synuclein formed aggregates faster than wild-type alpha-synuclein and at a lower alpha-synuclein concentration. Here, we characterized alpha-synuclein's aggregation pattern further in live cells to determine the threshold protein concentration needed to seed aggregation. When moderately expressed (pNMT4 vector), alpha-synuclein began forming aggregates, but at just slightly lower expression (pNMT1 vector), it remained completely soluble, indicating that the concentration threshold was in between these two concentrations. Despite alpha-synuclein's extensive aggregation, it was surprisingly non-toxic to fission yeast; future genetic dissection may yield molecular insight into this protection against toxicity. Unexpectedly, different from budding yeast, wild-type and A53T alpha-synuclein did not localize to the plasma membrane in fission yeast, not even at low alpha-synuclein concentrations or as an early precursor to forming aggregates. We speculate that alpha-synuclein toxicity may be linked to its membrane binding capacity. Thus, fission yeast sheds provocative insight into alpha-synuclein's role in PD pathogenesis. (Supported by NSF-MRI, NSF-CCLI & NIH R15)

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Intrabodies as Potential Therapeutics for Parkinson’s Disease
S. M. Lynch, 1 C. Zhou, 1 S. Emadi, 2 M. Siekiers, 1 A. Messer 2; 1Wadsworth Center, New York State Department of Health, Albany, NY, 2Department of Biomedical Sciences, University at Albany, Albany, NY
Misfolded proteins have been implicated in a number of progressive neurodegenerations, including Parkinson’s Disease (PD). In PD, alpha-synuclein probably plays a critical pathologic role, since it accumulates, causes toxicity when overexpressed, or when harboring specific mutations. In its native form, alpha-synuclein has an unstructured conformation, but can assume a beta-sheets configuration that predisposes it to an aggregated oligomeric form, thought to be involved in toxic gain of function. Therapeutic approaches involving the prevention of misfolding or aggregation of alpha-synuclein, or precluding it from reaching its toxic state, should prove valuable as direct therapeutics and rational drug design tools. Single-chain Fv (scFv) intracellular antibodies (intrabodies) against alpha-synuclein can be selected, engineered, and delivered as genes. They have the potential to bind and stabilize monomeric alpha-synuclein, reduce the presence of higher oligomeric forms, and counteract toxicity. Screening involved using transient transfections of cell lines ST14A, BI03, and human cells H4, with wild-type or mutant alpha-synuclein +/- the intrabody of interest, followed by Toxicity and MTT assessment of toxicity and viability, in 96-well plates. Transfection of A53T alpha-synuclein into ST14A cells (a conditionally-immortalized rat striatal cell line) gave the most consistent results. We have also tested for correction of synuclein-mediated growth impairment in HEK-293 cell lines stably transfected with alpha-synuclein. Intracytochemistry to localize a-syn and intrabody, plus Western blots, are used to more fully assess effects of the intrabodies on overexpressed alpha-synuclein. The intrabodies selected and tested to date target monomeric alpha-synuclein (apparent conformational epitope), oligomerized alpha-synuclein (selected using atomic force microscopy), the putative hydrophobic interaction region of alpha-synuclein, and peptides covering the A30P mutation region. Protection in one or more of the assays has been observed with one intrabody from each of the first three categories, validating a role for engineered antibody fragments in neurological therapeutics. (Support: National Parkinson Foundation; NIH)

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Identification of Putative Genetic Susceptibility Factors Associated with Parkinson’s Disease
R. N. Rivas, S. Hamamichi, K. A. Caldwell, G. A. Caldwell; Biological Sciences, The University of Alabama, Tuscaloosa, AL
Investigations into familial forms of Parkinson’s Disease (PD) have identified several mutated single gene products associated with protein mishandling and oxidative stress. For example, mutant or overexpressed alpha-synuclein results in the formation of characteristic protein inclusions called Lewy bodies. Human torsinA (torA), a protein linked to another hereditary movement disorder termed early-onset torsion dystonia, is highly expressed in dopaminergic neurons, and has been detected in Lewy bodies. We investigated the functional relationship between alpha-synuclein
and torA by generating an isogenic line of C. elegans that co-expresses TOR-2 (C. elegans torA homolog) and human alpha-synuclein fused to GFP in the body wall muscles. We found that overexpressed TOR-2 could ameliorate the formation of alpha-synuclein aggregates in vivo. We then employed RNAi feeding methods to systematically screen for factors that affect alpha-synuclein misfolding at a threshold of misfolding maintained by TOR-2 co-expression. A total of 900 candidates were analyzed including worm homologs of known PD genes, gene products co-expressed or interacting with these proteins, and gene targets with roles in protein folding and degradation. We identified 20 positives that result in increased alpha-synuclein misfolding and determined that 14 of these may function in a TOR-2-independent manner. We previously reported that tormis expressed in C. elegans dopamine neurons protect these cells from neurodegeneration. By generating transgenic worms in which cDNAs encoding prioritized candidates from these 20 positive gene products are overexpressed in dopamine neurons, we have thus far demonstrated that at least two of these proteins also display neuroprotection from alpha-synuclein-induced neurodegeneration in vivo. Further analysis is underway to determine if additional gene products identified through our screen function in a similar manner. These data provide a paradigm for investigating factors that influence protein misfolding and the management of intracellular stress associated with movement disorders.

The E46K Mutation in Alpha-Synuclein Associated with Early-Onset Parkinson's Disease Modulates SERCA2b Levels
S. Keller, Y. Yu, O. Platoshyn, J. Yuan; Medicine, University of California, San Diego, La Jolla, CA
Three mutations (A30P, A53T and E46K) in the gene encoding the pre-synaptic protein alpha-synuclein are associated with inherited early-onset Parkinson’s disease, which like the common age-related sporadic form, has the hallmark of dopaminergic neuron decline in the substantia nigra. The mechanisms associated with expression of the mutant alpha-synucleins and dopaminergic neuron death have not been fully resolved. The E46K alpha-synuclein was discovered in a large Spanish family, with complete segregation for disease onset in the heterozygous configuration. Previous studies have demonstrated that E46K alpha-synuclein has a higher affinity for phospholipid membranes compared to wild-type alpha-synuclein and other mutant alpha-synucleins. We performed heterologous expression experiments in HEK-293 and CHO cells to elucidate characteristics of E46K alpha-synuclein that may elicit pathological mechanisms leading to Parkinson’s disease. Although lacking a signal sequence, E46K alpha-synuclein associates with the endoplasmic reticulum (ER) membrane, as well as the cell surface membrane, as detected in immunofluorescent microscopy images. Expression of E46K alpha-synuclein appears to increase cell surface membrane permeation, deplete intracellular calcium stores and elevate store-operated calcium influx, as detected by calcium imaging technology. Furthermore, expression of E46K alpha-synuclein reduces the steady-state expression levels of the SR/ER calcium ATPase SERCA2B, which is involved in replenishing the ER with calcium. Based on this heterologous cell expression model, a combination of dysregulated calcium fluxes from the cell surface and a reduced ability to regulate ER calcium levels may impair the longevity of dopaminergic neurons.

Mitochondrial Localization of Mutant SOD1 Recapitulates the Mitochondrial Dysfunction Associated with ALS
I. Hervias, M. S. Henning, M. Dumont, G. Manfredi; Neurology/Neuroscience, Weill Medical College of Cornell University, New York, NY
Amyotrophic lateral sclerosis (ALS) is a progressive, neurological disorder characterized by motor neuron degeneration. Mutations in Cu,Zn-superoxide dismutase (sOD1) are responsible for 20% of familial ALS cases, via a toxic gain of function mechanism. SOD1 is mostly cytosolic, but a portion is localized in the mitochondrial intermembrane space (IMS). In this study, we have attempted to elucidate whether the IMS localization is responsible for the mitochondrial abnormalities observed in SOD1-ALS, or if a secondary mechanism via cytosolic SOD1 is at fault. We have stably transfected NSC34 motor neuronal cells with wild-type sOD1, as well as G93A and G85R mutant sOD1, either with or without an IMS targeting signal. The toxic effects of various cellular substrates were compared in cells expressing IMS and those in which IMS was deleted. Under metabolic stress conditions, such as serum deprivation and growth in poorly fermentable substrates, both IMS and untargeted sOD1 mutants resulted in increased toxicity. Mitochondrial oxidative stress with rotenone, coupled with glutathione depletion, also caused increased toxicity, in both targeted and untargeted mutants. We have recently generated transgenic mice expressing IMS-targeted G93A sOD1, and performed an initial phenotypic and biochemical characterization. Despite very low protein expression in the IMS, male transgenic mice (but not females) were chronically hypoxic and displayed a moderate motor impairment, as compared to non-transgenic littermates. Similar to their non-IMS targeted counterparts, these mice do not have a cognitive impairment. At ten months of age, male transgenic mice had impaired respiratory chain function in neural mitochondria. The difference between males and females is intriguing and needs to be further elucidated. These results indicate that IMS localization of mutant sOD1 can recapitulate the mitochondrial dysfunction found in cells and mice expressing mutant sOD1, and suggest that the IMS portion of the mutant protein is responsible for this dysfunction.

Nuclear Import-related Protein Expression at Multiple Steps Is Altered Following Inhibition of Long-term Potentiation (LTP) in a Rat Model
L. G. Sheffield; Pathology, SUNY Downstate Medical Center, Brooklyn, NY
In Alzheimer’s disease (AD) brain, a close association between nuclear pores and the paired helical filaments that form neurofibrillary tangles has been observed ultrastructurally. In addition, neuronal contour irregularity was more common in AD than controls and was frequently associated with neurofibrillary tangles. Moreover, cytoplasmic accumulation of nuclear transport factor 2 (NTF2) was found in a subset of hippocampal neurons in AD cases (J Neuropath Ex Neurol 65:45-54, 2006). In ongoing studies of the distribution of cytoplasmic transport-related proteins in brain, sections from rat brain were immunohistochemically labeled using antibodies directed against four nuclear import-related proteins: Nup62 (nucleoporin p62, BD Transduction), NTF2 (BD Transduction), karyopherin-α (NPI-1, Zymed), and importin-β1 (NTFp97, Affinity BioReagents). In an earlier study (J Neurophysiol Exp Neurophysiol 64:443, 2005), we reported that sparse neurons in the dentate gyrus and cortex were labeled with karyopherin-α, while importin-β1 immunoreactivity was found in neuronal nuclei throughout the rat brain. In the present study, Nup62 labeling was consistent with that found in human brain (e.g., all nuclei exhibited punctate labeling of the nuclear contour), however NTF2 labeling was not visible in normal cells. For all four antibodies, immunoreactivity was much more extensive in areas affected by prior induction and subsequent inhibition of LTP. Strong immunopositive labeling was observed in neuronal cytoplasm extending with the dendrites of numerous neurons in the ipsilateral treated hemisphere as well as in the most medial hippocampal neurons of the contralateral hemisphere. These results lend support to the hypothesis that neuronal expression of nuclear import-related proteins may be activity dependent. Changes in neuronal activity during neurodegeneration may lead to alterations in expression and localization of the nuclear import system, and contribute to formation of pathophysiological markers such as neurofibrillary tangles.

Abundance of Gigaxonin in the Nervous System and Determination of the Ubiquitination Activity of the Gigaxonin Mediated E3 Ligase in Disease
P. Bonnot, K. Yamazaki, D. Cleveland; Cell Biology, LICR, La Jolla, CA
Mutations in the gene encoding gigaxonin are causative for the fatal, early-onset recessive neurodegenerative disorder Giant Axonal Neuropathy (GAN). A crucial role for gigaxonin in neuronal maintenance was suggested by impairment of the motor/sensory tracts of the peripheral and central nervous systems. GAN, as many other neurodegenerative disorders points to the essential role of cytoskeleton architecture in neuronal function, and especially to the implication of Intermediate Filament disorganization in neurodegeneration. We studied here the expression pattern and the abundance of gigaxonin in various nervous tissues and compared the activity of the gigaxonin-E3 ligase with the wild type protein and the disease associated mutants. Using monoclonal antibodies directed against gigaxonin and extracts from lymphoblast cell lines derived from GAN patients, multiple disease causing GAN mutants are shown to be unstable, demonstrating that GAN is caused by the loss of function of gigaxonin. Gigaxonin is shown to accumulate only to an extremely low level throughout the peripheral and central nervous systems and to be unaffected in multiple mouse models that develop progressive motor neuron disease from expression of an ALS-linked mutation in SOD1. In order to determine the activity of the
A Metabolic Insight into the Dopaminergic, Glutamatergic, and Na+-K+-ATPase Interactions of Schizophrenia

E. K. Nanitos1, T. Wallis2, S. B. Parekh3, W. A. Bubba4, C. Race,5 V. J. Balcar2; 1Anatomy and Histology, The University of Sydney, Camperdown, Australia, 2School of Molecular and Microbial Sciences, The University of Sydney, Camperdown, Australia, 3Pathology, The University of Sydney, Camperdown, Australia, 4Prince of Wales Medical Research Institute, The University of New South Wales, Randwick, Australia

The neuronal death in schizophrenia is thought to involve the interactions of both the dopaminergic and glutamatergic systems. This is partly because various dopamine antagonists are able to alleviate some of the symptoms of schizophrenia and there have been some shown to down-regulate glutamate transport (GLuT). Additionally the glutamate N-methyl-D-aspartate (NMDA) receptor antagonism ketamine was able to show some of the positive and negative symptoms of schizophrenia. The present study explores the effect of dopamine agonist bromocriptine mesylate and antagonists (remoxipride hydrochloride and haloperidol) in order to determine the acute effect of the dopaminergic modulation on brain metabolism. Ouabain, a potent inhibitor of the Na+-K+-ATPase and also an inhibitor of GLuT was used to examine a possible link between these systems as dopamine antagonists have previously been shown to cause decreases in GLuT without direct interaction with the transporters. The distribution of 13C label into isomers of brain metabolites was studied in Sprague-Dawley rat brain cortical tissue slices using 13C and 1H NMR spectroscopy. Bromocriptine (10μM and 100μM) caused a significant dose-dependent decrease in the net flux of 13C into Krebs cycle intermediates and the glutamate-glutamine cycle. Decreases in metabolite pool sizes also occurred. Remoxipride hydrochloride (20μM) and haloperidol (10μM) caused significant increases in remoxipride and haloperidol (10μM) were greater than those at 20μM. Consequently the effects of ouabain could be placing the tissue in a state of metabolic deprivation due to a lack of glutamate entering the astrocytes upon clearance from the synaptic cleft, thus resulting in compensatory increases in 13C flux into Krebs cycle intermediates. This suggests that the link between dopaminergic and glutamatergic systems does not involve regulation of, or, signalling by, Na+-K+-ATPase.

SMN Knockdown in PC12 Cells: Assessing a Role for SMN in Neuronal Differentiation

M. Bowserman, D. Shalley, R. Kohary; Cellular and Molecular Medicine, Ottawa Health Research Institute, and the University of Ottawa, Ottawa, ON, Canada

Spinal muscular atrophy (SMA) is the most common genetic disease resulting in infant mortality due to severe loss of α-motor neurons. SMA is caused by mutations or deletions of the ubiquitously expressed survival motor neuron (SMN) gene. The SMN protein is found in all cells studied in both the cytoplasm and punctate nuclear structures called Gemini of coiled bodies, which play a role in RNA metabolism. The Smn protein is also a part of a multiprotein complex, an assembly of ribonucleoproteins (RNP). Thus, SMN is considered an important housekeeping gene playing a role in fundamental cellular events such as snRNP assembly and processing of most pre-mRNAs. Although this housekeeping function of SMN is very well described, why a motor neurons of SMA patients are specifically affected is not clear. Using PC12 cells as our model system for neurogenesis, we have established a clonal line (C-59) with an 85% decrease in Smn levels, achieved through the use of short hairpin RNA interference (shRNAi). We demonstrate that Smn knockdown in PC12 cells leads to defects in neurogenesis, an increased incidence of beading and swelling along the neurites, defects in modulation and localization of F-actin and GAP-43, an increase in RhoA activity, and a decrease in Cdc42 activity. Furthermore, we observed an alteration in profilin II mRNA splicing, resulting in 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. This leads to an increased formation of the ROCK/pIla complex and an inappropriate activation of the RhoA/ROCK pathway, resulting in the observed defects in neurogenesis and altered cytoskeletal integrity. Taken together, this study results in the first description of a molecular mechanism underlying SMA pathogenesis and highlights new targets for therapeutic intervention for this devastating disorder.

Episodic Ataxia Type 2 Mutants Disrupts the Membrane Trafficking of Human P/Q-type Ca2+ Channels

C. Jeng, 1M. Sun; 2T. O. Tang; 1Institute of Anatomy & Cell Biology, National Yang-Ming Univ., College of Medicine, Taipei, Taiwan, 2Dept. of Physiology, National Taiwan Univ., College of Medicine, Taipei, Taiwan

Episodic ataxia type 2 (EA2) is an autosomal dominant neurological disorder associated with mutations in the gene encoding pore-forming α1A subunits of human P/Q-type calcium (CaV2.1) channels. Virtually all CaV2.1 channels harboring EA2-related mutant α1A subunits display loss-of-function phenotypes. The exact mechanism of how mutant α1A subunits cause such clinical EA2 features as cerebellar dysfunctions, however, remains unclear. Our previous functional studies in Xenopus oocytes support the idea that both missense and nonsense EA2 mutants may exert prominent dominant-negative effects on wild-type CaV2.1 channels. To further pursue the mechanism underlying this dominant-negative effect, we examined the effect of EA2 mutants on the subcellular localization pattern of GFP-tagged wild-type CaV2.1 channels in HEK293T cells. In the presence of EA2 mutants, wild-type CaV2.1 channels displayed a significant deficiency in membrane targeting and a concurrent increase in cytoplasm retention. The cytoplasmic fraction of wild-type CaV2.1 channels co-localized with an endoplasmic reticulum (ER) marker, suggesting that a significant amount of wild-type CaV2.1 channels was trapped in ER in the presence of EA2 mutants. Taken together, our data suggest that EA2 mutants may induce significant ER-retention of wild-type α1A subunits, thereby disrupting the membrane trafficking property and suppressing the functional expression of CaV2.1 channels.

Studies of Hirano Bodies and Oxidative Stress in Neuroglioma Cells

R. Furukawa, C. Cubenas, M. Fechheimer; Cellular Biology, University of Georgia, Athens, GA

Hirano bodies are paracrine-stabilizing F-actin aggregations associated with a variety of conditions including aging, Alzheimer’s, Parkinson’s, diabetes, and other diseases. The physiological function of Hirano bodies is unknown. In an in vitro model for Hirano body formation was developed by expressing mutated forms of the 34 kDa actin-bundling protein (Maselli et al., 2002, 2003). The altered forms of the protein are termed α1A (aa 124-295) which lacks an amino terminal inhibitory region, and ΔEF1, which has mutations that affect intramolecular domain-domain interaction. Both forms exhibit activated actin bundling that causes formation of Hirano bodies. Because Hirano bodies are associated with aging and late onset diseases in which oxidative stress is implicated as a major factor, we investigated possible relations between oxidative stress and Hirano bodies. Experiments were conducted in WT (wild-type) H4 neuroglioma cells, and H4 cells transfected with either the enhanced green fluorescence protein (EGFP) or CT-EGFP. Application of oxidative stress results in formation of numerous Hirano bodies visualized by fluorescence microscopy. Later, large inclusions are present within large membrane bound organelles resembling autophagosomes. The viability of cells with and without Hirano bodies was assessed 24 hours after application of oxidative stress. All cell lines showed increased cell death with either increasing H2O2 or menadione sodium metabisulfitel concentrations. The cells expressing CT-EGFP had a significantly greater level of death, indicating that Hirano bodies increased cell susceptibility to oxidative stress. Pretreatment of the cells with the anti-oxidant N’N-dimethyl-thiothiourea restored cell viability to control levels in all cell lines, indicating that the cell death was caused by oxidative stress. Future experiments will investigate mechanistic details of these findings to better understand a possible relationship between Hirano bodies, oxidative stress, and late onset diseases. Supported by NIH NS 04645101.

The SPG20 Spastic Paraplegia Protein Spartin is Monoubiquitinated and Involved in the Degradation of Epidermal Growth Factor Receptor

J. C. Bakowska, 1H. J. Japil, 2P. Fatshedinn, 3R. Puertolato, 1C. Blackstone; 1NYU Medical Center, MD, 2NIHLAB, Bethesda, MD

Troyer syndrome (SPG20), one form of the hereditary spastic paraplegia (HSPs), characterized by spasticity of the lower limbs, dysarthria and shortness of stature is caused by a frameshift mutation in the spartin gene, which encodes a predicted 666-amino acid protein of unknown function. We have previously demonstrated by using yeast two-hybrid and pull-down assays that spartin interacts with the receptor protein tyrosine phosphatase (RPTP) and prompted us to test the function of spartin in the process of endocytosis and sorting of the cargo proteins. Immunoprecipitation experiments revealed that endogenous spartin is constitutively monoubiquitinated and examination of different deletion constructs suggested that spartin is polymonoubiquitinated. In addition, spartin binds to ubiquitin as shown by pull-down assays and yeast-two hybrid tests. Immunofluorescence microscopy studies demonstrated that over-expressed full-length spartin was predominantly cytoplasmic with distinct vesicular staining. The vesicular localization of spartin is a highly mobile fraction as revealed by fluorescence recovery after photobleaching analysis. We found that spartin is recruited to endosomes by the dominant form of GFP- Vps4-A and HA-tagged spartin co-localized with Flag-Exp15 within vesicular structures in HEK cells at steady state. We also investigated the localization of over-expressed spartin after EGF stimulation. We uncovered extensive co-localization of HA-spartin with Flag-Exp15 and EGF-Alexa at 5, 10, and 30 min after stimulation with labeled EGF. Over-expression of full-length 494
spartin in HeLa cells resulted in the significant decrease in the degradation of EGFR as demonstrated by Western blot and immunofluorescence staining. A similar effect to a lesser degree was observed after depletion of spartin by a specific siRNA. Our results suggest that spartin might play a role in the sorting of ubiquitinated endosomal cargo.

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**Cellular Localization and Interactions of the Hereditary Spastic Paraplegia (SPG21) Protein, ACP33/maspadrin**

M. C. Hanna, C. D. Blackstone; Cellular Biology Unit, NINDS/NIH, Bethesda, MD

Hereditary spastic paraplegias comprise a group of disorders characterized by lower extremity weakness and spasticity, sometimes accompanied by dementia and other neurological impairments. Most syndrome, one form of hereditary spastic paraplegia, is caused by a mutation in the ACP33/maspadrin gene, whose protein product, while ubiquitously expressed in most cell types, remains functionally unknown. Our objective is to gain insight into ACP33/maspadrin function by investigating its subcellular localizations, protein-protein interactions, and consequences of gene expression elimination. As a first step toward determining ACP33/maspadrin function, we investigated potential involvement of the protein in membrane trafficking in neurons. Using Western blot analysis in combination with specific antibodies the protein was detected in mouse, monkey, and lamprey brain extracts. Using immunofluorescence, ACP33/maspadrin was observed in punctate structures in neuronal cell bodies, where it partially co-localized with gamma-adaptin, a marker for the trans-Golgi network (TGN), as well as down the axon length, and at the axon terminal with where it colocalized with synaptophasin. Immunoprecipitation experiments followed by excision of Coomassie-stained protein bands and analysis by matrix assisted laser desorption ionization (MALDI) mass spectrometry identified a possible interaction with ALDH1H1A, an aldehyde dehydrogenase family member of unknown function. Co-immunoprecipitation of myc-ALDH1H1A with endogenous ACP33 using various ACP33 antibodies confirmed the interaction, and negative results with other antibodies against other aldehyde dehydrogenase subtypes suggests relative specificity. As an attempt to create a marine model of SPG21 and further investigate the loss of function of the protein an ACP33/maspadrin knockout mouse has been generated, which is currently being characterized. Our results show subcellular localization of ACP33 in the TGN and at the axon terminal in neurons. These results support the protein a role in membrane trafficking events and may function in conjunction with a metabolic enzyme, ALDH1H1A, to fulfill this function.

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**Differential Synaptic Protein Expression Profiling of Rats Fed with a High Fat Diet and Correlation with Brain-derived Neuronal Diseases Using a Proteomics Approach**

W. Lan, J. Guhaniyogi, M. J. Horn, B. Graham, J. Xia; 1Prospect Biosystems, LLC, Newark, NJ, 2Pel-Freez Biologicals, Rogers, AR

It is well known that a diet high in saturated fat (HF) not only leads to hyperlipidemia and hypercholesterolemia which increase the risk of cardiovascular diseases, but also increases oxidative stress in brains and leads to synaptic dysfunction. Despite tremendous progress, there are still some limitations for completely explaining the correlation of neuronal diseases and HF diet. Recently developed neuroproteomics has made it possible to understand the brain function in system-based approaches. However, the current proteomics technologies face a huge challenge due to the brain complexity, such as large numbers of post-translational modifications and the wide dynamic range of protein abundance. We have developed a powerful and robust technology (Edge™) for proteomics sample fractionation to reduce sample complexity and increase importance for low abundance protein isolation and biomarker discovery. In this study, we applied Edge technology and other proteomics techniques to perform a comparative analysis of synaptic protein expression profiles of rat brains with a HF diet and a control diet. Both rat brain cortex and control diet were homogenized and fractionated using Edge technology. Synaptosomal protein fractions were analyzed by western blot. The amounts of oxidized proteins containing carbonyl groups were measured by an Oxyblot kit. Selected fractions were further separated by two-dimensional gel electrophoresis. Interesting protein spots were excised, digested and identified by MALDI-TOF/TOF. The results demonstrated a possible new avenue to understand the correlation between HF diet and many neuronal diseases. In addition, Edge technology combined with other proteomics techniques likely will result in the discovery of novel biomarkers for HF diet related neuronal diseases.

2699

**Neuregulin Enhances Nerve Regeneration through Synthetic Biocompatible Tubes**

I. Alcalde, I. De la Fuente-Ayuso, O. L. Gamboa; 1Dept. of Cell Biology, Histology and Pharmacology, University of Valladolid, Valladolid, Spain, 2Incyel, University of Valladolid, Valladolid, Spain

**Objectives:** When in a sectioned nerve the proximal and distal stumps are separated due to traumas, retractions, surgical resections, etc., and surgical approximation is not possible, it is necessary a bridge so that regeneration of nerve fibers can occur. In this study we have used tubes of biocompatible material embedded in Neuregulin (NRG-1) as a bridge between sectioned nerve stumps. **Methods:** Sciatic nerves from New Zealand rabbits were sectioned under anaesthesia and a segment was removed to create a 3 cm gap between proximal and distal stumps. In this gap we inserted and sutured a poly (L-lactide-co-glycolide) tube coated with NRG-1 10 nM. The animals survived for 75 days after which they were perfused under anaesthesia using paraformaldehyde fixative and tissue was processed for immunohistochemistry and semithin sections. Results: Rabbits treated with NRG-1 exhibits great Schwann cell proliferation from proximal and distal stumps. Schwann cells invade the tubes and form bands of Bügner. Axonal regeneration is enhanced in these treated animals and great amounts of remyelinating fibers are present. Conclusions: A poly (L-lactide-co-glycolide) tube filled only with NRG-1 is an interesting model of prosthesis to achieve correct axonal regeneration and remyelination in long distances gaps. (Supported by a grant FIS PI03/1533)

2700

**Lethal Giant Larvae (Lgl) Controls the Polarized Delivery of Fragile X Protein and mRNA in Drosophila Neurons**

M. Pinter, P. Estes, S. Srivivasan, D. C. Zarsenus; Molecular and Cellular Biology, University of Arizona, Tucson, AZ

Fragile X protein (Fmrp) is an RNA binding protein thought to function in neural development and plasticity by controlling the transport and translation of target mRNAs. Fmrp deficiency leads to defects in neural morphology and function and causes Fragile X syndrome, the most inherited form of mental retardation. Recently, we took a forward genetic approach to identify novel functional interactors of Fmrp in Drosophila and identified lethal giant larvae (lgl), a tumor suppressor involved in the establishment and maintenance of cell polarity. Through a combined genetic, biochemical and cell biological approach, we have shown that Lgl as well as the PAR cell polarity complex are novel functional partners of Fmrp. Furthermore, we have identified a subset of mRNAs associated with the Fmrp/Lgl complex. Taken together, our previous work suggests that Lgl functions with Fmrp and a subset of target mRNAs during synaptic development and/or function. To elucidate the molecular mechanism by which Lgl regulates Fmrp/mRNA complexes during neural development we have developed an imaging system for Lgl/Fmrp/mRNA in Drosophila neurons. Primary neurons derived from the larval central nervous system exhibit well-developed and polarized neurites, which contain motile Fmrp/Lgl particles. Furthermore, Fmrp colocalizes with target mRNAs within developing neurites. We examined the distribution Fmrp granules in an lgl mutant background and found that despite the presence of normal neuronal morphology, most Fmrp is distributed in the cell soma and excluded from neurites. Interestingly, overexpression of a phospho-mutant form of Lgl (LglDA) specifically abolishes the localization of Fmrp in the axon but not in the dendrites. Together, our data suggest that we have uncovered a novel mechanism for the polarized delivery of Fmrp and associated target mRNAs in neurons, which may provide new insights into the molecular mechanisms of neuronal development and Fragile X syndrome.

2701

**Investigating Potential Bacterial Sources of Dopaminergic Neurotoxicity in C. elegans**

J. Armagost, J. Blalock, T. Hodges, J. B. Olson, G. A. Caldwell, K. A. Caldwell; Biological Sciences, The University of Alabama, Tuscaloosa, AL

Parkinson’s disease (PD) involves the progressive loss of dopamine (DA) neurons from the substantia nigra pars compacta (SNCs). Genetic forms of PD account for only 5-10% of known cases and environmental factors appear pivotal to sporadic causality. Evidence from both environmental and genetic studies of PD suggests that overloading the ubiquitin-proteasome system is a causative risk factor for PD. Furthermore, when proteasome inhibitors were injected directly into the brains of rats, PD-like symptoms resulted (McNaught et al., 2004; Ann Neurol. 56:149-162). Many proteasome inhibitors are isolated from bacterial strains within the order Streptomycetales, which are well known for the production of secondary metabolites. To determine if exposure to Actinomycetes could cause DA neurodegeneration in C. elegans, we exposed worms to four species of Streptomycetes (S. lividans, S. venezuelae, S. griseus, and S. coelicolor). When worms were exposed to either S. griseus or S. coelicolor, worms exposed to S. lividans showed no detectable neuronal degeneration. However, worms exposed to S. venezuelae displayed neuronal degeneration that increased over time and was specific to DA neurons. The causal factor is excreted by these species, as conditioned media are sufficient for inducing the neurodegenerative effect. Initial efforts to purify the factor(s) revealed that the same factor may be excreted by both species. Furthermore, it is lipophilic and does not fit the molecular profile of known proteasome inhibitors. A systematic analysis of various enriched media is underway to determine which formulations elicit production of this secondary metabolite. We are also analyzing the effect of the excreted factor in combination with known genetic factors linked to PD, such as excess endogenous DA production, as well as LRKK2 and alpha-synuclein expression in C. elegans DA neurons.
2702

Genetic Interactions Among Cortical Malformation Genes Mediate Susceptibility to Epileptic-Like Convulsions in C. elegans
C. J. Locke, S. N. Williams, E. M. Schwarz, G. A. Caldwell, K. A. Caldwell; 1Biological Sciences, The University of Alabama, Tuscaloosa, AL, 1Division of Biology, California Institute of Technology, Pasadena, CA

Haplosufficiency of LIS1 results in isolated lissencephaly sequence (ILS) or Miller-Dicker syndrome (MDS), neurological disorders characterized by intractable seizures long associated with defects in neuronal migration. Notably, MDS is typically more severe than ILS due to an additional deletion of a LIS1-localizing gene, I4-3-4episilop. Along with I4-3-4episilon, other genes have been shown to function in the ILS pathway, such as those encoding Nde1, dynec, CDK5, and p35. Accordingly, we have demonstrated that lis-1 mutants and dynec (dc-1) RNAi-treated escapee animals of C. elegans can exhibit epileptic-like convulsions in the presence of low concentrations of pentyleneetetrazeol (PTZ), an antagonist of GABAreceptor. Moreover, such worms do not have overt neuronal migration defects. Instead, LIS-1 pathway defective worms expressing unc-25::SNB-1::GFP (synaptobrevin) reproducibly exhibit a presynaptic clustering of vesicles, implicating aberrant release of GABA as causative for the convulsive phenotype. To determine if other genes functioning in the LIS1 pathway regulate vesicle trafficking and neuronal excitability, we identified putative orthologous worms of ILS1 pathway members through bioinformatics resources, such as CarpeDB (http://www.carpedb.ur.edu), and targeted them for phenotypic knockdown by RNAi feeding. Indeed, we observed significant changes in the distribution of unc-25::SNB-1::GFP at GABAergic synapses with multiple RNAi targets, including putative LIS1-1 pathway members, in wild-type (N2) and sensitized (lis-1 and unc-25 mutant) backgrounds. We hypothesize that diminished rates of GABA release contribute to postsynaptic overexcitation, which, like in LIS-1 pathway defective worms, is manifested as enhanced susceptibility to PTZ-induced convulsions. These findings illustrate an evolutionary conservation of LIS1 pathway function between C. elegans and humans and implicate cytoskeletal genes in the epileptic consequences of classical lissencephaly.

2702A

A Defect in the Metabolism of the Signaling Lipid, PI3,5P2, Causes Neurodegeneration in Mice
Y. Zhang, C. Y. Chow, S. C. Richardson, R. C. Piper, J. R. Westrick, M. H. Meisler, L. S. Weissman; 1Department of Cell and Developmental Biology, Life Sciences Inst., University of Michigan, Ann Arbor, MI, 2Department of Human Genetics, University of Michigan, Ann Arbor, MI, 3Department of Physiology, University of Iowa, Iowa City, IA

The low abundance signaling lipid, phosphatidylinositol 3,5-bisphosphate (PI3,5P2) is produced in the endocytic pathway. Its physiological functions are not known. PI3,5P2 is unique in that its levels change dramatically in response to specific stimuli. For example, in the yeast, S. cerevisiae, exposure to hyperosmotic shock, causes PI3,5P levels to transiently rise more than 20 fold within 5 minutes. The return to basal levels, even in the continued presence of osmolyte, is achieved by 30 minutes. As an approach to determine the physiological roles of PI3,5P2 in mammals, we have tested and found that proteins required for synthesis and turnover of PI3,5P2, are present in all tissues examined. This wide distribution suggests that PI3,5P2 plays multiple roles in signaling events that occur on internal membranes. Here we report that mice hypomorphic for the PI3,5P2 regulator, Vac14, die soon after birth. While development appears to be normal, there is massive neurodegeneration in both the central and peripheral nervous system. Cell bodies of multiple neurons are vacuolated, and large holes are present in areas where neurons should be present. Vacuolation appears specifically in neurons, however, other cell-types have the potential to form large vacuoles. These vacuoles appear first in neurons that suggest that either PI3,5P2 functions in a neuronal-specific compartment and/or that neurons by virtue of their long processes are more sensitive to general defects in membrane transport. These findings strongly suggest that the general signaling lipid, PI3,5P2, is specifically essential for neuronal survival, and raise the possibility that selected human neuropathies are caused by mutations in PI3,5P2 signaling pathways.

2703

Matrix Metalloproteinase Inhibition Modulates the Correlation between Lipid-induced Atherosogenesis and Lipid-induced Platelet Activation
D. F. Saladin; Chemistry/Biochemistry, College Misericordia, Dallas, PA

Our model of lipid-induced early atherosogenesis in 300 g male Sprague-Dawley rats has been well characterized by transmission electron microscopy (TEM), showing typical myofoibroelastic changes in the aorta. These changes are seen after the rats receive IV infusions of Lipofundin-S dosed at 6 g fat/day/kg body wt for 10 days. Hyperglycieridemia results after one day. At day 10, platelet hyperactivity (hyperaggregation) is noted in rats receiving lipids vs. controls given an equivalent volume of saline (p<0.001), cAMP (a second messenger platelet inhibitor) generation measured in pmol/mg ptoein/min was generated linearly from isolated platelet membranes. However, cAMP levels were lower (P<0.05) vs. those observed in controls. A forskolin probe demonstrated that these lowered levels were caused by a triglyceride-induced change in the catalytic subunit of adenylyl cyclase. A separate series of experiments were performed with 25 rats. Five were no-treatment controls, 5 were saline controls, 5 received the 10-day lipid treatment, 5 received a MMP inhibitor intraperitoneally, and 5 were given lipid plus MMP inhibitor. As expected, the aortas showed no changes in both of the control groups, but neither did the MMP inhibitor alone. The lipid-treated animals showed the previously-described focal myofibroelastic changes indicative of early atherosogenesis. However, almost no such changes were seen in the rats receiving lipid plus MMP inhibitor. Although we have not yet tested the effect of the MMP inhibitor on the platelets directly, the MMP inhibitor was shown to significantly inhibit the atherosogenesis, well-known to be enhanced by activated (hyperactive) platelets, in the present model induced by hyperglycieridemia.

2704

Glucocorticoid-induced TNFR Family-related Receptor Signaling Exacerbates Hapten-induced Collitis by CD4+ T Cells
B. Choi, S. Lee, S. Lee, Y. Kim, W. Kang, K. Kim, S. Sakaguchi, J. Suh, T. Kim, B. Kwon; 1Immunology, Immunomodulation Research Center, Ulsan, Republic of Korea, 2Experimental Pathology, Institute for Frontier Medical Science, Kyoto, Japan, 3Pathology, Ulsan University Hospital, Ulsan, Republic of Korea, 4LSU Eye Center, New Orleans, LA

Glucocorticoid-induced tumor necrosis factor receptor family-related gene (GTR) has been reported to be expressed on the activated T and CD4/CD25 regulatory T cells (Treg). GTR stimulation not only neutralizes the suppressive effect of Treg, but also augments activation, proliferation and cytokine production of effector T cells. To test the role of GTR in 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis, TNBS-injected Balb/c mice were treated with agonistic anti-GITR mAb. Anti-GITR treatment increased the death rate compared to rat IgG- treated mice. Typically the death occurred within 4 days after the TNBS injection when the mice were treated with anti-GITR. The mice that survived anti-GITR treatment suffered from severe inflammation in their entire intestines. CD4+ T-depletion protected the mice from colitis; even anti-GITR effect was not apparent. In contrast, CD8+ T-depletion showed less protective effect than CD4+ T-depletion did. Stimulation of GITR enhanced the production of pro-inflammatory cytokines including IFN-γ, TNF-α, IL-6, and IL-12. It also enhanced the humoral response such as serum levels of IgG, which was completely dependent on CD4+ T cells. Taken together, this study demonstrated that GTR signaling on CD4+ T cells is involved in the development and progression of colitis by enhancing both Th1 and Th2 type responses.

2705

E-Selectin Overexpression in HUVECs of Healthy Newborns from Mothers with Inactive Systemic Lupus Eritematosus Is Not Downregulated by Estradiol
E. Rodriguez, M. Collados, E. Zapata, A. Paez, L. Montano; 1Hoy Celular, Instituto Nacional de Cardiología, Mexico, Mexico, 2Instituto Tecnológico de Monterrey, Mexico, Mexico, 3Biotecnología, Instituto Tecnológico de Monterrey, Mexico

Systemic lupus erythematosus (SLE) is a severe autoimmune disease that affects mainly young adult women in reproductive age. There is ample evidence showing that a family history of SLE development and progress of colitis by enhancing both Th1 and Th2 type responses.

Novel Chemotherapeutic Agents for Readthrough of Nonsense Mutations
M. Shiouzka, K. Shimada, M. Arakawa, Z. Shijun, A. MacKerell, Y. Takahashi, D. Ikeda, Y. Nonomura, R. Matsuda; 1Dept. of Life Sci., The Univ. of Tokyo, Tokyo, Japan, 2The Institute of Microbial Chemistry, Tokyo, Japan, 3Dept. of Pharmacological Sci., Univ. of Maryland, Baltimore, MD

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Translation of rudimentary pathway induction is a promising method of therapy in a large variety of genetic disorders. We previously reported that megaminogly, a dipetide antibiotic, that binds to the ribosomal decoding site and alters translational accuracy, successfully restored dystrophi expression with less toxicity than gentamicin in mdx mouse which carries a premature termination codon (PTC) in the dystrophin gene. In order to investigate more potent pathway inducer with less toxicity and measure readthrough efficiency with quantitative accuracy, we have established three transgenic mouse strains containing dual-reporter gene composed of beta-galactosidase and luciferase connected with different PTC (Ochre, Amber, Opal) region. Using these Tg mice, we confirmed the evaluation of readthrough activity in each tissue in vivo and the efficacy of antibiotic treatment in a dose-dependent manner by transdermal administration. Moreover, we found five novel compounds which targets nonsense mutations, such as C10H11N3O4, C6H10N2O4, C10H14N2O7, C8H15N3O4, and C2H15N3O8. These low-molecular weight drug candidates are sterically related negaminoglycine-like molecules identified via in silico screening from over one million compounds, and allows the cellular machinery to bypass the nonsense mutation and continue the translation process with subcutaneous injection daily for 7 days at 4mg/kg. The first and second series presented had similar efficacy to gentamicin in a dose-dependent fashion without subacute toxicity. The fifth series resulted in the most potent compounds with efficiency. The chemotherapeutic agents that overrides PTCs and its translational drug delivery may provide significant value in treating genetic diseases caused by 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.

2707 Characterization of Alternatively Spliced Transcripts of the NOD2 Gene: Intrinsic Modulation of the NOD2/RIP2 Signaling Pathway

P. C. Rosenstein,1 K. Huse,2 A. Till,3 S. Billmann,4 C. Sina,3 M. Platter,5 S. Schreiber;6 Institute for Clinical Molecular Biology, Kiel, Germany, 2Genome Research, FLI Leibniz Institute Jena, Jena, Germany, 3FLI Leibniz Institute Jena, Jena, Germany

Background: Alternative splicing contributes to the regulation of gene function by generating endogenous inhibitor or activator molecules. NOD2 is an intracellular receptor for the bacterial cell wall component muramyl dipeptide and plays an important role in initiating immune responses against cytovasive pathogens. NOD2 overexpression sensitizes intestinal epithelial cells towards bacterial cell wall components, activates the pro-inflammatory transcription factor NF-kB and induces the subsequent release of the chemotactic cytokine IL-8. Aims&Results: We assessed the function and regulation of several transcription isoforms of NOD2 generated by the skipping of the exons in the CARD-CARD and LRR region. It is shown that NOD2 splice forms are preferentially expressed in the normal colon and are upregulated by the anti-inflammatory cytokine IL-10 (CARD exon splicing) or infection with intracellular bacteria (LRR exon splicing). Most spliceforms lacking LRR exons interfere with the NOD2-induced NF-kB activation (reporter gene assay) and IL-8 release (ELISA). A short CARD-only isoform generated by skipping of exon3 with a subsequent frameshift interferes with the assembly of the “nodosome”, as it interacts with both, NOD2 and RIP2 and inhibits NOD2/RIP2 oligomerization upon ligand sensing. Conclusions: These data unveil another level of complexity in the regulation of intracellular innate immunity and may have important implications for the molecular understanding of NOD/NALP protein-driven disease pathophysiology.

2708 Sabiporide, a Specific Na+/H+ Exchanger Inhibitor, Attenuates the Blood-Brain Barrier Damage through Intracellular Ca2+ Overload

S. Patr1,2,3, S. Y. Lee,4 Y. Y. Jung1,4, E. Bak,5,6 C. Moon,1,4, Y. Jung1,4, 1Department of Physiology, Ajou University, Suwon, Republic of Korea, 2Brain Korea 21 Project, Brain Korea 21 for Molecular Science and Technology, Suwon, Republic of Korea, 3Medical Science Division, Korea Research Institute of Technology, Daejon, Republic of Korea, 4Brain Korea 21 for Medical Science, School of Medicine, Suwon, Republic of Korea

Cerebral ischemia induces disruption of blood-brain barrier (BBB), which leads to increased vascular permeability and the formation of brain edema. Formation of cerebral edema caused by vascular leakage is a major problem in various injuries of the central nervous system (CNS), such as stroke. Therefore, we examined the effect of a selective NHE inhibitor, sabiporide, on the BBB damage using in vitro and in vivo models. In mouse brain microvessel endothelial cells (bEnd.3 cells) exposed to oxygen-glucose deprivation (OGD), a significant increase in intracellular calcium ([Ca2+]i) was observed, and this was inhibited by sabiporide. Sabiporide and Ca2+ chelator treatment attenuates OGD-induced BBB permeability increase. Disruption of TJ proteins (ZO-1, Claudin-1) during OGD were significantly inhibited by treatment with sabiporide and Ca2+ chelator. In addition, sabiporide treatment decreased the BBB permeability and inhibited TJ proteins disruption in rat brain middle cerebral artery (MCA) occlusion model. These results suggest that sabiporide, has an important role in regulating [Ca2+]i during ischemia and thereby, preventing BBB damage. Therefore, sabiporide might be a potential therapeutic agent for cerebral edema. This work was supported by a grant (CRB2-300-001-1-0-02) from the Center for Biomedical Innovators of the 21st Century Frontier R&D Program, the Ministry of Science and Technology and by a grant (R-01-2005-000-10510-0) from Basic Research Program of the Korea Science and Engineering Foundation.

2710 Genetic Pathways That Regulate Stress Response, Aging, and Immunity in Caenorhabditis elegans

A. Aballay; Molecular Genetics and Microbiology, Duke University, Durham, NC

Genetic studies have led to the identification of Caenorhabditis elegans signaling pathways that are required for defense response in both nematodes and mammals, suggesting that despite the vast evolutionary gulf between nematodes and mammals, some of the underlying mechanisms of defense response may be similar. Our laboratory has recently dissected two conserved pathways required for C. elegans immunity. A p38 MAPK/CEG-3 pathway and a heat shock transcription factor HSF-1 pathway that is elicited by increased temperature independently of p38 MAPK/CEG-3. A number of genes encoding conserved effector molecules of the innate immune system as well as potentially relevant signaling molecules and transcription factors were identified. Transgenic lines carrying promoter-gfp reporters indicate that many of these genes are expressed in tissues that are in direct contact with pathogens and are crucial to prevent bacterial invasion. The HSF-1 defense response regulates heat-shock-mediated immunity independently of the p38MAPK/CEG-3 pathway and through a system of chaperones including small and 90 kDa inducible heat shock proteins. In addition, HSF-1 is needed for the effects of the DAF2 insulin-like pathway in defense to pathogens, indicating that interacting pathways control stress response, aging, and immunity. Our results also show that the HSF-1 pathway is required for C. elegans immunity against Pseudomonas aeruginosa, Salmonella enterica, Terixia pestis, and Enterobacter aerogenes, indicating that HSF1 is part of a pan-pathogen defense pathway. Fever is an ancient immune mechanism used by metazoans in response to microbial infections. While homothoraces are capable of internally increasing the body temperature, poikilothersms migrate towards warmer environments to increase their temperature in response to infections. The activation of the HSF-1 pathways by heat shock and its function in C. elegans immunity provides both a molecular explanation for the beneficial role of behavioral fevers in poikilothersms and a mechanism by which fever works in metazoans.

2711 Apoptosis and Achalasia in Pneumomiasis

M. G. Pacheco-Tovar,1 J. J. Bollain-y-Goytia,1 E. Vega-Memije,2 L. López-Robles,1 R. Herrera-Esparza,1 E. Avalos-Díaz;2 1Immunology, UABE, Universidad Autónoma de Zacatecas, Zacatecas, Mexico, 2Department of Dermatology, Hospital "Sanborns" Doctor Manuel Gonzalez, Mexico, DF, Mexico

Pneumomiasis is an autoimmune disease, characterized by intraepidermal blisters due to anti-desmoglein 1 and 3 autoantibodies. These autoantibodies target the desmosomal antigens inducing epithelial cell detachment, such phenomenon is called achalasia. Pneumomiasis autoantibodies are pathogenic because cause achalasia, their binding on the epidermal cell surface triggers a kinase mediated down signal transduction that results in cell detachment and desmosomal breaking down. Another mechanism involved in blistering is inflammatory cytokines such as TNF that possible induce apoptotic features on keratinocytes near to the blisters. Present work assesses the role of apoptosis in the blister formation in pneumomiasis. Twenty Pneumomiasis biopsies were studied by H&E staining, apoptotic features were defined by TUNEL. Expression of pro-apoptotic molecules such as Fas, Fasl and Caspase 3 and Hax were studied by in situ hybridization and immunohistochemistry. Cells subset infiltrated were studied by direct immunofluorescence with anti-CD3, CD4 and CD8 antibod...


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The Heat-Shock Factor HSF-1 Pathway Regulates Stress Response and Innate Immunity in C. elegans

V. Singh, A. Aballay; Mol Genetics & Microbiol, Duke University, Durham, NC

The heat shock transcription factor HSF-1 boosts immunity. HSF-1 is required for immunity against four bacterial pathogens—both insect and vertebrate hosts. This led us to examine other conserved pathways which might regulate immunity in C. elegans. Our work opens up the possibility that HSF-1 activators could be used for boosting immunity against bacterial infections, possibly also in an immunodeficient background.

2714

E2 of Hepatitis C Virus Modulates Cellular Proteins Related with TNF-Signaling

S. Lee, M. Lee, S. Jang; Dept. of Life Science, POSTECH, Pohang, Republic of Korea

Hepatitis C virus (HCV) is a major human pathogen to which about 170 million people are succumbing currently. HCV is a causative agent of acute and chronic hepatitis, which often leads to liver cirrhosis and hepatocellular carcinoma. It has been speculated that HCV possesses mechanisms capable of modulating host defense systems such as innate and adaptive immunity since it causes chronic disease in most of infected patient (about 80%). Recently, we reported that E2 of HCV augments persistent infection of HCV by blocking apoptosis of infected cells (Lee et al., 1995). In order to understand the molecular basis of the anti-apoptotic activity of HCV, we monitored cellular proteins related with TNF-signaling that is responsible for cell survival and pro-inflammatory responses. The subcellular localizations of the proteins related with TNF-signaling (TNFR1 and TNFR2) were monitored by immunocytochemical methods and a fractionation of cell extracts. TNFR1 and TRAF2 were enriched in lipid raft together with E2 in an E2-expressing cell line. Moreover, the amounts of these proteins and other TNF-signaling-related proteins (XIAP and survivin) were greatly increased in the E2-expressing cell. We also observed activation of NF-κB in the E2-expressing cell. We speculate that E2 of HCV modulates innate immunity of host cells by triggering pro-inflammatory response through the activation of TNF-signaling.

2715

Bafilomycin A1-Sensitive Pathway Is Required for the Maturation of Cystic Fibrosis Transmembrane Conductance Regulator

A. Niibori, T. Okiyoneda, K. Harada, T. Kohno, H. Kusuhara, T. Takada, T. Shuto, M. Suico, Y. Sugiyama, H. Kai; Molecular Medicine, Kumamoto University, Kumamoto, Japan

The maturation pathway of CFTR is thought to be non-conventional since CFTR maturation is inhibited by dysfunction of syntaxin 13, which is involved in protein recycling via endosomal pathway. In this study, to clarify whether the endosomal trafficking is required for CFTR maturation, we utilized a specific vacuolar H+ ATPase inhibitor, bafilomycin A1 (BafA1), which inhibits the protein trafficking from early endosomes. Our data showed that BafA1 decreased mature CFTR and induced accumulation of immature CFTR. Pulse-chase analysis showed that BafA1 inhibited the maturation of CFTR, but it slightly stabilized immature CFTR. Immunocytochemical analyses revealed that BafA1 induced the accumulation of CFTR in the juxta-nuclear region containing an endosomal marker. These results indicate that BafA1-sensitive pathway is required for CFTR maturation and emphasize that endosomal trafficking pathway is involved in the maturation of CFTR.

2716

Ectopic Calcification in mdx Mouse Skeletal Muscle III

N. Kikkawa, M. Shiozuka, T. Kogure, M. Suico, T. Takada, T. Shuto, H. Kusuhara, H. Kai; Molecular Medicine, Kumamoto University, Kumamoto, Japan

Duchenne muscular dystrophy (DMD) is a lethal, progressive muscle disorder caused by mutations in dystrophin gene. Structures of high electron density were observed in skeletal muscles of mdx mouse, an animal model of DMD, using an x-ray micro CT scanner. We referred to this structure as “ectopic calcification”, as its component was identified as hydroxyapatite from the x-ray diffraction pattern. No such calcification was found in B10 mouse, the normal control. Serum of mdx mice, compared with B10 mice, contained high level (40%, p < 0.05) of inorganic phosphate (Pi), while serum calcium level showed no significant difference. Pi being perceived as a factor for the ectopic calcification in mdx mice, cells obtained from mdx skeletal muscle tissue were cultured in media containing high level of Pi. Expression of osteopontin, an osteogenic marker, was detected by indirect immunofluorescence staining. Osteopontin expression in cells cultured in the presence of high Pi concentrations increased over time, whereas the expression was unchanged in cells cultured in media containing normal Pi level, suggesting that Pi induces ectopic calcification. As macrophage accumulation was found to be colocalized with ectopic calcification in mdx mice, relations between ectopic calcification and inflammation or degeneration/regeneration of muscle were suggested. The calcification is able to observe in live animals using x-ray micro CT, therefore it may serve as a non-invasive diagnostic marker to evaluate the therapeutic effects of DMD therapy.

2717

Antibodies against C-Terminus of Dengue Virus Nonstructural Protein 1 Show Cross-Reactivity with Endothelial Cells via a Molecular Mimicry Mechanism

S. Wan, C. Lin, Y. Lin; Microbiology and Immunology, National Cheng Kung University Medical College, Tainan, Taiwan

Infection with dengue virus (DV) causes diseases ranging from self-limited dengue fever (DF) to life-threatening dengue hemorrhagic fever and dengue shock syndrome (DHF/DDS). Vascular leakage, thrombocytopenia, and bleeding are the clinical manifestations associated with DHF, yet the mechanisms are not fully defined. Previous studies in our laboratory showed that anti-DV nonstructural protein 1 (NS1) antibodies (Abs) cross-reacted with endothelial cells. The potential candidate proteins recognized by anti-DV NS1 Abs include ATP synthase β-chain, vimentin, heat shock protein 60, and protein disulfide isomerase. The C-terminal amino acids (a.a.) 311-352 of DV NS1 show sequence homology with the candidate proteins. In this study, the role of NS1 C-terminus on dengue autoimmunity was investigated. We have deleted the a.a. 277-352 of DV NS1 to prepare truncated NS1 (nNS1), and generated anti-DV nNS1 Abs in mice. The endothelial cell binding activity of anti-DV nNS1 Abs was lower than that of anti-DV NS1 Abs. In addition, the endothelial cell binding activity of anti-DV NS1 Abs was inhibited by preabsorption with DV NS1 but not with DV nNS1 proteins. The anti-P311 (a.a. 311-330) and P331 (a.a. 331-350) titers of dengue patient sera were positively correlated with the binding
activity of dengue patient sera IgM with endothelial cells. Dengue patient sera showed lower binding activity to DV IN5N1 than to DV IN5N1 proteins. The endothelial cell binding activity of dengue patient sera could be inhibited by preabsorption with DV IN51, P311, and P331. These studies help to understand the molecular mechanisms of autoimmunity mediated by anti-DV NS1 Abs, and the potential implications of IN51 in dengue vaccine development.

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Identification of the Transcription Factors Responsible for Enhancement of Endothelin-1 Expression in Spontaneously Hypertensive Rats Vascular Smooth Muscle T. Yang,1,2 C. Chen-Kung,1 L. Mei-Hua,1 C. Jang-Yi,1 C. Chuang-Fay,1 C. Li-Tze,1 Graduate Institute of Basic Medical Sciences, Chang Chung University, Kwei-Shan Tao-Yuan, Taiwan, 1Institute of Biology and Anatomy, National Defense Medical Center, Taipei, Taiwan

Endothelin-1 (ET-1) expression in vascular smooth muscle cells (VSMC) of spontaneously hypertensive rats (SHR) at prehypertensive stage is higher than age matched Wista-Kyoto rats (WKY). ET-1 promoter and deletion mutants activity assay demonstrated at region -1309 to -1143 of ET-1 promoter was responsible for higher expression of ET-1 in SHR than WKY rats. At the region -1309 to -1143 contains GHF-Iand C/EBP binding sequence. However, there is no GHF-I expression in SHR VSMC, the other transcription factors POU homeodomain family will concern. By RT-PCR and EMSA assay, we found that the Oct-2, and CEBPβ1 expression in VSMC of SHR higher than WKY rats but no different expression of Oct-1. By EMSA and super shift assay, the results showed the Oct-1, Oct-2 and CEBPβ1 can all bind to the -1309 to -1143 of ET-1 promoter probe. It is conclude that the over expression of ET-1 in VSMC SHR than WKY rats were due to over expression of Oct-2 and CEBPβ1 in VSMC SHR.

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Apolipoprotein F Secreted by Antigen-presenting Cells Regulates the Secretion of Interleukin-1β in Human Endothelial Cells J. Jin,1 M. Rubin,1 R. Haeussler,1 H. Lehrauch,1 P. Rosenberg1, Cell Biology, Institute of Clinical Molecular Biology, Kiel, Germany

Apolipoprotein F (ApoF), a 10 residue protein, is secreted by antigen-presenting cells upon TLR/IL-1 receptor engagement. ApoF is a witness of the endoplasmic reticulum (ER) and is involved in the homeostasis of the protein quality control network. Our previous studies demonstrated that ApoF binds to the extracellular domain (ECD) of TLR4/5 (lipopolysaccharide) and IL-1R1, and regulates the synthesis and secretion of IL-1β. The objective of the present study was to further examine the role of ApoF in the regulation of IL-1β secretion. Human umbilical vein endothelial cells (HUVECs) were treated with LPS in the presence or absence of ApoF and the levels of IL-1β and other pro-inflammatory cytokines were measured by ELISA. Our results showed that treatment of HUVECs with LPS in the presence of ApoF, significantly inhibited the secretion of IL-1β. In contrast, treatment of HUVECs with LPS in the absence of ApoF, increased the secretion of IL-1β. These findings suggest that ApoF is a negative regulator of IL-1β secretion in human endothelial cells.

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PYPAF3, a PYRIN-containing Apaf-1-like Protein, is a Feedback Regulator of Caspase-1-dependent Interleukin-1β Secretion M. Rubin,1 R. Haeussler,1 J. Jin,1 J. P. Hafner,1 T. Suda,1 Immunology and Molecular Biology, Kanazawa University Cancer Research Institute, Kanazawa, Japan

PYPAF3 is a member of the PYPAF family of proteins which includes PYPAF1, 2, 5, 7, and 10 and are thought to play a role in the regulation of inflammation. PYPAF3 inhibits caspase-1-mediated IL-1β secretion in THP-1 cells. Previous studies have shown that PYPAF3 binds to caspase-1 and interferes with its activation. In the current study, we examined the role of PYPAF3 in the regulation of IL-1β secretion in THP-1 cells. THP-1 cells were differentiated into macrophages with PMA. The cells were then treated with LPS in the presence or absence of ApoF and the levels of IL-1β and other pro-inflammatory cytokines were measured by ELISA. Our results showed that treatment of THP-1 cells with LPS in the presence of ApoF, significantly inhibited the secretion of IL-1β. In contrast, treatment of THP-1 cells with LPS in the absence of ApoF, increased the secretion of IL-1β. These findings suggest that ApoF is a negative regulator of IL-1β secretion in human endothelial cells.

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Systematic Analysis of NOD2-dependent Transcriptome and Proteome Signature Patterns upon Muramyl-dipeptide Stimulation T. Kinoshita, Y. Wang, M. Hasegawa, R. Imauma, T. Suda; Immunology and Molecular Biology, Kanazawa University Cancer Research Institute, Kanazawa, Japan

The NOD2-dependent global cellular proteome characterized previously (Weichart et al., J Biol Chem 2005). These data identify a complex cellular network of proinflammatory and autoregulatory mechanisms that regulate the expression of NOD2, and the potential implications of tNS1 in dengue vaccine development.

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Associative Gag Multimers with Filamentous Actin Is Required for Equine Infectious Anemia Virus Production C. Chen,1 J. Jin,2 M. Rubin,1 L. Huang,1 T. Sturgeon,1 K. M. Wexel,1 D. B. Stolz,1 S. C. Watkins,1 J. R. Banburg1, D. A. Weiss,2 R. C. Montelaro2; 1Biochemistry and Molecular Biology, Colorado State University, Fort Collins, CO, 2Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, PA

A role for the actin cytoskeleton in retrovirus assembly and budding has long been speculated. However, the molecular and cellular mechanisms by which retroviruses exploit actin dynamics to produce progeny virion remain largely undefined. Our previous studies demonstrated differential effects of experimentally modified actin dynamics on virion production of equine infectious anemia virus (EIAV), a lentivirus related to HIV-1, suggesting an involvement of Gag-actin association in retrovirus production. In the current study, we have employed bimolecular fluorescence complementation (BIFC) to reveal intimate (<10nm) and specific interactions between EIAV Gag and actin, but not tubulin. A specific interaction between Gag and filamentous actin (F-actin) was also demonstrated by co-immunoprecipitation experiments involving the common seier protein gelsolin to solubilize F-actin. Mutagenesis analyses of Gag polyprotein revealed that deletion of capsid (CA) or nucleocapsid (NC) genes reduced Gag association with F-actin by 40% and 95%, respectively. Interestingly, a leucine zipper motif derived from a yeast transcription factor of the PAMPs within human cells. Variants in NOD2/CARD15, a member of the NLR gene family, which is pivotal in innate immune responses by recognition of pathogen-associated molecular patterns (PAMPs) within human cells. Variants in NOD2/CARD15 are thought to function in apoptotic and inflammatory pathways, and are characterized by three distinct domains: an amino-terminal pyrin domain, a nucleotide-binding and oligomerization domain, and a variable number of carboxy-terminal leucine-rich repeat domain. Recent studies have shown that Gag association with F-actin was increased by the PYD domain of Gag, which is essential for Gag association with F-actin. CA-mediated homologous dimerization of Gag contributed to the formation of Gag multimers as well. The transcription factor GCN4 could substitute for the NC domain function in mediating F-actin association, indicating that multimerization of Gag polyproteins, induced by the NC function but not specific sequence of the NC domain, is essential for F-actin association. CA-mediated homologous dimerization of Gag contributed to the formation of Gag multimers as well. The transcription factor GCN4 could substitute for the NC domain function in mediating F-actin association, indicating that multimerization of Gag polyproteins, induced by the NC function but not specific sequence of the NC domain, is essential for F-actin association. CA-mediated homologous dimerization of Gag contributed to the formation of Gag multimers as well. The transcription factor GCN4 could substitute for the NC domain function in mediating F-actin association, indicating that multimerization of Gag polyproteins, induced by the NC function but not specific sequence of the NC domain, is essential for F-actin association. CA-mediated homologous dimerization of Gag contributed to the formation of Gag multimers as well.

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Identification of C. elegans Genes Linked to Sensory Cilia Formation and Function
O. E. Blasco, M. R. Leroux; Molecular Biology and Biochemistry, Simon Fraser University, Burnaby, BC, Canada.
Sensory cilia play essential functions in the monitoring and physical environment and are now associated with several critical signaling and developmental processes. To uncover novel genes that are required for sensory cilia biogenesis/function, we took several complementary approaches using C. elegans as a model system. The studies include (i) comparison of the expression profiles of ciliated cells versus non-ciliated cells, (ii) bioinformatic identification of genes regulated by the ciliogenic transcription factor DAF-19, (iii) identification of DAF-19 target genes by microarray analysis comparisons of wild-type and daf-19 mutant strains, and (iv) screening for mutants with abrogated cilia function (e.g., dyf, or dye-filling defective mutants, where the cilia are abrogated and are not exposed to the external environment). To validate if the candidate genes identified possess roles in cilia biogenesis or function, we then assess if their expression is specific to ciliated sensory neurons, determine the localization of the protein using time-lapse microscopy, or for example, if they encode ciliary/infraflagellar transport components, and finally, analyze the corresponding mutant strains for ciliary defects and behavioral (e.g., sensory) phenotypes. Our studies have led to the identification of many novel putative ciliary genes, and we have gathered evidence for ciliary functions for several of these. Two basic classes of ciliary genes appear to exist, namely those that are required for the formation of cilia, and others that likely play specific roles in the sensory functions of cilia or perhaps in the differentiation of the various ciliated sensory neuron types. The ultimate aim of these studies is to identify all of the components required for cilia biogenesis, to define the components and molecular pathways used by cilia for its sensory functions, and finally, to uncover new ciliary proteins associated with human diseases.

Role of ErbB4 in the Regulation of Cardiomyocyte Biology
B. Icli,1 X. Peng,2 D. Sawyer3; 1Molecular Medicine, Boston University School of Medicine, Boston, MA, 2Medicine, Vanderbilt University, Nashville, TN
ErbB4 is essential for cardiac development, and transmits a prosurvival signal in cardiac myocytes when activated by its ligand Neuregulin. However, alternatively spliced ErbB4 isoforms are known to exist that can transactivate a proapoptotic signaling. The purpose of this study was to examine whether these isoforms exist in rat ventricular myocytes and allow for reciprocal regulation of cell fate by ErbB4 ligands. We demonstrate that adult rat cardiomyocytes express all known ErbB4 isoforms: J1M-a, J1M-b, CYT-1 and CYT-2. Cloning of the full-length ErbB4 showed that JM-b and CYT-1 are the dominant isoforms in adult rat ventricular myocytes (ARVM). Treatment of ARVMs with PMA or Doxorubicin induced ErbB4 nuclear translocation. However, we did not observe the proteolytic cleavage of ErbB4 following the PMA or Doxorubicin treatments, and a gamma-secretase inhibitor did not prevent nuclear translocation of ErbB4. In addition to ErbB4 nuclear translocation, doxorubicin treatment induced p53 and p21 levels without increasing Bax expression. AG1478, an EGFR and ErbB4 kinase inhibitor, inhibited doxorubicin-induced induction. Hence ErbB4 may not be a classical EGFR. However, ErbB4 functions as a novel EGFR and its role in cardiac disease remains to be determined.

Progerin, the Abnormal Protein Expressed in Hutchinson-Gilford Progeria Syndrome, Interferes with Mitosis in Both Progeria and Normal Cells
K. Cao,1 B. Capell,1 M. Erdos,1 K. Djabali,2 P. Collins1; 1National Human Genome Research Institute, Bethesda, MD, 2Columbia University, New York, NY
Classic Hutchinson-Gilford progeria syndrome (HGPS) is caused by a de novo point mutation (G600G) in exon 11 of the LMNA gene. This mutation activates a cryptic splice donor, resulting in a mutant lamin A protein (known as “progerin”) with a 50 amino acid internal deletion near the carboxy-terminus. During interphase, progerin anchors to the nuclear membrane and causes characteristic nuclear blebbing. Progerin’s localization and activity during mitosis, however, are completely unknown. We have analyzed progerin during mitosis in GFP-progerin-transfected HeLa cells and in human HGPS fibroblasts. In contrast to the diffuse cytoplasmic signal of GFP-lamin A in metaphase, GFP-progerin associated with membrane structures and formed large insoluble aggregates in the cytoplasm. Fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP) studies revealed slower protein turnover and the presence of an insoluble fraction of GFP-progerin during mitosis. Blocking farnesylation with farnesyltransfase inhibitors (FTIs) or the farnesylate incompetent CAAAX mutant Progerin-SLAM effectively restored the localization and dynamics of GFP-progerin to those of normal lamin A. In HGPS fibroblasts, double immunofluorescence studies with a lamin A/C antibody and a progerin specific antibody revealed similar abnormal aggregation of progerin in metaphase, and a delay in the reassembly of progerin into the nuclear envelope compared with wildtype lamin A in late mitosis. In addition, we found an increase in lagging chromosomes and binucleation in HGPS fibroblasts. Last, we present evidence that small amounts of progerin exist in normal cells as indicated by immunofluorescence, RT-PCR and Western blotting. Importantly, a significant portion of the normal cells that express progerin are also binucleated, implicating progerin causing similar mitotic defects in normal aging. Our findings provide the first evidence of mitotic abnormality in HGPS, and may shed light on the general phenomenon of normal aging.

Loss of Usp14 Alters ISG15 Levels
P. C. Chen,1 D. E. Zhang,2 G. A. Korbel,3 H. L. Ploegh,3 S. M. Wilson1; 1Department of Neurobiology, University of Alabama at Birmingham, Birmingham, AL, 2Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, CA, 3Department of Pathology, Harvard Medical School, Boston, MA
Usp14 is a member of the ubiquitin-specific protease (UBP) family of deubiquitinating enzymes. Usp14 has been shown to associate with the proteasome and its ubiquitin-hydrolyase activity is greatly enhanced following proteasome binding. In axiata mice, loss of Usp14 results in reduced level of ubiquitin, which suggests that Usp14 is required for recycling ubiquitin at the proteasome. Since ubiquitin and ubiquitin-like proteins have similarity in both sequence and structure, we investigated if Usp14 also has activity toward other ubiquitin-like proteins. Here we show that Usp14 has catalytic activity toward ISG15-VS in vitro, which suggests that Usp14 may function to de-conjugate ISGylated proteins in vivo. Consistent with this, immunoblot analysis of tissues extracts from axiata mice shows that monomeric ISG15 levels are significantly reduced in lung, kidney, and spleen. To further study the role of Usp14 as a de-conjugation enzyme for ISGylated proteins, we use mouse embryonic fibroblasts (MEFs) to study ISG15 signaling. Both monomeric and conjugated forms of ISG15 levels are reduced in the Usp14−/− MEFs. To investigate the relationships between ISG15, Usp14, and the proteasome, MEFs were treated with proteasome inhibitor MG132. In wild-type MEF, MG132 dramatically enhanced the production of ISG15 in wild-type MEFs. In contrast, inhibition of proteasome in Usp14−/− MEFs only resulted in a slight increase of monomeric ISG15 levels. These results suggest that the decrease of ISG15 in Usp14 deficient cells is not only caused by proteasome-dependent degradation of ISG15. Furthermore, semi-quantitative RT-PCR shows that mRNA levels of both ISG15 and UBE1L, the ISG15 conjugating enzyme, are decreased in the Usp14−/− MEFs. These results suggest that Usp14 may function to control both protein and mRNA levels of ISG15. Our findings provide a new evidence for a link between Usp14 and ISG15 regulation in the ubiquitin-proteasome system.

Silencing of the Desmosomal Protein Plakophilin-2 in Neonatal Rat Cardiac Fibroblasts Affects Localization of the Gap Junction Protein Connexin43
E. M. Oxford, W. Coombs, S. Taffet, M. Delmar; Pharmacology, SUNY Upstate Medical University, Syracuse, NY
The cardiac intercalated disc is comprised of 3 different macromolecular protein complexes. Desmosomes and adherens junctions maintain mechanical stability between cardiac myocytes, while gap junctions are responsible for electrical and metabolic continuity. It has been hypothesized that a disruption of the mechanical junctions may interfere with the formation of gap junctions at the intercalated disc. However, the possible cross-talk between specific desmosomal proteins and the gap junction protein Connexin43 remains to be addressed. Of recent relevance, generic analysis has linked a number of cases of Arrhythmogenic Right Ventricular Dysplasia (ARVD/C; an inherited disease associated with sudden cardiac death) to mutations in the gene coding for plakophilin 2 (PKP2), an integral desmosomal protein. Here, we tested the hypothesis that loss of PKP2 leads to disruption in gap junction structure and function. Stealth RNAi (Invitrogen) was used to silence PKP2 expression in primary cultures of neonatal rat cardiac fibroblasts. A “scrambled” RNAi (same bases; random sequence) served as control. Western blot analysis demonstrated loss of detectable PKP2 from cells treated with the pertinent RNAi. Subcellular distribution of PKP2 and Cx43 was assessed by immunofluorescence. As expected, silencing of PKP2 led to the loss of detectable PKP2 protein. Interestingly, the absence of PKP2 signal was accompanied by redistribution of the Cx43-reactive signal. Gap junction plaques were absent, and Cx43 was detected only in the intracellular space. Western blot analysis revealed significant reduction (p<0.05) in the fraction of phosphorylated Cx43 in PKP2-deficient cells, as compared to control. Our data show that loss of PKP2 expression in neonatal rat cardiac fibroblasts causes significant Cx43 remodeling. These results support the notion that disruption of molecular integrity in the desmosome may lead to significant changes in intercellular communication. The latter may represent an important substrate in the genesis of ventricular arrhythmias in patients afflicted with ARVD/C.

Role of Autophagy in CFTR Degradation
L. Fu, E. Itzhak; Department of Cell Biology, University of Alabama at Birmingham, Birmingham, AL
Cystic Fibrosis (CF) is a lethal disease caused by mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR). Most CF patients (>70%) have the ΔF508 mutation that leads to CFTR misfolding and prevents the protein from reaching the plasma membrane to function as a chloride channel. ΔF508 CFTR is retained in the endoplasmic reticulum (ER) and eventually degraded. We have used yeast S. cerevisiae as a model system to study mechanisms regulating CFTR degradation. GFP-tagged CFTR was introduced into yeast under the control of a copper-inducible promoter. The expression, localization and degradation of GFP-CFTR were followed by time-lapse imaging in live yeast. We show that in wild-type cells, GFP-CFTR first appears along the ER ring 30-45 min after induction. Subsequently, GFP-CFTR follows two distinct pathways: it either gradually disappears from the ER, or is sequestered into structures called ER-associated complexes (ERACs). ERACs containing GFP-CFTR persist till about an hour after formation, and then rapidly disappear. This behavior suggests that CFTR may be degraded by multiple pathways. CFTR is known to be degraded by the proteasome. We compared the GFP-CFTR degradation pattern in wild-type yeast with that in the pre1-1 yeast deficient in proteasomal degradation. In pre1-1 strain, GFP-CFTR is synthesized along the ER ring and then concentrated in ERACs. There appears to be no degradation of GFP-CFTR from the ER, but degradation from ERACs still occurs. These results suggest that degradation of CFTR from the ER is proteasome-dependent, while degradation from the ERACs is proteasome-independent. To explore whether the proteasome-independent pathway for CFTR degradation involves autophagy, we tested GFP-CFTR behavior in autophagy mutants. We found that GFP-CFTR is sequestered into ERACs in the atg6Δ and vps38Δ strains. However, CFTR ERACs are not degraded in these strains. These results suggest that autophagy, specifically the VPS30 complex II, participates in CFTR degradation.

HIV-1 and Influenza Virus Exit from Cells via Separate Microdomains
M. Thali; University of Vermont, Burlington, VT

Tetraspanins are thought to laterally organize cellular membranes via specific associations with each other and with distinct integrins. Utilizing fluorescence microscopy and electron microscopy, we recently mapped tetraspanin-enriched microdomains (TEMs) containing CD9, CD81, CD82 and CD63 (JCB 173: 795-807). Our data also documented size, composition and distribution of these surface TEMs. Further, human immunodeficiency virus type 1 (HIV-1) components, together with constituents of the mammalian ESCRT1 complex which is part of the cellular extravesiculation machinery critical for HIV-1 budding, were demonstrated to accumulate at surface TEMs, suggesting that HIV-1 egress is gated through these microdomains. In vivo, HIV-1 is thought to be transmitted predominantly at virological synapses, for example at cell-cell junctions within lymph nodes. In contrast, influenza virus is released into the airways of the respiratory tract from free surfaces at the apical side of epithelial cells. Using various imaging techniques and also by applying release blockades with anti-tetraspanin antibodies, we now demonstrate that such differential release of HIV-1 and influenza virus is paralleled, at the subcellular level, by particle egress from different segments of free surface areas in non-polarized cells. The surface TEMs which we find to function as exit ports for HIV-1 are always located adjacent to or even overlapping with clathrin-coated areas of the plasma membrane. We are thus testing the hypothesis that these sub-micron sized membrane segments, together with viral components, can be sequestered in the endosomal compartment to be mobilized for the formation of a virological synapse upon attachment to a target cell. supported by NIH grants RO1 AI 47727 and R03 AI 060679 to M.T., SNSF 3100A0-104489/1 to M.F.