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Pearl Victoria Ryder Date

Actin Cytoskeleton Regulators Interact with the Hermansky-Pudlak Syndrome Complex BLOC-1 and its Cargo Phosphatidylinositol-4-kinase Type II Alpha

By

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Doctor of Philosophy

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Abstract

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By Pearl Victoria Ryder

Vesicle biogenesis machinery components such as coat proteins can interact with the actin cytoskeleton for cargo sorting into multiple pathways. However, whether or not these interactions are a general requirement for the diverse endosome traffic routes is unknown. In this dissertation, I identified actin cytoskeleton regulators as previously unrecognized interactors of complexes associated with the Hermansky-Pudlak syndrome. Two complexes mutated in the Hermansky-Pudlak Syndrome, AP-3 and BLOC-1, interact with and are regulated by the lipid kinase PI4KIIα. I therefore hypothesized that PI4KIIα interacts with novel regulators of these complexes. To test this hypothesis, I immunoaffinity purified PI4KIIα from isotope-labeled cell lysates (SILAC) to quantitatively identify interactors. Strikingly, PI4KIIα isolation preferentially co-enriched proteins that regulate the actin cytoskeleton, including guanine exchange factors for Rho family GTPases such as RhoGEF1 and several subunits of the WASH complex. I biochemically confirmed several of these PI4KIIα interactions. Importantly, BLOC-1 complex, WASH complex, RhoGEF1, or PI4KIIα depletions altered the content and/or subcellular distribution of the BLOC-1-sensitive cargoes PI4KIIα, ATP7A, and VAMP7. I conclude that the Hermansky-Pudlak syndrome complex BLOC-1 and its cargo PI4KIIα interact with regulators of the actin cytoskeleton. Exploring these interactions will provide insight into the regulation of actin polymerization at endosomes and the pathogenic mechanisms of human diseases such as pigmentation disorders, neurocutaneous syndromes, and neurodegeneration.

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Of course, I must thank my family and friends for their support during this experience. My friends from high school, college, medical school, and Atlanta have enriched my life and made graduate school a joyful process. My sisters are incredible role models and as a younger sibling, I have looked to their experiences to shape and guide my own. Finally, my parents have always pushed me and encouraged me to do good work. Through their own examples as boatbuilder and craftswoman, they have shown me that attention to detail and teamwork matters and to try to do it right the first time. My mother is the original storyteller in my family and has taught me to speak and write as clearly as possible. Finally, their support and love have enabled me to leap for new experiences at University of Chicago and Emory University with the security that I can fall back on them if needed.

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CHAPTER I

GENERAL INTRODUCTION

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Overview and significance

The segregation of membrane proteins and lipids to distinct domains of the cell is critical for cellular function (De Matteis and Luini, 2011). One mechanism by which this segregation is achieved is the sorting and targeting of specific membrane protein and lipids from a donor organelle to a target organelle by means of small, membrane-enclosed vesicular intermediates (Bonifacino and Glick, 2004). Cytosolic factors known as coats transiently polymerize at intracellular membranes to both scaffold vesicle biogenesis protein machinery and to concentrate cargo into nascent vesicles (Robinson, 2004). At the plasma membrane, local and coordinated polymerization of actin is associated with membrane deformation and scission. The adaptor protein complex-2 (AP-2) and clathrin coat recruit nucleators of actin polymerization to the site of vesicle budding as one component of the vesicle biogenesis machinery (Robertson *et al.*, 2009). However, a fundamental unanswered question is the extent to which cytoskeletal interaction is a general principal for cytosolic coat function. In this dissertation research, I tested this principle by isolating novel interactors of a vesicle biogenesis pathway defined by two protein complexes mutated in the Hermansky-Pudlak syndrome, the adaptor protein complex-3 (AP-3) and biogenesis of lysosome-related organelles complex-1 (BLOC-1). My central hypothesis was that:

> **The BLOC-1 complex and a cargo and regulator of the AP-3-BLOC-1 pathway, the phosphatidylinositol 4-kinase type IIα (PI4KIIα), interact with regulators of the actin cytoskeleton.**

My dissertation research tested this hypothesis and led to the following discoveries, detailed in Chapter 2:

1) The Hermansky-Pudlak syndrome protein complex, BLOC-1, and an associated regulatory lipid kinase, PI4KIIα, co-isolate with actin cytoskeleton regulators. These regulators include guanine exchange factors, such as RhoGEF1 and DOCK7, and subunits of an Arp2/3 activator, the WASH complex.
2) The AP-3 and BLOC-1 complexes genetically interact with both PI4KIIα, the guanine exchange factor RhoGEF1, and a subunit of the WASH complex.
3) Actin filaments, WASH complex subunits, and a BLOC-1 complex subunit co-reside at endosomes.
4) Depletion of BLOC-1 or the WASH complex alters PI4KIIα-containing endosomal morphology.
5) Cargoes of the AP-3 and BLOC-1 pathway mis-localize upon depletion of the WASH complex.

My dissertation research expands the conception of vesicular trafficking within the AP-3 and BLOC-1 complex pathway. My work supports a role for the actin cytoskeleton and its regulators as previously unrecognized participants within the vesicle biogenesis and sorting processes regulated by these complexes. In addition, my research contributes novel frameworks to conceptualize the regulation of actin polymerization at endosomes and the pathogenesis mechanisms of neurodegenerative diseases. In the following introduction, I will provide context to support these contributions. I will first review fundamental principles of membrane

trafficking, with a focus on the known role for the actin cytoskeleton within the canonical, AP-2-dependent vesicle budding process at the plasma membrane. Second, I will introduce the AP-3 and BLOC-1 complexes and their known regulator, the lipid kinase PI4KIIα. Finally, I will discuss the actin cytoskeleton and its regulators, including the small regulator GTPases of the Rho family, the Arp2/3 complex, and the nucleation-promoting factors including N-WASP and the WASH complex.

Fundamental Principles of Membrane Trafficking

Endocytic and secretory organelles exchange components by membrane trafficking

Organelles in eukaryotic cells maintain unique protein and lipid compositions, allowing for the compartmentalization of biochemical reactions and functions. In the endocytic and secretory pathways this compartmentalization is created and maintained by transport of molecular components between donor and target organelles within membrane-enclosed vesicles (Bonifacino and Glick, 2004). Transported molecular components include peripheral and integral membrane proteins, secreted proteins, intralumenal soluble proteins required for organelle function such as lysosomal hydrolases, and lipids. In total, estimates suggest that up to one-third of the human proteome is trafficked by vesicular trafficking pathways (De Matteis and Luini, 2011). Many human diseases are associated with genetic mutations affecting membrane trafficking proteins, highlighting the critical role this system plays in normal function and human disease (De Matteis and Luini, 2011). Vesicles are created by the coordinated action of cytosolic factors that are recruited to particular membranes upon specific signals and cues. These cytosolic factors

include coat complexes and their accessory adaptors, as well as small GTPases, BAR domain proteins, and lipid-modifying enzymes such as phosphatidylinositol kinases and phosphatases (Brodsky *et al.*, 2001; Bonifacino and Glick, 2004; Lippincott-Schwartz and Phair, 2010; Donaldson and Jackson, 2011; Qualmann *et al.*, 2011; Mayinger, 2012). Coat complexes play a particularly critical role in the vesicle budding process (Brodsky *et al.*, 2001). Upon specific recruitment cues, cytosolic coat proteins polymerize on intracellular membrane surfaces. At the membrane, coats act as scaffolds to concentrate the machinery and cargo required for vesicle budding. These complexes both bind to location-specific determinants and interact with motifs in the cytosolic domain of cargo to concentrate them into budding vesicles (Traub, 2009). In addition, coats recruit factors required for fusion at the target organelle, such as vesicular SNAREs and tethers (Bonifacino and Glick, 2004). Thus, individual coat complexes are important determinants of the sorting and segregation of molecular components within the cell. As such, many unique types of coat complexes exist in the cell and act to coordinate vesicular trafficking between different pairs of donor and target organelles (Robinson, 2004). My work focuses on the vesicular trafficking pathway defined by the adaptor protein complex-3 (AP-3) and an associated complex, the biogenesis of lysosome-related organelles complex-1 (BLOC-1). Before I introduce the AP-3 complex in detail, I will first introduce other adaptor complexes with a focus on the heterotetrameric adaptors and their relationship with the clathrin coat complex.

Adaptor protein complexes link coat complexes with cargo at specific subcellular domains

Adaptor complexes are so named for their ability to interact with the clathrin coat and subcellular membrane domains. The clathrin coat is composed of three heavy chains and three light chains that form a triskelion structure (Pearse, 1976; Brodsky *et al.*, 2001). The polymerization of these triskelia into a polyhedral lattice promotes deformation of the membrane and vesicle budding (Brodsky *et al.*, 2001). However, clathrin-coated vesicles are formed at multiple subcellular locations, including the plasma membrane, early endosome, and trans-Golgi network. Further, the clathrin-coated vesicles formed at these distinct subcellular localizations must contain unique cargo in order to induce and maintain the compartmentalization of membrane proteins and lipids introduced above. This cargo and localization selectivity is created by clathrin adaptors, which act as cargo selection modules at specific subcellular compartments (Robinson, 2004). These adaptors bind to both the clathrin lattice and to specific motifs within the cytosolic domain of cargo proteins, known as sorting signals (Traub, 2009). In addition, adaptors bind to membrane-recruitment signals such as phosphoinositides and activated small GTPases of the Arf family (Donaldson and Jackson, 2011; Mayinger, 2012). There are several different types of clathrin adaptors, including heterotetrameric adaptor complexes, monomeric Golgi-localized, γ-ear containing, Arf-binding proteins (GGAs), and cargo-specific adaptors, including, for one example, β-arrestin, a G protein coupled receptor-specific adaptor (Traub, 2003) (Fig. 1). The heterotetrameric adaptor complexes are particularly important coordinators of

vesicular trafficking pathways, as they can interact with both clathrin, cargo, and with accessory adaptors for specific cargo recruitment (Robinson, 2004).

To date, five heterotetrameric adaptor complexes have been described. These complexes are composed of two large subunits or adaptins (β1-5 and γ, α, δ, ε, ζ in AP-1-5), a medium chain subunit (μ1-5), and a small chain subunit (σ1-5). All together, these subunits form stable complexes of approximately 250 – 350 kDa, with disruption of one subunit leading to destabilization of the other subunits. The AP-1 adaptor recruits cargo into clathrin-coated vesicles at the trans-Golgi network and early endosome, while AP-2 acts at the plasma membrane for internalization and endocytosis. Unlike AP-1 and AP-2, the other heterotetrameric complexes, AP-3, AP-4, and AP-5, may not function as clathrin adaptors (Borner *et al.*, 2012). The AP-3 complex localizes to early endosomes and will be discussed extensively below, as it is a major focus for this work. The AP-4 and AP-5 complexes are ubiquitously expressed at low levels in mammalian cells and localize to the trans-Golgi network and late endosomes, respectively. While these proteins are implicated in vesicular trafficking, they are not present in clathrin-coated vesicle biochemical fractionations, suggesting that clathrin does not play a structural role in AP-4- and AP-5-dependent budding. One possibility is that another protein fulfills this scaffolding role, although this hypothesis has not been thoroughly tested to date. The process of AP-2-dependent clathrin-coated vesicle budding at the plasma membrane is best characterized. For this reason, I will first introduce the budding process by this canonical pathway before discussing the AP-3-BLOC-1 pathway.

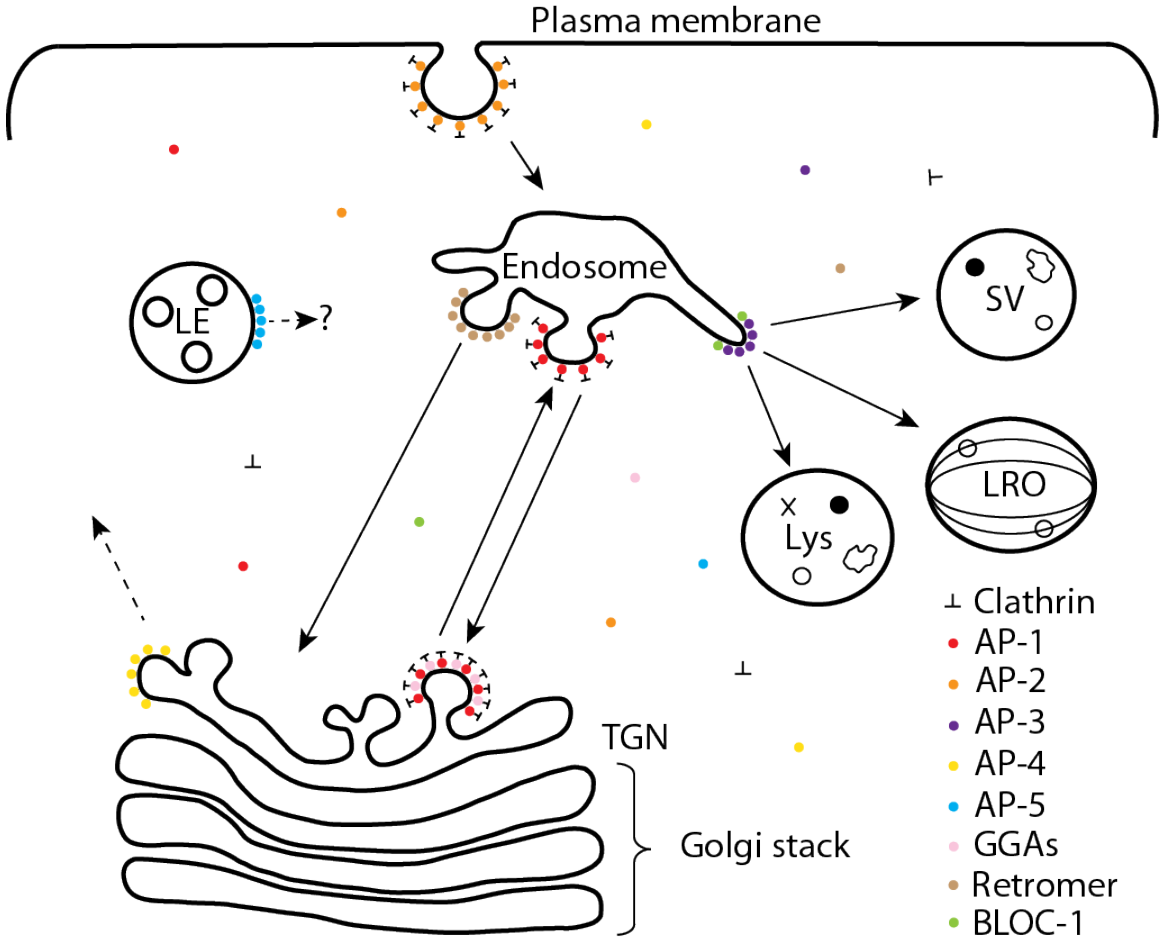

Figure 1. Adaptor-mediated vesicular transport creates and maintains unique organelle composition. Five heterotetrameric adaptor protein complexes (AP-1 to AP-5), the monomeric Golgi-localized, γ-ear containing, Arf-binding proteins (GGAs), the retromer complex, and the biogenesis of lysosome-related organelles complex-1 (BLOC-1) coordinate to mediate transport between unique combinations of donor and target organelles. The AP-3 and BLOC-1 complexes target membrane proteins and lipids from endosomes to synaptic vesicles (SV), lysosomes (Lys) and lysosome-related organelles (LROs). Other abbreviations used: LE, late endosome; TGN, trans-Golgi network. Figure inspired by (Robinson, 2004).

AP-2-dependent vesicle budding at the plasma membrane

There are several stages of AP-2-dependent clathrin-coated vesicle budding from the plasma membrane, which is also known as clathrin-mediated endocytosis. An initial nucleation event can lead to either failure or progression through

stabilization of a clathrin-coated pit, invagination, and eventual membrane scission (Fig. 2). The nucleation event is initiated by the random convergence and binding of two AP-2 adaptors to phosphatidylinositol-4,5-diphosphate (PI4,5P2). Four PI4,5P2 binding regions are present in AP-2: one in each large subunit, α and β2, and two in the medium chain μ2 (Jackson *et al.*, 2010). Binding of the adaptor complex to PI4,5P2 results in large scale conformational changes that expose pockets for cargo binding (detailed movie model from (Jackson *et al.*, 2010) available at http://tinyurl.com/AP2-docking). The μ2 subunit binds cargoes containing a YxxΦ motif (where x is any amino acid and Φ is a bulky hydrophobic residue) while an interface between the large α and small σ2 subunits binds cargoes containing a [ED]xxxL[LI] motif. These binding sites can be engaged simultaneously, which stabilizes the open conformation of AP-2 and promotes its retention at the membrane, thereby promoting progression to a stable clathrin-coated pit (Jackson *et al.*, 2010). The large subunits, α and β2, contain appendage domains that are separated from the core of the complex by flexible linker or hinge domains. These appendages bind the clathrin coat and other accessory proteins to scaffold these components at sites of vesicle budding. Many of these accessory proteins coordinate clathrin-coated vesicle endocytosis with the actin cytoskeleton, including the huntingtin-interacting proteins (HIP1 and HIP1-related) and the Bin/Amphiphysin/Rvs (BAR) domain proteins. In addition to interacting with the clathrin-AP-2 adaptor coat, HIP-1 and HIP-1R bind the actin cytoskeleton and lipids of the plasma membrane, which is shown to couple the force of actin polymerization with membrane deformation in the yeast model system (Wilbur *et al.*, 2008;

Skruzny *et al.*, 2012). The BAR domain proteins sense and/or induce membrane curvature (Qualmann *et al.*, 2011). Within the BAR domain superfamily, several classes exist with differential preference for high or low curvature membranes (Peter *et al.*, 2004; Henne *et al.*, 2010). Thus, these proteins are recruited at many stages of clathrin-coated vesicle internalization, particularly to the clathrin-coated pit and the neck formed just prior to membrane scission (Fig. 2). In addition to stabilizing membrane curvature, several BAR domain proteins bind and recruit the actin nucleation-promoting factor N-WASP, thereby coordinating polymerization of actin with membrane curvature (Merrifield *et al.*, 2004; Yarar *et al.*, 2007).

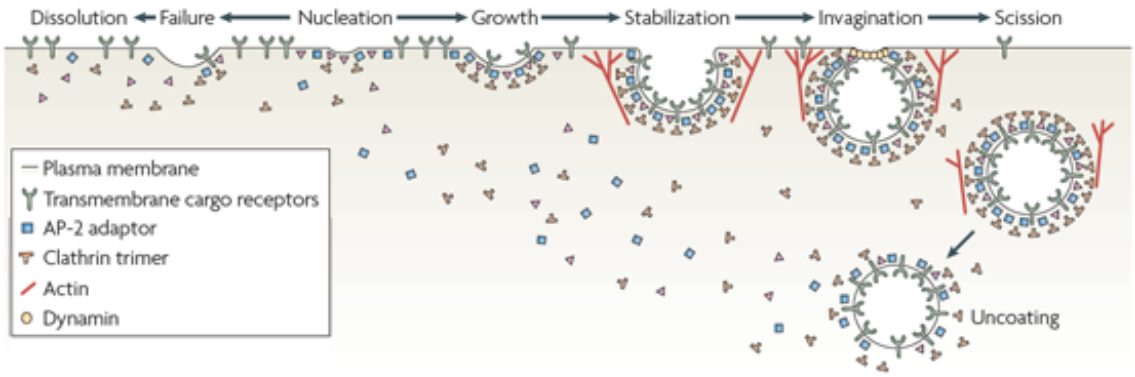

Figure 2. The canonical vesicle budding process: AP-2-dependent clathrin-mediated endocytosis. Clathrin-mediated endocytosis at the plasma membrane is initiated by the random convergence of two adaptor protein complexes and a single clathrin triskelion (Jackson *et al.*, 2010). Binding of the adaptor complex AP-2 to membrane proteins concentrates these cargoes into clathrin-coated pits. Invagination of these coated pits leads to membrane scission and budding of a clathrin- and AP-2-coated vesicle. Actin polymerization at late stages of clathrin-mediated endocytosis is implicated in the invagination and membrane scission of clathrin-coated vesicles. In addition, actin filaments are one suggested recruitment signal for the dynamin GTPase, a key step in membrane scission. Figure modified from (Traub, 2009).

The role of actin polymerization in AP-2-dependent vesicle budding

Actin polymerization is clearly required for clathrin-coated vesicle internalization in yeast cells, yet the role of actin polymerization in clathrin-mediated endocytosis in mammalian cells remains controversial (Galletta *et al.*, 2010; Mooren *et al.*, 2012). Membranes under high tension, such as the apical plasma membrane of epithelial cells, appear to require actin polymerization for internalization (Gottlieb *et al.*, 1993; Boulant *et al.*, 2011). Studies in cultured mammalian cells under low membrane tension report conflicting conclusions that actin polymerization is either necessary (Lamaze *et al.*, 1997; Merrifield *et al.*, 2002; Yarar *et al.*, 2005) or dispensable (Fujimoto *et al.*, 2000; Boucrot *et al.*, 2006) for clathrin-mediated endocytosis. However, electron microscopy studies of clathrin-coated vesicle internalization at low-tension membranes demonstrate the presence of short, highly branched actin filaments at most CCV budding profiles (Collins *et al.*, 2011). Correlative light microscopy analysis of these budding profiles often failed to detect these actin networks, suggesting that studies relying only on light microscopy approaches may underestimate the presence of actin at sites of clathrin-coated vesicle budding (Collins *et al.*, 2011). Thus, actin dynamics contribute to clathrin-mediated endocytosis but may not be obligatory in all cell types. Cells treated with drugs that perturb actin dynamics have an increased number of clathrin-coated pits stalled at a late stage in their maturation, suggesting roles for the actin cytoskeleton during invagination and membrane scission (Fig. 2) (Lamaze *et al.*, 1997; Merrifield *et al.*, 2005; Yarar *et al.*, 2005; Boulant *et al.*, 2011). A prominent role for actin polymerization during invagination was revealed in cells deficient in the dynamin

GTPase, which is required for membrane scission during clathrin-mediated endocytosis (Ferguson *et al.*, 2009; Mettlen *et al.*, 2009; Ramachandran, 2011). These cells formed exaggerated tubular clathrin-coated pits in an actin- and BAR protein-dependent manner (Ferguson *et al.*, 2009). Further, in a cell-free reconstitution of vesiculation from isolated plasma membrane sheets inhibition of actin polymerization by treatment with latrunculin resulted in the formation of stalled clathrin-coated pits that failed to form tubules (Wu *et al.*, 2010). In addition to this role during invagination, several reports have demonstrated a role for the actin cytoskeleton and associated motors in membrane scission during clathrin-mediated endocytosis (Fig. 2). One clear potential role for actin polymerization is the generation of force required to excise a vesicle, and *in vitro* reconstitution experiments support the sufficiency of actin polymerization to mediate membrane scission, albeit in a clathrin-independent pathway (Römer *et al.*, 2010). In addition to force generation, however, dynamin interacts with both actin and actin-binding proteins. Thus, the actin cytoskeleton and its regulators may be a recruitment cue and/or activator for the scission machinery (Schafer, 2004; Krendel *et al.*, 2007; Gu *et al.*, 2010). Consistent with these potential roles, dynamin is not recruited to stalled clathrin-coated pits in cells treated with the actin perturbing drugs latrunculin and jaskplakinolide (Boulant *et al.*, 2011). Further, *in vitro* activity assays show that addition of the actin regulatory proteins SNX9 or cortactin increase the GTPase activity of dynamin, which is fundamental for its function in membrane scission (Soulet *et al.*, 2005; Mooren *et al.*, 2009).

In summary, the adaptor protein complex-2 selectively binds the plasma membrane in an inositol phospholipid-dependent manner. In addition, AP-2 acts as a scaffold to recruit accessory proteins for vesiculation, which include the BAR domain-containing curvature sensors and actin regulatory proteins. While the role of actin during mammalian clathrin-mediated endocytosis has been controversial, an increasing number of reports support roles for actin polymerization during the late stages of clathrin-mediated endocytosis, including invagination and scission. Importantly, the AP-2 and clathrin coat complex recruit these regulators of actin polymerization. My work draws parallels with the conceptual framework provided by the mechanism of AP-2-dependent vesicle biogenesis at the plasma membrane. The recent identification of an activator of actin polymerization that localizes to the early endosome suggests actin polymerization may also contribute to budding from this organelle. My work explores this possibility by focusing on a vesicular trafficking pathway defined by the adaptor protein complex-3 (AP-3) and an associated complex, the biogenesis of lysosome related organelles complex-1 (BLOC-1). In the next section I introduce these two complexes and their roles in mammalian cells.

The adaptor protein complex-3 (AP-3)

AP-3 is a heterotetrameric complex composed of two large subunits, β3 and δ, a medium subunit μ3, and a small subunit, σ3 (Fig. 3). (Simpson *et al.*, 1996; 1997; Dell'Angelica *et al.*, 1997a). Two isoforms of the β3 and μ3 subunits exist in mammalian cells and are expressed either ubiquitously (μ3A, β3A) or neuronal-specifically (μ3B, β3B) (Simpson *et al.*, 1997; Dell'Angelica *et al.*, 1997b). AP-3

subunits were initially cloned and described as targets of autoimmune antibodies that cause neurodegeneration, suggesting an important role for the AP-3 complex in nervous system function (Newman *et al.*, 1995). These initial reports recognized the homology of the newly cloned proteins to subunits of the previously characterized adaptor complexes AP-1 and AP-2, which led to the identification of AP-3 as a third type of heterotetrameric adaptor complex (Simpson *et al.*, 1996; 1997). In addition, an independent group searching for heterotetrameric adaptor complex homologs identified the σ3 subunit by database searches and used this information to isolate the rest of the complex (Dell'Angelica *et al.*, 1997a; 1997b).

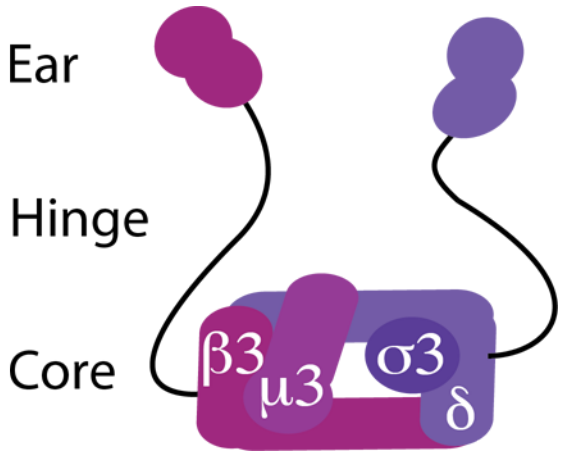

Figure 3. Composition of the adaptor protein complex-3 (AP-3). The AP-3 complex consists of two large subunits, β3 and δ, a medium subunit, μ, and a small subunit, σ3. All four subunits interact to form a core domain, which mediates binding to membranes and cargo. The hinge and ear domains of the large subunits interact with other cytosolic complexes, such as the clathrin coat molecule.

As an adaptor complex, AP-3 cycles between the cytosol and membranes in an Arf1-dependent and brefeldin A-sensitive manner (Simpson *et al.*, 1996; Faúndez *et al.*, 1998; Ooi *et al.*, 1998). In addition, AP-3 function is regulated by phosphatidylinositol 4-kinase type IIα binding and kinase activity (discussed in more detail in the section titled "PI4KIIα is a cargo and regulator of AP-3 and BLOC-

1”). AP-3 localizes to the early endosomal compartments at the tips of tubular structures (Dell'Angelica *et al.*, 1998; Peden *et al.*, 2004). Like other adaptor protein complexes, AP-3 concentrates membrane protein cargo into budding vesicles, although unlike AP-1 and AP-2, the role of clathrin in this process remains controversial. The AP-3 complex interacts with clathrin (Dell'Angelica *et al.*, 1998), 50% of AP-3 budding profiles in HepG2 liver cells are coated with clathrin (Peden *et al.*, 2004), and AP-3 is present in biochemical enrichments of clathrin-coated vesicles (Borner *et al.*, 2006), suggesting some functional interaction between clathrin and AP-3. However, data suggest that AP-3 does not interact with clathrin in the same fashion as the classical clathrin adaptors AP-1 and AP-2. First, unlike AP-1 and AP-2, AP-3 is not enriched several fold in clathrin-coated vesicle preparations (Simpson *et al.*, 1996; Borner *et al.*, 2012). Second, *in vitro* reconstitution of AP-3-dependent budding does not require clathrin (Faúndez *et al.*, 1998). Finally, rapid perturbation of clathrin function depletes AP-1 and AP-2 from clathrin-coated vesicle preparations, while AP-3 increases in these fractions, suggesting again a unique relationship with clathrin (Zlatic *et al.*, 2013). Thus, formally the AP-3 complex may not be a clathrin adaptor complex, but rather a vesicular coat complex.

AP-3 deficiency causes the Hermansky-Pudlak syndrome

Insight into the function of AP-3 arose with the recognition that spontaneous mutations to subunits of the AP-3 complex cause hypopigmentation and an increased bleeding tendency in animal models (Ooi *et al.*, 1997; Simpson *et al.*, 1997; Swank *et al.*, 1998; Li *et al.*, 2004). These symptoms are the core symptoms of a heterogeneous group of genetic human diseases known as the Hermansky-Pudlak

syndrome (HPS; OMIM 203300) (Hermansky and Pudlak, 1959). In addition to hypopigmentation and increased bleeding tendency, some HPS subtypes include impaired visual acuity, colitis, immune cell dysfunction, recurrent lung infections, pulmonary fibrosis, and occasionally cardiomyopathy and renal failure (see (Spritz, 2000; Huizing *et al.*, 2008) for extensive reviews of HPS symptoms). Further, some of the animal models of HPS, including the AP-3-deficient *mocha* mouse, have neurological phenotypes such as epilepsy and hyperactivity, suggesting an important role for the Hermansky-Pudlak syndrome pathway in neurological function (Kantheti *et al.*, 1998; Nakatsu *et al.*, 2004; Seong *et al.*, 2005; Newell-Litwa *et al.*, 2007). The protein products of the 15 genes described to cause a Hermansky-Pudlak syndrome phenotype in humans and/or animal models organize into five complexes: the adaptor protein complex-3 (AP-3; HPS2) (Dell'Angelica *et al.*, 1999; Feng *et al.*, 1999), biogenesis of lysosome-related organelles complex-1 (BLOC-1; HPS7-9) (Falcon-Perez, 2002; Moriyama and Bonifacino, 2002), BLOC-2 (HPS3, HPS5-6) (Di Pietro *et al.*, 2004; Gautam *et al.*, 2004), BLOC-3 (HPS1, HPS4) (Martina *et al.*, 2003; Nazarian *et al.*, 2003), and the homotypic fusion and protein sorting complex (HOPS) (Huizing *et al.*, 2001a; Suzuki *et al.*, 2003). The symptoms that characterize the Hermansky-Pudlak syndrome largely reflect impaired biogenesis or function of lysosome-related organelles, such as melanosomes (Nguyen and Wei, 2004), platelet dense granules (Novak *et al.*, 1984), cytotoxic T cell lytic granules (Clark *et al.*, 2003), and lung lamellar bodies (Lyerla *et al.*, 2003). A major focus of my dissertation work is the function of the AP-3 coat complex and an associated complex BLOC-1.

AP-3 vesicles are targeted from early endosomal compartments to lysosomes, lysosome-related organelles such as melanosomes and platelet dense granules, and synaptic vesicles in neuronal tissues (Dell'Angelica, 2009). Membrane proteins are considered AP-3 cargo if they meet three criteria: (1) direct binding to an AP-3 subunit, (2) *in vivo* localization to AP-3-vesicle budding profiles, and (3) functional consequence of AP-3 deficiency. To date, several AP-3 cargo have been identified, with known functions at lysosomes (Lamp1), lysosome-related organelles (neutrophil elastase (Benson *et al.*, 2003), tyrosinase (Huizing *et al.*, 2001b)), and synaptic vesicles (ZnT3 (Salazar *et al.*, 2004; 2005a), PI4KIIα (Guo *et al.*, 2003; Craige *et al.*, 2008; Newell-Litwa *et al.*, 2009), VGlut1 (Salazar *et al.*, 2005a)). In addition, AP-3 binds directly with the lysosomal SNARE Vamp7 and regulates its targeting to late endosomes, lysosomes, and synaptic vesicles from early endosomes (Martinez-Arca *et al.*, 2003; Newell-Litwa *et al.*, 2009; 2010; Kent *et al.*, 2012). AP-3 deficiency causes several types of functional consequences to cargo, including loss of the cargo at the target organelle, decrease in the total cellular content of the cargo (Salazar *et al.*, 2006), and/or an increase in the cell surface localization of the cargo (Dell'Angelica *et al.*, 1999; Peden *et al.*, 2002). This phenotype at the cell surface allows a sensitive, selective, and quantitative measurement of perturbation to the AP-3 pathway. Proteomic analyses of AP-3-coated vesicles and AP-3-interacting proteins have provided further insight into the function of AP-3 (Salazar *et al.*, 2005b; 2009). These analyses have revealed that subunits of the biogenesis of lysosome related-organelles complex-1 (BLOC-1) are a major component of the AP-3 interactome.

The biogenesis of lysosome-related organelles complex-1 (BLOC-1)

Subunits of the biogenesis of lysosome-related organelles complex-1 (BLOC-1) were first characterized as the gene products of causative genes for mouse models of Hermansky-Pudlak syndrome (Falcon-Perez, 2002; Moriyama and Bonifacino, 2002; Ciciotte *et al.*, 2003; Li *et al.*, 2003; Starcevic and Dell'Angelica, 2004). BLOC-1 is a stable complex of eight subunits, with loss of one subunit sufficient for destabilization of the other components of the complex (Fig. 4). Thus, the majority of the cellular content of each subunit appears to incorporate into stable complexes. This hypothesis is supported by the similarity of the systemic phenotypes of BLOC-1 subunit deficiencies. Unlike the AP-3 complex, where homology to proteins with known function was immediately apparent, the molecular function of the BLOC-1 complex has remained far more elusive. BLOC-1 subunits are 15-50 kDa in molecular weight and contain no apparent protein domains. The assembled complex has an elongated, curved, and flexible structure, as revealed by negative stain electron microscopy of purified recombinant complexes (Lee *et al.*, 2011). Endogenous BLOC-1 localizes to tubular domains of early endosomes in MNT1 melanoma cells (Di Pietro *et al.*, 2006). In neuronal cells, BLOC-1 subunits localize to pre- and post-synaptic compartments (Talbot *et al.*, 2006; Larimore *et al.*, 2013). In addition, BLOC-1 subunits are present in AP-3-derived synaptic-like microvesicles and synaptic vesicles (Salazar *et al.*, 2005b; 2006).

Figure 4. Subunit composition of the biogenesis of lysosome-related organelles complex-1 (BLOC-1). The BLOC-1 complex is comprised of eight subunits, which are organized into three functional subcomplexes, denoted above by columns. Dotted lines depict interactions between subunits as identified by yeast two-hybrid binding analysis. Note the elongated structure of the complex, which reflects structural analysis of purified recombinant complexes by negative stain electron microscopy. Abbreviations used: BLOS1-3, biogenesis of lysosome-related organelles complex subunit 1-3; Cpo, cappuccino. Figure inspired by (Lee *et al.*, 2011).

Possible functions of the BLOC-1 complex

The BLOC-1 complex is proposed to function in membrane fusion and in vesicular trafficking of membrane proteins. A role in membrane fusion is suggested by the multiple studies and approaches that have demonstrated binding of BLOC-1 subunits with SNARE proteins (Huang *et al.*, 1999; Ilardi *et al.*, 1999; Salazar *et al.*, 2006; Gokhale *et al.*, 2012a). In addition, the BLOC-1 complex co-purifies with tethering complexes such as the exocyst complex, which functions at the plasma membrane, and the COG complex, which functions within the Golgi complex (Gokhale *et al.*, 2012a). These tethering complexes bring together membranes undergoing fusion in a step upstream of SNARE mediated fusion (Bröcker *et al.*, 2010; Wickner, 2010). Multiple lines of evidence support a role for BLOC-1 in vesicular trafficking. Numerous reports from independent laboratories document an interaction between BLOC-1 and the coat complex AP-3 (Di Pietro *et al.*, 2006; Salazar *et al.*, 2006; Hashimoto *et al.*, 2009; Hikita *et al.*, 2009; Newell-Litwa *et al.*, 2009; Salazar *et al.*, 2009; Taneichi-Kuroda *et al.*, 2009; Mead *et al.*, 2010; Newell-Litwa *et al.*, 2010; Lee *et al.*, 2011; Gokhale *et al.*, 2012a). Two AP-3 cargoes, the

lysosomal proteins CD-63 and Lamp1, are mis-localized to the cell surface in BLOC-1 deficiencies, suggesting that this interaction is functional (Di Pietro *et al.*, 2006; Salazar *et al.*, 2006). Further, in neuronal cells BLOC-1 deficiency alters the targeting of two well-characterized AP-3 cargoes to synaptic vesicles and pre-synaptic compartments, the lysosomal SNARE Vamp7 and the phosphatidylinositol 4-kinase type IIα (Salazar *et al.*, 2006; 2009; Larimore *et al.*, 2011). However, combined genetic deficiencies in AP-3 and BLOC-1 are more severe than the single deficiencies, suggesting that these two complexes act independently in at least some functions (Di Pietro *et al.*, 2006; Cheli and Dell'Angelica, 2010). Supporting this hypothesis, BLOC-1 is implicated in AP-3-independent targeting of at least two proteins to the melanosome, a lysosome-related organelle: the copper transporter ATP7A and the tyrosinase-related protein-1 (TYRP1) (Setty *et al.*, 2007; 2008). In the absence of BLOC-1, ATP7A and TYRP1 accumulate in early endosomes and fail to reach melanosomes, suggesting a defect in exit from early endosomes.

Possible molecular mechanisms of BLOC-1 function

The precise molecular mechanisms by which BLOC-1 regulates membrane protein sorting remain unresolved. Several non-exclusive mechanisms are possible. First, the BLOC-1 complex may act as a coat complex and generate vesicles independently of AP-3. As discussed above, several types of evidence support this role, including: (1) binding of BLOC-1 with membrane protein cargo, (2) *in vivo* localization of BLOC-1 and cargo proteins to tubulo-vesicular budding profiles, and (3) functional consequences to BLOC-1-interacting proteins that reflect defective vesiculation at early endosomes. To fully establish BLOC-1 as a coat complex, however, requires *in*

vitro reconstitution of an AP-3-independent, ATP-dependent, and GTPase-dependent vesicle budding reaction. A second possibility is that the BLOC-1 complex facilitates AP-3-dependent vesicle budding at early endosomes. For example, BLOC-1 could bind to both cargo and to the AP-3 complex, to facilitate the concentration of cargo into AP-3-coated vesicles. This type of "accessory coat" mechanism would potentially regulate only the subset of AP-3 cargoes capable of interacting with BLOC-1. Alternatively, BLOC-1 could act as a structural support to facilitate the tubulation process required for AP-3-dependent vesiculation. Consistent with this possibility, BLOC-1 localizes along the length of endosomal tubules *in vivo* and is precipitated from cytosol by filamentous actin (Falcon-Perez, 2002). The complexity of BLOC-1 interactions and consequences of BLOC-1 deficiency suggest that this complex may have multiple roles within the endocytic pathway. Precise characterization of its molecular function will therefore require multi-pronged approaches, including the characterization of its regulators, such as the phosphatidylinositol-4-kinase type IIα.

In summary, the AP-3 and BLOC-1 complexes coordinate to regulate vesicular trafficking from early endosomes to lysosomes, lysosome-related organelles, and to synaptic vesicles. Mutations to these complexes cause the Hermansky-Pudlak syndrome, which is characterized by hypopigmentation, an increased bleeding tendency, and pulmonary fibrosis. These symptoms represent defects in the biogenesis of lysosome-related organelles. To date, the best-characterized regulator of AP-3 and BLOC-1 vesicle trafficking is the lipid kinase PI4KIIα. My work expands known interactors and regulators by defining the

interaction network of this regulatory lipid kinase. In the next section, I will introduce this membrane lipid kinase, its function and the modulation of its activity within the Wnt signaling pathway, and its role within the AP-3 and BLOC-1 vesicle trafficking pathway.

The phosphatidylinositol 4-kinase type IIα is a cargo and a regulator of AP-3 and BLOC-1

Insight into the function and regulation of the AP-3 and BLOC-1 complexes arose from the identification and characterization of cargo within this pathway. These cargo were identified by mass spectrometry analysis of AP-3-coated vesicles derived from neuronal cells (Salazar *et al.*, 2005b) and reverse coating reactions *in vitro* (Baust *et al.*, 2008). In addition, immunoprecipitation of AP-3 complexes from *in vivo* crosslinked cell lysates followed by mass spectrometry analysis of interacting proteins expanded our knowledge of the AP-3 interaction network (Salazar *et al.*, 2009). This crosslinking technique preserves the low affinity and transient interactions characteristic of coat-cargo interactions and allows for rigorous biochemical purifications. These approaches led to the identification of the phosphatidylinositol 4-kinase type IIα (PI4KIIα) as a prominent interactor of both the AP-3 complex and the BLOC-1 complex (Craige *et al.*, 2008; Salazar *et al.*, 2009; Larimore *et al.*, 2011). In the next section, I will discuss the history of the identification of phosphoinositides and phosphatidylinositol kinases with a focus on PI4KIIα. I will then describe currently known functions of PI4KIIα, including the role of PI4KIIα in regulation of AP-3 and BLOC-1.

Initial characterization of phosphatidylinositol kinases

Interest in phosphatidylinositol kinases arose upon purification and identification of membrane lipids in mycobacteria, the brain, and plants (Anderson and Roberts, 1930; Klenk and Sakai, 1939). In the early 1940s, phosphatidylinositol phosphate was described as a component of brain lipids, along with phosphatidylserine and phosphatidylethanolamine (Folch, 1949). Further characterization demonstrated that the primary brain phosphatidylinositol phosphate was phosphorylated at the 4 position of the inositol ring (PI4P) (Brockerhoff and Ballou, 1961). Biochemical characterization of the phosphatidylinositol 4-kinases in mammalian tissues demonstrated that they were ubiquitously expressed and active despite the fact that the phospholipid product PI4P accumulated to sufficient levels for biochemical detection in brain tissue only (Colodzin and Kennedy, 1965; Michell *et al.*, 1967). Prior to molecular cloning, three classes of phosphatidylinositol 4-kinases were described on the basis of their biochemical and pharmacological properties leading to the type I-III distinctions. The type I kinase was later realized to be a phosphatidylinositol 3-kinase (PI3K) (Endemann *et al.*, 1987; Whitman *et al.*, 1987; 1988). Type II phosphatidylinositol 4-kinases are approximately 55 kDa in molecular weight, inhibited by adenosine but not wortmannin, and have higher affinities for ATP and phosphatidylinositol than the type III kinases (Husebye *et al.*, 1990). Type III phosphatidylinositol 4-kinases are soluble proteins and are structurally related to the PI3K family (Yoshida *et al.*, 1994; Nakagawa *et al.*, 1996a; 1996b; Meyers and Cantley, 1997). While the phosphatidylinositol 4-kinases generate the same lipid product, PI4P, they act at unique subcellular domains,

interact with diverse pathways, and thus appear to have independent cellular functions.

The type II phosphatidylinositol 4-kinases

There are two mammalian type II PI 4-kinases encoded by unique genes, PI4KIIα and PI4KIIβ (Barylko *et al.*, 2001; Minogue, 2001). The protein sequence for the human type II PI 4-kinases are 61% identical and 73% similar by BLASTp analysis, with the C-terminal catalytic domain especially well conserved. PI4KIIβ distributes between the cytosol and intracellular membranes of the trans-Golgi network, endosomes, and plasma membrane (Balla *et al.*, 2002; Wei *et al.*, 2002). PI4KIIα, the major focus of this work, is palmitoylated and as a result is tightly membrane associated (Fig. 5). While some reports have described localization of PI4KIIα to the trans-Golgi network (Wang *et al.*, 2003; 2007), independent reports from different groups demonstrate a predominant localization to the endocytic pathway (Balla *et al.*, 2002; Salazar *et al.*, 2005b; Minogue, 2006; Salazar *et al.*, 2006; Craige *et al.*, 2008; Salazar *et al.*, 2009) and to vesicles derived from this compartment, including synaptic vesicles (Guo *et al.*, 2003; Salazar *et al.*, 2005b; Takamori *et al.*, 2006; Larimore *et al.*, 2011) and GLUT4-containing vesicles (Jia *et al.*, 2010). In the sections below, I will highlight two known functions of PI4KIIα. First, I will discuss the role of PI4KIIα in canonical Wnt signaling, a pathway in which modulation of PI4KIIα activity is described. Second, I will thoroughly introduce the role of PI4KIIα in regulation of the AP-3 and BLOC-1 complexes.

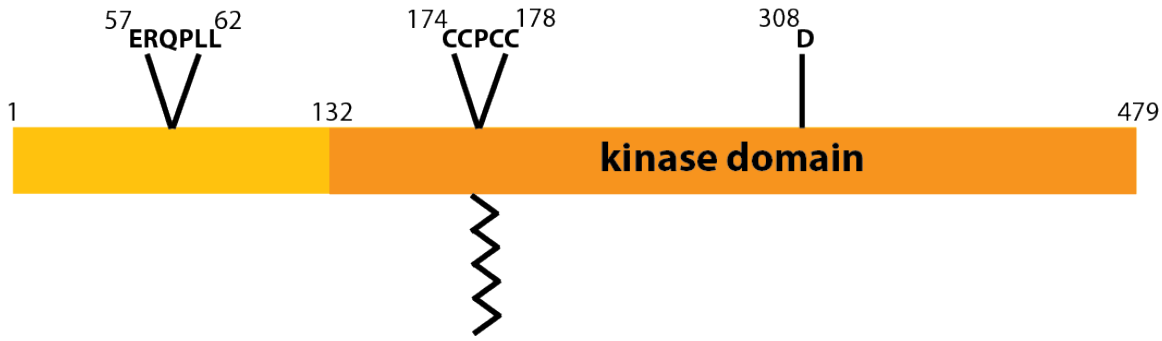

Figure 5. Domain architecture of phosphatidylinositol 4-kinase type IIα (PI4KIIα). PI4KIIα is comprised of 479 amino acids and contains a highly conserved dileucine sorting motif at the N-terminal domain. This motif, ERQPLL, mediates direct binding between PI4KIIα and the AP-3 heterotetrameric sorting complex. A motif within the kinase domain, CCPCC, is necessary for palmitoylation and tight membrane association. Finally, an aspartic acid residue within the kinase domain is necessary for kinase activity.

PI4KIIα is a component of the canonical Wnt signaling pathway

The canonical Wnt signaling pathway is a cell-to-cell communication mechanism with critical roles in embryonic patterning and tissue healing (Rottner *et al.*, 2010). Secreted Wnt proteins bind two co-receptors, the frizzled protein and the low-density lipoprotein receptor-related protein-6 (LRP6) (Fig. 6). Binding of Wnt3a to frizzled recruits the dishevelled signaling adaptor to the cytoplasmic tail of frizzled (Rotty *et al.*, 2012). Dishevelled forms a tripartite complex with both PI4KIIα and the phosphatidylinositol 4-phosphate 5-kinase (PIP5K), an enzyme that catalyzes the conversion of PI4P to PI4,5P2 (Pan *et al.*, 2008; Qin *et al.*, 2009). The production of PI4,5P2 promotes phosphorylation of the cytoplasmic tail of the LRP6 co-receptor, possibly by serving as a membrane recruitment signal for LRP6 kinases (Salazar *et al.*, 2009). This phosphorylation is a critical step in the Wnt signaling cascade, and thereby leads to the accumulation of β-catenin and the transcription of target genes. While the secreted Wnt protein engages frizzled at the plasma membrane, the

complex is rapidly internalized, and the interaction between dishevelled and PI4KIIα appears to predominantly occur at early endosomes (Salazar *et al.*, 2009). One aspect of this pathway of high interest is the modulation of PI4KIIα kinase activity. First, binding of dishevelled to PI4KIIα results in a two-fold increase in kinase activity *in vitro*, thereby promoting Wnt signaling *in vivo*. Second, the ubiquitin ligase Itch participates and regulates this pathway via PI4KIIα (Mössinger *et al.*, 2012). Binding of Itch to PI4KIIα decreases the kinase activity of PI4KIIα *in vitro* and thereby attenuates Wnt signaling via the frizzled-LRP6 co-receptors *in vivo*. In addition, both PI4KIIα and Itch are necessary for the degradative sorting of internalized frizzled receptors to lysosomes. Thus, protein-protein interactions can mediate both increases and decreases in PI4KIIα kinase activity.

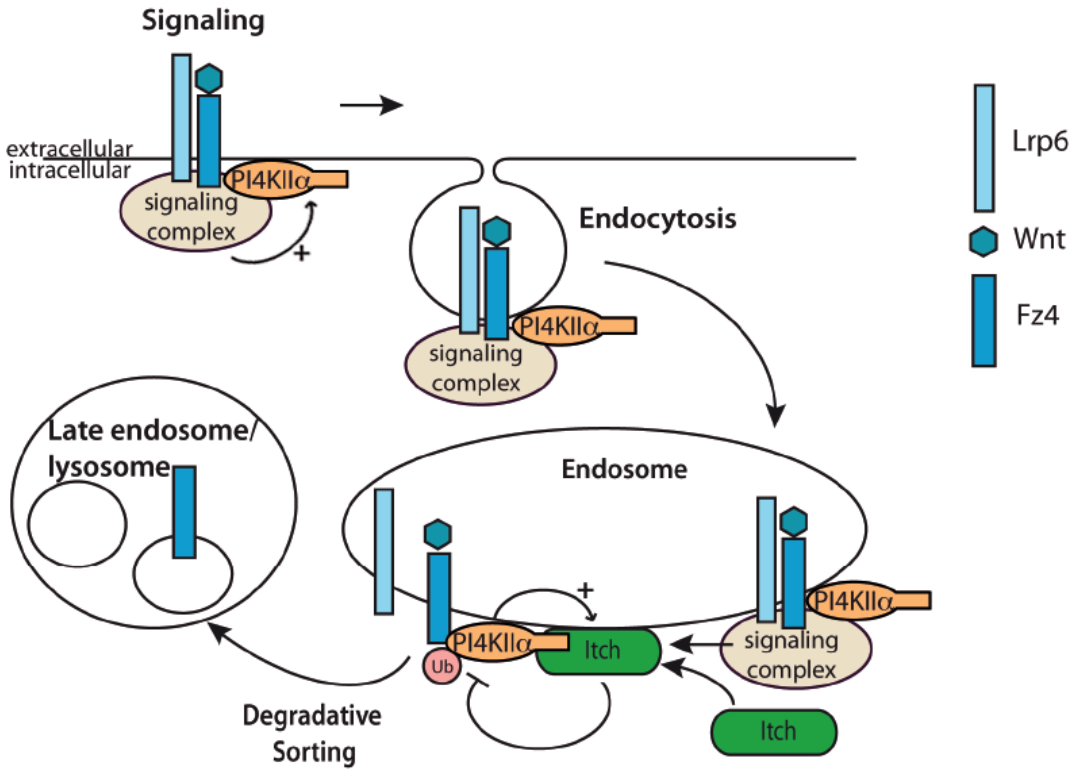

Figure 6. Model for regulation of PI4KIIα activity by the Wnt signaling pathway. The Wnt signaling pathway provides the best-characterized example of

regulation of PI4KIIα kinase activity. Binding of the secreted Wnt ligand to its co-receptors the low-density lipoprotein receptor-related protein-6 (Lrp6 and frizzled-4 (Fz4) recruits PI4KIIα and a cytosolic signaling complex. This signaling complex promotes PI4KIIα kinase activity via protein-protein interactions. Following internalization, the ubiquitin ligase Itch associates with PI4KIIα and negatively regulates is kinase activity via protein-protein interactions and ubiquitin (Ub) modification. Figure modified from (Mössinger *et al.*, 2012).

PI4KIIα is a cargo and a regulator of the BLOC-1 and AP-3 complexes

A major function for PI4KIIα and the focus of this work is its role as both a cargo and a regulator of the AP-3 and BLOC-1 complexes (Fig. 7). Several lines of evidence support the model that PI4KIIα is a cargo of the AP-3 and BLOC-1 complexes. First, PI4KIIα contains a highly conserved dileucine sorting motif, which fits the consensus sequence (DER)XXL(L/I) (Larimore *et al.*, 2011). These dileucine sorting motifs mediate direct binding of heterotetrameric adaptor complexes with their membrane protein cargoes (Gokhale *et al.*, 2012a). In the case of PI4KIIα, this motif is required for direct binding with the AP-3 complex (Larimore *et al.*, 2011). In addition, BLOC-1 protein complexes contain PI4KIIα and the association of AP-3 with PI4KIIα decreases upon loss of BLOC-1 (Salazar *et al.*, 2009; Larimore *et al.*, 2011). In addition to biochemical interactions, by immunofluorescence analysis approximately 50% of PI4KIIα co-localizes with AP-3 subunits in a dileucine motif-dependent manner (Salazar *et al.*, 2005b; Craige *et al.*, 2008). Finally, localization of PI4KIIα to Lamp1-positive organelles, synaptic-like microvesicles, and hippocampal nerve terminals requires both the AP-3 and BLOC-1 complexes (Salazar *et al.*, 2005b; Craige *et al.*, 2008; Larimore *et al.*, 2011). However, PI4KIIα acts as more than just a passive cargo of this pathway. Heterotetrameric adaptors are recruited to membranes in part by binding phosphatidylinositol phosphate lipids such as

PI4,5P2 at the plasma membrane and PI4P at the trans Golgi network. This model suggests that localized production of PI4P by PI4KIIα at the early endosome may facilitate the recruitment of AP-3 to the membrane. Consistent with this model, depletion of PI4KIIα by siRNA leads to a decrease in AP-3 membrane association and a dispersion of AP-3 particles within the cell (Salazar *et al.*, 2005b; Craige *et al.*, 2008). In addition, the kinase activity of PI4KIIα is required for the co-localization between AP-3 and PI4KIIα and the localization of PI4KIIα to lysosomes labeled by Lamp1 (Craige *et al.*, 2008). Thus, PI4KIIα behaves as both a cargo and a regulator of the AP-3 and BLOC-1 complexes and is a prime target for discovery of novel interactors and regulators of these complexes.

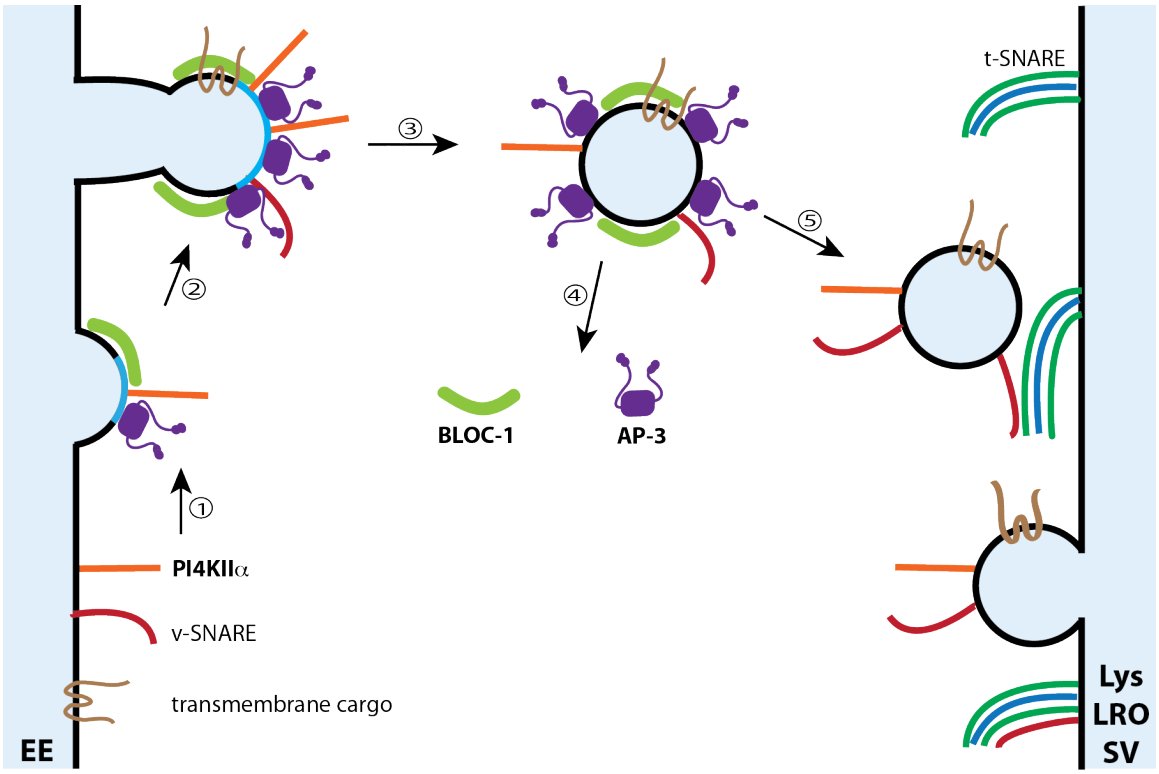

Figure 7. Model for regulation of the AP-3 and BLOC-1 complexes by PI4KIIα. Modification of membrane lipids by PI4KIIα (step 1 above) recruits the AP-3 and BLOC-1 complexes from the cytosol to polymerize on early endosomal (EE) membranes (step 2). Direct binding of the AP-3 complex to the PI4KIIα dileucine

sorting motif concentrates this enzyme in budding vesicles and promotes the vesicle biogenesis process. Following membrane scission (step 3), AP-3 and BLOC-1 depolymerize from vesicles by an unknown process (step 4) to maintain the cytosolic population of these complexes. Vesicles are targeted to fuse at lysosomes (Lys), lysosome-related organelles (LRO), and synaptic vesicles (SV). As predicted, mutations to the PI4KIIα dileucine sorting motif or kinase domain increase the cytosolic fraction of AP-3 and BLOC-1 and decrease the generation of AP-3-coated vesicles. Figure inspired by (Bonifacino and Glick, 2004).

My dissertation research described in Chapter 2 characterizes the interaction network of PI4KIIα and identifies regulators of the actin cytoskeleton as predominant components of this network. These regulators include guanine exchange factors for regulatory small GTPases, such as RhoGEF1 and DOCK7, and an activator of the Arp2/3 complex, the WASH complex. In addition to interacting with PI4KIIα, these actin cytoskeleton regulators physically and genetically interact with the BLOC-1 complex and modulate the localization of BLOC-1 cargoes, suggesting a functional role for these interactions. In the following section, I will introduce the actin cytoskeleton and the regulation of the polymerization of actin filaments by small regulatory GTPases, guanine exchange factors, and the Arp2/3 complex. In addition, I will introduce the known activators of the Arp2/3 complex, with a focus on the well-characterized N-WASP and WAVE proteins and the recently identified endosomal activator, the WASH complex.

The actin cytoskeleton and its regulators

Actin monomers polymerize into structural units critical for cellular function

Actin is a 42 kDa globular protein that can spontaneously polymerize into filaments to create a structural framework and generate force in all eukaryotic cells. Many prokaryotes have structurally and functionally similar proteins, suggesting that actin is an ancient and fundamental component of biological systems (Thanbichler

and Shapiro, 2008). Actin filaments are asymmetric in their structural and kinetic properties, with a pointed (-) and a barbed end (+) (Pollard and Borisy, 2003). Actin monomers are preferentially incorporated into filaments at the barbed end and slowly dissociate from the pointed end. While monomeric actin can spontaneously polymerize into filaments, formation of the initial dimer required for filament polymerization is energetically unfavorable (Pollard and Borisy, 2003). As a result, spontaneous polymerization of actin filaments proceeds slowly both *in vitro* and *in vivo*. A diversity of mechanisms have evolved in eukaryotic cells to overcome this energetic barrier and to allow for the local, rapid, and regulated polymerization of actin filaments. Proteins complexes known as actin nucleators are the keystone of cellular control of actin polymerization. Three major actin nucleators exist in eukaryotic cells: (1) the Arp2/3 complex, (2) formins, and (3) WH2 domain nucleators (Campellone and Welch, 2010). The formins and WH2 domain nucleators catalyze the polymerization of unbranched actin filaments. Many of these nucleators also bind and bundle microtubules, allowing for crosstalk between cytoskeletal elements (Rosales-Nieves *et al.*, 2006; Chesarone *et al.*, 2010). Roles for formins and WH2 domain nucleators have been described at multiple subcellular locations, with particularly important roles in function of phagocytic structures, stress fibers, and membrane protrusions such as lamellipodia and filopodia (Campellone and Welch, 2010). Activation of these processes is regulated in time and space, in large part by small GTPases of the Rho family. My work focuses on an activator of the Arp2/3 complex, the WASH complex (Wiskott-Aldrich syndrome protein and SCAR Homolog) and its role in regulating vesicle trafficking at early endosomes. In the

next sections, I will introduce aspects of actin polymerization regulation relevant to this work, including the Rho family GTPases, the Arp2/3 actin nucleator, its activators, and the WASH complex.

Regulation of actin polymerization in vivo: small GTPases and guanine exchange factors

The small GTPase superfamily contains more than 80 members, which are organized into five broad families on the basis of function and structure (Rojas *et al.*, 2012). These families are named for their canonical founding members: Ras, Ran, Rab, Arf, and Rho. Broadly speaking, the Rab and Arf families are traditionally associated with endosomes and vesicle trafficking (Itzen and Goody, 2011). The Rho family of small GTPases contains 18 members organized into 10 groups by homology, including three members that have been best characterized: RhoA, Cdc42, and Rac1 (Madaule and Axel, 1985; Hall, 1998). Small GTPases act as bimolecular switches that cycle between a GTP-bound and a GDP-bound form (Fig. 8). GTP-bound Rho family members bind to and activate effectors, leading to cytoskeletal rearrangements (Ridley and Hall, 1992; Hall, 2012). Rho family GTPase effectors regulate the polymerization of actin for diverse cellular functions, including cell migration, clathrin-independent internalization at the plasma membrane, movement of endosomes and endocytic vesicles along cytoskeletal tracks, and sorting within the late endosomal pathway (Ellis and Mellor, 2000). The nucleation-promoting factors discussed below are one important class of Rho GTPase effector (Rohatgi *et al.*, 1999; Eden *et al.*, 2002).

The cycle of GTP and GDP binding is critical for the regulation of actin polymerization by Rho GTPases. As mentioned above, Rho-GTP activates effectors, while Rho-GDP is inactive (Fig. 8). Three classes of molecules regulate this nucleotide binding cycle. The GTPase-activating proteins, or GAPs, stimulate the GTPase activity of Rho-GTP (Hall, 2012). Guanine dissociation inhibitors (GDIs) bind to Rho-GDP to prevent the dissociation of GDP and thereby inhibit Rho-GTP function (Matsui *et al.*, 1990). Guanine or GTP-exchange factors (GEFs) bind to RhoGDP and cause conformational changes that disrupt the nucleotide binding cleft, leading to the release of GDP (Rossman *et al.*, 2005). GTP rapidly diffuses into the nucleotide binding cleft due to its relatively high concentration in the cytosol. Signaling and feedback pathways act upon these regulators of the nucleotide binding cycle to modulate the activity of the Rho GTPases. Therefore, regulation of the expression, localization, and activity of guanine exchange factors is a critical aspect of cellular control of actin polymerization.

There are two classes of Rho family guanine exchange factors in mammals, the DOCK family and the Dbl family. Members of the DOCK family of guanine exchange factors contain a characteristic DOCK homology fold 2 domain (DHR-2) that binds to the Rho GTPase to promote nucleotide exchange (Meller *et al.*, 2005). All known members also contain a highly conserved DHR-1 domain, which for some members has been shown to bind to phosphatidylinositol-3,4,5-triphosphate as a signaling and localization mechanism (Côté *et al.*, 2005; Kanai *et al.*, 2008). There are 11 mammalian DOCK180 family members. Interestingly, one member of this family, DOCK7, has been considered as a potential component of the Hermansky-

Pudlak syndrome pathway on the basis of the phenotype of mice deficient in this gene (Swank *et al.*, 1998; Wei and Li, 2012). The *misty* mouse carries a spontaneous insertion in the *DOCK7* gene on the DBA/J background, while the *moonlight* mouse carries a deletion in the *DOCK7* gene on the C57 background (Sviderskaya *et al.*, 1998; Blasius *et al.*, 2009). Both of these mutations affect the majority of the coding region and destabilize the DOCK7 gene product. Both *misty* and *moonlight* mice have a generalized hypopigmentation phenotype coupled with white spotting. In addition, the *misty* mouse has a prolonged bleeding time associated with platelet abnormalities (Sviderskaya *et al.*, 1998). To date, however, no molecular mechanisms have been described to account for these systematic phenotypes that represent impaired lysosome-related organelles. Instead, DOCK7 function has been primarily described in neuronal cells, where it is proposed to regulate microtubule dynamics and to be required for axonal outgrowth (Watabe-Uchida *et al.*, 2006).

The Dbl family of Rho guanine exchange factors contains 71 members in the human genome (Hall, 2012). Dbl is the canonical member of the family and was initially cloned and characterized as a transforming gene in diffuse B-cell lymphoma (Hart *et al.*, 1991). Dbl family members are characterized by the presence of a Dbl homology (DH) domain flanked by a lipid binding pleckstrin homology (PH) domain. The DH domain binds Rho GTPases to disrupt the conformation of the nucleotide binding cleft, while the PH domain is proposed to bind lipid bilayers to facilitate membrane recruitment and orient the GEF to facilitate exchange activity (Kaksonen *et al.*, 2006). One member of this family, the RhoA guanine exchange factor RhoGEF1, is a focus of my work (Fig. 8) (Hart *et al.*, 1996). In addition to the DH-PH module,

RhoGEF1 contains a regulator of G protein signaling (RGS) domain at its N-terminus (Aittaleb *et al.*, 2010). G protein coupled receptor signaling activates $G_{\alpha 12/13}$ proteins, which then bind RhoGEF1, promoting its translocation from the cytosol to the plasma membrane and stimulating its nucleotide exchange activity (Hart *et al.*, 1998; Meyer *et al.*, 2008; Siehler, 2009). As a consequence, RhoA is activated at the plasma membrane and its effectors modulate the actin cytoskeleton. In addition, the RhoGEF1 RGS domain acts as a GTPase-activating protein for $G_{\alpha 12/13}$ proteins to limit the extent of signaling (Meyer *et al.*, 2008). Diverse receptors signal through $G_{\alpha 12/13}$ proteins, including dopamine D_5, M_1 and M_5 muscarinic acetylcholine, and α_1 adrenergic receptors (Siehler, 2009). In addition to regulation by G protein coupled receptors, the C-terminus of RhoGEF1 mediates homo-oligomerization, which negatively regulates guanine exchange activity (Chikumi *et al.*, 2004). Thus, diverse mechanisms can regulate RhoGEF1 activity, and thereby the activation of actin polymerization effectors by the small GTPase RhoA.

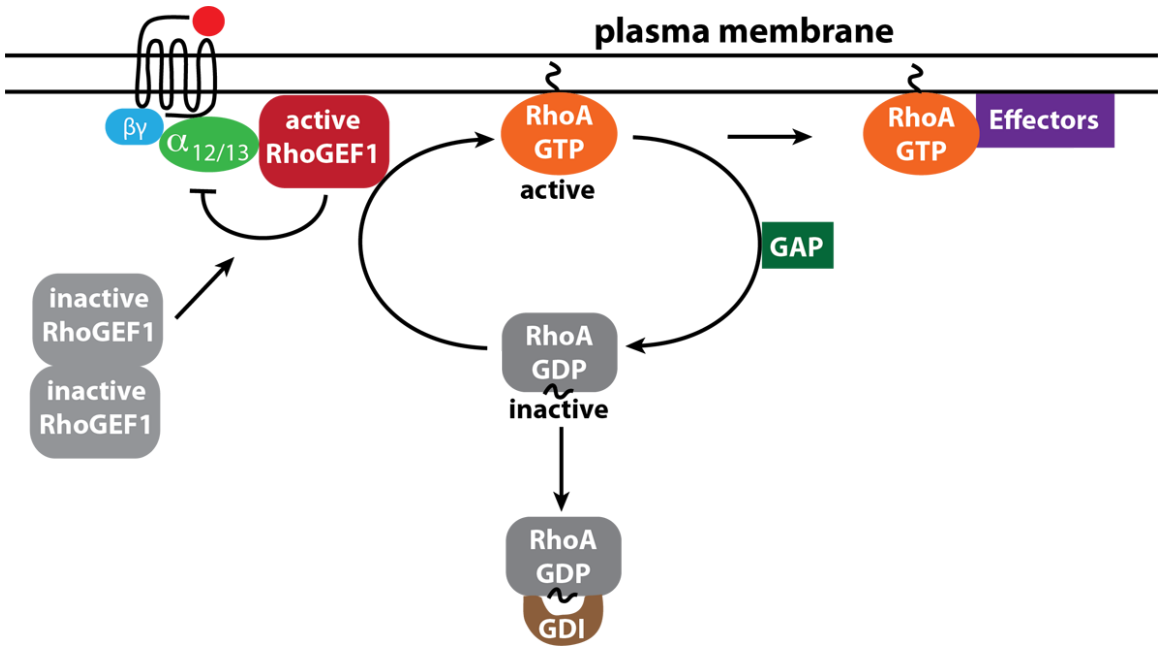

Figure 8. The RhoA GTPase cycle and regulation by Rho guanine exchange factor-1 (RhoGEF1). Small GTPases such as RhoA cycle between an active GTP-bound state and an inactive GDP-bound state. Guanine exchange factors such as RhoGEF1 promote conformational changes that lead to the release of GDP and binding of GTP. Activated RhoA recruits effectors to modify the cytoskeleton. GTPase-activating proteins (GAPs) accelerate the GTPase activity of RhoA, thereby leading to inactivation and cessation of signaling. Guanine dissociation inhibitors (GDI) bind GDP-RhoA to retain the molecule in its inactive state. RhoGEF1 activity is regulated by G-protein coupled receptor signaling. Activation of $G\alpha_{12/13}$ proteins by ligand binding then recruits and activates RhoGEF1 at the plasma membrane. RhoGEF1 activates RhoA, thereby leading to cytoskeletal changes. In addition, activated RhoGEF1 negatively regulates signaling by $G\alpha_{12/13}$ proteins. Figure inspired by (Koh, 2006).

The Arp 2/3 complex nucleates and organizes actin filaments

One mechanism by which small GTPases such as RhoA stimulate actin polymerization is via activation of the Arp2/3 complex, a highly conserved 220 kDa complex of 7 subunits (Machesky *et al.*, 1994). In contrast to the formins and WH2 domain nucleators, the Arp2/3 complex both nucleates filament polymerization and organizes filaments into branched networks (Fig. 9) (Mullins *et al.*, 1998; Amann and Pollard, 2001). Arp2 and Arp3 form a dimer within the complex that resembles

the barbed end of an actin monomer, thereby likely acting as the first two subunits of a newly formed daughter filament (Robinson *et al.*, 2001). Two other complex members, ArpC2 and ArpC4, form the predominant contacts with the parent actin filament, which orients the Arp2/3 surface for daughter filament polymerization at approximately a 70° angle from the parent filament (Rouiller *et al.*, 2008). While the purified Arp2/3 complex alone does enhance the polymerization of actin monomers *in vitro*, this activity can be considerably enhanced by the addition of protein complexes known as nucleation-promoting factors (Mullins *et al.*, 1998; Higgs *et al.*, 1999). These factors bind the Arp2/3 complex to change its conformation to favor nucleation and are fundamental for cellular regulation of Arp2/3 complex activity (Campellone and Welch, 2010).

Nucleation-promoting factors (NPFs)

Nucleation-promoting factors (NPFs) bind the Arp2/3 complex to regulate its activity at diverse subcellular compartments (Fig. 9). Two major classes of NPF exist. Class I NPFs are defined by the presence of a carboxy-terminal WCA module. Class II NPFs contain N-terminal domains to bind the Arp2/3 complex, multiple binding sites for filamentous actin, and regulatory C-terminal domains. In addition, class II NPFs can interact with class I NPFs to regulate their function. The WASH complex, a major focus of my work, is a class I NPF. Other class I NPFs include the Wiskott-Aldrich Syndrome Protein (WASP) and bacterial proteins that hijack actin polymerization such as the ActA protein of *Listeria monocytogenes* (Machesky and Insall, 1998; Welch *et al.*, 1998). These NPFs are defined by the presence of a WCA module, which contain three functional domains: the WASP homology 2 (WH2),

connector or central, and acidic domains. WH2 domains bind actin while the acidic region binds the Arp2/3 complex. The connector region interacts with the Arp2/3 complex and induces conformational changes to promote actin polymerization (Rodal *et al.*, 2005). The WCA module of nucleation-promoting factors is the minimal region required to promote Arp2/3 complex-mediated actin polymerization *in vitro* (Machesky *et al.*, 1999). In contrast to this highly conserved C-terminal module, the N-terminal regions of the known nucleation-promoting factors are highly divergent, allowing for diverse cellular roles for these factors (Burianek and Soderling, 2013). The mammalian class I NPFs are organized into five groups on the basis of N-terminal homology: (1) WASPs (Wiskott-Aldrich Syndrome Protein); (2) WAVEs (WASP and Verprolin homolog); (3) WASH (WASP and Scar Homolog); (4) WHAMM (WASP Homolog associated with Actin, Membranes, and Microtubules); and (5) JMY (Junction-Mediating regulatory protein). The WASP and WAVE groups were the first recognized eukaryotic NPFs and as such are best characterized (Machesky and Insall, 1998). For this reason, I will briefly introduce these two NPF groups before discussing WASH in more detail.

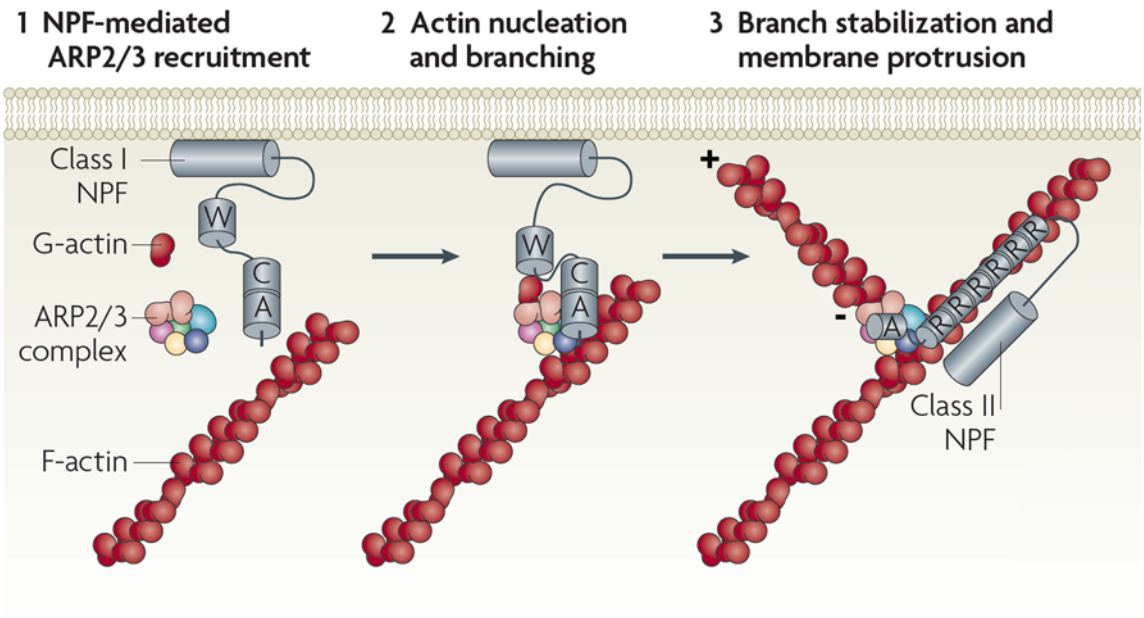

Figure 9. Nucleation of actin polymerization by the Arp2/3 complex and nucleation-promoting factors. The actin-related protein-2/3 (Arp2/3) complex nucleates the polymerization of actin filaments (F-actin) from the globular monomeric form (G-actin). This nucleation overcomes the kinetic barrier to polymerization of actin molecules. In addition, the Arp2/3 complex binds existing actin filaments and thereby forms actin branches. Arp2/3-mediated actin polymerization is regulated in time and space by Class I nucleation-promoting factors (NPFs). These factors bind both actin monomers and the Arp2/3 complex to promote actin polymerization. The Wiskott-Aldrich Syndrome Protein (WASP) and WASP- and Scar Homolog (WASH) are examples of Class I NPFs that promote branched actin polymerization at the plasma membrane and endosomes, respectively. Figure modified from (Campellone and Welch, 2010).

The Wiskott-Aldrich Syndrome Proteins

Mammalian genomes encode two highly related WASP proteins. These proteins are named for the Wiskott-Aldrich syndrome, a human disease syndrome characterized by eczema, thrombocytopenia, and immunodeficiency (Bosticardo *et al.*, 2009). This syndrome is caused by a deficiency of the WASP protein, which is expressed only in hematopoietic cells (Derry *et al.*, 1994). In mammals, the closely related protein neural-WASP (N-WASP; also known as Wiskott Aldrich syndrome-like or WASL) is ubiquitously expressed (Miki *et al.*, 1996). Targeted deletion of N-

WASP in mouse models is embryonic lethal and causes neural and cardiac abnormalities (Snapper *et al.*, 2001). N-WASP regulates actin dynamics at the plasma membrane and is critical for a variety of cellular functions, including filopodia formation, dorsal membrane ruffling, and membrane deformation during phagocytosis and clathrin-mediated endocytosis (Burianek and Soderling, 2013). In addition, N-WASP-dependent actin polymerization can drive movement of endosomes and vesicles (Schafer *et al.*, 2000; Taunton *et al.*, 2000). In addition to the C-terminal WCA module, N-WASP contains domains for binding to small regulatory GTPases and phosphatidylinositol (4,5)-bisphosphate (PI4,5P2). N-WASP is autoinhibited by intramolecular binding of the GTPase binding domain to the connector and acidic domains of the WCA module (Rohatgi *et al.*, 2000). Binding of N-WASP to the small GTPase cdc42 releases this autoinhibition and this activation is enhanced by binding to PI4,5P2 (Rohatgi *et al.*, 1999).

The WASP and Verprolin Homolog (WAVE) Proteins

Three WAVE isoforms exist in mammals, with WAVE2 ubiquitously expressed while WAVE1 and WAVE3 are predominantly expressed in neuronal tissues (Suetsugu *et al.*, 1999). The main function of these isoforms is activation of the Arp2/3 complex during plasma membrane protrusion and cell motility. Targeted disruption of individual isoform genes *WAVE1* or *WAVE2* leads to decreased viability and brain and cardiovascular system abnormalities, indicating that despite high homology and seemingly overlapping functions, these isoforms cannot fully compensate for each other (Dahl *et al.*, 2003; Soderling *et al.*, 2003; Yan *et al.*, 2003). WAVE proteins contain domains for binding phosphatidylinositol

(3,4,5)-phosphate (PI3,4,5P3), a plasma membrane component that aids in membrane association and subcellular localization. In contrast to the WASPs, the WAVE proteins do not have an autoinhibitory mechanism. Instead, WAVE proteins are incorporated into a regulatory complex, with most signaling and regulation occurring through interactions with complex members (Gautreau *et al.*, 2004). This regulatory complex has four members: BRICK1, ABL interactor 1 (ABI1), NCK-associated protein 1 (NAP1), and specifically Rac-associated 1 (SRA1). Binding of non-catalytic kinase-1 and -2 (NCK1 and NCK2) and the small GTPase Rac1 with components of this regulatory complex lead to the release of inhibition and activation of actin polymerization (Ismail *et al.*, 2009). Of note, a structurally analogous complex regulates the WASH nucleation-promoting factor (Jia *et al.*, 2010).

WASP and SCAR Homolog (WASH)

The Wiskott-Aldrich Syndrome Homolog (WASH) was first characterized as the gene product of the most telomerically duplicated gene in the human genome (Linardopoulou *et al.*, 2007). Subtelomeric regions of the human genome are highly dynamic regions for DNA breaks and repair. As a result, many of the genes encoded at subtelomeres are duplicated and diverse repertoires of alleles exist in humans (Linardopoulou *et al.*, 2005). In the case of *Wash*, seven isoforms are encoded in the human genome, yet only one of these genes encodes a full-length protein, as the other isoforms contain frame-shifts or nonsense mutations (Linardopoulou *et al.*, 2007). WASH duplication appears to be unique to primates, with other metazoans containing only a single *Wash* gene. Despite its recent description, WASH is the most

ancient of the known class I NPFs, with homologs present in all eukaryotic kingdoms (Veltman and Insall, 2010). Of note, one important model system, the yeast *Saccharomyces cerevisiae*, does not contain a WASH homolog (the only yeast nucleation-promoting factor is a WASP homolog encoded by the *Las17* gene). Amongst vertebrates, WASH is highly conserved and is approximately 50 kDa in size. In addition to the C-terminal WCA domain that characterizes the class I NPFs, WASH has several well-conserved functional domains: an N-terminal WASH homology domain 1 (WAHD1) that binds WASH regulatory complex members; a more central tubulin binding region (TBR) (also known as WAHD2); and a proline-rich domain. Initial characterization of *Wash* in *Drosophila melanogaster* demonstrated that it is an essential gene (Linardopoulou *et al.*, 2007). Further, engineered deletion of *Wash* in mice results in early embryonic lethality, at day E7.5 (Gomez *et al.*, 2012). Thus, other Class I nucleation-promoting factors cannot compensate for WASH function despite high conservation at the WCA domain.

Localization

Initial assessment of WASH protein function in cell culture by overexpression of fluorescent protein tagged constructs suggested a role for WASH in membrane protrusions such as filopodia and lamellipodia (Linardopoulou *et al.*, 2007). However, localization studies using antibodies to the endogenous protein later revealed that WASH is present at intracellular structures and colocalizes with markers of early endosomes, including both sorting and recycling types (Derivery *et al.*, 2009; Gomez and Billadeau, 2009; Duleh and Welch, 2010). In addition, WASH colocalizes predominantly with early but not late endosomes as labeled by

internalized epidermal growth factor receptor (Gomez and Billadeau, 2009) or transferrin receptor (Derivery *et al.*, 2009). No colocalization is observed with markers of the *cis*- and *trans*-Golgi complex (Derivery *et al.*, 2009). Consistently, colocalization analysis of WASH with endosomal markers or proteins that it functionally regulates demonstrates a partial overlap or juxtaposition of the two signals rather than complete colocalization (Derivery *et al.*, 2009; Gomez and Billadeau, 2009; Duleh and Welch, 2010).

Function

WASH recruits the Arp2/3 complex to early endosomes where both are required for actin polymerization (Derivery *et al.*, 2009; Carnell *et al.*, 2011; Gomez *et al.*, 2012). WASH-nucleated actin polymerization at early endosomes regulates endosomal shape as well as cargo sorting. WASH-dependent actin polymerization is implicated in numerous endosomal pathways. Most pathways described to date involve trafficking from the endosome to the plasma membrane or *trans* Golgi network. For example, depletion of WASH leads to functional defects in the recycling of transferrin receptor (Derivery *et al.*, 2009; Zech *et al.*, 2011) and α5-integrin (Zech *et al.*, 2011; Duleh and Welch, 2012) from early endosomes to the plasma membrane. Further, WASH is associated with actin-stabilized endosomal tubules required for the sequence-dependent sorting of the β2-adrenergic receptor (β2AR) for recycling to the plasma membrane by the retromer complex (Puthenveedu *et al.*, 2010; Temkin *et al.*, 2011). In addition, WASH complex activity is required for the endosome-to-Golgi retrograde transport of the cation-independent mannose 6-phosphate receptor (CI-MPR) by the retromer complex (Gomez and Billadeau, 2009;

Harbour *et al.*, 2010). WASH activity is also linked to lysosomal function. Deficiency of WASH in fibroblasts results in enlarged lysosomes with altered morphology (Gomez *et al.*, 2012). In the amoeba *Dictyostelium discoideum*, WASH activity regulates the removal of the V-ATPase proton pump from lysosomes, which is required for lysosomal neutralization. Finally, WASH activity is required for the degradative sorting of the epidermal growth factor receptor (Duleh and Welch, 2010).

Mechanism of action

As described above, WASH-dependent actin polymerization is required for the function of multiple endosomal pathways. The exact mechanisms by which WASH-dependent actin polymerization is coupled to function are still being elucidated. One hypothesis is that WASH-dependent actin polymerization generates force that is required for membrane scission (Fig. 10) (Derivery *et al.*, 2009; Gomez and Billadeau, 2009). This hypothesis is supported by the appearance of elongated tubules upon WASH depletion and the co-precipitation of WASH with the dynamin GTPase (Gomez and Billadeau, 2009). Tubular structures are reported to be marked by internalized transferrin (Derivery *et al.*, 2009), internalized CI-MPR (Gomez and Billadeau, 2009), and the retromer complex (Harbour *et al.*, 2010). However, these tubules do not appear in cells with an engineered deficiency in the WASH complex (Gomez *et al.*, 2012) or cells treated with latrunculin to induce actin depolymerization (Derivery *et al.*, 2012). Further, in some pathways and cell types, depletion of WASH leads to sorting defects that are not coupled to altered tubular morphology (Carnell *et al.*, 2011; Zech *et al.*, 2011). Thus, future studies, including *in*

vitro reconstitution assays, are required to conclusively determine if WASH-dependent actin polymerization generates force required for membrane scission.

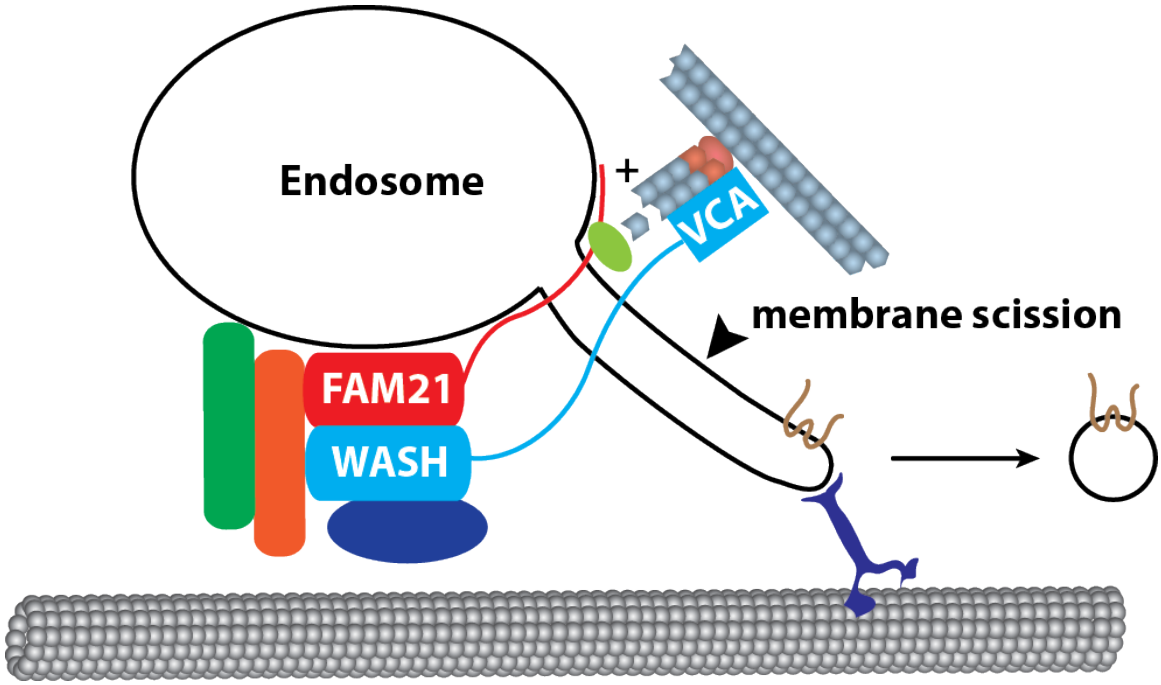

Figure 10. Proposed mechanism of action for WASH-dependent membrane scission. The Class I nucleation-promoting factor WASH is incorporated into a complex four proteins (see Figure 12 for details). The FAM21 subunit of this complex binds to endosomal membranes and recruits WASH to these domains. WASH promotes the polymerization of a branched actin network by activating the Arp2/3 complex. New actin subunits are incorporated adjacent to the endosomal membrane, which can thus oppose a pulling force provided by kinesin motors moving along microtubules. These opposing forces are proposed to generate tension that leads to membrane scission and release of a vesicle from endosomes. Figure inspired by (Rottner *et al.*, 2010).

In addition to membrane scission, several alternative, non-exclusive roles for WASH-dependent actin polymerization have been proposed (Fig. 11). Actin polymerization may facilitate the segregation of endosomal membranes into unique subdomains. These subdomains could act as sorting structures on the basis of physical interaction between cargo and actin, as has been proposed for the actin-binding V-ATPase proton pump (Carnell *et al.*, 2011). Alternatively, polymerized

actin stabilizes endosomal tubules that provide the basis for kinetic sorting of the slowly diffusing β2AR (Puthenveedu *et al.*, 2010). WASH associates with these structures, suggesting that it may induce polymerization of actin to stabilize tubules, although this hypothesis remains untested (Puthenveedu *et al.*, 2010). Finally, WASH-dependent actin polymerization may be required to induce membrane deformation required for vesicle biogenesis. This hypothesis is analogous with the coupling of N-WASP-dependent actin polymerization at the plasma membrane with membrane deformation during clathrin-mediated endocytosis (Anitei and Hoflack, 2012). The enlargement of early endosomes and the absence of membrane tubulation in WASH-deficient cells are consistent with this hypothesis. Of note, these proposed mechanisms are non-exclusive and multiple mechanisms may be at play in diverse endosomal pathways.

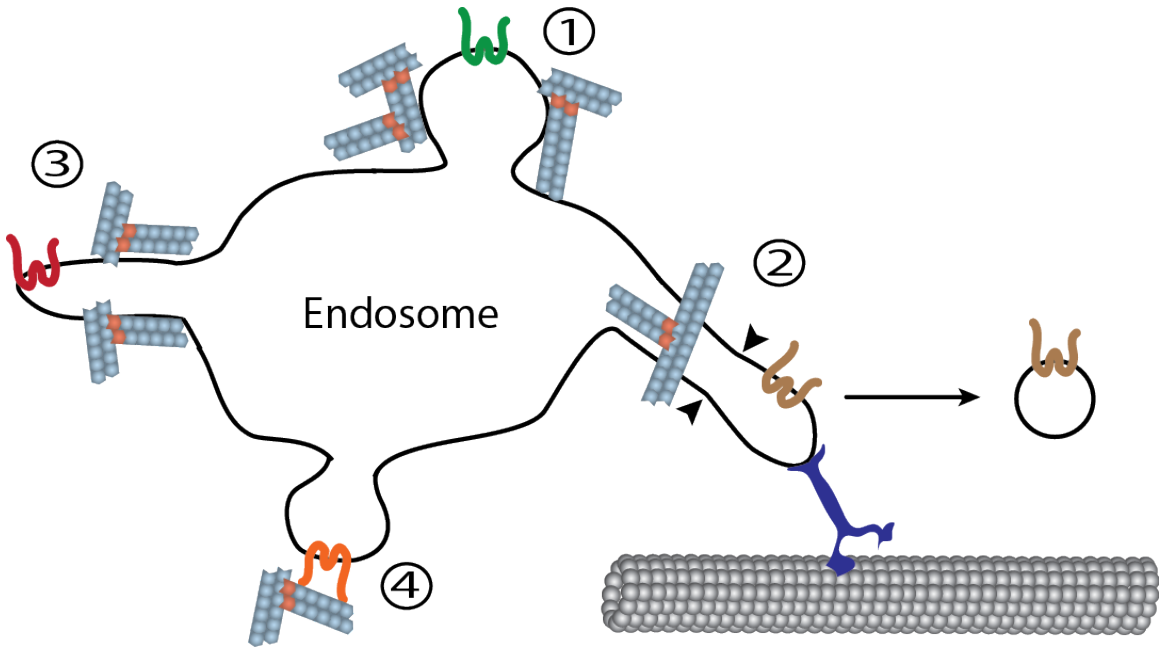

Figure 11. Potential roles for the actin cytoskeleton in endosomal sorting. There are several non-exclusive potential roles for branched actin filaments at endosomes. First, actin filaments may provide a structural support that supports deformation of membranes into vesicles (#1, above). Second, actin filaments may

provide a structural support and mechanical force that opposes pulling of endosomal membranes by kinesin motors and thus leads to membrane scission (#2). Third, actin filaments provide a structural support to endosomal tubules, which stabilizes these tubules, allowing for slowly diffusing cargo to enter the tubule and thereby leads to a kinetic sorting of cargo (#3). Finally, direct binding of actin filaments to cargo may play a physical sorting role to promote the retention of cargo within discrete sorting domains.

Regulation

WASH exists within a stable complex of four proteins: FAM21 (Family with sequence similarity 21; also known as VPEF or *Vaccinia* Penetration Factor (Huang *et al.*, 2008)), SWIP (Strumpellin- and WASH-interacting protein; also known as KIAA1033), Strumpellin, and CCDC53 (coiled-coiled domain containing 53) (Fig. 12) (Derivery *et al.*, 2009; Harbour *et al.*, 2010; Jia *et al.*, 2010). FAM21, Strumpellin, and SWIP appear to form a stable core of this complex, as depletion of any one of these proteins destabilizes the entire WASH complex (Jia *et al.*, 2010). However, these three components remain, albeit at lower levels, in deficiency of WASH or depletion of CCDC53, suggesting a possible role for these proteins outside their regulation of WASH (Jia *et al.*, 2010; Gomez *et al.*, 2012). This hypothesis is supported by the presence of FAM21, Strumpellin, SWIP, and CCDC53 in the genome of the marine diatom *Thalassiosira,* in the absence of WASH or Arp2/3 complex homologs (Veltman and Insall, 2010). One major role for the subunits of the WASH complex appears to be the regulation of subcellular localization of WASH. The FAM21 subunit is largely responsible for localization to endosomes labeled by the retromer complex and mannose 6-phosphate receptor (M6PR) (Gomez and Billadeau, 2009; Jia *et al.*, 2012). A predominant membrane recruitment factor is binding of FAM21 to the retromer complex (Harbour *et al.*, 2012; Helfer *et al.*, 2013). In addition, FAM21

binds phosphoinositides *in vitro*, including PI4P, the product of PI4KIIα enzymatic activity, and PI3P, which is enriched in early endosomal membranes (Jia *et al.*, 2010). Binding to phosphoinositides by FAM21 may stabilize the WASH complex at membranes or promote its segregation into subdomains. However, to date, the regulation of the WASH complex *in vivo* by phosphoinositides and phosphatidylinositol kinases and/or phosphatases remain unexplored.

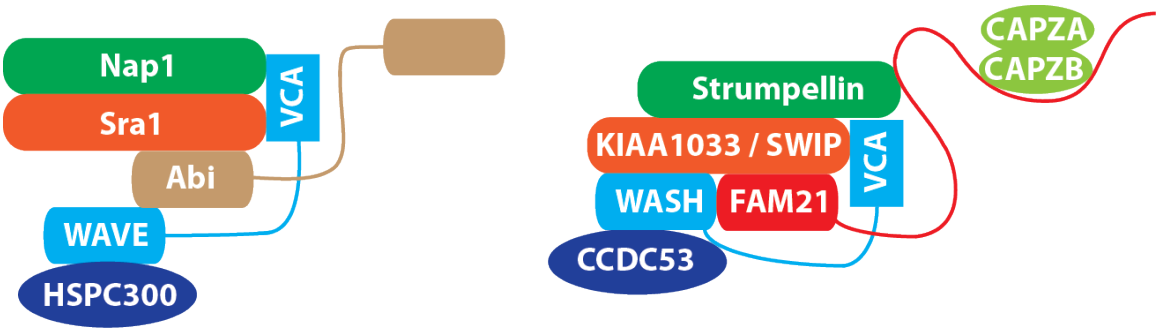

Figure 12. The WAVE and WASH actin nucleation-promoting factors are incorporated into structurally analogous complexes. The WAVE and WASH class I nucleation-promoting factors are both incorporated into complexes that regulate their localization and activation. In addition, subunits within these complexes display distant structural homology, such as the Nap1 subunit of the WAVE complex and the Strumpellin subunit of the WASH complex. The FAM21 subunit of the WASH complex is particularly important for localization to endosomes. The capping protein heterodimer (CAPZA, CAPZB) may promote the generation of highly branched networks of short actin filaments that rapidly turnover, although this remains to be tested within the context of WASH activity. While the small GTPases Rac1 and cdc42 are known to activate WAVE activity via subunits of the WAVE complex, to date no analogous mechanism is described for the regulation of WASH activity. Figure inspired by (Jia *et al.*, 2010).

The role of the WASH complex in regulating the actin polymerization activity of WASH remains controversial. Profile-against-profile alignment revealed distant homology between components of the WASH complex and the WAVE regulatory complex, suggesting that actin polymerization of WASH and WAVE may be regulated by similar mechanisms (Jia *et al.*, 2010). Studies *in vitro* show that the WASH WCA

domain is as efficient a nucleator of actin polymerization as the WASP WCA domains and more efficient than the WAVE and WHAMM WCA domains (Duleh and Welch, 2010). Further, full length WASH polymerizes actin *in vitro* with similar efficiency as the WCA domain, suggesting that the full length protein is active and not autoinhibited, similar to the WAVE NPFs and unlike the WASP NPFs (Duleh and Welch, 2010). While full-length WAVE-2 is capable of actin polymerization, in cells the WAVE regulatory complex holds WAVE-2 in an inactive conformation (Ismail *et al.*, 2009). To date, studies on the actin polymerization activity of purified or recombinant WASH complex *in vitro* have demonstrated conflicting results, which have yet to be resolved (Derivery *et al.*, 2009; Derivery and Gautreau, 2010; Jia *et al.*, 2010). However, a recent study found that the WASH complex regulates enzymatic activity by recruiting an ubiquitin ligase that targets WASH itself (Hao *et al.*, 2013). Polyubiqutination of WASH releases the protein from an inhibited state and promotes the polymerization of actin. Perturbation of this activation mechanism disrupted the endosome-to-Golgi trafficking of the mannose 6-phosphate receptor by the retromer complex. Whether or not this mechanism regulates the activity of the WASH complex within other trafficking pathways is unknown. Another possible mechanism of regulation is the binding of small GTPases. The WAVE complex is activated upon binding of the small GTPase Rac1, which changes the conformation of the complex to release the conformational inhibition of the WAVE protein. To date, no small GTPase regulatory mechanism has been fully elucidated for the WASH complex. While the small GTPase RhoA genetically and physically interacts with WASH in *Drosophila melanogaster* (Liu *et al.*, 2009), a study in mammalian cells did

not find an interaction between recombinant WASH complex and RhoA (Jia *et al.*, 2010). However, the possibility remains that a small GTPase regulates the WASH complex only upon specific cellular activation cues or within particular trafficking pathways. My research facilitates the testing of this hypothesis by identifying guanine exchange factors for Rho family GTPases as novel interactors of the WASH complex.

Contributions of this dissertation research

A fundamental unanswered question is the degree to which the cytoskeleton contributes to the vesicle biogenesis process in diverse membrane trafficking pathways. Within a well-defined vesicle biogenesis mechanism, AP-2-dependent vesicle budding at the plasma membrane, the polymerization of actin filaments promotes membrane deformation and scission. My dissertation research used this mechanism as a conceptual framework to hypothesize that:

> **The BLOC-1 complex and a cargo and regulator of the pathway, the phosphatidylinositol 4-kinase type IIα (PI4KIIα), interact with regulators of the actin cytoskeleton.**

In Chapter 2 I provide a detailed test of this hypothesis. I took an unbiased approach to identify previously unrecognized interactors of the BLOC-1 pathway by characterizing the interaction network of its regulator, the lipid kinase PI4KIIα. I isolated PI4KIIα by immunoprecipitation followed by peptide elution to release PI4KIIα protein complexes and performed quantitative mass spectrometry analysis to identify co-isolating proteins. Bioinformatic analysis of this interaction network revealed regulators of the actin cytoskeleton as predominant network components.

These regulators included the guanine exchange factors for small regulatory GTPases, RhoGEF1 and DOCK7, and subunits of an endosomal Arp2/3 activator, the WASH complex. I demonstrated that these actin regulators co-isolate with both PI4KIIα and the BLOC-1 complex and that these components genetically interact. I found co-residence of actin, WASH complex subunits, BLOC-1 complex subunits, and PI4KIIα at endosomes by sucrose velocity sedimentation, deconvolution immunofluorescence microscopy of fixed cells, and spinning disk confocal microscopy of live cells. Further, depletion of BLOC-1 or the WASH complex changed the morphology of PI4KIIα-containing endosomes. In addition, two BLOC-1 complex cargoes, the copper transporter ATP7A and the lysosomal SNARE Vamp7, mis-localize in WASH complex deficiency. Together, these data support a functional interaction between BLOC-1, PI4KIIα, and actin cytoskeleton regulators.

My dissertation research supports a role for the actin cytoskeleton as a core participant in the BLOC-1 vesicle biogenesis pathway. In addition, my work expands the known interactors of the recently identified Arp2/3 activator, the WASH complex, introducing novel hypotheses regarding its regulation. Finally, this work provides a framework to understand the pathogenesis of neurodegenerative diseases that are genetically linked to these protein complexes. In the next chapter, I will describe my dissertation research findings in further detail.

CHAPTER II

The WASH Complex, an Endosomal Arp2/3 Activator, Interacts with the Hermansky-Pudlak Syndrome Complex BLOC-1 and its Cargo Phosphatidylinositol-4-kinase Type II Alpha

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Abstract

Vesicle biogenesis machinery components such as coat proteins can interact with the actin cytoskeleton for cargo sorting into multiple pathways. However, whether or not these interactions are a general requirement for the diverse endosome traffic routes is unknown. In this study, we identify actin cytoskeleton regulators as previously unrecognized interactors of complexes associated with the Hermansky-Pudlak syndrome. Two complexes mutated in the Hermansky-Pudlak Syndrome, AP-3 and BLOC-1, interact with and are regulated by the lipid kinase PI4KIIα. We therefore hypothesized that PI4KIIα interacts with novel regulators of these complexes. To test this hypothesis, we immunoaffinity purified PI4KIIα from isotope-labeled cell lysates (SILAC) to quantitatively identify interactors. Strikingly, PI4KIIα isolation preferentially co-enriched proteins that regulate the actin cytoskeleton, including guanine exchange factors for Rho family GTPases such as RhoGEF1 and several subunits of the WASH complex. We biochemically confirmed several of these PI4KIIα interactions. Importantly, BLOC-1 complex, WASH complex, RhoGEF1, or PI4KIIα depletions altered the content and/or subcellular distribution of the BLOC-1-sensitive cargoes PI4KIIα, ATP7A, and VAMP7. We conclude that the Hermansky-Pudlak syndrome complex BLOC-1 and its cargo PI4KIIα interact with regulators of the actin cytoskeleton.

Introduction

Vesicular trafficking is a general cellular mechanism by which secretory and endocytic pathway organelles selectively exchange components. This exchange mechanism requires coordinated steps, which include sorting and concentration of membrane protein cargo into nascent vesicles, membrane deformation and scission, directional movement through the cytosol, and fusion at the target organelle (Bonifacino and Glick, 2004). Many of these steps require mechanical force, which is generated within vesicular trafficking pathways by association of specialized molecular machines. These molecular machines include: coat proteins that sort membrane protein cargo, BAR-domain proteins to sense or induce membrane deformation, the dynamin GTPase for membrane scission, and tethers and SNARE complexes for membrane fusion (Kaksonen *et al.*, 2005; Schmid and McMahon, 2007; Wickner and Schekman, 2008; Bröcker *et al.*, 2010; McMahon and Boucrot, 2011). While some of these machines are intrinsically capable of force generation, such as dynamin and SNARE fusion complexes (Schmid and Frolov, 2011; Ferguson and De Camilli, 2012; Gao *et al.*, 2012), others require association with the actin or microtubule cytoskeleton. For example, coat proteins sort membrane protein cargo into nascent vesicles. In addition, however, some coat proteins also bind cytoskeletal components for both vesicle biogenesis and directional movement (Nakagawa *et al.*, 2000; Styers *et al.*, 2004; Kaksonen *et al.*, 2006; Delevoye *et al.*, 2009; Anitei *et al.*, 2010; Anitei and Hoflack, 2012; Mooren *et al.*, 2012).

A fundamental unanswered question is the extent to which this cytoskeletal interaction is a general principle for coat protein function. One of the best-

characterized interactions between vesicle trafficking machinery and the cytoskeleton is the formation of vesicles at the plasma membrane by the clathrin and AP-2 adaptor coats. In this vesicle biogenesis pathway, the coat and coat-associated factors, such as BAR-domain proteins, locally regulate the polymerization and organization of actin filaments. Actin polymerization is nucleated in large part by the Arp2/3 complex and its activator, the nucleation-promoting factor N-WASP (neural-Wiskott Aldrich Syndrome Protein) (Conner and Schmid, 2003; Kaksonen *et al.*, 2005; 2006; Schmid and McMahon, 2007; McMahon and Boucrot, 2011; Taylor *et al.*, 2011; Mooren *et al.*, 2012). The recent identification of a nucleation-promoting factor that specifically localizes to endosomes, the WASP and SCAR Homolog (WASH) (Derivery *et al.*, 2009; Gomez and Billadeau, 2009), suggests that coat association with the actin cytoskeleton may be a general principle operating in the generation of vesicles. Supporting this possibility, depletion of WASH leads to functional defects in recycling, endosome-to–Golgi, and endosome-to-lysosome trafficking pathways (Derivery *et al.*, 2009; Gomez and Billadeau, 2009; Duleh and Welch, 2010; Carnell *et al.*, 2011; Zech *et al.*, 2011; Gomez *et al.*, 2012; Harbour *et al.*, 2012; Hao *et al.*, 2013; Piotrowski *et al.*, 2013). However, to date, WASH has been only been described to interact with one endosomal coat complex, the retromer complex, which primarily sorts cargo into the endosome-to-Golgi pathway (Gomez and Billadeau, 2009; Gomez *et al.*, 2012; Harbour *et al.*, 2012; Jia *et al.*, 2012; Seaman, 2012; Hao *et al.*, 2013; Helfer *et al.*, 2013). Therefore, whether or not endosomal coats coordinate with the actin cytoskeleton as a general principle remained untested.

We provide evidence supporting this hypothesis through the unbiased identification of an interaction between the WASH complex and sorting complexes associated with the Hermansky-Pudlak syndrome. This syndrome is genetically defined by defects in four molecular complexes: the adaptor protein complex-3 (AP-3) coat, and the biogenesis of lysosome-related organelles complexes-1 to -3 (BLOC-1 to BLOC-3). In addition, genetic defects in specific Rabs and regulatory enzymes trigger Hermansky-Pudlak syndrome-like phenotypes (Bultema and Di Pietro, 2012). Defects in these complexes in mammals and invertebrates lead to the hypopigmentation, platelet dysfunction, pulmonary fibrosis, and, in some cases, immune dysfunction and neurologic phenotypes (Newell-Litwa *et al.*, 2007; Raposo and Marks, 2007; Raposo *et al.*, 2007; Huizing *et al.*, 2008; Dell'Angelica, 2009; Wei and Li, 2012). These phenotypes are a consequence of impaired cargo trafficking between early endosomes and target organelles such as lysosome-related organelles and synaptic vesicles (Newell-Litwa *et al.*, 2007; Raposo *et al.*, 2007; Raposo and Marks, 2007; Huizing *et al.*, 2008; Dell'Angelica, 2009; Wei and Li, 2012). Therefore, exploring the molecules and functional mechanisms of these complexes both increases our understanding of the molecular basis of the Hermansky-Pudlak syndrome and allows us to test fundamental questions concerning principles of vesicular trafficking processes.

In order to identify functional mechanisms of complexes associated with Hermansky-Pudlak syndrome, we focused on a membrane-anchored and endosome-localized lipid kinase, phosphatidylinositol-4-kinase type II alpha (PI4KIIα) (Balla *et al.*, 2002; Guo *et al.*, 2003; Minogue, 2006; Craige *et al.*, 2008). Biochemical and

genetic evidence indicate that PI4KIIα regulates and binds two complexes mutated in Hermansky-Pudlak syndrome, AP-3 and BLOC-1 (Craige *et al.*, 2008; Salazar *et al.*, 2009; Larimore *et al.*, 2011; Gokhale *et al.*, 2012a). We therefore hypothesized that PI4KIIα would interact with novel proteins modulating the function of BLOC-1 and AP-3. To test this hypothesis, we isolated PI4KIIα and its interactors by quantitative immunoaffinity purification from *in vivo* crosslinked cell lysates. PI4KIIα preferentially co-isolated with proteins that regulate actin cytoskeleton polymerization, including the WASH complex and RhoGEF1. Depletion of PI4KIIα, BLOC-1 subunits, RhoGEF1 or the WASH complex modified the content of other components in the PI4KIIα interactome, thereby establishing genetic interactions in addition to physical interactions. Further, depletion of the WASH complex resulted in changes in PI4KIIα endosomal morphology and mis-localization of two BLOC-1 cargoes, the Menkes disease copper transporter, ATP7A, and a lysosomal SNARE, VAMP7. We propose that diverse coats such as clathrin-AP-2, retromer, and AP-3-BLOC-1 use common principles to garner force or create discrete membrane domains by localized actin polymer assembly.

Results

The PI4KIIα interactome enriches actin regulatory proteins

PI4KIIα binds the AP-3 and BLOC-1 complexes and these interactions regulate its subcellular distribution (Craige *et al.*, 2008; Salazar *et al.*, 2009; Larimore *et al.*, 2011; Gokhale *et al.*, 2012a). In addition, the lipid kinase activity of PI4KIIα regulates the recruitment of the AP-3 and BLOC-1 coat complex to endosomal membranes (Craige *et al.*, 2008; Salazar *et al.*, 2009). Therefore, we hypothesized that interactors of PI4KIIα are previously unrecognized regulators of the Hermansky-Pudlak syndrome-associated AP-3 and BLOC-1 complexes. We tested this hypothesis by isolation of endogenous PI4KIIα and interacting proteins. To isolate PI4KIIα, we raised an antibody to a peptide containing the dileucine sorting motif that is required for interaction of PI4KIIα with the AP-3 and BLOC-1 complexes (Fig. S1A) (Craige *et al.*, 2008). Mutation of the dileucine sorting motif abrogates the recognition of PI4KIIα by this antibody by immunoblotting (Fig. S1A). Therefore, we premised that this antibody would compete with AP-3 for binding to PI4KIIα and thereby preferentially enrich for PI4KIIα that is not bound to AP-3. This design strategy allowed for the enrichment of PI4KIIα-interacting proteins that are upstream or downstream of AP-3 binding. After establishing that this antibody specifically recognizes PI4KIIα, we immunoprecipitated PI4KIIα from minimally crosslinked SHSY-5Y neuroblastoma cell lysates (Figs. S1A, 1A). As we have extensively characterized, this crosslinking strategy: (1) stabilizes protein interactions to allow rigorous biochemical isolation strategies as described below; (2) uses a cell permeable and chemically reducible crosslinker, dithiobis

succinimidyl propionate (DSP) (Lomant and Fairbanks, 1976), that allows for *in vivo* crosslinking but is amenable to protein identification by mass spectrometry and immunoblot; and (3) is non-saturating to minimize stabilization of unrelated protein complexes (Salazar *et al.*, 2009; Zlatic *et al.*, 2010; Perez-Cornejo *et al.*, 2012; Gokhale *et al.*, 2012a; 2012b). Silver stain of immunoprecipitated protein complexes revealed several polypeptides that co-isolate with PI4KIIα (Fig. 1A, lane 5). As expected, several of these co-isolated polypeptides were not detected in control reactions where an excess of the PI4KIIα antigenic peptide outcompeted the endogenous protein for binding to the immunoprecipitation antibody (Fig. 1A, lane 4). However, eluting protein complexes from antibody-coated beads with Laemmli sample buffer released background polypeptides that precluded the identification of PI4KIIα and interacting proteins by silver stain (Fig. 1A, lanes 3-5). For this reason, we eluted protein complexes following immunoprecipitation by incubating the washed beads with an excess of the PI4KIIα antigenic peptide (Fig. 1A, lane 7). This immunoaffinity purification strategy greatly reduced background contaminants and immunoglobulins, as revealed by silver stain and immunoblot (Fig. 1A, compare lanes 4-5 to lanes 6-7). Finally, immunoblot demonstrated that our immunoaffinity purification approach highly enriched for PI4KIIα (Fig. 1A, compare lanes 2 and 7).

Having analytically established a selective approach to isolate PI4KIIα and interacting proteins, we performed a preparative immunoaffinity purification of crosslinked PI4KIIα complexes. We identified interactors by quantitative mass spectrometry using stable isotope labeling with amino acids in cell culture (SILAC) (Ong *et al.*, 2002; Mann, 2006). SHSY-5Y neuroblastoma cells were grown to

equilibrium in either a light-label media where the amino acids arginine and lysine contain ^{12}C and ^{14}N isotopes ("R0K0 media") or a heavy-label media where these amino acids contain ^{13}C and ^{15}N ("R10K8 media," Fig. S1B). Following incorporation of these amino acid isotope labels, PI4KIIα was immunoprecipitated from light-labeled cell lysates. As a control, immunoprecipitations from heavy-labeled cell lysates were performed in the presence of an excess of PI4KIIα antigenic peptide (Fig. S1B). As described above, both samples were then competitively eluted from the beads with an excess of PI4KIIα antigenic peptide. Light- and heavy-labeled eluted polypeptides were mixed 1:1 for MS/MS analysis. This purification enriched PI4KIIα by ~55 fold and its relative abundance was represented 220 spectral counts (Fig. 1B-D, green dot). In addition, we identified 701 co-isolating proteins with two or more unique peptides. Of the total 702 proteins, 268 were enriched more than 2 fold by PI4KIIα immunoaffinity purification over control, a SILAC cutoff we previously demonstrated enriches for interactions that can be confirmed genetically (Perez-Cornejo *et al.*, 2012; Gokhale *et al.*, 2012a; 2012b). However, one known limitation of bead-based purifications is the tendency of proteins to bind non-specifically to the antibody-coated beads (Trinkle-Mulcahy *et al.*, 2008). To overcome this limitation, we filtered out any polypeptides that bind non-specifically to either beads alone or beads coated with unrelated antibody. Applying these filters identified 123 co-isolating proteins in addition to PI4KIIα for further study (Fig. 1B-D, F and Table 1).

We first characterized the PI4KIIα interactome by performing functional annotation analysis of the 124 identified proteins using Gene Ontology annotations

(Fig. 1E). This analysis revealed that cytoskeleton proteins (30/124) and actin cytoskeleton proteins (13/124) were enriched in the PI4KIIα interactome as compared to a random sampling of the human genome (p-value 1.62 x 10^{-7} and 7.67 x 10^{-7}, respectively). For example, actin-related proteins within the PI4KIIα interactome include regulatory proteins for small GTPases, such as RhoGEF1 (ARHGEF1), dedicator of cytokinesis 7 (DOCK7), and GEF-H1 (ARHGEF2), which are guanine exchange factors for RhoA, cdc42, and Rho-Rac GTPases, respectively (Fig. 1C, teal dots) (Rossman *et al.*, 2005; Birkenfeld *et al.*, 2008; Blasius *et al.*, 2009; Aittaleb *et al.*, 2010). In addition, functional annotation analysis indicated a highly significant enrichment of proteins annotated as "vesicle-mediated transport proteins" (13/124), as predicted given the role of PI4KIIα in regulating AP-3-dependent vesicle biogenesis (Fig. 1E, p-value 1.19 x 10^{-3}, Supplementary Table 2). These vesicle-mediated transport proteins include clathrin heavy chain (CLTC), the beta subunit of the AP-3 complex (AP3B1), and dynamin-2 (DYN-2) (Fig. 1D, blue dots). In addition to functional annotation, we performed network analysis to identify previously published direct interactions amongst these co-purifying proteins. Our goal was to identify functional sub-complexes and hubs of high network connectivity that co-purify with PI4KIIα and may play a role in AP-3- and BLOC-1-mediated vesicle biogenesis (Fig. 1F) (Gokhale *et al.*, 2012b). For this reason, we included the eight subunits of the BLOC-1 complex in this analysis. Network analysis revealed the presence of three of the five subunits of the WASH complex (FAM21B, KIAA0196, and KIAA1033) and three known interactors of the WASH complex (FKBP15, CAPZA1, and CAPZB) (Fig. 1C, red dots) (Campellone and Welch,

2010; Jia *et al.*, 2010; Rottner *et al.*, 2010; Rotty *et al.*, 2012). The WASH complex localizes to early endosomes where it is suggested to polymerize actin for cargo sorting or vesicle scission, suggesting a role of the WASH complex in PI4KIIα-regulated BLOC-1-mediated vesicle biogenesis.

Biochemical and genetic confirmation of PI4KIIα -interacting proteins

We confirmed PI4KIIα interactions identified by SILAC mass spectrometry by independent immunoaffinity purification of PI4KIIα followed by immunoblotting to test for the presence of proteins of interest. We found that PI4KIIα interacts with vesicle-mediated transport proteins such as clathrin heavy chain (CHC) and the pallidin subunit of BLOC-1 (Fig. 2A, lanes 6 and 7). Interacting proteins were found only in PI4KIIα isolations and not in controls where an excess of antigenic peptide was used to outcompete immunoprecipitation of PI4KIIα complexes (Fig. 2A, lanes 4 and 5). Consistent with our previous reports, detection of interactions between PI4KIIα and vesicle-mediated transport proteins required treatment with the crosslinker DSP, presumably because these associations are labile and/or transient. Further, we found that PI4KIIα interacts with two known interactors of the BLOC-1 and AP-3 complexes, respectively, the antioxidant enzyme peroxiredoxin-1 (Prdx-1) and an ubiquitin ligase Nedd4-1 (Salazar *et al.*, 2009; Mössinger *et al.*, 2012) (Fig. 2A, lanes 6 and 7). Nedd4-1 represents the lower boundaries of our significance thresholds in terms of spectral count and enrichment cut-offs for our SILAC quantification, thereby validating that these thresholds minimize inclusion of false negatives in the PI4KIIα interactome (3 spectral counts, 3.69 fold enrichment, Fig. 1B). Finally, we tested the interaction between PI4KIIα and proteins that regulate

the actin cytoskeleton, since functional annotation and network analysis suggested that these interactions were highly significant. We confirmed the interaction between PI4KIIα and the strumpellin subunit of the WASH complex and two guanine exchange factors for small GTPases RhoGEF1 and Dock7. Of note, these co-purifications did not require the DSP crosslinker, suggesting that PI4KIIα interacts with these proteins with either higher affinity or a longer half-life than with the membrane-trafficking related proteins (Fig. 2A lanes 6 and 7). To further test the specificity these interactions, we isolated HA-tagged PI4KIIα from HEK293T cells by HA immunoprecipitation. We confirmed the presence of RhoGEF1 in these protein complexes, demonstrating that these interactions can be detected regardless of purification strategy (Fig. 2B, lane 3). All together, these results indicate that the PI4KIIα interactome enriches proteins whose gene ontology annotation significantly implicates them in actin function.

Our network analysis of the PI4KIIα interactome and BLOC-1 subunits suggested connections between PI4KIIα-interacting proteins and BLOC-1 subunits (Fig. 1F). Moreover, BLOC-1 regulates the subcellular localization of PI4KIIα (Larimore *et al.*, 2011). For these reasons, we predicted that these previously unrecognized actin-related PI4KIIα interactors would co-isolate with BLOC-1. To test this prediction we exploited an SHSY-5Y cell line that stably expresses a Flag-tagged dysbindin subunit of BLOC-1. This tagged subunit incorporates into functional BLOC-1 complexes (Larimore *et al.*, 2011; Gokhale *et al.*, 2012a). First, we confirmed that this BLOC-1 subunit could be detected in PI4KIIα immunoaffinity purifications (Fig. 2C, lane 3). We next tested if PI4KIIα interactors would be present

in BLOC-1 protein complexes isolated by Flag immunoaffinity purification (Fig. 2D, lanes 4-5). As previously reported, PI4KIIα can be detected by this method (Fig. 2D, lane 5) (Larimore *et al.*, 2011). In addition, two PI4KIIα interactors, RhoGEF1 and the WASH complex subunit strumpellin, co-isolate with BLOC-1 complexes (Fig. 2D, lane 5). Further, we also detected the presence of two known BLOC-1 cargoes, the Menkes disease copper transporter ATP7A and the lysosomal SNARE VAMP7 (Salazar *et al.*, 2006; Setty *et al.*, 2008; Newell-Litwa *et al.*, 2009; 2010). These interactions were not detected in controls where an excess of Flag peptide was included in the reaction to outcompete Flag-dysbindin for binding to the immunoprecipitation antibody (Fig. 2D, compare lanes 5 and 7). Our results indicate that isolating PI4KIIα or BLOC-1 protein complexes robustly co-purifies an actin regulatory machinery. The co-purification of PI4KIIα, strumpellin, RhoGEF1, and BLOC-1 subunits suggests that these proteins may act in concert to regulate BLOC-1-dependent traffic.

Mutation or depletion of a membrane trafficking complex subunit usually correlates with down-regulation of the other complex subunits and with changes in the total cellular content of pathway cargoes and associated factors (Peden *et al.*, 2002; Kantheti *et al.*, 2003; Li *et al.*, 2003; Starcevic and Dell'Angelica, 2004; Jia *et al.*, 2010). We used this principle to independently test components of the PI4KIIα interactome. We predicted that perturbation of either PI4KIIα or BLOC-1 would affect the content of PI4KIIα interactome components if these proteins were regulators of a BLOC-1-dependent vesicle biogenesis pathway. PI4KIIα and two BLOC-1 subunits, muted and pallidin, were independently depleted by shRNA in

HEK293T cells (Fig. 3A-B, and D). Semi-quantitative immunoblotting analysis demonstrated that PI4KIIα depletion up-regulated the content of RhoGEF1 as well as subunits of AP-3 and BLOC-1. The latter observation is consistent with the existence of a tri-partite complex between PI4KIIα, AP-3, and BLOC-1 (Salazar *et al.*, 2009) (Fig. 3A and D). These relationships amongst components of the PI4KIIα interactome were also observed after depletion of the BLOC-1 complex (Fig. 3B and D). The total cellular content of PI4KIIα, RhoGEF1 and BLOC-1 subunits was significantly reduced in response to the depletion of either muted or pallidin (Fig. 3B and D). We then analyzed the content of two well-characterized BLOC-1 cargoes, a lysosomal SNARE, VAMP7, and the copper transporter mutated in Menkes disease, ATP7A (OMIM 309400) (Salazar *et al.*, 2006; Setty *et al.*, 2008; Newell-Litwa *et al.*, 2009; 2010). The total cellular content of both of these cargoes was significantly decreased in BLOC-1-depleted cells but not in PI4KIIα-depleted cells (Mann-Whitney U test p-value < 0.05, Fig. 3A-B and D, red boxes). The normal content of VAMP7 and ATP7A in PI4KIIα-depleted cells suggests that the up-regulation of BLOC-1 complex subunits may be compensatory.

Next, we tested if shRNA-mediated depletion of BLOC-1, strumpellin, or RhoGEF1 would affect the total cellular content of other PI4KIIα interactome components. First, we explored the consequences of BLOC-1 depletion in the melanoma cell line, MNT-1, a cell type where localization of BLOC-1 to tubular endosomes has been defined at the subcellular level by electron microscopy (Di Pietro *et al.*, 2006). Consistent with our finding in HEK293T cells, the total cellular content of the PI4KIIα interactor RhoGEF1 decreased in MNT1 cells depleted of

BLOC-1 (Fig. 3C and E). Depletion of the PI4KIIα interactome components RhoGEF1 and strumpellin did not modify the total cellular content of BLOC-1 subunits (Fig. 3C and E). However, the BLOC-1 cargoes VAMP7 and ATP7A were significantly changed (Fig. 3C and E). The total cellular content of ATP7A increased in response to BLOC-1, RhoGEF1, or strumpellin depletion in MNT1 cells (Fig 3E, 2.3 ± 0.12, 2.2 ± 0.46, and 1.6 ± 0.13 fold of increase compared to controls, respectively). In addition, VAMP7 levels were significantly decreased in BLOC-1 depletions, significantly increased in RhoGEF1 depletions, yet unchanged in strumpellin depletions. These data suggest a disruption to the regulation of steady state levels of these BLOC-1 cargoes upon depletion of PI4KIIα interactome components, and thereby establish genetic interactions between PI4KIIα interactome components.

The WASH complex and filamentous actin reside on PI4KIIα-positive early endosomes

The WASH complex is an actin regulatory complex that localizes to early endosomes (Derivery *et al.*, 2009; Gomez and Billadeau, 2009; Duleh and Welch, 2010; Carnell *et al.*, 2011; Zech *et al.*, 2011; Gomez *et al.*, 2012; Harbour *et al.*, 2012). Similarly, PI4KIIα localizes to early endosomes where it binds to BLOC-1 (Balla *et al.*, 2002; Guo *et al.*, 2003; Minogue, 2006; Craige *et al.*, 2008; Salazar *et al.*, 2009). Therefore, we predicted that PI4KIIα-positive endosomes contain BLOC-1, the WASH complex, and actin. To test this prediction, we first separated endosomal compartments from HEK293T cell homogenates by sucrose velocity sedimentation (Clift-O'Grady *et al.*, 1998; Lichtenstein *et al.*, 1998). As expected, PI4KIIα co-sedimented to 35% sucrose with transferrin receptor, an endosomal marker (Fig. 4A-B). In addition, the WASH

complex subunit strumpellin, the BLOC-1 complex subunit pallidin, and actin were present in these fractions (Fig. 4A-B). Pallidin and actin were predominantly found at the top of gradients where free cytosolic proteins sediment (Clift-O'Grady *et al.*, 1998; Lichtenstein *et al.*, 1998). The presence of filamentous actin on PI4KIIα-containing endosomes was confirmed by live cell imaging of fluorescently tagged EGFP-PI4KIIα with the LifeAct fluorescent probe (Fig. 4C) (Riedl *et al.*, 2008). We tracked fluorescently labeled Flag antibodies bound to an exofacial tag engineered in the beta-2 adrenergic receptor (SSF-B2AR, Fig. 4D). We stimulated internalization of receptor-Flag antibody complexes by addition of the adrenergic receptor agonist isoproterenol to functionally define early endosomes (Puthenveedu *et al.*, 2010). In these early endosomes, branched actin networks were detected by cortactin-dsRed (Fig. 4D-G), which was concentrated at the base of small digit-like projections that were enriched for PI4KIIα. The small amounts of pallidin and actin co-sedimenting and/or co-localizing with PI4KIIα and endosomal markers reflects the transient nature of these associations when analyzed in the absence of the cross-linker DSP. Thus, we unequivocally identified that PI4KIIα and branched actin co-localize in functionally defined early endosomes.

We tested the presence of the WASH complex in PI4KIIα-positive endosomes by visualizing EGFP-tagged subunits of the WASH complex and endogenous PI4KIIα using high-resolution deconvolution microscopy. Wash1-, strumpellin-, SWIP-, and Fam21-EGFP were all closely apposed to the PI4KIIα signal (Fig. 5A and C). This physical proximity was especially apparent in three-dimensional projections of the z-stack data, where the WASH complex subunits appear to wrap about PI4KIIα-

containing organelles (Fig. 5B and D). We observed a low degree of overlap between the highest intensity signals and thus measured the co-localization of strumpellin-EGFP and Wash1-EGFP with endogenous PI4KIIα using Pearson's co-localization coefficient. The Pearson's coefficient was 9.1± 2.5 and 13.1± 1.9 for strumpellin-EGFP and Wash1-EGFP, respectively, confirming that there was a low correlation of intensity between overlapping signals (Fig. 5E). However, we verified the specificity of this calculated coefficient by rotating one channel 15° and by analyzing the co-localization of PI4KIIα with the mitochondrial probe Mitotracker. The Pearson's co-localization coefficient between PI4KIIα and Mitotracker was negative, reflecting a complete lack of overlap of these signals (Fig. 5E). This observation was confirmed because channel rotation did not modify the negative Pearson's coefficient between PI4KIIα and Mitotracker. In contrast, rotating either strumpellin-EGFP or Wash1-EGFP by 15° dropped the Pearson's coefficient to values indistinguishable from those of Mitotracker co-localization (p-value ≥ 0.05; Fig. 5E). These results indicate that the WASH complex and actin filaments reside in subdomains of PI4KIIα-containing endosomes. These findings suggest that the Wash1-actin machinery is poised to regulate the trafficking of PI4KIIα and other BLOC-1 cargoes from early endosomes.

The WASH complex modulates the targeting of BLOC-1 cargoes

WASH complex depletion is characterized by the appearance of enlarged cargo-laden tubules from endosomes and cargo mis-sorting (Gomez and Billadeau, 2009; Gomez *et al.*, 2012; Harbour *et al.*, 2012; Jia *et al.*, 2012; Seaman, 2012; Helfer *et al.*, 2013). Therefore, we hypothesized that PI4KIIα is present in tubular structures

whose length is regulated by the WASH complex. To test this hypothesis, we expressed PI4KIIα-EGFP in HEK293T cells stably expressing Flag-tagged beta-2 adrenergic receptor and imaged cells by live cell microscopy. Endosomes were unequivocally identified by ligand-induced internalization of fluorescent Flag antibodies bound to the exofacial domain of the beta-adrenergic receptor. Tubules containing PI4KIIα and/or beta-2 adrenergic receptor emerged from early endosomes and their size increased in WASH complex depleted cells (Fig. 6A and C). Overall, the size of early endosomes containing PI4KIIα and beta-adrenergic receptor was unaffected by WASH complex deficiency (Fig. 6A). In contrast, BLOC-1 depletion by shRNA targeting of pallidin was characterized by an enlargement of these early endosomes without enlargement of tubules (Fig. 6A-B). The changes in the architecture of early endosomes containing PI4KIIα and beta-adrenergic receptor by depletion of either BLOC-1 or WASH complexes support a model whereby WASH complexes in concert with BLOC-1 regulate the targeting of BLOC-1 cargoes from early endosomes.

We predicted that WASH complex deficiencies would alter the subcellular distribution of BLOC-1 cargoes if these complexes act on the same pathway. To test this prediction, we treated MNT1 melanoma cells with shRNA targeting the strumpellin subunit of the WASH complex or scramble control. We assessed the cell surface level of two BLOC-1 cargoes: the Menkes copper transporter ATP7A and the lysosomal SNARE protein VAMP7 (Salazar *et al.*, 2006; Setty *et al.*, 2008; Newell-Litwa *et al.*, 2009; 2010). As previously described, we observed a ~ 1.5-fold increase in the total cellular content of ATP7A in WASH complex depletion (Fig. 7A, lanes 1 –

4). However, cell surface levels of ATP7A increased 3.3 ± 0.8 fold in WASH complex depletion (Fig. 7A, lanes 5 and 6, Fig. 7C average ± SEM). Similarly, cell surface levels of VAMP7 increased 2.5 ± 0.6 fold in WASH complex depletions as compared to control, while the total cellular levels of VAMP7 were moderately but not significantly affected (Fig. 7A, lanes 5 and 6, and see Fig. 3C and D). These cell surface increases were specific to BLOC-1 cargoes rather than global perturbations, as the total cellular content and cell surface levels of transferrin receptor were unaffected in WASH complex depleted cells (Fig. 7B and C). Moreover, silver stain of total biotinylated cell surface protein showed no global increases in cell surface proteins from WASH complex depletions (Fig. 7B). Thus, BLOC-1 cargoes are mis-sorted to the cell surface in WASH complex depletion. These data support a model where WASH complexes modify the traffic of BLOC-1 cargoes.

Discussion

In this study we identified previously unrecognized components of a membrane protein sorting pathway affected in the Hermanksy-Pudlak syndrome by isolating the membrane-anchored lipid kinase PI4KIIα. PI4KIIα binds and regulates two complexes mutated in the Hermansky-Pudlak syndrome, the AP-3 and BLOC-1 complexes, by means of a dileucine sorting motif and its kinase activity (Craige *et al.*, 2008; Salazar *et al.*, 2009; Larimore *et al.*, 2011; Gokhale *et al.*, 2012b). We therefore hypothesized that PI4KIIα interacts with unrecognized components of a vesicle transport pathway requiring the BLOC-1-AP-3-PI4KIIα complex. PI4KIIα could interact with these components either concurrently with binding to AP-3 and BLOC-1 and/or at steps either preceding or following the binding to these complexes. We therefore designed a strategy to enrich PI4KIIα interactors upstream and downstream of the PI4KIIα dileucine sorting motif recognition event. To achieve this goal, we raised an antibody against a twenty-residue peptide of PI4KIIα that contains the ERQPLL sorting motif. Mutation of this motif to ERQPAA is sufficient to prevent recognition of PI4KIIα by this antibody by immunoblot. While the ERQPLL motif is present in five other proteins encoded in the human genome, none of these proteins were enriched in thePI4KIIα interactome, ensuring that this antibody specifically recognizes PI4KIIα (http://www.mrc-lmb.cam.ac.uk/genomes/madanm/harvey/). We used this antibody to immunoaffinity purify PI4KIIα from isotope-labeled cell lysates (SILAC), allowing for the quantitative identification of interacting proteins over non-specific contaminants and immunoglobulins (Ong *et al.*, 2002; Mann, 2006; Zlatic *et al.*,

2010; Gokhale *et al.*, 2012b). Strikingly, PI4KIIα isolation preferentially co-enriched proteins that regulate the actin cytoskeleton, including guanine exchange factors for Rho family GTPases, RhoGEF1, DOCK7, and GEF-H1, and several subunits of the WASH complex. In addition, PI4KIIα co-isolated with membrane trafficking protein complexes, such as clathrin heavy chain and the β3A subunit of the AP-3 complex. These membrane trafficking complexes co-isolated with PI4KIIα at a lower stoichiometry as compared to actin regulators, as intended with our isolation strategy. We biochemically confirmed several of these PI4KIIα interactions. Further, we predicted that if PI4KIIα interactome components interact, then perturbing these proteins would change the total cellular content of other interactome components. With this guiding rationale, we established that genetic depletion of PI4KIIα itself, the BLOC-1 complex, RhoGEF1, or the WASH complex altered the total cellular content of either PI4KIIα interactome components or BLOC-1 cargoes, the Menkes disease copper transporter ATP7A and the lysosomal SNARE VAMP7 (Salazar *et al.*, 2006; Setty *et al.*, 2008; Newell-Litwa *et al.*, 2009; 2010). We conclude that these novel PI4KIIα interactors biochemically and genetically interact with components of the actin cytoskeleton. We favor a model whereby membrane protein cargoes are sorted by a BLOC-1-dependent mechanism into tubular carriers whose size is controlled by the WASH complex, possibly by generating force for membrane scission.

We focused our efforts to characterize the consequences of these interactions on the WASH complex. Wash1 is a type I nucleation-promoting factor that binds the Arp2/3 complex to locally nucleate and organize the polymerization of branched

actin filaments (Derivery *et al.*, 2009; Jia *et al.*, 2010; Veltman and Insall, 2010; Rotty *et al.*, 2012). The WASH complex localizes to tubular domains of early endosomes where actin polymerization is proposed to either create localized membrane domains for cargo sorting and/or regulate tubule morphology by membrane scission (Gomez and Billadeau, 2009; Duleh and Welch, 2010; 2012; Gomez *et al.*, 2012; Harbour *et al.*, 2012; Helfer *et al.*, 2013). Our work identifies the WASH complex as a previously unrecognized interactor of BLOC-1 and PI4KIIα. This assertion is founded on the following findings and rationale. We found that WASH complex subunits localize to PI4KIIα-containing organelles and co-precipitate with BLOC-1 complex subunits, which is consistent with a previous report that Wash1 interacts with the BLOS2 subunit of the BLOC-1 complex by yeast two-hybrid analysis and immunoprecipitation of tagged subunits (Monfregola *et al.*, 2010). Further, we found that PI4KIIα is contained in small tubular structures that emanate from early endosomes, which were functionally labeled by internalized beta-adrenergic receptors. If BLOC-1 and the WASH complex coordinate to regulate early endosomal tubule morphology and/or cargo sorting, then we predicted morphological and mis-sorting phenotypes would arise upon perturbation of protein complexes. First, we predicted early endosomal morphological changes in BLOC-1 and WASH complex depletions, leading to either enlarged endosomes or endosomal tubules. Indeed, we found that BLOC-1 depletion increases the size of functionally identified early endosomes without increasing tubule size. This phenotype is consistent with our previous findings that endosomes increase in size following AP-3, BLOC-1, or PI4KIIα depletion (Salazar *et al.*, 2009). Further, in

WASH complex depletion we found elongation of early endosomal PI4KIIα-containing tubules, which is consistent with previous reports demonstrating increases in endosomal tubule length upon Wash1 depletion (Derivery *et al.*, 2009; Gomez and Billadeau, 2009). Second, we predicted mis-sorting of BLOC-1 cargo in WASH complex depletions, which would suggest an impaired flow of cargoes or membrane out of endosomes. We concentrated on the mis-sorting of specific endo-lysosomal cargoes to the cell surface, which is a characteristic phenotype in AP-3, BLOC-1, and PI4KIIα deficiencies (Dell'Angelica *et al.*, 1999; Peden *et al.*, 2002; Di Pietro *et al.*, 2006; Salazar *et al.*, 2006; Setty *et al.*, 2007; Baust *et al.*, 2008). Two BLOC-1 cargoes, ATP7A and VAMP7, mis-localize to the cell surface in WASH complex depletions. This phenotype was specific to endo-lysosomal cargo, as cell surface levels of a receptor that undergoes constitutive recycling, the transferrin receptor, were unchanged.

How do BLOC-1 and the WASH complex mechanistically coordinate to regulate endosomal morphology and cargo sorting? Actin polymerization fulfills many roles in membrane protein sorting and trafficking, including creating domains to spatially organize sorting machinery, providing mechanical support for membrane deformation during vesicle budding, generating force to aid in membrane scission of vesicles, and propelling carriers after budding is completed (Qualmann *et al.*, 2000). The BLOC-1 cargo mis-sorting phenotype observed after WASH complex depletion suggests a role for Wash1 in steps occurring at or upstream of the membrane scission of vesicles. Actin polymerization by the WASH complex is proposed to generate force required for membrane scission, a hypothesis

that still awaits testing by *in vitro* biochemical reconstitution (Bear, 2009; Derivery *et al.*, 2009; Gomez and Billadeau, 2009; Rotty *et al.*, 2012). However, this model is consistent with the binary biochemical association of WASH complex subunits, PI4KIIα, BLOC-1, and the BLOC-1 cargoes ATP7A and VAMP7 documented here and in our previous work (Salazar *et al.*, 2009; Larimore *et al.*, 2011). In addition, this model predicts that these components interact concurrently, which remains to be documented. An alternative hypothesis is that PI4KIIα and the BLOC-1 complex independently associate with the WASH complex to regulate its activity. Irrespective of whether BLOC-1 and PI4KIIα act in concert or as independent factors, we speculate that the WASH complex acts as a generic mechanism to nucleate branched actin polymerization in endosomes, with spatial or cargo specificity dictated by different upstream accessory factors such as coats and/or lipid modifications.

PI4KIIα is an interesting candidate upstream activator of the WASH complex because of its capacity to produce PI4P (Balla *et al.*, 2002; Barylko, 2002). The WASH complex Fam21 subunit, a component of the PI4KIIα interactome, is proposed to localize WASH to endosomal membranes by binding phosphoinositides and membrane protein sorting complex subunits, such as the Vps35 subunit of the retromer complex (Gomez and Billadeau, 2009; Harbour *et al.*, 2012; Helfer *et al.*, 2013). Fam21 binds PI4P *in vitro*, the lipid product of PI4KIIα (Jia *et al.*, 2010). PI4KIIα and PI4P may thereby either recruit the WASH complex to early endosomal membranes or stabilize the complex at these membranes for function within the AP-3-BLOC-1 or multiple sorting pathways. As discussed below, genetic disruption of a WASH complex subunit and *PI4K2A* cause common neurodegenerative phenotypes

in human patients and mouse models, supporting a regulatory role for PI4KIIα in WASH localization (Valdmanis *et al.*, 2007; Simons *et al.*, 2009; Clemen *et al.*, 2010). In addition, PI4KIIα interactome components may regulate WASH complex activation. A common feature of nucleation-promoting factors such as Wash1 is their regulation and activation by small GTPases. The regulatory GTPase for the WASH complex has remained elusive in mammalian cells. In *Drosophila melanogaster* genetic interactions between Rho and Wash1 have been reported (Liu *et al.*, 2009). The high stoichiometry association of a RhoA guanine exchange factor, RhoGEF1, with PI4KIIα and the similarities between strumpellin depletion and RhoGEF1 depletion in MNT1 cells suggests a regulatory mechanism between these two PI4KIIα interactors. Future experiments are needed to test precise molecular and functional relationships between PI4KIIα, RhoGEF1, the WASH complex, and the BLOC-1 complex.

In addition to expanding our conception of the vesicular trafficking pathway controlled by the Hermansky-Pudlak syndrome complex BLOC-1, our findings have novel implications for the understanding of neurodegenerative diseases that affect central or peripheral motor neuron axons such as hereditary spastic paraplegia (HSP). Point mutations in the WASH complex subunit strumpellin cause autosomal dominant HSP (OMIM 610657), which is characterized by the degeneration of axons projecting to the spinal cord from the brain motor cortex (Valdmanis *et al.*, 2007; Clemen *et al.*, 2010). More than 50 genes have been linked to HSP in humans (Finsterer *et al.*, 2012). The common theme of this disease is degeneration of central neurons with long axons, presumably due to the high burden of maintaining a

synapse distant from the corresponding cell body. Consistent with this theme, many of the 50 identified genes are endosomal membrane trafficking complexes (Blackstone, 2012). To date, no interactions between strumpellin and other HSP-causative genes have been described, thereby making it difficult to place strumpellin and the WASH complex within a cellular framework to understand how mutations in the strumpellin protein lead to disease. A recent study found that strumpellin subunits containing HSP disease-causing mutations assemble into WASH complexes that localize to early endosomes in neurons and have no apparent consequence for retromer complex-mediated recycling of the beta-adrenergic receptor (Freeman *et al.*, 2013). Our data suggest that PI4KIIα interactome components could be an alternative pathway disrupted by these mutations. This hypothesis is supported by the HSP-like phenotype that results following targeted disruption of PI4KIIα in mice (Simons *et al.*, 2009). In addition to a role of the PI4KIIα interactome in degeneration of central motor neurons, our data suggest connections with disease mechanisms in peripheral motor neuron axonal degeneration. Point mutations to the BLOC-1 cargo ATP7A in human patients cause a degeneration of peripheral motor axons (Kennerson *et al.*, 2010; Yi *et al.*, 2012). In this report, we found that ATP7A is mis-localized in strumpellin-depleted cells. In addition, PI4KIIα itself is a BLOC-1 cargo, suggesting it too may mis-localize in strumpellin perturbations that cause HSP in humans. Therefore, we propose that disruption to PI4KIIα interactome components may contribute to the pathophysiology of central and peripheral motor neuron axon degeneration.

Materials and Methods

Antibodies and cell culture

Antibodies used in this study are listed in supplementary Table 3. The PI4KIIα antibody used here for immunoprecipitation, Western blot, and immunofluorescence microscopy experiments was raised against the following sequence: 51-PGHDRERQPLLDRARGAAAQ-70 and was described in Larimore et al. (Larimore *et al.*, 2011). We obtained this antigenic peptide by custom peptide synthesis (Bio-Synthesis). The antigenic peptide was diluted to a 20 mM stock in 0.5M MOPS pH 7.4 and stored at -80°C.

HEK293T and SHSY-5Y cells (American Type Culture Collection) were maintained in Dulbecco's modification of Eagle's medium with 4.5 g/liter glucose, L-glutamine, and sodium pyruvate (DMEM) (Cellgro) supplemented with 10% fetal bovine serum (FBS) (Hyclone) and 100 µg/mL of penicillin/streptomycin (Hyclone) at 37°C and 10% CO_2. MNT1 cells were maintained in DMEM media supplemented with 20% AIM-V medium (Life Technologies), FBS 10% FBS (heat inactivated at 65°C for 60 min) and 100 µg/mL of penicillin/streptomycin at 37°C and 5% CO_2.

For SILAC labeling, cells were grown in either a "light" medium containing only C^{12} and N^{14} in amino acids arginine and lysine (R0K0) or in a "heavy" medium containing C^{13} and N^{15} in these amino acids (R10K8). All reagents for SILAC labeling were obtained from Dundee Cell Products. Cells were grown for seven passages to ensure maximal incorporation of these amino acids. Our previous work has found that these conditions lead to greater than 97.5% incorporation of these amino acids

into the total cellular pool (Ong and Mann, 2006; Perez-Cornejo *et al.*, 2012; Gokhale *et al.*, 2012a).

DNA Expression Constructs

The following PI4KIIα expression constructs were used: PI4KIIα-EGFP WT, PI4KIIα-HA WT, PI4KIIα-HA D308A, PI4KIIα-HA LL60,61AA (Craige *et al.*, 2008). The Flag-tagged dysbindin construct is described in our previous work (Gokhale *et al.*, 2012a). Wash1-EGFP, Strumpellin-EGFP, KIAA1033-EGFP, and FAM21-EGFP were described previously (Harbour *et al.*, 2010). Cortactin-dsRed and Signal-Sequence Flag tagged β-2 adrenergic receptors (SSF-B2AR) have been previously described (Kaksonen *et al.*, 2000; Puthenveedu *et al.*, 2010).

Immunoprecipitation and immunoaffinity chromatography

SHSY-5Y neuroblastoma cells were grown to confluence and minimally crosslinked as previously described (Zlatic *et al.*, 2010; Perez-Cornejo *et al.*, 2012; Gokhale *et al.*, 2012a; 2012b). Briefly, cells were rapidly moved from the incubator to an ice bath and washed two times with ice-cold PBS supplemented with $CaCl_2$ (0.1 mM) and $MgCl_2$ (1 mM) (PBS-Ca-Mg). Following this wash, cells were incubated for 2 hours in 1 mM DSP diluted in PBS-Ca-Mg or a DMSO vehicle-only control. After crosslinking, the DSP reaction was quenched by addition of a 25mM Tris pH 7.4 solution for 15 min. Cells were then washed two times in ice-cold PBS-Ca-Mg and lysed for 30 min in Buffer A (150 mM NaCl, 10 mM HEPES, 1 mM EGTA, 0.1 mM $MgCl_2$) with 0.5% Triton-X-100 supplemented with Complete anti-protease (Roche). Following lysis, cell debris were scraped from the plates and lysates were spun at 16,100 x *g* for 15 min. The supernatant was recovered and diluted to 1 mg/mL. For

immunoprecipitation, 500 µg of this clarified cell lysate was applied to 30 µL of Dynal magnetic beads (Invitrogen) coated with the immunoprecipitation antibody of interest. These immunoprecipitation reactions were incubated for 2 hours with end-over-end rotation at 4°C. For PI4KIIα peptide competition controls, the PI4KIIα antigenic peptide was included in the immunoprecipitation reaction at a concentration of 40 µM. For FLAG competition controls, the 3X FLAG peptide was included at 340 µM, as previously described (Perez-Cornejo *et al.*, 2012; Gokhale *et al.*, 2012a). For HA competition controls, the HA peptide was included at 10 µM. Following the 2h incubation, beads were washed 5 times in Buffer A with 0.1% Triton X-100. Proteins were then eluted from the beads either by boiling in Laemmli sample buffer at 75°C for 5 min or by incubating with the antigenic peptide for 2 hours on ice. Peptides were diluted in lysis buffer to their elution concentration (PI4KIIα antigenic peptide: 200 µM; 3X FLAG peptide: 340 µM). Samples were resolved by SDS-PAGE electrophoresis followed by either silver stain or immunoblotting to detect individual proteins. For SILAC analysis, 18 immunoaffinity chromatography and 18 control reactions were conducted. Eluates from these reactions were combined and TCA precipitated to concentrate the samples for SDS-PAGE and mass spectrometry analysis. Samples were analyzed for SILAC protein identification by MS Bioworks (MI, USA) and the Emory CND Proteomics Facility (GA, USA). SILAC-labeled samples were separated on a 4-12% Bis-Tris Novex mini-gel (Invitrogen, CA, USA) using the MOPS buffer system, stained with Coomassie and the lane was excised into 20 equal segments using a grid. Gel pieces were processed

using a robot (ProGest, DigiLab) as described (Perez-Cornejo *et al.*, 2012; Gokhale *et al.*, 2012a).

Transient protein knockdown and immunoblot analysis

For shRNA-mediated protein knockdown, constructs in the pLKO.1 vector were obtained from Open Biosystems. Clone IDs for the targeting constructs are as follows: muted (Bloc1s5): TRCN0000129117; pallidin (Bloc1s6): TRCN0000122781; strumpellin: TRCN0000128018; and RhoGEF1: TRCN0000033567. A scramble control vector was obtained from Addgene (Vector 1864). HEK293T cells seeded in 6 well plates were transfected with 1 μg of shRNA construct DNA overnight by Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions. MNT1 and SHSY-5Y cells were seeded in 6-well plates and treated with 1-2 μL of high-titer lentiviral particles containing the appropriate lentiviral construct. The Emory NINDS Viral Vector Core prepared all high titer lentiviruses. Two days after delivery of shRNA construct, transformed cells were selected by treatment with 2 μg/mL puromycin (Invitrogen) in the appropriate growth media as described above. The total length of treatment was 5-10 days to allow for efficient knockdown of the proteins of interest. siRNA oligonucleotide sequences against PI4KIIα and controls as well as procedures were described previously (Craige *et al.*, 2008; Salazar *et al.*, 2009).

For immunoblot analysis of protein levels, cells were grown to confluence in 6-well plates. Cells were treated with lentiviruses for 7 days. Plates were rapidly moved to an ice bath and immediately washed two times in ice-cold PBS. Cells were then lifted in Buffer A and spun at 16,100 x *g* for 1 min to pellet. MNT1 cells were incubated for

10 min in Buffer A prior to lifting to facilitate release from the tissue culture plate. Cell pellets were resuspended in 100 μL Buffer A supplemented with Complete anti-protease. Cells were then lysed by sonication (3 rounds of 5 one-second bursts). Lysates were diluted to 1 mg/mL and resolved by SDS-PAGE and immunoblotting. Five to 15 μg of cell lysate was loaded per well.

Immunofluorescence microscopy

HEK293T cells were transfected in 6-well plates with 1 μg of the DNA construct by Lipofectamine 2000 for 4 hours and then switched into growth medium. After 24 hours, cells were seeded on glass coverslips coated with Matrigel. The next day, coverslips were moved to ice, washed 2 times in ice-cold PBS, and then fixed with 4% paraformaldehyde in PBS for 20 min. For cells stained with the A488 Mitotracker dye (Invitrogen), cells were incubated with 500 nM mitotracker dye diluted in growth medium for 30 min. Following this incubation, cells were immediately fixed at 37°C in 4% paraformaldehyde. Following fixation, all coverslips were washed 2 times in PBS and then blocked and permeabilized for 30 min at room temperature in a solution of 0.02% saponin (Sigma), 15% horse serum (Hyclone), 2% bovine serum albumin (Roche), and 1% fish skin gelatin (Sigma) in PBS. Primary antibodies (Table 3) were diluted in blocking solution and applied for 30 min at 37°C. After primary antibody incubation, coverslips were washed 3 times in blocking solution. Coverslips were incubated for 30 min at 37°C with fluorophore-conjugated secondary antibodies (Table 3) diluted in blocking solution. Coverslips were washed 2 times in blocking solution, 1 time in PBS, 1 time in

ultrapure water, and then mounted on glass slides in Gelvatol mounting media (Sigma).

For fixed-cell deconvolution microscopy, images were collected on a Zeiss Axiovert 200M inverted microscope (Carl Zeiss Group) with a Sedat filter set (Chroma Technology). Slidebook 4.0 OSX software was used to run a multi-wavelength, widefield, three-dimensional microscopy system (Intelligent Imaging Innovations). Images were visualized with a 100X oil DIC objective with numerical aperture 1.4 and captured using a scientific-grade cooled charge-coupled Cool-Snap HQ camera (Photometrics) with an ORCA-ER chip (Hamamatsu Photonics). Images were captured with 200 nm between z-planes. A nearest neighbor, constrained iterative deconvolution algorithm with Gaussian smoothing was used to remove out-of-focus light (Swedlow *et al.*, 1997; 2002). Images were exported from the Slidebook 4.0 software as 8-bit TIF series. For co-localization analysis, in-focus image planes were background subtracted with a 10-pixel rolling ball algorithm in Fiji (ImageJ). A region of interest immediately surrounding individual cells was selected and the Pearson coefficient was calculated by the Coloc 2 plugin. Three-dimensional reconstructions were created from deconvolved 8-bit TIF series images imported into the Imaris 6.3.1 software using manual thresholding and a minimal volume limit of 4 voxels (Bitplane Scientific Software).

For live-cell imaging of eGFP-tagged PI4KIIα and mCherry-tagged LifeAct peptide, HEK293T cells expressing fluorescent-tagged protein constructs were seeded in Matrigel-coated glass-bottom culture dishes (Matek Corporation). Prior to imaging, cells were maintained in HEK293T growth media as described above at 37ºC and

10% CO_2. Immediately before imaging, an imaging medium consisting of Hank's balanced salt solution minus phenol red and $NaHCO_2$ (Sigma) supplemented with 10% FBS (HyClone) was applied. Cells were imaged using a Nikon TE2000 inverted microscope (Nikon Instruments) equipped with a hybrid scanner, Perfect Focus, and an environmental chamber for temperature regulation to 37ºC and 5% CO_2. Images were captured with a 100X, 1.49 numerical aperture oil objective and 500–550/570–620 filter sets using NIS-Elements AR 3.1 (Nikon) software. Cells were alternately excited with 488 and 568 nm wavelength lasers every 4.25 s and a single confocal plane image was captured. Images were exported as an 8-bit TIF series in the NIS-Elements software and figures were compiled in Adobe Photoshop.

For live cell imaging of PI4KIIα with labeled functionally labeled endosomes, fluorescent labels were from expressed protein constructs (GFP and dsRed) or anti-flag M1 antibody conjugated to Alexa-647 dye. Cells were grown on coverslips and maintained in 10%FBS (Gibco) high glucose DMEM (HyClone) and imaged in Opti-MEME (Gibco) with 10% FBS and buffered to pH 7.4 with 40 mM HEPES. Live cell confocal images were collected on an Andor Revolution XD Spinning Disk system with a Nikon Eclipse Ti inverted microscope. Objective used for capture was a 100x 1.49 NA TIRF objective from Nikon. Both microscope and objectives are housed in a temperature control case maintained at 37°C. Light sources were 488 nm, 561 nm and 647 nm solid state lasers and images were captured on an iXON + 897 EM-CCD Camera using Andor iQ. Image analysis was performed on raw images in ImageJ (NIH). Images were not modified in anyway other than color and brightness/contrast assignment for multi-channel images. For tubule scoring, cells

were blindly selected in the beta2-dregenergic receptor channel. We considered a cell to have the tubuled phenotype if it contained more than one object ~4 times longer than wide in the size range of endosomes. Cells were set in focus using only the beta2-dregenergic receptor channel before agonist to avoid bias selecting for cells with the phenotype. All file names were randomized for blind assessment.

Cell fractionation

Confluent HEK293T cells were washed two times in ice-cold PBS and then lifted in intracellular buffer (38 mM potassium aspartate, 38 mM potassium glutamate, 38 mM potassium gluconate, 20 mM MOPS-KOH, pH 7.2, 5 mM reduced glutathione, 5 mM sodium carbonate, 2.5 mM magnesium sulfate, 2 mM EGTA). Cells were spun at 800 x *g* to pellet and then resuspended in a minimal volume of intracellular buffer supplemented with 5X concentrated Complete anti-protease. Cells were homogenized using a cell cracker with a 12-µm clearance (Clift-O'Grady *et al.*, 1998; Lichtenstein *et al.*, 1998). Between 200 – 250 µl of homogenate (450 – 800 µg) was layered on 10-45% sucrose gradients buffered with 20 mM MOPS (pH 7.2), which were prepared using the Gradient Master (Biocomp Instruments). Sucrose gradients were centrifuged for 1 h at 210,000 x g at 4°C in a Beckman SW55Ti rotor and fractions analyzed by immunoblot. Sucrose refractive indices for each fraction were measured by refractometer (Leica).

Surface labeling and streptavidin pulldowns

Following 7 day treatment with scramble or strumpellin-targeted lentiviruses, plates of confluent MNT1 cells were moved to an ice bath and washed two times with ice-cold PBS-Ca-Mg (0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ in PBS). All solutions used

were ice-cold when applied to cells and cells were maintained on an ice bath throughout. Following washes, cells were incubated in a 1 mM sodium periodate (Sigma) solution prepared in PBS-Ca-Mg for 30 min, after which the periodate solution was removed and a quenching solution of 1 mM glycerol in PBS-Ca-Mg was added (Zeng *et al.*, 2009). Cells were then washed two times with PBS-Ca-Mg and a biotin ligation solution of 100 μM aminooxy biotin (Biotium) and 10 mM aniline (Sigma) was applied for 90 min. Following biotin ligation, cells were washed two times in PBS-Ca-Mg and lysed for 30 min in Buffer A (150 mM NaCl, 10 mM HEPES, 1 mM EGTA, 0.1 mM $MgCl_2$) with 0.5% Triton-X-100 supplemented with Complete anti-protease (Roche). This biotinylation procedure is based on a periodate-generated aldehyde on sialic acids. This process is catalyzed by aniline-dependent ligation with an appropriate tag. Aniline catalysis accelerates ligation, facilitating the reaction with low concentrations of aminooxy-biotin at neutral pH to label the most cell-surface sialylated glycoproteins (Zeng *et al.*, 2009). Following lysis, cell debris were scraped from the plates and lysates were spun at 16,100 x *g* for 15 min. The supernatant was recovered and diluted to 0.2 mg/mL. A small volume (50 μL) of neutravidin-coated agarose beads (Thermo Scientific) was pre-washed two times in Buffer A with 0.1% Triton X-100 and 100 μg of cell lysate was incubated with the beads for two hours with end-over-end rotation at 4°C. Beads were then washed 5 times in Buffer A with 0.1% Triton X-100 for 5 min with end-over-end rotation at 4°C. Proteins were eluted from the beads by boiling in Laemmli sample buffer at 75°C for 5 min and samples were analyzed by SDS-PAGE followed by silver stain or immunoblot.

Computational and statistical analysis

Functional gene annotation for the set of PI4KIIα-interacting proteins (Table 2) was performed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID, version 6.7) and gene ontology terms (Dennis *et al.*, 2003; Huang *et al.*, 2007). The Cytoscape plugin Enrichment Map was used to display overlap between gene ontology terms within Cytoscape (version 2.8.3) (Merico *et al.*, 2010). Node size was mapped to number of genes within a GO category and node color was mapped to the p-value for enrichment of a given category. For network analysis, a network interaction map was built using GeneGo Metacore (version 6.11 build 41105) for PI4KIIα-interacting proteins (Table 1) and BLOC-1 subunits. The network was visualized in Cytoscape and node color was mapped to proteins of interest.

Experimental conditions were compared using KaleidaGraph v4.1.3 (Synergy) or StatPlus Mac Built5.6.0pre/Universal (AnalystSoft). Data are represented as box plots 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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Figures

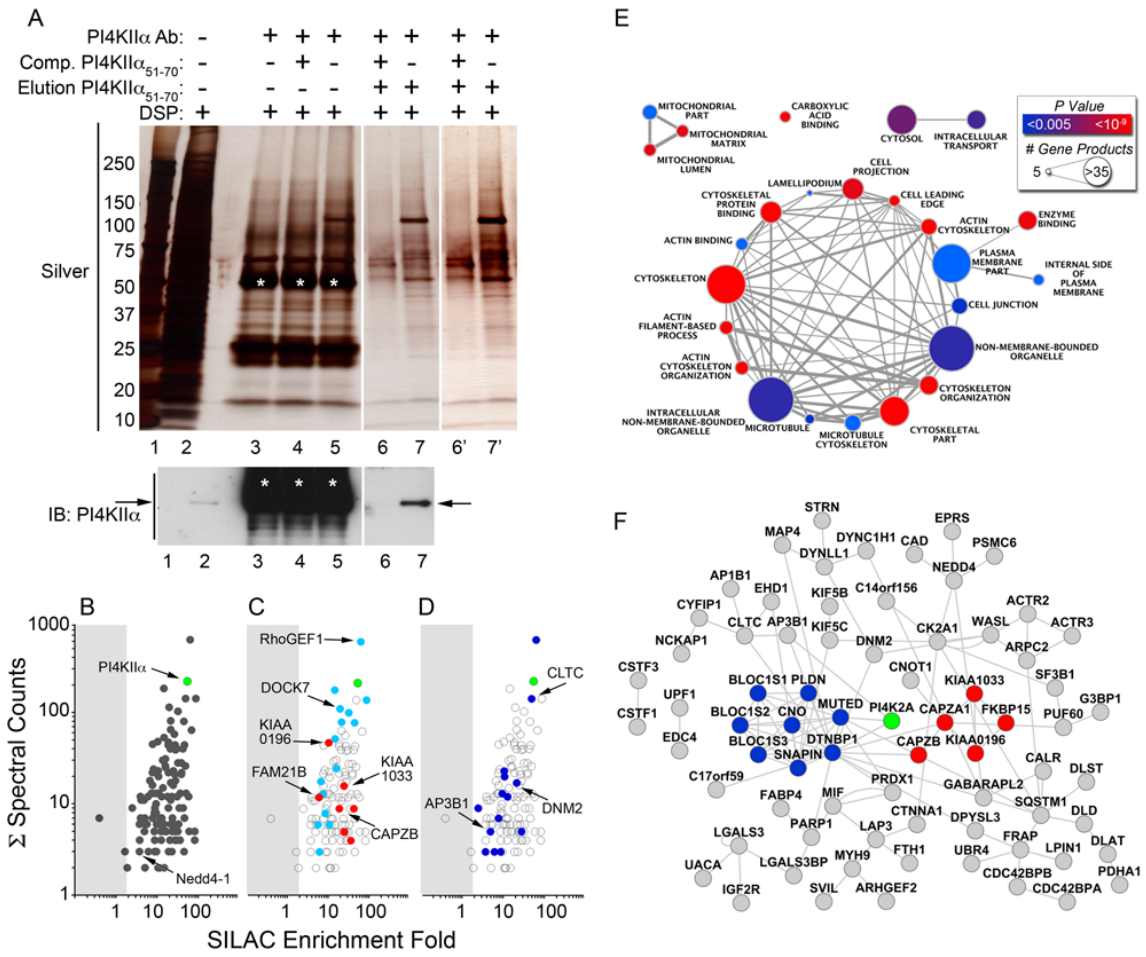

Fig 1

Figure 1. The PI4KIIα interactome enriches actin regulatory proteins. (A) PI4KIIα was immunoaffinity purified from DSP-crosslinked SHSY-5Y neuroblastoma cell homogenates. PI4KIIα and co-isolating proteins were resolved by SDS-PAGE and silver stained (lane 5). Some polypeptides are absent from controls, which include antibody-coated beads without lysate (lane 3) or incubations where an excess of antigenic PI4KIIα peptide was included (lane 4). Bead elution was performed with Laemmli sample buffer (lanes 3 to 5). Lanes 6 and 7 depict antigenic peptide eluates from beads similar to those in lanes 4 and 5. Immunoaffinity purification allowed for the selective elution of PI4KIIα co-isolating proteins against low background (compare lanes 6 and 7). An arrow marks the band containing PI4KIIα. Silver stain is a representative image of two independent experiments. The bottom panel is an immunoblot of PI4KIIα in a parallel set of assays as those in the silver-stained SDS-PAGE. IgG chains are marked by asterisks. Input represents 1% and 0.33% for immunoprecipitation and immunoaffinity purifications, respectively. (B) Plots represent PI4KIIα and co-purifying proteins (see Supplementary Table 1 for details).

X- and Y-axes show SILAC fold of enrichment and the total number of spectral counts used for protein identification, respectively. Reference dots highlight PI4KIIα (green) and Nedd4-1. (C) Teal and red dots highlight actin-related proteins co-isolated with PI4KIIα. These include GDP exchange factors RhoGEF1, DOCK7, and GEF-H1. Red dots indicate the actin-related proteins that are subunits or interactors of the WASH complex: strumpellin (KIAA0196), SWIP (KIAA1033), Fam21B, FKBP15, capping protein alpha, and capping protein beta. (D) Blue dots highlight membrane-trafficking related proteins co-isolated with PI4KIIα, including clathrin heavy chain (CLTC), dynamin-2 (DNM2), and β3A subunit of the AP-3 complex (AP3B1). (E) Functional annotation analysis of the identified proteins using Gene Ontology annotations revealed a preponderance of actin-related proteins. Enrichment of categories such as cytoskeleton (30/124 interactors) and actin cytoskeleton (13/124) were significant at p-values of 1.62×10^{-7} and 7.67×10^{-7}, respectively. See supplementary table 2 for details. (F) Network analysis of putative PI4KIIα interactome components and BLOC-1 subunits. PI4KIIα (green), the BLOC-1 subunits (blue) and WASH complex subunits (red) are highlighted.

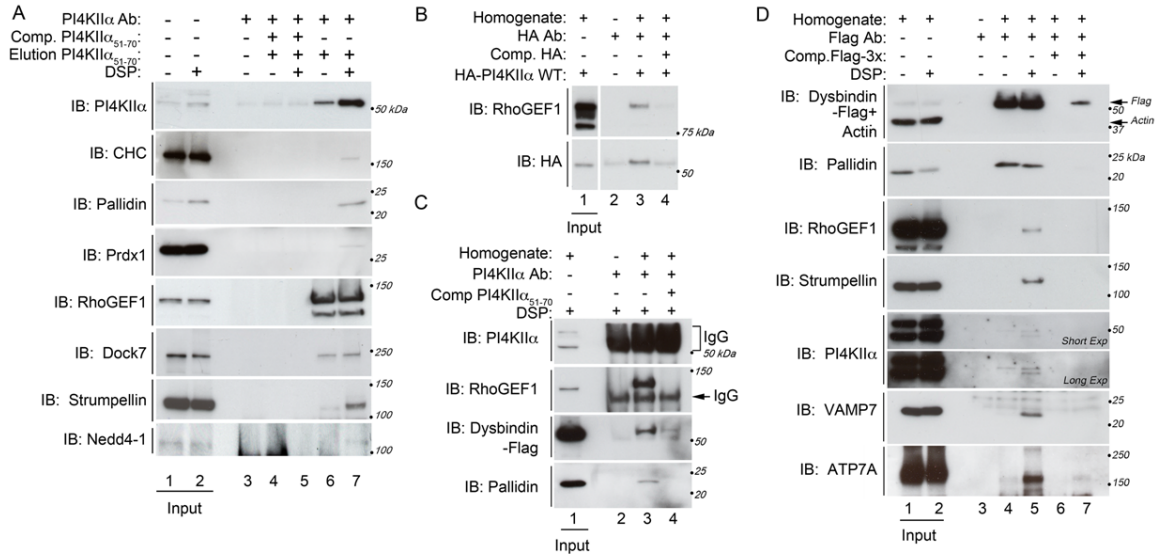

Fig 2

Figure 2. Biochemical confirmation of PI4KIIα-interacting proteins. (A) PI4KIIα was immunoaffinity purified from uncrosslinked (odd lanes) and DSP-crosslinked (even lanes) detergent-soluble SHSY-5Y neuroblastoma cell extracts. Immune complexes were resolved by SDS-PAGE and analyzed by immunoblot with antibodies against PI4KIIα, clathrin heavy chain (CHC), pallidin, peroxiredoxin-1 (Prdx-1), RhoGEF1, Dock7, and the WASH complex subunit strumpellin and Nedd4-1. Co-isolating proteins were not detected in control reactions where an excess of PI4KIIα antigenic peptide was included to outcompete the endogenous PI4KIIα (lanes 4 and 5). Lane 3 shows antibody-coated beads incubated in the absence of cell lysate. Inputs represent 0.25%. (B) HEK293T cells transiently expressing HA-

tagged PI4KIIα were DSP-crosslinked (lanes 3-4) and detergent soluble extracts were immunoprecipitated with HA antibodies. Immune complexes were resolved by SDS-PAGE and analyzed by immunoblot with the indicated antibodies. RhoGEF1 is present in HA immunoprecipitations (lane 3) but not in peptide outcompetition controls (lane 4). Lane 2 shows antibody-coated beads incubated in the absence of cell lysate. Inputs represent 0.5% and n = 3. (C and D) SHSY-5Y cells stably expressing a Flag-tagged subunit of the BLOC-1 complex (dysbindin) were incubated in the absence (D lanes 1, 4, 6) or presence (C all lanes and D lanes 2, 5, 7) of DSP crosslinker and detergent soluble extracts were immunoprecipitated with antibodies against PI4KIIα (C) or the Flag epitope (D). Immune complexes were resolved by SDS-PAGE and analyzed by immunoblot with antibodies against the indicated proteins identified in the PI4KIIα interactome. Also included are two BLOC-1 membrane protein cargoes, the v-SNARE VAMP7 and the Menkes disease copper transporter ATP7A. As in A and B, controls are antigenic peptide outcompetition (C, lane 4 and D, lanes 6-7). Lanes C2 and D3 show antibody-coated beads incubated in the absence of cell lysate. Inputs represent 0.5%.

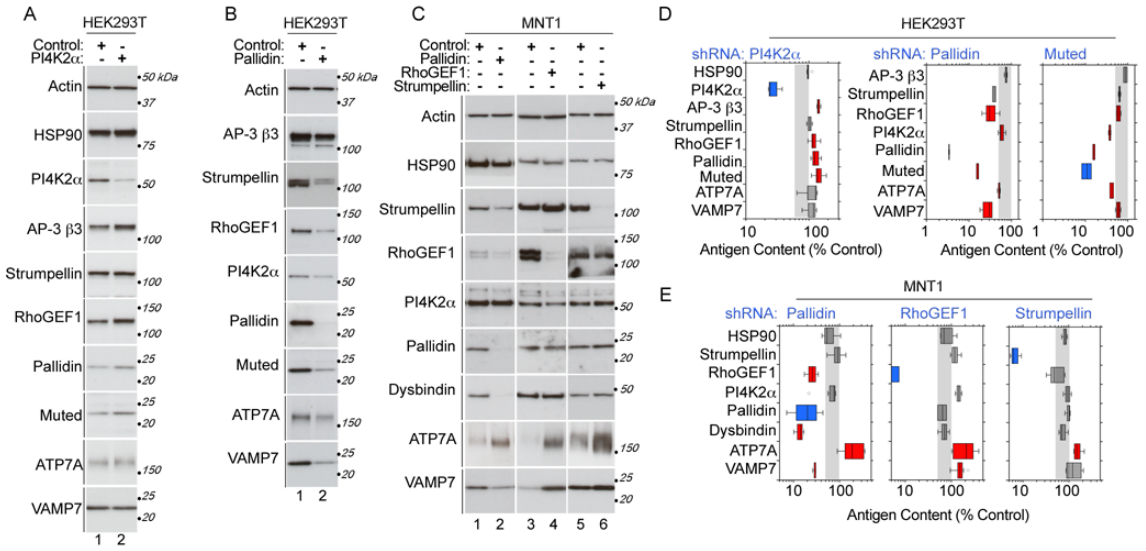

Fig 3

Figure 3. Genetic confirmation of PI4KIIα-interacting proteins. (A and D) HEK293T cells were treated with scramble siRNA control or siRNA targeting PI4KIIα. (B and D) HEK293T cells were treated with lentiviruses carrying scramble shRNA control or shRNA targeting the pallidin or muted subunits of the BLOC-1 complex. (C and E) MNT-1 human melanoma cells were treated with lentiviruses carrying scramble shRNA control or shRNA targeting the BLOC-1 subunit pallidin, RhoGEF1, or the WASH complex subunit strumpellin. HEK293T and MNT-1 cell lysates were resolved by SDS-PAGE and the total cellular content of proteins of interest was measured by semi-quantitative immunoblot. Box plots in D and E depict quantifications where the targeted protein is highlighted in blue and antigens

significantly changed are labeled in red. Grey shading represents 50-100% of control. All experiments were done in biological triplicates with at least one determination per replicate. Significant p-values are all below 0.025 and were determined by a non-parametric analysis using a group Kruskal-Wallis Rank Sum Test followed by Wilcoxon-Mann-Whitney Rank Sum Test. The AP-3 subunit β3A (D) and Hsp90 (D-E) were used as controls for comparisons.

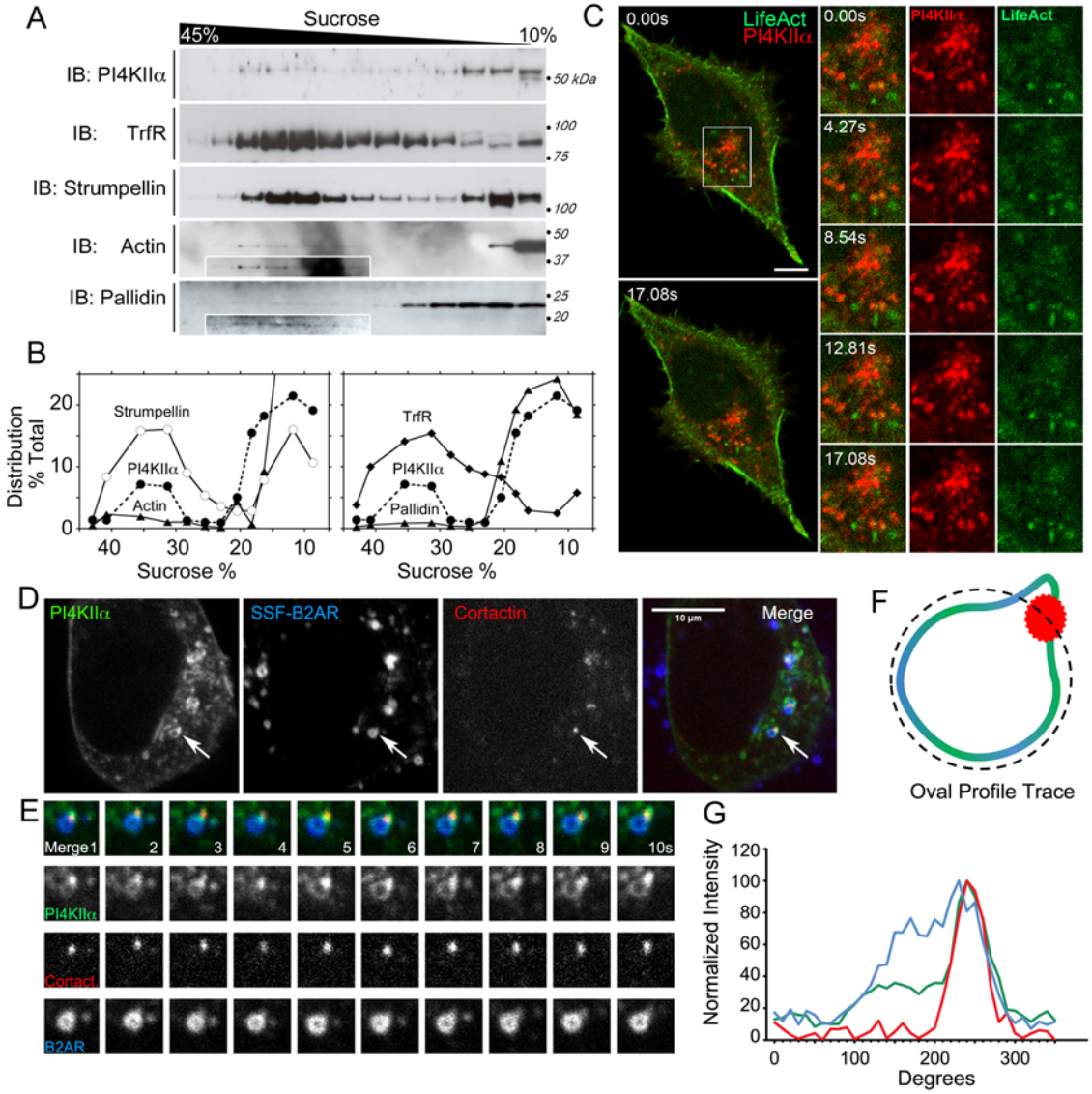

Fig 4

Figure 4. PI4KIIα, the WASH complex subunit strumpellin, and actin cytoskeleton components co-reside at early endosomes. (A-B) HEK293T cells were homogenized and low-speed supernatants were resolved by sucrose velocity sedimentation on a 10-45% sucrose gradient. PI4KIIα and the WASH complex

subunit strumpellin co-sedimented to 35% sucrose with endosomal organelles, as identified by the transferrin receptor (TrfR). Cytosolic fractions sediment at the top of the gradient (10% sucrose). (B) Relative distribution of the indicated proteins for the fractionation shown in A. Images and distribution profile are representative of two independent experiments. (C) Live cell imaging of fluorescent-tagged PI4KIIα and LifeAct to visualize filamentous actin in HEK293T cells confirmed the presence of actin at PI4KIIα-positive organelles (n = 12 cells). (D) HEK293T cells stably expressing PI4KIIα-GFP and Signal-Sequence Flag tagged β-2 adrenergic receptors (SSF-B2AR) were transiently transfected with mCortactin-dsRed and imaged 10 minutes after addition of 10 μM isoproterenol. A single confocal stack was captured in three channels at 1 second intervals. (E) Expanded image of endosome indicated by an arrow in A over at 10 second period. (F) Diagram of the oval profile used to generate traces of fluorescence intensity around endosomes. (G) Representative trace of normalized fluorescence intensity for each protein in the endosome above.

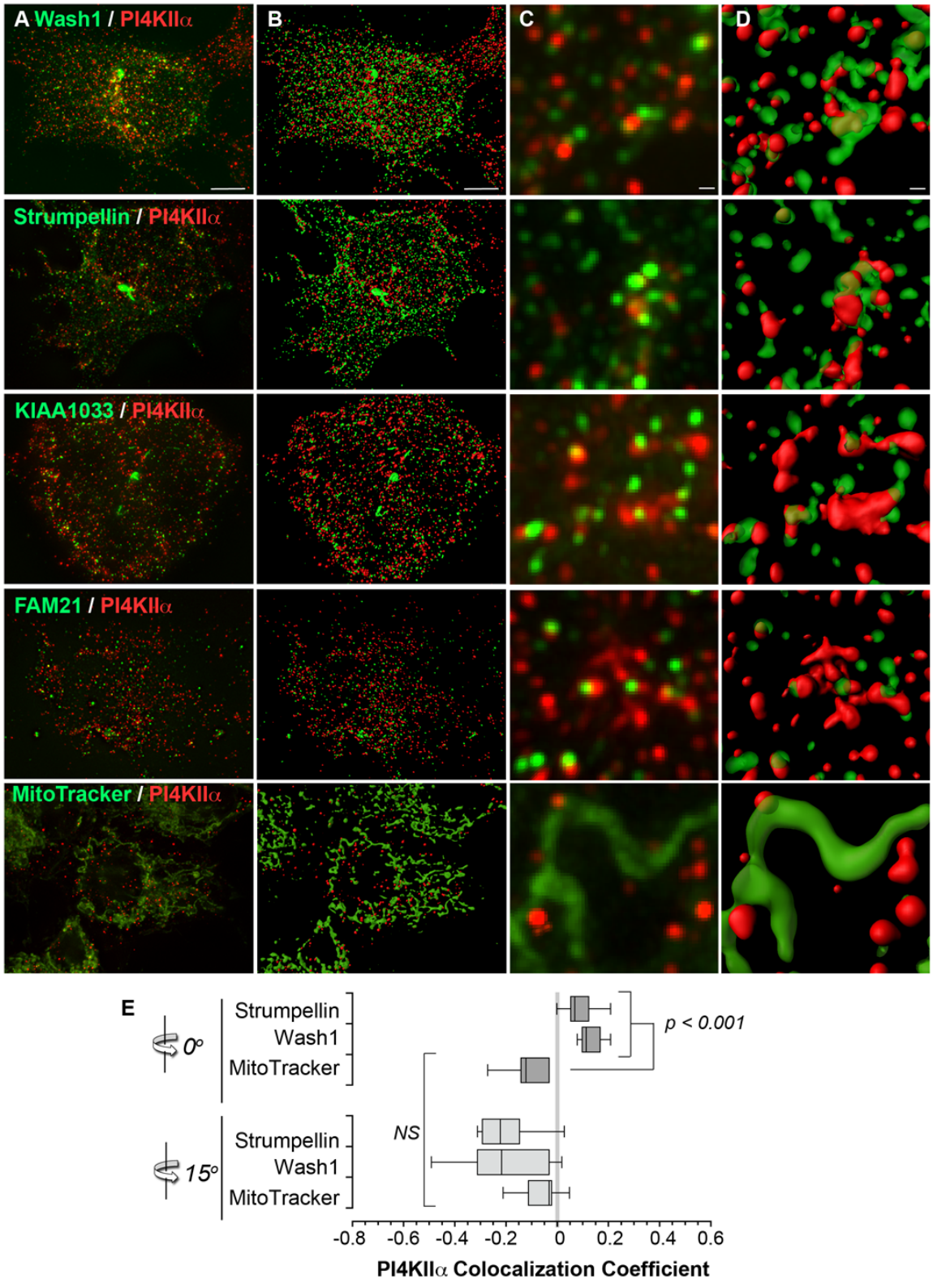
A Wash1 / PI4KIIα
B
C
D
Strumpellin / PI4KIIα
KIAA1033 / PI4KIIα
FAM21 / PI4KIIα
MitoTracker / PI4KIIα
E
0°
15°
Strumpellin
Wash1
MitoTracker
Strumpellin
Wash1
MitoTracker
p < 0.001
NS
-0.8 -0.6 -0.4 -0.2 0 0.2 0.4 0.6
PI4KIIα Colocalization Coefficient

Fig 5

Figure 5. PI4KIIα co-localizes with the WASH complex. (A and C) HEK293T cells were either transiently transfected with EGFP-tagged subunits of the WASH complex or stained with Mitotracker green, fixed, and processed for indirect immunofluorescence microscopy. Images were acquired by high-resolution deconvolution microscopy. Z-stack projections are shown. (B and D) Isosurface renderings of the z-stack data created using Imaris software. (E) Calculation of the Pearson's correlation coefficient between signal intensities of strumpellin-EGFP, WASH-EGFP, or Mitotracker and endogenous PI4KIIα. Correlation was lost when one channel was rotated by 15°. n = 6 for WASH-EGFP, n = 7 for strumpellin-EGFP, and n = 9 for Mitotracker-A488. Statistical comparisons were performed by non-parametric analysis using a group Kruskal-Wallis Rank Sum Test followed by individual Wilcoxon-Mann-Whitney Rank Sum Tests.

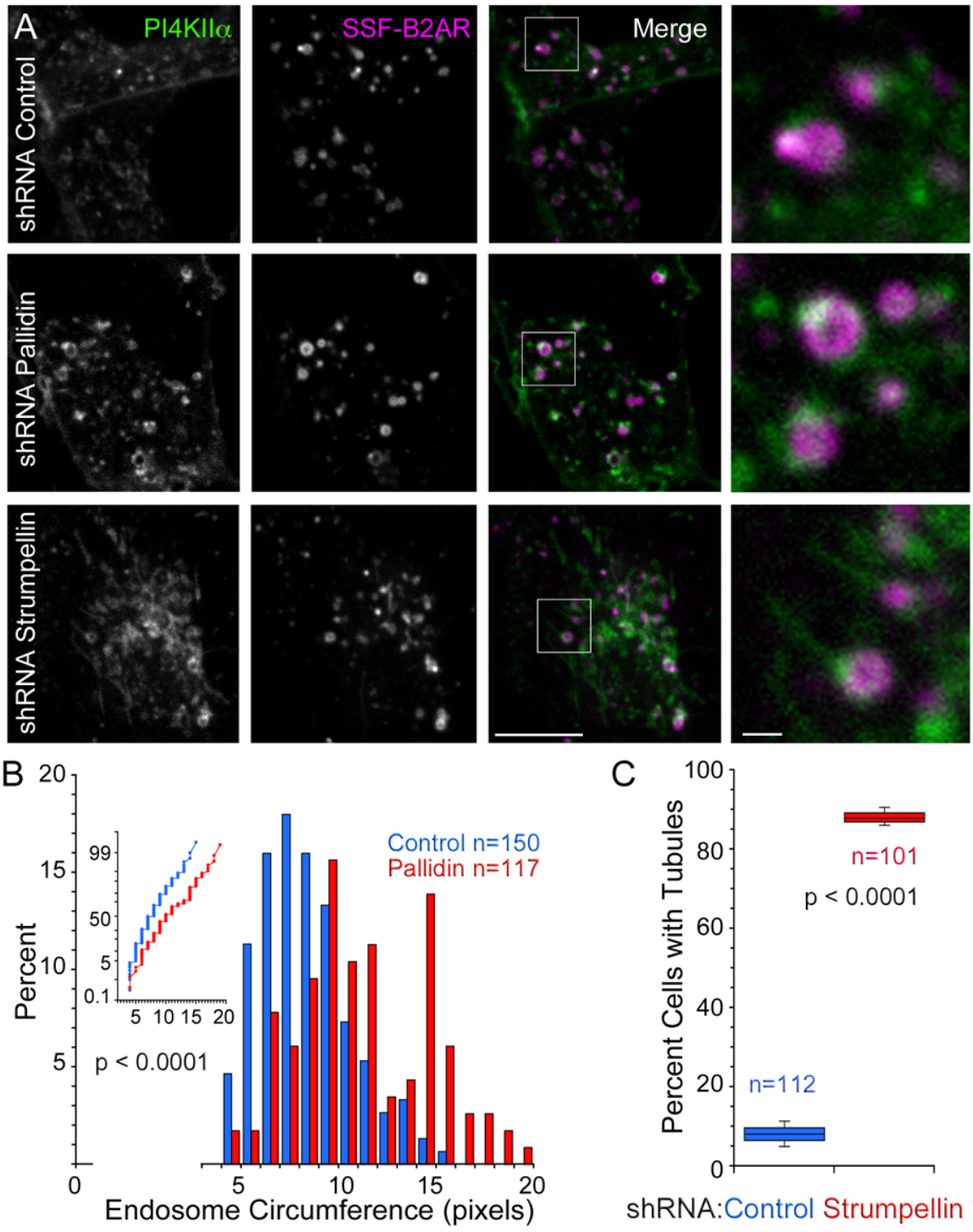

Fig 6

Figure 6. Depletion of pallidin and strumpellin alter endosomal morphology. (A) HEK293T cells stably expressing Signal-Sequence Flag tagged β-2 adrenergic

receptors (SSF-B2AR) and PI4KIIα-EGFP were transduced with scrambled, pallidin, or strumpellin shRNA lentiviruses. Pallidin-depleted cells were imaged 8 days from initiation of transduction and strumpellin-depleted cells were imaged 5 days after transduction. Cells were grown under selection after day 2 of transduction. All knockdowns were compared to parallel transduction with scrambled shRNA. Images are max projections of 3 confocal slices taken with 0.3 μm intervals. (B) Frequency histogram of endosomal circumference from cells treated with either scrambled (blue) or pallidin (red) shRNA. Insert to the left depicts a probability plot of the same data. P-value determined by non-parametric Kolmogorow-Smirnov test. n = 150 for control cells and n = 117 for pallidin-depleted cells. (C) Percentage of cells expressing scrambled or strumpellin shRNA displaying endosomal tubulation. Cell selection was performed in the SSF-B2AR channel and blinded for PI4KIIα prior to Flag antibody internalization, then imaged 15 minutes after addition of 10 μM isoproterenol. P-value determined by non-parametric Fisher exact test. n = 112 for control cells and n = 101 for pallidin-depleted cells.

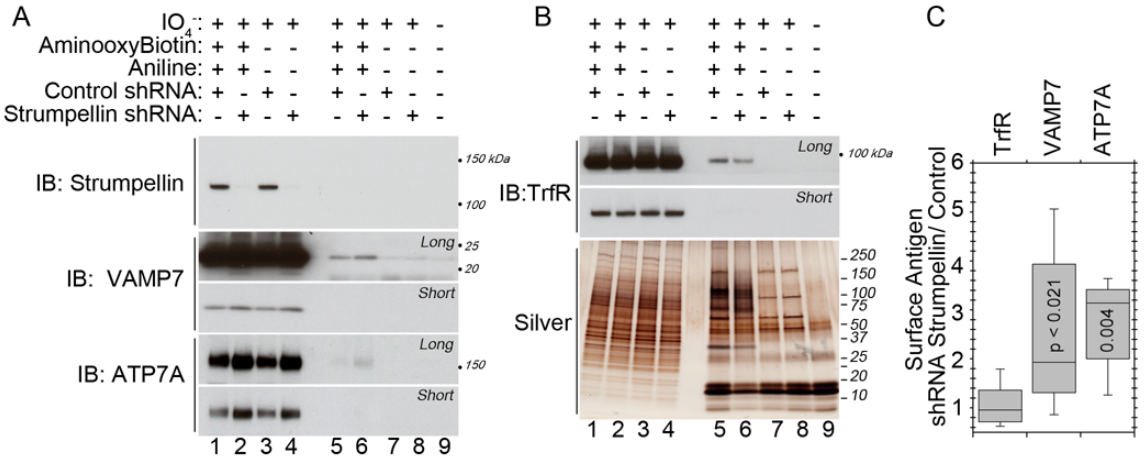

Fig 7

Figure 7. WASH complex depletion alters the subcellular distribution of BLOC-1 cargoes. (A) MNT1 melanoma cells were treated with shRNA directed against the strumpellin subunit of the WASH complex or scramble control. Cell surface proteins were labeled by surface biotinylation and isolated by streptavidin bead pulldown. Representative immunoblots show an increase in the surface content of ATP7A and Vamp7, two BLOC-1 cargoes, in WASH complex depletions. (B) Transferrin receptor (TrfR) was measured as control. Silver stain of isolated proteins revealed no global decreases in the cell surface content of proteins (compare lanes 5 and 6). (C) Quantifications of the surface expression of proteins of interest relative in WASH complex depletion relative to scramble control. Data are averages ± SEM. We found significant increases in the BLOC-1 cargoes ATP7A and Vamp7 of 3.3 ± 0.8 fold and 2.5 ± 0.6 fold, respectively. Transferrin receptor was not significantly changed. P-value determined by non-parametric Wilcoxon-Mann-Whitney Rank Sum Test. n = 8 determinations from four independent experiments.

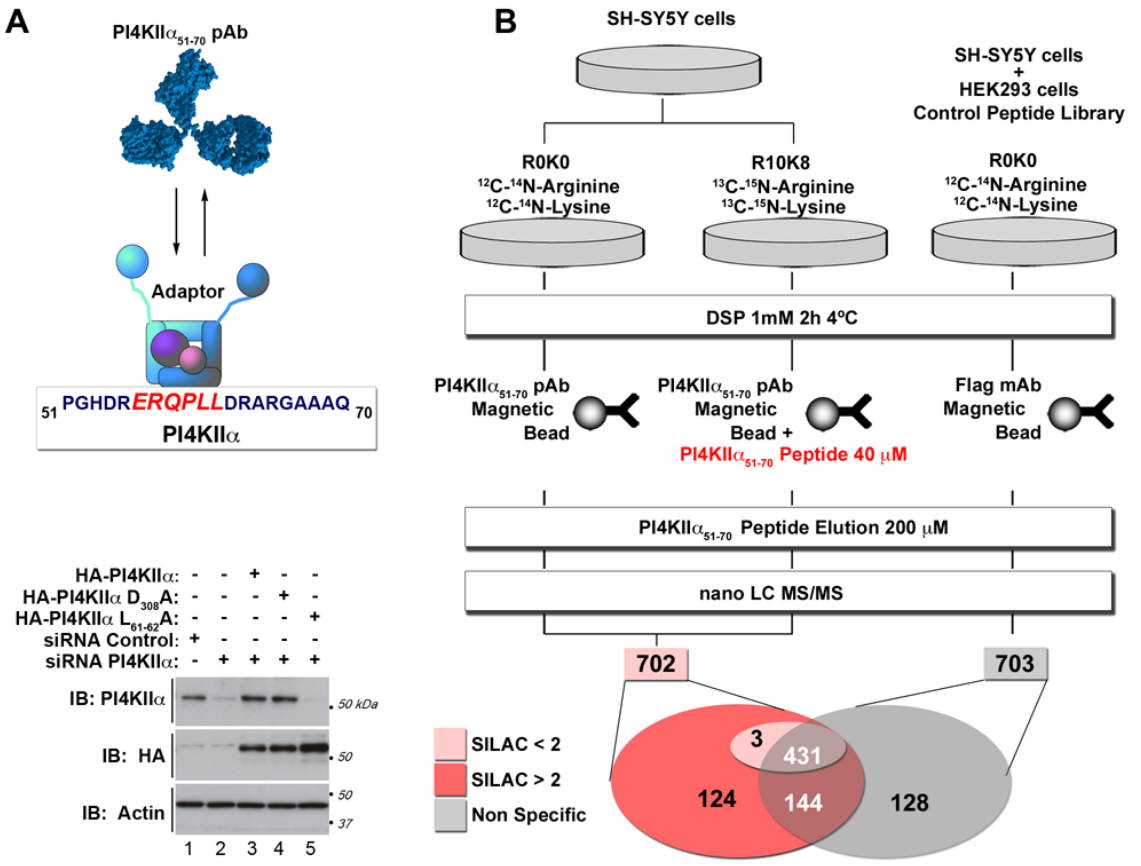

Fig S1

Supplementary Figure 1. (A) The PI4KIIα antibody specifically binds to the dileucine sorting signal in PI4KIIα. A polyclonal antibody was raised against residues 51-70 of human PI4KIIα, a region that encodes an AP-3-BLOC-1 interaction-based sorting signal. HEK293T cells were treated with non-targeting siRNA (lane 1) or siRNA directed against PI4KIIα (lanes 2-5). Cells were rescued by expression of HA-PI4KIIα (lane 3), a mutant lacking the ERQPLL sorting signal (lane 4), or a mutant in the ATP binding site (lane 5). Cell extracts were resolved by SDS-PAGE and analyzed by immunoblot with the indicated antibodies. Note that the dileucine sorting signal mutant PI4KIIα is not recognized by the PI4KIIα polyclonal antibody. (B) Diagram for the SILAC experimental design: SH-SY5Y neuroblastoma cells were isotopically labeled with either "light" (R0K0) or "heavy" (R10K8) media followed by *in vivo* chemical crosslinking using DSP. Detergent soluble lysates were then immunoprecipitated either using immunomagnetic beads decorated with the PI4KIIα antibody (R0K0 lysates) or with immunomagnetic beads decorated with the PI4KIIα antibody in the presence of the antigenic peptide (R10K8 lysates; outcompetition control). PI4KIIα interacting protein complexes were eluted from the beads using the PI4KIIα peptide (immunoaffinity purification). The samples were then combined at a 1:1 ratio and analyzed by nano LS MS/MS. Peptides whose

labeling with heavy and light isotopes differed by a factor of 2 or more were considered. As an additional control the entire experiment was repeated with SH-SY5Y cells labeled with R0K0 media to identify cellular proteins that non-specifically bound to the immunomagnetic beads decorated with an irrelevant antibody. The Venn diagram represents an overall profile of the number of proteins identified in the experiment after each curation of the dataset.

Supplementary Table 1. SILAC Mass spectrometry Data for PI4KIIα Interactors.

	IPI Accession Number	Gene Name	Protein Name	Total Spectra	Ratio H/L	Ratio H/L Significance	Quantitation Events Used to Calculate Ratio
1	IPI00395605;]	ARHGEF1	Rho guanine nucleotide exchange factor	626	0.015789	0.049982	357
2	IPI00020124;]	PI4K2A	Phosphatidylinositol 4-kinase type 2-al	220	0.018091	0.07883	112
3	IPI00640957;]	CDC42BPA	Serine/threonine-protein kinase MRCK	183	0.068854	0.38818	78
4	IPI00913924;]	LRRC15	Leucine-rich repeat-containing protein	142	0.011507	0.01156	78
5	IPI00024067;]	CLTC	Clathrin heavy chain 1;CLH-17	142	0.020299	0.086607	88
6	IPI00513791;]	DOCK7	Dedicator of cytokinesis protein 7	114	0.051258	0.38885	51
7	IPI00472160;]	ARHGEF2	Rho guanine nucleotide exchange factor	103	0.031229	0.23358	54
8	IPI00853400;]	FKBP15	FK506-binding protein 15	81	0.046855	0.38908	29
9	IPI00029111;]	DPYSL3	Collapsin response mediator protein 4 l	80	0.022409	0.13378	59
10	IPI00007797;]	FABP5	Fatty acid-binding protein, epidermal;E	65	0.046996	0.33144	36
11	IPI00215746;]	FABP4	Fatty acid-binding protein, adipocyte;A-	64	0.014672	0.027979	42
12	IPI00019502;]	MYH9	Myosin-9;Myosin heavy chain 9;Myosin	58	0.038189	0.36472	36
13	IPI00913848;]	FERMT2	cDNA FLJ77570, highly similar to Homc	53	0.068265	0.38983	27
14	IPI00748472;]	KIAA0196	Protein KIAA0196 variant;Putative uncl	48	0.096929	0.31267	23
15	IPI00023555;]	OSBPL3	Oxysterol-binding protein-related prote	47	0.053418	0.48843	29
16	IPI00410103;]	KCTD3	BTB/POZ domain-containing protein K(	45	0.12888	0.18178	17
17	IPI00012303;]	SELENBP1	Selenium binding protein 1;Selenium-b	44	0.053655	0.45931	19
18	IPI00376274;]	VSIG8	V-set and immunoglobulin domain-cont	44	0.021638	0.091394	28
19	IPI00456969;]	DYNC1H1	Cytoplasmic dynein 1 heavy chain 1;Cyt	44	0.036736	0.20587	20
20	IPI00013031	DUSP14	Dual specificity protein phosphatase 14	40	0.047603	0.38116	25
21	IPI00000874;]	PRDX1	Peroxiredoxin-1;Thioredoxin peroxidas	40	0.023752	0.11552	19
22	IPI00301263;]	CAD	CAD protein;Glutamine-dependent carb	40	0.032762	0.23868	24
23	IPI00299063	STIM1	Stromal interaction molecule 1	39	0.035984	0.28998	19
24	IPI00012837;]	KIF5B	Kinesin-1 heavy chain;Ubiquitous kines	31	0.063104	0.41603	15

25	IPI00925719;|AMDHD2	Putative N-acetylglucosamine-6-phospl	30	0.07338	0.22321	21
26	IPI00034049;|UPF1	Regulator of nonsense transcripts 1;ATI	28	0.031236	0.2022	19
27	IPI00470610;|PYCR2	Pyrroline-5-carboxylate reductase 2;cD	28	0.025846	0.13323	19
28	IPI00419237;|LAP3	Cytosol aminopeptidase;Leucine amino	26	0.063471	0.4722	8
29	IPI00031618;|DDI1	Protein DDI1 homolog 2	26	0.02053	0.20527	17
30	IPI00011676;|WASL	Neural Wiskott-Aldrich syndrome prote	25	0.062313	0.44758	12
31	IPI00166010;|CNOT1	CCR4-NOT transcription complex subur	24	0.12144	0.16184	6
32	IPI00163403;|SHKBP1	SH3KBP1-binding protein 1;cDNA FLJ5	23	0.09413	0.30813	12
33	IPI00013452;|EPRS	Bifunctional aminoacyl-tRNA synthetas	23	0.040211	0.33344	13
34	IPI00021369;|CRYAB	Alpha-crystallin B chain;Alpha(B)-cryst	22	0.052993	0.43494	9
35	IPI00017283 IARS2	Isoleucyl-tRNA synthetase, mitochondr	22	0.13036	0.15013	10
36	IPI00739343;|RTL1	Retrotransposon-like protein 1;Retrotra	22	0.025438	0.15254	11
37	IPI00216984 CALML3	Calmodulin-like protein 3;Calmodulin-r	21	0.037428	0.27216	18
38	IPI00009305;|GNPDA1	Glucosamine-6-phosphate isomerase 1;	21	0.025731	0.21143	13
39	IPI00289819;|IGF2R	Cation-independent mannose-6-phospl	20	0.092354	0.24976	6
40	IPI00550069 RNH1	Ribonuclease inhibitor;Ribonuclease/a	18	0.036062	0.22594	11
41	IPI00412650;|SVIL	Supervillin;Archvillin;p205/p250	18	0.14948	0.074247	5
42	IPI00033022;|DNM2	Dynamin-2;cDNA FLJ40556 fis, clone TI	17	0.046838	0.47444	13
43	IPI00103242;|POF1B	Protein POF1B;Premature ovarian failu	16	0.13053	0.24154	8
44	IPI00298991;|KIAA1033	UPF0681 protein KIAA1033	16	0.040973	0.34741	8
45	IPI00746934;|UBR4	E3 ubiquitin-protein ligase UBR4;N-rec	15	0.072683	0.47835	6
46	IPI00026513 RPIA	Ribose-5-phosphate isomerase;Phosph	13	0.019839	0.30756	9
47	IPI00384265;|FAM120A	Constitutive coactivator of PPAR-gamm	13	0.13592	0.204	7
48	IPI00001466;|EML4	Echinoderm microtubule-associated pr	13	0.12733	0.17974	8
49	IPI00903062;|CYFIP1	cDNA FLJ43948 fis, clone TESTI401492	13	0.13736	0.20172	4
50	IPI00307259;|DNAJC13	DnaJ homolog subfamily C member 13;I	13	0.10585	0.20873	5
51	IPI00032388;|LPIN1	Lipin-1;cDNA FLJ51642, highly similar t	13	0.092624	0.25834	6
52	IPI00465431;|LGALS3	Galectin-3;Galactose-specific lectin 3;M	13	0.027336	0.40628	3
53	IPI00019329;|DYNLL1	Dynein light chain 1, cytoplasmic;Dynei	12	0.25572	0.073971	5
54	IPI00304082;|ISOC1	Isochorismatase domain-containing pro	12	0.017121	0.24377	7

55	IPI00328257;	AP1B1	AP-1 complex subunit beta-1;Adapter-r	12	0.078142	0.40903	5
56	IPI00383581;	GANAB	Neutral alpha-glucosidase AB;Glucosida	12	0.043672	0.43235	9
57	IPI00884375;	FAM21B	Protein FAM21B;Protein FAM21A;cDNA	12	0.16688	0.093232	5
58	IPI00026089;	SF3B1	Splicing factor 3B subunit 1;Pre-mRNA-	12	0.056146	0.48223	9
59	IPI00376317;	EDC4	Enhancer of mRNA-decapping protein 4	12	0.032734	0.19751	3
60	IPI00554521;	FTH1	Ferritin heavy chain;Cell proliferation-i	11	0.24464	0.061452	10
61	IPI00301434;	BOLA2	BolA-like protein 2	11	0.047123	0.49755	7
62	IPI00173359;	UACA	Uveal autoantigen with coiled-coil dom	11	0.2075	0.055667	3
63	IPI00020599	CALR	Calreticulin;CRP55;Calregulin;HACBP;E	10	0.10627	0.18624	4
64	IPI00293276	MIF	Macrophage migration inhibitory factor	9	0.029117	0.16864	7
65	IPI00026358	GABARAPL2	Gamma-aminobutyric acid receptor-ass	9	0.40113	0.024308	3
66	IPI00031410;	FRAP	Serine/threonine-protein kinase mTOR	9	0.11325	0.35277	3
67	IPI00473136;	CTNNA1	Catenin alpha-1;Cadherin-associated pr	8	0.10679	0.2864	4
68	IPI00306301;	PDHA1	cDNA FLJ59461, highly similar to Pyruv	7	0.19981	0.063463	7
69	IPI00028561;	KIF5C	Kinesin heavy chain isoform 5C;Kinesin	7	0.25265	0.045038	3
70	IPI00419433;	RAB11FIP1	Rab11 family-interacting protein 1;Rab	7	0.12836	0.2233	5
71	IPI00294610;	DNAJA3	DnaJ homolog subfamily A member 3, n	7	0.021694	0.28045	5
72	IPI00383439;	CEP97	Centrosomal protein of 97 kDa;Leucine	7	2.5387	2.97E-05	2
73	IPI00783118;	NT5DC2	5'-nucleotidase domain-containing prot	7	0.084215	0.19069	4
74	IPI00880007;	MAP4	Microtubule-associated protein 4;Putat	7	0.077275	0.31593	2
75	IPI00013079;	EMILIN1	EMILIN-1;Elastin microfibril interface-l	7	0.042758	0.29468	6
76	IPI00021338;	DLAT	Dihydrolipoyllysine-residue acetyltrans	6	0.19679	0.068274	6
77	IPI00105598;	PSMD11	26S proteasome non-ATPase regulatory	6	0.10286	0.30189	5
78	IPI00005198;	ILF2	Interleukin enhancer-binding factor 2;N	6	0.084667	0.21247	6
79	IPI00795611;	GIT1	cDNA FLJ59232, highly similar to ARF G	6	0.26807	0.034977	2
80	IPI00216088;	CRABP2	Cellular retinoic acid-binding protein 2;	6	0.030243	0.14734	4
81	IPI00018206;	GOT2	Aspartate aminotransferase, mitochond	6	0.046199	0.40828	6
82	IPI00449049;	PARP1	Poly [ADP-ribose] polymerase 1;ADPRT	6	0.067997	0.42804	5
83	IPI00409684;	NCKAP1	Nck-associated protein 1;p125Nap1;Me	6	0.17777	0.10635	2
84	IPI00182757;	KIAA1967	Protein KIAA1967;Deleted in breast car	6	0.038493	0.28696	5

85	IPI00477763;	CDC42BPB	Serine/threonine-protein kinase MRCK	6	0.09393	0.29016	3
86	IPI00014456;	STRN	Striatin	5	0.080326	0.30018	3
87	IPI00783097;	GARS	Glycyl-tRNA synthetase;Glycine--tRNA l	5	0.11735	0.23854	3
88	IPI00788907;	PGAM5	Phosphoglycerate mutase family memb	5	0.060166	0.48553	5
89	IPI00009922	C14orf156	SRA stem-loop-interacting RNA-binding	5	0.031565	0.1823	3
90	IPI00011528;	CSTF1	Cleavage stimulation factor 50 kDa subu	5	0.01226	0.041963	2
91	IPI00023673;	LGALS3BP	Galectin-3-binding protein;Lectin galact	5	0.016594	0.067916	4
92	IPI00024097;	TES	Testin;TESS	5	0.065326	0.31923	4
93	IPI00017184;	EHD1	EH domain-containing protein 1;Testili	5	0.035863	0.48195	3
94	IPI00016613;	CK2A1	cDNA, FLJ92904, highly similar to Hom	5	0.043081	0.45592	4
95	IPI00216008;	G6PD	Glucose-6-phosphate 1-dehydrogenase	5	0.03995	0.46886	2
96	IPI00021129;	AP3B1	AP-3 complex subunit beta-1;Adapter-r	5	0.20417	0.093231	2
97	IPI00015195;	CSTF3	Cleavage stimulation factor 77 kDa subu	4	0.036701	0.29881	3
98	IPI00011770;	NDUFA4	NADH dehydrogenase [ubiquinone] 1 a	4	0.26705	0.069069	4
99	IPI00015911;	DLD	Dihydrolipoyl dehydrogenase, mitochor	4	0.11477	0.16169	4
100	IPI00329748;	C17orf59	Uncharacterized protein C17orf59	4	0.079908	0.28018	2
101	IPI00001539;	ACAA2	3-ketoacyl-CoA thiolase, mitochondrial;	4	0.048637	0.47944	4
102	IPI00008832	GAS1	Growth arrest-specific protein 1	4	0.012145	0.10785	2
103	IPI00023591	PURA	Transcriptional activator protein Pur-al	4	0.033985	0.3434	4
104	IPI00455348;	KCTD2	BTB/POZ domain-containing protein K(	3	0.60351	0.00079678	2
105	IPI00009322;	NEDD4	E3 ubiquitin-protein ligase NEDD4;Neu	3	0.27047	0.034247	3
106	IPI00420108;	DLST	Dihydrolipoyllysine-residue succinyltra	3	0.10445	0.1536	3
107	IPI00295004;	ALS2	Alsin;Amyotrophic lateral sclerosis prot	3	0.16357	0.15007	2
108	IPI00305438;	RP11-12M19.	Vacuolar protein sorting-associated pro	3	0.1144	0.18356	2
109	IPI00550689;	C22orf28	UPF0027 protein C22orf28;cDNA FLJ5E	3	0.052575	0.35506	3
110	IPI00012442;	G3BP1	Ras GTPase-activating protein-binding	3	0.022061	0.24928	2
111	IPI00926977;	PSMC6	26S protease regulatory subunit S10B;F	3	0.044076	0.42131	3
112	IPI00069750;	PUF60	Poly(U)-binding-splicing factor PUF60;(	2	0.53153	0.0080325	2
113	IPI00166144	LYG1	Lysozyme g-like protein 1	2	0.1979	0.11675	2
114	IPI00030255;	PLOD3	Procollagen-lysine,2-oxoglutarate 5-dio	2	0.067564	0.39028	2

115	IPI00297452;	TFG	Tyrosine-protein kinase receptor;Prote	2	0.086704	0.26637	2
116	IPI00964675;	BCKDHA	cDNA FLJ45695 fis, clone FEBRA20135	2	0.088804	0.24348	2
117	IPI00300074	FARSB	Phenylalanyl-tRNA synthetase beta cha	2	0.094358	0.28254	2
118	IPI00179473;	SQSTM1	Sequestosome-1;Phosphotyrosine-inde	2	0.067299	0.37646	2
119	IPI00470573;	ACTR2	cDNA FLJ51656, highly similar to Actin-	4	0.027794	0.25176	3
120	IPI00005969;	CAPZA1	F-actin-capping protein subunit alpha-1	9	0.023473	0.12956	7
121	IPI00218782;	CAPZB	Capping protein (Actin filament) muscl	9	0.053118	0.42312	5
122	IPI00028091;	ACTR3	Actin-related protein 3;Actin-like protei	8	0.11663	0.13665	5
123	IPI00005161;	ARPC2	Actin-related protein 2/3 complex subu	5	0.040495	0.31901	5

Supplementary Table 2. Functional Ontology Terms Enriched in the PI4KIIα Interactome.

Category	Term	Count	%	PValue	Genes
GOTERM_CC_FAT	cytoskeleton	30	1.697	1.62E-07	CEP97, ALS2, CAPZA1, FERMT2, STRN, CAPZB, ACTR3, ACTR2, DYNLL1, ARPC2, DYNC1H1, DNAJA3, GIT1, ARHGEF2, KIF5B, CRYAB, KIF5C, UBR4, DPYSL3, MYH9, CTNNA1, EML4, UACA, G6PD, SVIL, MAP4, FKBP15, WASL, DNM2, CDC42BPB
GOTERM_CC_FAT	actin cytoskeleton	13	0.735	7.67E-07	CRYAB, FERMT2, CAPZA1, CTNNA1, MYH9, CAPZB, ACTR3, ACTR2, ARPC2, SVIL, FKBP15, WASL, DNAJA3
GOTERM_CC_FAT	cytoskeletal part	23	1.301	1.58E-06	ALS2, CEP97, ARHGEF2, KIF5B, CRYAB, CAPZA1, FERMT2, KIF5C, STRN, MYH9, CAPZB, EML4, ACTR3, ACTR2, G6PD, DYNLL1, ARPC2, SVIL, MAP4, FKBP15, DYNC1H1, DNAJA3, DNM2
GOTERM_MF_FAT	cytoskeletal protein binding	17	0.962	9.09E-07	GABARAPL2, ARHGEF2, KIF5B, CRYAB, CAPZA1, C22ORF28, MYH9, CTNNA1, CAPZB, ACTR3, ACTR2, ARPC2, SVIL, CYFIP1, POF1B, WASL, DNM2
GOTERM_CC_FAT	cell leading edge	9	0.509	8.98E-06	ALS2, ACTR3, ARPC2, CDC42BPA, CYFIP1, WASL, MYH9, CAPZB, CDC42BPB
GOTERM_MF_FAT	carboxylic acid binding	9	0.509	1.19E-05	DLST, NEDD4, PLOD3, CRABP2, DLD, FABP4, CAD, DLAT, FABP5
GOTERM_CC_FAT	cell projection	17	0.962	5.71E-05	ALS2, KIF5B, KIF5C, STRN, DOCK7, DPYSL3, MYH9, CAPZB, NCKAP1, ACTR3, ACTR2, ARPC2, SVIL, DLD, FKBP15, CYFIP1, WASL

GOTERM_CC_FAT	mitochondrial matrix	10	0.566	5.09E-05	GOT2, BCKDHA, DLST, DLD, GARS, KIAA1967, PDHA1, IARS2, DLAT, DNAJA3
GOTERM_CC_FAT	mitochondrial lumen	10	0.566	5.09E-05	GOT2, BCKDHA, DLST, DLD, GARS, KIAA1967, PDHA1, IARS2, DLAT, DNAJA3
GOTERM_MF_FAT	enzyme binding	15	0.848	3.21E-05	ALS2, GABARAPL2, ARHGEF2, STRN, CAD, DOCK7, FTH1, SQSTM1, NEDD4, IGF2R, CYFIP1, DNAJA3, DNM2, AP3B1, CDC42BPB
GOTERM_BP_FAT	actin cytoskeleton organization	11	0.622	7.07E-06	ARHGEF2, DYNLL1, FERMT2, CAPZA1, CDC42BPA, WASL, MYH9, CALR, LPIN1, CAPZB, CDC42BPB
GOTERM_BP_FAT	actin filament-based process	11	0.622	1.24E-05	ARHGEF2, DYNLL1, FERMT2, CAPZA1, CDC42BPA, WASL, MYH9, CALR, LPIN1, CAPZB, CDC42BPB
GOTERM_BP_FAT	cytoskeleton organization	15	0.848	4.20E-06	ARHGEF2, CRYAB, FERMT2, CAPZA1, DOCK7, MYH9, CALR, CAPZB, LPIN1, DYNLL1, SVIL, CDC42BPA, WASL, DYNC1H1, CDC42BPB
GOTERM_CC_FAT	cytosol	23	1.301	2.78E-04	ALS2, GABARAPL2, ARHGEF2, AP1B1, G3BP1, GARS, EPRS, SELENBP1, CAD, MYH9, CALR, FTH1, UACA, G6PD, DYNLL1, PSMD11, SQSTM1, NEDD4, FARSB, FABP4, RPIA, DYNC1H1, DNAJA3

GOTERM_CC_FAT	non-membrane-bounded organelle	35	1.980	4.03E-04	CEP97, ALS2, CAPZA1, FERMT2, STRN, CAPZB, ACTR3, ACTR2, DYNLL1, ARPC2, DYNC1H1, DNAJA3, GIT1, ARHGEF2, UPF1, KIF5B, CRYAB, KIF5C, UBR4, DPYSL3, MYH9, CTNNA1, EML4, PURA, UACA, G6PD, ILF2, NEDD4, SVIL, MAP4, FKBP15, WASL, PARP1, CDC42BPB, DNM2
GOTERM_CC_FAT	intracellular non-membrane-bounded organelle	35	1.980	4.03E-04	CEP97, ALS2, CAPZA1, FERMT2, STRN, CAPZB, ACTR3, ACTR2, DYNLL1, ARPC2, DYNC1H1, DNAJA3, GIT1, ARHGEF2, UPF1, KIF5B, CRYAB, KIF5C, UBR4, DPYSL3, MYH9, CTNNA1, EML4, PURA, UACA, G6PD, ILF2, NEDD4, SVIL, MAP4, FKBP15, WASL, PARP1, CDC42BPB, DNM2
GOTERM_CC_FAT	cell junction	13	0.735	4.86E-04	GIT1, ARHGEF2, FERMT2, STRN, CTNNA1, MYH9, ACTR3, ARPC2, CDC42BPA, CYFIP1, DNM2, CDC42BPB, TES
GOTERM_CC_FAT	microtubule cytoskeleton	13	0.735	8.12E-04	ALS2, CEP97, ARHGEF2, KIF5B, CRYAB, KIF5C, MYH9, EML4, G6PD, DYNLL1, MAP4, DYNC1H1, DNM2
GOTERM_CC_FAT	plasma membrane part	30	1.697	1.20E-03	AP1B1, FERMT2, STRN, CALR, CLTC, SHKBP1, ACTR3, ARPC2, EHD1, AP3B1, TES, GIT1, ARHGEF2, STIM1, KCTD3, KCTD2, GAS1, MYH9, CTNNA1, NCKAP1, G6PD, FAM120A, NEDD4, IGF2R, SVIL, PI4K2A, CDC42BPA, CYFIP1, DNM2, CDC42BPB
GOTERM_CC_FAT	lamellipodium	5	0.283	1.88E-03	ALS2, ACTR3, CYFIP1, WASL, CAPZB

GOTERM_CC_FAT	internal side of plasma membrane	9	0.509	2.57E-03	G6PD, FAM120A, AP1B1, SVIL, CLTC, EHD1, CTNNA1, NCKAP1, AP3B1
GOTERM_MF_FAT	actin binding	10	0.566	7.30E-04	ACTR3, ACTR2, ARPC2, SVIL, CAPZA1, POF1B, CYFIP1, WASL, MYH9, CAPZB
GOTERM_CC_FAT	microtubule	8	0.452	4.53E-03	ARHGEF2, KIF5B, DYNLL1, KIF5C, MAP4, DYNC1H1, EML4, DNM2
GOTERM_CC_FAT	mitochondrial part	12	0.679	4.93E-03	NDUFA4, GOT2, BCKDHA, DLST, ACAA2, DLD, GARS, KIAA1967, PDHA1, IARS2, DLAT, DNAJA3
GOTERM_BP_FAT	intracellular transport	15	0.848	3.61E-04	ALS2, GABARAPL2, ARHGEF2, UPF1, KIF5B, AP1B1, MYH9, CALR, CLTC, FTH1, SQSTM1, NEDD4, EHD1, DNM2, AP3B1
GOTERM_CC_FAT	adherens junction	6	0.339	6.20E-03	GIT1, ARPC2, FERMT2, MYH9, CTNNA1, TES
GOTERM_CC_FAT	microtubule associated complex	5	0.283	7.25E-03	KIF5B, DYNLL1, KIF5C, MAP4, DYNC1H1
GOTERM_BP_FAT	regulation of protein complex disassembly	5	0.283	5.59E-04	ARHGEF2, UPF1, CAPZA1, MAP4, CAPZB
GOTERM_MF_FAT	microtubule binding	5	0.283	2.04E-03	GABARAPL2, ARHGEF2, KIF5B, CRYAB, DNM2
GOTERM_CC_FAT	cell-substrate junction	5	0.283	1.00E-02	GIT1, ACTR3, ARPC2, FERMT2, TES
GOTERM_CC_FAT	anchoring junction	6	0.339	9.51E-03	GIT1, ARPC2, FERMT2, MYH9, CTNNA1, TES
GOTERM_MF_FAT	Ras GTPase binding	5	0.283	4.77E-03	ALS2, ARHGEF2, CYFIP1, DOCK7, CDC42BPB
GOTERM_CC_FAT	mitochondrion	16	0.905	1.52E-02	BCKDHA, NDUFA4, DLST, ACAA2, GARS, KIAA1967, C14ORF156, DLAT, IARS2, CLTC, PRDX1, LAP3, GOT2, DLD, PDHA1, DNAJA3

GOTERM_MF_FAT	small GTPase regulator activity	8	0.452	4.41E-03	ALS2, GIT1, ARHGEF2, ARHGEF1, CDC42BPA, WASL, DNAJA3, CDC42BPB
GOTERM_BP_FAT	carbohydrate catabolic process	6	0.339	1.33E-03	GNPDA1, G6PD, LYG1, PDHA1, RPIA, DLAT
GOTERM_BP_FAT	vesicle-mediated transport	13	0.735	1.19E-03	ALS2, GABARAPL2, AP1B1, GARS, CLTC, FTH1, SQSTM1, NEDD4, IGF2R, PI4K2A, EHD1, DNM2, AP3B1
GOTERM_MF_FAT	small GTPase binding	5	0.283	6.88E-03	ALS2, ARHGEF2, CYFIP1, DOCK7, CDC42BPB
GOTERM_MF_FAT	motor activity	6	0.339	4.20E-03	KIF5B, DYNLL1, KIF5C, MYH9, DYNC1H1, DNM2
GOTERM_BP_FAT	cell projection organization	10	0.566	1.71E-03	ALS2, NEDD4, KIF5C, STRN, CYFIP1, DOCK7, GAS1, MYH9, LPIN1, CAPZB
GOTERM_BP_FAT	monosaccharide catabolic process	5	0.283	1.94E-03	GNPDA1, G6PD, PDHA1, RPIA, DLAT
GOTERM_MF_FAT	tubulin binding	5	0.283	6.64E-03	GABARAPL2, ARHGEF2, KIF5B, CRYAB, DNM2
GOTERM_MF_FAT	nucleotide binding	27	1.527	1.23E-02	TFG, CAD, IARS2, ACTR3, ACTR2, EHD1, DYNC1H1, UPF1, KIF5B, KIF5C, G3BP1, GARS, EPRS, C14ORF156, DOCK7, MYH9, PSMC6, G6PD, ILF2, PI4K2A, DLD, FARSB, CDC42BPA, PARP1, PUF60, DNM2, CDC42BPB
GOTERM_MF_FAT	nucleoside binding	21	1.188	1.47E-02	UPF1, KIF5B, G3BP1, KIF5C, GARS, EPRS, TFG, CAD, IARS2, MYH9, ACTR3, ACTR2, PSMC6, ILF2, PI4K2A, DLD, FARSB, CDC42BPA, DYNC1H1, EHD1, CDC42BPB

GOTERM_MF_FAT	adenyl ribonucleotide binding	20	1.131	1.40E-02	UPF1, KIF5B, G3BP1, KIF5C, GARS, EPRS, TFG, CAD, IARS2, MYH9, ACTR3, ACTR2, PSMC6, ILF2, PI4K2A, FARSB, CDC42BPA, DYNC1H1, EHD1, CDC42BPB
GOTERM_MF_FAT	purine nucleoside binding	21	1.188	1.37E-02	UPF1, KIF5B, G3BP1, KIF5C, GARS, EPRS, TFG, CAD, IARS2, MYH9, ACTR3, ACTR2, PSMC6, ILF2, PI4K2A, DLD, FARSB, CDC42BPA, DYNC1H1, EHD1, CDC42BPB
GOTERM_CC_FAT	plasma membrane	39	2.206	2.22E-02	AP1B1, FERMT2, STRN, CALR, CLTC, FTH1, SHKBP1, GOT2, ACTR3, DYNLL1, ARPC2, EHD1, AP3B1, TES, GIT1, DLST, ARHGEF2, ARHGEF1, LGALS3, CRYAB, G3BP1, STIM1, KCTD3, DPYSL3, KCTD2, GAS1, MYH9, CTNNA1, NCKAP1, G6PD, FAM120A, NEDD4, IGF2R, SVIL, PI4K2A, CDC42BPA, CYFIP1, CDC42BPB, DNM2
GOTERM_MF_FAT	GTPase binding	5	0.283	8.96E-03	ALS2, ARHGEF2, CYFIP1, DOCK7, CDC42BPB
GOTERM_MF_FAT	ATP binding	20	1.131	1.22E-02	UPF1, KIF5B, G3BP1, KIF5C, GARS, EPRS, TFG, CAD, IARS2, MYH9, ACTR3, ACTR2, PSMC6, ILF2, PI4K2A, FARSB, CDC42BPA, DYNC1H1, EHD1, CDC42BPB
GOTERM_CC_FAT	cytoplasmic vesicle	11	0.622	2.21E-02	GANAB, AP1B1, IGF2R, DLD, PI4K2A, GARS, CLTC, EHD1, RAB11FIP1, PRDX1, AP3B1
GOTERM_MF_FAT	nucleoside-triphosphatase regulator activity	9	0.509	1.20E-02	ALS2, GIT1, ARHGEF2, ARHGEF1, CDC42BPA, DOCK7, WASL, DNAJA3, CDC42BPB

GOTERM_MF_FAT	GTPase regulator activity	9	0.509	1.07E-02	ALS2, GIT1, ARHGEF2, ARHGEF1, CDC42BPA, DOCK7, WASL, DNAJA3, CDC42BPB
GOTERM_MF_FAT	adenyl nucleotide binding	21	1.188	1.17E-02	UPF1, KIF5B, G3BP1, KIF5C, GARS, EPRS, TFG, CAD, IARS2, MYH9, ACTR3, ACTR2, PSMC6, ILF2, PI4K2A, DLD, FARSB, CDC42BPA, DYNC1H1, EHD1, CDC42BPB
GOTERM_BP_FAT	microtubule-based process	8	0.452	2.85E-03	KIF5B, DYNLL1, CRYAB, KIF5C, DOCK7, MYH9, DYNC1H1, EML4
GOTERM_BP_FAT	alcohol catabolic process	5	0.283	3.14E-03	GNPDA1, G6PD, PDHA1, RPIA, DLAT
GOTERM_BP_FAT	cellular carbohydrate catabolic process	5	0.283	3.73E-03	GNPDA1, G6PD, PDHA1, RPIA, DLAT
GOTERM_BP_FAT	regulation of cytoskeleton organization	6	0.339	3.49E-03	ACTR3, ARHGEF2, ARPC2, CAPZA1, MAP4, CAPZB
GOTERM_CC_FAT	vesicle	11	0.622	2.86E-02	GANAB, AP1B1, IGF2R, DLD, PI4K2A, GARS, CLTC, EHD1, RAB11FIP1, PRDX1, AP3B1
GOTERM_CC_FAT	endosome	7	0.396	3.12E-02	ALS2, SQSTM1, IGF2R, PI4K2A, FKBP15, EHD1, RAB11FIP1
GOTERM_CC_FAT	cell fraction	15	0.848	3.11E-02	ALS2, DLST, GABARAPL2, CRYAB, GARS, EPRS, CALR, UACA, DYNLL1, NEDD4, IGF2R, PI4K2A, FARSB, CYFIP1, FABP4
GOTERM_MF_FAT	purine nucleotide binding	23	1.301	2.42E-02	UPF1, KIF5B, G3BP1, KIF5C, GARS, EPRS, TFG, DOCK7, CAD, IARS2, MYH9, ACTR3, ACTR2, PSMC6, ILF2, PI4K2A, DLD, FARSB, CDC42BPA, DYNC1H1, EHD1, CDC42BPB, DNM2

GOTERM_BP_FAT	cell morphogenesis	9	0.509	5.13E-03	ALS2, ARHGEF2, KIF5C, CYFIP1, C22ORF28, DOCK7, GAS1, MYH9, CDC42BPB
GOTERM_MF_FAT	purine ribonucleotide binding	22	1.244	2.85E-02	UPF1, KIF5B, G3BP1, KIF5C, GARS, EPRS, TFG, DOCK7, CAD, IARS2, MYH9, ACTR3, ACTR2, PSMC6, ILF2, PI4K2A, FARSB, CDC42BPA, DYNC1H1, EHD1, CDC42BPB, DNM2
GOTERM_MF_FAT	ribonucleotide binding	22	1.244	2.85E-02	UPF1, KIF5B, G3BP1, KIF5C, GARS, EPRS, TFG, DOCK7, CAD, IARS2, MYH9, ACTR3, ACTR2, PSMC6, ILF2, PI4K2A, FARSB, CDC42BPA, DYNC1H1, EHD1, CDC42BPB, DNM2
GOTERM_CC_FAT	neuron projection	7	0.396	4.38E-02	ALS2, KIF5B, KIF5C, STRN, FKBP15, DPYSL3, DOCK7
GOTERM_BP_FAT	monosaccharide metabolic process	7	0.396	6.35E-03	AMDHD2, GNPDA1, G6PD, CRYAB, PDHA1, RPIA, DLAT
GOTERM_MF_FAT	cofactor binding	6	0.339	3.87E-02	GOT2, DLST, G6PD, DLD, DLAT, PARP1
GOTERM_MF_FAT	RNA binding	11	0.622	4.16E-02	CSTF3, UPF1, FAM120A, ILF2, G3BP1, FARSB, EPRS, C14ORF156, CALR, CSTF1, PUF60
GOTERM_BP_FAT	cell morphogenesis involved in differentiation	7	0.396	9.88E-03	ALS2, KIF5C, CYFIP1, C22ORF28, DOCK7, GAS1, MYH9
GOTERM_MF_FAT	coenzyme binding	5	0.283	4.66E-02	DLST, G6PD, DLD, DLAT, PARP1
GOTERM_BP_FAT	cellular component morphogenesis	9	0.509	9.66E-03	ALS2, ARHGEF2, KIF5C, CYFIP1, C22ORF28, DOCK7, GAS1, MYH9, CDC42BPB
GOTERM_BP_FAT	neuron projection development	7	0.396	1.23E-02	ALS2, NEDD4, KIF5C, STRN, CYFIP1, DOCK7, GAS1
GOTERM_BP_FAT	Golgi vesicle transport	5	0.283	1.67E-02	GABARAPL2, AP1B1, CLTC, FTH1, DNM2

GOTERM_BP_FAT	regulation of cell morphogenesis	5	0.283	1.67E-02	ACTR3, ARHGEF1, FERMT2, CYFIP1, MYH9
GOTERM_BP_FAT	regulation of cellular component size	7	0.396	1.59E-02	ALS2, ACTR3, ARPC2, CRYAB, CAPZA1, CYFIP1, CAPZB
GOTERM_BP_FAT	membrane invagination	6	0.339	2.45E-02	AP1B1, NEDD4, IGF2R, EHD1, DNM2, AP3B1
GOTERM_BP_FAT	endocytosis	6	0.339	2.45E-02	AP1B1, NEDD4, IGF2R, EHD1, DNM2, AP3B1
GOTERM_BP_FAT	cell motion	9	0.509	2.56E-02	ACTR3, ACTR2, ARPC2, KIF5C, CAPZA1, WASL, GAS1, MYH9, CAPZB
GOTERM_BP_FAT	negative regulation of cellular component organization	5	0.283	2.17E-02	ARHGEF2, ARHGEF1, CAPZA1, MAP4, CAPZB
GOTERM_BP_FAT	membrane organization	8	0.452	2.39E-02	AP1B1, NEDD4, IGF2R, CLTC, EHD1, FTH1, DNM2, AP3B1
GOTERM_BP_FAT	regulation of organelle organization	6	0.339	2.32E-02	ACTR3, ARHGEF2, ARPC2, CAPZA1, MAP4, CAPZB
GOTERM_BP_FAT	coenzyme metabolic process	5	0.283	2.76E-02	DLST, G6PD, DLD, RPIA, DLAT
GOTERM_BP_FAT	glucose metabolic process	5	0.283	2.76E-02	G6PD, CRYAB, PDHA1, RPIA, DLAT
GOTERM_BP_FAT	cell death	11	0.622	4.19E-02	ALS2, ARHGEF2, DYNLL1, SQSTM1, KIAA0196, GARS, KIAA1967, GAS1, DNAJA3, PUF60, NCKAP1
GOTERM_BP_FAT	death	11	0.622	4.36E-02	ALS2, ARHGEF2, DYNLL1, SQSTM1, KIAA0196, GARS, KIAA1967, GAS1, DNAJA3, PUF60, NCKAP1
GOTERM_BP_FAT	positive regulation of hydrolase activity	5	0.283	4.50E-02	ALS2, GABARAPL2, UACA, DOCK7, DNAJA3

GOTERM_BP_FAT	regulation of apoptosis	12	0.679	3.72E-02	ARHGEF2, UACA, DYNLL1, SQSTM1, CRYAB, IGF2R, GAS1, CALR, PRDX1, DNAJA3, MIF, DNM2
GOTERM_BP_FAT	neuron development	7	0.396	4.13E-02	ALS2, NEDD4, KIF5C, STRN, CYFIP1, DOCK7, GAS1
GOTERM_BP_FAT	regulation of cell death	12	0.679	4.04E-02	ARHGEF2, UACA, DYNLL1, SQSTM1, CRYAB, IGF2R, GAS1, CALR, PRDX1, DNAJA3, MIF, DNM2
GOTERM_BP_FAT	regulation of programmed cell death	12	0.679	3.95E-02	ARHGEF2, UACA, DYNLL1, SQSTM1, CRYAB, IGF2R, GAS1, CALR, PRDX1, DNAJA3, MIF, DNM2

Supplementary Table 3. Antibodies Used in this Study.

Antigen	*Host species*	*Catalog Number*	*Source*	*Immuno-precipitation*	*Dilutions*	
					Immunoblot	*Immunofluorescence*
A488-conjugated anti-chicken IgG	goat	A-11039	Molecular Probes	-	-	1:1000
A555-conjugated anti-rabbit IgG	goat	A-21428	Molecular Probes	-	-	1:1000
Actin (AC-15)	mouse	A5451	Sigma	-	1:5000	-
AP-3β	rabbit	13384-1-AP	Proteintech Group	-	1:500	-
AP-3δ (SA4)	mouse	-	Developmental Studies Hybridoma Bank	-	1:500	-
ATP7A (Menkes)	mouse	Q04656	NeuroMab	-	1:1000	-
Clathrin heavy chain	mouse	610499	BD Transduction Laboratories	-	1:1000	-
Dock7	rabbit	13000-1-AP	Proteintech Group	-	1:1000	-
Dysbindin	rabbit	HPA029616	Sigma	-	1:500	-
FLAG (M2)	mouse	F3165	Sigma	Yes	1:1000	-
FLAG	rabbit	A190-102A	Bethyl Laboratories	-	1:1000	-

GFP	mouse	A11120	Molecular Probes	Yes	-	-
GFP	rabbit	132002	Synaptic Systems	-	1:2000	-

CHAPTER III

DISCUSSION

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The goal of this dissertation research was to test the hypothesis that the actin cytoskeleton contributes to vesicle biogenesis mechanisms at the early endosome. This principle draws on the conceptual framework provided by the well-defined adaptor protein complex-2, clathrin-coated vesicle biogenesis mechanism at the plasma membrane. In this pathway, the local polymerization of branched actin networks by the Arp2/3 complex contributes to membrane deformation and scission (Gottlieb *et al.*, 1993; Lamaze *et al.*, 1997; Merrifield *et al.*, 2002; Yarar *et al.*, 2005; Boulant *et al.*, 2011). I postulated that actin cytoskeleton dynamics similarly contribute to vesicle biogenesis at the early endosome. I focused my research on two protein complexes mutated in the Hermansky-Pudlak syndrome, the adaptor protein complex-3 (AP-3) and the biogenesis of lysosome-related organelles complex-1 (BLOC-1). My central hypothesis was that:

> **The BLOC-1 complex and its cargo and regulator, the phosphatidylinositol 4-kinase type IIα (PI4KIIα), interact with regulators of the actin cytoskeleton.**

I tested this hypothesis by isolating PI4KIIα protein complexes and identified co-isolating proteins by quantitative mass spectrometry. This approach led to the following research findings, described in detail in Chapter II:

1) The Hermansky-Pudlak syndrome protein complex, BLOC-1, and an associated regulatory lipid kinase, PI4KIIα, co-isolate with actin cytoskeleton regulators. These regulators include guanine exchange factors, such as RhoGEF1 and DOCK7, and subunits of an Arp2/3 activator, the WASH complex.

2) The AP-3 and BLOC-1 complexes genetically interact with both PI4KIIα, the guanine exchange factor RhoGEF1, and a subunit of the WASH complex.
3) Actin filaments, WASH complex subunits, PI4KIIα, and a BLOC-1 complex subunit co-reside at endosomes.
4) Depletion of BLOC-1 or the WASH complex alters PI4KIIα-containing endosomal morphology.
5) Cargoes of the AP-3 and BLOC-1 pathway mis-localize upon depletion of the WASH complex.

In the following sections, I will first summarize my findings and implications for our understanding of the function of the AP-3 and BLOC-1 complexes. In addition, I will discuss novel hypotheses this dissertation research raises, including:

1) PI4KIIα and BLOC-1 concurrently recruit the WASH complex to AP-3-BLOC-1 vesicle biogenesis pathway
2) The Arp2/3- and WASH complex-dependent polymerization of actin regulates the cargo sorting into and/or membrane scission of tubular vesicle intermediates
3) The BLOC-1 complex acts as a tubule-stabilizing factor
4) PI4KIIα and BLOC-1 recruit guanine exchange factors as activators of actin polymerization
5) Disruption to AP-3- and BLOC-1-dependent vesicle biogenesis contributes to the pathogenesis of neurodegenerative diseases

Summary of findings and contributions to the field

In this dissertation, I describe a previously unrecognized participation of the actin cytoskeleton within a vesicle biogenesis pathway defined by the AP-3 and BLOC-1 complexes. I hypothesized that the actin cytoskeleton participates in AP-3- and BLOC-1-dependent vesicle biogenesis by drawing a conceptual parallel with AP-2- and clathrin-dependent vesicle biogenesis at the plasma membrane. In the AP-2-clathrin pathway, actin filaments are locally polymerized during late stages of vesicle biogenesis and participate in membrane deformation and scission (Lamaze *et al.*, 1997; Merrifield *et al.*, 2002; 2005; Yarar *et al.*, 2005; Boulant *et al.*, 2011). Prior to my dissertation research, however, very little was known about the role of the actin cytoskeleton within the AP-3- and BLOC-1 pathway and accepted mechanisms for this vesicle biogenesis pathway did not include roles for actin polymerization (Figure 1).

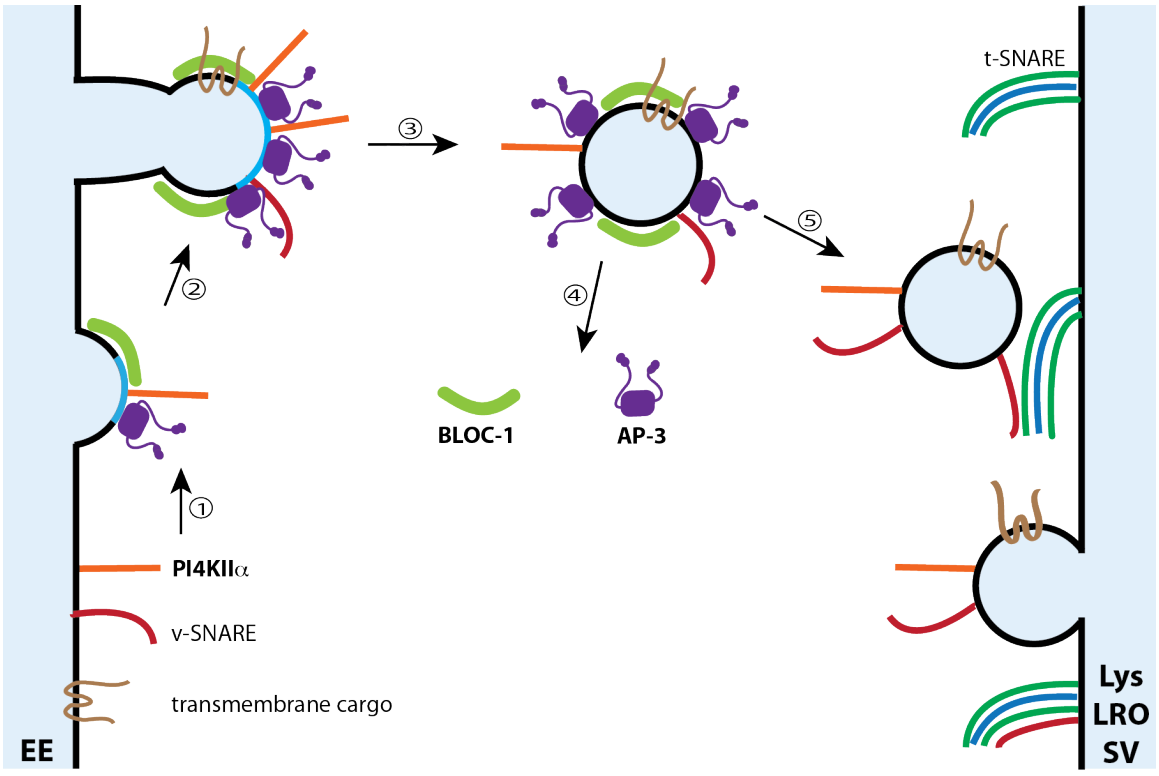

Figure 1. Model of AP-3- and BLOC-1-dependent vesicle biogenesis prior to this dissertation research. The accepted model for the generation of AP- and BLOC-1-coated vesicles prior to this research did not include a role for the actin cytoskeleton. These cytosolic coats are hypothesized to polymerize at early endosomal membranes in order to scaffold proteins required for vesicle biogenesis and to concentrate cargo into nascent vesicles.

However, an initial report described co-isolation and co-localization between the BLOC-1 complex and actin filaments (Falcon-Perez, 2002). Prior to my dissertation research, many unanswered questions remained, including: (1) which actin regulatory proteins interact with the AP-3-BLOC-1 pathway, (2) whether or not the actin cytoskeleton plays a functional role in this sorting pathway, and (3) if components of the AP-3-BLOC-1 pathway regulate actin cytoskeleton dynamics at early endosomal membranes. My work has expanded the conception of AP-3- and BLOC-1-dependent vesicle biogenesis to include regulation of the actin cytoskeleton as a component of this process (Fig. 2). As described in Chapter II, PI4KIIα, a cargo and regulator of this pathway, co-isolates with the WASH complex, an activator of Arp2/3-dependent branched actin polymerization. In other vesicular trafficking pathways, the polymerization of actin nucleated by the WASH complex is suggested to play multiple roles during vesicle biogenesis, including cargo sorting and membrane scission (see Chapter I, Fig. 11).

My data are consistent with a role for the WASH complex during the vesicle biogenesis step of AP-3- and BLOC-1-dependent trafficking at the early endosome, as proposed in Figure 2. First, I detected interactions between the WASH complex and BLOC-1 subunits in addition to the interaction with PI4KIIα. The WASH complex is proposed to polymerize actin at small endosomal tubules to mediate cargo sorting and/or membrane scission (Derivery *et al.*, 2009; Gomez and Billadeau, 2009;

Puthenveedu *et al.*, 2010). Consistent with this proposed role, PI4KIIα is contained within small tubules that co-localize with actin at functionally labeled early endosomes. In addition, these tubules elongate upon WASH complex depletion by shRNA. Finally, two BLOC-1 cargoes, the lysosomal SNARE Vamp7 and the copper transporter ATP7A, mis-localize in WASH complex depletions, supporting a functional interaction between these complexes.

Based on these data, I propose a novel model of AP-3- and BLOC-1-dependent vesicle biogenesis, which is depicted in Figure 2. AP-3 and BLOC-1 are upstream factors required for the formation of small tubules from which vesicles bud. In this model, AP-3 is recruited to the endosomal membrane by activated Arf1-GTP (not depicted) and by binding PI4P generated by PI4KIIα (Fig. 2, Step #1). To date, AP-3 has not been shown to bind PI4P directly, yet this hypothesis is consistent with the model of AP-1 recruitment at the Golgi apparatus and the decreased amount of AP-3 associated with membranes following siRNA-mediated depletion of PI4KIIα (Salazar *et al.*, 2005b; Craige *et al.*, 2008; Ren *et al.*, 2013). Moreover, I suggest that the binding of AP-3 to the dileucine sorting motif in PI4KIIα serves to scaffold and concentrate this enzyme at a vesicle nucleation site, which is consistent with the requirement of this motif for direct interaction between AP-3 and PI4KIIα (Craige *et al.*, 2008). In this dissertation, I identified isolated PI4KIIα from *in vivo* crosslinked cell lysates using an antibody directed against this dileucine sorting motif. This strategy suggests the identified co-isolating proteins interact with PI4KIIα at a distinct step from AP-3 binding, and thus, I propose that BLOC-1 and PI4KIIα concurrently bind and recruit the WASH complex (Fig. 2, Step #2).

Polymerization of actin then plays a functional yet undefined role in either tubule formation, sorting of membrane proteins, vesicle budding, and/or the movement of AP-3-BLOC-1 carriers away from donor compartments. In this model, the WASH complex may act as an effector of RhoA-GTP activated by RhoGEF1 (Fig. 2, Step #3). This hypothesis is supported by the high stoichiometry association of RhoGEF1 with PI4KIIα and the genetic interactions between this factor and PI4KIIα, BLOC-1, and the BLOC-1 cargoes ATP7A and Vamp7 described in Chapter 2.

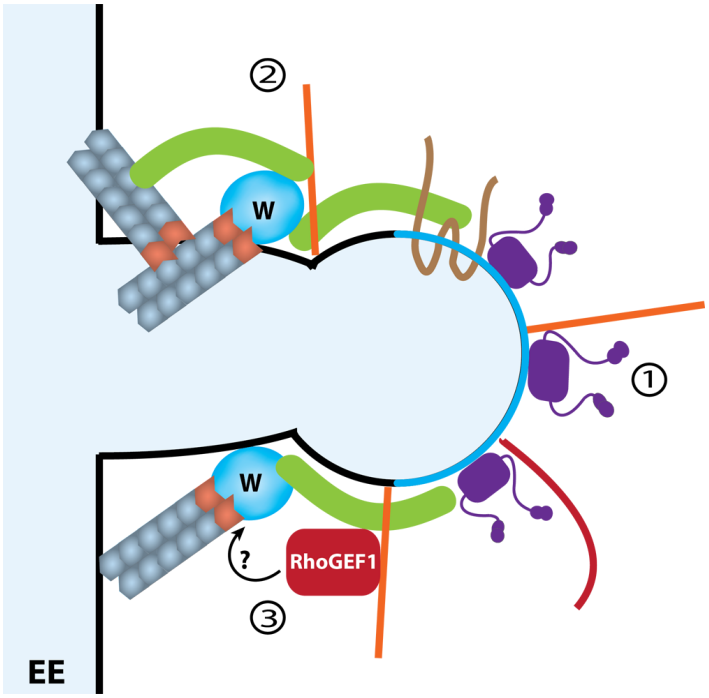

Figure 2. Model for AP-3- and BLOC-1 vesicle biogenesis based on this dissertation research. In this model I propose that the AP-3 complex is recruited to early endosomal membranes by activated Arf1-GTP (not shown) and by binding PI4P synthesized by PI4KIIα (#1). My research suggests that PI4KIIα and the BLOC-1 complex concurrently bind the WASH complex to recruit this actin nucleation-promoting factor to sites of vesicle biogenesis (#2). In addition, PI4KIIα may recruit RhoGEF1, a guanine exchange factor for RhoA, which could then provide an activation signal for the WASH complex (#3 and see Fig. 4).

This model both encompasses the data presented in Chapter II and proposes several new testable hypotheses, including: (1) PI4KIIα and BLOC-1 concurrently

recruit the WASH complex to the AP-3-BLOC-1 vesicle biogenesis pathway, (2) the Arp2/3- and WASH complex-dependent polymerization of actin regulates the cargo sorting into and/or membrane scission of tubular vesicular intermediates, (3) the BLOC-1 complex acts as a tubule stabilizing factor, and (4) PI4KIIα and BLOC-1 recruit guanine exchange factors as activators of actin polymerization to the sites of AP-3-BLOC-1 vesicle biogenesis. In addition, mutations to several of these components are genetically linked to neurodegenerative diseases yet pathogenic mechanisms remain elusive (Simons *et al.*, 2009; Blackstone *et al.*, 2010; Kennerson *et al.*, 2010; Blackstone, 2012; Yi *et al.*, 2012). Thus, another novel hypothesis arising from my dissertation research is that disruption to AP-3- and BLOC-1-dependent vesicle biogenesis contributes to the pathogenesis of neurodegenerative disease. In the next sections I will discuss these five novel hypotheses in detail.

Novel hypothesis #1: PI4KIIα and BLOC-1 concurrently recruit the WASH complex to AP-3-BLOC-1 vesicle biogenesis pathway

As described above and in Chapter II, the unbiased approach I took to identify novel PI4KIIα interactors predominantly identified interactors that bind the population of PI4KIIα that is not bound to AP-3. In addition, I performed *in vivo* crosslinking, a technique our laboratory has carefully calibrated to stabilize only a small fraction of protein interactions and thereby avoid extended protein complexes of biologically meaningless interactions (Salazar *et al.*, 2009; Zlatic *et al.*, 2010). As demonstrated in Chapter II and our other published work, this approach identifies novel interactions which can be genetically confirmed and are functionally relevant (Perez-Cornejo *et al.*, 2012; Gokhale *et al.*, 2012a). However, this technique does not

provide information about the architecture of the identified interactions. For example, components of the PI4KIIα interactome likely form networks of interactions with other co-isolating proteins, and thus, may not directly interact with PI4KIIα. In addition, the crosslinker may stabilize PI4KIIα with proteins that bind to its lipid product, PI4P. Thus, my dissertation research documents a set of functional protein interactions of unknown molecular architecture. Determining the precise architecture of these interactions in the future will provide mechanistic insight into the regulation of vesicle biogenesis at the early endosome.

I focused on the WASH complex in part because a previous report documented an interaction between the WASH protein and the BLOS2 subunit of the BLOC-1 complex (Monfregola *et al.*, 2010). This interaction was demonstrated in part by yeast 2-hybrid analysis, suggesting a direct interaction between these two proteins (Fig. 3, #1). In addition, a subunit of the WASH complex, FAM21, binds to PI4P *in vitro* (Jia *et al.*, 2010), suggesting that PI4KIIα enzymatic activity may contribute a membrane recruitment signal for the WASH complex (Fig. 3, #2). Based upon these previously known interactions and those documented in Chapter II, I hypothesize that PI4KIIα and BLOC-1 concurrently recruit the WASH complex to early endosomes at the site of AP-3- and BLOC-1-dependent vesicle biogenesis (Fig. 3). There are several testable predictions of this hypothesis. First, I predict that BLOC-1, PI4KIIα, and the WASH complex interact concurrently as a tripartite complex. This model predicts direct interactions between some of the components of these complexes, such as the BLOS2 subunit of BLOC-1 and the WASH protein. Yeast two-hybrid analysis and direct binding assays using recombinant proteins

could test this prediction and further refine the model proposed in Figure 3. In addition, I predict that the interaction between these subunits will be sensitive to the cellular levels of the other components. For example, if BLOC-1 directly binds WASH, loss of BLOC-1 may decrease the association between PI4KIIα and the WASH complex. Further, mutations affecting PI4KIIα enzymatic activity may decrease binding of the Fam21 subunit and decrease association of the WASH complex with early endosomal membranes. As discussed thoroughly in the next section, subunits of the BLOC-1 complex may bind actin filaments, which would further stabilize these complexes at early endosomes (Fig. 3, #3). Finally, if PI4KIIα and BLOC-1 are concurrent upstream factors for the recruitment of the WASH complex to sites of AP-3-BLOC-1 vesicle budding, then the interaction between these two factors may not be affected by WASH complex deficiency. The model presented in Figure 3 will require further experimental testing and refinement. However, defining the precise architecture of the interactions between this membrane lipid kinase, cytosolic coat component, and activator of actin polymerization would provide mechanistic insight into the regulation of actin polymerization during vesicle biogenesis. In the next section, I will discuss hypotheses regarding the functional role of actin polymerization during AP-3- and BLOC-1 vesicle biogenesis.

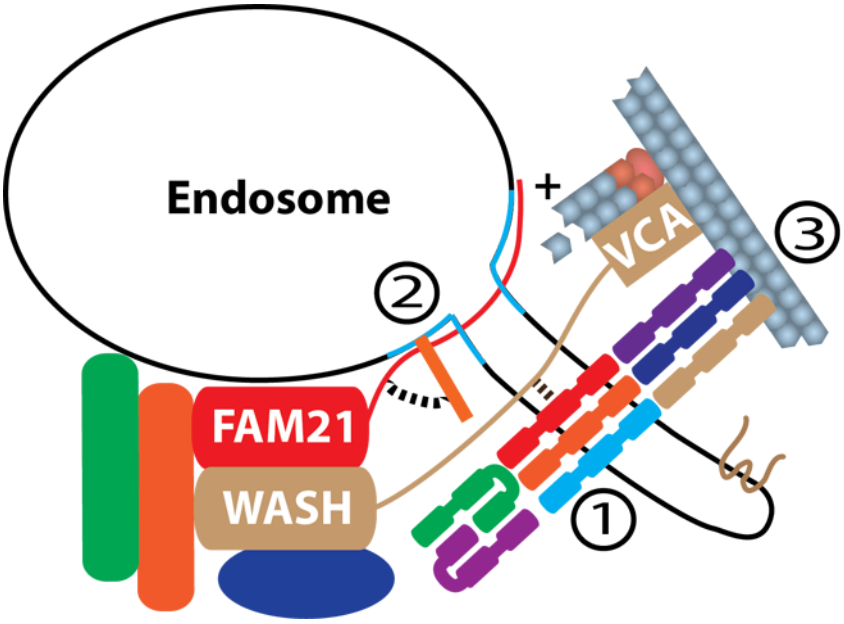

Figure 3. Proposed interactions between PI4KIIa, PI4P, the WASH complex, and BLOC-1. My dissertation research suggests that several components of the PI4KIIα interactome interact directly. The BLOC1S2 subunit binds WASH by yeast two-hybrid analysis (#1). The Fam21 WASH complex subunit binds the lipid PI4P *in vitro*, suggesting that PI4KIIα enzymatic activity may facilitate WASH complex recruitment. In addition, BLOC-1 complex subunits co-precipitate with actin filaments, suggesting the untested hypothesis that BLOC-1 directly binds actin (#3). Further defining these interactions would expand our understanding of the regulation of actin polymerization during vesicle biogenesis at early endosomes.

Novel hypothesis #2: The Arp2/3- and WASH complex-dependent polymerization of actin regulates the cargo sorting into and/or membrane scission of tubular vesicle intermediates

Analysis of the PI4KIIα interaction network revealed a preponderance of actin regulatory proteins (Chapter II, Table 2). As described in Chapter II, my work focused on the interaction between PI4KIIα, BLOC-1, and subunits of the WASH complex. Three of the five core components of this complex (Strumpellin, SWIP, and Fam21) are present in the PI4KIIα interactome (Table 1). In addition, three proteins that interact with the WASH complex, FKBP15 and the capping protein heterodimer, co-isolated with PI4KIIα. Depletion of the WASH complex affected PI4KIIα

endosomal morphology and resulted in mis-localization of BLOC-1 cargoes, suggesting that this complex has a functional role during AP-3- and BLOC-1-dependent vesicle biogenesis. Since the WASH complex acts to nucleate the polymerization of branched actin networks via the Arp2/3 complex in other endosomal trafficking pathways, I hypothesize that the active polymerization plays a functional role within the AP-3-BLOC-1 pathway. This hypothesis is supported by the presence of three of the seven subunits of the Arp2/3 complex within the PI4KIIα interactome (Table 1). In addition, the total cellular content of Arp2/3 subunits change in BLOC-1 deficiencies, supporting a functional relationship between these complexes (Gokhale A, Ryder PV, and Faundez V, unpublished observations). However, the precise molecular function of polymerized actin within sorting processes at endosomes remains elusive. Thus, studying the role of polymerized actin during AP-3- and BLOC-1-dependent vesicle biogenesis may provide insight and models to test for other pathways.

There are two dominant hypotheses for the role of WASH-dependent actin polymerization during vesicle biogenesis at endosomes. The first hypothesis is that actin polymerization at membranes creates microdomains critical for localization of coat complexes and sorting of cargo (Zech *et al.*, 2011; Duleh and Welch, 2012; Gomez *et al.*, 2012; Seaman *et al.*, 2013). Such sorting could be either predominantly a kinetic mechanism or a physical mechanism. A kinetic mechanism could involve the stabilization of structures that support sorting processes, such as tubules. Longer half-lives of these structures could allow the concentration of slowly diffusing cargo, as has been proposed for sorting of the β-adrenergic receptor

(Puthenveedu *et al.*, 2010). Alternatively, a physical interaction between actin filaments and transmembrane protein cargoes and/or cytosolic coat complexes could segregate these components. As described in Chapter II, the AP-3 and BLOC-1 cargo PI4KIIα localizes to early endosomal tubules that co-localize with actin and appear to be vesicle-budding intermediates. Depletion of the WASH complex resulted in elongated tubules that contain PI4KIIα. One model to explain this phenotype is that branched actin polymerization regulates membrane scission, as will be thoroughly discussed below. However, treating cells with brefeldin A, which interferes with Arf GTPase-dependent recruitment of cytosolic coat complexes to endosomes and the Golgi complex, induces a dramatic and extensive tubulation of the endosomal and secretory membrane systems (Lippincott-Schwartz *et al.*, 1991). Thus, actin polymerization may fulfill roles other than exclusively membrane scission at endosomes.

One possibility is that actin polymerization facilitates sorting and segregation of AP-3 and BLOC-1 cargoes into tubules and budding vesicles. The presence of PI4KIIα along the length of tubules in WASH complex-depleted cells may be due to diffusion of this palmitoylated protein rather than active sorting into the tubule. Visualization of a transmembrane cargo, such as the lysosomal SNARE Vamp7, would allow for testing of the hypothesis that WASH-dependent actin polymerization is required for sorting of AP-3-BLOC-1 cargoes into tubule intermediates and subsequent budded vesicles. If polymerized actin contributes to sorting, then this transmembrane protein cargo would remain concentrated in the body of endosomes in WASH complex depletions rather than entering elongated

tubules. Further, inhibition of actin polymerization such as by treatment with the depolymerization agent cytochalasin D or by acute and local inactivation of the Arp2/3 complex would result in decreased entry of Vamp7 into tubules. As described above, this sorting mechanism may require physical interactions between AP-3-BLOC-1 cargo and/or kinetic stabilization of sorting tubules. Future experiments should therefore test the ability of AP-3-BLOC-1 cargoes to bind actin filaments. Additionally, actin polymerization may actively promote stabilization of short (100-200 nm) and relatively long-lived tubules (> 30s), characteristics required for recycling of the β-adrenergic receptor from endosomes (Puthenveedu *et al.*, 2010). Precise kinetic characterization of tubules containing AP-3-BLOC-1 cargoes such as PI4KIIα and Vamp7 is needed to determine if a similar mechanism contributes to sorting in this pathway. Acute and local perturbation of the Arp2/3 and WASH complexes would then test the hypothesis that the kinetics of these tubules depends upon polymerization of branched actin networks.

The role of these branched actin networks in membrane scission remains controversial. One model for force generation by actin polymerization is that incorporation of actin monomers adjacent to a membrane by an anchored complex would then provide a pushing force on the membrane (Galletta *et al.*, 2010). Anchoring to the membrane would require scaffolding proteins capable of binding both actin filaments and the membrane, a mechanism documented in clathrin-mediated endocytosis in yeast (Skruzny *et al.*, 2012). Alternatively, filamentous actin and nucleation-promoting factors could provide a scaffold to bind and recruit other proteins intrinsically capable of generating force, such as the dynamin GTPase.

Dynamin co-immunoprecipitates with the WASH complex and is a component of the PI4KIIα interactome, suggesting this mechanism may function in the AP-3-BLOC-1 vesicle budding (Derivery *et al.*, 2009). The AP-3-BLOC-1 pathway provides assays and tools to facilitate testing if actin polymerization by WASH contributes to force generation for membrane scission. AP-3-coated synaptic-like microvesicles containing Vamp7 can be biochemically isolated from PC12 cells (Clift-O'Grady *et al.*, 1998; Lichtenstein *et al.*, 1998). This cell line is easily manipulated and biochemically tractable, allowing for precise testing of the role of various components in this process. If polymerized actin contributes to force generation, then AP-3-coated vesicles should decrease following depolymerization of actin, inhibition of the Arp2/3 complex, and depletion of the WASH complex. Further, AP-3 budding can be reconstituted from purified endosomal membranes, allowing for more precise biochemical characterization of this budding reaction and the dependence on polymerized actin. Overall, defining the roles for actin polymerization during AP-3- and BLOC-1-dependent vesicle biogenesis will both contribute to our understanding of this process and provide testable models for other pathways that utilize the WASH complex.

Table 1. Subunits of the Arp2/3 and WASH complexes co-isoslate with PI4KIIα. Three of seven Arp2/3 complex subunits and three of five WASH complex subunits co-isolate with PI4KIIα. In addition, three known WASH complex interactors co-isolated with PI4KIIα. The number of unique spectra identified by mass spectrometry analysis and the relative enrichment as determined by analysis isotope-labeled samples are included for each complex subunit.

Symbol	Name	Proposed role	Spectra	Ratio L/H
ACTR2	Actin-Related Protein 2	Arp2/3 complex subunit; proposed to complex with Arp3 to form the surface for daughter	4	35.9

		filament polymerization.		
ACTR3	Actin-Related Protein 3	Arp2/3 complex subunit; proposed to complex with Arp2 to form the surface for daughter filament polymerization.	8	8.5
ARPC2	Actin-Related Protein 2/3 Complex Subunit 2	Arp2/3 complex subunit; unknown function within the complex.	5	24.6
KIAA0196	Strumpellin	Subunit of the WASH complex. Point mutations to the strumpellin protein cause autosomal dominant hereditary spastic paraplegia, yet a specific role within the WASH complex remains unknown.	48	10.3
FAM21B	Family with Sequence Similarity 21	Subunit of the WASH complex. Primarily responsible for localization of the complex to endosomal membranes. Binds PI3P and PI4P lipids *in vitro.*	12	5.9
SWIP	Strumpellin- and WASH-Interacting Protein	Subunit of the WASH complex. Candidate gene for non-syndromic autosomal recessive intellectual disability.	16	24.4
CAPZA1	Capping Protein Alpha	Forms heterodimer with CAPZB. Binds to the barbed end of actin filaments to promote polymerization of short, highly branched actin networks. Co-isolates with the WASH complex.	9	42.6
CAPZB	Capping Protein Beta	Forms heterodimer with CAPZA. Binds to the barbed end of actin filaments to promote polymerization of short, highly branched actin networks. Co-isolates with the WASH complex.	9	18.8
FKBP15	FK506 Binding Protein 15	WASH complex interactor. Involved in transport of early endosomes at the level of transition between actin filament-based and microtubule-based movement.	81	21.3

Novel hypothesis #3: The BLOC-1 complex acts as a tubule-stabilizing factor

Bending endosomal membranes into highly curved tubular and vesicular structures requires both energy and structural support to maintain curvature (McMahon and Gallop, 2005; Zimmerberg and Kozlov, 2005). As discussed above, actin polymerization may contribute force to the AP-3 and BLOC-1 trafficking pathway to promote this membrane deformation. Within many vesicular trafficking pathways, proteins containing BAR domains are required to induce and/or stabilize these curved membranes. These proteins contribute to many stages of vesicle biogenesis, such as nucleation, inward progression, and membrane scission by acting as scaffolds to recruit and coordinate the activity of coat proteins and actin nucleators (Qualmann *et al.*, 2011; Rao and Haucke, 2011). The retromer complex incorporates and functionally requires two BAR domain-containing proteins for endosome-to-Golgi trafficking (Teasdale and Collins, 2011; Cullen and Korswagen, 2012). This pathway represents the canonical endosomal trafficking pathway that incorporates the WASH complex and thus provides conceptual frameworks and models for the AP-3-BLOC-1 pathway (Seaman *et al.*, 2013). The retromer complex consists of a cargo-selective trimer and a heterodimer of sorting nexin proteins that contain BAR domains (McGough and Cullen, 2011). The cargo-selective trimer binds cargo to concentrate them into budding vesicles and recruits the WASH complex for actin polymerization (Attar and Cullen, 2010; Seaman *et al.*, 2013). The sorting nexin heterodimer transiently interacts with the cargo-selective trimer and primarily binds highly curved membranes to structurally support tubule and vesicle formation (Teasdale and Collins, 2011; Cullen and Korswagen, 2012). Thus, one surprising aspect of the PI4KIIα interactome is the lack of recognizable BAR

domain-containing proteins. In the next section I will discuss a novel untested hypothesis: BLOC-1 acts as an AP-3-specific stabilizer of membrane tubules in an analogous fashion to the role of BAR-domain containing proteins within other vesicular trafficking pathways.

Several pieces of evidence support the hypothesis that BLOC-1 acts to stabilize membrane tubules. First, the BLOC-1 complex decorates the sides of endosomal tubules by electron microscopy (Di Pietro *et al.*, 2006) while the AP-3 complex appears to primarily localize to the tip of such tubules (Peden *et al.*, 2004). Second, the flexible, elongated, and curved structure of BLOC-1 as demonstrated by negative stain electron microscopy suggests that it may laterally polymerize on curved membranes (Lee *et al.*, 2011). Third, subunits of the BLOC-1 complex demonstrate distant homology to the sorting nexins as determined by the HHPred homology prediction detection. The HHPred approach was developed as a highly sensitive method to detect distant homologs and structurally similar proteins that are not detected by sequence-based searching such as BLAST and FASTA (Söding *et al.*, 2005; Hildebrand *et al.*, 2009). This approach first considers all known homologs of a given protein in order to determine motifs or patterns of well-conserved residues. The software then searches these highly conserved motifs against highly conserved motifs within other known proteins or protein domains in order to find structurally related proteins and/or distant homologs. Using this method, the cappuccino and BLOC1S1 subunits of BLOC-1 demonstrate homology to the BAR domains of sorting nexin 1 and 2 with greater than 85% probability of a true homologous relationship (Ryder PV and Faundez V, unpublished observations).

Thus, homology prediction suggests that components of BLOC-1 may function as BAR domains to support and/or induce curved membranes. Finally, the co-localization and co-isolation of BLOC-1 with actin filaments may contribute to a scaffolding and structural role (Falcon-Perez, 2002). To date, no experiments have tested the prediction of the model proposed in Figure 2 that BLOC-1 interacts directly with actin filaments. However, the HHPred homology detection software predicts homology between the BLOC1S2 subunit of BLOC-1 and tropomyosin with greater than 90% probability of a true homologous relationship. Tropomyosin directly binds actin filaments along their length. Thus, this homology supports the possibility that BLOC-1 interacts directly with actin filaments, which could thereby physically link the cytoskeleton and the membrane to stabilize tubules and vesicles.

There are several testable predictions of the hypothesis that BLOC-1 acts as an AP-3-specific stabilizer of membrane tubules. First, BLOC-1 would be required for the generation and stability of tubules. The enlarged endosomes and AP-3 pathway trafficking disruptions observed upon BLOC-1 depletion are consistent with this prediction. An additional prediction is that the elongated tubules visualized upon depletion of the WASH complex would collapse in cells lacking both BLOC-1 and the WASH complex. One fundamental characteristic of BAR domain-containing proteins is the ability to tubulate membranes *in vitro*. Thus, if BLOC-1 does act to induce and stabilize membrane tubules, the complex should demonstrate this property *in vitro*. Experimental data testing this hypothesis will no doubt lead to a refinement of the model. However, as discussed in the Introduction of this dissertation, the molecular function of BLOC-1 remains unclear. Thus, my

dissertation research contributes a novel, testable hypothesis regarding BLOC-1 function.

Novel hypothesis #4: PI4KIIα and BLOC-1 recruit guanine exchange factors as activators of actin polymerization

Small GTPases of the Rho family activate actin nucleation-promoting factors such as the WASH complex (Hall, 2012). While RhoA genetically interacts with WASH in the model organism *Drosophila melanogaster* (Liu *et al.*, 2009), to date no small GTPase is described to regulate the WASH complex in mammalian cells. Both upstream and downstream elements of Rho family GTPase signaling co-isolate with PI4KIIα, suggesting that these factors could regulate WASH complex activation (Table 2). In this section, I will discuss the hypothesis that PI4KIIα and BLOC-1 recruit components of Rho family GTPase signaling to regulate actin polymerization at the site of AP-3- and BLOC-1-dependent vesicle biogenesis.

Rho family GTPases cycle between GTP- and GDP-bound states (Hall, 2012). In the GTP-bound state, these small GTPases translocate to the membrane from the cytosol to activate effector proteins, leading to cellular changes. Enzymes known as guanine exchange factors interact with these small GTPases to promote their activation and subsequent signaling (Rossman *et al.*, 2005). Several guanine exchange factors for Rho GTPases co-isolated with PI4KIIα at high stoichiometry, being represented by more than 100 spectra counts and enrichment ratios of approximately 20-fold to 60-fold (Table 2). For comparison, PI4KIIα was represented by 220 spectral counts and was enriched 55-fold. Two of these guanine exchange factors, RhoGEF1 and RhoGEF2, are known to activate RhoA, which

genetically interacts with WASH in *Drosophila melanogaster* (Rossman *et al.*, 2005; Liu *et al.*, 2009). RhoGEF2 also activates Rac1, another Rho family member, and associates with microtubules, thereby connecting the actin and microtubule cytoskeletons (Birkenfeld *et al.*, 2008). Both RhoGEF1 and RhoGEF2 are guanine exchange factors of the Dbl family, and as such, contain a characteristic pleckstrin homology (PH) domain (Zheng, 2001). This domain binds to membrane lipids to localize the guanine exchange factor to the membrane and facilitate a conformation that promotes guanine exchange activity (Rossman *et al.*, 2003; Viaud *et al.*, 2012). Thus, the lipid-modifying activity of PI4KIIα may serve as a recruitment factor for translocation of RhoGEF1 and RhoGEF2 to the membrane for subsequent activation of small GTPases.

In addition, RhoGEF1 is activated by G-protein receptor signaling via Gα12/13 proteins (Hart *et al.*, 1998; Meyer *et al.*, 2008; Siehler, 2009; Aittaleb *et al.*, 2010). Receptors that signal through these G proteins include the M_1 and M_5 muscarinic acetylcholine and D_5 dopamine receptors (Siehler, 2009). While the work that describes this signaling and activation of RhoGEF1 is described at the plasma membrane, many receptors are internalized and signaling continues at endosomes (Gonnord *et al.*, 2012). Further, there are no reports of the subcellular localization of endogenous RhoGEF1 protein. Attempts to document the localization of RhoGEF1 during this dissertation research were limited by lack of suitable reagents. However, the biochemical and genetic interactions between RhoGEF1, PI4KIIα, and the BLOC-1 complex described in Chapter II support a functional relationship between these components. One model is that activation of RhoGEF1

via G protein coupled receptor signaling could then activate RhoA and actin polymerization at early endosomes within the context of the AP-3 and BLOC-1 pathways (Fig. 4). In this model, binding of an agonist to its G protein coupled receptor would induce internalization of the receptor and localization of the cytosolic G proteins to early endosomes (Fig. 4, #1). Activation of $G\alpha_{12/13}$ proteins would then activate and recruit RhoGEF1 to early endosomes (Fig. 4, #2). PI4KIIα and its lipid product, PI4P, could serve to facilitate and promote the localization of RhoGEF1 to early endosomes (Fig. 4, #3). RhoGEF1 activates RhoA-GTP (Fig. 4, #4), which potentially could facilitate the activation and/or recruitment of the WASH complex to early endosomes and subsequent AP-3- and BLOC-1-dependent vesicular trafficking of G protein coupled receptors (Fig. 4, #5). Consistent with this proposed model, the AP-3 complex binds the M_5 muscarinic receptor and is required for sustained signaling via this receptor (Bendor *et al.*, 2010). A testable prediction of this model is that RhoGEF1 and RhoA-GTP are required components for sustained signaling via the M_5 muscarinic receptor. If this requirement proceeded via recruitment of the AP-3, BLOC-1 and WASH complexes, then combined depletion of RhoGEF1 or RhoA with PI4KIIα, BLOC-1, or the WASH complex would have no synergistic effect.

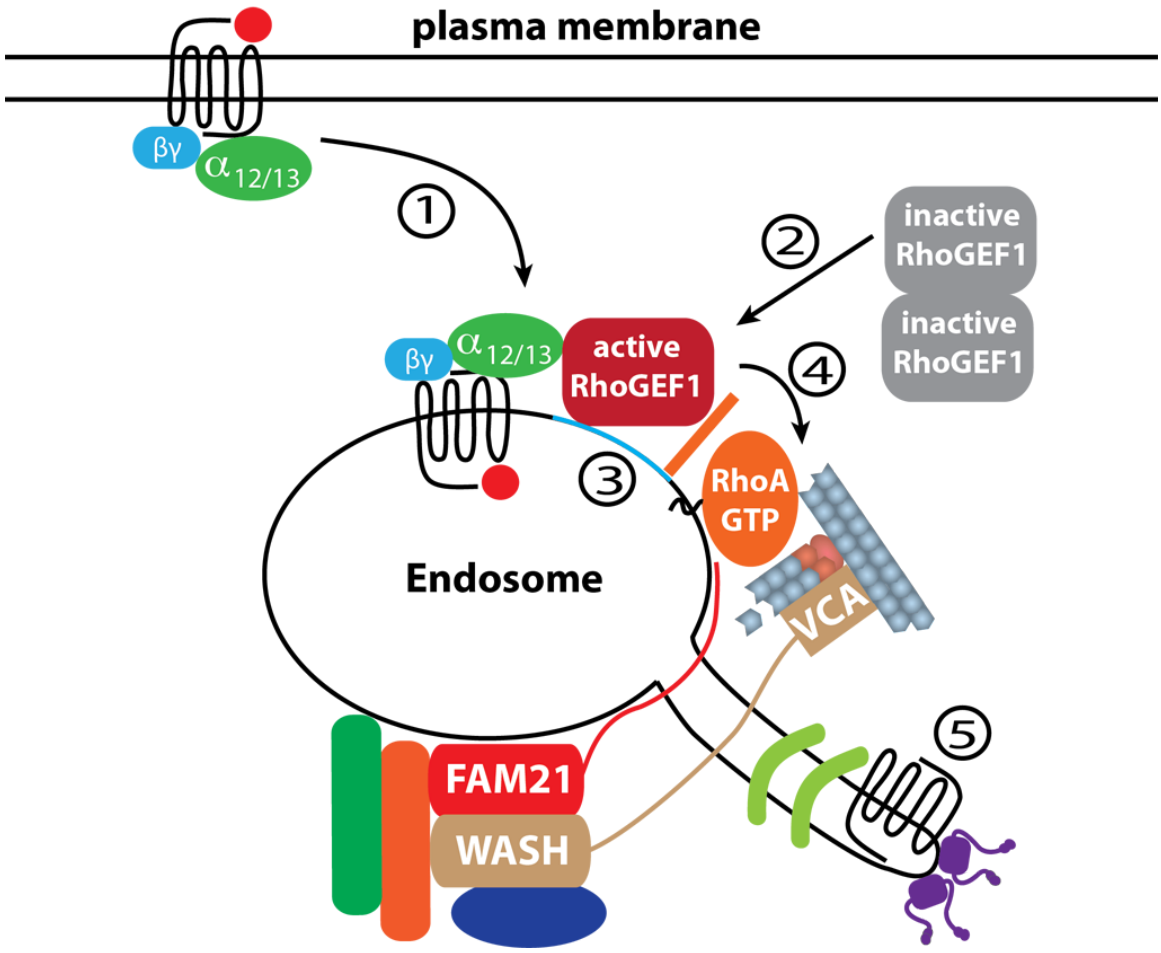

Figure 4. Model for RhoGEF1 activation of the WASH complex. G protein coupled receptor activation by an agonist may lead to internalization of this receptor (#1), localizing the receptor and activated G proteins to early endosomes. Activated $G\alpha_{12/13}$ proteins could then activate RhoGEF1 and promote its translocation to early endosomal membranes (#2). PI4KIIα and its lipid product PI4P may facilitate the localization of RhoGEF1 to early endosomes. The guanine exchange factor activity of RhoGEF1 could then activate RhoA (#4), which may recruit the WASH complex as an effector to contribute to AP-3- and BLOC-1-dependent vesicular trafficking of this G protein coupled receptor (#5).

Another guanine exchange factor of high interest is dedicator of cytokinesis 7 (DOCK7). DOCK7 activates the Rac1 and Rac3 small GTPases of the Rho family (Watabe-Uchida *et al.*, 2006). Deficiency of DOCK7 in the mouse model organism cause a Hermansky-Pudlak syndrome-like phenotype of hypopigmentation and increased bleeding tendency (Sviderskaya *et al.*, 1998; Swank *et al.*, 1998; Blasius *et*

al., 2009; Wei and Li, 2012). To date, however, no molecular mechanism accounts for this phenotype. One hypothesis that arises from my dissertation research is that the phenotype of DOCK7 deficiency arises due to a disruption in AP-3 and BLOC-1 function. Another possibility is that AP-3- and BLOC-1-dependent vesicular trafficking targets DOCK7 to target lysosome-related organelles such as melanosomes and platelet-dense granules where it plays critical roles for organelle function. Thus, exploring the relationship between PI4KIIα, BLOC-1, and DOCK7 may further our understanding of lysosome-related organelle function. In addition, DOCK7 contributes to synapse function by regulating cytoskeleton reorganization (Watabe-Uchida *et al.*, 2006). Loss of DOCK7 at synapses may therefore contribute to the neurological phenotypes of AP-3 deficiency.

Table 2. Upstream and downstream elements of Rho family GTPase signaling co-isolate with PI4KIIα. Three guanine exchange factors for Rho family GTPases and two Rho family GTPase effectors co-isolate with PI4KIIα. These components of the PI4KIIα interactome suggest that PI4KIIα may contribute to regulation of Rho family GTPases (see Fig. 4).

Symbol	Name	Proposed role	Spectra	Ratio L/H
ARHGEF1	Rho Guanine Exchange Factor 1	RhoA guanine exchange factor. Couples signaling via G-protein coupled receptors to changes in actin cytoskeleton dynamics.	626	63.3
ARHGEF2	Rho/Rac Guanine Exchange Factor	Rho family guanine exchange factor. Proposed to activate RhoA and Rac1.	103	32.0
DOCK7	Dedicator of Cytokinesis 7	Guanine exchange factor for Rac1 and Rac3 small GTPases. Plays a role in axon formation and neuronal polarization. Mutations in DOCK7 cause a Hermansky-Pudlak-like syndrome in mouse models.	114	19.5

CDC42BPA	CDC42 Binding Protein Kinase Alpha	Proposed CDC42 effector. Serine/threonine protein kinase targets actin modulatory proteins including myosin light chain 9.	183	14.5
CDC42BPB	CDC42 Binding Protein Kinase Beta	Proposed CDC42 effector. Serine/threonine protein kinase.	6	10.6

Novel hypothesis #5: Disruption to AP-3- and BLOC-1-dependent vesicle biogenesis contributes to the pathogenesis of neurodegenerative diseases

In addition to providing mechanistic insight into fundamental cell biological processes, my dissertation raises hypotheses that may contribute to the understanding of neurodegenerative disease processes. Mutations to several of the protein complexes in the PI4KIIα interactome are genetically linked to neurodegenerative diseases (Simons *et al.*, 2009; Blackstone *et al.*, 2010; Kennerson *et al.*, 2010; Blackstone, 2012; Yi *et al.*, 2012). However, the precise mechanistic roles of these proteins in disease pathogenesis remain elusive, in part because the known interactions of these proteins with other neurodegenerative disease-causing proteins are limited. In the next section, I detail and describe hypotheses and models related to the proteins that are the focus of my dissertation research: PI4KIIα, the strumpellin subunit of the WASH complex, the Menkes copper transporter, and the BLOC-1 complex.

Targeted disruption of PI4KIIα in mice leads to neurodegeneration of central nervous system axons within the corticospinal track (Simons *et al.*, 2009). This phenotype resembles the human disease syndrome known as hereditary spastic

paraplegia (HSP). HSP is characterized by degeneration of long central nervous system axons connecting the motor cortex with the spinal cord leading to progressive muscle spasticity and weakness (Blackstone *et al.*, 2010). More than 50 causative genes have been identified, yet pathogenic mechanisms are limited in scope (Blackstone, 2012). Many of these causative genes contribute to membrane trafficking pathways, suggesting that these pathways are necessary to deliver components required to maintain long axons and synapses distant from cell bodies (Blackstone *et al.*, 2010). Intriguingly, one HSP locus for which the causative gene is yet to be identified has been mapped to the genetic locus where PI4KIIα is encoded, suggesting that PI4KIIα could be a causative gene for HSP in humans (Simons *et al.*, 2009). In addition, disease severity as represented by symptom severity and age of onset can vary between patients with the same genetic variant of HSP. Thus, another possibility is that subtle changes to components of the PI4KIIα interactome network could act as disease-modifying factors that influence severity.

One of the most exciting connections arising from my work is the interaction between PI4KIIα and the strumpellin subunit of the WASH complex. Point mutations to the strumpellin protein are causative mutations of autosomal dominant HSP in humans (Valdmanis *et al.*, 2007). However, analysis of these mutants has not yet revealed any causative mechanism (Valdmanis *et al.*, 2007; Freeman *et al.*, 2013). One difficulty in determining a disease mechanism for strumpellin mutations is the lack of known interactions with other HSP-causative genes. Thus, a novel hypotheses arising from my dissertation research is that mutations to strumpellin specifically disrupt the trafficking and function of PI4KIIα and other BLOC-1 cargoes.

Supporting this hypothesis, the BLOC-1 cargo ATP7A is independently linked to neurodegeneration of peripheral motor axons (Kennerson *et al.*, 2010; Yi *et al.*, 2012). Thus, strumpellin mutations may mis-localize this copper transporter, which could contribute to the development of HSP. In normal cellular physiology, ATP7A localizes to the Golgi complex and specialized lysosome-related organelles such as melanosomes where it loads metalloenzymes with copper, a necessary co-factor for certain enzymatic processes (Hasan and Lutsenko, 2012). Excess cellular copper causes ATP7A to move to the plasma membrane where it effluxes copper from the cytosol (Hasan and Lutsenko, 2012). Thus, mis-localization of ATP7A may affect both the function of metalloenzymes and the total cellular content of copper. Finally, the documented increases in the surface content of two BLOC-1 cargoes, ATP7A and the lysosomal SNARE Vamp7, suggest an assay by which to screen for other causative proteins. This biochemical assay is an extremely sensitive method to identify proteins affected by disrupted endosomal trafficking. A quantitative surface analysis and comparison between cells expressing the dominant strumpellin mutations and cells expressing the WT strumpellin subunit would allow for the identification of novel disease components which may also be trafficked via the BLOC-1 pathway.

Conclusion

My dissertation research reveals an unexpected role for the actin cytoskeleton in AP-3- and BLOC-1-dependent vesicle biogenesis. I drew upon the conceptual framework of AP-2- and clathrin-dependent vesicle budding at the plasma membrane and hypothesized that the actin cytoskeleton contributes to vesicle

biogenesis at early endosomes. I tested this hypothesis by identifying proteins that co-isolate with a cargo and regulator of AP-3 and BLOC-1, the membrane lipid kinase PI4KIIα. Actin regulatory proteins including nucleation-promoting factors and guanine exchange factors for Rho family GTPases are predominant components of the PI4KIIα interactome. In addition, in Chapter II, I showed that some of these actin regulators genetically interact with PI4KIIα and that depletion of these components leads to mis-localization of BLOC-1 cargo. This work expanded our understanding of the AP-3-BLOC-1 pathway and provided several new hypotheses for future study. Exploring the role of the actin cytoskeleton in AP-3- and BLOC-1-dependent vesicle biogenesis may reveal several different aspects of insight into this process, including: recruitment mechanisms for actin nucleators, the functional roles for actin polymerization, and the regulation of actin polymerization. In addition, studying these mechanisms may lead to new conceptions of the roles of these proteins in human diseases such as pigmentation disorders, neurocutaneous syndromes, and neurodegeneration.

CHAPTER IV

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