The Schizophrenia Susceptibility Factor Dysbindin and its Associated Complex Sort Cargoes from Cell Bodies to the Synapse.

Jennifer Larimore¹, Karine Tornieri¹, Pearl V. Ryder¹-², Avanti Gokhale¹, Stephanie A. Zlatic¹-², Branch Craige¹-²#, Joshua D. Lee⁴, Konrad Talbot⁴, Jean-Francois Paré⁵, Yoland Smith⁵, Victor Faundez¹-³

¹Department of Cell Biology, ²Graduate Program in Biochemistry, Cell, and Developmental Biology; ³Center for Neurodegenerative Disease; ⁴Center for Neurobiology and Behavior, Department of Psychiatry, University of Pennsylvania; ⁵Department of Neurology and Yerkes National Primate Research Center. Emory University.

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*Address correspondence to: Victor Faundez  
Department of Cell Biology  
Emory University School of Medicine  
615 Michael Street, Room 446  
Atlanta, GA 30322  
Telephone: 404-727-3900  
Fax: 404-727-6256  
E-Mail: vfaunde@emory.edu

#Current address: Department of Cell Biology, University of Massachusetts Medical School.

Dysbindin assembles into the biogenesis of lysosome related organelles complex 1 (BLOC-1), which interacts with the adaptor protein complex 3 (AP-3) mediating a common endosome trafficking route. Deficiencies in AP-3 and BLOC-1 affect synaptic vesicle composition. However, whether AP-3-BLOC-1-dependent sorting events that control synapse membrane protein content take place in cell bodies, upstream nerve terminals, remains unknown. We tested this hypothesis analyzing the targeting of phosphatidylinositol-4-kinase type II α (PI4KIIα), a membrane protein present in pre and postsynaptic compartments. PI4KIIα co-purified with BLOC-1 and AP-3 in neuronal cells. These interactions translated into a decreased PI4KIIα content in the dentate gyrus of dysbindin-null BLOC-1 deficiency, and AP-3-null mice. Reduction of PI4KIIα in the dentate reflects a failure to traffic from the cell body. PI4KIIα was targeted to processes in wild type primary cultured cortical neurons and PC12 cells, but failed to reach neurites in cells lacking either AP-3 or BLOC-1. Similarly, disruption of an AP-3 sorting motif in PI4KIIα impaired its sorting into processes of PC12 and primary cultured cortical neuronal cells. Our findings indicate a novel vesicle transport mechanism requiring BLOC-1 and AP-3 complexes for cargo sorting from neuronal cell bodies to neurites and nerve terminals.

Introduction

Cell polarity is established and maintained, in part, by selective targeting of membrane components to distinct organelles or plasma membrane domains. This is particularly evident in neurons where membrane components transported in vesicular carriers generated in the cell body are selectively targeted to distal compartments of axons or...
dendrites. Alternatively vesicle carriers are generated locally at synaptic domains. Vesicular carriers are generated by cytosolic coats, which specify their protein and lipid composition (Bonifacino and Glick, 2004). Diverse coats and accessory proteins generate a multitude of vesicles in eukaryotic cells (Robinson, 2004). While the diversity of vesicle carriers and their pathways have been intensely studied in yeast and mammalian fibroblastoid cell lines, our knowledge about the diversity and specializations of vesicle transport pathways in polarized cells is limited. Clathrin and clathrin-binding adaptors orchestrate vesicle biogenesis and in polarized cells they contribute to specialized transport mechanisms (Folsch et al., 1999; Folsch et al., 2003; Deborde et al., 2008). For example, heterotetrameric clathrin adaptors AP-1-4 participate in the biogenesis of synaptic vesicles at the nerve terminal (Voglmaier et al., 2006; Glyvuk et al., 2010; Haucke et al., 2011), delivery of receptors to dendrites (Dwyer et al., 2001; Matsuda and Yuzaki, 2009), binding of synaptic vesicle membrane proteins (Horikawa et al., 2002), processing of the amyloid precursor protein (Burgos et al., 2010), and trafficking to autophagosomes in axons (Matsuda et al., 2008). Human mutations in clathrin adaptors isoforms trigger neuropathology ranging from cerebral palsy to mental retardation, emphasizing central roles played by adaptor complexes in neuronal homeostasis (Tarpey et al., 2006; Saillour et al., 2007; Borck et al., 2008; Montpetit et al., 2008; Moreno-De-Luca et al., 2011). Vesicle carriers from the cell body must deliver membrane proteins that will later recycle locally in pre- or post-synaptic compartments. Surprisingly, the diversity of cell body-derived vesicle carriers bound to synapses, the coat-adaptor mechanisms that assemble these vesicles, and their contents remains largely unexplored (Lasiecka and Winckler, 2011; Winckler and Choo Yap, 2011). Defects in vesicle trafficking mechanisms may also promote pathogenesis of major psychoses, including schizophrenia (Ryder and Faundez, 2009; Karayiorgou et al., 2010). Genome wide analyses reveal strong associations between genes encoding adaptor binding proteins and schizophrenia (Ryder and Faundez, 2009). Of particular interest is dysbindin, the product encoded by the DTNBP1 locus. DTNBP1 ranks high among all genes studied thus far in their strength of association with schizophrenia risk. Importantly, dysbindin protein levels are reduced in the prefrontal cortex, superior temporal gyrus, and hippocampal formation (hippocampus plus dentate gyrus) of schizophrenia patients further underscoring the association between dysbindin function and schizophrenia pathogenesis (Talbot et al., 2004; Tang et al., 2009a; Talbot et al., 2011). However, DTNBP1 genetic association with disease is not universal among all human populations (Ross et al., 2006; Allen et al., 2008; Sun et al., 2008; Talbot et al., 2009; Ghiani and Dell'angelica, 2011; Mullin et al., 2011).

Dysbindin is a subunit of the octameric BLOC-1 complex (Biogenesis of Lysosome-Related Organelles Complex 1)(Li et al., 2003; Starcevic and Dell'Angelica, 2004; Di Pietro and Dell'Angelica, 2005). The BLOC-1 complex is assembled by dysbindin, pallidin, muted, snapin, cappuccino, and BLOS1-3 subunits (Li et al., 2004; Di Pietro and Dell'Angelica, 2005; Wei, 2006; Raposo and Marks, 2007; Dell'angelica, 2009). The BLOC-1 complex binds to the clathrin adaptor protein complex AP-3 (Di Pietro et al., 2006; Newell-Litwa et al., 2010); a heterotetramer constituted of δ, β3, μ3 and σ3 subunits. The genes encoding the component proteins in AP-3 and BLOC-1 belong to a group of fifteen genetic loci in mice. Mutations in some of these loci trigger Hermansky-Pudlak syndrome in humans, which is characterized by, but not limited to, pigment...
dilution, bleeding diathesis, and pulmonary fibrosis; phenotypes that are recapitulated in mouse models of this syndrome. These mutated genes encode products that belong to five distinct cytosolic complexes: AP-3, BLOC-1, BLOC-2, BLOC-3, and the HOPS complex (Li et al., 2004; Di Pietro and Dell'Angelica, 2005; Wei, 2006; Raposo and Marks, 2007; Dell'angelica, 2009). At the cellular level, AP-3 and BLOC-1 subunits localize to nerve terminals and/or axons and deficiencies in these complexes alter the composition of synaptic vesicles and the surface expression of neurotransmitter receptors (Talbot et al., 2006; Iizuka et al., 2007; Ji et al., 2009; Newell-Litwa et al., 2009; Tang et al., 2009b; Marley and von Zastrow, 2010; Newell-Litwa et al., 2010). These cellular defects trigger neurobehavioral phenotypes from Drosophila to mouse, some of which resemble those found in schizophrenia patients (Hattori et al., 2008; Bhardwaj et al., 2009; Cox et al., 2009; Dickman and Davis, 2009; Talbot, 2009; Cheli et al., 2010; Papaleo et al., 2010). These observations highlight fundamental vesicle transport pathways controlled by BLOC-1 and AP-3 in neurons. However, a central question not yet addressed is whether BLOC-1 and AP-3 perform sorting functions uniquely restricted to the synapse (Voglmaier et al., 2006) and/or whether these complexes assemble vesicle carriers in cell bodies destined to deliver membrane proteins that are later incorporated into synapses.

Here we explore polarized neuronal membrane trafficking routes requiring BLOC-1 and AP-3 using the membrane anchored lipid kinase, phosphatidylinositol-4-kinase type IIα (PI4KIIα) as a reporter. Our focus on PI4KIIα is based on its capacity to bind the AP-3 complexes and to regulate AP-3 recruitment to membranes (Craigie et al., 2008; Salazar et al., 2009). We demonstrate that PI4KIIα copurifies with BLOC-1 complexes assembled with either tagged dysbindin or muted subunits as well as with AP-3. These biochemical interactions were confirmed genetically since a PI4KIIα synaptic depletion phenotype in the dentate gyrus of dysbindin-null sandy mice was phenocopied in that area of the brains of AP-3-null mocha mice and in mice lacking the muted or pallidin components of BLOC-1. PI4KIIα synaptic depletion suggested that BLOC-1 and AP-3 regulate delivery of membrane proteins from cell bodies to nerve terminals. Consistent with this hypothesis, analysis of the subcellular localization of wild type PI4KIIα or a mutant form unable to bind AP-3 and BLOC-1, indicated that the interaction of PI4KIIα with AP-3-BLOC-1 was required for PI4KIIα export from cell bodies to neurites. Similarly, wild type PI4KIIα failed to reach neurites in neurons of AP-3 or BLOC-1 mutant mice. Our findings reveal a novel vesicle transport mechanism in which BLOC-1, in association with the AP-3 complex, deliver specific cargos from neuronal cell bodies to neurites and nerve terminals. We propose that defects in the dysbindin/BLOC-1 vesicle trafficking pathway and the resulting mislocalization of specific cargo molecules contribute to the pathogenesis of complex psychiatric disorders.

Results

**Phosphatidylinositol-4-kinase type IIα biochemically and genetically interacts with BLOC-1 and AP-3.**

We have previously shown that phosphatidylinositol-4-kinase type IIα (PI4KIIα) binding to AP-3 is sensitive to the dosage of the dysbindin-containing BLOC-1 complex (Salazar et al., 2009). Precipitation of AP-3 complexes with AP-3 δ antibodies specifically co-precipitated PI4KIIα from wild type fibroblasts (Fig. 1A compare lanes 3 and 5). In contrast, the association of PI4KIIα to AP-3 complexes was decreased in BLOC-1-null cells carrying the Pldn<sup>padj</sup> allele (Fig. 1A compare lanes 5 and 6). Thus, we tested the
hypothesis that dysbindin associates with the adaptor complex AP-3 and PI4KI\(\alpha\), in addition to its interactions within the BLOC-1 complex. We expressed N-terminal FLAG tagged dysbindin in SH-SY5Y neuroblastoma cells. Protein complexes co-precipitating with FLAG-dysbindin were isolated from cells treated in the absence or presence of DSP (dithiobis[succinimidy] propioniate)(Fig. 1). DSP is a cell permeable and reducible crosslinker used here to stabilize protein-protein interactions labile to stringent purification (Lomant and Fairbanks, 1976; Salazar et al., 2009; Zlatic et al., 2010). Isolation of FLAG-dysbindin protein complexes with FLAG antibodies coprecipitated the BLOC-1 subunits muted and pallidin (Fig. 1B, lane 7) as well as the AP-3 subunits \(\delta\), \(\beta_3\), and \(\sigma_3\) (Fig. 1B, lane 8). Dysbindin’s associations with AP-3 subunits required stabilization with DSP (Fig. 1B, lane 8). These associations with FLAG-dysbindin beads were specific as determined by their absence from beads alone (Fig. 1B, lanes 3-4), beads coated with control SV2 antibody (Fig. 1B, lanes 5-6), and competition with an excess FLAG antigenic peptide during bead incubation with cell extracts (Fig. 1B, lanes 9-10).

We next tested whether endogenous PI4KI\(\alpha\) copurified with BLOC-1 subunits by PI4KI\(\alpha\) immunoaffinity chromatography. PI4KI\(\alpha\) was isolated with an antibody directed against amino acids 51-70 from human PI4KI\(\alpha\) and PI4KI\(\alpha\) protein complexes were selectively eluted with the PI4KI\(\alpha\) peptide 51-70 used to generate the PI4KI\(\alpha\) antibody (Supp. Fig.1). PI4KI\(\alpha\) proteins complexes were purified from DSP-treated FLAG-dysbindin (Fig. 1C) or FLAG-muted expressing cells (Fig. 1D). FLAG-tagged BLOC-1 subunits dysbindin or muted specifically eluted from PI4KI\(\alpha\) immunoaffinity chromatography matrices with the PI4KI\(\alpha\) peptide 51-70 but not by buffer alone (Fig. 1C-D, compare lanes 3’ and 5’). Out-competition with a molar excess of PI4KI\(\alpha\) peptide during the incubation of cell extracts with PI4KI\(\alpha\) antibody matrices abrogated the association of PI4KI\(\alpha\) and associated proteins such as endogenous BLOC-1 subunits (Fig. 1C-D, compare lanes 3 and 4) as well as FLAG-tagged dysbindin and muted (Fig. 1C-D, compare lanes 4’ and 5’). Conversely, FLAG immunoaffinity chromatography from FLAG-muted expressing cell extracts isolated PI4KI\(\alpha\) (Fig. 1E, lanes 7-8). The specificity of these associations was determined by performing FLAG immunoaffinity chromatography from untransfected SH-SY5Y cells (Fig. 1E, lanes 5-6). These data indicate that dysbindin forms complexes with AP-3 and the membrane protein cargo PI4KI\(\alpha\), in addition to dysbindin’s inclusion into the BLOC-1 complex.

Dysbindin’s interactions with other BLOC-1 components, as well as with AP-3, and PI4KI\(\alpha\) suggest that these proteins act together on a common trafficking pathway. We tested this hypothesis genetically by determining whether PI4KI\(\alpha\) phenotypes observed in dysbindin-null sandy brain (\(Dtnbp1^{sdy/sdy}\)) were phenocopied in other BLOC-1 deficient mice muted (\(Muted^{mu/mu}\)) and pallid (\(Pldnpa^{pa/pa}\)), and the AP-3 deficient allele mocha (\(Ap3d1^{mh/mh}\))(Kantheti et al., 1998; Huang et al., 1999; Zhang et al., 2002; Li et al., 2003). We analyzed PI4KI\(\alpha\) by quantitative confocal immunofluorescence microscopy of the dentate gyrus in the hippocampal formation (Fig. 2). The dentate gyrus is where we previously observed a strong decrease in the levels of the AP-3 cargo VAMP7 in BLOC-1 deficient muted and pallid mice (Newell-Litwa et al., 2010). PI4KI\(\alpha\) content was decreased in the neuropil of dysbindin-null sandy dentate gyrus (Fig. 2A and E). This phenotype was similar in BLOC-1-null mice, either by deficiency of muted or pallidin (Fig. 2B-C and E), and the AP-3 deficient mocha allele (Fig. 2D and
The selectivity of this PI4KIIα phenotype is highlighted by the absence of an effect on synaptophysin, a membrane protein targeted to synaptic vesicles by mechanisms independent of AP-3 or BLOC-1 (Salazar et al., 2004b; Salazar et al., 2006; Newell-Litwa et al., 2009; Newell-Litwa et al., 2010). The biochemical interactions between the BLOC-1 complex, AP-3, and PI4KIIα as well as the common hippocampal PI4KIIα phenotypes in BLOC-1 and AP-3 deficiencies demonstrate that these components belong to common trafficking pathway in neuronal tissue.

**PI4KIIα is depleted in nerve terminals of mice deficient in the AP-3 complex.**

The subcellular compartment affected by reduced levels of PI4KIIα in the mutant mice carrying deficiencies in either BLOC-1 or AP-3 was identified by subcellular fractionation and quantitative immuno electron microscopy of brain tissue. We focused on AP-3-null brains to facilitate these experiments because AP-3-null brain phenotypes are widespread in contrast to BLOC-1 deficiency phenotypes, which are predominantly observed in the dentate gyrus of the hippocampus (Kantheti et al., 1998; Newell-Litwa et al., 2010). High-speed pellets (P2) from whole brain homogenates were sedimented in Percoll density gradients to resolve fractions enriched in pinched-off nerve terminals or heavy synaptosomes (Fig. 3, HS). Levels of SV2 and synaptophysin, two synaptic vesicle markers, remained unaffected in AP-3 mocha (Ap3d1mhmh) heavy synaptosomes. In contrast, the content of PI4KIIα and the synaptic vesicle AP-3 cargoes VAMP7 and ZnT3 were reduced in Ap3d1mhmh heavy synaptosomes. ZnT3 reduction in synaptosomes was due to decreased ZnT3 levels in Ap3d1mhmh homogenates. In contrast, PI4KIIα was significantly diminished in synaptosomes of Ap3d1mhmh brains without changes in total homogenate levels (Fig. 3). This pattern is similar to that of VAMP7 (Fig. 3), a membrane protein present in synaptic vesicles that we and others demonstrated to be decreased in nerve terminals of AP-3-null brains (Scheuber et al., 2006; Newell-Litwa et al., 2010).

We further explored the subcellular localization of PI4KIIα in the hippocampal formation of wild type and Ap3d1mhmh mice by immunocytochemistry with a PI4KIIα-specific antibody (Fig. 4 and Suppl. Fig. 1 and 2). At the light microscopic level, PI4KIIα displayed a distinctive distribution in different regions of the hippocampal formation. Immunoreactivity was most prominent in cell bodies and dendrites of hippocampal pyramidal cells and in three presynaptic fields: (1) the inner molecular layer of the dentate gyrus, which is the terminal field of axons from large neurons in the dentate hilus, (2) the dentate hilus, and (3) the stratum lucidum of CA3, which (along with the dentate hilus) is the terminal field of dentate granule cells (Suppl. Fig. 2). PI4KIIα subsynaptic distribution was determined by immuno electron microscopy of the dentate gyrus. Immunoreactivity was present in dendrites (not shown) and presynaptic elements of asymmetric axodendritic and axospinous synapses (Fig. 4A-A1, and C). PI4KIIα immunoreactivity in presynaptic elements is consistent with previous reports of PI4KIIα localization on synaptic vesicles (Guo et al., 2003; Salazar et al., 2005; Takamori et al., 2006). We quantified the number of PI4KIIα-positive synaptic elements in the dentate gyrus hilus of wild type and mutant mice (Fig. 4C-F). Most PI4KIIα-labeled elements were asymmetric axospinous terminals in the mouse dentate gyrus hilus (Fig. 4C). Three quarters of those nerve terminals were PI4KIIα immunoreactive in wild type mice.
(Ap3d1<sup>+/+</sup>). In contrast, only 35% of such terminals were PI4KIIα-positive in AP-3-null Ap3d1<sup>mh/mh</sup> (Fig. 4D). Since AP-3 deficiency did not affect the density of asymmetric axospinous terminals in the dentate (Fig. 4E), the reduction in the density of such terminals positive for PI4KIIα reflects depletion of the enzyme in them.

**Disruption of AP-3, BLOC-1, and PI4KIIα interactions impairs PI4KIIα targeting from cell body to processes.**

PI4KIIα depletion in nerve terminals may reflect failed export of this cargo from the parent cell bodies. We addressed this by expressing EGFP tagged PI4KIIα or a dileucine sorting mutant version of PI4KIIα, which is unable to bind AP-3 or coprecipitate with the BLOC-1 complex, PI4KIIαL60-61A (Craig et al., 2008; Salazar et al., 2009). PI4KIIα is targeted to brain synaptic vesicles and PC12 cells synaptic-like microvesicles by AP-3 and BLOC-1 mechanisms (Salazar et al., 2005; Newell-Litwa et al., 2009). Moreover, synaptic-like microvesicles are coated with AP-3 and BLOC-1 complexes (Salazar et al., 2005; Salazar et al., 2006). Thus, we determined the targeting of tagged PI4KIIα to PC12 synaptic-like microvesicles isolated by sucrose velocity sedimentation (Lichtenstein et al., 1998). Endosomes and synaptic-like microvesicles sediment in fractions 5-7 and 15 in these gradients, respectively (Cleft-O’Grady et al., 1998; Lichtenstein et al., 1998). Wild type EGFP-PI4KIIα was present in endosome and synaptic-like microvesicle fractions isolated from PC12 cells (Suppl. Fig. 3). In contrast, EGFP-PI4KIIαL60-61A targeting to synaptic-like microvesicle fractions was reduced (Suppl. Fig. 3A and B) concomitantly with an increased EGFP-PI4KIIαL60-61A content in endosomes (Suppl. Fig. 3B, compare open and closed circles). We then used NGF-differentiated PC12 cells and assessed EGFP-PI4KIIα distribution by imaging live and fixed specimens (Fig. 5). Wild type PI4KIIα was enriched at neurite tips of differentiated PC12 cells (Fig. 5A and A1). In contrast, the EGFP-PI4KIIαL60-61A mutant signal was faint in neurites and their tips (Fig. 5C and C1). We depicted these dramatic differences in subcellular localization as X,Y coordinates where each dot depicts an individual cell defined by its PI4KIIα fluorescence intensity in cell bodies –X axis- and processes –Y axis- (Fig. 5E-F).

Decomposition of these X,Y coordinates plots into cell body and process PI4KIIα fluorescence intensities (Fig. 5G-H), revealed that cells expressed similar cell body levels per voxel of wild type or mutant EGFP-PI4KIIα (Fig. 5G). However, they differed significantly in the amount of PI4KIIα present in cell processes and their tips (Fig. 5H). Similar results were obtained with fixed differentiated PC12 cells where the EGFP-PI4KIIαL60-61A mutant levels in processes were selectively reduced when compared to VAMP2, a synaptic vesicle marker whose targeting is not affected by AP-3 or BLOC-1 deficiencies (Fig. 5I)(Salazar et al., 2004a; Newell-Litwa et al., 2009). Next, we performed fluorescence recovery after photobleaching (FRAP) of PC12 cell neurite tips expressing recombinant PI4KIIα to determine: a) whether EGFP-PI4KIIα is delivered in an anterograde fashion and b) whether the EGFP-PI4KIIα delivery mechanism could be distinguished from EGFP-PI4KIIαL60-61A, as predicted by the reduced targeting of this mutant to synaptic-like microvesicles (Suppl. Fig. 3). EGFP-PI4KIIα FRAP of the neurite tip reached a plateau by 45 minutes (Fig. 6A-B). In contrast, the EGFP-PI4KIIαL60-61A mutant recovery was accelerated attaining steady-state by 10 minutes (Fig. 6 A-B). Rapid fluorescence recovery of PI4KIIαL60-61A could be due to diffusion of a plasma membrane EGFP-PI4KIIαL60-61A pool. This hypothesis
stems from the observation that AP-3 cargoes are misrouted to the cell surface when the cargo-adaptor association is perturbed (Dell'Angelica et al., 1999; Peden et al., 2004). To address this, we compared EGFP-PI4KIIαL60-61A FRAP to an EGFP targeted to the plasma membrane by the palmitoylation signal of GAP43 (EGFP-GAP43-ps) since PI4KIIα is anchored to membranes by palmitate (Fig. 6 A and C) (Matsuda and Cepko, 2007; Barylko et al., 2009). EGFP-PI4KIIαL60-61A and EGFP-GAP43-ps FRAP were similar suggesting that EGFP-PI4KIIαL60-61A rapid recovery is due to plasma membrane diffusion. These results indicate that wild type PI4KIIα uses a vesicular delivery mechanism to neurite tips that is high capacity and low speed. However, a PI4KIIα mutant unable to bind AP-3 reaches PC12 neurite tips by a low capacity mechanism compatible with diffusion in the plane of the membrane.

These experiments indicated that impairment of an AP-3, BLOC-1, and PI4KIIα interaction prevents cargo inclusion into synaptic-like microvesicles and thereby prevents PI4KIIα delivery to neurite tips in PC12 cells either assessed in vivo or in fixed specimens. To complement the results obtained with PC12 cells, we analyzed primary cultured neurons to determine if PI4KIIα targeting to neurites was sensitive to the ablation of AP-3, the dileucine-sorting motif in PI4KIIα, and/or BLOC-1. We analyzed the distribution of PI4KIIα and the synaptic vesicle protein VAMP2 in 7-day in vitro cultures of cortical neurons (7 DIV). Fluorescence intensity in cell bodies and processes was scored for PI4KIIα and VAMP2, and values were represented as X, Y coordinates (Fig. 7 and 8). In wild type neurons, endogenous PI4KIIα and VAMP2 were present in cell bodies and neuronal processes, but in Ap3d1mhmh neurons the content of PI4KIIα in cell bodies and processes was selectively reduced (Fig. 7A, B, E1). VAMP2 distribution was not affected in this AP-3 deficiency (Fig. 7A, B, E2). We also expressed EGFP-PI4KIIα in wild type and AP-3-null mocha cells (Fig. 7C, D, F). Targeting of EGFP-PI4KIIα to processes was impaired in Ap3d1mhmh neurons despite cell body expression levels of EGFP-PI4KIIα spanning one order of magnitude (compare Fig. 7E1 and F1). Processes of wild type neurons were efficiently populated by EGFP-PI4KIIα in that range of cell body expression levels (Fig. 7F1). As in neurons lacking AP-3, those expressing mutant PI4KIIα unable to bind AP-3 and coprecipitate with BLOC-1 (EGFP-PI4KIIαL60-61A)(Craig et al., 2008; Salazar et al., 2009) failed to populate cell processes (Fig. 7G1-H1). This EGFP-PI4KIIαL60-61A phenotype was observed irrespective of the culture age and recombinant protein expression level in cell bodies (Fig. 7G1-H1). The selectivity of these phenotypes was demonstrated by unaltered VAMP2 distribution (Fig. 7G2-H2). Our data demonstrates that integrity of interaction between the AP-3 adaptor and its cargo, PI4KIIα, is required for cargo export from cell bodies to neurites.

PI4KIIα association to AP-3 requires BLOC-1 and deficiencies in BLOC-1 complex subunits phenocopied a PI4KIIα phenotype in the AP-3-null dentate gyrus (Fig. 2). Thus, we tested whether endogenous PI4KIIα, EGFP-PI4KIIα, and EGFP-PI4KIIαL60-61A export from cell bodies to neurites was impaired in dysbindin-BLOC-1 deficient sandy Dtnbp1sdy/sdy neurons (Fig. 8). We focused on dysbindin-null neurons because of strong association of dysbindin gene polymorphisms with schizophrenia (Ross et al., 2006; Allen et al., 2008; Sun et al., 2008; Talbot et al., 2009). Control (Dtnbp1+/+) and dysbindin-BLOC-1-null neurons (Dtnbp1sdy/sdy) expressed either endogenous (Fig. 8E) or
EGFP-PI4KIIα to a similar extent in cell bodies (Fig. 8A, C, F). However, PI4KIIα or EGFP-PI4KIIα did not populate neurites in Dtnbp1<sup>sdy/sdy</sup> neurons (Fig. 8E and 8F, compare closed -Dtnbp1<sup>+/+</sup>- and open circles -Dtnbp1<sup>sdy/sdy</sup>-). EGFP-PI4KIIαL60-61A remained constrained to cell bodies both in control and BLOC-1-null sandy cells (Fig. 8G). All PI4KIIα phenotypes were selective since the distribution of VAMP2 in neurons was not affected by the Dtnbp1<sup>sdy/sdy</sup> allele or the expression of wild type or mutant forms of PI4KIIα (Fig. 8E1 to F1, compare closed -Dtnbp1<sup>+/+</sup>- and open triangles -Dtnbp1<sup>sdy/sdy</sup>-). Therefore, the dysbindin-containing BLOC-1 complex is required for the delivery of PI4KIIα to neurites from neuronal perikarya.

**Discussion**

We show here that the schizophrenia susceptibility gene product dysbindin, contained in the BLOC-1 complex, and its interacting AP-3 adaptor, are required to target cargoes into vesicles assembled at cell bodies for delivery into neurites and nerve terminals. These carriers selectively deliver a subset of synaptic vesicle membrane proteins to the synapse. In contrast, synaptic vesicles proteins such as synaptophysin and VAMP2 are not affected by null alleles of these protein complexes. We explored the BLOC-1-AP-3 transport mechanism by assessing the subcellular distribution and movement of PI4KIIα, a lipid kinase that both binds to AP-3--by a BLOC-1-dependent mechanism--and regulates AP-3 recruitment to endosomes (Craige et al., 2008; Salazar et al., 2009). PI4KIIα is depleted in dentate gyrus neuropil of dysbindin-null sandy mice, a trait phenocopied in mice deficient in the dysbindin-interacting BLOC-1 subunits, muted and pallidin, and in AP-3-null mice carrying the Ap3d1<sup>mh/mh</sup> mocha allele. The common PI4KIIα phenotype among these four mutant mice results from disrupted association of PI4KIIα with BLOC-1 and AP-3. PI4KIIα was retained in the cell body of neurons lacking AP-3 or BLOC-1 protein complexes, a phenotype emulated by mutagenesis of the dileucine sorting motif in PI4KIIα. This motif is necessary for PI4KIIα association with AP-3, its co-purification with BLOC-1, and PI4KIIα exit from early endosomes in human fibroblastoid cells (Craige et al., 2008; Salazar et al., 2009).

We predicted the existence of a BLOC-1-AP-3 pathway that delivers vesicles from cell bodies to nerve terminals from our ‘seesaw’ sorting hypothesis (Newell-Litwa et al., 2009). This seesaw model was postulated to explain the opposing effects that ubiquitous and neuronal AP-3-null alleles had in cargo delivery to synaptic vesicle fractions (Newell-Litwa et al., 2009). A BLOC-1-AP-3 route between cell bodies and nerve terminals was further supported by the existence of 1) BLOC-1 and AP-3-decorated vesicle carrier in PC12 cells (Salazar et al., 2005; Salazar et al., 2006), 2) the presence of a BLOC-1-AP-3 supracomplex in isolated nerve terminals –synaptosomes–(Newell-Litwa et al., 2010) and 3) BLOC-1- and AP-3-null nerve terminal phenotypes in brain tissue in toto (Newell-Litwa et al., 2010). However, points 1-3 were similarly compatible with a local role of BLOC-1 and AP-3 in nerve terminals. Moreover, such a local role is additionally supported by the observation that AP-3-dependent mechanisms acutely regulate vesicle traffic in nerve terminals (Voglmaier et al., 2006). Thus, the origin of BLOC-1 and AP-3 vesicles in neurons remained an untested prediction from our previous work. Our data, presented here, demonstrate that the cell body is the most upstream site where BLOC-1 and AP-3 are required for membrane protein sorting toward the synapse. This is clearly illustrated by the trapping of endogenous and exogenously expressed PI4KIIα in cell bodies of neurons defective either in BLOC-1 or AP-3. In addition to the
assembly and loading of vesicular carriers in the cell body, it is possible that BLOC-1 and AP-3 may perform functions along axons and dendrites, because subunits of these complexes are found in these subcellular compartments (Zakharenko et al., 1999; Talbot et al., 2006; Newell-Litwa et al., 2010). Although our data clearly indicate that BLOC-1 and AP-3 function in a common vesicular transport pathway, especially in the transport of the PI4KIIα cargo, it is also possible that BLOC-1 complexes may generate vesicles independent of AP-3. This possibility is supported by the observation that ATP7A targeting to melanosomes requires only BLOC-1 (Setty et al., 2008).

It is likely that PI4KIIα is just one of the proteins trafficked from cell bodies to neurites via the pathway identified here. We predict that other vesicular cargoes are also affected in BLOC-1 and AP-3 deficiencies. The SNARE protein VAMP7, also known as TI-VAMP, is a probable candidate for trafficking with PI4KIIα in vesicles assembled by BLOC-1-AP-3 (Salazar et al., 2006; Scheuber et al., 2006; Newell-Litwa et al., 2010). VAMP7 is present in a subset of nerve terminals in the brain, which in the hippocampal formation (Muzerelle et al., 2003) are distributed in a manner very similar to that described here for PI4KIIα. Moreover, VAMP7 is reduced in the dentate gyrus of AP-3-null mocha mice and BLOC-1-null muted mice (Scheuber et al., 2006; Newell-Litwa et al., 2010). The spectrum of vesicles and their cargoes may also include those targeted to dendritic compartments. Dendritic carriers assembled by BLOC-1 and/or AP-3 could deliver membrane cargoes bound to dendritic compartments from cell bodies. This hypothesis is supported by the presence of PI4KIIα in both cell bodies and dendritic compartments as demonstrated here, as well as AP-3 and dysbindin as demonstrated previously (Talbot et al., 2004; Seong et al., 2005; Talbot et al., 2006; Newell-Litwa et al., 2010; Talbot et al., 2011). Alternatively, BLOC-1 and/or AP-3 could participate in the local recycling of specific membrane proteins, such as the dopamine D2 receptor and the NR2A subunit of the NMDA receptor, to and from the synaptic plasma membrane (Iizuka et al., 2007; Ji et al., 2009; Tang et al., 2009b; Marley and von Zastrow, 2010). Impaired cargo delivery not only could affect membrane dynamics in mature neurons but also impair the development of neuronal processes (Ghiani et al., 2009; Ito et al., 2010). Pre- or post-synaptic functional deficits in neurons lacking either BLOC-1 or AP-3 complexes likely result from the type of proteins depleted from synapses and the extent of their depletion. For example, vesicle fusion may be impaired as a result of reduced PI4KIIα and SNAREs, including VAMP7. Consistent with this, dysbindin sandy mice, and a knock-out of the BLOC-1 subunit snapin, reveal a role for BLOC-1 complex subunits in modulating calcium-regulated exocytosis of chromaffin granules and synaptic vesicles (Ildardi et al., 1999; Tian et al., 2005; Chen et al., 2008; Pan et al., 2009).

Similarly, Drosophila dysbindin functions presynaptically in adaptive homeostatic modulation of vesicle release (Dickman and Davis, 2009). Importantly, these changes in the physiology of presynaptic secretory organelles occur concomitantly with changes in organelle morphology. Chromaffin granules from dysbindin-null mice are larger, a phenotype observed in both chromaffin granules and synaptic vesicles from dentate gyrus glutamatergic terminals of the AP-3 mocha mouse (Grabner et al., 2006; Chen et al., 2008; Newell-Litwa et al., 2010). These similar morphological changes in dysbindin and AP-3 null cells further support that BLOC-1 and AP-3 participate in the same pathway. Dysbindin, its interacting partner pallidin (unpublished data), and AP-3 are widely expressed in the brain. Judging from the synaptic fields in which dysbindin is
concentrated and the synaptic effects of dysbindin loss (Talbot et al., 2004; Talbot et al., 2006; Iizuka et al., 2007; Chen et al., 2008; Ji et al., 2009; Talbot et al., 2009; Tang et al., 2009b; Papaleo et al., 2010), multiple neurotransmitter systems (especially dopamine, GABA, and glutamate) may be affected by deficiencies in the dysbindin-BLOC-1-AP-3 pathway. Since vesicle trafficking mechanisms control the subcellular delivery of multiple membrane proteins to nerve terminals, defects in specific membrane protein sorting events are almost certainly involved in the diverse neurochemical phenotypes observed in schizophrenia given widespread synaptic dysbindin reductions observed in the disorder (Talbot et al., 2004; Tang et al., 2009a; Talbot et al., 2011). We propose that pathways defined by schizophrenia susceptibility genes, such as the dysbindin-BLOC-1-AP-3 pathway, offer encompassing mechanisms to explain multiple, pleitropic neurochemical and neurodevelopmental phenotypes observed in schizophrenia brains.

**Materials and Methods**

**Antibodies, Plasmids, Recombinant Proteins, and Mice**

The following antibodies were used in this study: mouse monoclonals against AP3-δ (SA4) and SV2 from Developmental Studies Hybridoma Bank at the University of Iowa, synaptophysin (SY38) from Chemicon International/Millipore (Billerica, MA, USA), and VAMP7-T1 (generous gift of Dr. Andrew Peden, Cambridge University), as well as rabbit polyclonals against PI4KIIα peptide 51-71 PGHDRERQPLLDRARGAAAQ (Uniprot/Swiss-Prot entry Q99M64 -P4K2A_RAT- UniProtKB/Swiss-Prot) generated in this study and VAMP-2 from Synaptic Systems (Göttingen, Germany). Chicken anti-GFP was obtained from Aves Labs, INC (Tigard, Oregon). Rat monoclonal anti-HA was from Roche (Indianapolis, IN). GST-full fusion protein of PI4KIIα was prepared as previously described (Craige et al., 2008). pEGFP-C2-wild type rat PI4KIIα and pEGFP-C2-PI4KIIαL60-61A mutant plasmids were previously described (Craige et al., 2008). C-terminally FLAG tagged mutated (Cat No. EXT4795-M14) and N-terminal tagged FLAG-tagged Dysbindin (Cat No. EX-Mm12550-M12) were obtained from Genecopoeia (Rockville, MD). Both constructs were in a pReceiver vector backbone and sequences were independently confirmed. EGFP-GAP43-ps was expressed from pCAG-mGFP Addgene plasmid 14757 (www.addgene.org/14757)(Matsuda and Cepko, 2007).

**Mocha** (STOCK gr +/+ Ap3d1mhm/J, here referred to as Ap3d1mhm) and its control **grizzled** (STOCK gr +/+ Ap3d1+/J, here referred to as Ap3d1+/+) and its control **pallid** (B6.Cg-Pldnpa/J, here referred to as Pldnpa) breeding mouse pairs were obtained from Jackson Labs (Bar Harbor, Maine) and bred in house following IUCAC approved protocols. Muted mice and their controls (B6C3 Aw+/A-Mutedmu/J, Mutedmu and CHMU+/mu) (Zhang et al., 2002) were obtained from Dr. Richard Swank (Roswell Park Cancer Institute, Buffalo, NY, USA) and bred in-house. Sandy mice in C57B6 background were previously described (Cox et al., 2009). All mice were bred in-house following IUCAC approved protocols.

**Cell Culture**

Cerebrocortical neurons were cultured from post-natal day 4 (P4) mice (Ap3d1+/+, Ap3mhm, CHMU and muted) and maintained in neurobasal media containing B27, L-glutamine, and 100µg/mL penicillin and streptomycin (Hyclone, Logan, UT, USA) at 10%CO₂ and 37°C. Dissociated neurons were plated on poly-L-lysine (Sigma Aldrich, St. Louis, MO) coated glass cover-slips and cultured for 3-7 days in vitro (DIV). Dissociated neurons (2 x 10⁶) were transfected with 3 µg plasmid DNA using Amaxa
nucleofection electroporation (Lonza Walkersville Inc, Koeln, Germany). Neurons were plated at a density of 6 x 10^4 cells per well in a 12-well plate.

HEK-293, SH-SY5Y, wild type and pallidin-deficient mouse skin primary culture fibroblasts, and PC12 cells (ATCC, Manassas, VA, USA) were cultured in DMEM supplemented with either 10% Fetal Bovine Serum or, in the case of PC12 cells, a mix of 5% Fetal Bovine Serum plus 10% Equine Serum, respectively (Hyclone, Logan, UT, USA). Media was supplemented with 100 μg/mL penicillin and streptomycin (Hyclone, Logan, UT, USA). Cells were maintained at 37°C with 10% CO2. PC12 cells were transfected with 3μg/μL DNA by nucleofection using Amaxa Cell Line Nucleofector Kit V (Lonza Walkersville Inc, Koeln, Germany), and plated in PC12 culture media supplemented with 100 ng/mL NGF 2.5S (Murine, natural) (Invitrogen, Carlsbad, CA, USA) on matrigel coated glass bottom culture dishes (Matek, Ashland, MA, USA). PC12 cells were differentiated for 48-72 hours at 37°C with 10% CO2.

**Immunohistochemistry**

We analyzed PI4KIIα distribution using two immunoperoxidase labeling protocols. First, 11 male and 11 female C57BL6/J mice 3-6 months old were deeply anesthetized with sodium pentobarbital (1.0 mg/kg) and perfused transcardially with saline followed by neutral buffered formalin. Their brains were then postfixed in the same fixative overnight, embedded in paraffin, and sectioned coronally at 6 μm. The sections were mounted and air dried. They were next dewaxed in xylenes, rehydrated in descending concentrations of ethanol, and immersed for 30 min in 5% hydrogen peroxide dissolved in absolute methanol to quench endogenous peroxidase activity. Following a 10 min water rinse, the tissue was subjected to antigen retrieval by boiling in 1 mM EDTA in 0.1 M Tris buffer, pH 8.0 for 10 min (Pileri et al., 1997). After cooling and rinsing, the sections were reacted immunohistochemically with the PI4KIIα antibody (1:300) generated for this study using a standard avidin-biotin-peroxidase method with nickel sulfate amplification of the DAB reaction product (Talbot et al., 2004). Results were analyzed on a BX61 Olympus research microscope equipped with an Olympus DP71 cooled CCD digital camera.

**Immuno electron microscopy**

Tissue used in brain slice preparations was obtained from either male or female mice between 6 to 8 weeks of age, unless otherwise indicated. Following deep anesthesia with Nembutal, animals were transcardially perfused with fixative (4% paraformaldehyde with 0.1% glutaraldehyde). Brains were post-fixed in 4% paraformaldehyde, which was replaced with PBS within 12-18 hours. Using a vibrating microtome, brains were cut into 60 μm thick sections and stored in antifreeze (0.1M sodium phosphate monobasic, 0.1M sodium phosphate dibasic heptahydrate, 30% ethylene glycol, 30% glycerol) at -20°C until immunohistochemical preparation.

The 60 μm-thick brain sections were rinsed in PBS then incubated in sodium borohydride, followed by 100% cryoprotectant (phosphate buffer 0.05M, pH 7.4, 25% sucrose, 10% glycerol) for 20 minutes at -80°C and returned to decreasing amounts of cryoprotectant. Sections were pre-incubated in PBS with 1% normal horse serum (NHS) and 1% bovine serum albumin (BSA), followed by primary antibody incubation with 1:500 affinity purified anti-PI4KIIα. Sections were rinsed in PBS then incubated in a secondary goat anti-rabbit IgGs antibody solution (1:1000 dilution) (Jackson ImmunoResearch Labs, West Grove, PA) for 90 minutes at room temperature. Sections
were rinsed with PBS, and then incubated in a 1:1000 dilution of rabbit peroxidase anti-
peroxidase (PAP; Jackson ImmunoResearch Labs, West Grove, PA). Sections were
rinsed in PBS with a final rinse in Tris buffer (50mM, pH 7.6) before a 10 minute
incubation in 0.025% 3,3’-diaminobenzidine, 1mM imidazole, and 0.005% hydrogen
peroxide in Tris buffer at room temperature. At this point sections were mounted and
analyzed by light microscopy or they were further processed for electron microscopy after
rinsing sections in phosphate buffer (0.1M, pH 7.4). Sections were incubated in 1%
oxidation for 10 minutes, and then returned to phosphate buffer before being
dehydrated in increasing concentrations of ethanol. At the 70% ethanol incubation, 1%
uranyl acetate was added, and sections were incubated in the dark for 35 minutes. After
dehydration, sections were treated with propylene oxide, and embedded in epoxy resin
overnight (Durcupan ACM; Fluka, Buschs, Switzerland). Next, tissues were mounted
onto slides and incubated at 60°C for 48 hours. Tissue samples of the dentate gyrus were
removed from the slides, mounted on resin blocks, cut into 60 nm-thick sections,
collected on Pioloform-coated copper grids and stained with lead citrate for 5 minutes.
Electron microscopy was performed with a Zeiss EM-10C electron microscope with a
CCD camera (DualView 300W; Gatan, Inc., Pleasanton, CA, USA). Images were
acquired with Gatan Digital Micrograph Software (v. 3.10.1; Gatan, Inc.). Analysis was
focused on the interface between the granule cells and hilar neuropil, where PI4KIIα was
concentrated across mice strains. In the electron microscope, only tissue areas from the
surface of the blocks with optimal antibody penetration were examined. These sections
were scanned at 10,000X, and random fields of view containing at least one asymmetic
synapse were photographed at 75,000X. A total of 190 micrographs from 3 blocks of
tissue in 3 animals covering 773 μm² were examined for the control and 172 micrographs
from 3 blocks of tissue in 3 animals covering 700 μm² were examined. From this
material, the total number of PI4KIIα-immunoreactive or immunonegative terminals
forming asymmetric axo-spinous synapses were counted to estimate the relative
percentage of positively-labeled terminals. Also, in wild type (Ap3d1+/+)
all positive immunoperoxidase-labeled neuronal elements in the field were counted and
categorized as axons, spines, dendrites or glia, based on ultrastructural criteria defined by
Peters et al. (Peters et al., 1991). Light microscopy of brain sections was performed with a
Leica DMRB microscope with a 10x/0.3 DiC objective (Leica Microsystems, Inc.,
Bannockburn, IL, USA) and images were captured with a CCD camera and the Leica
IM50 software (Leica DC500).

**Immunofluorescence Labeling for Confocal Microscopy**

Confocal microscopy was performed with an Axiovert 100M microscope (Carl Zeiss)
coupled to an Argon and HeNe1 lasers. Images were acquired using Plan Apochromat
10x/0.5 dry, 20X/0.5 dry, and 40x/1.3 and 63x/1.4 DiC oil objectives. Emission filters
used for fluorescence imaging were BP 505-530 and LP 560. Images were acquired with
ZEN and LSM 510 software (Carl Zeiss). Hippocampal formation 60 μm-thick brain
sections were first rinsed with PBS and then incubated in 1% sodium borohydride in PBS
for 20 minutes at room temperature, followed by extensive washing with PBS. Samples
were pre-incubated in a solution of PBS with 5% NHS and 1% BSA and 0.3% Triton X-
100 for 60 minutes at RT. Samples were incubated overnight at 4°C in primary antibody
solutions of PBS with 1% NHS and 1% BSA and anti-PI4KIIα with anti-synaptophysin
(SY38) (dilutions of 1:500 and 1:10,000, respectively). After rinsing in PBS, sections
were incubated for 60 minutes in a secondary antibody PBS solution with 1% NHS and 1% BSA and 1:500 dilutions of the following Alexa-conjugated secondary antibodies: anti-mouse 555 (for anti-synaptophysin) and anti-rabbit 488 (for anti-PI4KII\(\alpha\)) (Invitrogen Molecular Probes, Carlsbad, CA, USA). Following PBS rinses, sections were incubated in cupric sulfate (3.854 W/V Ammonium Acetate, 1.596 W/V Cupric Sulfate in distilled water, pH 5) for 30 minutes. Sections were washed with PBS and mounted on slides with Vectashield (Vector Laboratories).

HEK293 cells, PC12 cells and cortical neurons were washed in ice cold PBS and fixed in 4% paraformaldehyde for 20 minutes on ice. Cells were then incubated in blocking solution (2% BSA + 1% fish skin gelatin + 15% horse serum + 0.02% saponin in PBS) for 30 minutes at RT. Next, cells were incubated with antibody for 30 minutes at 37\(^\circ\), rinsed, then incubated with secondary antibody diluted in block for 30 minutes at 37\(^\circ\). Cells were then rinsed and mounted with gelvatol and sealed. Cortical neurons were selected from a field of neurons using the following criteria. Neurons were chosen where a single neuron could be discerned from neighboring neurons, where the morphology met the criteria for the proper development in vitro (Goslin and Baker, 1989), and only chosen based on the staining of the control vesicle marker illuminating the entire neuron. Micrographs were analyzed by creating a region of interest (ROI) outlining only the axon or only the cell body and then determining total number of fluorescent pixels in that specific ROI for the VAMP2 channel and the PI4KIIalpha channel.

Live imaging of PC12 cells expressing PI4KII\(\alpha\)-GFP, PI4KII\(\alpha\)L60-61A-GFP or EGFP-GAP43-ps was performed on an A1R Laser Scanning Confocal Microscope (Nikon, Melville, NY, USA) equipped with a hybrid scanner, Perfect Focus, and an environmental chamber for regulation of temperature to 37\(^{\circ}\)C and 10% CO\(_2\). Confocal images with a Z-step of 1\(\mu\)m were captured for 10 minutes (no delay) with an APO TIRF 60x/1.49 Oil DIC objective on NIS-Elements AR 3.1 (Nikon, Melville, NY, USA) software. Neurite tips of PC-12 cells were photobleached for 2 seconds at 30% laser power. Images were captured for 15 seconds (no delay) before photobleaching and for 15 minutes every 5 minutes after photobleaching followed by 2 hours every 15 minutes. PC12 cell imaging was performed in Hank’s balanced salt solution minus phenol red and NaHCO\(_3\) (Sigma) and supplemented with 10% Donor Equine Serum (Hyclone, Logan, UT, USA), 5% Fetal Bovine Serum (Hyclone, Logan, UT, USA) and 100 ng/mL NGF 2.5S (Murine, natural) (Invitrogen, Carlsbad, CA, USA). Imaris 6.3.1 (Bitplane, St. Paul, MN, USA), and Image J 1.41(NIH) software were used for image analysis. For FRAP experiments a region of interest (ROI) representing neurite tips was selected, fluorescent intensity was measured using Image J, and normalized to a second ROI in the cell body to compensate for photobleaching due to imaging. Voxel fluorescent intensity was measured in neurites and cell bodies of PC12 cells using Imaris software.

**Synaptosome preparation**

Synaptosomes were prepared according to Nagy and Delgado-Escueta (Nagy and Delgado-Escueta, 1984) from 4 week old mice. Briefly, mice were anesthetized by CO\(_2\) and brains quickly transferred to ice cold PBS. Tissue was homogenized by 16 strokes of a Potter-Elvehjem homogenizer at 800 rpm in 0.32M sucrose, 5 mM Heps and 0.5 mM EDTA supplemented with Complete\textsuperscript{TM} anti-protease inhibitor (Roche Molecular Biochemicals; Indianapolis, IN). Homogenates were spun at 1,000 x g for 10min and S1 supernatants were further sedimented at 12,000xg for 20 min. This P2 pellet was
resuspended in 8.5% Percoll (Sigma-Aldrich) and then loaded on a discontinuous gradient comprised of 10 and 16% Percoll. Gradients were spun at 15,000xg for 20 minutes.

**Microvesicles Isolation**

PC12 cell microvesicles were prepared as described (Clift-O'Grady et al., 1998). Briefly, cells were lifted from culture dishes with PBS 5mM EDTA on ice. Cells were spun at 800 RPM for 5 minutes and resuspended in bud buffer (38 mM potassium aspartate, 38 mM potassium glutamate, 38 mM potassium gluconate, 20 mM 4-morpholinepropanesulfonic acid (Mops) pH 7.2, 5 mM reduced glutathione, 5 mM sodium carbonate, 2.5 mM magnesium sulfate) and spun again for 5 minutes at 800 RPM. Cells were then passed through the cell homogenizer (Isobiotec, Heidelberg, Germany) for 16 passes. Cell homogenates were spun for 5 minutes at 1,000 x g and supernatant resolved 10-45% sucrose gradient. Gradient were spun in a SW55 rotor for 1 h at 116,000 × g (Lichtenstein et al., 1998).

**Immuno-affinity Chromotography**

To assess low-affinity interactions between PI4KIIα, AP-3 and BLOC-1 subunits, we performed cross-linking in intact cells with DSP [dithiobis(succinimidylpropionate)](Craige et al., 2008; Salazar et al., 2009; Zlatic et al., 2010). Briefly, SHSY5Y cells stably transfected either with FLAG-dysbindin or FLAG-mutted were placed on ice, rinsed twice with PBS, and incubated either with 10 mM DSP (Pierce, Rockford, IL), or as a vehicle control DMSO, diluted in PBS for 2 h on ice. Tris, pH 7.4, was added to the cells for 15 min to quench the DSP reaction. The cells were then rinsed twice with PBS and lysed in buffer A (150 mM NaCl, 10 mM HEPES, 1 mM EGTA, and 0.1 mM MgCl2, pH 7.4) with 0.5% TritonX-100 by incubation for 30 min on ice. Cells were scraped from the dish, and cell homogenates were centrifuged at 16,100 X g for 10 min. The clarified supernatant was recovered, and at least 500 μg was applied to 30 microliters Dynal magnetic beads (Invitrogen) coated with antibody, and incubated for 2 hours at 4°C. In some cases, immunoprecipitations were done in the presence of the antigenic peptide as a control. The beads were then washed 4-6 times with buffer A with 0.1% TritonX-100. Proteins were eluted from the beads either with sample buffer or by 2 hours incubation with 200 μM antigenic peptide (either PI4KIIα peptide 51-71 or 3X FLAG peptide) on ice. Samples were resolved by SDS-PAGE and contents analyzed by immunoblot.

**Statistical Analysis**

Experimental conditions were compared with the non-parametric Wilcoxon-Mann-Whitney Rank Sum Test or One Way ANOVA, Dunnett's Multiple Comparison using Synergy KaleidaGraph v4.03 (Reading, PA) or StatPlus Mac Built5.6.0pre/Universal (AnalystSoft, Vancouver, Canada). Data are presented as boxplots displaying the four quartiles of the data, with the "box" comprising the two middle quartiles, separated by the median. The upper and lower quartiles are represented by the single lines extending from the box.


Starcevic, M., and Dell'Angelica, E.C. (2004). Identification of snapin and three novel proteins (BLOS1, BLOS2, and BLOS3/reduced pigmentation) as subunits of


Figure Legends

Figure 1: Dysbindin coprecipitates BLOC-1 subunits, AP-3 complexes and PI4KIIα

A) Wild type (Pldn<sup>+/+</sup> odd lanes) or pallidin-deficient (Pldn<sup>pa/pa</sup> even lanes) mouse skin primary culture fibroblasts were treated with DSP, detergent solubilized, and extracts precipitated with magnetic beads with antibodies against AP-3 delta (lanes 3-6) either in the absence (lanes 5-6) or presence (lanes 3-4) of delta antigenic peptide as an immunoprecipitation control. B) SH-SY5Y stably expressing triple FLAG dysbindin treated in the absence or presence of DSP were solubilized in detergent and extracts precipitated with magnetic beads alone as controls (lanes 3-6), with antibodies against FLAG tag (lanes 7-10). Precipitations were performed in the absence or presence of an excess of FLAG peptide (lanes 9-10). An irrelevant antibody (SV2, lanes 5 and 6) was
used to confirm specificity. C) SH-SY5Y FLAG dysbindin or D) SH-SY5Y FLAG muted cell extracts were precipitated with PI4KIIα antibodies (lanes 3-4 and 3'-5') either in the absence or presence of PI4KIIα peptide 51-70 to outcompete binding of PI4KIIα complexes to beads (1st Comp., lanes 4 and 4'). Protein complexes bound to beads were eluted with SDS-PAGE sample buffer (lanes 2-4). The band detected in PI4KIIα blots in lanes 1 and 4 corresponds to the rabbit anti-PI4KIIα IgG also used for immunoprecipitation. Alternatively, PI4KIIα protein complexes were eluted from beads with buffer either in the absence (lane 3') or presence of 200 μM PI4KIIα peptide 51-70 (2nd Elusion, lane 5'). E) Untransfected SH-SY5Y (lanes 1-2 and 5-6) or SH-SY5Y FLAG muted cells treated in the absence or presence of DSP were solubilized in detergent and extracts were precipitated with FLAG antibodies (lanes 1-4) and FLAG muted protein complexes were eluted from beads with 200 μM FLAG peptide (lanes 5-8). Note that PI4KIIα coprecipitates with FLAG tagged Muted even in the absence of DSP (lane 7). Specificity was determined by using cell extracts from non-transfected cells (lanes 5-6). Immune complexes resolved by SDS-PAGE were analyzed by immunoblot with antibodies against FLAG, the BLOC-1 subunits pallidin and muted, and AP-3 subunits (δ, β3, σ3), and PI4KIIα. Inputs are 10% and in panels B-D inputs are lanes 1-1'.
Figure 2: Dentate gyrus PI4KIIα content is reduced in the neuropil of BLOC-1- and AP-3-deficient mice.

The dentate gyrus of the hippocampal formation from four to six week old control (A, B, C, D), BLOC-1-deficient sandy (Dtnbp1sdy/sdy), muted (Mutedmu/mu), pallid (Pldnpa/pa) and AP-3 deficient mocha (Ap3d1mh/mh) mice was stained with antibodies against PI4KIIα (green) and the synaptic vesicle marker synaptophysin (red). E. Total pixels for synaptophysin and PI4KIIα were quantified by Metamorph analysis and expressed as a ratio of PI4KIIα to synaptophysin pixel counts. Numbers in parentheses represent the number of independent sections stained from three animals. Asterisk p<0.0001, double asterisk p<0.005. Bar represents 50 μm.
Figure 3: PI4KIIα content in synaptosomes of AP-3-deficient mocha mice is reduced.

A. Synaptosome fractions from control brains (lanes 1-8) and AP-3 deficient mocha (Ap3d1mh/mh) brains (lanes 1’-8’) were resolved on SDS-PAGE and the contents were analyzed by immunoblot with antibodies against synaptic vesicle markers (SV2, synaptophysin), AP-3-dependent synaptic vesicle cargoes (PI4KIIα, VAMP7, ZnT3) and AP-3 σ3 subunit. B. Depicts quantification of antigen content expressed as a ratio of the heavy synaptosome fraction from control and AP-3 deficient mocha (Ap3d1mh/mh) brains. Numbers in parentheses represent the number of independent immunoblots performed from three independent fractionations. Asterisk p< 0.0001, double asterisk p= 0.0157. P1 and P2 represent low and high-speed pellets. LS, light synaptosomes; HS, heavy synaptosomes; and Mit mitochondrial enriched fractions obtained from Percoll gradients. Fractions were generated as per Nagy and Delgado-Escueta (Nagy and Delgado-Escueta, 1984).
Figure 4: PI4KIIα is present in synapses and its presynaptic levels are decreased in AP-3-null brains (Ap3d1<sup>mh/mh</sup>).

A and A1 show PI4KIIα immuno-peroxidase labeling in the active zone of axon terminals forming asymmetric axosynaptic synapses in the dentate gyrus of a wild type mouse (Ap3d1<sup>+/+</sup>) whereas B and B1 illustrate the lack of such labeling in a AP-3-null mocha mouse (Ap3d1<sup>mh/mh</sup>). Note that the spine in A also displays a low level of immunoreactivity. See arrows. C. Relative prevalence of neural elements immunoreactive for PI4KIIα in random fields of view of the dentate gyrus taken at a
magnification of 75 000X in three wild type animals. A total of 134 dentate gyrus fields were analyzed from three mice. D. Percentage of PI4KIIα-positive terminals over the total number of asymmetric synapses counted in control and AP-3-null mocha (Ap3d1mh/mh) dentate gyrus sections. n1 corresponds to number of animals and n2 is the number of terminals scored per genotype. Note the significant difference in percent of labeled terminals between WT and AP-3-null mocha mice. F. Total number of axon terminals forming axo-spinous asymmetric synapses per square micron of dentate gyrus tissue in three control and three AP-3-null mocha (Ap3d1mh/mh) mice. Numbers in the box plot are the total area of tissue analyzed. Scale bars are 200 nm.
Figure 5: PI4KIIα targeting to neurites in PC12 cells requires the PI4KIIα dileucine sorting motif.

PC12 cells expressing wild type EGFP-PI4KIIα (A,B; n=17 cells) or EGFP-PI4KIIαL60-61A (C,D; n=15 cells) tagged with EGFP were NGF-differentiated for 48-72 hr post-transfection and cells were imaged in vivo. B, D) DIC images. A1, C1) Enlarged view of neurite tips in A and C. E and F, Fluorescent intensity per voxel was measured for wild type PI4KIIα (closed circles) and PI4KIIαL60-61A (open circles) expressing cells both in cell bodies and their processes. (H) and (G) compare fluorescence intensity between PI4KIIα and PI4KIIαL60-61A expressing cells from panels E and F for cell bodies and processes, respectively. I) Transfected cells were stained for VAMP2 and EGFP and
imaged by confocal fluorescence microscopy. Fluorescent pixels present in cell body and processes were quantified for both VAMP2 and transfected PI4KIIα. Closed symbols depict data from cells expressing wild type PI4KIIα (n=26 cells) whereas open symbols depict fluorescent pixels from cells expressing PI4KIIαL60-61A (n= 26). Circles and triangles represent EGFP and VAMP2 fluorescence values, respectively. In E, F, and I, each point depicts the fluorescence intensity in processes and cell body of individual cells as a X,Y coordinates. Data were collected from three independent experiments. Bars equal 10 μm.
Figure 6: Distinct mechanisms mediate the delivery of wild type and dileucine mutant PI4KIIα into neurites.

(A) Look-up table (LUT) of photobleached neurite tips of PC12 cells expressing EGFP-PI4Kα, EGFP-PI4KIIαL60-61A or EGFP-GAP43-ps. In vivo images were taken before (Pre), during photobleaching (0’) and every 5 min thereafter for 30 min, after which an image was acquired every 15 min for an additional 45 min. (B and C) Time course of neurite tips fluorescent intensity (%) during FRAP, normalized to their fluorescent intensity before photobleaching. (B) EGFP-PI4KIIαL60-61A (n=14 cells) recovers faster than EGFP-PI4Kα (n= 23 cells) following photobleaching reaching a plateau within 10 min compared to 45 min respectively. (C) No differences are observed in the time course of recovery between EGFP-PI4KIIαL60-61A and EGFP-GAP43-ps (n= 8 cells). Scale bars are 20 μm.
Figure 7: PI4KIIα targeting to neuronal processes is impaired in AP-3 deficient mocha (Ap3d1<sup>mh/mh</sup>) neurons.

Primary cultured forebrain P4 neurons from wild type (Ap3d1<sup>+/+</sup> A,C) or AP-3 deficient mocha mice (Ap3d1<sup>mh/mh</sup> B,D) either untransfected (A,B) or transfected (C,D) with EGFP-PI4KIIα were cultured for 7DIV. Cells were stained for VAMP2 (red) and either EGFP (green) or endogenous PI4KIIα (green) and imaged by confocal fluorescence microscopy. Fluorescent pixels present in the cell body and processes were quantified for both VAMP2 and either endogenous (E) or transfected PI4KIIα (F) and presented as cell body to processes fluorescent intensity X,Y coordinates. Closed symbols depict data from wild type neurons whereas open symbols depict fluorescent pixels from AP-3-null mocha neurons. Circles and triangles represent PI4KIIα and VAMP2 fluorescence values, respectively. Each point represents an individual neuron. (G-H) Primary cultured forebrain P4 neurons from wild type mice expressing PI4KIIα or PI4KIIαL60-61A tagged with EGFP were cultured for either 7DIV (H) or 3DIV (G). Cells were stained for VAMP2 and EGFP and imaged by confocal fluorescence microscopy. Fluorescent pixels present in cell body and processes were quantified for both VAMP2 and either transfected PI4KIIα (G-H). Closed symbols depict data from cells expressing wild type PI4KIIα whereas open symbols depict fluorescent pixels from cells expressing PI4KIIαL60-61A. Circles and triangles represent EGFP and VAMP2 fluorescence values, respectively. E1-2 n=20 cells, F1-2 n=20 wild type and AP-3-null cells (Ap3d1<sup>mh/mh</sup>), G1-2 n=24 EGFP-PI4KIIα transfected cell and n=20 EGFP-PI4KIIαL60-61A transfected cells. H1-2 n=49 EGFP-PI4KIIα transfected and n=45 EGFP-PI4KIIαL60-61A transfected cells. All neurons were obtained from three independent experiments. Scale bars are 50 μm.
Fig. 8
Figure 8: PI4KIIα targeting to neuronal processes is impaired in BLOC-1 deficient neurons.
Primary cultured forebrain neurons from control (*Dtnbp1*+/+. A, B) or dysbindin-BLOC-1 deficient *sandy* mice (*Dtnbp1*^*sd/sd^+. C, D) either not transfected (E), transfected with EGFP-PI4KIIα (A, C), or EGFP-PI4KIIαL60-61A (B, D) were cultured for 7DIV. Cells were stained for VAMP2 (red) and PI4KIIα for untransfected cells or VAMP2 and EGFP (green) for transfected cells. Fixed cells were imaged by confocal fluorescence microscopy. Fluorescent pixels present in cell body and processes were quantified for both VAMP2 (E1, F1, G1) endogenous PI4KIIα (E), and transfected PI4KIIα (F-G). Closed circles depict PI4KIIα or EGFP fluorescent pixels from wild type neurons whereas open circles depict pixels from dysbindin-BLOC-1-null *sandy* neurons. Closed triangles depict VAMP2 fluorescent pixels from wild type neurons whereas open triangles depict pixels from dysbindin-BLOC-1-null *sandy* neurons. Each point represents an individual neuron. 20 neurons per condition were obtained from three independent experiments. Scale bars are 50 μm.