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The schizophrenia susceptibility factor dysbindin and its associated complex sort cargoes from cell bodies to the synapse

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ABSTRACT 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 of nerve terminals remains unknown. We tested this hypothesis by analyzing the targeting of phosphatidylinositol-4-kinase type II \(\alpha\) (PI4KII\(\alpha\)), a membrane protein present in presynaptic and postsynaptic compartments. PI4KII\(\alpha\) copurified with BLOC-1 and AP-3 in neuronal cells. These interactions translated into a decreased PI4KII\(\alpha\) content in the dentate gyrus of dysbindin-null BLOC-1 deficiency and AP-3–null mice. Reduction of PI4KII\(\alpha\) in the dentate reflects a failure to traffic from the cell body. PI4KII\(\alpha\) 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 PI4KII\(\alpha\) 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). Although the diversity of vesicle carriers and their pathways have been intensely studied in yeast and mammalian fibroblastoid cell lines, 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, 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.,...
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 adaptor 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 presynaptic or postsynaptic compartments. It is surprising that the diversity of cell body–derived vesicle carriers bound to synapses, the coat-adapter mechanisms that assemble these vesicles, and their contents remain largely unexplored (Lasichecka 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 terms of their strength of association with schizophrenia risk. Of importance, 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, 2011; Tang et al., 2009a). 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 biogenesis of lysosome-related organelles complex 1 (BLOC-1 complex; Li et al., 2003; Starcevic and Dell’Angelica, 2004; Di Pietro and Dell’Angelica, 2005). The BLOC-1 complex is assembled by dysbindin, pallidin, mutated, 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 heterotrimer constituted of δ, β3, μ3, and ε3 subunits. The genes encoding the component proteins in AP-3 and BLOC-1 belong to a group of 15 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; Izuka et al., 2007; Ji et al., 2009; Newell-Litwa et al., 2009, 2010; Tang et al., 2009b; Marley and von Zastrow, 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 complex and to regulate AP-3 recruitment to membranes (Craige et al., 2008; Salazar et al., 2009). We demonstrate that PI4KIIα copurifies with BLOC-1 complexes assembled with either tagged dysbindin or mutated 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 mutated 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 neuron-null or BLOC-1–mutant mice. Our findings reveal a novel vesicle transport mechanism in which BLOC-1, in association with the AP-3 complex, delivers 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 previously showed that PI4KIIα binding to AP-3 is sensitive to the dose 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 (Figure 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 Pldhr+/pa allele (Figure 1A, compare lanes 5 and 6). Thus we tested the hypothesis that dysbindin associates with the adaptor complex AP-3 and PI4KIIα in addition to its interactions within the BLOC-1 complex. We expressed N-terminal FLAG-tagged dysbindin in SH-SYSY neuroblastoma cells. Protein complexes coprecipitating with FLAG-dysbindin were isolated from cells treated in the absence or presence of dithiobis(succinimidyl proprionate) (DSP; Figure 1). DSP is a cell-permeable and reducible cross-linker used here to stabilize protein–protein interactions liable to stringent purification (Lomant and Fairbanks, 1976; Salazar et al., 2009; Zlatic et al., 2010). Isolation of FLAG-dysbindin protein complexes with FLAG antibodies co precipitated the BLOC-1 subunits mutated and pallidin (Figure 1B, lane 7), as well as the AP-3 subunits δ, β3, and ε3 (Figure 1B, lane 8). Dysbindin’s associations with AP-3 subunits required stabilization with DSP (Figure 1B, lane 8). These associations with FLAG-dysbindin beads were specific, as determined by their absence from beads alone (Figure 1B, lanes 3 and 4), beads coated with control SV2 antibody (Figure 1B, lanes 5 and 6), and competition with an excess FLAG antigenic peptide during bead incubation.
with cell extracts (Figure 1B, lanes 9 and 10). We next tested whether endogenous PI4KIIα copurified with BLOC-1 subunits by PI4KIIα immunofinity chromatography. PI4KIIα was isolated with an antibody directed against amino acids 51–70 from human PI4KIIα, and PI4KIIα protein complexes were selectively eluted with the PI4KIIα peptide 51–70 used to generate the PI4KIIα antibody (Supplemental...
Dysbindin-dependent protein targeting 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, 2006; Newell-Litwa et al., 2009, 2010). The biochemical interactions among 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 immunoelectron 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 (Figure 3, HS). Levels of SV2 and synaptophysin—two synaptic vesicle markers—remained unaffected in AP-3 mocha (Ap3d1mhs/mhs)–heavy synaptosomes. In contrast, the content of PI4KIIα and the synaptic vesicle AP-3 cargoes VAMP7 and ZnT3 were reduced in AP3d1mhs/mhs–heavy synaptosomes. ZnT3 reduction in synaptosomes was due to decreased ZnT3 levels in Ap3d1mhs/mhs homogenates. In contrast, PI4KIIα was significantly diminished in synaptosomes of Ap3d1mhs/mhs brains without changes in total homogenate levels (Figure 3). This pattern is similar to that of VAMP7 (Figure 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 Ap3d1mhs/mhs mice by immunocytochemistry with a PI4KIIα–specific antibody (Figure 4 and Supplemental Figures S1 and S2). At the light-microscopic level, PI4KIIα displayed a distinctive distribution in different regions of the...
Disruption of AP-3, BLOC-1, and PI4KIIα interactions impairs PI4KIIα targeting from cell body to processes

PI4KIIα depletion in nerve terminals might reflect failed export of this cargo from the parent cell bodies. We addressed this by expressing enhanced green fluorescent protein (EGFP)-tagged PI4KIIα or a dileucine-sorting mutant version of PI4KIIα that is unable to bind AP-3 or coprecipitate with the BLOC-1 complex, PI4KIIαL60-61A (Claire et al., 2008; Salazar et al., 2009). PI4KIIαα is targeted to brain synaptic vesicles and PC12 cell 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, 2006). Thus we determined the targeting of tagged PI4KIIαα 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 (Clift-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 (Supplemental Figure S3). In contrast, EGFP-PI4KIIαL60-61A targeting to synaptic-like microvesicle fractions was reduced (Supplemental Figure S3, A and B) concomitantly with an increased EGFP-PI4KIIαL60-61A content in endosomes (Supplemental Figure S3B; compare open and closed circles). We then used nerve growth factor (NGF)-differentiated PC12 cells and assessed EGFP-PI4KIIα distribution by imaging live and fixed specimens (Figure 5). Wild-type PI4KIIα was enriched at neurite tips of differentiated PC12 cells (Figure 5, A and A1). In contrast, the EGFP-PI4KIIαL60-61A mutant signal was faint in neurites and their tips (Figure 5, C 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; Figure 5, E and F). Decomposition of these x, y-coordinate plots into cell body and process PI4KIIα fluorescence intensities (Figure 5, G and H) revealed that cells expressed similar cell body levels per voxel of wild-type or mutant EGFP-PI4KIIα (Figure S5). However, they differed significantly in the amount of PI4KIIα present in cell processes and their tips (Figure 5H). Similar results were obtained with fixed differentiated PC12 cells, where the EGFP-PI4KIIαL60-61A mutant levels in processes were selectively reduced compared with VAMP2, a synaptic vesicle marker whose targeting is not affected by AP-3 or BLOC-1 deficiencies (Figure 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 PI4KIIαα to determine 1) whether EGFP-PI4KIIα was delivered in an anterograde manner and 2) 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 (Supplemental Figure S3). EGFP-PI4KIIα FRAP of the neurite tip reached a plateau by 45 min (Figure 6, A and B). In contrast, the EGFP-PI4KIIαL60-61A mutant recovery was accelerated, attaining steady state by 10 min (Figure 6, A and 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.
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 whether 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-d 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 (Figures 7 and 8). In wild-type neurons, endogenous PI4KIIα and VAMP2 were present in cell bodies and neuronal processes, but in Ap3d1<sup>tm1/mh</sup> neurons the content of PI4KIIα in cell bodies and processes was selectively reduced (Figure 7A, B, and E1). VAMP2 distribution was not affected in this AP-3 deficiency (Figure 7A, B, and E2). We also expressed EGFP-PI4KIIα in wild-type and AP-3–null mocha cells (Figure 7, C, D, and F). Targeting of EGFP-PI4KIIα to processes was impaired in Ap3d1<sup>tm1/mh</sup> neurons despite cell body expression levels of EGFP-PI4KIIα spanning one order of magnitude (compare Figure 7, E1 and F1). Processes of wild-type neurons were efficiently populated by EGFP-PI4KIIα in that range of cell body expression levels (Figure 7F1). As in neurons lacking AP-3, those expressing mutant PI4KIIα unable to bind AP-3 and coprecipitate with BLOC-1 (EGFP-PI4KIIαL60-61A; Craige et al., 2008; Salazar et al., 2009) failed to populate cell processes (Figure 7, G1–H1). This EGFP-PI4KIIαL60-61A phenotype was observed irrespective of the culture age and recombinant protein expression level in cell bodies (Figure 7, G1–H1). The selectivity of these phenotypes was demonstrated by unaltered VAMP2 distribution (Figure 7, G2–H2). Our data demonstrate that integrity of interaction between the AP-3 adaptor and its cargo, PI4KIIα, is required for cargo export from cell bodies to neurites.

(EGFP-GAP43-ps), since PI4KIIα is anchored to membranes by palmitate (Figure 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.
endogenous (Figure 8E) or EGFP-Pi4KIIα to a similar extent in cell bodies (Figure 8, A, C, and F). However, Pi4KIIα or EGFP-Pi4KIIα did not populate neurites in Dnbp1<sup>+/+;+/y</sup> neurons (Figure 8, E and F; compare closed circles [Dnbp1<sup>+/+</sup>] and open circles [Dnbp1<sup>+/y</sup>]). EGFP-Pi4KIIαL60-61A remained constrained to cell bodies both in control and BLOC-1–null sandy cells (Figure 8G). All Pi4KIIα phenotypes were selective, since the distribution of VAMP2 in neurons was not affected by the Dnbp1<sup>+/+;+/y</sup> allele or the expression of wild-type or mutant forms of Pi4KIIα (Figure 8, E1–F1; compare closed triangles [Dnbp1<sup>+/+</sup>] and open triangles [Dnbp1<sup>+/y</sup>]). Therefore, the dysbindin-containing BLOC-1 complex is required for the delivery of Pi4KIIα to neurites from neuronal perikarya.

### DISCUSSION

We showed here that the schizophrenia susceptibility gene product dysbindin, which is 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 vesicle 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 (Craigie 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 munt and palladin and in AP-3–null mice carrying the Ap3d1<sup>m/n</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 copurification with BLOC-1, and its exit from early endosomes in human fibroblastoid cells (Craigie 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 carriers in PC12 cells (Salazar et al., 2005 2006), 2) the presence of a BLOC-1–AP-3 supercomplex 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 (Vogtmaier 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 in either 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 melanosome requires only BLOC-1 (Setty et al., 2008; Pan et al., 2009). Similarly, Drosophila dysbindin functions presynaptically in adaptive homeostatic modulation of vesicle release (Dickman and Davis, 2009). Of importance, these changes in the physiology of presynaptic secretory organelles occur concomitant 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, 2006, 2009; 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 could impair the development of neuronal processes (Ghiani et al., 2009; Ito et al., 2010).

Presynaptic or postsynaptic 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 knockout 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 (Iardi et al., 1999; Tian et al., 2005; Chen et al., 2009b; Pan et al., 2009).

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-PI4KIIα, 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, C) Time course of neurite tip fluorescence intensity (%) during FRAP, normalized to their fluorescence intensity before photobleaching. (B) EGFP-PI4KIIαL60-61A (n = 14 cells) recovers faster than EGFP-PI4KIIα (n = 23 cells) following photobleaching, reaching a plateau within 10 min vs. 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, 20 μm.
Figure 7: PI4KIIα targeting to neuronal processes is impaired in AP-3–deficient mocha (Ap3d1<sup>1<sup>mh/mh</sup></sup>) neurons. Primary cultured forebrain P4 neurons from wild-type (Ap3d1<sup>+/+</sup>) (A, C) or AP-3–deficient mocha mice (Ap3d1<sup>1<sup>mh/mh</sup></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 fluorescence 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 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, E2, n = 20 cells; F1, F2, n = 20 wild-type and AP-3–null cells (Ap3d1<sup>1<sup>mh/mh</sup></sup>), G1, G2, n = 24 EGFP-PI4KIIα-transfected cells and n = 20 EGFP-PI4KIIαL60-61A–transfected cells; H1, H2, n = 49 EGFP-PI4KIIα–transfected and n = 45 EGFP-PI4KIIαL60-61A–transfected cells. All neurons were obtained from three independent experiments. Scale bars, 50 μm.

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 the Developmental Studies Hybridoma Bank at the University of Iowa (Iowa City, IA), synaptophysin (SY38) from Chemicon International/Millipore (Billerica, MA), and VAMP7-T1 (a generous gift of Andrew Peden, Cambridge University, Cambridge, United Kingdom), as well as rabbit polyclonals against PI4KIIα peptide 51-71 PGHDREQPLLDRARGAAAQ (UniProt/Swiss-Prot entry Q9MM64 [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 (Tigard, OR).

Rat monoclonal anti-HA was from Roche (Indianapolis, IN). Glutathione S-transferase full fusion protein of PI4KIIα was prepared as previously described (Craig et al., 2008). pEGFP-C2 wild-type rat PI4KIIα and pEGFP-C2-PI4KIIαL60-61A mutant plasmids were previously described (Craig et al., 2008). C-Terminal FLAG-tagged mused (EXT4755-M14) and N-terminal–tagged FLAG-tagged Dysbindin (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 (Cambridge, MA) plasmid 14757 (Matsuda and Cepko, 2007).

mocha (STOCK gr<sup>+/+</sup> Ap3d1<sup>1<sup>mh/mh</sup></sup>), here referred to as Ap3d1<sup>1<sup>mh/mh</sup></sup> and its control grizzled (STOCK gr<sup>+/+</sup> Ap3d1<sup>+/+</sup>), here referred to as Ap3d1<sup>+/+</sup>) and pallid (B6.Cg-Pldn<sup>+/+</sup>) mice were obtained from Jackson Labs (Bar Harbor, ME) and bred in-house following Institutional Animal Care and Use Committee (IUCAC)–approved protocols. Muted mice and their controls (B6.C3 A<sup>w</sup>–/A-Mutec<sup>md1</sup>)(J, here referred to as Ap3d1<sup>+/+</sup>) and Pallid mice (B6C3 A<sup>w</sup>–/A-Mutec<sup>md1</sup>)(J, Muted<sup>md1</sup>), and CHMU.<sup>m</sup> (<sup>md1</sup>) (Zhang et al., 2002) were obtained from Richard Swank (Roswell Park Cancer Institute, Buffalo, NY) and bred in-house. Sandy mice in C57/6 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 postnatal day 4 (P4) mice (Ap3d1<sup>1<sup>+/+</sup></sup>, Ap3d1<sup>1<sup>mh/mh</sup></sup>, CHMU, muted, C57 and sandy) and maintained in neurobasal media containing B27, l-glutamine, and 100 μg/ml penicillin and streptomycin (Hyclone, Logan, Utah).
UT) at 5% CO₂ and 37°C. Dissociated neurons were plated on poly-l-lysine (Sigma-Aldrich, St. Louis, MO)–coated glass coverslips and cultured for 3–7 d in vitro (DIV). Dissociated neurons (2 × 10⁶) were transfected with 3 μg of plasmid DNA using Amaxa nucleofection electroporation (Lonza, Cologne, Germany). Neurons were plated at a density of 6 × 10⁴ 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 (American Type Culture Collection, Manassas, VA) were cultured in DMEM supplemented with either 10% fetal bovine serum (FBS) or, in the case of PC12 cells, a mix of 5% FBS plus 10% equine serum, respectively (HyClone). Media were supplemented with 100 μg/ml penicillin and streptomycin (HyClone). Cells were maintained at 37°C with 10% CO₂. PC12 cells were transfected with 3 μg/μl DNA by nucleofection using Amaxa Cell Line Nucleofector Kit V (Lonza) and plated in PC12 culture media supplemented with 100 ng/ml NGF 2.5S (murine, natural) (Invitrogen, Carlsbad, CA) on Matrigel-coated, glass-bottom culture dishes (MaTek, Ashland, MA). PC12 cells were differentiated for 48–72 h 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 mo 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 ascending 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 3,3'-diaminobenzidine (DAB) reaction product (Talbot et al., 2004). Results were analyzed on a BX61 Olympus (Center Valley, PA) research microscope equipped with an Olympus DP71 cooled charge coupled device (CCD) digital camera.

Immuno–electron microscopy
Tissue used in brain slice preparations was obtained from either male or female mice between 6 and 8 wk of age, unless otherwise indicated. Following deep anesthesia with Nembutal, animals were transcardially perfused with fixative (4% paraformaldehyde with 0.1% glutaraldehyde). Brains were postfixed in 4% paraformaldehyde, which was replaced with phosphate-buffered saline (PBS) within 12–18 h. With use of a vibrating microtome, brains were cut into 60-μm-thick sections and stored in antifreeze (0.1 M sodium phosphate monobasic, 0.1 M 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 and then incubated in sodium borohydride, followed by 100% cryoprotectant (phosphate buffer 0.05 M, pH 7.4, 25% sucrose, 10% glycerol) for 20 min at −80°C and returned to decreasing amounts of cryoprotectant. Sections were preincubated in PBS with 1% normal horse serum (NHS) and 1% BSA, followed by primary antibody incubation with 1:500 affinity-purified anti-PI4KIIα. Sections were rinsed in PBS and then incubated in a 1:1000 dilution of rabbit peroxidase antiperoxidase (Jackson ImmunoResearch Labs). Sections were rinsed in PBS with a final rinse in Tris buffer (50 mM, pH 7.6) before a 10-min incubation in 0.025% DAB, 1 mM 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 (0.1 M, pH 7.4). Sections were incubated in 1% osmium tetroxide for 10 min 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 min. 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 h. 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 min.

Electron microscopy was performed with a Zeiss EM-10C electron microscope with a CCD camera (DualView 300W; Gatan, Pleasanton, CA). Images were acquired with Gatan Digital Micrograph Software, version 3.10.1 (Gatan). Analysis was focused on the interface between the granule cells and hilar neuropil, where PI4KIIα was concentrated across mice brains. In the electron microscope, only tissue areas from the surface of the blocks with optimal antibody penetration were examined. These sections were scanned at 10,000×, and random fields of view containing at least one asymmetric synapse were photographed at 75,000×. One hundred ninety micrographs from three blocks of tissue in three animals covering 773 μm² were examined for the control and 172 micrographs from three blocks of tissue in three animals covering 700 μm² were examined from this material, the total number of PI4KIIα-immunoreactive or immunonegative terminals forming asymmetric axosomatic synapses was counted to estimate the relative percentage of positively labeled terminals. In addition, in wild-type (Ap3d1Δ/Δ) mice, 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. (1991). Light microscopy of brain sections was performed with a Leica DMRB microscope with a 10×/0.3 differential interference contrast (DIC) objective (Leica Microsystems, Bannockburn, IL), and images were captured with a CCD camera and the Leica IMS50 software (Leica DC500).

**Immunofluorescence labeling for confocal microscopy**

Confocal microscopy was performed with an Axiovert 100M microscope (Carl Zeiss, Jena, Germany) coupled to Ar and He–Ne lasers. Images were acquired using Plan Apochromat 10×/0.5 dry, 20×/0.5 dry, and 40×/1.3 and 63×/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 min at room temperature, followed by extensive washing with PBS. Samples were preincubated in a solution of PBS with 5% NHS and 1% BSA and 0.3% Triton X-100 for 60 min at room temperature. 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 min in a secondary antibody PBS solution with 1% NHS and 1% BSA and anti-PI4KIIα with anti-synaptophysin (SY38) (dilutions of the following Alexa-conjugated secondary antibodies: anti–mouse S55 (for anti-synaptophysin) and anti–rabbit 488 (for anti-PI4KIIα) (Invitrogen Molecular Probes, Carlsbad, CA). Following PBS rinses, sections were incubated in cupric sulfate (3.854 wt/vol ammonium acetate, 1.596 wt/vol cupric sulfate in distilled water, pH 5) for 30 min. Sections were washed with PBS and mounted on slides with Vectashield (Vector Laboratories, Burlingame, CA).

HEK293 cells, PC12 cells, and cortical neurons were washed in ice-cold PBS and fixed in 4% paraformaldehyde for 20 min on ice. Cells were then incubated in blocking solution (2% BSA plus 1% fish skin gelatin plus 15% horse serum plus 0.02% saponin in PBS) for 30 min at room temperature. Next cells were incubated with antibody for 30 min at 37°C, rinsed, and then incubated with secondary antibody diluted in block in 30 min at 37°C. 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 Banker, 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 PI4KIIα channel.

Live imaging of PC12 cells expressing PI4KIIα-GFP, PI4KIIαL60-61A-GFP, or EGFP-GAP43-p5 was performed on an A1R Laser Scanning Confocal Microscope (Nikon, Melville, NY) equipped with a hybrid scanner, Perfect Focus, and an environmental chamber for regulation of temperature to 37°C and 10% CO2. Confocal images with a Z-step of 1μm were captured for 10 min (no delay) with an Apo total internal reflection fluorescence 60×/1.49 oil differential interference contrast (DIC) objective on NIS-Elements AR 3.1 (Nikon) software. Neurite tips of PC12 cells were photobleached for 2 s at 30% laser power. Images were captured for 15 s (no delay) before photobleaching and for 15 min every 5 min after photobleaching, followed by 2 h every 15 min. PC12 cell imaging was performed in Hank’s balanced salt solution minus phenol red and NaHCO3 (Sigma-Aldrich) and supplemented with 10% Donor Equine Serum (Hyclone), 5% fetal bovine serum (Hyclone), and 100 ng/ml NGF 2.5S. Imaris 6.3.1 (Bitplane, St. Paul, MN) and ImageJ 1.41 (National Institutes of Health, Bethesda, MD) software were used for image analysis. For FRAP experiments an ROI representing neurite tips was selected, and fluorescence intensity was measured using ImageJ and normalized to a second ROI in the cell body to compensate for photobleaching due to imaging. Voxel fluorescence 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 (1984) from 4-wk-old mice. Briefly, mice were anesthetized by CO2 and brains quickly transferred to ice cold PBS. Tissue was homogenized by 16 strokes of a Potter-Elvehjem homogenizer at 800 rpm in 0.32 M sucrose, 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 0.5 mM EDTA supplemented with Complete antiprotease inhibitor (Roche). Homogenates were spun at 1000 × g for 10 min, and S1 supernatants were further sedimented at 12,000 × g 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 15,000 × g for 20 min.

Microvesicle isolation

PC12 cell microvesicles were prepared as described (Clift-O’Grady et al., 1998). Briefly, cells were lifted from culture dishes with PBS plus 5 mM EDTA on ice. Cells were spun at 800 rpm for 5 min and resuspended in bud buffer (38 mM potassium aspartate, 38 mM potassium glutamate, 38 mM potassium gluconate, 20 mM 4-morpholinepropanesulfonic acid, pH 7.2, 5 mM reduced glutathione, 5 mM sodium carbonate, 2.5 mM magnesium sulfate) and spun again for 5 min at 800 rpm. Cells were then passed through the cell homogenizer (Isobiotec, Heidelberg, Germany) for 16 passes. Cell homogenates were spun for 5 min at 1000 × 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).

Immunofluorescence microscopy

To assess low-affinity interactions between PI4KⅢα, AP-3, and BLOC-1 subunits, we performed cross-linking in intact cells with DSP (Clift et al., 2008; Salazar et al., 2009; Zlatic et al., 2010). Briefly, SHSY5Y cells stably transfected either with FLAG-dysbindin or FLAG-mutated 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 ethylene glycol tetraacetic acid, and 0.1 mM MgCl2, pH 7.4) with 0.5% Triton X-100 by incubation for 30 min on ice. Cells were scraped from the dish, and cell homogenates were centrifuged at 16,100 × g for 10 min. The clarified supernatant was recovered, and at least 500 μg was applied to 30 μl of Dynal magnetic beads (Invitrogen) coated with antibody and incubated for 2 h at 4°C. In some instances, immunoprecipitations were done in the presence of the antigenic peptide as a control. The beads were then washed four to six times with buffer A with 0.1% Triton X-100. Proteins were eluted from the beads either with sample buffer or by 2 h of incubation with 200 μM antigenic peptide (either PI4KⅢα peptide 51-71 or 3× FLAG peptide) on ice. Samples were resolved by SDS-PAGE and contents analyzed by immunoblot.

Statistical analysis

Experimental conditions were compared with the nonparametric Wilcoxon–Mann–Whitney rank sum test or one-way analysis of variance, Dunnett’s multiple comparison using Synergy KaleidaGraph, version 4.03 (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.

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