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Targeting of the Lysosomal Tether, The HOPS Complex, By Clathrin-Dependent Mechanisms

By

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Doctor of Philosophy

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Targeting of The Lysosomal Tether, The HOPS Complex, By Clathrin-Dependent Mechanisms

By

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A.B., Washington University in St. Louis, 2006

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Abstract

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By Stephanie A. Zlatic

Localization of cytosolic protein between the cytoplasm and lysosomal membranes is critical for the biogenesis and maintenance of lysosome molecular composition and function. My dissertation examines how the cytosolic tether, HOPS complex, required for organelle fusion with the lysosome is localizes to subcellular domains in polarized and non-polarized mammalian cells. Current models of membrane traffic segregate vesicle formation machinery from fusion machinery spatially and functionally. In this model, tethers such as the HOPS complex localize to their target organelle through selective recruitment from the cytoplasm to the limiting membrane of the lysosome. However, this model does not account for the localization of HOPS complex subunits to early and late endosomes in mammalian cells described in my dissertation.

In my dissertation I hypothesize cytosolic HOPS complex subcellular localization is regulated by a vesicular coat-dependent mechanism of membrane traffic along the endocytic route. Here, I present biochemical and immunomicroscopy evidence that HOPS subunits interact and colocalize with the coat proteins AP-3 and clathrin and localize to sites reminiscent of AP-3/clathrin vesicle and clathrin plaque formation at early endosomes. Biochemical fractionation reveals HOPS complex subunits cofractionate with clathrin-coated vesicles. Furthermore, distribution of HOPS complex subunits to both early and late endosomes/lysosome subcellular locations is rapidly perturbed by acute inhibition of clathrin function through a chemical-genetic strategy in non-polarized cells. In polarized cells of neuronal origin, HOPS complex subunits localize to the tips of neurites. HOPS subunit localization to the proximal region of neurites decreased following acute chemical-genetic disruption of clathrin function.

Together, these data lead me to propose a novel model of HOPS localization in mammalian cells in which HOPS complexes localize to sites of AP-3/clathrin vesicle formation and flat clathrin plaques at early endosomes for their delivery as cargo to the late endosome/lysosome either through vesicular transport and/or endosomal maturation. My findings provide novel insight into the possible roles that HOPS complex subunits may have in regulation of lysosome and lysosome-related organelle trafficking in mammalian cells. Overall, my dissertation challenges the current conception that vesicle formation coats and vesicle fusion tethers are spatially and functionally segregated.

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CHAPTER I

GENERAL INTRODUCTION

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Overview

While several models of protein targeting along the endocytic pathway are accepted, the precise mechanism(s) that individual proteins follow in their transit to lysosomes remains unexplored. In my dissertation I specifically focus on the mechanisms that define localization of the lysosomal tethering complex, the HOPS (**HO**motypic fusion and **P**rotein **S**orting) complex, subunit in mammalian cells. Knowing how HOPS subunits are targeted in mammalian cells will provide insight into normal and pathological mechanisms of lysosome targeting such as those affected in human syndromes resulting from mutations to genes encoding HOPS subunits. In this Introduction I will review: 1) The importance of protein localization in cells and specifically localization to the lysosome, 2) a description of relevant molecular machinery for lysosomal localization (including AP-3, clathrin) 3) an analysis of the molecular and cellular biology of the HOPS complex, 4) the current models of membrane trafficking between endosomes and lysosomes which include vesicular and maturation models, 5) the importance of HOPS complex in localization of cargoes to lysosomes and mutations of HOPS subunits that result in phenotypes for higher order eukaryotes including mammals, and 6) current views of HOPS complex localization in *S. cerevisiae* compared to mammalian systems.

Significance

The overall goal of my dissertation is to understand the mechanism of cytosolic localization of mammalian HOPS complex tethering/fusion proteins (Seals *et al.* 2000) in mammalian cells. As discussed below, cells segregate

specific functions through the selective localization of proteins and lipids to specific membrane enclosed organelles (Figure 1). Eukaryotic cells have evolved elegant mechanisms of carrier/vesicle-mediated trafficking to localize membrane and membrane-associated proteins.

In yeast, a key observation was made that HOPS subunits are primarily localized to the vacuole, the equivalent of the lysosome in mammals (Nakamura *et al.* 1997; Wang 2003; Wang 2003; LaGrassa and Ungermann 2005; Angers and Merz 2009; Cabrera *et al.* 2009). Both clathrin and **A**daptor **P**rotein Complex **3** (**AP-3**) participate in membrane trafficking pathways to the lysosomal compartment in higher order eukaryotes (Braulke and Bonifacino 2009). Several models have been proposed for the localization of HOPS subunits including: localization of HOPS subunits from the cytosol to the vacuolar membrane compartment through lipid interactions (Hickey *et al.* 2009) where incoming AP-3 vesicles could interact with HOPS at the vacuole (Angers and Merz 2009), or a model where the individual HOPS subunit, Vps41, is used as a transport coat for AP-3 vesicles instead of the vesicle coat protein clathrin (Rehling *et al.* 1999). These models will be discussed in further detail in Section 6 of this Introduction. In contrast to yeast data, immunofluorescent microscopy from mammalian cells suggested HOPS complex is localized at endosomal compartments other than the late endosome or lysosome (Richardson *et al.* 2004). This suggested a more complex model of HOPS localization would be required in higher eukaryotes (Kim 2004; Richardson *et al.* 2004). Results from this dissertation specifically lead me to propose a more elaborate model of HOPS

localization to endosomal compartments in mammalian cells that challenge some hypotheses derived from yeast studies. Here, I focus on two main questions:

1) Where are HOPS subunits localized in mammalian cells?
2) Can the localization of HOPS subunits be modulated by coat-dependent mechanisms of membrane traffic along the endocytic route?

My dissertation work has lead to the following discoveries about HOPS subunits (Chapter 2):

1) HOPS subunits are recruited to early endosomal membranes at locations containing the molecules AP-3 and clathrin required for vesicle-dependent trafficking to the lysosome.
2) HOPS subunits form a macro-molecular complex with AP-3 and clathrin.
3) HOPS subunits are found in clathrin-coated vesicular intermediates.
4) HOPS subunits localize to early and late endosomes/lysosomes in a clathrin-dependant manner.
5) HOPS subunits localized to neurites in polarized neuronal cells through clathrin-dependant mechanisms.

Taken together, these data strongly support a novel model of HOPS subunit localization where subunits are localized to the early endosome as cargo for inclusion into clathrin-dependant vesicular and/or endosomal maturation pathways. For the purposes of this dissertation, cargoes are defined as proteins present within or on the transport organelle membrane for localization purposes. The function of coats then determines delivery of HOPS subunits from early endosomes to late endosomes/lysosomes, and neurites of polarized neuronal cell

types. These results indicate that localization of HOPS subunits is more complex than originally described by research from yeast model organisms. In fact, this novel model of HOPS localization in mammalian cells reveals new hypotheses about how mutations to genes encoding these subunits may lead to human syndromes, provide the field with a more accurate perception of how proteins can be localized along the endo-lysosomal pathway, and possible roles for HOPS complex subunits at multiple stages of vesicular trafficking in the cell.

Section 1. The Importance of Protein Targeting to the Lysosome is Critical for the Maintenance of Cellular Function

The lysosome is an acidic and digestive organelle. The lysosome functions to accept proteins, lipids, and other molecules for degradation and recycle the resulting peptides, amino acids, carbohydrates, and lipids back to the cell (Meel and Klumperman 2008). Additionally, lumenal enzymes providing degradative properties and lysosomal regulatory proteins must be localized to the lysosome (Figure 1). Examples of lysosomal regulatory proteins are the cytosolic HOPS complex subunits. As will be discussed below HOPS complex subunits help establish and maintain the molecular identity of lysosomes by regulating fusion events that localize lysosomal specific proteins and molecules bound to lysosomes.

The lysosome is a membrane bound organelle that regulates and maintains separate molecular environments inside and outside the limiting membrane. For example in lysosomes, membrane proteins regulate pH, ionic concentrations, and metabolic product recycling in the lumen whereas the

limiting membrane defines the lysosomes morphological identity while separating lumenal environment with the cytoplasm (Meel and Klumperman 2008). Directed protein sorting to the limiting membrane of the lysosome regulates fusion events with incoming vesicles or other membranous organelles.

Proteins are recruited to organelles in a variety of mechanisms depending on whether or not they localize to membranes (Figure 1). Cytosolic proteins may be recruited directly from the cytoplasm to the organelle or bind to membranes and then be delivered through membrane traffic pathways to a target destination. Membrane trafficking pathways are used to target lumenal and membrane bound proteins to specific organelles and will be discussed in Section 4. The mechanisms that provide selectivity in the fusion of incoming membranes with lysosomes are critical for the degradation of signaling molecules, fusion with autophagosomes, lysosome secretion, recycling of old or malfunctioning proteins, and the delivery of lysosomal enzymes (Seaman *et al.* 1996; Andrews 2000; Meel and Klumperman 2008). Specific recruitment of HOPS subunits for the regulation of fusion events at the lysosome provides one proofreading mechanism for the correct delivery of lysosomal proteins (Starai *et al.* 2008). A variety of mutations in genes that encode for both lysosomal proteins and regulators of lysosome trafficking illustrate the importance of regulating localization of lysosomal proteins. There are approximately 50 human syndromes characterized by the inability of lysosomal proteins to localize or function to/at the lysosome and 15 human syndromes resulting from mutations to genes encoding proteins responsible for membrane trafficking to the lysosome (Gieselmann 1995; Aridor and Hannan 2000). The 15 human syndromes includes

mutations of genes encoding HOPS subunits in diverse eukaryotic organisms such as *C. elegans* (Ruan *et al.* 2009; Zhu *et al.* 2009), *D. melanogaster* (Warner *et al.* 1998; Pulipparacharuvil *et al.* 2005; Lindmo *et al.* 2006; Akbar *et al.* 2009; Chi *et al.* 2010; Akbar *et al.* 2011), *D. rerio* (Matthews *et al.* 2005; Sadler *et al.* 2005; Maldonado *et al.* 2006; Schonthaler *et al.* 2008; Cullinane *et al.* 2009), *M. musculus* (Suzuki *et al.* 2003; Kim *et al.* 2006), *R. norvegicus* (Kim *et al.* 2006), and human syndromes (Gissen *et al.* 2006; Huizing *et al.* 2008). Thus, the HOPS complex subunits in metazoans are highly conserved and illustrate the importance of HOPS subunits in maintaining lysosome and lysosome-related organelle morphology and function. In addition, the wide range of tissues affected in mutation of HOPS subunits in metazoans, ranging from neurons to epidermis, highlights the universality of the molecules and biological processes I have studied in this dissertation.

HOPS proteins regulate fusion of membranes with lysosomes and lysosome-related organelles in higher eukaryotes (Warner *et al.* 1998; Eastham *et al.* 2001; Suzuki *et al.* 2003; Lo *et al.* 2005; Pulipparacharuvil *et al.* 2005; Lindmo *et al.* 2006; Maldonado *et al.* 2006; Akbar *et al.* 2009; Zhu *et al.* 2009). Lysosome-related organelles include melanosomes, platelet dense granules, lamellar bodies in neumocytes and keratinocytes, lytic granules immune cells, *D. melanogaster* pigment granules, gut granules and the fibrous body-membranous organelles in *C. elegans* sperm (Dell'Angelica *et al.* 2000; Zhu and L'Hernault 2003; L'Hernault 2006; L'Hernault 2009). Lysosome-related-organelles maintain high acidity and share lumenal and membrane proteins found in lysosomes, can fuse with the plasma membrane releasing contents to the

extracellular space, and also perform cell type specific functions in addition to lysosomal functions (Dell'Angelica *et al.* 2000). For example, melanocytes produce melanosomes (Dell'Angelica *et al.* 2000). Melanosomes store the pigment that determines hair, skin and eye color as well as providing protection against UV irradiation (Dell'Angelica *et al.* 2000). Lysosome-related-organelles will be discussed in further detail in this dissertation since mutations in HOPS proteins as well as vesicle coats associated with HOPS proteins produced pigmentation and lamellar body phenotypes derived from defects in lysosome-related organelles.

While it is apparent that membrane proteins use vesicle mediated membrane trafficking pathways for localization in cells, it is unclear how some lysosomal cytosolic proteins, like HOPS complex subunits, localize to membrane compartments. One possibility is that HOPS proteins are directly localized to the lysosome membrane from the cytosol (Figure 2A). However, another possibility considered here entails HOPS protein localized to membranes distinct from the lysosomal target location and trafficked as membrane associated cargoes via membrane trafficking pathways (Figure 2B). To distinguish between these two models of HOPS protein localization I formulated the central hypothesis guiding this dissertation:

Cytosolic HOPS subunit subcellular localization is localized to early endosomal compartments and their lysosomal localization is regulated by a clathrin-dependent mechanism of membrane traffic.

A test of this hypothesis is described in Chapter 2 of my dissertation. I demonstrate that HOPS protein subcellular localization is regulated by the coat

clathrin. These findings support a model whereby HOPS protein localizes to early endosomes and are transported as membrane-associated cargoes via membrane trafficking pathways (Figure 2B).

A familiarization with relevant molecular complexes involved in membrane trafficking pathways to the lysosome will be discussed in the next two sections followed, in Section 4, by a presentation of two models of membrane protein trafficking toward lysosomes.

Section 2. Fundamentals of Molecular Complexes Required for Lysosome Protein Localization

Vesicles are packets of proteins enclosed in a membrane generated at a donor organelle that will be delivered to a target location in the cell (Bonifacino and Glick 2004). Vesicle trafficking is frequently thought of in two distinct steps: 1) cargo recruitment and vesicle formation, 2) vesicle docking and fusion (Figure 3; Bonifacino and Glick 2004; Cai *et al.* 2007). In this section I will detail the structure and composition of vesicle formation proteins required for cargo concentration and membrane deformation at vesicle donor sites in addition to membrane fusion proteins required for vesicle tethering, docking and fusion.

Section 2.01 ARFs and Adaptors

Specific proteins must be localized to the lumen, membrane and cytosolic face of each specific membrane compartment. One way this can be achieved is by localizing and concentrating target specific proteins, or cargoes, into membrane patches where vesicle formation will occur (Figure 3). **A**DP **R**ibosylation **F**actor,

ARF, is activated at the cytoplasmic face of a donor compartment prior to cargo enrichment at sites of vesicle formation (Figure 4; Gillingham and Munro 2007). ARF activation along with phosphoinositol lipids at the membrane surface enhance interactions of adaptor protein complexes at the vesicle formation site (Figure 3 and 4; Robinson 2004; Behnia and Munro 2005; Di Paolo and De Camilli 2006). Specific adaptor proteins then interact with selective motifs on the cytoplasmic tails of a subset of membrane proteins (Ohno *et al.* 1995; Ohno *et al.* 1998; Owen and Evans 1998; Bonifacino and Glick 2004; Robinson 2004). The result is a domain of membrane that is highly concentrated with adaptors and cargo proteins that will later be incorporated into a newly forming vesicle (Figure 3; Bonifacino and Glick 2004). Tetrameric and monomeric adaptors are examples of vesicle formation machinery recruited by ARF proteins.

Tetrameric protein complexes including adaptor protein complexes AP-1, AP-2, AP-3, and AP-4, form a coat on newly forming vesicles (Kirchhausen 1999; Bonifacino and Glick 2004; Robinson 2004). Each of these adaptors contains four subunits: a small σ subunit, a medium μ subunit, a large β subunit, and a second large subunit γ, α, δ, or ε (AP-1 to -4 respectively; Figure 5; Kirchhausen 1999). Tetrameric adaptors are composed of a main core region, which recognizes motif signals present in specific transmembrane cargo proteins, two flexible hinge regions, and an ear region at the end of the flexible hinge region (Figure 5; Jackson *et al.* 2010).

In addition to interactions with cargoes, adaptors also interact with the coat protein clathrin (Figure 6). Adaptors are so named since their interactions with cargos and clathrin form the link between the clathrin coat and the cargo in

newly forming vesicles. Clathrin has been shown to be critical in AP-1 and AP-2 vesicle trafficking while, as described below in detail, clathrin's role in AP-3 trafficking has been controversial (Kirchhausen 1999; Kirchhausen 2000; Duden 2001; Conner and Schmid 2003; Pagano *et al.* 2004; Borner *et al.* 2006; Salazar *et al.* 2009). AP-3 has been shown to bind clathrin, but there are conflicting reports as to the enrichment of AP-3 in clathrin-coated vesicles (Pagano *et al.* 2004; Borner *et al.* 2006; Salazar *et al.* 2009). Despite the controversy of clathrin's role in AP-3 vesicle trafficking, research in mammalian cells described in this dissertation and elsewhere has demonstrated an interaction between AP-3 and clathrin and their presence in clathrin-coated vesicles (Borner *et al.* 2006; Salazar *et al.* 2009). Part of the theme developed in this dissertation is that a subset of AP-3-clathrin coated vesicles contains HOPS complex subunits and that this subset may be responsible for the traffic of HOPS subunits to the lysosomal target location.

Section 2.02 Clathrin: Plaques, and Vesicles

Clathrin molecules consist of three heavy chains and three light chains (Figure 6; Kirchhausen 2000; Conner and Schmid 2003). These six molecules form a three-legged structure known as the clathrin triskelion (Kirchhausen 2000; Conner and Schmid 2003). When several clathrin triskelion oligomerize they can arrange to form either a plaque at the membrane surface or a dome shaped scaffold that forms a shell at sites of vesicle budding (Figure 6; Kirchhausen *et al.* 1986; Schmid 1997; Kirchhausen 2000; Conner and Schmid 2003). In both clathrin plaques and domes clathrin triskelia assemble into

higher order quaternary structures with pentameric or hexameric patterns. In these polygons, the hubs of the clathrin triskelion organize into vertices while trans interactions between clathrin triskelion legs form lines linking the vertices (Figure 6; Kirchhausen *et al.* 1986; Schmid 1997; Ybe *et al.* 1998; Ybe *et al.* 1999; Kirchhausen 2000; Conner and Schmid 2003). A domain found in the proximal leg region of clathrin heavy chain, referred to as the clathrin repeat domain, is used to bind clathrin light chain with clathrin heavy chain (Kirchhausen *et al.* 1987; Nathke *et al.* 1992; Ybe *et al.* 1999; Kirchhausen 2000). The clathrin repeat domain also stabilizes clathrin cage assembly (Kirchhausen *et al.* 1987; Nathke *et al.* 1992; Ybe *et al.* 1999; Kirchhausen 2000).

Adaptor interactions with clathrin result in domed shells at vesicle formation sites. Mammalian adaptors AP-1, AP-2, and AP-3 directly interact with clathrin molecules (Kirchhausen 1999; Boehm and Bonifacino 2001). Adaptor interactions with clathrin result in: 1) a deformation of the donor compartment membrane culminating in vesicle budding and 2) the incorporation of cargoes into the budding vesicle (Figure 3; Bonifacino and Glick 2004). Scission proteins can then be recruited to the budding vesicle, pinching the vesicle from the donor compartment (Kirchhausen 2000). Scission of the vesicle from the donor compartment results in an adaptor/clathrin coated vesicle containing cargo specific for a target organelle.

Following vesicle scission at the donor organelle, the vesicle will undergo an uncoating step (Kirchhausen 2000). During uncoating, the same molecular switch, which recruited adaptors to the site of vesicle formation, ARF, undergoes GTP hydrolysis. ARF GTP hydrolysis results in the release of adaptors and coats

from the membrane (Figure 4; Behnia and Munro 2005). Uncoating results in exposure of the cytosolic regions of cargo proteins, which were previously interacting with coat proteins (Figure 3; Bonifacino and Glick 2004).

Section 2.03 SNAREs

One such cargo packaged into vesicles is the vesicular **SNA**P (Soluble NSF Attachment Protein) **RE**ceptor (**SNARE**; Figure 3, 7; Bonifacino and Glick 2004; Jahn and Scheller 2006). SNAREs are α-helical domain containing proteins (Hong 2005). SNAREs are often membrane anchored, though some cytosolic SNAREs localize to membrane surfaces through interactions with membrane attached SNAREs (Figure 7; Hong 2005). Cytosolically exposed α-helical regions of a SNARE at a vesicular surface (v-SNARE or R-SNARE) and two to three SNAREs at a target surface (t-SNAREs or Qa, Qb, and Qc SNAREs) will interact together to form a helical bundle (Figure 7; Fasshauer 2003; Brunger 2005; Hong 2005; Jahn and Scheller 2006). This interaction is referred to as a trans-SNARE pair. By preferentially binding into a specific trans-SNARE pair, the cell increases the fidelity of specific cargo delivery during fusion (Fasshauer 2003; Bonifacino and Glick 2004; Hong 2005; Jahn and Scheller 2006; Cai *et al.* 2007). Importantly, it has been noted that SNAREs can interact promiscuously leading to non-preferential binding partners and non-specific fusion events (Brunger 2005; Cai *et al.* 2007). The fidelity and specificity of SNARE pairing depends on target specific fusion machinery, such as the HOPS complex, which aid in the preferential binding of specific SNARE pairs and will be discussed in Section 2.04 (Cai *et al.* 2007). In mammalian lysosomal trafficking, v-SNAREs,

VAMP7 or VAMP8, will preferentially interact with t-SNAREs, VTI1b, Syntaxin7, and Syntaxin8, localized at the late endosome/lysosome (Hong 2005; Jahn and Scheller 2006).

Section 2.04 Rabs and Tethers

Before trans-SNARE interactions can be made, incoming vesicles must first be tethered to the target location (Bonifacino and Glick 2004; Cai *et al.* 2007; Brocker *et al.* 2010). Tethering is the initial distant interaction of target specific vesicles with the target membrane. Tethering complexes are either extended coiled coils or multi-subunit complexes. During vesicle tethering, tethering complexes interact with Rab proteins for the initial stages of vesicle fusion (Figure 3, 8; Bonifacino and Glick 2004; Cai *et al.* 2007; Stenmark 2009; Brocker *et al.* 2010). Rabs are small G-proteins of the ras superfamily that when activated interact with tethers resulting in the recognition of a target and aid in trans-SNARE pair recognition (Figure 3; Bonifacino and Glick 2004; Behnia and Munro 2005; Cai *et al.* 2007; Barr and Lambright 2010; Brocker *et al.* 2010). Tethering is accomplished in part by the localization of Rab proteins to specific target membranes (Pfeffer and Aivazian 2004; Behnia and Munro 2005). In mammals there are approximately 60 known Rabs with individual localization patterns, some ubiquitously expressed and some expressed in tissue dependant manner (Pereira-Leal and Seabra 2001; Pfeffer and Aivazian 2004; Behnia and Munro 2005; Barr and Lambright 2010). Rab GTPases are considered inactive in their GDP bound form and active in their GTP bound form (Figure 8; Pfeffer and Aivazian 2004; Behnia and Munro 2005; Stenmark 2009; Barr and Lambright

2010). In mammals, Rab7 (Stenmark 2009; Barr and Lambright 2010; Wang *et al.* 2011) and more recently Rab7b (Yang *et al.* 2004; Wang *et al.* 2007; Yao *et al.* 2009; Bucci *et al.* 2010; Progida *et al.* 2010) have been shown to localize at and regulate transport to late endosome/lysosomes. When Rabs are in an active GTP form they interact with effector tethering molecules (Figure 3, 8; Bonifacino and Glick 2004; Behnia and Munro 2005; Cai *et al.* 2007; Brocker *et al.* 2010). Following Rab GTP hydrolysis, tethering complexes aid in arrangement of SNAREs to form trans-SNARE pairs (Figure 3, 7; Cai *et al.* 2007; Stenmark 2009; Brocker *et al.* 2010). After trans-SNARE pair formation, the opposing membranes are brought into close proximity and fusion results (Figure 3, 7; Bonifacino and Glick 2004; Brunger 2005; Hong 2005; Jahn and Scheller 2006; Cai *et al.* 2007).

The tethering complex referred to as **HO**motypic fusion and **P**rotein **S**orting (**HOPS**) complex has been shown to be a critical Rab effector in lysosomal protein trafficking (Figure 3, 9; Cai *et al.* 2007; Nickerson *et al.* 2009; Brocker *et al.* 2010). In addition to effector activities for vacuolar Rab tethering, the HOPS complex subunits interact directly with SNAREs to ensure the proper assembly of vacuole specific SNARE pairs required for fusion at the vacuole (Figure 3; Eitzen *et al.* 2000; Price *et al.* 2000; Price *et al.* 2000; Sato *et al.* 2000; Seals *et al.* 2000; Poupon *et al.* 2003; Cai *et al.* 2007; Starai *et al.* 2008; Nickerson *et al.* 2009; Brocker *et al.* 2010; Wang *et al.* 2011). These functions seem to have been conserved as inferred from analysis of mutations in metazoans.

Section 3. Comparative Molecular and Cell Biology of the HOPS Complex

The HOPS complex was originally discovered in yeast *Saccharomyces cerevisiae*. Genetic screens in *S. cerevisiae* for mutant strains deficient in the localization of lysosomal enzymes revealed phenotypes in vacuole morphology (Banta *et al.* 1988; Robinson *et al.* 1988; Raymond *et al.* 1992; Wada and Anraku 1992). These mutants displayed lysosomal proteins in the extracellular media, representing a targeting defect of lysosomal proteins. The gene products that were missing or defective in these strains are referred to as the **v**acuole **p**rotein **s**orting (**Vps**) proteins (Raymond *et al.* 1992). Forty-one Vps mutant strains were discovered and classified as class A-F based on vacuolar characteristics and morphology (Banta *et al.* 1988; Raymond *et al.* 1992). Referenced in this dissertation are the classes A, B, C, and D Vps strains. The HOPS complex is composed of class B and C Vps proteins while the CORVET complex, a complex that will be discussed later in this dissertation, consists of class A, C and D Vps proteins.

The class B proteins Vps39 and Vps41 and the class C proteins Vps11, Vps16, Vps18, and Vps33 form a stable complex referred to as the HOPS complex (Figure 9; Nakamura *et al.* 1997; Seals *et al.* 2000; Wurmser *et al.* 2000). In the HOPS complex each of the class B and C subunits seems to play a specific role in the tethering, docking, and fusion stages of vesicle fusion (Figure 3). Importantly, it has been shown that homologues of HOPS Vps proteins in *S. cerevisiae* are present in higher eukaryotes (Pevsner *et al.* 1996; Warner *et al.* 1998; Caplan *et al.* 2001; Huizing *et al.* 2001; Kim *et al.* 2001). Furthermore, in higher eukaryotes

the Vps33 subunit appears to have two homologues in the genome (Huizing *et al.* 2001). In mammals these homologues are referred to as Vps33a and Vps33b (Huizing *et al.* 2001). From *S. cerevisiae* to mammals the class C Vps subunits Vps11, Vps16, Vps18, and Vps33 in *S. cerevisiae* and Vps33a or Vps33b in higher eukaryotes make up the core subunits of the HOPS complex along with two class B subunits Vps39 and Vps41 (Raymond *et al.* 1992; Sato *et al.* 2000; Seals *et al.* 2000; Wurmser *et al.* 2000; Peplowska *et al.* 2007; Nickerson *et al.* 2009; Brocker *et al.* 2010; Ostrowicz *et al.* 2010; Wang *et al.* 2011). In addition, it was recently shown in *C. elegans* and mammalian models that a subset of the HOPS complex directly interacts with an additional complex member, SPE-39 (Zhu *et al.* 2009).

Recently, the interaction of the four core class C Vps subunits with the class A subunit Vps8 and class D subunit Vps3 has been shown to form a distinct complex referred to as the class **C** c**OR**e **V**acuole/**E**ndosome **T**ethering (**CORVET**) complex (Figure 9; Raymond *et al.* 1992; Peplowska *et al.* 2007; Nickerson *et al.* 2009; Brocker *et al.* 2010). While the HOPS complex has been shown to be responsible for tethering and fusion events at the late endosome/lysosome, less is known about the role of the CORVET complex. Studies have suggested a role for the CORVET complex in Golgi to endosome traffic or in retrograde traffic from the vacuole to the Golgi (Peplowska *et al.* 2007; Markgraf *et al.* 2009; Nickerson *et al.* 2009; Brocker *et al.* 2010). Furthermore, there are conflicting reports that the CORVET complex can act as a tethering complex, like the HOPS complex (Peplowska *et al.* 2007; Markgraf *et al.* 2009; Nickerson *et al.* 2009; Brocker *et al.* 2010). Despite these conflicting

reports, the CORVET subunit Vps8 has been shown to interact with the endosomal Rab5 homologue Vps21 in *S. cerevisiae* (Markgraf *et al.* 2009; Nickerson *et al.* 2009; Brocker *et al.* 2010). To date, the CORVET complex has only been identified in *S. cerevisiae*.

Two recent papers suggest how the HOPS subunits interact to form a complex based on genetic and biochemical interactions in *S. cerevisiae* (Ostrowicz *et al.* 2010; Plemel *et al.* 2011). In this organizational model, the class C subunits reside as an extended core of the complex with Vps33 interacting with membrane SNAREs while the class B subunits Vps39 and Vps41 interact with the Rab7-GTPase (Figure 9; Ostrowicz *et al.* 2010; Plemel *et al.* 2011).

The proposed organization for HOPS complex subunits makes sense considering the known domains and tethering functions of individual subunits. One of the core subunits, Vps33 and its higher eukaryotic homologues Vps33a and Vps33b, are members of the Sec1/Munc18 protein family (Gissen *et al.* 2005). The Sec1/MUNC18 domain is known to bind Q-SNAREs or t-SNAREs (Figure 7; Jahn and Sudhof 1999; Waters and Hughson 2000; Cai *et al.* 2007). The class C Vps33 subunit is known to bind the SNAREs vam3p and vam7p in *S. cerevisiae* (Seals *et al.* 2000; Laage and Ungermann 2001; Wang *et al.* 2001; Stroupe *et al.* 2006). Interactions of Vps33 with SNAREs through the Sec1/MUNC18 domain may have a role in the recognition of target-specific SNAREs and aid in trans-SNARE pairing or inhibit non-specific trans-SNARE pairs (Figure 7). The class B HOPS subunits Vps39 and Vps41 have specificity for interactions with the Rab7 *S. cerevisiae* homologue Ypt7 (Figure 8). In *S. cerevisiae*, the HOPS subunit Vps39 has been shown to interact with Ypt7 and act

as the GEF for the vacuolar Rab (Figure 8; Wurmser *et al.* 2000) though more recent studies *in vivo* suggest Vps39 is not the Rab7 GEF (Peralta *et al.* 2010; Angers and Merz 2011). The subunit Vps41 has also been shown to directly bind Ypt7 and act as the effector for Ypt7 in vacuolar tethering events (Figure 8; Brett *et al.* 2008; Nickerson *et al.* 2009).

In addition to interactions with the Rab7 homologue, Vps41 has also been shown to interact with the vesicle coat protein and adaptor AP-3 in *S. cerevisiae* (Rehling *et al.* 1999; Darsow *et al.* 2001; Angers and Merz 2009; Angers and Merz 2011). In contrast with my data, *S. cerevisiae* studies suggest that the interaction between Vps41 and AP-3 is occurring at the fusion site and not at sites of vesicle formation (Figure 10; Angers and Merz 2009; Angers and Merz 2011). The difference in these two sets of findings may occur due to fundamental differences in *S. cerevisiae* versus mammalian AP-3 vesicle trafficking pathways and will be discussed further in Section 6.

All subunits of the HOPS complex, except Vps33, share similar secondary structure with an amino terminal β-propeller and an α-solenoid toward the carboxy terminus (Figure 11; Nickerson *et al.* 2009; Dokudovskaya *et al.* 2011; Plemel *et al.* 2011). Interestingly, this general structure is also found in the vesicle coat protein clathrin heavy chain (ter Haar *et al.* 1998; Kirchhausen 2000; Devos *et al.* 2004; Dokudovskaya *et al.* 2011). The HOPS subunits Vps41, Vps39, Vps18, and Vps11 also contain the clathrin repeat domain found in clathrin heavy chain (Figure 11; Radisky *et al.* 1997; Rehling *et al.* 1999; Ybe *et al.* 1999; Darsow *et al.* 2001; Huizing *et al.* 2001). As mentioned in Section 2.02, clathrin repeat domains are utilized for clathrin interaction between heavy and light chain and

interactions of clathrin triskelions with each other to form clathrin cages (Nathke *et al.* 1992; Ybe *et al.* 1999; Kirchhausen 2000). The function of clathrin repeats in HOPS complex subunits is not fully understood and will be discussed further in Chapter 3 of my dissertation.

In the next section I will describe four current models of membrane trafficking and how the molecular components described in Sections 2 and 3 are utilized in the specific localization of proteins to the lysosome.

Section 4. Models of Membrane Trafficking Pathways Along the Endocytic Route

My dissertation tested the hypothesis that cytosolic class B and C Vps HOPS subunit localization is regulated in clathrin-dependent traffic mechanisms. Two main models of endosomal membrane trafficking pathways have been suggested for eukaryotic cells:

1) Vesicle mediated (Figure 12)

2) Maturation (Figure 12)

As will be described in this section, the vesicle mediated and maturation models of membrane trafficking describe a set of steps used for localizing membranes and proteins along a series of organelles. The actual mechanisms of protein localization can most likely be explained by a combination of these models, though specific cell types or cargoes may preferentially utilize one mechanism over another.

Section 4.01 Vesicular Model of Membrane Traffic to the Lysosome

One model of trafficking to the lysosome is through a vesicle mediated trafficking pathway that requires coats such as clathrin, AP-1, and AP-3. Vesicle mediated mechanisms are thought to be parallel to an endosome maturation pathway (Figure 12; Stoorvogel *et al.* 1996; Peden *et al.* 2004; Meel and Klumperman 2008). In the vesicular model lysosomal proteins are sorted into vesicles at early stages of endosomal traffic and transported directly to the late endosome/lysosome (Meel and Klumperman 2008). As will be discussed in Section 4.02, this model differs from the maturation model where lysosomal proteins progressively concentrate in endosomal compartments. The composition of membrane and lumenal contents of endosomes defines the organelle as either an early, recycling, late endosome, and lysosome (Meel and Klumperman 2008).

In the vesicle-mediated model, lysosomal-destined cargoes are recruited into membrane budding profiles at donor organelles where vesicle formation will occur (Figure 3). In *S. cerevisiae* the adaptors AP-1 and AP-3 are important for Golgi derived vesicle formation (Phan *et al.* 1994; Stepp *et al.* 1995; Cowles *et al.* 1997; Piper *et al.* 1997; Stepp *et al.* 1997; Angers and Merz 2009). In mammals, evidence supports a role for AP-1 in Golgi derived vesicle formation for delivery to the lysosome, and AP-1 and AP-3 in early endosome derived vesicle formation to the lysosome (Ghosh and Kornfeld 2004; Peden *et al.* 2004; Robinson 2004; Meel and Klumperman 2008). Once vesicles are formed they are transported directly to the late endosome/lysosome (Figure 12). Most models of vesicle trafficking postulate that vesicles are uncoated soon after the formation stage (Figure 3). However, recent evidence from *S. cerevisiae* suggests that AP-3

derived vesicles remain coated or partially coated until reaching the vacuolar target (Figure 10; Angers and Merz 2009; Angers and Merz 2011).

Once vesicles arrive at the lysosome, the HOPS complex provides a platform for a series of molecular interactions with lysosome-specific Rabs and SNAREs required for fidelity of fusion (Figure 3; Cai *et al.* 2007; Nickerson *et al.* 2009; Brocker *et al.* 2010; Wang *et al.* 2011). Target-specific v-SNAREs are recruited to vesicle formation sites and included onto newly forming vesicles at the vesicle formation step (Figure 3). Lysosomal SNARE interaction with HOPS tethering complex in turn defines that vesicle contents will be delivered to the lysosome.

An important point to consider is that the vesicular model follows the canonical vesicle traffic mechanism with two major steps, vesicle formation and subsequent fusion. With one exception (Angers and Merz 2009; Angers and Merz 2011), the canonical view of vesicle trafficking emphasizes that vesicle formation machinery is predominantly localized to formation site, while vesicle fusion machinery remains at the fusion site (Figure 2A and 3; Bonifacino and Glick 2004). In the canonical model, these steps are not only molecularly distinct but also physically segregated. My studies establish the alternate possibility that in mammalian cells, HOPS fusion machinery and vesicle coat machinery such as AP-3 and clathrin are molecularly and spatially associated. In contrast with data from Angers *et al.*, I provide evidence that the HOPS complex subunits are localized to sites of vesicle formation and interact with vesicle formation machinery (Figure 2B). Furthermore, I demonstrate that localization of HOPS complex subunits to a late endosome/lysosome is regulated by clathrin-coated

vesicle formation machinery (Figure 2B). My results provide a novel way of considering the spatial and temporal organization vesicle formation and fusion machineries in eukaryotic cells.

Section 4.02 Maturation Model of Lysosome Traffic

In contrast to the vesicular trafficking model, the maturation model is characterized by lysosomal cargoes which remain at the endosomal compartment while proteins associated with the cytosolic leaflet of the limiting membrane are exchanged over time, resulting in the enrichment of lysosome specific proteins over time (Figure 12; Meel and Klumperman 2008; Segev 2010). In the maturation model, vesicles change the integral membrane protein content that defines the characteristics of early and late endosomes (Meel and Klumperman 2008).

Early, and late endosomes/lysosomes are characterized in part by the localization of Rab5 and Rab7 respectively, and the number of intralumenal vesicles (Figure 12 and 13; Mari *et al.* 2008; Meel and Klumperman 2008; Segev 2010). The exchange of Rabs at these endosomes is a key feature of the maturation model (Behnia and Munro 2005; Del Conte-Zerial *et al.* 2008; Segev 2010; Wang *et al.* 2011). For instance, as an endosome loses Rab5 and accumulates Rab7 it has matured from an early to recycling endosome. In mammals Mon1 and *C. elegans* SAND1 exchange early endosomal Rab5 for the late endosomal Rab7 allowing for maturation from early endosomes to late endosomes/lysosomes (Figure 13; Brocker *et al.* 2010; Poteryaev *et al.* 2010). Furthermore, HOPS complex subunits have also been shown to interact with

Mon1/SAND1 (Figure 13; Poteryaev *et al.* 2010). Since Rabs only interact with specific tethers and thus form specific trans-SNARE pairs, by changing the Rab content endosomes also change the specificity of membrane fusion (Cai *et al.* 2007; Brocker *et al.* 2010).

Section 5. The Importance of the HOPS Complex for Lysosome and Lysosome-Related Organelle Function

As mentioned in Section 1, there are a large number of human diseases resulting from the mislocalization of lysosomal proteins (Gieselmann 1995; Aridor and Hannan 2000). In this section I will introduce the human syndromes that reveal an importance for HOPS subunits in regulating lysosome and lysosome-related organelle morphology and function. Syndromes discussed include: **A**rthrogryposis, **R**enal Dysfunction and **C**holestasis (**ARC**), **H**ermansky **P**udlak **S**yndrome (**HPS**). Additionally, reports indicate the regulation of Notch signaling and a *C. elegans* model of Parkinson's disease through HOPS subunits (Wilkin *et al.* 2008; Ruan *et al.* 2009).

The foundational screens for vacuolar trafficking defects in yeast provided the first evidence for involvement of subunits of the HOPS complex in lysosomal formation (Banta *et al.* 1988; Robinson *et al.* 1988; Raymond *et al.* 1992; Wada and Anraku 1992). Since then, *S. cerevisiae* studies have been instrumental in understanding the important role HOPS subunits have in regulating tethering, SNARE pair formation, and fusion of lysosomaly destined membrane with the lysosomal compartment (see Section 3 for detailed review; Banta *et al.* 1988; Banta *et al.* 1990; Raymond *et al.* 1992; Wada and Anraku 1992; Cowles *et al.*

1997; Nakamura *et al.* 1997; Gerhardt *et al.* 1998; Rehling *et al.* 1999; Sato *et al.* 2000; Seals *et al.* 2000; Wurmser *et al.* 2000; Darsow *et al.* 2001; Laage and Ungermann 2001; Mcvey Ward 2001; Wang *et al.* 2002; Iwaki *et al.* 2003; Poupon *et al.* 2003; Wang 2003; Bugnicourt *et al.* 2004; LaGrassa and Ungermann 2005; Starai *et al.* 2008; Angers and Merz 2009; Cabrera *et al.* 2009; Hickey *et al.* 2009; Nickerson *et al.* 2009; Brocker *et al.* 2010; Ostrowicz *et al.* 2010; Wang *et al.* 2011).

The human syndrome, ARC, results from mutations to the gene responsible for encoding the class C HOPS subunit Vps33b, or HOPS interacting protein SPE-39 (Gissen *et al.* 2006; Cullinane *et al.* 2010). Patients with ARC syndrome display a variety of symptoms including severe contracture of joints referred to as arthrogryposis, defects in renal function, reduced flow of bile from the liver referred to as cholestasis, bleeding disorders, dry scaly skin referred to as ichthyosis, defects in metabolic absorption, an absence or severe decrease in size of the corpus callosum which is the brain region bridging the left and right hemispheres, and defects in neuronal pathfinding in the anterior horn of the spinal cord (Horslen *et al.* 1994; Abdullah *et al.* 2000; Denecke *et al.* 2000; Eastham *et al.* 2001; Howells and Ramaswami 2002; Gissen *et al.* 2004; Hayes *et al.* 2004; Abu-Sa'da *et al.* 2005; Choi *et al.* 2005; Tekin *et al.* 2005; Bull *et al.* 2006; Gissen *et al.* 2006; Parsch and Pietrzak 2007; Taha *et al.* 2007; Hershkovitz *et al.* 2008; Arhan *et al.* 2009; Cullinane *et al.* 2009; Jang *et al.* 2009; Cullinane *et al.* 2010; Kim *et al.* 2010; Jang *et al.* 2011). Neurological defects in arthrogryposis in addition to the reports of patients with an absence or severe decrease in size of the corpus callosum (Abdullah *et al.* 2000; Denecke *et*

al. 2000; Eastham *et al.* 2001; Hayes *et al.* 2004; Gissen *et al.* 2006) and defects in neuronal pathfinding in the anterior horn of the spinal cord (Di Rocco *et al.* 1995; Hayes *et al.* 2004) strongly suggest a role for Vps33b/SPE-39 in development and maintenance of neuronal cells. Importantly, severe joint contracture in patients with ARC syndrome results from a neurological defect as opposed to muscular defects (Eastham *et al.* 2001; Hayes *et al.* 2004; Gissen *et al.* 2006). Furthermore, analysis of ichthyosis in ARC patients suggests defects in lamellar granular secretion in epithelial cells as shown by increased amounts of lamellar granules in patients with ARC syndrome by electron microscopy (Choi *et al.* 2005; Bull *et al.* 2006; Hershkovitz *et al.* 2008). Microscopic analysis of kidney and liver cells show a loss of targeted apical protein distribution to the entirety of the plasma membrane (Gissen *et al.* 2004). Also, microscopic analysis of liver hepatocytes shows increased intracellular bile containing organelles, suggesting defects in bile secretion, which could lead to cholestasis phenotypes (Gissen *et al.* 2004). A zebrafish model of ARC syndrome has been used to study cholestasis phenotypes (Matthews *et al.* 2005). Vps33b knockdown in zebrafish also lead to an increase in cytoplasmic vesicles in hepatocytes, also suggesting of defects in bile secretion from these cells (Matthews *et al.* 2005). Cholestasis symptoms might also be the result of bile secretion due to a decrease in the number of bile ducts (Matthews *et al.* 2005). Matthews *et al.* suggest that the build up of cytoplasmic vesicles in developing hepatocytes might lead to cell death resulting in the decreased number of bile ducts observed (Matthews *et al.* 2005). All of these phenotypes are consistent with a role for Vps33b/SPE-39 in regulation of lysosomes, lysosome-related organelles, and secretion in polarized

mammalian cell types. Knockdown of zebrafish Vps33b causes bile secretory defects that are remarkably similar to human patients with ARC syndrome, suggesting that this model system will be useful for functional studies (Matthews *et al.* 2005).

Another human syndrome associated with mutations in subunits of the HOPS complex is HPS. HPS is characterized by a decrease in pigmentation of the skin, hair, and eyes, prolonged bleeding, and pulmonary fibrosis (Hermansky and Pudlak 1959; Bonifacino 2004; Pierson *et al.* 2006). Symptoms of HPS arise from defects in function and sorting to lysosomes and lysosome-related organelles such as the melanosome and platelet dense granules (Huizing *et al.* 2002; Bonifacino 2004; Pierson *et al.* 2006). In humans, a subset of HPS patients display mutations of the gene encoding the β subunit of AP-3 (Huizing *et al.* 2002; Bonifacino 2004; Pierson *et al.* 2006). Models for HPS have been discovered in mouse and Drosophila (Huizing *et al.* 2002; Bonifacino 2004; Pierson *et al.* 2006). In mice, mutations to the genes that encode AP-3β1 AP-3δ1 and the class C HOPS subunit Vps33a result in decreased coat color, prolonged bleeding, and defects in lysosomal protein targeting (Swank *et al.* 1998; Huizing *et al.* 2002; Suzuki *et al.* 2003; Bonifacino 2004). Drosophila homologues of class B and C HOPS subunits Vps33a, Vps41, Vps18, and adaptor protein AP-3β1 result in defects of lysosome and pigment granule trafficking (Shestopal *et al.* 1997; Warner *et al.* 1998; Mullins *et al.* 1999; Sevrioukov *et al.* 1999; Mullins *et al.* 2000; Huizing *et al.* 2002; Akbar *et al.* 2009). These studies provide further evidence for the importance of HOPS complex subunits in delivery, maintenance, and function of proteins to lysosomes and lysosome-related organelles.

ARC and HPS have well characterized symptoms with several studies showing an importance of HOPS subunits in lysosome, lysosome-related organelles, and secretion defects. Here I will describe less well characterized models for HOPS subunits involvement in Notch signaling and a *C. elegans* model of Parkinson's disease. Parkinson's disease is primarily characterized by the loss of dopaminergic neurons with the presence of Lewy bodies (Braak and Braak 2000). Lewy bodies are cytoplasmic inclusions composed primarily of the protein α-synuclein. α-synuclein in Lewy bodies is misfolded and accumulates in part due to decreased degradation (Braak and Braak 2000; Venda *et al.* 2010). An RNAi screen for increased misfolded α-synuclein in *C. elegans* yielded 20 candidates, one of which being the class B HOPS subunit Vps41 (Hamamichi *et al.* 2008). Following this study, Ruan *et al.* showed that expression of human HOPS subunit Vps41 in *C. elegans* and the human neuroblastoma cell line, SH-SY5Y, had a protective effect on dopaminergic neurons under toxic conditions (Ruan *et al.* 2009). Ruan *et al.* suggest that Vps41 provides a protective effect against α-synuclein accumulation through degradation of misfolded or aggregated proteins (Ruan *et al.* 2009) providing further evidence for the importance of HOPS subunits in lysosomal degradation.

A study by Wilkin *et al.* in 2008 suggested a role for HOPS subunits in regulating the degradation of the cell signaling molecule, Notch (Wilkin *et al.* 2008). Notch signaling is critical for embryonic development of neural and epidermal cell fates and neuromuscular junction plasticity (Lai 2004; de Bivort *et al.* 2009). Notch is a transmembrane receptor found at the plasma membrane (Lai 2004). In a canonical Notch signaling pathway, Notch binds extracellular

ligands and undergoes cleavage at the extra- and intracellular domains (Figure 14; Lai 2004; Wilkin *et al.* 2008). Following cleavage, the intracellular domain is transported to the nucleus where it undergoes interaction with transcription factors for gene expression (Lai 2004; Wilkin *et al.* 2008). Wilkin *et al.* also describe an endosomal pathway for Notch activation where Notch receptor is internalized prior to activation and recruited away from the degradation pathway (Wilkin *et al.* 2008). Following internalization, Notch undergoes inclusion into intralumenal vesicles in multi-vesicular bodies where the entire receptor will be degraded (Figure 14; Wilkin *et al.* 2008). However Wilkin *et al.* also describe an alternative path where Notch is sorted away from intralumenal vesicles, possibly through interaction with AP-3, and remains at the endosomal limiting membrane (Figure 14; Wilkin *et al.* 2008). At the lysosome, the lumenal domain of Notch is degraded while retaining the intracellular domain (Wilkin *et al.* 2008). The intracellular domain then undergoes cleavage releasing the intracellular domain for translocation to the nucleus (Lai 2004; Wilkin *et al.* 2008). The proposed role of HOPS subunits in this study was in regulating the fusion of late endosomes with the lysosome (Wilkin *et al.* 2008). An interesting alternative is that AP-3 interactions with HOPS complex subunits may be regulating the ability of Notch receptor to be sorted away from intralumenal vesicles in a maturation model of lysosome trafficking. Regardless of these two possibilities, this study shows the importance of HOPS subunits in regulating a key molecule for development. A HOPS dependent mechanism of Notch regulation could provide an explanation for severe neuronal and neuromuscular symptoms in patients with ARC syndrome.

As mentioned, HOPS complex is a lysosomal/late endosomal fusion protein that orchestrates Rab activation, SNARE pairing, and fusion. One can postulate that perturbations to the function and/or localization of HOPS subunits could lead to severe consequences for lysosome trafficking. If HOPS function was compromised, lysosome targeted membranes/cargoes may be unable to localize to the lysosome. Or, in the absence or mislocalization of HOPS proofreading mechanisms for trans-SNARE pairs may not be properly orchestrated and lysosome could inappropriately fuse with other organelles leading to abnormal degradation. These possibilities could be occurring in cells containing mutations of genes encoding HOPS subunits in ARC and HPS and are discussed in detail in Chapter 3. In my dissertation, I address the localization of HOPS complex on membrane compartments found along the endocytic route in mammalian cells and how perturbations of this pathway by altering coat dynamics lead to the mislocalization of HOPS subunits.

Section 6. Comparison of *S. cerevisiae* and Mammalian HOPS Localization Mechanisms and the Central Contribution of This Dissertation

As mentioned in Section 3, the class B and C Vps subunits that make up the HOPS complex were originally discovered in *S. cerevisiae* aided by the use of genetic and biochemical techniques (Banta *et al.* 1988; Robinson *et al.* 1988; Raymond *et al.* 1992; Wada and Anraku 1992). Fine detail has been revealed about HOPS complex conservation and individual roles and interactions of HOPS Vps subunits in Rab GTPase activity and SNARE pair formation at the vacuole

(Eitzen *et al.* 2000; Price *et al.* 2000; Price *et al.* 2000; Sato *et al.* 2000; Seals *et al.* 2000; Poupon *et al.* 2003; Cai *et al.* 2007; Starai *et al.* 2008; Nickerson *et al.* 2009; Brocker *et al.* 2010; Wang *et al.* 2011). However, based on the nature of early experimentation in *S. cerevisiae,* the complete localization of HOPS complex was unknown. Immunofluorescent localizations of HOPS subunits in *S. cerevisiae* showed HOPS to be primarily localized to a vacuolar compartment (Nakamura *et al.* 1997; Wang *et al.* 2002; Wang 2003; LaGrassa and Ungermann 2005; Angers and Merz 2009; Cabrera *et al.* 2009). HOPS complex subunits have not been identified at the Golgi or endosomal intermediates in *S. cerevisiae* (Angers and Merz 2009). Thus it was largely assumed that in *S. cerevisiae*, cytosolic HOPS complex cycled on and off the membrane at the site of activity (Figure 2). The HOPS complex presumably localized to the vacuole in response to either interactions with Rabs or SNAREs at the vacuole or with changes in membrane lipid composition (Figure 2A, 3).

Early experiments of HOPS localization by Richardson *et al.* in mammalian cells yielded a strikingly different localization of HOPS subunits compared to *S. cerevisiae* cells (Richardson *et al.* 2004). In mammalian cells, subunits of the HOPS complex were found to colocalize highly with early endosomal markers but only minimally with late endosomal and lysosomal markers (Richardson *et al.* 2004). Despite the localization data in mammalian models, biochemical data still suggested HOPS complex subunits interacted with late endosomal and lysosomal fusion proteins such the SNARE syntaxin-7 (Kim *et al.* 2001) and as a Rab7 effector (Caplan *et al.* 2001) suggesting a role of HOPS complex subunits in late endosome/lysosome fusion events. These observations

raised the proposition that HOPS localization in higher eukaryotic organisms required a more complex model of localization to late endosomes/lysosomes than was previously appreciated in *S. cerevisiae* or that yeast was not a useful model organism for resolving additional details. I hypothesized that HOPS subunits are recruited to early endosomes and trafficked through the vesicle trafficking and/or maturation models pathways to arrive at the target destination, the late endosome/lysosome (Figure 2B).

One possibility in discrepancies between *S. cerevisiae* HOPS localization primarily to the vacuole and the mammalian punctate distribution of HOPS at endosomal compartments could result from fundamental differences in *S. cerevisiae* membrane trafficking pathways and mammalian membrane trafficking pathways. In *S. cerevisiae*, the adaptor AP-3 recruits cargoes at the Golgi compartment for inclusion into vesicles that will be targeted to the vacuole, whereas in mammals, AP-3 functions at the early endosomal for vesicle formation (Cowles *et al.* 1997; Piper *et al.* 1997; Stepp *et al.* 1997; Peden *et al.* 2002; Robinson 2004). Studies of clathrin and AP-3 in *S. cerevisiae* indicate that clathrin neither binds AP-3 nor is clathrin required for the proper localization of proteins in trafficking pathways (Cowles *et al.* 1997; Stepp *et al.* 1997; Vowels and Payne 1998). However in mammals, clathrin does bind AP-3 and clathrin-coated vesicles contain AP-3 subunits (Dell'Angelica *et al.* 1998; Peden *et al.* 2004; Theos *et al.* 2005; Borner *et al.* 2006; Salazar *et al.* 2009).

Early studies of HOPS subunits suggested interaction of AP-3 with the HOPS subunit Vps41 (Rehling *et al.* 1999; Darsow *et al.* 2001). Direct interaction of the ear domain of AP-3 with Vps41 was presumed to occur in the context of the

Vps subunit and not with the entire HOPS complex (Rehling *et al.* 1999; Darsow *et al.* 2001). Furthermore, isolation of AP-3 vesicles from *S. cerevisiae* did not show coat structures by electron micrography nor was a clathrin identified by immunoblot of isolated AP-3 vesicles (Rehling *et al.* 1999). These data along with data showing similar secondary structure of Vps41 with clathrin and the clathrin repeat domain in Vps41 primary structure lead to a model where AP-3 vesicles would require Vps41 as a coat complex in lieu of clathrin (Figure 11; Darsow *et al.* 2001). However, recent evidence in *S. cerevisiae* indicates HOPS subunits do not localize to Golgi compartments where AP-3 vesicle formation occurs, nor could they be found to localize to AP-3 vesicular intermediates (Angers and Merz 2009). Instead, a model was suggested where coated or partially coated AP-3 vesicles docked at the vacuole interact with HOPS through the subunit Vps41 (Figure 10; Angers and Merz 2009). However, in mammals the AP-3 delta ear domain is known to bind clathrin (Dell'Angelica *et al.* 1998; Kirchhausen 1999) and as Richardson *et al.* show and in my studies I found HOPS to localize to early endosomes (Richardson *et al.* 2004) where AP-3 vesicle formation occurs (see Chapter 2; Peden *et al.* 2004; Robinson 2004).

Taken together, the fundamental differences in membrane trafficking pathways and localization of HOPS subunits in *S. cerevisiae* and mammalian cells suggested that the localization of HOPS to the lysosome in higher order eukaryotes was more complex than suspected in *S. cerevisiae*. Based on these differences, I proposed a model where HOPS complex subunits are recruited to vesicular membrane trafficking pathways in a coat-dependent manner for the specific localization to the target organelle (Figure 2B). This novel model of

HOPS targeting makes several fundamental contributions to the trafficking mechanisms among membrane bound compartments:

1) It provides a novel coat-dependent modality of localization for the HOPS complex subunits along the endocytic route.
2) It provides the first evidence of a molecular integration of the vesicle budding and vesicle fusion processes by early interactions between coats and cytosolic tethers.
3) It describes a principle by which organelle specific tethers are selectively localized to specific compartments
4) It describes a mechanism for long range delivery of tethers in polarized cells and cells of complex architectures such as neurons.

Section 7. Figures

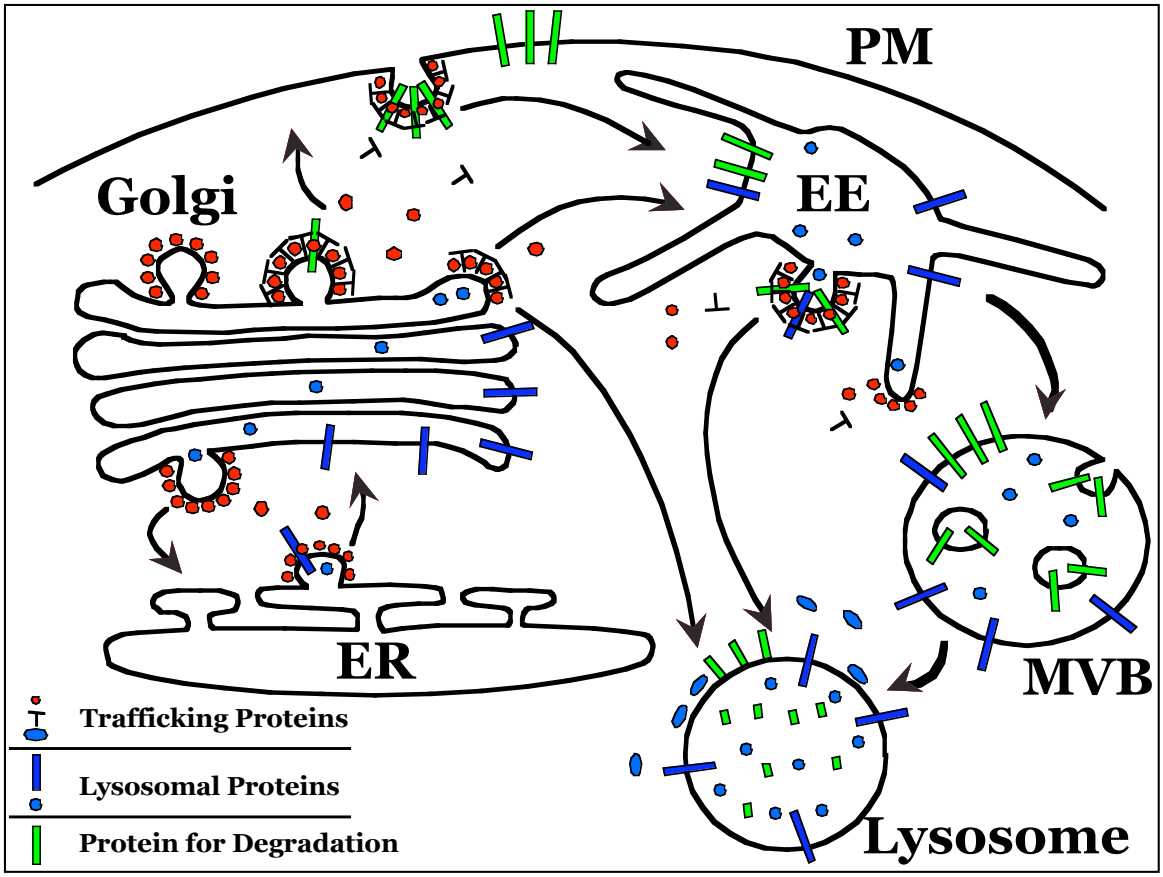

Figure 1. Organelles Maintain Unique Protein Compositions Through Protein Localization and Membrane Transport Mechanisms

Organelles maintain separate lumenal and cytoplasmic environments using membrane bilayers. The unique makeup of each organelle provides the organelle with a distinct identity and provides cellular functionality. Cytosolic vesicle proteins (red circle, black T, and blue oval) localize to the membrane surface of organelles. Lysosomal degradation enzymes (blue circle, blue line) are packaged into vesicles and trafficked to the lysosome as lumenal cargoes or progress through endosome-lysosome maturation. Membrane proteins for lysosomal degradation (green lines) are trafficked through the endocytic maturation

pathway and can be included into lumenal vesicles in the multi-vesicular body for degradation. ER, Endoplasmic Reticulum, PM, Plasma Membrane, EE, Early Endosome, MVB, Multi-Vesicular Body/Late Endosome.

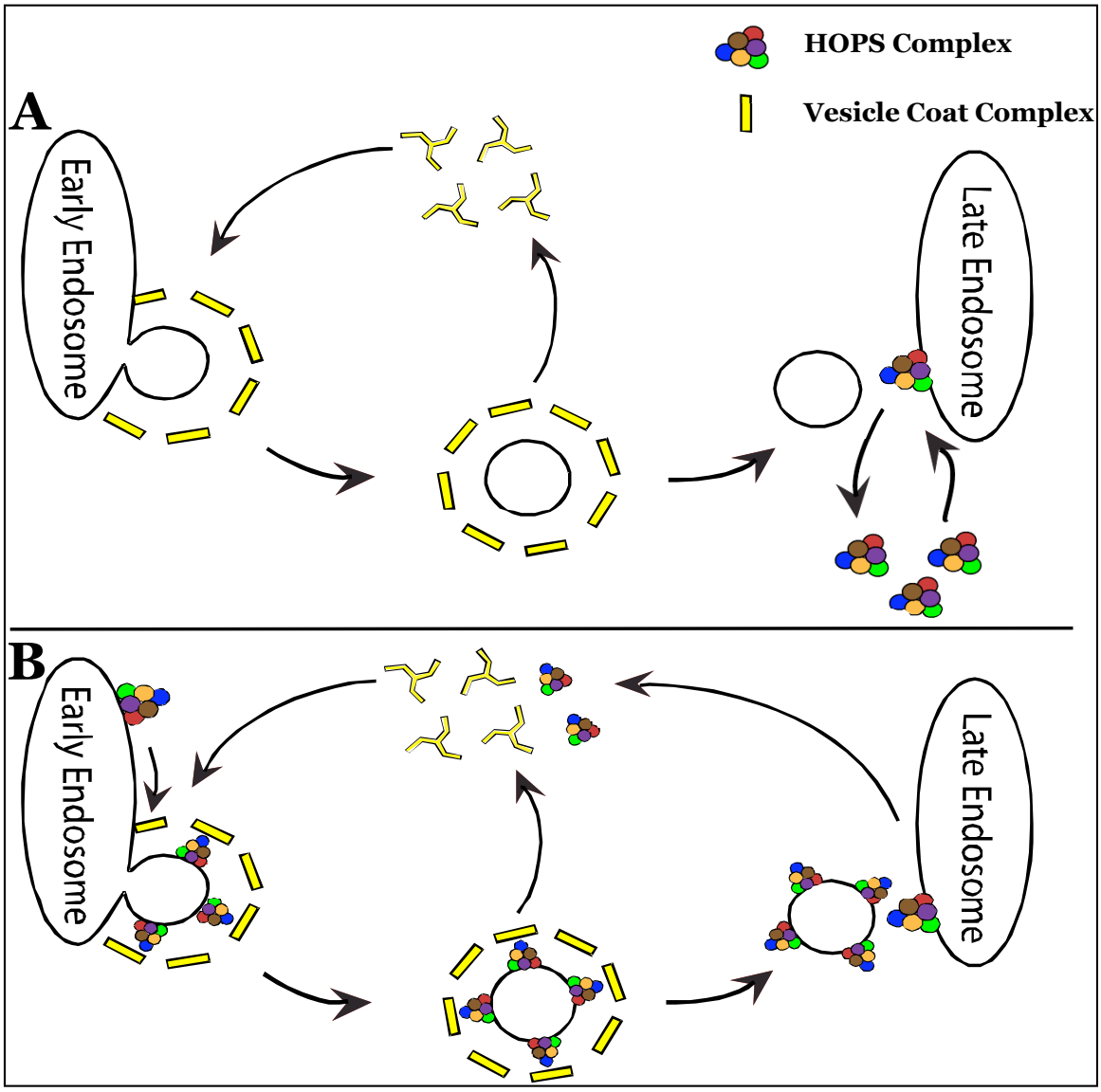

Figure 2. Two Models of HOPS Complex Localization to a Late Endosome/Lysosome Compartment

A) In one model of HOPS complex localization, cytosolic HOPS complexes cycle on and off of the membrane in response to incoming vesicle fusion activity or changes in lipid content at the lysosome. B) In a second model of HOPS complex localization, HOPS complex subunits are incorporated into newly forming vesicles and localized along vesicle trafficking pathways. This second model of

HOPS localization provides lysosomal identity through out the entire vesicle trafficking process by retaining HOPS fusion machinery at all stages. The hypothesis for this dissertation follows with this later model of HOPS subunit localization.

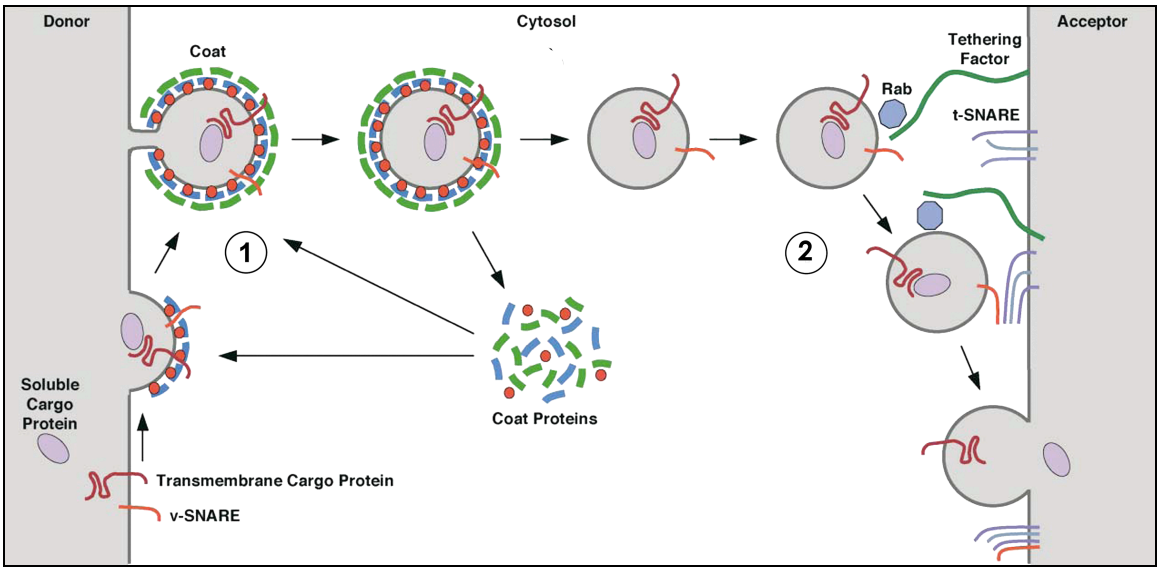

Figure 3. Model of Vesicle Transport

Vesicles carry lumenal and membrane cargoes from donor organelles to acceptor organelles. Vesicle transport is frequently thought of in two distinct steps: 1) cargo recruitment and vesicle formation, and 2) vesicle docking and fusion. 1) Membrane cargoes, including vesicular v-SNAREs, are recruited to patches where vesicle formation will occur. ARFs (red circle) when activated localize to the donor compartment and lead to recruitment of adaptor proteins (blue rectangle) like AP-1 to -4 and coat proteins (green rectangle) such as clathrin. Membrane curvature results in vesicle formation and the scission of the vesicle from the donor compartment. Following vesicle scission, vesicle formation machinery such as ARFs, adaptors, and clathrin are released to the cytosol where they can be recycled back to other vesicle formation events. 2) When vesicles containing Rabs come into close proximity with the acceptor organelle, tethering factors are activated initiating vesicle tethering. Importantly, Rabs preferentially interact with acceptor organelle tethering factors resulting in specific fusion

events only at the target organelle. Tethering results in the close association of vesicle and donor organelle to allow trans-SNARE pairing to occur between the v-SNARE and target t-SNAREs. Following trans-SNARE pairing, vesicle and acceptor membranes come into close contact resulting in vesicle fusion. As the vesicle fuses, lumenal proteins in the vesicle are released into the lumen of the target organelle and vesicular membrane cargo is incorporated into the acceptor organelle. Figure modified from (Bonifacino and Glick 2004).

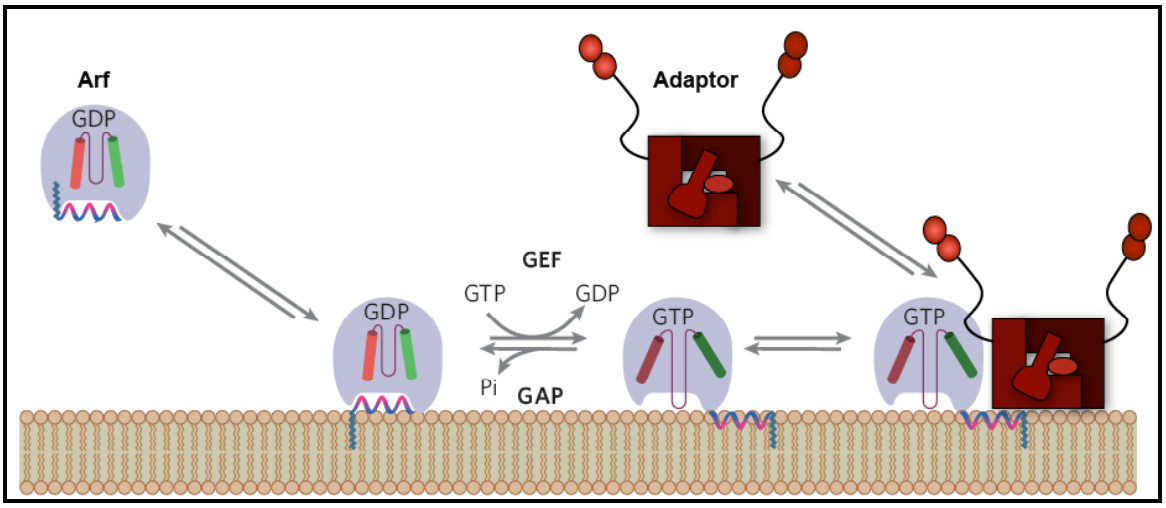

Figure 4. GTP-ARF Recruits Cytosolic Adaptor to Membranes

ADP **R**ibosylation **F**actor, **ARF**, is a small GTPase of the ras superfamily of GTPases. GDP-ARF is recruited to the donor membrane surface and undergoes exchange of a GDP for a GTP through interactions with a Guanosine Exchange Factor (GEF). In its active GTP form, ARF leads to the recruitment of adaptor proteins from the cytosol. Figure modified from (Behnia and Munro 2005).

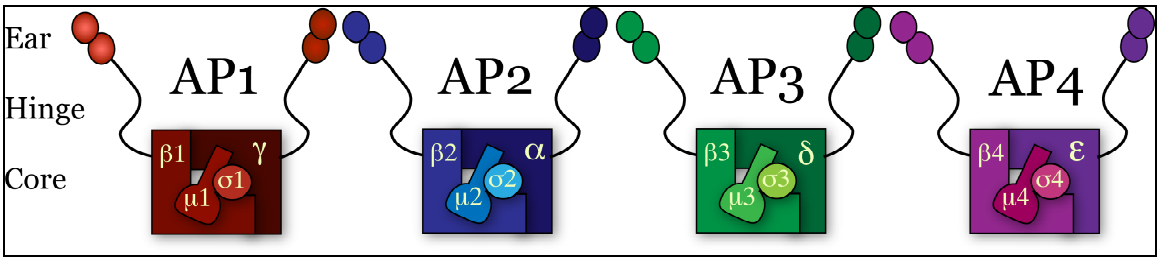

Figure 5. Adaptor Protein Complexes

Tetrameric adaptor proteins AP-1, AP-2, AP-3, and AP-4. Each complex has an adaptor specific small σ subunit, medium μ subunit, large β subunit, and a large γ, α, δ, or e subunit (AP-1 to -4 respectively). The four subunits form a core region closest to the membrane, a flexible hinge region and two ear regions.

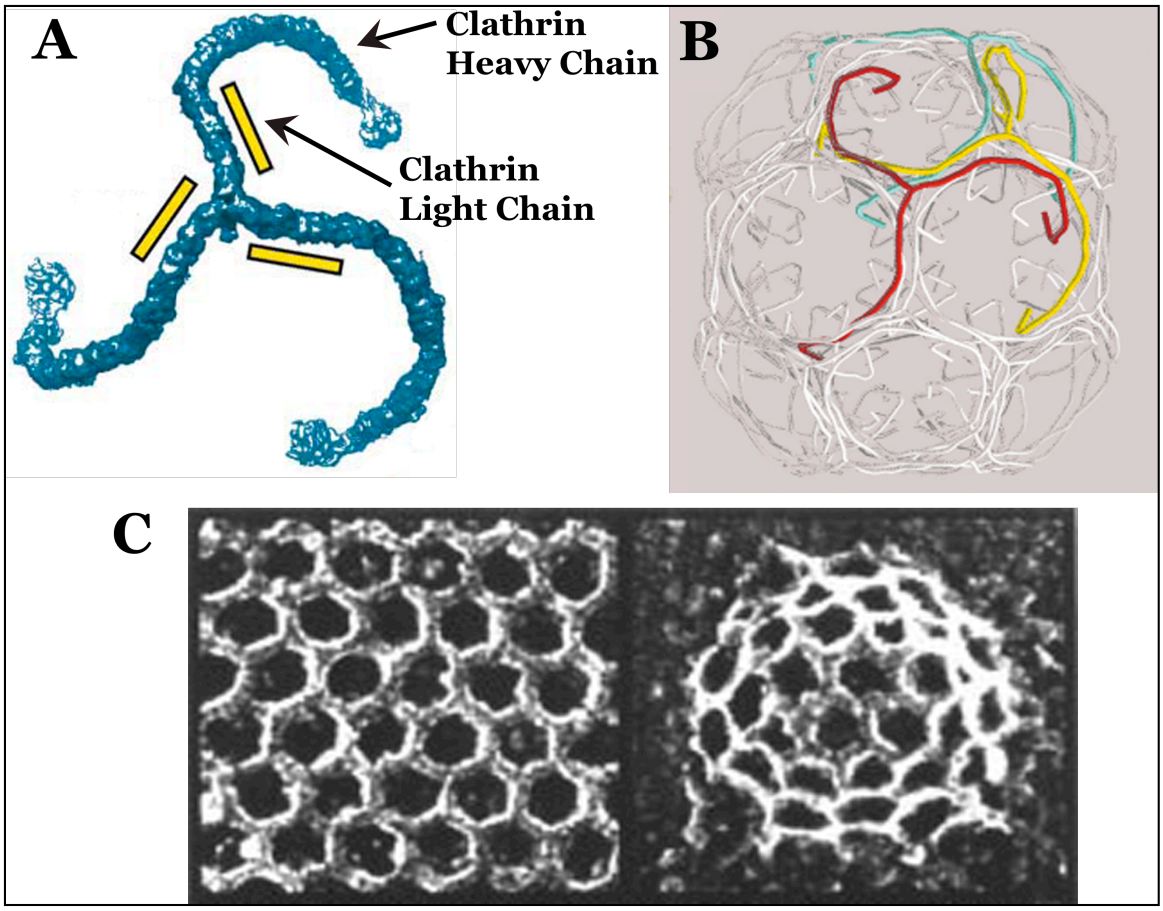

Figure 6. Clathrin Structures

A) Clathrin consists of three heavy chains and three light chains. These six molecules form a three-legged structure known as the clathrin triskelion. Clathrin heavy chains shown as a model from electron cryo-microscopy and crystal structure, light chains inserted as schematics. Figure 6A modified from (Fotin *et al.* 2004). B) Hubs of the clathrin triskelion organize into vertices and trans interactions between clathrin triskelion legs form lines linking the vertices. Clathrin is shown as a tracing of clathrin barrel structure from electron cryo-microscopy. Figure 6B modified from (Kirchhausen 2000). C) Trans-clathrin interactions form pentameric or hexameric patterns resulting in either clathrin

plaque formation (left) or a domed shell at sites of vesicle budding (right). Image is of quick-freeze-deep-etch electron microscopy. Figure 6C modified from (Schmid 1997).

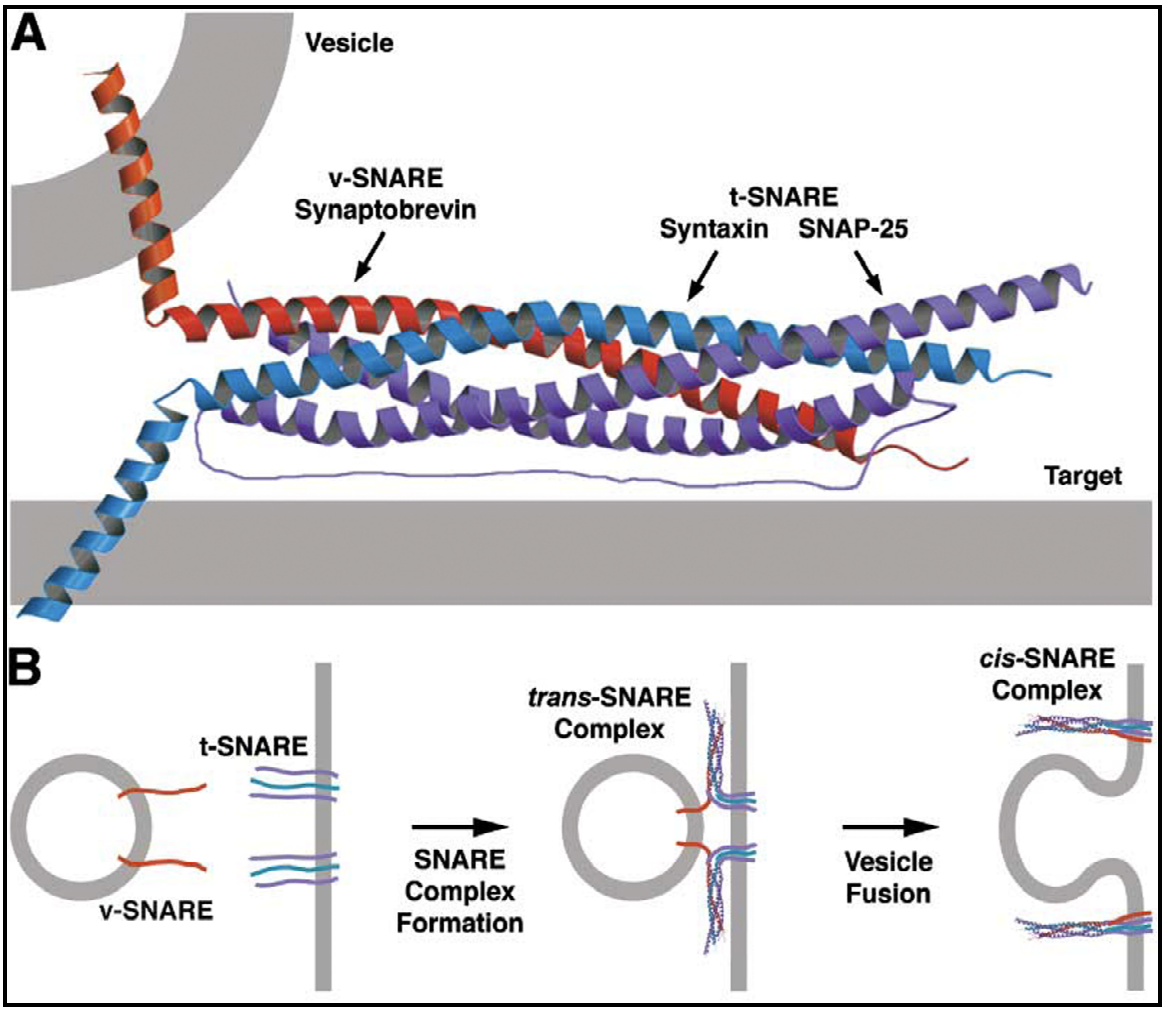

Figure 7. SNARE Interactions

Interactions of α-helical SNARE proteins results in bringing donor and target membranes into close proximity prior to fusion. A) SNAREs are often membrane anchored (red and blue helical representations), though some cytosolic SNAREs have been identified to localize to membrane surfaces through interactions with membrane attached SNAREs (purple helical representation) B) Cytosolically exposed α-helical regions of a SNARE at a vesicular surface (v-SNARE) and two to three SNAREs at a target surface (t-SNAREs) will interact together to form a helical bundle referred to as a trans-SNARE complex. Once fusion occurs and the

SNAREs are in the same membrane the complex is referred to a cis-SNARE complex. Figure modified from (Bonifacino and Glick 2004).

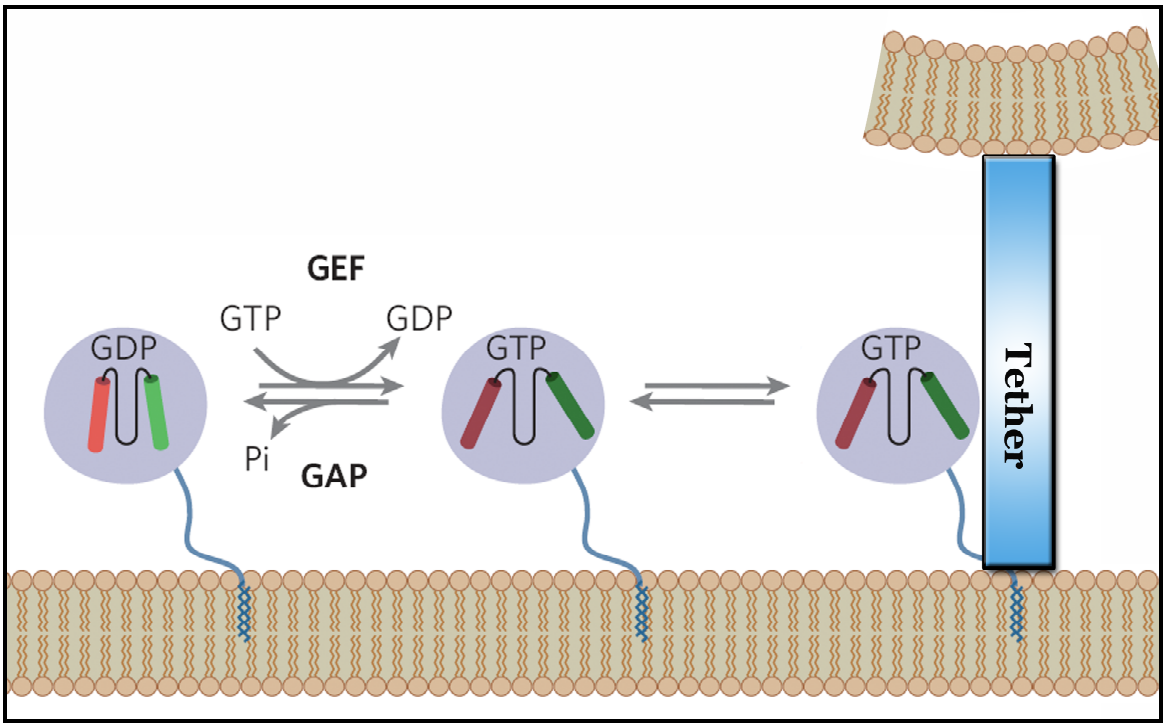

Figure 8. Rab GTPase

Rab GTPases are inactive in their GDP bound form and active in their GTP bound form. When Rabs are in an active GTP form they interact with effectors also commonly known as tethering factors or tethering complexes. The HOPS complex is an effector for the late endosomal Rab7. Figure modified from (Behnia and Munro 2005).

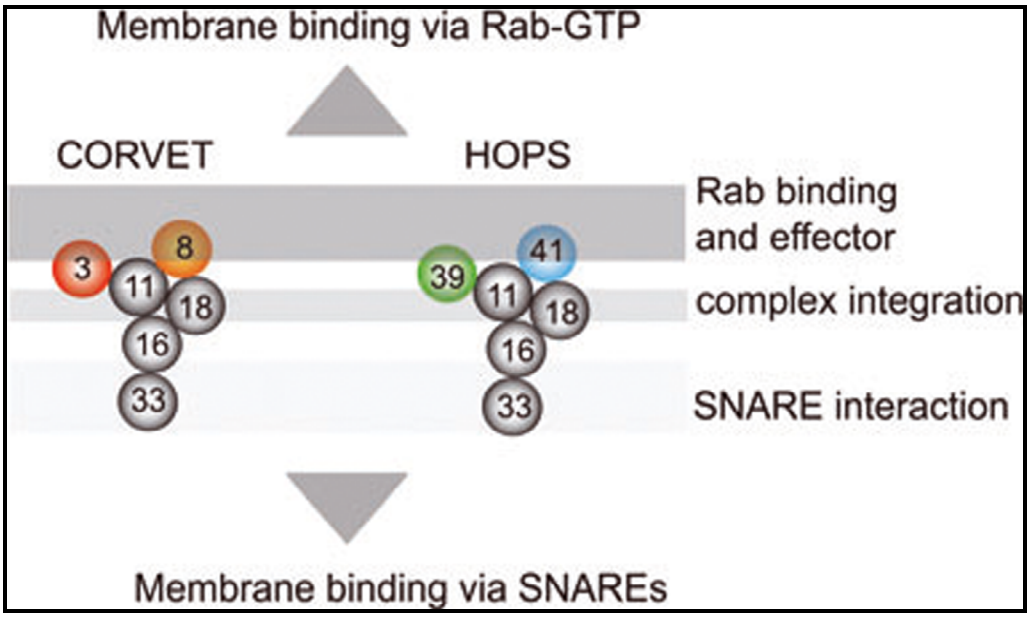

Figure 9. HOPS and CORVET Organization

The HOPS complex (HOmotypic fusion and Protein Sorting) and CORVET complex (class C cORe Vacuole/Endosome Tethering) share the core Vps subunits Vps11, Vps16, Vps18, and Vps33. HOPS specific Vps proteins include Vps39 and Vps41. CORVET specific subunits include Vps3 and Vps8. Higher order eukaryotes contain two homologues of Vps33, Vps33a and Vps33b. Only one Vps33a/b subunit is presumed incorporated into the core complex at a time. The HOPS complex subunits Vps39 and Vps41 have are known to bind the Rab7 homologue YPT7 in yeast. The Vps33 subunits are Sec1/Munc18 proteins. These subunits bind to SNAREs and may be responsible for isolating t-SNAREs at the acceptor compartment from non-specific v-SNARE interactions. Figure modified from (Ostrowicz *et al.* 2010).

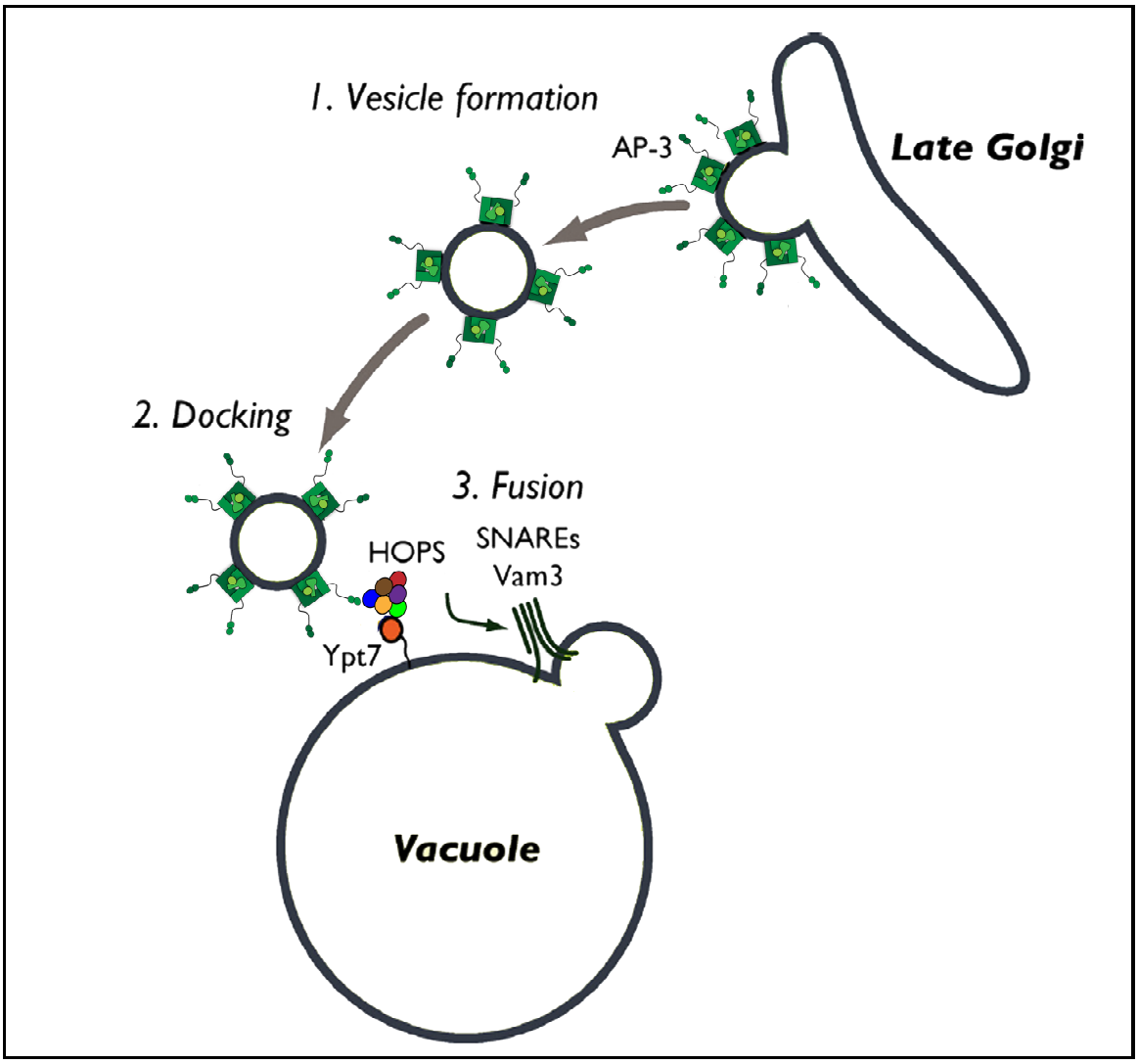

Figure 10. Model of HOPS/AP-3 Interaction in the yeast *S. cerevisiae*

HOPS complex subunits localize primarily to the vacuole, the equivalent of the lysosome in *S. cerevisiae*. In yeast, HOPS complex subunits do not localize to the site of AP-3 vesicle formation, the late Golgi. Yet, HOPS subunits and AP-3 are known to colocalize and interact with one another. Angers and Merz proposed a model in which AP-3/HOPS interactions occur at the sites of vesicle fusion. Figure modified from (Angers and Merz 2009).

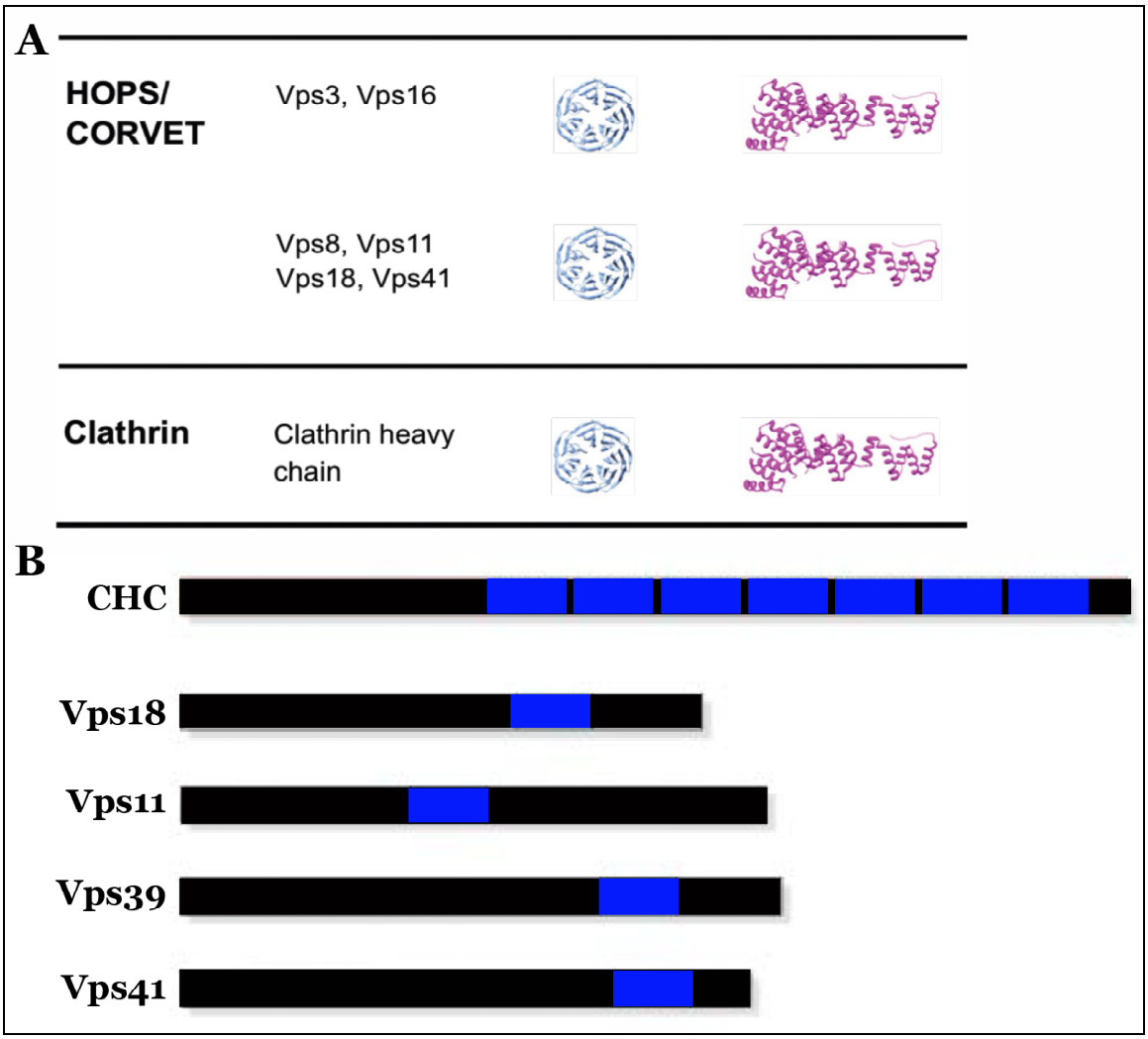

Figure 11. Homologous Structures in HOPS Subunits and Clathrin Heavy Chain

A) Subunits of the HOPS complex and clathrin share similar secondary structure with an amino terminal β-propeller and an α-solenoid toward the carboxy terminus. Figure 12A modified from (Dokudovskaya *et al.* 2011). B) HOPS subunits Vps41, Vps39, Vps18, and Vps11 contain a clathrin repeat domain (blue box) found in clathrin heavy chain (CHC). Figure 12B modified from (Ybe *et al.* 1999).

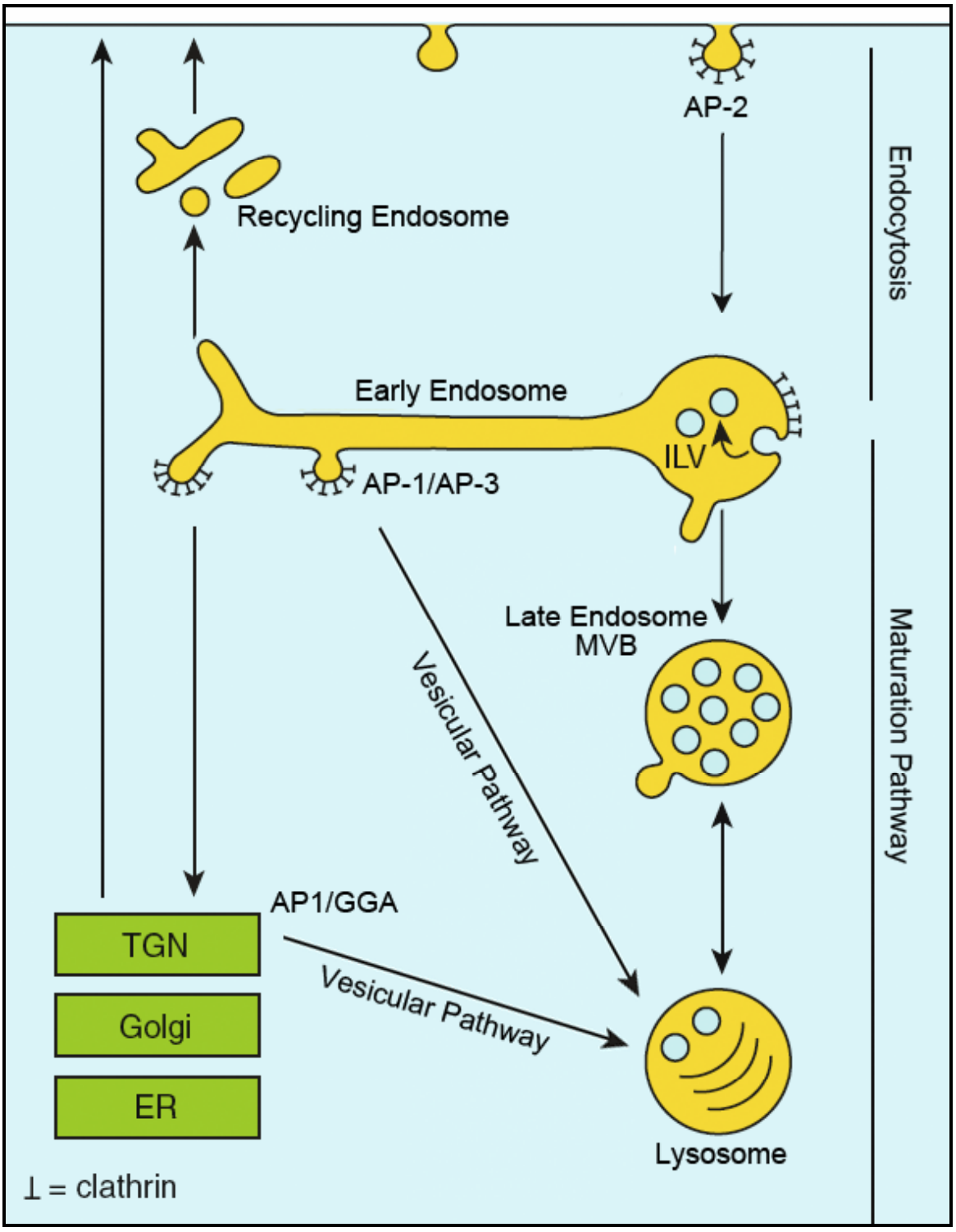

Figure 12. Vesicular and Maturation Pathway of Lysosome Delivery

Proteins can be delivered to the lysosome from a direct vesicular pathway and from an endosomal maturation pathway. In mammals, AP-1 and GGA vesicles

from the trans Golgi network (TGN), or AP-1 and AP-3 vesicles from the early endosome can be packaged with lysosomal cargoes and directly transported to the lysosome. Lysosomal cargoes can also be endocytosed from the plasma membrane in AP-2 vesicles for delivery to the early endosome. Once at the early endosome, cargoes can be recycled back to the plasma membrane via transport to the recycling endosome, loaded into AP-1 or AP-3 vesicles for direct transport to the lysosome, or progress through endosome maturation where ESCRT complexes recruit membrane proteins for degradation into intralumenal vesicles. Along with increase in intralumenal vesicle content, Rab content on early endosomes changes from Rab5 to Rab7, and early endosomes mature to late endosomal multi-vesicular bodies (MVB) and finally to lysosomes. Figure modified from (Meel and Klumperman 2008).

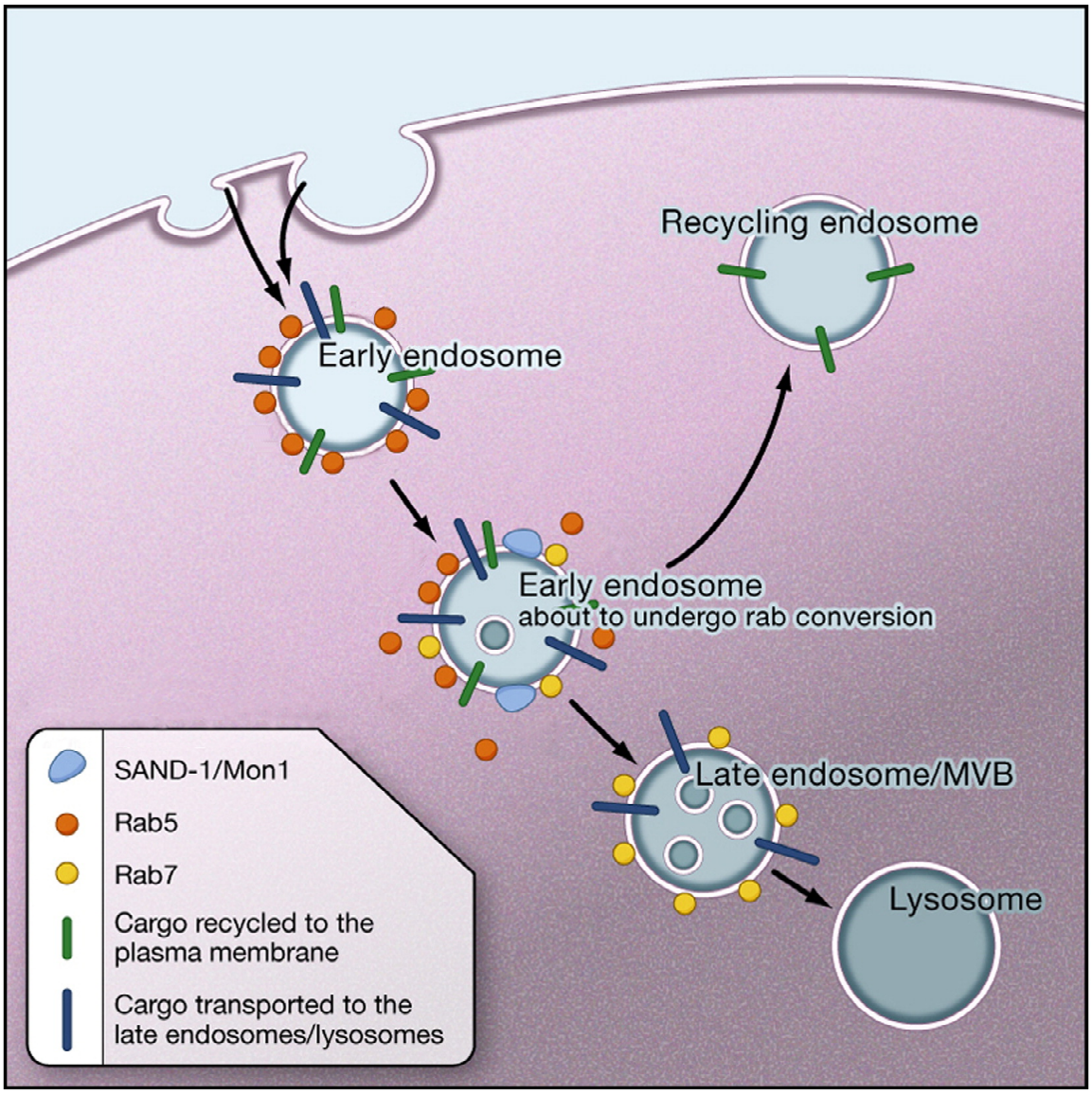

Figure 13. Rab Conversion

The exchange of Rab5 to Rab 7 occurs as the early endosome matures into a late endosome. Early endosomes contain the Rab GTPase Rab5. As the early endosome matures, Mon1 (mammals) SAND1 (*C. elegans*) in complex with Ccz1 is recruited to the limiting membrane. Recruitment of Mon1-Ccz1 results in the dissociation of Rab5 and the recruitment and activation of Rab7. Recruitment of

Rab7 is associated with the maturation of an early endosome to a late endosome. Figure modified from (Poteryaev *et al.* 2010).

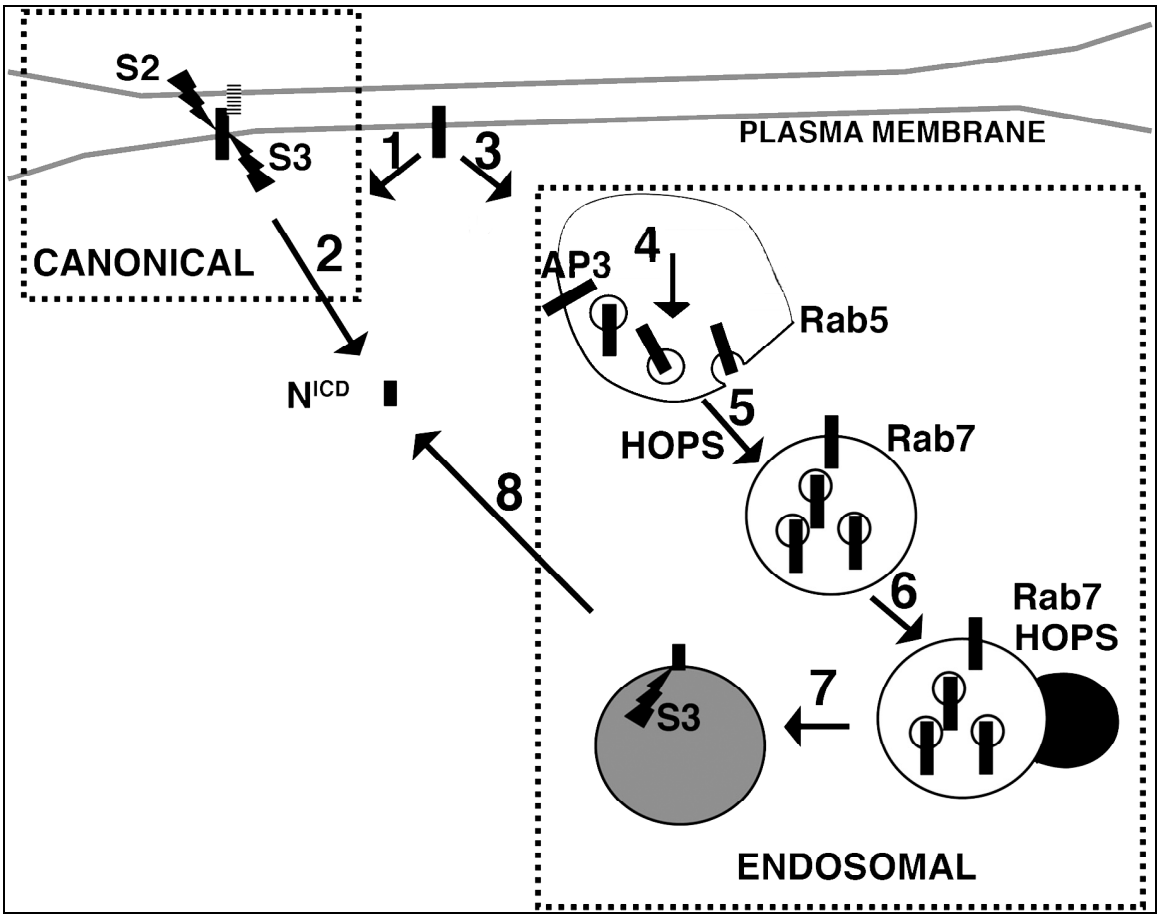

Figure 14. Notch Activation and Degradation Pathways

In a canonical pathway of Notch activation, 1) Notch (black bar) is localized to the plasma membrane and interacts with its receptor (grey bar). 2) Interaction signals cleavage at the extracellular membrane (S2) and intracellular membrane (S3). 3) Once cleaved, the Notch intracellular domain (N^{ICD}) will be transported to the nucleus for signal activation. In the endosomal pathway the Notch signaling molecule can go through a degradation pathway or a second non-canonical activation pathway. 3) Notch is internalized to a Rab5 positive early endosomal compartment. 4) Notch is recruited into intra-luminal vesicles for degradation or sorted away from the degradation pathway and remain at the

limiting membrane. 5) HOPS complex is involved in the Rab5 to Rab7 switch in early to late endosome maturation. 6) HOPS complex promotes fusion with the lysosome. 7) Notch present in intralumenal vesicles is degraded. The lumenal domain of Notch is degraded from Notch present at the limiting membrane of the lysosome. 8) The cytosolic domain of Notch, N^{ICD}, is then transported to the nucleus as in the canonical pathway. Figure modified from (Wilkin *et al.* 2008).

CHAPTER II

Clathrin-Dependent Mechanisms Modulate the Subcellular Distribution of Class C Vps/HOPS Tether Subunits in Polarized and Nonpolarized Cells

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Abstract

Coats define the composition of carriers budding from organelles. In addition, coats interact with membrane tethers required for vesicular fusion. The yeast AP-3 (Adaptor Protein Complex 3) coat and the class C Vps/HOPS (HOmotypic fusion and Protein Sorting) tether follow this model as their interaction occurs at the carrier fusion step. Here we show that mammalian Vps class C/HOPS subunits and clathrin interact and that acute perturbation of clathrin function disrupts the endosomal distribution of Vps class C/HOPS tethers in HEK293T and polarized neuronal cells. Vps class C/HOPS subunits and clathrin exist in complex with either AP-3 or hepatocyte growth factor receptor substrate (Hrs). Moreover, Vps class C/HOPS proteins cofractionate with clathrin-coated vesicles, which are devoid of Hrs. Expression of FK506 binding protein (FKBP)–clathrin light chain chimeras, to inhibit clathrin membrane association dynamics, increased Vps class C/HOPS subunit content in rab5 endosomal compartments. Additionally, Vps class C/HOPS subunits were concentrated at tips of neuronal processes, and their delivery was impaired by expression of FKBP–clathrin chimeras and AP20187 incubation. These data support a model in which Vps class C/HOPS subunits incorporate into clathrin-coated endosomal domains and carriers in mammalian cells. We propose that vesicular (AP-3) and nonvesicular (Hrs) clathrin mechanisms segregate class C Vps/HOPS tethers to organelles and domains of mammalian cells bearing complex architectures.

Introduction

Membranous organelles maintain their steady-state structural and functional identity despite the continuous flow of protein and lipids through them. Flow is dictated by departing carriers budding off organelles and incoming vesicles fusing with their targets. Thus fidelity in vesicle budding and fusion is central to generating and maintaining organelle identity. Coats and adaptors specify vesiclebudding events by selecting cargoes loaded into vesicle carriers. In contrast, Soluble NSF Attachment Protein REceptors (SNAREs), rabs, and tethers determine vesicle fusion fidelity (Bonifacino and Glick 2004). Vesicular and nonvesicular mechanisms regulate flow of macromolecules between endosomes in mammals. Flow between compartments along the endocytic pathway occurs by vesicle-mediated mechanisms (Stoorvogel *et al.* 1996; Peden *et al.* 2004), and the processes of tubule-mediated transfer of cargoes (Delevoye *et al.* 2009), kiss-and-run (Bright *et al.* 2005), and endosome maturation (Stoorvogel *et al.* 1991; Rink *et al.* 2005; Poteryaev *et al.* 2010). Here we show that the coat clathrin interacts with endosomal tethers involved in endosome maturation and fusion (class C Vps/HOPS subunits) by means of vesicular (clathrin and the clathrin adaptor AP-3 [Adaptor Protein Complex 3]) and nonvesicular mechanisms (clathrin and hepatocyte growth factor receptor substrate, Hrs).

Mammalian endosomes possess clathrin domains in the form of budding profiles that contain AP-1/AP-3 clathrin adaptors (Stoorvogel *et al.* 1996; Peden *et al.* 2004; Theos *et al.* 2005) or as flat lattices defined by the presence of Hrs (Raiborg *et al.* 2002; Raiborg *et al.* 2006). Of these coats, buds containing AP-3

and clathrin mature into vesicles that transport their contents between early endosomes and late endosomal/lysosomal compartments (Di Pietro and Dell'Angelica 2005; Dell'Angelica 2009). In contrast, *Saccharomyces cerevisiae* generates AP-3 vesicles at the Golgi complex destined for the lysosomal equivalent, the vacuole (Odorizzi *et al.* 1998). This process is independent of clathrin in yeast (Seeger and Payne 1992; Anand *et al.* 2009). Mutation of the human clathrin adaptor AP-3 causes Hermansky Pudlak Syndrome (HPS), in which lysosome-related organelles such as melanosomes, dense-platelet granules, and lamellar bodies are defective. HPS patients exhibit pigment dilution, bleeding diathesis, and pulmonary fibrosis, and this pathology is directly attributable to defects in vesicular biogenesis (Di Pietro and Dell'Angelica 2005; Dell'Angelica 2009). HPS phenotypes are triggered in mice when any one of 15 genes is defective. These 15 gene products assemble into protein complexes: AP-3, BLOC-1 to BLOC-3 (biogenesis of lysosome-related organelles complex), and tethering complexes constituted by class C Vps subunits (Li *et al.* 2004). Vps33a mutant mice possess a phenotype similar to other HPS mutants, which might be explained by interactions of Vps33a with other HPS complexes (Suzuki *et al.* 2003). Whether these genetic similarities underlie associations of Vps33a with HPS complexes, such as AP-3, into common complexes is presently unknown. This hypothesis is supported, however, by genetic and biochemical interactions between yeast class C Vps proteins and AP-3 (Anand *et al.* 2009; Angers and Merz 2009; Salazar *et al.* 2009).

The mammalian class C Vps protein Vps33a/b assembles into a core complex of class C Vps proteins including Vps11, Vps16, and Vps18. This core

associates with Vps39 and Vps41 to form the HOmotypic fusion and Protein Sorting (HOPS) complex (Kim *et al.* 2001; Nickerson *et al.* 2009; Zhu *et al.* 2009). The class C Vps core complex could also associate with Vps8/KIAA0804 and Vps3/TGFBRAP1 to constitute a hypothetical mammalian CORVET complex, a molecular species so far only documented in yeast (Peplowska *et al.* 2007; Markgraf *et al.* 2009). Class C core subunits and accessory HOPS subunits are critical for fusion events at the vacuole, a lysosome equivalent in the yeast *S. cerevisiae*. In this organism, HOPS orchestrates interactions with the GTPase ypt7, a rab7 ortholog, SNARES, and, recently, AP-3 to achieve selective membrane fusion (Ostrowicz *et al.* 2008; Angers and Merz 2009; Nickerson *et al.* 2009; Wickner 2010). Association of AP-3 and HOPS occurs at the vesicle fusion step with the vacuole when incoming Golgi-derived AP-3–coated vesicles reach the vacuole, the compartment where HOPS complexes reside in yeast (Angers and Merz 2009).

Here we show that mammalian Vps class C/HOPS subunits coimmunoprecipitated and/or colocalized with the endosomal coats clathrin, AP-3, and Hrs. Isolated clathrin-coated vesicles had Vps class C/HOPS subunits on them, yet they were depleted of Hrs. We also found that acute perturbation of clathrin dynamics on and off membranes using FKBP–clathrin light chain (CLC) chimeras (Moskowitz *et al.* 2003; Deborde *et al.* 2008) altered the distribution of class C Vps/HOPS proteins in two different types of mammalian cells. HEK293T cells with reduced clathrin function had elevated levels of Vps class C proteins in rab5-positive endosomes, suggesting that clathrin dynamics at vesicular AP-3– or nonvesicular Hrs-positive endosomal domains regulate Vps class C protein con-

tent on early endosomes. We hypothesized that coats in endosomal tether-coat complexes could contribute to polarized distribution of tethers, such as the HOPS complex. In support of this idea, differentiated neuroendocrine cells accumulated Vps class C/HOPS subunits at the growing tip of processes, where they were concentrated with clathrin. Targeting of Vps class C/HOPS subunits from cell bodies to neurites was sensitive to clathrin function perturbation. Our data suggest that vertebrate cells differ from yeast in that the vertebrates engage in novel interactions between class C Vps–containing tethers and coats, perhaps for specialized demands such as polarized targeting in neuroendocrine and epithelial cells.

Results

Subunits of the Vps class C/HOPS tethering complexes associate with clathrin–AP-3 adaptor subunits

We initially explored molecular interactions between tethering complexes and coats taking advantage of protein complexes affected in HPS. Mouse mutations on genes encoding the class C-Vps protein Vps33a or AP-3 subunits trigger a phenotype that recapitulates HPS (Suzuki *et al.* 2003; Li *et al.* 2004), suggesting that AP-3 and complexes containing Vps33a act on a common pathway. Consistent with this interpretation, subunits of the tethering HOPS complex interact with AP-3 in both *S. cerevisiae (Angers and Merz 2009)* and mammalian cells (Salazar *et al.* 2009). Despite this similarity, in mammalian cells, but not yeast, AP-3 shows interactions with clathrin (Seeger and Payne 1992; Dell'Angelica *et al.* 1998; Anand *et al.* 2009). This observation suggests

that Vps class C/HOPS tethers interact with coats differently in vertebrates as compared with yeast.

We tested whether all four class C Vps proteins, class C Vps33a-b isoforms, and the two HOPS-specific subunits associated with AP-3 and clathrin complexes isolated from HEK293T cells. As described in *Materials and Methods*, we used in vivo cross-linking with dithiobissuccinimidyl propionate (DSP) coupled to immunoaffinity chromatography with monoclonal antibodies (mAbs) against the AP-3 δ subunit or clathrin chains. DSP treatment decreases immunoreactivity of AP-3 δ subunit detected with a mAb against δ adaptin (see *Materials and Methods*). Vps class C and HOPS subunits present in AP-3 or clathrin immune complexes were detected with antibodies either against endogenously expressed Vps33b or tags engineered in recombinantly expressed subunits (Vps11, Vps16, Vps18, Vps33a, Vps33b, Vps39, and Vps41). We expressed tagged recombinant proteins because available class C Vps or HOPS subunit antibodies failed to detect endogenous proteins in multiple cell types, including HEK293T cells (our unpublished data).

We first determined whether phenotypic similarities between mice carrying mutations in either the class C Vps protein Vps33a or AP-3 subunits were predictors of a biochemical interaction between Vps33a and AP-3. Recombinantly expressed Vps33a selectively coprecipitated with AP-3 immunocomplexes (Figure 1A, compare lanes 3 and 5). This association was not restricted to Vps33a; similar results were obtained with tagged Vps33b (Figure 1A, compare lanes 4 and 6). The association of Vps33 isoforms with AP-3 could be due either to isolated Vps33 polypeptides interacting with AP-3 or Vps33

assembled into a Vps class C protein complex, such as HOPS. To discern between these hypotheses, we explored whether protein constituents of the HOPS complex were coisolated with AP-3 complexes together with endogenous Vps33b. Recombinantly expressed class C Vps proteins (Vps11, Vps16, and Vps18) or HOPS-specific subunits (Vps41 and Vps39) copurified with AP-3 and the endogenously expressed class C Vps protein, Vps33b. These associations were preferentially detected in the presence of DSP (Figure 1A, lanes 5 and 6; Figure 1B, lanes 8 and 10; Figure 1C, lane 10; Figure 1D, lanes 14, 16, and 18; Figure 1, E and F, lanes 10 and 12). The association specificity of AP-3 and these Vps subunits was probed using magnetic beads conjugated with anti–mouse immunoglobulin (Ig)G (beads alone, Figure 1B). Vps18-Myc and Vps33b coisolated with AP-3 when these beads contained mouse AP-3 δ antibodies, yet none of these proteins were detected with beads alone (Figure 1B, compare lanes 5 and 6 with 7–10). Second, we out-competed AP-3 δ binding to magnetic beads linked to mouse δ-antibodies with the δ peptide antigen (Salazar *et al.* 2009). We monitored peptide competition by assessing the presence of AP-3 subunits (β3A or δ) and clathrin heavy chain (CHC; an AP-3 binding partner) in immunoisolated cross-linked AP-3 complexes (Dell'Angelica *et al.* 1998; Salazar *et al.* 2009). Pretreating beads displaying δ-antibodies with the δ-peptide abolished binding of AP-3 subunits, clathrin, and all Vps subunits to these beads (Figure 1A, compare lanes 3 and 4 with lanes 5 and 6; Figure 1, C, E, and F, compare lanes 6 and 8 with 10 and 12; Figure 1D, compare lanes 8, 10, and 12 with lanes 14, 16, and 18). Abolished binding indicates that class C Vps and HOPS subunits interact with the beads by binding to intact AP-3 and not via some

nonspecific mechanism. As a control, we showed that AP-1 complexes were not cross-linked to Vps33b, Vps16-HA (hemagglutinin), or Vps41-Myc, showing that the association of class C Vps and HOPS subunits with AP-3 was selective (Figure 1G). AP-3-Vps subunit associations could result simply from abundantly expressed tagged proteins in endosomal compartments. To test this hypothesis, we asked whether the Vps33b-interacting protein Spe39 coisolates with AP-3 and clathrin complexes. We selected Spe39 because we previously demonstrated that Spe39 defines a subclass of Vps class C/HOPS complexes (Zhu *et al.* 2009). In fact, Spe39 is present in a discrete pool of the total Vps33b and other class C protein as determined by quantitative immunomicroscopy (Zhu *et al.* 2009). Importantly, endogenous Spe39 does not colocalize with AP-3 or CLCs (Supplemental Figure 1, A and B). Consistent with our immunomicroscopy, neither endogenous nor recombinant green fluorescent protein (GFP)-tagged Spe39 coprecipitated with AP-3 immunocomplexes (Supplemental Figure 1, C and D). These results argue against nonselective DSP cross-linking of recombinantly expressed proteins to AP-3.

Others have proposed that the HOPS subunit Vps41 can associate with AP-3 as a clathrin-like coat (Rehling *et al.* 1999; Darsow *et al.* 2001). Consequently, we next looked for association of clathrin with Vps class C/HOPS in AP-3 cross-linked complexes. Cross-linked samples were treated with antibodies against CLC. Both Vps33 isoforms selectively coisolated with clathrin complexes isolated with CLC antibodies (Figure 2A, lane 3). We found that CLC consistently coimmunoprecipitated with CHC, the AP-3 δ subunit, endogenous Vps33b, and Vps41-Myc (Figure 2B, lane 7). Neither coats nor Vps proteins were detected with

beads alone or beads coated with nonimmune IgG (Figure 2A, compare lanes 3 and 5 with lane 7). Immunoprecipitation of CLC after siRNA-mediated down-regulation of CHC not only showed lowered CHC levels, but also greatly diminished the abundance of the AP-3, Vps33b, and Vps41-Myc proteins (Figure 2B, compare lanes 7 and 8). We further assessed whether clathrin, AP-3, and Vps41-Myc form a complex together by performing sequential immunopurification of clathrin-containing AP-3 complexes from HEK293T cells stably expressing Vps41-Myc (Figure 2C). First, cross-linked AP-3 complexes were immunoaffinity purified with AP-3 δ antibodies in either the absence or presence of δ antigenic peptide as a control of binding selectivity to beads (Figure 2C, compare lanes 3 and 4 with lanes 5 and 6). Cross-linked AP-3 complexes bound to beads (Figure 2C, lane 6) were then eluted in native conditions from magnetic beads using the δ antigenic peptide. These eluted AP-3-cross-linked complexes were subjected to a second round of immunomagnetic isolation with either nonimmune mouse IgG (control) or a monoclonal anti-CLC. The composition of complexes bound to CLC antibody-coated beads was assessed by immunoblot. CLC antibodies allowed coimmunoprecipitation of the AP-3 δ subunit and Vps41-Myc (Figure 2C, compare lanes 7 and 8), indicating that at least a subset of AP-3, clathrin, and the HOPS subunit Vps41 exist in a multiprotein complex.

The HOPS subunit Vps41 is known to directly interact with AP-3 through its δ subunit in yeast, whereas mammalian CHC interacts with AP-3 through its β3 subunit (Dell'Angelica *et al.* 1998; Rehling *et al.* 1999; Angers and Merz 2009). These interactions suggest that AP-3 could bridge between the HOPS complex and clathrin in mammalian cells. We examined this possibility by

performing shRNA down-regulation of either AP-3 δ or CHC, cross-linking in vivo with DSP, and immunoprecipitating cell extracts with CLC or AP-3 δ antibodies (Figure 2, D and E). As compared with control cells, AP-3 δ subunit shRNA treatment caused a ~50% drop in the level of AP-3 δ polypeptide (49 ± 4.6%), whereas CHC shRNA eliminated nearly all CHC (95.5 ± 0.9% reduction, n = 4, Figure 2, D and E, compare lanes 1 and 2). Immunoaffinity purification with CLC antibodies from mock shRNA treated control cells recovered clathrin complexes that were cross-linked to AP-3 subunits, Vps33b, and Vps41-Myc (and Vps39; unpublished data; Figure 2D, lane 5). Although CLC antibodies failed to isolate AP-3 subunits from AP-3 δ shRNA-treated cells, there were no discernible effects on the levels of Vps33b or Vps41-Myc present in CLC-isolated cross-linked complexes (Figure 2D, compare lanes 5 and 6). These results suggest that other tetrameric adaptors, such as AP-1, could mediate the association between Vps class C and HOPS subunits with clathrin. We did not detect, however, an association of Vps class C or HOPS subunits to cross-linked AP-1 complexes isolated from HEK293T cells (Figure 1G). Furthermore, cross-linked AP-3 complexes isolated from CHC-depleted cells, although devoid of clathrin, contained both endogenous Vps33b and recombinantly expressed Vps41-Myc (Figure 2E, compare lanes 8 and 10) or Vps39-GFP (unpublished data). In fact, cross-linked AP-3 complexes isolated from CHC shRNA–treated cells copurified with increased levels of Vps41-Myc and Vps33b. This effect may be attributable to an increased expression of Vps41-myc but not Vps33b in CHC-shRNA–treated cells (Figure 2E, compare lanes 1 and 2; Vps41-Myc 255 ± 18.5% of control shRNA, n = 2; Vps33b 138.5 ± 42%, n = 4). These data indicate that, despite the

fact that clathrin–AP-3–HOPS subunits form a complex, clathrin is not required for the binding of Vps class C and HOPS subunits to AP-3 complexes, nor is AP-3 necessary for their binding to clathrin complexes. In addition to AP-3, other clathrin-interacting molecules present in endosomes may interact with Vps class C/HOPS subunits. One such mechanism is the Hrs-clathrin flat coat present in endosomes (Raiborg *et al.* 2002; Raiborg *et al.* 2006). Endogenous Hrs or recombinantly expressed Hrs-Myc coprecipitated endogenous CHC and Vps33b (Figure 2F, lanes 5–6 and 5′-6′). These interactions were selective as judged by the absence of Vps33b and CHC in GFP control immunoprecipitations as well as by the absence of transferrin receptor in all immunocomplexes (Figure 2F, lanes 3–4 and 3′-4′). Collectively, our results indicate that Vps class C/HOPS subunits copurify with at least two pools of clathrin present in early endosomes: clathrin–AP-3 and clathrin–Hrs complexes.

Vps class C and HOPS subunits are present in clathrin-coated organelles

Vps class C and HOPS subunits association with clathrin–AP-3 and clathrin–Hrs predicts that these Vps proteins should be present in domains of early endosomes as well as clathrin-coated vesicle carriers. The presence of Vps class C and HOPS subunits in early endosomes and clathrin-coated carriers would be consistent with the observation that AP-3, AP-3–clathrin budding profiles, clathrin–Hrs flat coats, Vps class C, and HOPS subunits are found in early endosomal compartments (Raiborg *et al.* 2002; Peden *et al.* 2004; Richardson *et al.* 2004; Theos *et al.* 2005; Raiborg *et al.* 2006). We determined the subcellular

distribution of Vps class C and HOPS subunits by immunofluorescence using quantitative Delta deconvolution microscopy. We used antibodies against coats (clathrin, AP-1 γ, and AP-3 δ) and either the early endosome marker, rab5, or the late endosomal markers rab7, rab7b, and LAMP-1. Our rationale for including the late endosomal marker rab7b is that its depletion selectively increases the protein levels of AP-3 (Progida *et al.* 2010). We focused on endogenously expressed Vps33b and used Vps16-HA to further validate Vps33b results. In addition, we analyzed the subcellular localization of the HOPS subunits Vps39–GFP and Vps41–Myc in clathrin-positive compartments and endosomes. Endogenous Vps33b partially overlapped with puncta positive for AP-3 (Figure 3, B and C) and/or clathrin (Figure 3, E and F). Although partial, of these two coats, fluorescent signal overlap was more pronounced between Vps33b and clathrin than Vps33b with AP-3. One third of all Vps33b-positive pixels were positive for CHC (Figure 3, E, F, and J). Similar to Vps33b, Vps16-HA was preferentially found in puncta positive for CHC (Figure 3, G and J) or AP-3 δ (Figure 3H), where coats signals partially overlapped with class C Vps subunits. In contrast to CHC localization, Vps33b and Vps16-HA signals minimally overlapped with AP-1 γ (Figure 3, I and J).

Vps33b was found in rab5 endosomes, in which we observed a partial overlap of fluorescent signals (Figure 3, A and J). In contrast, Vps33b was undetectable in late endosomes defined by rab7 and LAMP-1 staining (Figure 3J). Vps33b overlapped with rab7b (Figure 3, D and J), however, at levels similar to those of AP-3 (Figure 3J). Rab7b organelles were also positive for clathrin and the late endosomal SNARE vesicle-associated membrane protein 7 (VAMP7;

Figure 3J). Another class C Vps protein, Vps16–HA, showed a similar pattern of endosomal localization compared with Vps33b (Figure 3J). Analysis of the distribution of the HOPS subunits Vps41–Myc and Vps39–GFP revealed that the signal overlap with coats and endosomal markers was far less pronounced as compared with the class C Vps proteins Vps33b/Vps16–HA. Nonetheless, the two HOPS-specific Vps subunits, Vps41–Myc and Vps39–GFP, were localized to rab5- and CHC-positive compartments (Figure 3J).

The partial overlap between coats, Vps class C, and HOPS proteins suggests that domains of endosomes, rather than the whole organelle, contain these factors. We tested this hypothesis by enlarging rab5 compartments expressing the GTPase-deficient rab5Q79L. This tool has been successfully used to enlarge endosomes facilitating the identification of domains in the limiting membrane of endosomes by high-resolution optical microcopy (Raiborg *et al.* 2002; Raiborg *et al.* 2006; Craige *et al.* 2008). We determined the distribution of coats, HOPS subunits, and class C Vps proteins by deconvolution immunofluorescence microscopy and volume rendering of digitally reconstructed enlarged endosomes (Figure 4 and Supplemental Movies 1 and 2). Single optical sections revealed that endogenous Vps33b, Vps16–HA, and Vps41–Myc were present in the limiting membrane of enlarged rab5 endosomes in discrete domains often partially overlapping with either CHC- or AP-3 δ–decorated patches (Figure 4). Fifty and thirty-five percent of all Vps33b domains present on the limiting membrane of rab5Q79L-enlarged endosomes were positive for CHC and AP-3 δ subunit, respectively (52.4 ± 25.7, n = 68; 34.2 ± 24.3, n = 103). Digital volume rendering of rab5Q79L-enlarged endosomes confirmed that the

HOPS subunit Vps41–Myc was present in discrete domains that partially overlapped either with CHC (Supplemental Movie 1) or AP-3 δ (Supplemental Movie 2) at the limiting membrane of enlarged endosomes.

We isolated clathrin-coated vesicles from HEK293T cells or HEK293T cells stably expressing either Vps41–Myc or Vps16–HA and asked whether class C Vps/HOPS proteins were present in these organelles (Figure 5 and Supplemental Figure 2). Clathrin-coated vesicle isolation was monitored by the enrichment of CHC detected by Coomassie Blue dye (Figure 5A) or by immunoblot with antibodies CHC, AP-1 γ, AP-2 α, and AP-3 β3 subunits in clathrin-coated vesicle fractions (Figure 5B and Supplemental Figure 2, compare lanes 1 and 6). Clathrin-coated vesicles contained class C Vps proteins Vps33b and Vps16–HA and the HOPS subunit Vps41–Myc (Figure 5B and Supplemental Figure 2, compare lanes 1 and 6). Of these Vps proteins, Vps41–Myc and Vps16–HA were clearly enriched in clathrin-coated vesicle fractions. We isolated clathrin-coated vesicles from control or CHC RNAi–treated cells to define whether Vps class C and HOPS subunits cosedimenting with clathrin-coated vesicles correspond to true components of these carriers or just contaminants present on these membranes (Borner *et al.* 2006). Coomassie Blue staining revealed that clathrin-coated vesicle fractions isolated from CHC down-regulated cells decreased the content of several polypeptides, including CHC (Figure 5A, asterisks). Down-regulation of CHC precluded formation of clathrin-coated vesicles as determined by the absence or decreased levels of adaptor subunits from clathrin-coated vesicles (Figure 5B; Supplemental Figure 2, compare lanes 6 and 6′; Figure 5C). Importantly, the levels of Vps33b, Vps16–HA, or Vps41–Myc

were significantly reduced from clathrin-coated vesicles isolated from CHC RNAi–treated cells indicating that Vps33b, Vps16–HA, and Vps41–Myc specifically reside in clathrin-coated vesicles (Figure 5B; Supplemental Figure 2, compare lanes 6 and 6′; Figure 5C). Contamination of clathrin-coated vesicle fractions with early endosomes markers such as EEA1, rabaptin5, and Hrs was negligible (Figure 5B). Therefore these results indicate that clathrin, Vps class C, and HOPS subunits coreside both in coated domains of early endosomes as well as clathrin-coated vesicle carriers.

Acute perturbation of clathrin alters Vps class C/HOPS subunit subcellular distribution

We evaluated the effect of CHC shRNA on the distribution of Vps class C/HOPS subunits in rab5- and rab7b-positive endosomes. CHC down-regulation, which is achieved within several days of shRNA treatment, did not alter the distribution of Vps class C/HOPS subunits in rab5 or rab7b compartments, suggesting compensatory mechanisms controlling Vps class C/HOPS subunit subcellular distribution (unpublished data). To overcome possible compensatory mechanisms, we acutely perturbed clathrin function by using a chemical/genetic approach that uses chimeric CLCs carrying an oligomerization module, a modified FKBP 12 domain (Moskowitz *et al.* 2003; Deborde *et al.* 2008). FKBP domains selectively oligomerize upon addition of the cell-permeant bivalent chemical AP20187. CLC oligomerization that follows incubation with AP20187 halts vesicle formation from donor organelles, such as plasma membrane or *trans*-Golgi network by “freezing” clathrin onto membranes and aborted budding

profiles (Moskowitz *et al.* 2003; Deborde *et al.* 2008). Thus we used FKBP–CLC chimeras to trap clathrin at membranes. Tagged FKBP–CLC chimeras incorporated into 60.7 ± 18.5% (n = 30) of all CHC-positive organelles in HEK293T cells as determined by deconvolution microscopy (Supplemental Figure 3A). Like endogenous CLC (Figure 2), chimeric mCherry-FKBP–CLC specifically coprecipitated class C Vps/HOPS subunits (Supplemental Figure 3D). Cell treatment with AP20187 for 2 h induced enlargement of CHC/tagged-FKBP–CLC dual-labeled organelles and the accumulation of endogenous and recombinant clathrin chains at the perinuclear region (Supplemental Figure 3, B and C). The perinuclear accumulation of CHC in HEK293T cells indicates that clathrin-dependent processes are rapidly inhibited by FKBP–CLC chimeras (Moskowitz *et al.* 2003; Deborde *et al.* 2008).

We next determined the effect of chemically perturbing clathrin function in HEK293T- and NGF-differentiated PC12 cells on Vps class C/HOPS subunits (Figures 6–9 and Supplemental Movies 3–6). We determined the speed of the AP20187-induced Vps class C redistribution by in vivo, time-lapsed confocal microscopy. mCherry-FKBP-CLC– and Vps18-GFP–expressing cells were imaged 25 min before drug addition and for 2 h in the presence of AP20187. mCherry-FKBP–CLC oligomerization induced by AP20187 quickly caused an accumulation of recombinant clathrin and Vps18–GFP in the perinuclear region. Concomitantly, Vps18–GFP organelles distributed in the cell periphery grew in number and size (Figure 6C and Supplemental Movie 5 and 6). Vps18–GFP redistributed in <30 min after AP20187 addition and reached a plateau by 60 min. Addition of either AP20187 to cells expressing solely Vps18–GFP (Figure 6A

and Supplemental Movie 3) or ethanol vehicle to mCherry-FKBP-CLC and Vps18-GFP doubly expressing cells (Figure 6B and Supplemental Movie 4) did not alter the subcellular distribution of either fluorescently tagged protein. These results demonstrate a swift rearrangement of class C Vps proteins by acute chemical/genetic inhibition of clathrin-coated vesicle formation.

The effects of AP20187-induced perturbation of clathrin function were not just restricted to Vps18–GFP. Analysis of images from fixed specimens showed that drug addition caused redistribution of endogenous Vps33b, Vps18–GFP, or Vps41–Myc into larger puncta preferentially found at the perinuclear region as was seen with Vps18–GFP during live imaging (Figure 7). This change in Vps class C/HOPS subunits distribution occurred concomitantly with an increased colocalization of Vps class C/HOPS subunits with either CHC or mCherry-FKBP–CLC (Figure 7A). Importantly, AP20187 increased the colocalization of clathrin chains with AP-3 as well as rab5 and rab7b, suggesting that AP20187 similarly traps clathrin chains on both endosomes (Figure 7, B and C). These results are consistent with the presence of clathrin in AP-3–, rab5-, and rab7b-positive compartments, as was observed at steady state in HEK293T cells (Figure 3J).

Vps class C/HOPS subunit binding to both clathrin–AP-3 and clathrin–Hrs coats suggest that Vps class C/HOPS subunit accumulation should be preferentially observed in early endosomes as compared with other intracellular compartments, such as late endosomes or the *trans*-Golgi network. To test this hypothesis, we analyzed the affects of AP20187 on the colocalization of rab5, rab7b, and AP-1 with CHC and/or endogenous Vps33b in mCherry-FKBP-CLC–expressing cells using deconvolution microscopy. The amount of CHC-positive

puncta that were also positive for Vps33b doubled from 12.3 ± 5.3% (n = 120) to 20.8 ± 11.9% after drug addition (n = 120, $p < 0.0001$, Wilcoxon–Mann–Whitney test; Figure 8). AP20187 increased the colocalization of Vps33b with rab5 from 10.2 ± 5% to 20 ± 9.6% (n = 60, $p < 0.0001$, Wilcoxon–Mann–Whitney test; Figure 8; Supplemental Figure 4, compare A and B). In contrast, colocalization between rab7b and Vps33b modestly decreased after 2 h of clathrin perturbation (Figure 8; Supplemental Figure 4, compare C and D). Vps33b colocalization with clathrin in rab5-positive endosomes was selective because overlap between Vps33b and AP-1 γ remained at background levels before or after AP20187 incubation (Supplemental Figure 5 and Figure 8). Although AP20187 induced clathrin recruitment to rab5- and rab7b-positive compartments to a similar extent (Figure 8), the preferential association of Vps33b- to rab5-positive organelles after drug-induced clathrin perturbation supports the hypothesis that clathrin coats selectively regulate the localization of class C Vps proteins to early endosomes.

Clathrin and Vps class C proteins are targeted to neuronal processes by clathrin-dependent mechanisms

We tested whether class C Vps proteins present in clathrin-coated organelles undergo directional delivery in polarized cells. To this end, we used human cortical neurons HCN-1A, a cell type that extends processes (Ronnett *et al.* 1990), as well as NGF-differentiated PC12 cells. HCN-1A cells were double-labeled with antibodies against endogenous Vps33b and CHC, and cells were imaged by high-resolution Delta deconvolution microscopy (Figure 9A). Vps33b

was concentrated at the tip of neuronal processes where it preferentially colocalized with clathrin. This polarized distribution of a class C Vps/HOPS subunit was also observed by in vivo imaging of NGF-differentiated PC12 cells expressing recombinant mCherry-FKBP–CLC with either Vps39–GFP (unpublished data) or Vps18–GFP (Figure 9B).

We predicted that if class C Vps proteins associate with clathrin-positive organelles generated at the cell body for subsequent delivery to neurites, then inhibition of clathrin-dependent mechanisms by AP20187 should lead to a progressive decrease of mCherry-FKBP–CLC and Vps18–GFP fluorescent signals in the proximal segment of neurites. NGF-differentiated PC12 cells expressing mCherry-FKBP–CLC and Vps18–GFP were treated with vehicle or with AP20187 and continuously imaged by confocal microscopy for 2 h. The integrated fluorescence intensity per volume unit was measured in the proximal third of the neurite (Figure 9B, arrows). Addition of AP20187 decreased CLC and Vps18 fluorescence in the proximal segment of neurites after 2 h (Figure 9B). Quantification of CLC and Vps18 fluorescence intensity revealed a progressive reduction per unit of proximal neurite volume after AP20187 incubation (Figure 9C, open circles). This decrease was not observed in vehicle-treated cells (Figure 9C, closed circles). Changes in fluorescence intensity per voxel induced by drug incubation are due to neither changes in neurite length nor diameter. These results indicate that Vps18–GFP delivery to neurites of polarized neuroendocrine cells is sensitive to acute perturbation of clathrin function.

Discussion

Mouse mutants in subunits of HPS protein complexes and Vps33a, a Vps class C tether complex subunit, share phenotypes. The shared phenotypes suggests that associations between Vps33a and other HPS protein complexes participate in the same pathway, delivering membrane proteins from early endosomes to late endosomes/lysosomes and lysosome-related organelles (Suzuki *et al.* 2003; Li *et al.* 2004). Defects in this route trigger HPS in humans (Li *et al.* 2004; Di Pietro and Dell'Angelica 2005). Similarly, *S. cerevisiae* orthologues of Vps class C proteins and AP-3 subunits participate in the delivery of cargoes to the vacuole. Genetic and/or biochemical interactions between AP-3 and HOPS subunits in yeast and vertebrates provide mechanistic insight into the molecular organization of this genetic pathway (Angers and Merz 2009; Nickerson *et al.* 2009; Salazar *et al.* 2009). Our results are the first evidence of vertebrate Vps class C/HOPS proteins interacting with the early endosomal coats clathrin and AP-3 and the clathrin-binding scaffold Hrs. Moreover, we provide biochemical evidence of an interaction between Vps33a and AP-3 predicted from the phenotypic similarities between Vps33a- and AP-3-deficient mice. Clathrin and AP-3 highlight fundamental differences in the way that yeast and mammalian Vps class C and coats interact. In contrast with yeast AP-3, the mammalian ortholog of this adaptor complex interacts with clathrin (Seeger and Payne 1992; Dell'Angelica *et al.* 1998; Anand *et al.* 2009). These observations suggest differences in the biochemical and functional architecture of mechanisms controlled by vertebrate Vps class C/HOPS–containing tethers and coats.

Mammalian class C Vps proteins (Vps11, 16, 18, and 33a-b) and the HOPS-specific subunits Vps39 and Vps41 establish specific interactions with clathrin chains (Figure 2). We identified Vps class C/HOPS proteins in isolated clathrin-coated carriers, clathrin-positive domains of rab5-positive early endosomes, and rab7b-containing endosomes (Figures 3–5). The association of clathrin with Vps class C/HOPS proteins has at least two modalities: one occurring with the AP-3 complex and another in which Hrs participates. Clathrin–AP-3–Vps class C/HOPS and clathrin–Hrs–Vps class C/HOPS associations suggest vesicular and nonvesicular mechanisms controlling Vps class C/HOPS subunit subcellular distribution along the endocytic pathway, respectively.

We focused on the association between Vps class C/HOPS subunits and clathrin chains because quantitative fluorescence microscopy indicated a greater degree of overlap between Vps33b/Vps16 and CHC than with AP-3 (Figure 3J). The functionality of Vps class C/HOPS protein–clathrin interactions was demonstrated by acute perturbation of clathrin function (Moskowitz *et al.* 2003; Deborde *et al.* 2008). Chimeric CLC carrying the oligomerization module FKBP and AP20187 treatment rapidly redistributed CHC, class C Vps proteins (Vps18, Vps33b), and HOPS subunits (Vps39, Vps41) to organelles distributed throughout the cytoplasm and the perinuclear region (Figures 6–8). Quantitative immunofluorescence microscopy revealed that, upon clathrin function perturbation, Vps33b content preferentially increased in rab5-positive over either rab7b-containing endosomes or AP-1–positive organelles, such as the *trans*-Golgi (Figure 8 and Supplemental Figures 4 and 5). These findings support a model whereby clathrin-dependent mechanisms acutely define the subcellular

distribution of Vps class C/HOPS-containing tethers. We directly tested this model in human cortical neurons and in NGF-differentiated PC12 cells, two polarized cellular models. We observed enrichment of class C Vps/HOPS subunits and clathrin at the tip of neurites (Figure 9A). Moreover, acute perturbation of clathrin function led to a progressive depletion of Vps18–GFP and mCherry-FKBP–CLC from the proximal segment of neurites imaged in vivo (Figure 9, B and C). We interpret this decrease in neurite Vps18–GFP and mCherry-FKBP–CLC fluorescence as the result of two concomitant processes: (1) inhibition of newly formed Vps18-clathrin–positive organelles entering the proximal neurite from the cell body plus (2) Vps18-clathrin–positive organelles already present in the neurite proximal segment at the time of drug addition progressively moving downstream toward the neurite tip.

Clathrin, AP-3, and Vps41 form a tripartite complex (Figure 2C). The association of Vps class C/HOPS subunits with clathrin, however, is independent of AP-3 expression levels (Figure 2D). Conversely, association of AP-3 and class C Vps/HOPS proteins is independent of clathrin expression (Figure 2E). These independent associations suggest that Vps class C/HOPS subunits establish multipronged interactions with clathrin and adaptors, such as AP-3 and/or that other clathrin-interacting molecules in early endosomes may mediate clathrin–Vps class C/HOPS subunit associations. We identified the early endosomal clathrin–Hrs flat coat participating in clathrin–Vps class C subunit interactions. Endogenous Hrs as well as recombinant Hrs coprecipitated endogenous clathrin and vps33b. Hrs and AP-3 mechanisms are likely independent because AP-3 is present in clathrin-coated vesicles yet Hrs is excluded from these coated carriers

(Figure 5B). Therefore we propose that vesicular and nonvesicular clathrin–Vps class C/HOPS protein complexes regulate the subcellular distribution of class C Vps/HOPS subunits along the endocytic route. Structural predictions point to the presence of CHC homology domains in *S. cerevisiae* Vps 11, 18, 39, and 41, raising the possibility of multipronged associations between components of the coat and Vps class C/HOPS subunits (Darsow *et al.* 2001; Nickerson *et al.* 2009). Irrespective of whether multiple clathrin-binding molecules, such as AP-3 and Hrs, vesicular or nonvesicular mechanisms, or multipronged interactions between coats and Vps tethers exists, however, the redistribution of Vps class C/HOPS subunits upon of acute perturbation of clathrin function indicates that significant pools of Vps class C/HOPS tethers are under control of clathrin-dependent mechanisms.

Angers and Merz have put forward an attractive model in which interactions between a tether, HOPS, and the coat AP-3 mediate docking of vesicles with the vacuole in *S. cerevisiae*, a process that culminates with fusion of membranes. Donor compartment (Golgi) and incoming AP-3–coated vesicles are devoid of HOPS complex in this model. HOPS complexes reside in the acceptor vacuolar compartments where coat and tether encounters occur to facilitate vesicle consumption (Angers and Merz 2009; Angers and Merz 2011). Our data suggest that, in addition to this mechanism, mammalian Vps class C/HOPS tethers are included in clathrin- and clathrin-AP-3–coated carriers, suggesting a coat-dependent mechanism for delivering Vps class C/HOPS tethers. Perturbing clathrin function with FKBP-CLC/AP20187 caused an increase in the number and size of organelles positive for Vps class C/HOPS subunits and trapped

Vps33b in rab5-positive endosomes (Figures 6–8; Supplemental Figure 4). We attribute these effects to inhibition of clathrin-coated vesicle budding, consistent with published data (Moskowitz *et al.* 2003; Deborde *et al.* 2008) and possibly to changes in the dynamic of flat clathrin–Hrs coats in early endosomes. Alternatively, FKBP-CLC/AP20187 could possibly cause promiscuous recruitment of cytoplasmic Vps class C/HOPS protein pools to membranes by oligomerized FKBP–CLC. We do not favor this alternative hypothesis, however, because we do not detect an increased association of Vps33b to rab7b- or AP-1–positive organelles (Figure 8; Supplemental Figures 4 and 5).

AP-3 budding occurs mainly, if not exclusively, from transferrin receptor–positive endosomes (Peden *et al.* 2004; Theos *et al.* 2005; Craige *et al.* 2008). Quantitative electron microscopy indicates that half of these AP-3 budding profiles possess clathrin on them (Peden *et al.* 2004; Theos *et al.* 2005). Perhaps, the presence of clathrin allows coats and Vps class C/HOPS subunits to be recruited at early stages in the vesicle life cycle. In the absence of clathrin, tethers and coats could undergo late interactions at target organelles, as proposed previously for yeast (Angers and Merz 2009; Angers and Merz 2011). Early inclusion of Vps class C/HOPS tethers into clathrin-coated carriers could serve a role for long-range delivery of tethers to polarized domains in mammalian cells. This hypothesis is supported by our findings that endogenous Vps33b and recombinantly expressed Vps18 and Vps39 display polarized distribution in the tip of neurites in human cortical neurons or differentiated PC12 cells (Figure 9). An alternative yet nonexclusive model is that Vps class C/HOPS subunits could play a role in cargo selection either through indirect effects of Vps proteins on

cargo recognition by coats or by direct association of class C Vps/HOPS proteins with SNAREs and nonSNARE membrane proteins. Support for this interpretation was obtained recently when *ema*/CLEC16A, which is a lectin-type membrane protein, was shown to directly bind to Vps16A in *Drosophila melanogaster* (Kim *et al.* 2010).

At least four modalities of content delivery between stages of the endocytic route have been documented: vesicle-mediated (Stoorvogel *et al.* 1996; Peden *et al.* 2004), tubule-mediated transfer of cargoes (Delevoye *et al.* 2009), kiss-and-run (Bright *et al.* 2005), and endosome maturation (Stoorvogel *et al.* 1991; Rink *et al.* 2005; Poteryaev *et al.* 2010). Endosome maturation is kinetically defined by the conversion over time of the same endosome membrane from a rab5- to a rab7-decorated compartment (Stoorvogel *et al.* 1991; Rink *et al.* 2005; Poteryaev *et al.* 2010). This process depends on a switch mechanism in which a later acquisition of HOPS subunits promotes rab7 activation by interactors of the Vps39 subunit of HOPS (Nordmann *et al.* 2010; Poteryaev *et al.* 2010). Presently, it is unknown whether rab5 compartments can mature into rab7b compartments. We focused on rab7b because class C Vps/HOPS proteins were found in very low (background) levels in rab7- or LAMP-1–containing late endosomes (Figure 3J), suggesting that Vps class C/HOPS subunit binding may be short-lived on them. In contrast, class C Vps/HOPS associates to rab7b compartments. Rab7b has been implicated in delivery of Toll-like receptors from the cell surface to lysosomes in macrophages as well as in retrograde transport between endosomes and Golgi complex in HeLa cells (Wang *et al.* 2007; Progida *et al.* 2010). The presence of clathrin in rab7b-positive compartments and the observation that

down-regulation of rab7b increases the expression level of AP-3 (Progida *et al.* 2010) suggest a role of rab7b in vesicle-mediated transport between early endosomes and late endosomes/lysosomes or in a specialized retrograde transport between endosomes and the Golgi complex. Irrespective of whether rab7b-clathrin compartments represent vesicles, endosomes, or a combination thereof, our findings suggest that maturation of clathrin-coated membranes in transit among endosomal compartments could occur.

S. cerevisiae class C Vps proteins (Vps11, 16, 18, and 33) form a core that incorporates into CORVET and HOPS complexes (Peplowska *et al.* 2007; Nickerson *et al.* 2009; Ostrowicz *et al.* 2010; Wickner 2010). Vps8 and Vps3 constitute the CORVET complex whereas Vps39 and Vps41 establish specific interactions with the core defining the HOPS complex (Peplowska *et al.* 2007; Markgraf *et al.* 2009; Ostrowicz *et al.* 2010). The organization and subcellular localization of HOPS has been partially characterized in metazoans (Kim *et al.* 2001; Richardson *et al.* 2004; Zhu *et al.* 2009; Cullinane *et al.* 2010). In contrast, metazoan CORVET has not been studied. Although putative human orthologues of CORVET subunits, Vps8/KIAA0804 and Vps3/TGFBRAP1, are present in databases, it remains unknown whether these gene products assemble with class C Vps proteins to form a mammalian CORVET complex. It is formally possible that mammalian class C Vps proteins, such as Vps33b and Vps16, identified in clathrin-containing organelles by biochemical and immunolocalization studies, may be part of both CORVET and HOPS complexes. The presence of class C vps subunits in both complexes might explain why there was a relatively higher colocalization between Vps33b/Vps16 (found in both CORVET and HOPS) and

clathrin as compared with Vps41, Vps39, and clathrin (found only in HOPS; Figure 3). If this were the case, our data suggest that perturbing clathrin function affects Vps class C proteins along rab5 and rab7b compartments (Figure 8) by stalling maturation from a CORVET to a HOPS-positive-clathrin–coated vesicle or endosome.

Our results demonstrate a unique functional architecture of mechanisms controlled by vertebrate Vps class C/HOPS–containing tethers and clathrin coats. We postulate that clathrin-dependent mechanisms provide long-range and directional delivery of class C Vps/HOPS tethers to organelles and/or specialized domains of mammalian cells bearing complex architectures.

Materials and Methods

Antibodies

The following antibodies were used in this study: polyclonal anti-myc (A190105A) and anti-HA (A190108A; Bethyl Laboratories, Montgomery, TX); polyclonal anti-GFP (cat# GFP-1020, Aves Labs, Tigard, OR, and cat# 132002, Synaptic Systems, Gottingen, Germany); monoclonal anti–Lamp H4A3, anti-SV2, and anti-AP3δ SA4 (Developmental Studies Hybridoma Bank, Iowa City, IA); monoclonal anti–rab7 ab50533 (Abcam, Cambridge, MA); monoclonal anti–rab7b clone 3B3 (Abnova, Taipei, Taiwan); monoclonal anti-rab5, anti–AP1γ adaptin, anti–rabaptin 5, and anti-EEA1 (610724, A36120, 610676, and 610456; BD Bioscience Transduction Laboratories, Pasadena, CA); monoclonal anti–CHC X22 (Calbiochem, San Diego, CA); monoclonal anti–CLC CON.1 (MMS423P; Covance, Berkeley, CA); polyclonal anti–mCherry dsRed (632496; Clontech,

Mountain View, CA); monoclonal anti-AP2α adaptin and anti-actin (A4325 and A5441; Sigma, St. Louis, MO); polyclonal anti-AP3β1 (13384–1-AP; ProteinTech Group, Chicago, IL); monoclonal anti–GFP 3E6 (A11120; Molecular Probes, Eugene, OR); monoclonal anti–HRS A-5 (Enzo Life Sciences, Plymouth Meeting, PA); anti–synaptophysin clone SY38 (Millipore, Billerica, MA); and monoclonal anti–transferrin receptor H68.4 (136800; Zymed Laboratories, San Francisco, CA). Anti-Vps33b, anti-Spe39, and anti–AP3 β3 have been previously described (Faundez and Kelly 2000; Salazar *et al.* 2009; Zhu *et al.* 2009). Anti-VAMP7 was a gift from Andrew Peden (Department of Clinical Biochemistry, University of Cambridge, UK). Immunofluorescence secondary antibodies were Alexa Fluor 488, 555, 568, or 647 anti–rabbit, rat, chicken, or isotype specific mouse IgG (Molecular Probes). The secondary antibodies used on Western blots were horseradish peroxidase-goat anti-mouse or anti-rabbit (626420 and G21234; Invitrogen, Carlsbad, CA).

Plasmids, oligos, and peptides

Plasmids encoding C-terminally tagged murine Vps11–HA and Vps16–HA, and N-terminally tagged GFP-murine Vps18 and GFP–Vps39 were gifts from Robert Piper (Department of Molecular Physiology and Biophysics, University of Iowa, Iowa City, IA), Vps18–myc from Liping Wang (Human Nutrition Research Center, UC Davis, CA), N-terminally tagged myc–HRS from Harold Stenmark (Institute for Cancer Research; Oslo University Hospital) (Raiborg *et al.* 2002), and N-terminally tagged GFP–Rab5Q79L from Laura Volpicelli (University of Pennsylvania) (Volpicelli *et al.* 2001). Spe39-enhanced GFP and HA-tagged

Vps33a and Vps33b have been described (Zhu *et al.* 2009).

C-terminal Vps41–myc was created from human Vps41 cDNA clone (cat# SC111791; Origene, Rockville, MD) and the primers 5′ caccatggcggaagcagaggag and 3′ ttggagatgaaaaaagaacaaaaacttatttctgaagaagatctgtag using PCR. The PCR product was cloned into TOPO vector pcDNA 3.1 (Invitrogen), following the manufacturer's directions. The TOPO vector DNA was subsequently cut with enzymes *Bam*HI and *Eco*RV, and the Vps41–myc fragment was subcloned into pIREShyg3. The resulting clone was confirmed as error-free by DNA sequencing.

An N-terminally tagged HA–FKBP–CLC construct was the gift of Enrique Rodriguez-Boulan (Deborde *et al.* 2008). This construct was modified by replacing the HA tag with a fluorescent mCherry tag as follows. We designed PCR primers complementary to mCherry that were flanked by *Nhe*I 5′ and *Bgl*II 3′(5′ GCTAGCATGGTGAGCAAGGGC and 3′ AGATCTCTTGTACAGCTCGTCCATGC) sites to allow cloning from a pmCherry vector (Clontech) into the TOPO TA 2.1 vector (Invitrogen). The TOPO TA 2.1 mCherry vector and the HA-FKBP–CLC PCR product were each digested with *Nhe*I and *Bgl*II. Appropriately sized DNA bands were identified on an agarose gel, extracted, and ligated to create mCherry-FKBP–CLC. Coding sequence was verified as error-free by DNA sequencing. The ARGENT Regulated Homodimerization Kit containing FKBP plasmids and the dimerization drug AP20187 was purchased from ARIAD (www.ariad.com; Cambridge; MA).

All siRNA oligos were purchased from Dharmacon (Lafayette, CO). The siCONTROL Non-Targeting siRNA Pool #1 (D0012061305) was used for control knockdown. siGENOME human CLTC, NM_004859 siRNA was used for CHC

knockdown (D00400102) sense: GCAAUGAGCUGUUUGAAGAUU, antisense: 5′ pUCUUCAAACAGCUCAUUGCUU. siRNA oligos were transfected as described later in the text. AP3δ (RHS4533-NM_003938) and CHC (RHS39799577067) shRNA in a pLKO.1 vector for lentiviral infection were obtained from Open Biosystems (Huntsville, AL). Control shRNA in pLKO.1 was obtained from Addgene (vector 1864; Cambridge, MA).

The peptide against the epitope for AP3δ SA4 (AQQVDIVTEEM-PENALPSDEDDKDPNDPYRA) (Salazar *et al.* 2009) was purchased from the Emory Microchemical Facility (Atlanta, GA) and Invitrogen (EvoQuest Team, Carlsbad, CA).

Cell culture, transfection, and lenti viral infection

HEK293T and HCN-1A cells (American Type Culture Collection [ATCC], Manassas, VA) in DMEM (Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (FBS) (Hyclone) and 100 μg/ml penicillin and streptomycin (Hyclone) and PC12 cells (ATCC) in DMEM supplemented with 5% FBS, 10% equine serum (Hyclone), and 100 μg/ml penicillin and streptomycin (Hyclone) were incubated at 37°C with 10% CO2.

For recombinant DNA expression, HEK293T cells were transfected in six-well dishes with 0.5–2.0 μg DNA in 0.25% Lipofectamine 2000 (Invitrogen) diluted in Opti-Mem (Life Technologies, Grand Island, NY). Cells were transfected for 4 h followed by incubation in either culture medium alone or culture medium containing the selection drug(s) G418 (0.1 mg/ml) and/or hygromycin (0.1 mg/ml) for the maintenance of stable cell lines. PC12 cells were

transfected by nucleofection with 3 μg of DNA using Amaxa Cell Line Nucleofector Kit V (cat# VCA-1003; Lonza Walkersville, Koeln, Germany, www.lonza.com), and were plated on Matrigel-coated glass-bottom culture dishes (Matek, Ashland, MA) in PC12 culture medium supplemented with 100 ng/ml NGF 2.5S (murine, natural) (cat# 13257-019; Invitrogen). PC12 cells were differentiated for 48–72 h at 37°C with 10% CO2 For siRNA knockdown, HEK293T cells were transfected in six-well dishes with 50 nM oligo for 4 h, incubated for 20 h in culture medium, and transfected a second time with 50 nM oligo for 4 h followed by incubation for 3 d in culture medium. For lentiviral infection, HEK293T cells seeded in 10-cm plates were infected with 1 μl of high-titer lentivirus containing the shRNA constructs mentioned earlier in the text. Following a 24-h infection, cells were incubated for up to 6 d in culture medium supplemented with 4 μg/ml puromycin for selection. The Emory Neuroscience NINDS Viral Vector Core Facilities prepared all high-titer lentiviruses.

Cross-linking and immunoprecipitation

Cross-linking was performed as previously described using DSP (Craige *et al.* 2008; Salazar *et al.* 2009; Zlatic *et al.* 2010). DSP is a homobifunctional reversible and cell-permeable cross-linker with a 12-Å spacer arm that stabilizes labile protein interactions (Lomant and Fairbanks 1976). Plates of confluent HEK293T cells were placed on ice, washed twice with ice-cold phosphate-buffered saline (PBS)/1 mM MgCl2/0.1 mM CaCl2, and then incubated with 1 mM DSP (cat# 22585; Thermo Scientific, Rockford, IL) or dimethyl sulfoxide control in PBS/1 mM MgCl2/0.1 mM CaCl2 for 2 h on ice. DSP was then

quenched with 25 mM TRIS, pH 7.4, followed by two rinses with ice-cold PBS/1 mM MgCl2/0.1 mM CaCl2. Cells were lysed in Buffer A (150 mM NaCl, 10 mM HEPES, 1 mM EGTA, 0.1 mM MgCl2) + 0.5% Triton X-100 by incubation for 30 min at 4°C. Any remaining cellular debris was scraped from plates and centrifuged at 16,000 × *g* for 10 min. The supernatant was collected, diluted to 1 μg/ul in Buffer A + 0.5% Triton X-100, and incubated with Dynal immunomagnetic precipitation beads (Dynal, Oslo, Norway) in the absence or presence of a 10 μM SA4 peptide competitor. Beads and lysate were incubated at 4°C for 2 h and washed six times with Buffer A containing 0.1% Triton X-100 to remove nonspecifically bound material. The material that remained bound after these washes was then eluted by treatment with either the peptide antigen or SDS–PAGE sample buffer followed by incubation at 75°C for 5 min. Immunoprecipitated material was analyzed on SDS–PAGE Western blot.

Despite reduced detection of δ in SA4 immunoprecipitates, all other AP-3 subunits are readily detectable in these immunocomplexes (Salazar *et al.* 2009). Decreased detection of AP-3 δ by the SA4 mAb in immunoblots after DSP cross-linking likely reflects a chemical modification of the SA4 epitope by the cross-linking agent. The lysine and arginine present in this peptide are susceptible to modification by DSP. The SA4 antibody is used sequentially in immunoprecipitation and immunoblot, thus effectively magnifying the difference between (–) and (+) DSP samples.

Immunolocalization, microscopy, and quantification

Coverslips were prepared as previously described (Faundez *et al.* 1997).

Cells were seeded onto Matrigel (BD Bioscience, San Jose, CA)-coated glass coverslips, washed twice in PBS/1 mM MgCl2/0.1 mM CaCl2, and fixed using 4% paraformaldehyde in PBS. Cells were then permeabilized and blocked with 0.02% saponin (Sigma), 15% horse serum (Hyclone), 2% bovine serum albumin, and 1% fish skin gelatin (Sigma) in PBS. Blocked and permeabilized cells were incubated with the primary and secondary antibodies described earlier in the text and were mounted with Gelvatol onto slides. Fixed-cell confocal microscopy was performed on fixed cells as described (Deborde *et al.* 2008) using an Axiovert 100M microscope (Carl Zeiss, Thornwood, NY) with Argon/HeNe (488/543) laser excitation. Images were captured using a Plan Apochromat 63×/1.4 oil DIC objective, BP 505–550/LP 560 filter set, and LSM 510 3.2.0.104 software (Carl Zeiss). Deconvolution microscopy was performed as described (Deborde *et al.* 2008) with a 200M inverted microscope using 63×/1.4 and 100×/1.4 oil DIC objectives (Carl Zeiss) and a Sedat filter set. Images were collected using a scientific grade cooled charge-coupled Cool-Snap HQ camera with ORCA-ER chip on a multiwavelength, wide-field, three-dimensional microscopy system using Slidebook 4.0 OS X software (Intelligent Imaging Innovations, Denver, CO). Out-of-focus light was removed with a constrained iterative deconvolution algorithm (Swedlow *et al.* 1997). Confocal and deconvolution images were processed and analyzed using LSM Image Browser 4.0.0.157 (Carl Zeiss), Meta-morph software version 6.1 (Universal Imaging, Sunnyvale, CA), and Imaris 6.3.1 software (Bitplane, St. Paul, MN). Colocalization and puncta size were determined from three consecutive z-series focal planes per image. All channels were thresholded individually. Percent colocalization was determined by

calculation of pixel area containing fluorescent signals from two channels per total pixel area for a single fluorophore. Puncta size was determined by integrated morphometric analysis of total pixel area within puncta as determined by image thresholding.

Clathrin-coated vesicle isolation

HEK293T or HEK293T cells stably expressing tagged HOPS or class C Vps proteins were used to prepare clathrin-coated vesicles as described previously (alternate protocol 2 in Girard *et al.*, 2004). Briefly, cells were grown to confluence and washed twice with PBS/1 mM MgCl2/0.1 mM CaCl2. Cells were lifted in CCV buffer (100 mM MES, 1.0 mM EGTA, 0.5 mM MgCl2, pH 6.5), transferred to a Potter Elvehjem glass-teflon homogenizer, and homogenized for 10 strokes at 1500 rpm on a Tri-R Stir-R variable speed laboratory motor (Model S63C; Tri-R Instruments, Rockville Center, NY). Homogenate was centrifuged in an SS-34 fixed-angle rotor for 20 min at 17,000 × *g*, 4°C. The resulting supernatant was further centrifuged in a type 40 fixed-angle rotor for 60 min at 56,000 × *g*, 4°C. The resulting pellet was resuspended, homogenized, and transferred to polyallomer centrifuge tubes (Beckman, Palo Alto, CA). The sample was then underlaid with D2O-sucrose solution (8% sucrose, 100 mM MES, 1.0 mM EGTA, 0.5 mM MgCl2, D2O) (D2O cat# 364312-10G; Sigma) and was centrifuged in a SW-55 swing-bucket rotor for 2 h at 116,000 × *g*, 4°C. This final, clathrin-coated, vesicle-enriched pellet was resuspended and aliquoted along with reserves from previous fractionation steps and was run on SDS–PAGE gels for Western blotting or Coomassie stain.

Acute clathrin perturbation

HEK293T or HEK293T cells stably expressing tagged HOPS or class C Vps proteins and/or transiently expressing tagged FKBP–CLC were incubated for 2 h at 37°C, 10% CO2 in culture medium supplemented with 50 nM AP20187 (ARIAD) or 0.05% ethanol vehicle control. Culture dishes were then placed on ice and processed for either immunolocalization or cross-linking followed by immunoprecipitation.

Live cell imaging

HEK293T or PC12 cells expressing Vps18–GFP and/or mCherry-FKBP–CLC were grown on Matrigel-coated glass-bottom culture dishes (Matek). Imaging medium consisted of Hank's balanced salt solution minus phenol red and NaHCO2 (Sigma) and supplemented with 10% FBS (Hyclone) and 20 mM HEPES for HEK293T, and with 10% Donor Equine Serum (Hyclone), 5% FBS (Hyclone), and 100 ng/ml NGF 2.5S (murine, natural, cat# 13257–019; Invitrogen) for PC12 cells. Live imaging was performed on an A1R Laser Scanning Confocal Microscope (Nikon, Melville, NY) equipped with a hybrid scanner, Perfect Focus, and an environmental chamber for regulation of temperature to 37°C and 10% CO2. Fluorophores were alternately excited with 488 and 568 nm wavelength laser every 5 min for 2 h and 30 min. AP20187 (Ariad) or ethanol vehicle control was added to imaging medium at concentrations listed earlier in the text after the first 25 min of imaging. Images were captured with an APO

TIRF 60×/1.49 oil DIC objective and 500–550/570–620 filter sets on NIS-Elements AR 3.1 (Nikon) software. NIS-Elements AR 3.0, Imaris 6.3.1 (Bitplane), and Image J 1.41(NIH) software were used for image analysis.

Statistical analysis

Experimental conditions were compared with the nonparametric Wilcoxon–Mann–Whitney Rank Sum Test using Synergy KaleidaGraph v4.03 (Reading, PA) or StatPlus Mac Built5.6.0pre/Universal (AnalystSoft, Vancouver, Canada). Data are presented as boxplots displaying the four quartiles of the data, with the "box" comprising the two middle quartiles, separated by the median. The upper and lower quartiles are represented by single lines extending from the box. Circles correspond to outlier points defined by the statistical software as beyond the upper or lower quartile plus 1.5 times the value of the 2–3 interquartile distance.

Acknowledgements

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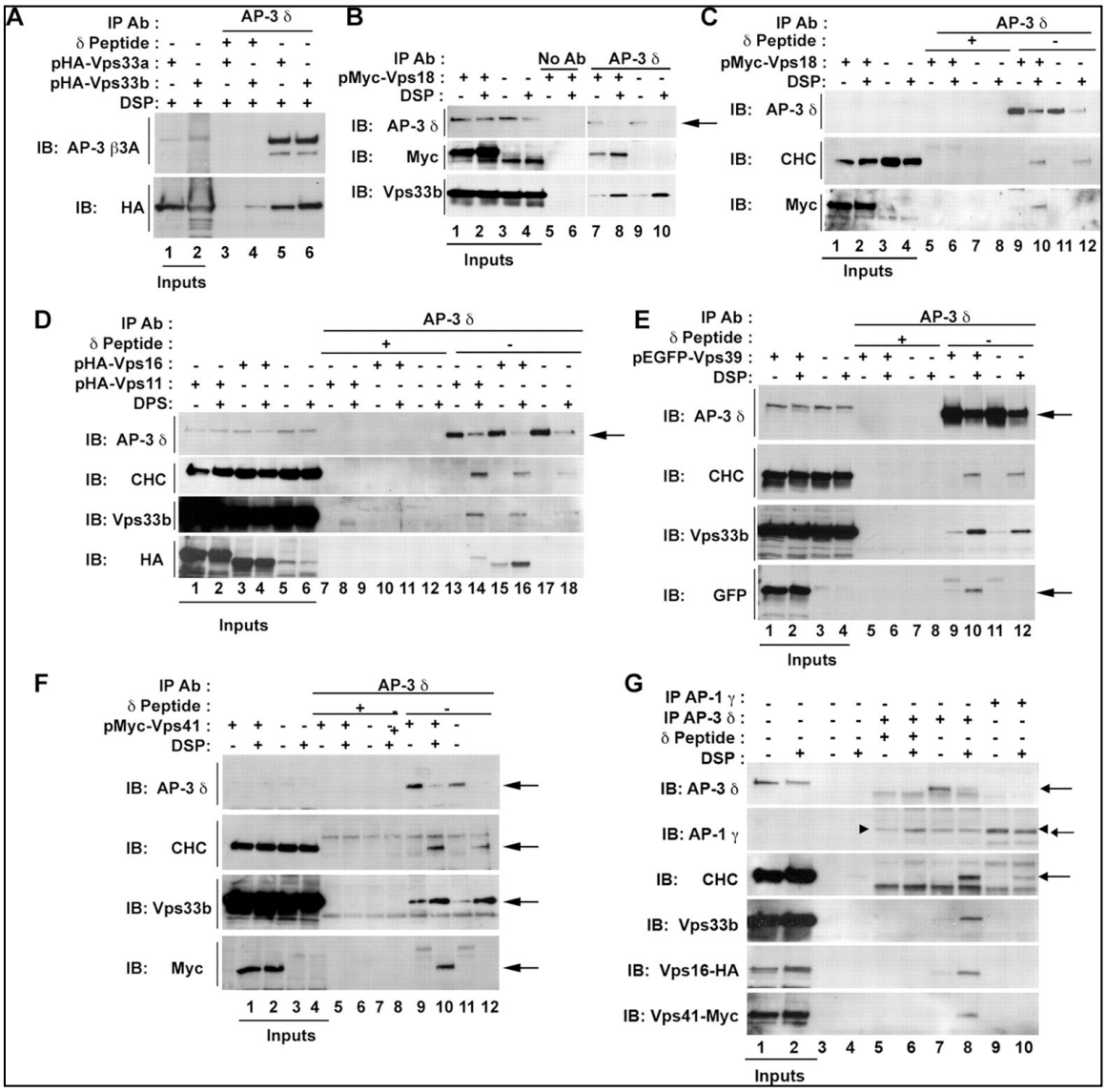
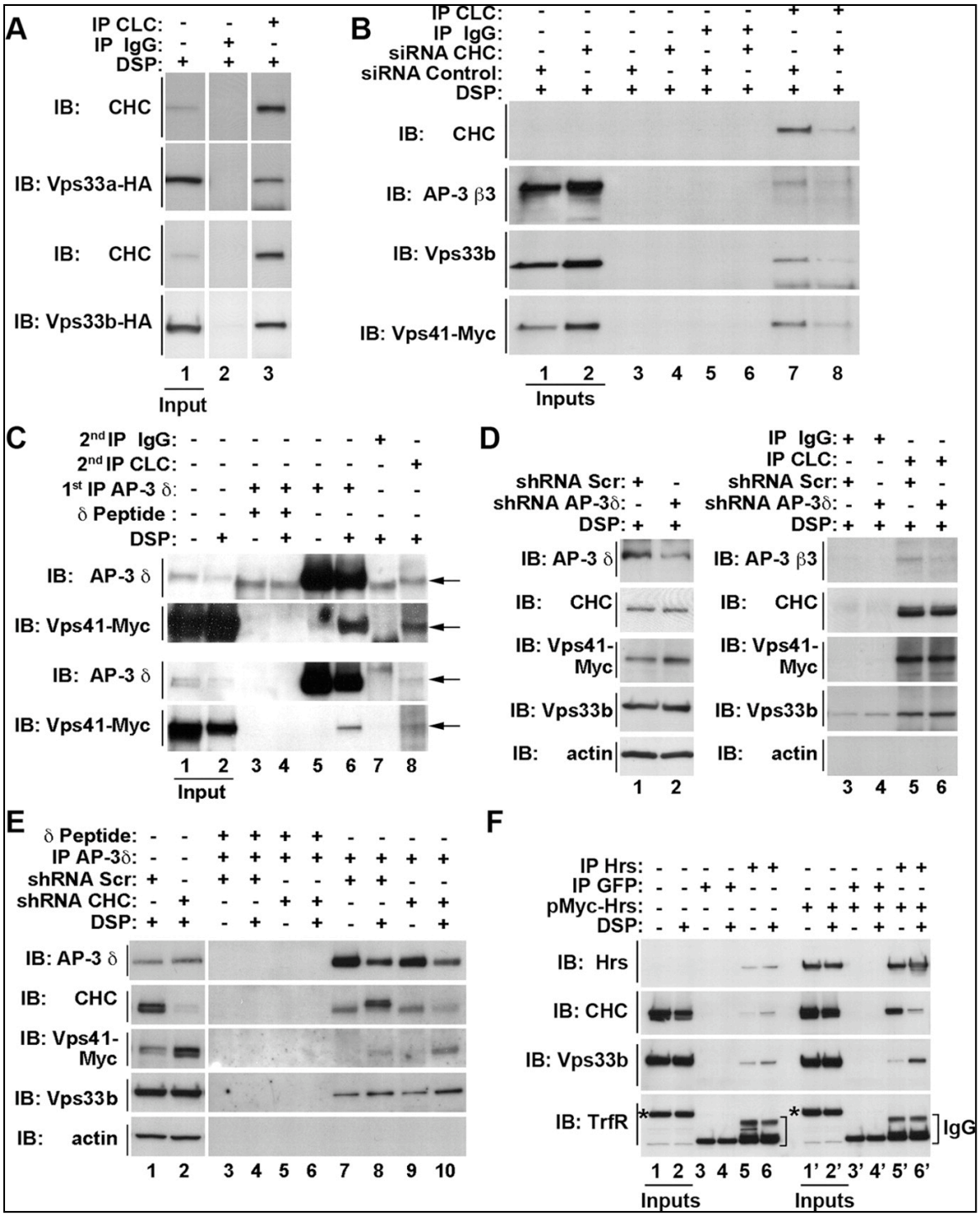


Figure 1. Association of class C Vps/HOPS subunits with the adaptor complex AP-3. HEK293T cells or cell lines expressing recombinant class C Vps/HOPS subunits (A–G) were left untreated (B–G, odd lanes) or treated (B–G, even lanes) with DSP at 4°C. Detergent-soluble cell extracts were incubated with sheep anti–mouse IgG-coated magnetic beads as follows: (A) Extracts of DSP-treated HEK293T cells transfected with Vps33A (odd lanes) or Vps33b (even

lanes) incubated with magnetic beads decorated with antibody directed against AP-3 δ (lanes 3–6). The peptide used to raise the AP-3 δ mAb was added in excess during the immunoprecipitation to determine the specificity of signals detected by immunoblot (lanes 3 and 4). (B) Extracts of HEK293T cells (lanes 3, 4, 9, and 10) and cells expressing Vps18–Myc (lanes 1, 2, and 5–8) were incubated with anti–mouse IgG magnetic beads either lacking mouse antibody (lanes 5 and 6, No antibody) or linked to mouse mAb directed against AP-3 δ (lanes 7–10). (C) Extracts of HEK293T cells (lanes 3, 4, 7, 8, 11, and 12) and cells expressing Vps18–Myc (lanes 1, 2, 5, 6, 9, and 10) were incubated with anti–mouse IgG magnetic beads decorated with antibody directed against AP-3 δ (lanes 5–12). The peptide used to raise the AP-3 δ mAb was used as in A (lanes 5–8). (D) Extracts of HEK293T cells (lanes 5, 6, 11, 12, 17, and 18), cells expressing Vps16–HA (lanes 3, 4, 9, 10, 15, and 16), or cells expressing Vps11–HA (lanes 1, 2, 7, 8, 13, and 14) were incubated with anti–mouse IgG magnetic beads decorated with antibody directed against AP-3 δ (lanes 7–18). Peptide competition was performed as in A (lanes 7–12). (E and F) Extracts of HEK293T cells (E and F lanes 3, 4, 7, 8, 11, and 12) and cells expressing Vps39–GFP (E lanes 1, 2, 5, 6, 9, and 10) or cells expressing Vps41–Myc (F lanes 1, 2, 5, 6, 9, and 10) were incubated with anti–mouse IgG magnetic beads bound to AP-3 δ antibodies (E and F lanes 5–12). AP-3 δ peptide competition was performed as in A (E and F lanes 5–8). (G) Cells expressing both Vps16–HA and Vps41–Myc were incubated with anti–mouse IgG magnetic beads only (lanes 3 and 4) or beads decorated with mAbs directed against: AP-3 δ subunit (lanes 5–8, AP-3 δ) or AP-1 γ subunit (lanes 9 and 10, AP-1 γ). Specificity of association with AP-3 complexes was

determined by peptide competition as in A (lanes 5 and 6). Immune complexes were resolved by SDS–PAGE, and their composition was assessed by immunoblot with antibodies against AP-3 δ, AP-3 β3A, AP-1 γ, CHC, endogenous Vps33b, Myc, GFP, or HA tags. Arrows in B, D, E, and F depict specific bands, and other bands correspond to background sheep or mouse IgG from beads or residual signal from previous immunoblot probings. Arrowhead in AP-1 γ blot strip depicts sheep or mouse IgG from beads or residual signal from previous immunoblot probing. All experiments were performed at least twice in stably or transiently expressing cells. For A–G, inputs represent 5%.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DSP: + + +
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IB: Vps33a-HA
IB: CHC
IB: Vps33b-HA
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IB: AP-3 β3
IB: Vps33b
IB: Vps41-Myc
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IB: Vps33b
IB: actin
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DSP: + + - + - + - + - +
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IB: CHC
IB: Vps41-Myc
IB: Vps33b
IB: actin
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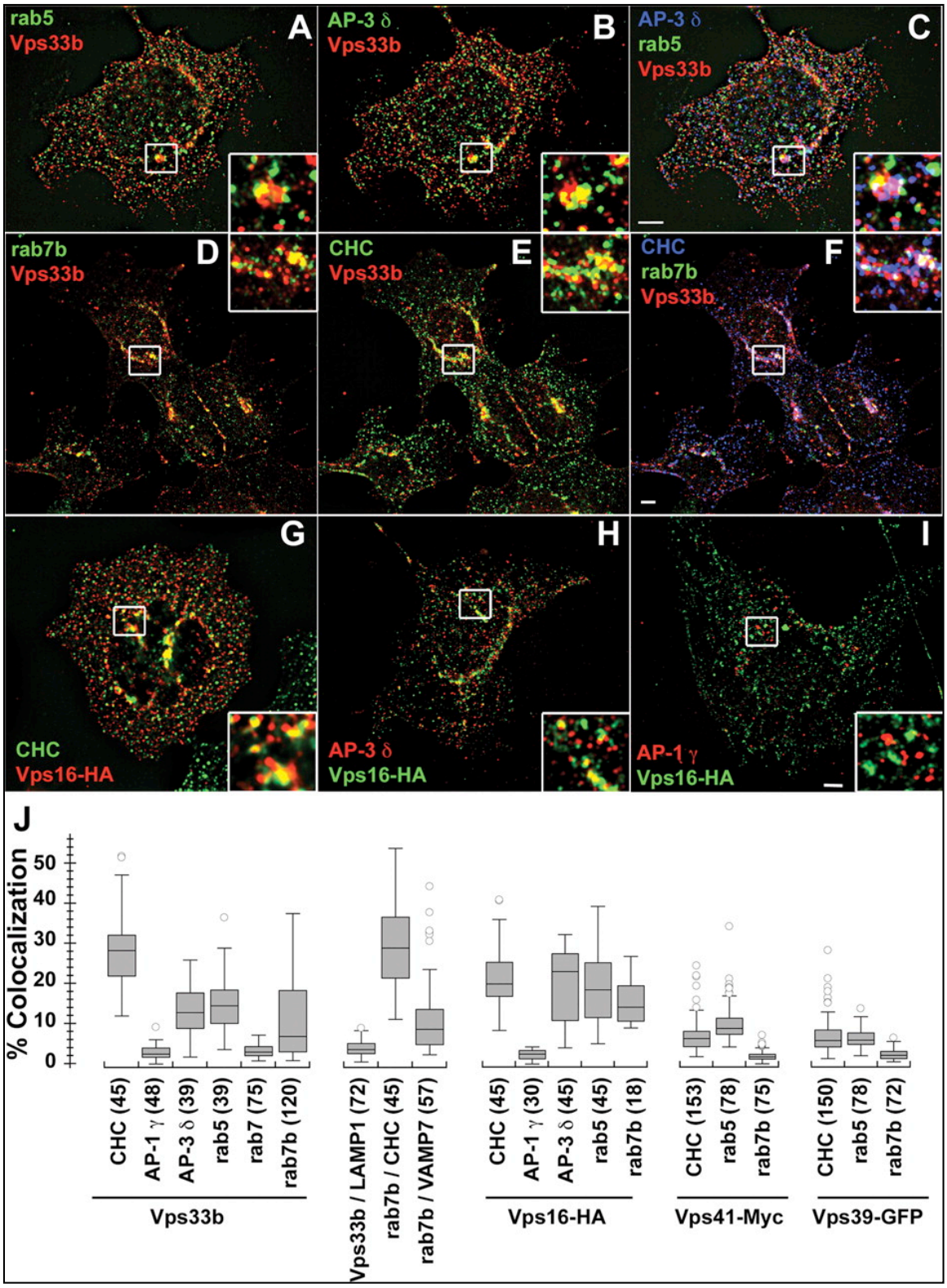
IB: Hrs
IB: CHC
IB: Vps33b
IB: TrfR
IgG
1 2 3 4 5 6 1' 2' 3' 4' 5' 6'
Inputs
Inputs

Figure 2. CHC and CLC associate with class C Vps/HOPS subunits. (A) Extracts of DSP-treated HEK293T cells transfected with Vps33A or Vps33b were incubated with magnetic beads decorated with antibody directed against control IgG (lane 2) or CLC (lane 3). Immunocomplexes were resolved by SDS–PAGE, and contents were analyzed by immunoblot with antibodies against the HA tag and CHC. Input represents 5%. (B) HEK293 cells stably expressing Vps41–Myc were treated with scramble (Control, odd lanes) and CHC siRNA (CHC, even lanes) for 5 d. siRNA-treated cells were incubated in the presence of DSP (all lanes) at 4°C. Detergent-soluble cell extracts were immunoprecipitated with magnetic beads lacking mouse IgG (lanes 3 and 4), control mouse IgG (monoclonal SV2 lanes 5 and 6), or antibodies against CLCs (lanes 7 and 8). Immunocomplexes were resolved by SDS–PAGE, and contents were analyzed by immunoblot with antibodies against CHC, AP-3 β3A, Vps33b, and Myc. Depletion of CHC prevents the precipitation of Vps33b and Vps41-Myc with CLC. Input represents 5%. (C) Vps41–Myc is in a complex with AP-3 and clathrin complexes. HEK293T cells stably expressing Vps41–Myc were treated in the absence (lanes 1, 3, and 5) or presence of DSP (lanes 2, 4, and 6–8). Clarified cell extracts were first immunoprecipitated with magnetic beads decorated with AP-3 δ antibodies (lanes 3–6) either in the absence (lanes 5 and 6) or presence of excess δ antigenic peptide as a control (lanes 3 and 4). Cross-linked AP-3 complexes bound to beads in lane 6 were eluted under native conditions using δ antigenic peptide. These eluted cross-linked AP-3 complexes were subjected to a second round of immunoprecipitations with control mouse IgG (mAb SV2 [top two panels] and SY38 [bottom two panels], lane 7) or CLC antibodies (lane 8). SV2 and SY38

antibodies recognize neuronal antigens absent in HEK293T cells. Immunocomplexes were resolved by SDS–PAGE, and contents were analyzed by immunoblot with antibodies against AP-3 δ and Myc epitope. Arrows mark specific bands for AP-3 δ and Vps41–Myc. Note the different background bands obtained with the two different negative control antibodies during the second round of immunoprecipitation. Input represents 5%. (D) Interaction of Vps class C/HOPS subunits with clathrin is not affected by down-regulation of AP-3. HEK293T cells stably expressing Vps41–Myc were infected with lentiviruses encoding nontargeting shRNA (Scr, lanes 1, 3, and 5) or shRNA targeting AP-3 δ (lanes 2, 4, and 6). After 6 d, cells were incubated in the presence of DSP (lanes 1–6) and homogenized. Detergent cell extracts were immunoprecipitated with magnetic beads decorated with control mouse IgG (monoclonal SY38, lanes 3 and 4), or antibodies against CLCs (lanes 5 and 6). Immunocomplexes were resolved by SDS–PAGE, and contents were analyzed by immunoblot with antibodies against AP-3 β3A, AP3 δ, CHC, Myc epitope, Vps33b, and β actin. Lanes 1 and 2 correspond to homogenates run on different gels to assess efficiency of down-regulation. (E) Vps class C/HOPS subunits interaction with AP-3 is not affected by down-regulation of CHC. HEK293T cells stably expressing Vps41–Myc were infected with lentiviruses encoding nontargeting shRNA (Scr, lanes 1, 3, 4, 7, and 8) or shRNA targeting CHC (lanes 2, 5, 6, 9, and 10). After 6 d, cells were incubated in the absence (lanes 3, 5, 7, and 9) or presence of DSP (lane 1 and even lanes). Cell extracts were immunoprecipitated with magnetic beads decorated with AP-3 δ antibodies (lanes 3–10) either in the absence (lanes 7–10) or presence of an excess of δ antigenic peptide as a control (lanes 3–6).

Immunocomplexes were resolved by SDS–PAGE, and contents were analyzed by immunoblot with antibodies against AP-3 δ, CHC, Myc epitope, Vps33b, and β actin. Lanes 1 and 2 correspond to homogenates run on different gels to assess efficiency of down-regulation. (F) Vps33b associates with clathrin–Hrs coats. HEK293T cells (lanes 1–6) or transiently transfected with Hrs–Myc (lanes 1′–6′) were treated in the absence (odd lanes) or presence of DSP (even lanes). Detergent-soluble cell extracts were immunoprecipitated with magnetic beads decorated with GFP antibodies as controls (all lanes 3 and 4) or Hrs antibodies (all lanes 5 and 6). Immunocomplexes were resolved in SDS–PAGE, and their composition was analyzed by immunoblot with antibodies against Hrs, CHC, Vps33b, and transferrin receptor (TrfR) as control. Asterisks denote TrfR band, and brackets denote IgGs. Inputs correspond to 5%.


rab5
Vps33b
A
AP-3 δ
Vps33b
B
AP-3 δ
rab5
Vps33b
C
rab7b
Vps33b
D
CHC
Vps33b
E
CHC
rab7b
Vps33b
F
CHC
Vps16-HA
G
AP-3 δ
Vps16-HA
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AP-1 γ
Vps16-HA
I
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% Colocalization
0
10
20
30
40
50
CHC (45)
AP-1 γ (48)
AP-3 δ (39)
rab5 (39)
rab7 (75)
rab7b (120)
Vps33b
Vps33b / LAMP1 (72)
rab7b / CHC (45)
rab7b / VAMP7 (57)
CHC (45)
AP-1 γ (30)
AP-3 δ (45)
rab5 (45)
rab7b (18)
Vps16-HA
CHC (153)
rab5 (78)
rab7b (75)
Vps41-Myc
CHC (150)
rab5 (78)
rab7b (72)
Vps39-GFP

Figure 3. Vps class C/HOPS are present in AP-3, clathrin, Rab5, and Rab7b-compartments. HEK293T cells were fixed, and either triple (A–F) or double (G–I) labeled for indirect immunofluorescence with antibodies against the following antigens: Vps33b, rab5, and AP-3 δ (A–C); Vps33b, rab7b, and CHC (D–F); HA epitopes to detect recombinantly expressed Vps16-HA (G–I), and either CHC (G), AP-3 δ (H), or AP-1 γ (I). Cells were imaged by Delta deconvolution fluorescence microscopy. Bar depicts 5 μm; images to the right are 300% magnifications of white-boxed inserts. (J) Quantitative analysis of Vps class C/HOPS subcellular distribution. HEK293T cells or those expressing recombinant Vps16–HA, Vps39–GFP, or Vps41–Myc were labeled with combinations of antibodies against endogenous Vps33b or tags engineered in the Vps class C/HOPS subunits Vps16, Vps39, Vps41; the coats CHC, AP-1 γ, AP-3 δ; the early endosome marker rab5; the late endosome markers VAMP7, rab7, rab7b, and LAMP-1. Cells were imaged by Delta deconvolution fluorescence microscopy, and the percentage of overlapping pixels of different antigen combinations was determined using Metamorph. The number of images analyzed appears in parentheses, and quantifications were obtained in at least three independent experiments.

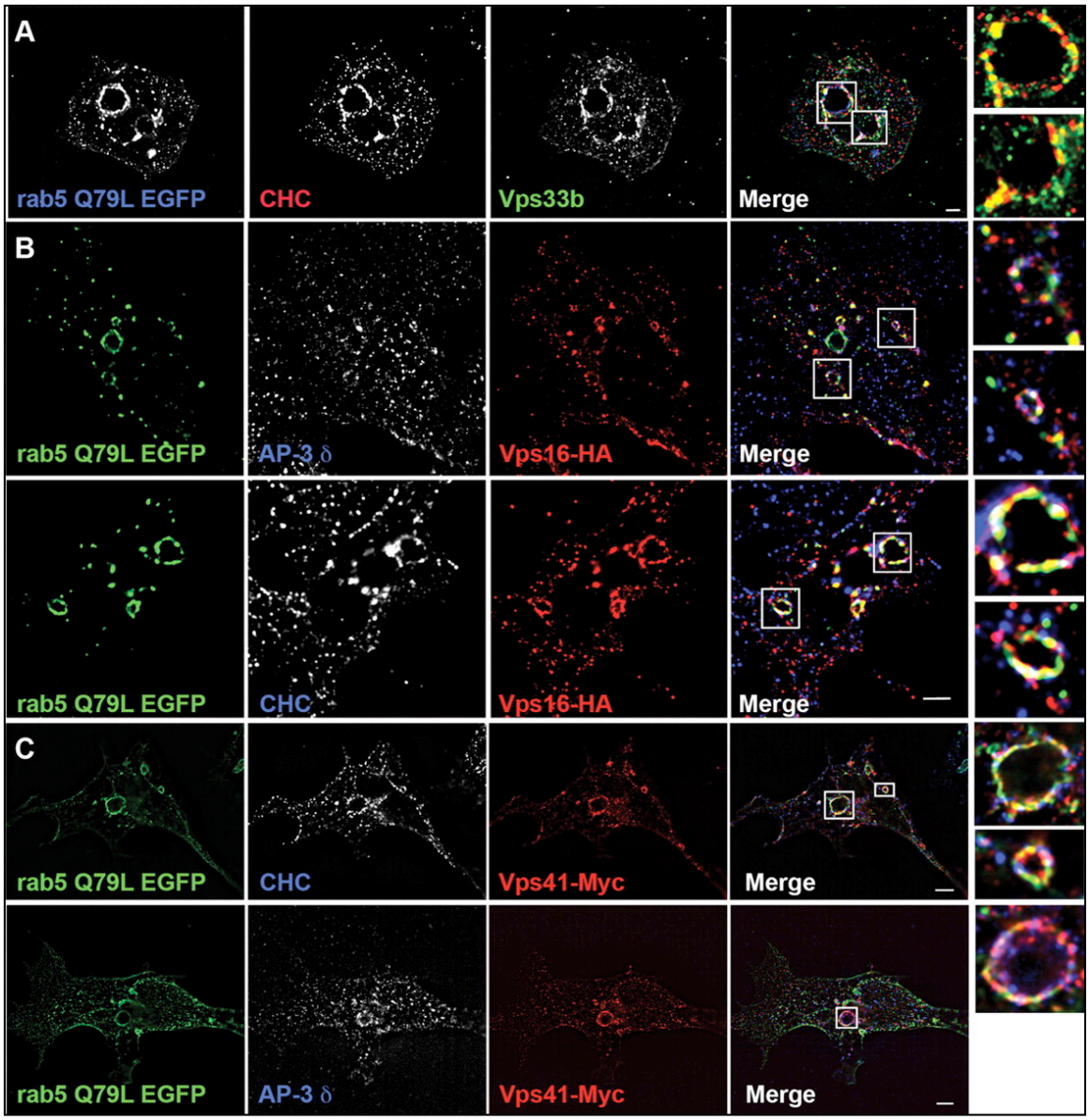


Figure 4. Vps class C/HOPS subunits localize to discrete domains of enlarged early endosomes. HEK293T cells transiently expressing recombinant rab5Q79L–GFP alone (A) or rab5Q79L–GFP plus either Vps16–HA (B) or Vps41–Myc (C) were fixed and triple labeled for indirect immunofluorescence with antibodies against GFP (A–C) plus: (A) antibodies against Vps33b and CHC, (B) antibodies against HA epitope plus either AP-3 δ or

CHC, or (C) antibodies against Myc epitope plus either AP-3 δ or CHC. Cells were imaged by Delta deconvolution fluorescence microscopy. Bar depicts 5 μm; images to the right are 300% magnifications of insets. Supplementary Movies 1 and 2 depicts a tridimensional rendering of enlarged endosomes shown in panel C.

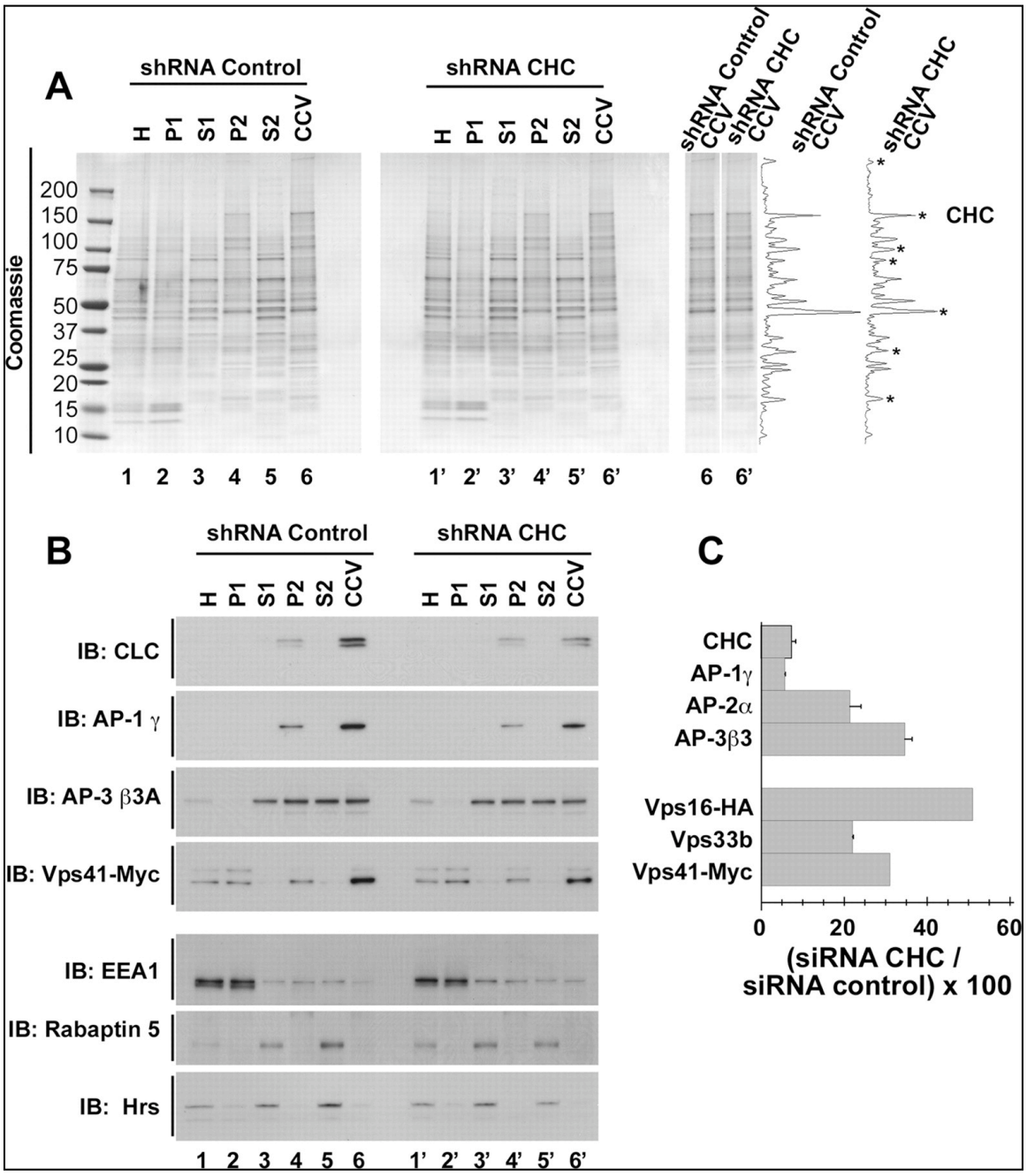


Figure 5. Vps class C/HOPS subunits cosediment with clathrin-coated vesicles. (A and B) HEK293T cells were infected with scramble (lanes 1–6) or CHC directed shRNA lentiviruses (CHC, lanes 1′–6′). Cells were homogenized, and clathrin-coated vesicle fractions were obtained (H, homogenate; P_n, pellets;

S_n, supernatants; and CCV, clathrin-coated vesicle-enriched fraction). Fractions were Coomassie stained (A). Right panel in A depicts densitometry traces of lanes 6 and 6′ where protein bands decreased by CHC knock-down are marked by asterisks. (B) Shows the same fractions as in (A) blotted with antibodies against the coats AP-1 γ, AP-3 β3A, CLCs, and the HOPS subunit Vps41–Myc. Endosomal contamination was assessed by blotting with antibodies against EEA1, rabaptin 5, and Hrs. (C) Quantification of CCV fractions from immunoblot of the coat proteins AP-1 γ, AP-2 α, AP-3 β3A, AP-3 δ, CHC; and the Vps class C/HOPS subunits Vps16–HA, Vps33b, and Vps41–Myc. The content of these antigens was determined as a percentage between CHC and scrambled knockdowns. Data depict the quantification of three experiments.

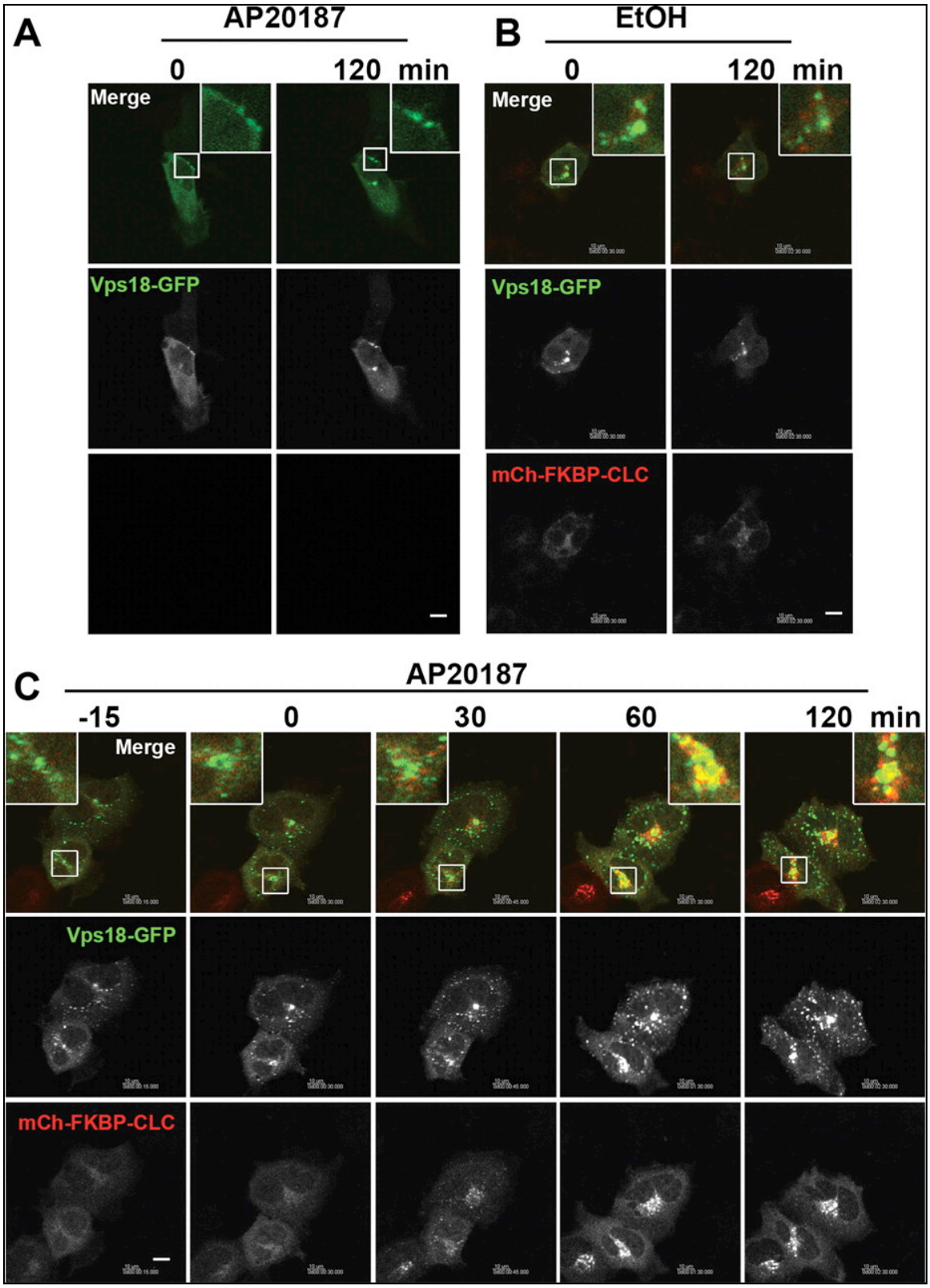

A
AP20187
0
120 min
Merge
Vps18-GFP
B
EtOH
0
120 min
Merge
Vps18-GFP
mCh-FKBP-CLC
C
AP20187
-15
0
30
60
120 min
Merge
Vps18-GFP
mCh-FKBP-CLC

Figure 6. In vivo chemical/genetic disruption of clathrin chains rapidly redistributes Vps18. HEK293T cells transiently expressing Vps18–GFP (A) and an mCherry-tagged (mCh, B and C) chimeric CLC carrying a modified FKBP 12, as an oligomerization module, were imaged by time-lapse confocal microscopy in the presence of ethanol vehicle (B, EtOH, 0.05% vol/vol) or AP20187 (A and C, 50 nM). Cells were imaged for 25 min before vehicle or drug additions and continuously for 2 h after drug addition. AP20187 effects upon Vps18–GFP require expression of mCh-FKBP-CLC. AP20187 induces redistribution of Vps18–GFP in <30 min. See Supplemental Movies 3–6.

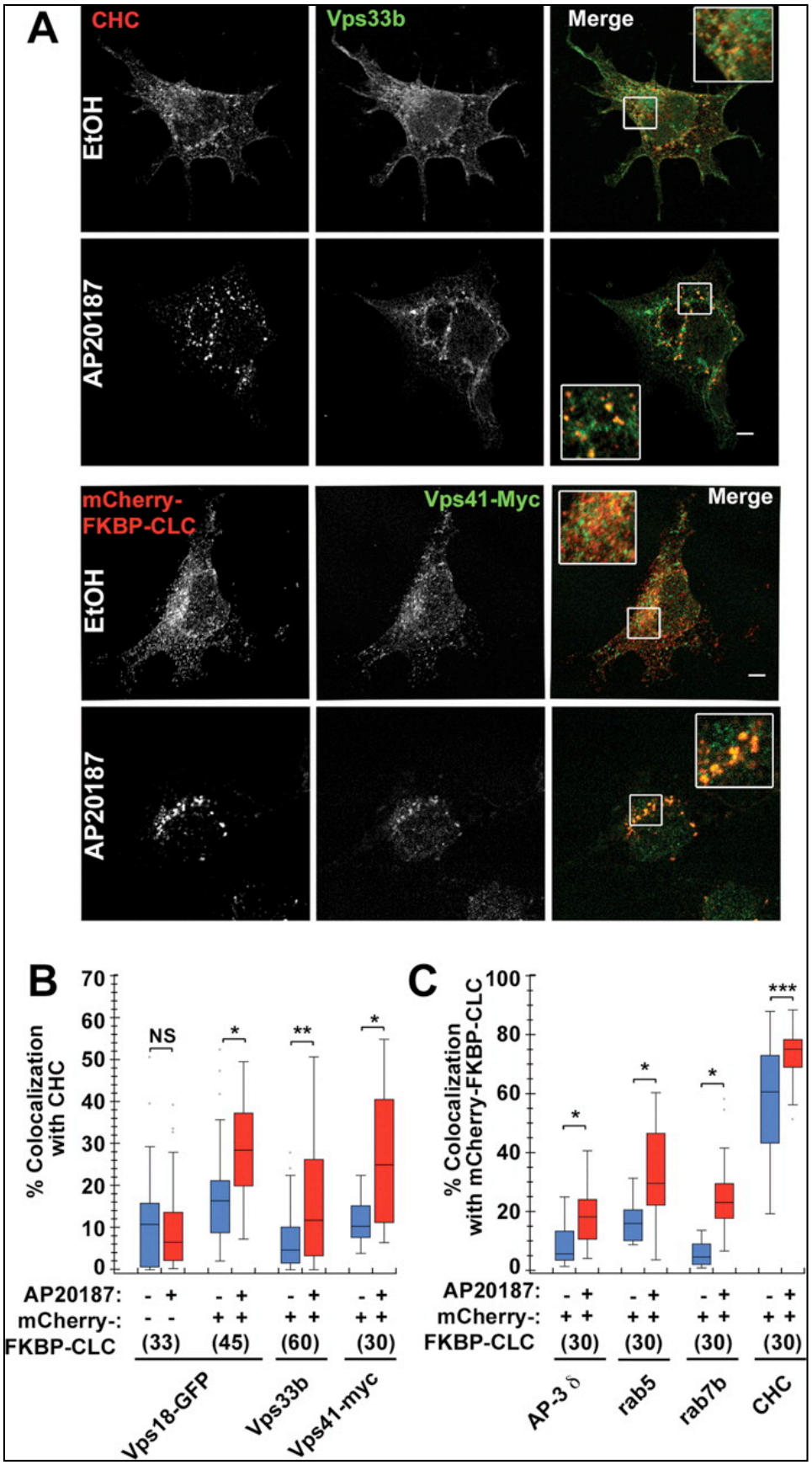

A
CHC
Vps33b
Merge
EtOH
AP20187
mCherry-
FKBP-CLC
Vps41-Myc
Merge
EtOH
AP20187
B
% Colocalization with CHC
NS
*
**
*
AP20187:
mCherry-:
FKBP-CLC
(33)
(45)
(60)
(30)
Vps18-GFP
Vps33b
Vps41-myc
C
% Colocalization with mCherry-FKBP-CLC
*
*
*
***
AP20187:
mCherry-:
FKBP-CLC
(30)
(30)
(30)
(30)
AP-3 δ
rab5
rab7b
CHC

Figure 7. Chemical/genetic disruption of clathrin chains affects Vps class C/HOPS subunits and coat distribution. HEK293T cells transiently expressing mCherry-FKBP–CLC were treated in the presence of ethanol vehicle (EtOH, 0.05% vol/vol) or AP20187 (50 nM) for 2 h. Cells were fixed, processed for indirect immunofluorescence microscopy, and imaged by confocal microscopy. (A) Top two panels, cells expressing mCherry-FKBP–CLC were probed with antibodies against endogenous CHC and Vps33b. Bottom two panels, cells expressing mCherry-FKBP–CLC and Vps41–Myc were probed with antibodies against mCherry and Myc epitopes. Note the redistribution of clathrin chains and Vps class C/HOPS subunits. (B) HEK293T cells mock transfected (–) or expressing mCherry-FKBP–CLC (+) were treated in the presence of ethanol vehicle (–) or AP20187 (+) for 2 h. Cells were stained with antibodies against CHC and one of the following antigens: GFP, to detect exogenously expressed Vps18–GFP; Myc, to detect exogenously expressed Vps41–Myc; or Vps33b to detect the endogenous protein. (C) HEK293T cells expressing mCherry-FKBP–CLC (+) were treated in the presence of ethanol vehicle (–) or AP20187 (+) for 2 h. Cells were stained with antibodies against mCherry and one of the following: AP-3 δ, rab5, rab7b, or CHC. (B and C) Percentage of overlapping pixels between different antigen combinations was determined using Metamorph. Numbers depicted in parentheses denote numbers of analyzed images obtained from at least three independent experiments. NS, not significant; * $p < 0.0001$, ** $p < 0.003$, *** $p < 0.0004$, Wilcoxon–Mann–Whitney Rank Sum Test.

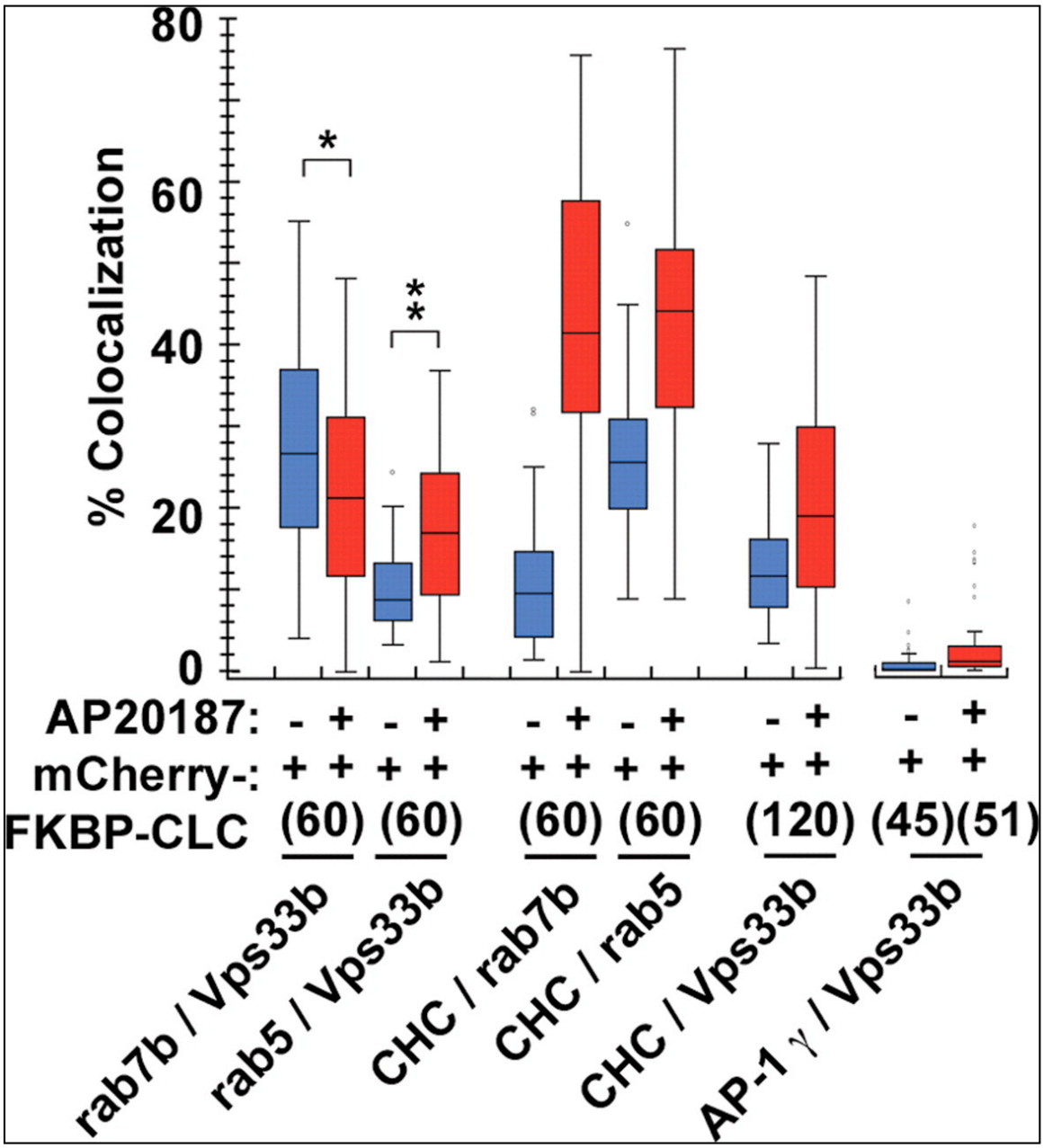


Figure 8. Chemical/genetic disruption of clathrin affects Vps33b distribution in Rab5 and Rab7b endosomal compartments. This figure depicts the quantification of experiments as those in Supplemental Figure 3. HEK293T cells expressing mCherry-FKBP–CLC (+) were treated in the presence

of 0.05% vol/vol ethanol vehicle (–) or 50 nM AP20187 (+) for 2 h. Cells were triple stained with antibodies against CHC, Vps33b, and one of the following: rab5 or rab7b. Alternatively, cells were double labeled with AP-1 γ and Vps33b antibodies. Percentage of overlapping pixels between different antigen combinations was determined using Metamorph. Numbers depicted in parentheses denote number of analyzed images obtained from at least three independent experiments. * $p = 0.0288$, ** $p < 0.0001$, Wilcoxon–Mann–Whitney Rank Sum Test.

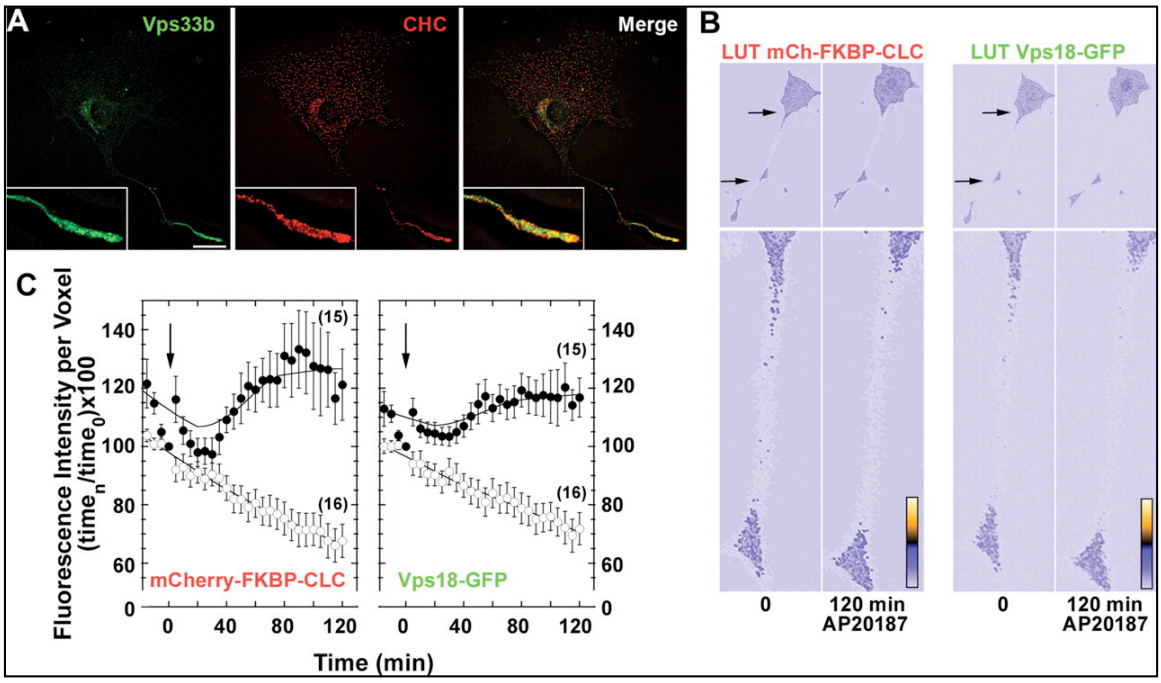
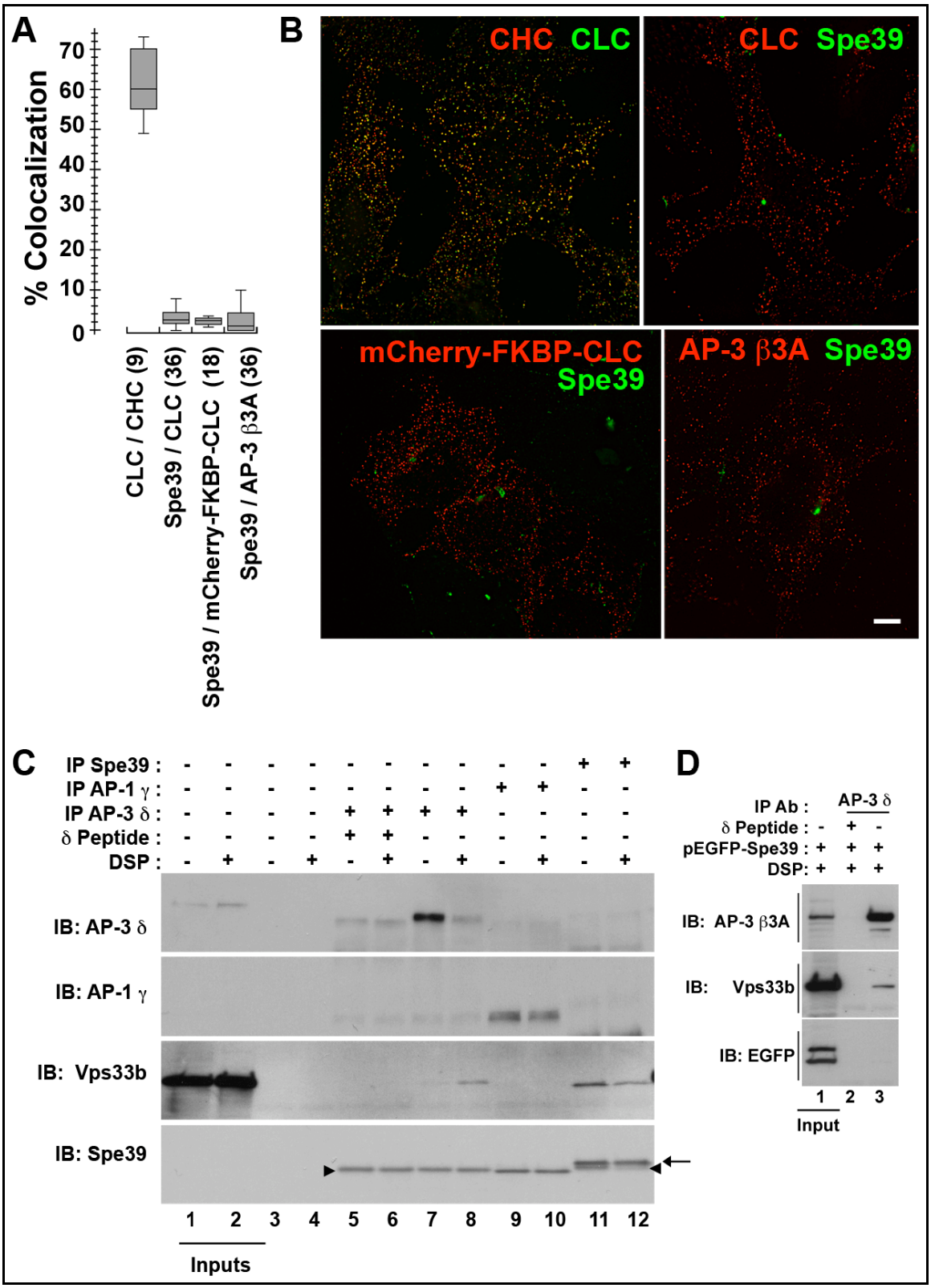
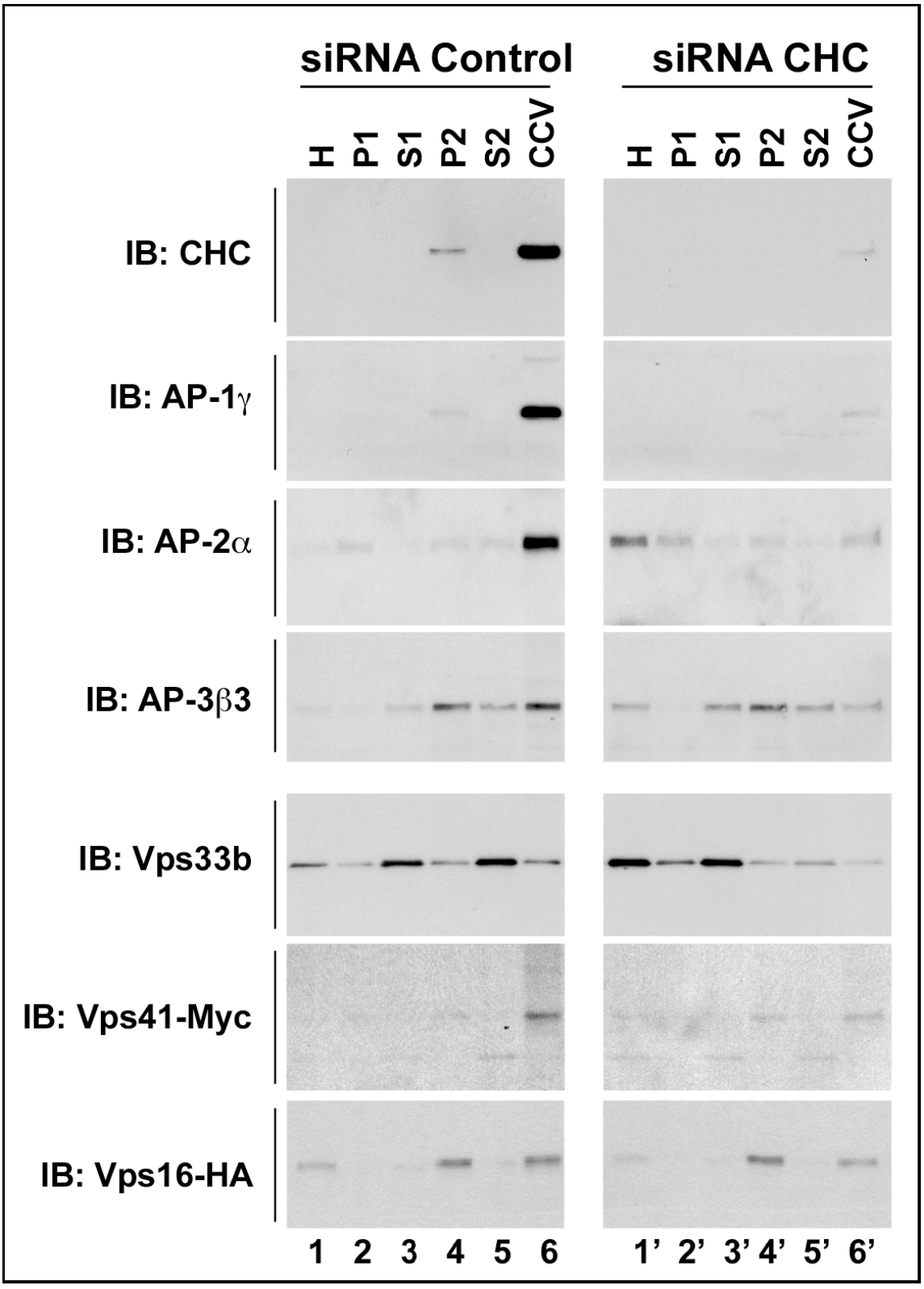


Figure 9. Polarized distribution of clathrin and Vps class C proteins in neuronal cells. (A) Human cortical neuronal cells, HCN-1, were fixed and processed for indirect immunofluorescence microscopy. Cells were double labeled with antibodies against endogenous Vps33b and CHC and imaged by high-resolution Delta deconvolution microscopy as previously described. HCN-1 cells that spontaneously extend processes in culture were imaged. (B) PC12 cells expressing recombinant mCherry-FKBP–CLC and Vps18–GFP were differentiated with NGF to induce process extension. Cells were imaged by live time-lapse confocal microscopy in the presence of 50 nM AP20187. Images of cells at time 0 and 120 min after drug addition are presented in (B). Panels depict images pseudocolored using the ICA LUT from Image J. Arrows mark the segment of the neurite where fluorescence intensities were measured over time. (C) Depicts a quantitative analysis of fluorescence intensity per voxel for

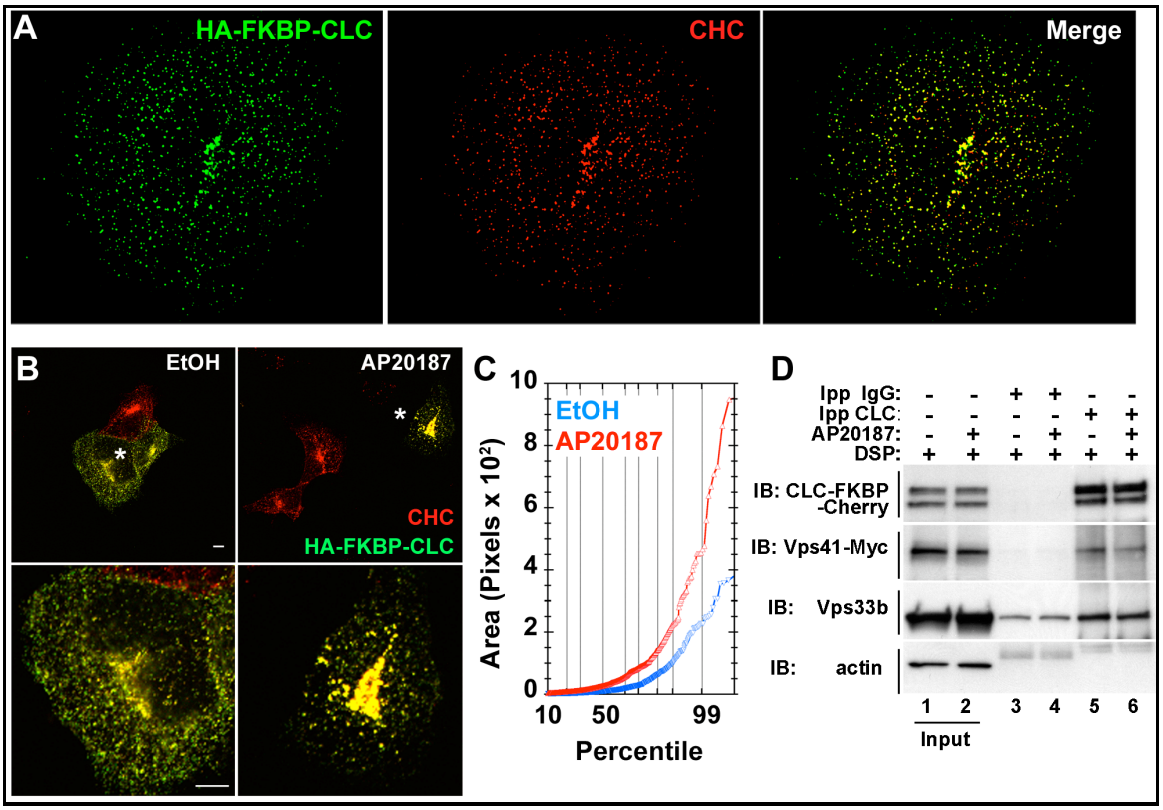
mCherry-FKBP–CLC and Vps18–GFP. Neurite volume was measured in the proximal third of neurites as indicated by the arrows in (B). Closed circles correspond to cells imaged in the presence of ethanol vehicle (0.05% vol/vol), and open circles correspond to cells imaged in the presence of AP20187 (50 nM). Addition of either ethanol or AP20187 is marked by an arrow and corresponds to time 0. All data were normalized to the fluorescence intensity at time 0. Fluorescence intensities for mCherry-FKBP–CLC or Vps18–GFP at 60, 90, and 120 min after drug addition are significantly different from those from ethanol-treated cells ($p < 0.00015$, Wilcoxon–Mann–Whitney Rank Sum Test). Bar represents 20 μm.


A
% Colocalization
70
60
50
40
30
20
10
0
CLC / CHC (9)
Spe39 / CLC (36)
Spe39 / mCherry-FKBP-CLC (18)
Spe39 / AP-3 β3A (36)
B
CHC CLC
CLC Spe39
mCherry-FKBP-CLC
Spe39
AP-3 β3A Spe39
C
IP Spe39 : - - - - - - - - - - + +
IP AP-1 γ : - - - - - - - - + + - -
IP AP-3 δ : - - - - + + + + - - - -
δ Peptide : - - - - + + - - - - - -
DSP : - + - + - + - + - + - +
IB: AP-3 δ
IB: AP-1 γ
IB: Vps33b
IB: Spe39
1 2 3 4 5 6 7 8 9 10 11 12
Inputs
D
IP Ab : AP-3 δ
δ Peptide : - + -
pEGFP-Spe39 : + + +
DSP: + + +
IB: AP-3 β3A
IB: Vps33b
IB: EGFP
1 2 3
Input

Supplementary Figure 1. The Vps33b-Interacting Potein Spe39 does not Associate with Clathrin or AP-3 Positive Organelles. (A-B) HEK293T cells or cells expressing mCherry-FKBP-CLC were fixed and double labeled for indirect immunofluorescence with combinations of antibodies against the following antigens: Clathrin heavy and light chains (CHC and CLC), mCherry and Spe39, and AP-3 β3A and Spe39. A) Quantitative Analysis of Vps Class C/HOPS colocalization in the indicated antigen pairs. Numbers in parentheses indicate the number of images analyzed using Metamorph software. In B) bar depicts 5 μm. C) HEK293T cells were treated in the absence (odd lanes) or presence of DSP (even lanes) and homogenized. Clarified detergent soluble cell extracts were immunoprecipitated with magnetic beads decorated with no antibodies (lanes 3-4), or monoblonal antibodies directed against AP-3 δ (lanes 5-8), AP-1 γ (lanes 9-10), or Spe39 (lanes 11-12). The peptide used to raise the AP-3 δ monoclonal antibody was added in excess to determine the specificity of protein coprecipitation with AP-3 (lanes 5-6). Immune complexes were resolved by SDS-PAGE and analyzed by immunoblot with antibodies against AP-3 δ, AP-1 γ, Vps33b, and Spe39. D) HEK293T cells transiently transfected with Spe39-GFP were treated in the presence of DSP and cells extracts immunoprecipiated with antibodies directed against AP-3 δ either in the absence (lane 3) or presence of antigenic peptide (lane 2). Immune complexes were resolved by SDS-PAGE and analyzed by immunoblot with antibodies against AP-3 β3A, Vps33b, and Spe39. All inputs represent 5%.

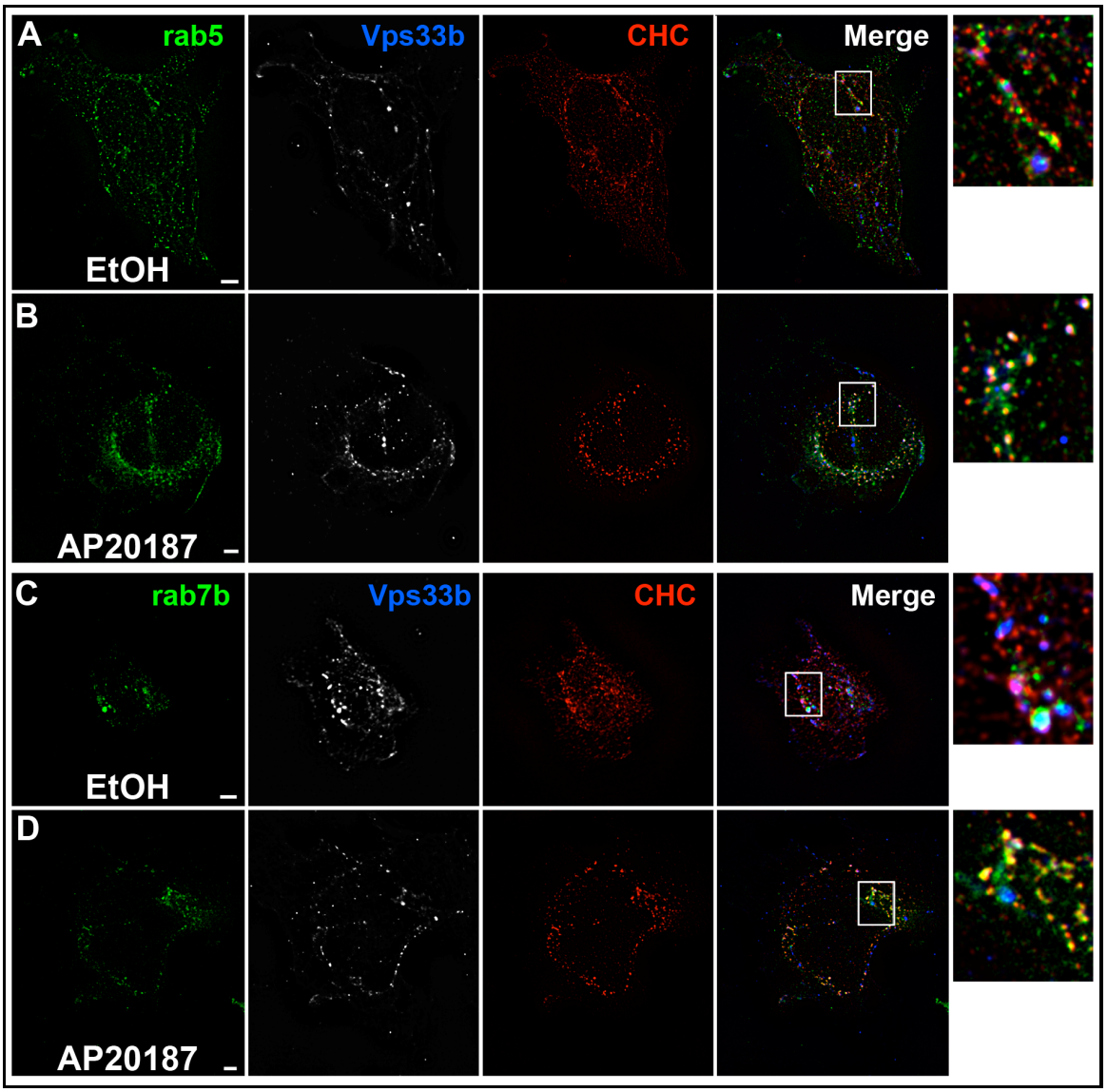

siRNA Control
siRNA CHC
H
P1
S1
P2
S2
CCV
IB: CHC
IB: AP-1γ
IB: AP-2α
IB: AP-3β3
IB: Vps33b
IB: Vps41-Myc
IB: Vps16-HA
1 2 3 4 5 6
1' 2' 3' 4' 5' 6'

Supplementary Figure 2. Vps Class C/HOPS Subunits Cosediment with Clathrin-Coated Vesicles. HEK293T cells or cells expressing Vps16-HA or Vps41-Myc were treated with scramble (Control, lanes 1-6) or clathrin heavy chain siRNA (CHC, lanes1'-6') for 5 days. Cells were homogenized and clathrin coated vesicle fractions were obtained as described (H, homogenate; P_n, pellets; S_n, supernatants; and CCV, clathrin-coated vesicle enriched fraction). Fractions were blotted with antibodies against the coats AP-1 γ, AP-2 α, AP-3 β3, clathrin heavy chain (CHC); and the Vps class C/HOPS subunits Vps16-HA, Vps33b, and Vps41-Myc.



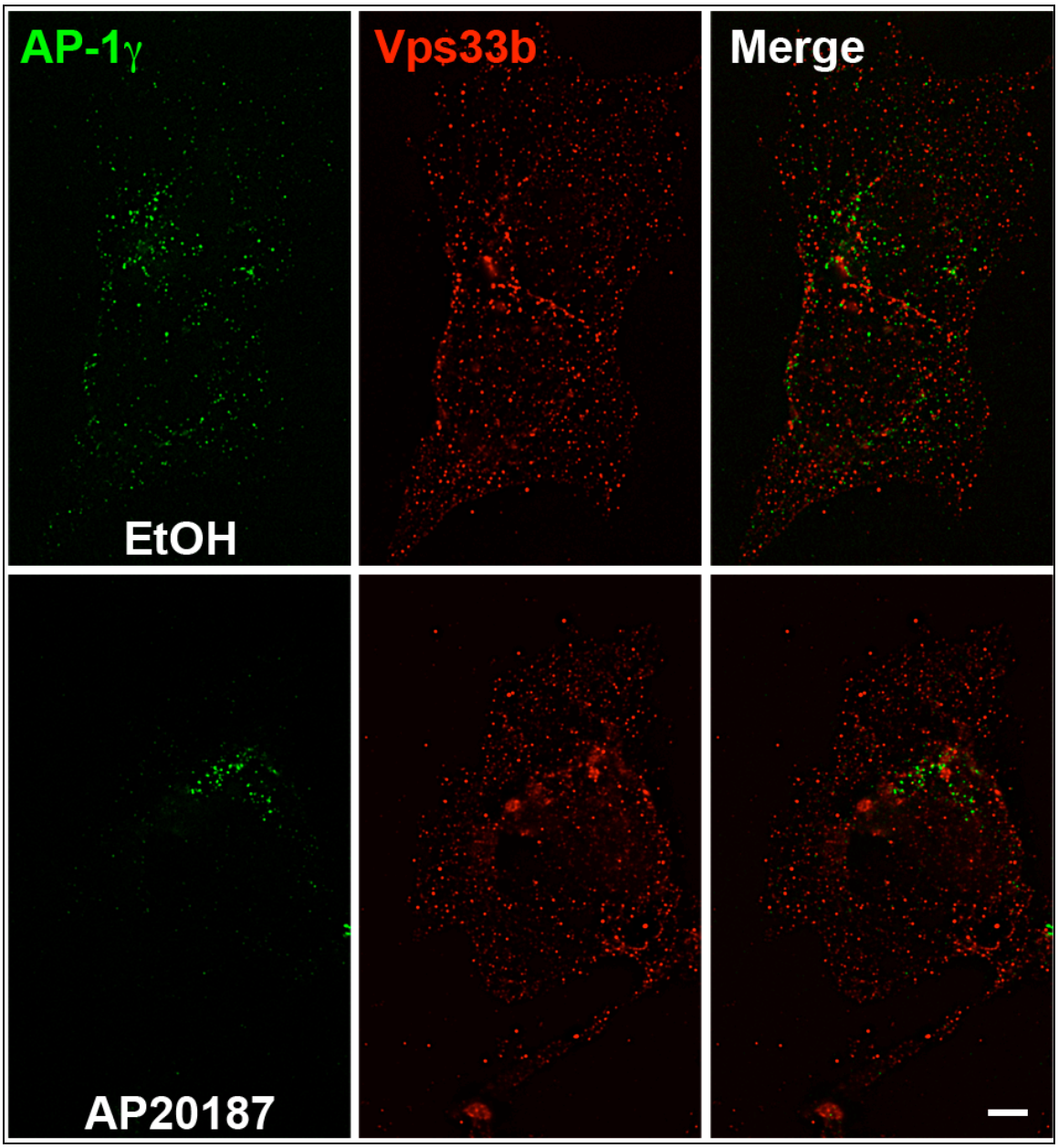
Supplementary Figure 3. Chemical-Genetic Disruption of Clathrin Chains Affects Coat Distribution. HEK293T cells transiently expressing HA-FKBP-CLC (A-C) were either not treated (A), treated with ethanol vehicle (B, EtOH, 0.05% v/v), or AP20187 (B, 50nM) for 2 hours. Cells were fixed, processed for indirect immunofluorescence microscopy, and imaged by Delta Vision deconvolution microscopy (A) or confocal microscopy (B-C). Cells expressing HA-FKBP-CLC were stained with antibodies against clathrin heavy chain (CHC) and the HA tag. C) Probability plot depicts the quantification of the area size (Y axis), in pixels, of CHC-positive organelles from cells treated with ethanol vehicle (blue trace) or AP20187 (red trace) for 2 hours. Blue trace and red traces were constructed with 1568 and 2953 CHC-positive structures, respectively. Object area was determined by Metamorph analysis. D) HEK293T cells stably

expressing Vps41-Myc were transiently transfected with mCherry-FKBP-CLC. Cells were treated in the absence (odd lanes) or presence of AP20187 (even lanes) for 2 hours followed by DSP treatment. Clarified cell extracts were incubated with magnetic beads decorated with control mouse IgG (monoclonal antibody SY38, lanes 3-4) or CLC antibodies (lanes 5-6). Crosslinked clathrin immunocomplexes were resolved by SDS-PAGE and contents analyzed by immunoblot with antibodies against mCherry, Myc epitope, Vps33b, and β actin. Input represents 5%.



Supplementary Figure 4. Chemical-Genetic Disruption of Clathrin Affects Vps33b Distribution in Endosomal Compartments. HEK293T cells transiently expressing mCherry-FKBP-CLC were treated in the presence of ethanol vehicle (A and C, EtOH, 0.05% v/v) or AP20187 (B and D, 50 nM) for 2 hours. Cells were fixed, processed for indirect immunofluorescence microscopy, and imaged by Delta deconvolution microscopy. Cells expressing mCherry-FKBP-CLC were triple stained with antibodies against endogenous clathrin heavy chain (CHC) to assess the effect of AP20187 treatment; rab5 to detect early

endosomes; and rab7b, to detect late endosomal compartments. Upon redistribution of clathrin chains, Vps33b immunoreactivity in rab5 compartments increases while it moderately decreases in rab7b compartments. Bar depicts 5 μm; images to the right are 300% magnifications of inserts.



Supplementary Figure 5. Chemical-Genetic Disruption of Clathrin does not Increase the Colocalization of Vps33b and the AP-1 Adaptor. HEK293T cells expressing mCherry-FKBP-CLC were treated in the presence of ethanol vehicle (EtOH, 0.05% v/v) or AP20187 (50 nM) for 2 hours. Cells were fixed, processed for indirect immunofluorescence microscopy, and imaged by Delta deconvolution microscopy. Cells expressing mCherry-FKBP-CLC were

double stained with antibodies against endogenous Vps33b and AP-1 γ. The effect of AP20187 was determined by the clustering of AP-1 around perinuclear regions. Transfection efficiency is close to 90%. Bar depicts 5 μm.

Supplementary Movie 1 and 2. Tridimensional Rendering of HOPS Subunit Vps41, AP-3, and Clathrin Localization to Enlarged Early Endosomal Compartments. Supplementary Movie 1 and 2 depicts a tridimensional rendering of a subset of enlarged early endosomes shown in Fig. 4 panel C. Tridimensional reconstruction and animation was performed in Imaris software. Tridimensional space was pseudocolored to match panel C where rab5Q79L-GFP is green, Vps41-Myc is red and coats are in blue. Colocalized domains in movies are depicted as overlapping diaphanized areas instead of the merged colors. Movie 1 shows enlarged endosomes positive for Vps41-Myc and AP-3. Movie 2 shows endosomes positive for Vps41-Myc and CHC.

Supplementary Movie 3-6. In Vivo Chemical-Genetic Disruption of Clathrin Chains Rapidly Redistributes Vps18. HEK293T cells transiently expressing Vps18-GFP alone (movie 3) or with a mCherry-tagged (mCh) chimeric clathrin light chain (CLC) carrying a modified FK506-binding protein 12 (FKBP, movie 4, 5 and 6), as an oligomerization module, were imaged by time lapse confocal microscopy. Movie 4 was imaged in the presence of ethanol vehicle (EtOH, 0.05% v/v) while movies 3, 5, and 6 were imaged in the presence of AP20187 (50 nM). Cells were imaged 25 min before vehicle or drug additions and continuously for 2 hours following treatment.

CHAPTER III

DISCUSSION

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A Novel Model of Class B and C Vps HOPS Complex Subunit Localization in Mammalian Cells: Overview of Findings and Significance

Canonical mechanisms of vesicle trafficking are broken down into two main steps: vesicle formation and vesicle fusion. The protein machinery required for the formation and fusion steps is considered spatially and temporally segregated (Figure 1, 2). My dissertation provides an alternate mechanism of vesicle trafficking where the vesicle formation machinery, clathrin, and fusion machinery, HOPS complex, interact at multiple stages of vesicle traffic (Figure 2A).

In my dissertation I hypothesized that:

Cytosolic HOPS complex subunit subcellular localization is regulated by membrane coat-dependent mechanisms in mammalian cells.

Experiments described in Chapter 2 tested this hypothesis and showed that:

1) Class B and C Vps HOPS subunits interact and colocalize on endosomes with vesicle coats, AP-3 and clathrin, which direct cargoes to lysosomes.
2) Class B and C Vps HOPS subunits localize with AP-3 and clathrin in puncta reminiscent of vesicle formation sites and clathrin plaques.
3) Class B and C Vps HOPS subunits form a macromolecular complex with AP-3 and clathrin.
4) Class B and C Vps HOPS subunits co-fractionate with clathrin-coated vesicles.

5) Class B and C Vps HOPS subunits localize to endosomal compartments in a clathrin-dependant manner.
6) Class B and C Vps HOPS subunits localize to subcellular regions of polarized cells in a clathrin-dependant manner.

My findings provide the first evidence of a mechanism governing organelle-specific tethering complex localization. Moreover, my findings challenge models proposed in *Saccharomyces cerevisiae*. Since the discovery of HOPS subunits, the predominant model of late endosomal/lysosomal tether localization was inferred from work in *S. cerevisiae*. The basic tenant of this model, which is challenged in my dissertation, is that HOPS subunits localize through recruitment from the cytoplasm directly to late endosome/lysosomes for vesicle fusion activities (Figure 2). This model predicts class B and C Vps HOPS subunits localization exclusively to the late endosome/lysosome in *S. cerevisiae*. However, my data is not consistent with this model. Rather, I found that HOPS subunits are present in multiple compartments of the endocytic pathway in mammalian cells and polarized localization in neuronal cells. These data along with others described in Chapter 2 lead to my novel model of class B and C Vps HOPS subunit localization in mammalian cells (Figure 3). In this model, subunits localize to early endosomes at sites of vesicle formation or clathrin plaques. HOPS complex subunits can then be included in clathrin-coated membranes for traffic to the late endosome/lysosome or polarized regions of cells bearing complex architectures, such as neurons (Figure 3).

One major question is: How does a transported vesicle "know" its target location (Behnia and Munro 2005)? As described in the General Introduction

(Chapter 1 Section 2), adaptors recruit target-specific cargoes to discrete membranes domains on organelles for inclusion into vesicles (Figure 1). Target-specific cargo recruitment creates carriers containing cargoes that are selectively delivered to a target membrane. However, adaptors do not provide any targeting information by themselves (Ohno *et al.* 1995; Ohno *et al.* 1998; Owen and Evans 1998; Bonifacino and Glick 2004; Robinson 2004). Furthermore, a vast majority of research suggests vesicle coats and adaptors are shed prior to arriving at the target location (Figure 1). Thus, during uncoating, any targeting information coats may have carried is lost mid-course (Bonifacino and Glick 2004). However, if fusion machinery required for target identification, such as the HOPS complex or SNAREs, were also included onto the vesicle during cargo recruitment, then newly formed vesicles would achieve target-specific identities early on and could be maintained throughout the "life" of the vesicle. This view has been underrepresented in canonical models of vesicle membrane traffic that conceptually segregate vesicle formation from vesicle fusion machineries for analytical purposes (Figure 1; Bonifacino and Glick 2004). My results depart from this canonical model in that vesicle formation coats and fusion tethers interact and maintain associations from the time of vesicle generation at a donor membrane to the time of vesicle fusion with its target organelle. The significance of these findings is that the lysosomal tethering complex, vesicle fusion machinery, is not spatially segregated to lysosomal fusion sites and could provide target specific identity at multiple stages of vesicle trafficking. Localization of a target specific tether at multiple stages of trafficking could provide a mechanism for the targeted delivery of organelle specific proteins. Furthermore, these

findings also lead to questions about the roles HOPS complex subunits could have at multiple stages of lysosomal vesicle trafficking.

Question #1: Could HOPS Complex Subunits Interact with Alternate Adaptors?

Class B and C Vps HOPS subunits interact and localize with the adaptor AP-3, but not with the adaptor AP-1. AP-1 was used as a control for selectivity of clathrin-adaptor-tether associations. However, a pressing question is whether clathrin-AP-3-HOPS interactions are restricted to this adaptor or whether they reflect a general organization principle shared by other adaptors.

The endocytic adaptor, AP-2, represents a clathrin-coated vesicle adaptor not tested in my dissertation. AP-2 localizes to the plasma membrane and is critical for clathrin-mediated endocytosis to the early endosome (Conner and Schmid 2003; Meel and Klumperman 2008; Doherty and McMahon 2009). As seen in Chapter 2 Figure 3D-F and Figure 7A (Figure 4 of this discussion), a portion of class C Vps HOPS subunit Vps33b localizes to the cell periphery, and more specifically at the junctions between opposing cells. Whether this localization at the cell periphery represents HOPS subunits present in the plasma membrane or closely apposed endosomes needs to be distinguished. Interestingly, acute perturbation of clathrin function yielded an apparent decrease of class B and C Vps HOPS subunits at the cell periphery (Figure 4). These data have not been quantified but are an intriguing result for HOPS complex localization. Similar to HOPS subunit localization to early endosomes for delivery to late endosome/lysosome, class B and C HOPS Vps subunits could

potentially interact with the adaptor AP-2 and clathrin at the plasma membrane for delivery to an early endosomal compartment during endocytosis. This dissertation supports a model where HOPS subunits are incorporated as cargo in clathrin-coated membrane carriers. Since AP-2 leads to clathrin-coated vesicle generation, I hypothesize that HOPS complex subunits will also be included in these vesicle populations. This could be tested using similar experimental design from Chapter 2 where HOPS subunits were colocalized and co-immunoprecipitated with the AP-3 adaptor and in sequential co-immunoprecipitations with AP-3 and clathrin.

Question #2: Are HOPS Subunits Involved in Cargo Recruitment During Vesicle Formation?

My dissertation shows that class B and C Vps HOPS subunits are localized with clathrin and adaptors at sites were vesicle formation could occur. However, it is unclear what role HOPS subunits perform at this location. One hypothesis described in Chapter 2 is that HOPS subunits are themselves cargoes that localize to subcellular compartments via a clathrin-mediated pathway. This model has been discussed extensively in my dissertation. Additionally, a non-exclusive role regards class B and C Vps HOPS subunits as a requirement for vesicle formation. In yeast, Vps41 was initially believed to be required for AP-3 vesicle formation, though recent evidence demonstrates an increase in AP-3 vesicles in yeast cells null for HOPS subunit Vps41 (Rehling *et al.* 1999; Darsow *et al.* 2001; Angers and Merz 2009). These data suggest Vps41 is not a direct requirement for AP-3 vesicle formation. However, the possibility exists that HOPS subunits play a role

in membrane protein recognition and loading of cargo into vesicles. Such a role would qualify HOPS subunits as "accessory adaptors."

The HOPS complex subunits Vps33a and Vps33b may have a role in the recruitment of SNAREs onto newly forming vesicles. The class C Vps subunit Vps33 in yeast, and Vps33a and Vps33b in mammals contains a Sec1/Munc18 domain known to bind t-SNAREs (Jahn and Sudhof 1999; Waters and Hughson 2000; Gissen *et al.* 2005; Cai *et al.* 2007). V-SNAREs are known to be included into newly forming vesicles (Bonifacino and Glick 2004). However, how do t-SNAREs localize to the target compartment? HOPS subunits may bind lysosomal SNAREs at the sites of vesicle formation to regulate recruitment into newly forming lysosomal vesicles. Additionally, early interaction of t-SNAREs with HOPS during vesicle formation may act to prevent promiscuous binding of SNAREs during transit, prior to arrival at the late endosomal/lysosomal target.

Question #3: Are HOPS Subunits Involved in Cargo Sorting During Endosome Maturation?

Vps HOPS subunits at early endosomes may also participate in a maturation model of lysosomal trafficking as opposed to a vesicular model. One study described an AP-3 and HOPS dependant mechanism of regulating an alternative Notch receptor activation pathway (Chapter 1 Section 5; Wilkin *et al.* 2008). In this study, the authors proposed that HOPS regulates an alternative Notch activation pathway by regulating fusion with the late endosome/lysosome while AP-3 recruits the Notch receptor and segregates it away from lumenal vesicle formation sites (Wilkin *et al.* 2008). Results described in Chapter 2 of this

dissertation demonstrate that HOPS interacts and localizes with clathrin and AP-3. My results presented in this dissertation can be integrated in a model where HOPS regulates an alternative Notch activation pathway through interactions with AP-3, to recruit Notch receptor away from lumenal vesicles, thus preventing the sorting of Notch receptor for lysosomal degradation.

Question #4: Can My Novel Model of HOPS Localization Give Insight Into CORVET Localization and Trafficking Mechanisms?

As discussed in the General Introduction, in yeast both the HOPS and CORVET complexes consist of four class C Vps core subunits: Vps11, Vps16, Vps18, and Vps33 (Figure 5; Peplowska *et al.* 2007). The CORVET complex has not been identified in mammals, though putative orthologues of the CORVET specific subunits Vps8 (Vps8 in mammals) and Vps3 (TRAPPI in mammals) are found on NCBI nucleotide searches (http://www.ncbi.nlm.nih.gov/pubmed). We presently do not know if the putative CORVET specific Vps8 and Vps3 orthologues interact as a complex with the class C core in mammals. Nor do we know whether CORVET specific subunits interact with clathrin or localize in a clathrin dependent manor as I demonstrated for HOPS specific subunits. Understanding whether putative CORVET subunits interact with coat proteins, as HOPS subunits have been shown to do in my dissertation, would provide a foundation for understanding a general principle of tether localization in mammalian cells. A redistribution of CORVET subunits subcellular distribution by clathrin perturbation would also provide evidence of a general principle organizing the subcellular distribution of multisubunit tethers in the endocytic

route and suggest a preponderant role of class C core subunits in defining tether-coat interactions.

The potential association of coats and CORVET specific subunits is suggested by results presented in Chapter 2, where the percentage of class C core Vps subunits localizing to Rab5-positive early endosomes is higher than the percentage of class B Vps HOPS specific subunits. I speculate that co-localization of core class C Vps subunits represents the dual localization of both the HOPS and CORVET complex class C Vps subunits with coats at early endosomes. I hypothesize that deconvolution microscopy of CORVET specific Vps subunits would colocalize at early endosomes similar to the localization of HOPS specific Vps subunits.

Question #5: Might HOPS Complex Have a Role in Lysosome-Related Organelle Fusion Events, and Polarized Mammalian Cells That Cannot Be Recognized in Yeast?

In Chapter 1 Section 5 of my dissertation I describe two human syndromes that illustrate the importance of HOPS Complex subunits: **H**ermansky **P**udlak **S**yndrome (**HPS**) and **A**rthrogryposis, **R**enal dysfunction, and **C**holestasis (**ARC**) syndrome (Swank *et al.* 1998; Huizing *et al.* 2002; Suzuki *et al.* 2003; Bonifacino 2004; Gissen *et al.* 2004; Gissen *et al.* 2006). Studies of class B and C Vps HOPS subunits in models of human syndromes in metazoan model genetic organisms further illustrate the importance of HOPS subunits in human syndromes and (Shestopal *et al.* 1997; Warner *et al.* 1998; Mullins *et al.* 1999; Sevrioukov *et al.* 1999; Mullins *et al.* 2000; Matthews *et al.* 2005; Pierson

et al. 2006; Parsch and Pietrzak 2007; Taha *et al.* 2007; Hamamichi *et al.* 2008; Hershkovitz *et al.* 2008; Wilkin *et al.* 2008; Akbar *et al.* 2009; Arhan *et al.* 2009; Cullinane *et al.* 2009; Jang *et al.* 2009; Ruan *et al.* 2009; Cullinane *et al.* 2010; Kim *et al.* 2010; Jang *et al.* 2011). Common themes in each of these syndromes are 1) defects in polarized cells and 2) perturbation of lysosome-related organelle biogenesis.

Clathrin mediated pathways are important for the localization and internalization of proteins at apical and basolateral membranes in polarized epithelial cells and vesicle traffic along axons in polarized neuronal cells (Altschuler *et al.* 1999; Deinhardt *et al.* 2007; Deborde *et al.* 2008; Weisz and Rodriguez-Boulan 2009). Discovering a clathrin-dependent mechanism of HOPS complex subunit localization suggests HOPS subunits may regulate defined subcellular domains that characterize polarized cells. This hypothesis is strengthened by phenotypes in patients with ARC syndrome carrying mutations in HOPS subunit Vps33b. Patients with ARC syndrome display defects in polarized cells such as neuroanatomical defects compatible with defective neuronal patterning, mislocalization of apical and basolateral proteins in kidney and liver epithelia, and lamellar body secretion from skin epithelia (Horslen *et al.* 1994; Abdullah *et al.* 2000; Denecke *et al.* 2000; Eastham *et al.* 2001; Howells and Ramaswami 2002; Gissen *et al.* 2004; Hayes *et al.* 2004; Abu-Sa'da *et al.* 2005; Choi *et al.* 2005; Tekin *et al.* 2005; Bull *et al.* 2006; Gissen *et al.* 2006; Parsch and Pietrzak 2007; Taha *et al.* 2007; Hershkovitz *et al.* 2008; Arhan *et al.* 2009; Cullinane *et al.* 2009; Jang *et al.* 2009; Cullinane *et al.* 2010; Kim *et al.* 2010; Jang *et al.* 2011).

As mentioned in Question #2 of this Discussion, a portion of HOPS subunit Vps33b localizes to the periphery of cells and is often concentrated at the junctions of opposing cells. These data could be an indicator of HOPS localization to sites of AP-2 clathrin-coated vesicle formation, or clathrin-independent mechanisms of endocytosis. An alternative hypothesis is that in mammalian cells HOPS subunits regulate fusion at the plasma membrane in addition to fusion at lysosomes. This hypothesis would also support lamellar body and bile secretion defects found in patients carrying mutations in Vps33b and is consistent with the proposed role of Vps33b in directing junctional proteins to the cell surface of polarized epithelial cells (Cullinane *et al.* 2010).

In Chapter 2 Figure 9 of this dissertation I show that HOPS subunits localize to the tips of neurites. I also demonstrate that localization of HOPS subunits to neurites occurs in a clathrin-dependant manner. HOPS localization to the tips of neurites provides additional support for a role of HOPS subunits in fusion activities at the plasma membrane or internalization as described above. Such localization and putative functions may explain the neurological defects in patients with ARC syndrome, such as the absence of the corpus callosum or path finding defects in the spinal cord (Gissen *et al.* 2006). For instance, class B and C Vps HOPS subunits might regulate polarized neuronal pathfinding phenotypes in a clathrin-dependant manner through localization and fusion of vesicles carrying axon guidance factors to the neuronal growth cone.

Mutations to Vps33a in models of HPS result in other defects in lysosome-related organelle biogenesis including melanosomes, dense platelet granules, and lamellar bodies (Swank *et al.* 1998; Dell'Angelica *et al.* 2000; Huizing *et al.* 2002;

Weaver *et al.* 2002; Suzuki *et al.* 2003; Bonifacino 2004). Lysosome-related organelles share many of the same characteristics as lysosomes (Dell'Angelica *et al.* 2000). For example, both lysosomes and lysosome-related organelles maintain a low pH, use similar vesicular trafficking mechanisms, and contain similar membrane protein compositions (Dell'Angelica *et al.* 2000). Mutations in class B and C Vps HOPS subunits provide a strong indication of HOPS' role in lysosome-related organelle biogenesis (Dell'Angelica *et al.* 2000; Bonifacino 2004; Huizing *et al.* 2008). However, the mechanism by which class B and C Vps HOPS subunits are involved in lysosome-related organelle biogenesis is less well understood. Mutations to HOPS subunits may lead to defects in vesicle formation, cargo recruitment, or interactions with vesicle formation machinery during lysosome-related organelle biogenesis.

Question #6: Can My Novel Model of Clathrin-Dependant HOPS Localization Explain How Defects in HOPS Genes Contribute to Lysosomal Human Diseases?

Prior to my dissertation, understanding how mutations in genes of class B and C Vps HOPS subunits resulted in disease relied on understanding the interactions during SNARE pairing, Rab7 interaction and activation, and HOPS complex regulation of fusion activities at the lysosome and lysosome-related organelle (Bonifacino 2004; Gissen *et al.* 2006; Huizing *et al.* 2008; Wilkin *et al.* 2008; Ruan *et al.* 2009). This dissertation provides a novel conceptual framework for understanding how HOPS complex subunits function and

distribute along the endocytic route and therefore, how mutational defects of HOPS complex subunits may lead to disease in multiple tissues.

Class C Vps33a and Vps33b subunits are altered in models of HPS and ARC syndrome respectively (Chapter 1 Section 5; Swank *et al.* 1998; Huizing *et al.* 2002; Suzuki *et al.* 2003; Bonifacino 2004; Gissen *et al.* 2004; Gissen *et al.* 2006). Both Vps33a and Vps33b contain a Sec1/Munc18 domain for interaction with fusion SNAREs (Jahn and Sudhof 1999; Waters and Hughson 2000; Gissen *et al.* 2005; Cai *et al.* 2007). These data suggest mutations to Vps33a and Vps33b might lead to abnormal SNARE pairing, and thus defective fusion of organelles with lysosome or lysosome-related organelles. However, based on data presented in this dissertation, we can postulate that cellular defects observed in these diseases occur at nascent stages of vesicle biogenesis such as cargo recruitment or the identity of SNAREs being loaded onto vesicles. An initial approach is to define subcellular localization of mutant versions of Vps33a and Vps33b and interactions of mutant Vps33a and Vps3b with vesicle formation machinery. There are at least 17 known mutations to Vps33b resulting in ARC syndrome and 2 mutations in Vps33a resulting in HPS (Sevrioukov *et al.* 1999; Suzuki *et al.* 2003; Gissen *et al.* 2006). Some of these mutations may result in defects of HOPS coordination of fusion events at the late endosome/lysosome. However, some mutations might result from defects in localization to early endosomes, or interaction with AP-3, clathrin or SNAREs, resulting in a defect of cargo recruitment or trafficking to the late endosome/lysosome. We are actively pursuing this avenue as some of the missense mutations may prove to be valuable tools to test vesicle formation roles of HOPS complex subunits.

Summary

My dissertation contributes a novel mechanism for the localization of multisubunit tethers, in particular the HOPS complex. This model includes the localization of HOPS complex subunits to early endosomal compartments, their presence on clathrin-coated vesicle intermediates, and the clathrin dependent localization of HOPS subunits to target fusion sites, lysosomes (Figure 3; Nickerson *et al.* 2009). Consistent with this model, I found that HOPS subunits interact and colocalize with vesicle formation machinery, localize to sites where vesicle formation could be occurring with vesicle formation machinery, are present and enriched in clathrin-coated vesicle fractions in a clathrin dependent manor and localize to endosomal membrane compartments in a clathrin dependent manor.

My novel mechanism of class B and C Vps HOPS subunit localization could apply to other tethering complexes and define a general principle for the localization of tethers to their respective sites of activity. The identification of vesicle formation and fusion machinery colocalization and interaction allows us to pursue new possibilities in lysosomal vesicle trafficking. The study of HOPS complex subunits as cargo recruitment factors in conjunction with AP-3 cargo recruitment mechanisms could be particularly important in the case of vesicle trafficking. The localization of HOPS complex into newly forming vesicles could provide a cargo recruitment mechanism of target-specific SNAREs at newly forming vesicle. Interaction of HOPS subunits with SNAREs at early stages of vesicle traffic could prevent non-specific fusion events prior to localization at the

late endosome/lysosome. Conversely, HOPS localization could enhance target-specific interactions of vesicles by providing lysosome and lysosome-related organelle identities early during the "lifetime" of the vesicle. Given these new findings on HOPS subunits' protein interactions with coats and effects of coat function on HOPS subcellular distribution, my dissertation offers new and complementary ways for understanding how human disease could result from mutations in genes encoding subunits of the HOPS complex.

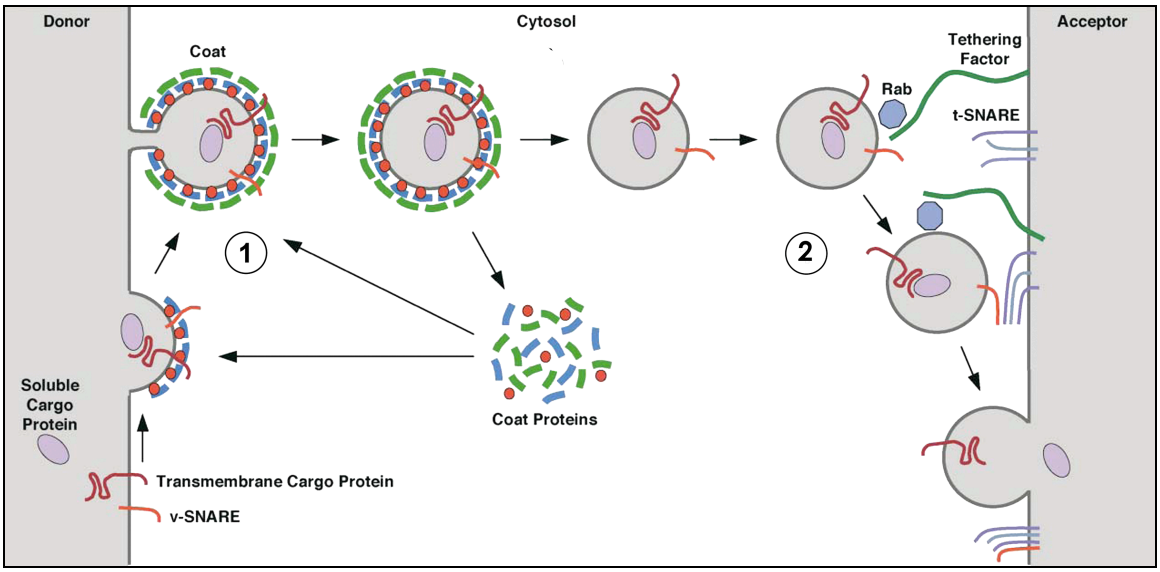

Figure 1. Model of Vesicle Transport

Vesicles carry lumenal and membrane cargoes from donor organelles to acceptor organelles. Vesicle transport is frequently thought of in two distinct steps: 1) cargo recruitment and vesicle formation, and 2) vesicle docking and fusion. In this model vesicle formation and vesicle fusion machinery are spatially and temporally segregated. Modified from (Bonifacino and Glick 2004).

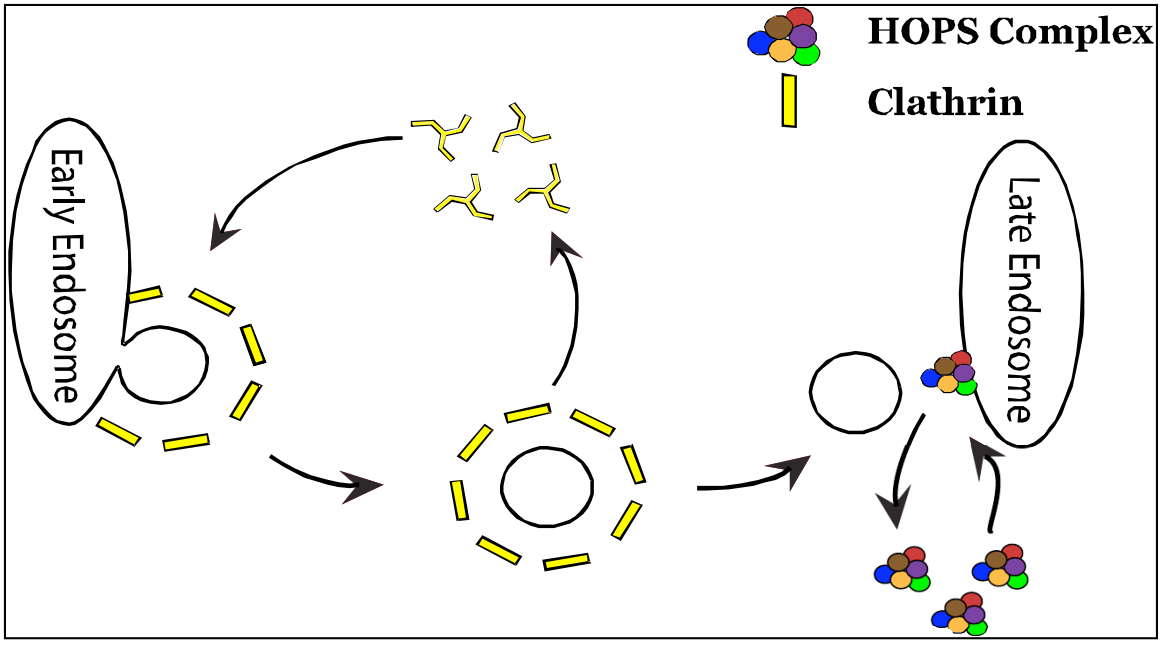

Figure 2. Canonical Model of HOPS Complex Localization

The predominant model of late endosomal/lysosomal tethering localization inferred cytoplasmic HOPS complex subunits cycled on and off the late endosome/lysosome membrane, where it is critical for fusion events. This model predicts:

1) HOPS subunits would retain either a cytoplsmic or late endosome/lysosome localization.
2) HOPS subunits would not localize to early endosomes.
3) HOPS subunits would not interact with vesicle formation machinery.
4) HOPS subunits would not be found in vesicular intermediates.
5) Vesicle formation and traffic events should have no effect on HOPS subunit localization.

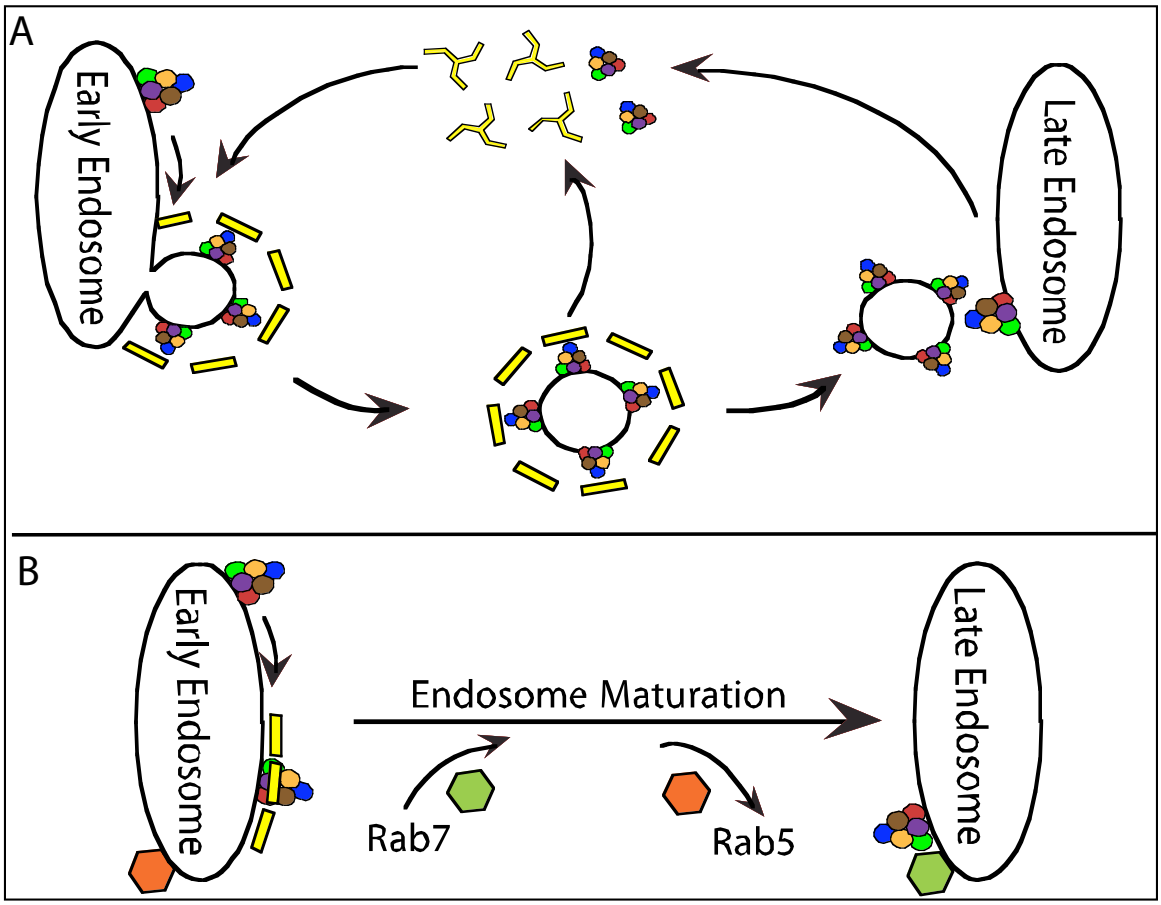

Figure 3. Novel Model Of HOPS Complex Localization in Mammalian Cells

This dissertation supports a novel model of HOPS complex subunit localization. In this novel model HOPS complex subunits are localized to early endosomes at sites of vesicle formation (A), or clathrin plaques (B). HOPS complex subunits can be included in clathrin-coated membranes for traffic to the late endosome/lysosome (A), or remain at the endosomal surface during endosomal maturation (B). This model is consistent with the findings of this dissertation:

1) Class B and C Vps HOPS subunits interact and colocalize with vesicle coats that direct cargoes to lysosomes, AP-3 and clathrin.

2) Class B and C Vps HOPS subunits localize with AP-3 and clathrin in puncta reminiscent of vesicle formation sites and clathrin/Hrs plaques.
3) Class B and C Vps HOPS subunits form a macromolecular complex with AP-3 and clathrin.
4) Class B and C Vps HOPS subunits co-fractionate with clathrin-coated vesicles.
5) Class B and C Vps HOPS subunits localized to endosomal compartments in a clathrin-dependant manner.

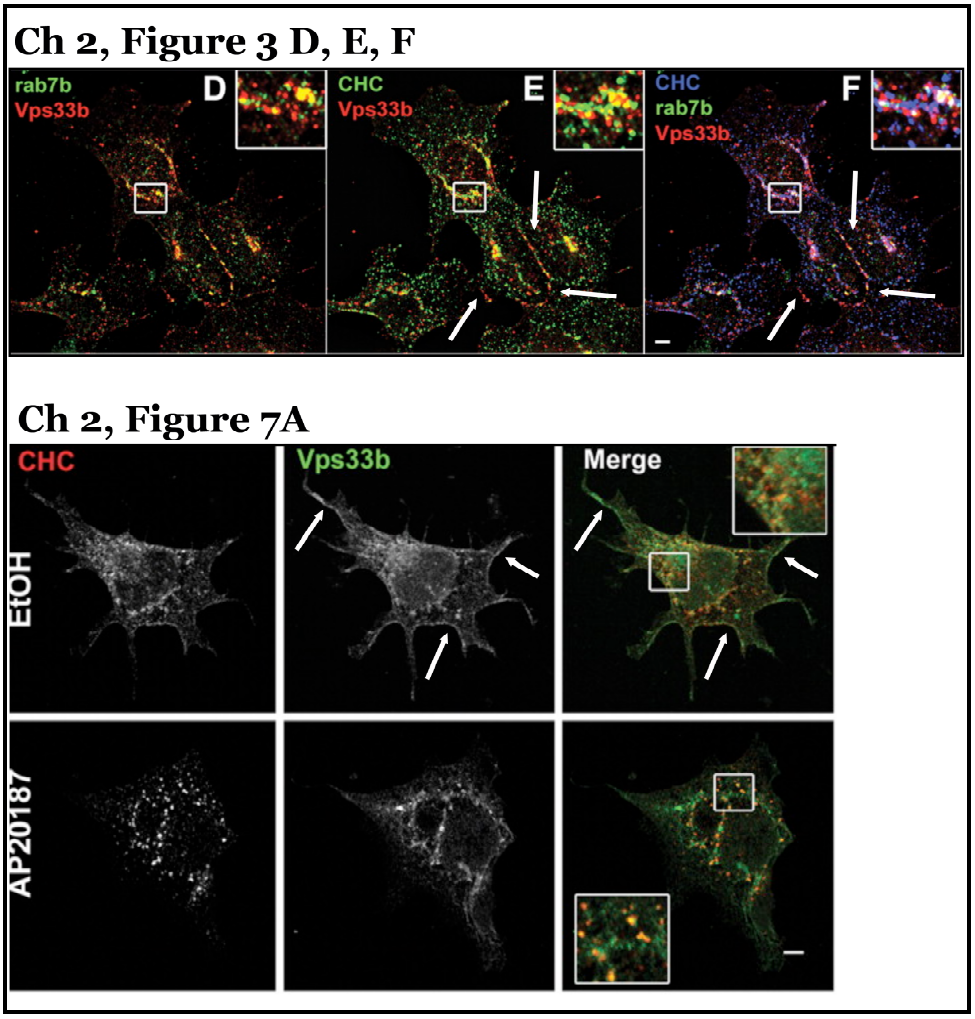

Figure 4. Vps33b Subunits Localize Near the Plasma Membrane

Figures from Chapter 2 are altered to show the localization of Vps33b subunits signal to the plasma membrane, or to endosomal compartments near the plasma membrane. Arrows point to regions of Vps33b signal at or near the plasma membrane. Figure modified from (Zlatic *et al.* 2011).

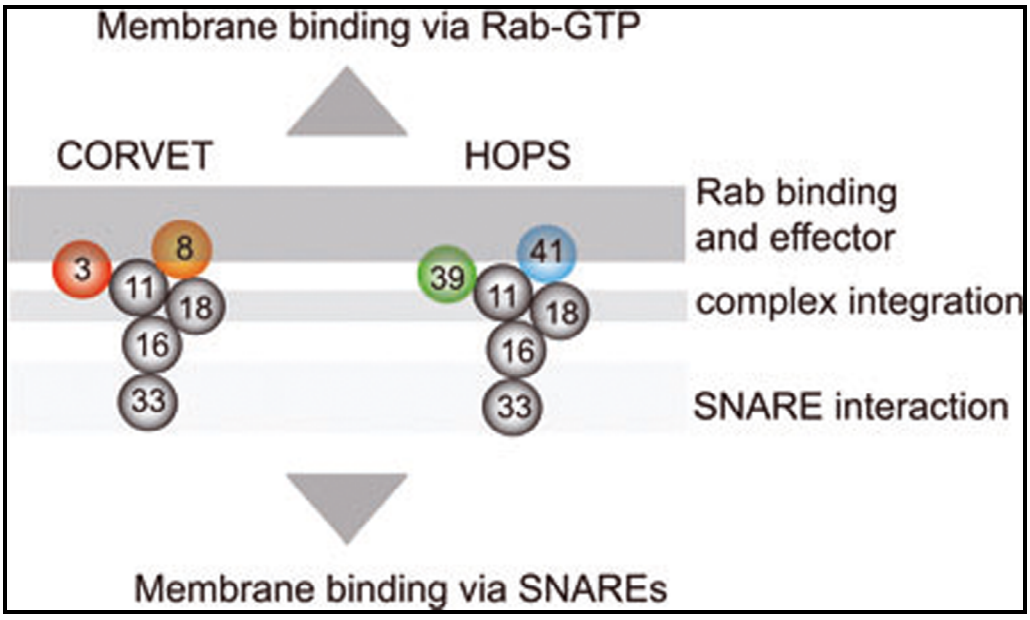

Figure 5. HOPS and CORVET Organization

The HOPS complex (HOmotypic fusion and Protein Sorting) and CORVET complex (class C cORe Vacuole/Endosome Tethering) share the core Vps subunits Vps11, Vps16, Vps18, and Vps33. HOPS specific Vps proteins include Vps39 and Vps41. CORVET specific subunits include Vps3 and Vps8. Higher order eukaryotes contain two homologues of Vps33, Vps33a and Vps33b. Only one Vps33a/b subunit is presumed incorporated into the core complex at a time. Figure modified from (Ostrowicz *et al.* 2010).

CHAPTER IV

REFERENCES

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Abdullah, M. A., Z. Al-Hasnan, et al. (2000). "Arthrogryposis, renal dysfunction and cholestasis syndrome." Saudi Med J **21**(3): 297-9.

Abu-Sa'da, O., M. Barbar, et al. (2005). "Arthrogryposis, renal tubular acidosis and cholestasis (ARC) syndrome: two new cases and review." Clin Dysmorphol **14**(4): 191-6.

Akbar, M. A., S. Ray, et al. (2009). The SM protein Car/Vps33A regulates SNARE-mediated trafficking to lysosomes and lysosome-related organelles. Mol Biol Cell. **20:** 1705-14.

Akbar, M. A., C. Tracy, et al. (2011). "The full-of-bacteria gene is required for phagosome maturation during immune defense in Drosophila." J Cell Biol **192**(3): 383-90.

Altschuler, Y., S. Liu, et al. (1999). "ADP-ribosylation factor 6 and endocytosis at the apical surface of Madin-Darby canine kidney cells." J Cell Biol **147**(1): 7-12.

Anand, V. C., L. Daboussi, et al. (2009). "Genome-wide analysis of AP-3-dependent protein transport in yeast." Mol Biol Cell **20**(5): 1592-604.

Andrews, N. W. (2000). "Regulated secretion of conventional lysosomes." Trends Cell Biol **10**(8): 316-21.

Angers, C. G. and A. J. Merz (2009). "HOPS interacts with Apl5 at the vacuole membrane and is required for consumption of AP-3 transport vesicles." Mol Biol Cell **20**(21): 4563-74.

Angers, C. G. and A. J. Merz (2011). "New links between vesicle coats and Rab-mediated vesicle targeting." Semin Cell Dev Biol **22**(1): 18-26.

Arhan, E., A. M. Yusufoğlu, et al. (2009). Arc syndrome without arthrogryposis, with hip dislocation and renal glomerulocystic appearance: a case report. Eur J Pediatr. **168:** 995-8.

Aridor, M. and L. A. Hannan (2000). "Traffic jam: a compendium of human diseases that affect intracellular transport processes." Traffic **1**(11): 836-51.

Banta, L. M., J. S. Robinson, et al. (1988). Organelle assembly in yeast: characterization of yeast mutants defective in vacuolar biogenesis and protein sorting. The Journal of Cell Biology. **107:** 1369-83.

Banta, L. M., T. A. Vida, et al. (1990). Characterization of yeast Vps33p, a protein required for vacuolar protein sorting and vacuole biogenesis. Mol Cell Biol. **10:** 4638-49.

Barr, F. and D. G. Lambright (2010). "Rab GEFs and GAPs." Curr Opin Cell Biol **22**(4): 461-70.

Behnia, R. and S. Munro (2005). "Organelle identity and the signposts for membrane traffic." Nature **438**(7068): 597-604.

Boehm, M. and J. S. Bonifacino (2001). "Adaptins: the final recount." Mol Biol Cell **12**(10): 2907-20.

Bonifacino, J. S. (2004). Insights into the biogenesis of lysosome-related organelles from the study of the Hermansky-Pudlak syndrome. Ann N Y Acad Sci. **1038:** 103-14.

Bonifacino, J. S. and B. S. Glick (2004). The mechanisms of vesicle budding and fusion. Cell. **116:** 153-66.

Borner, G. H., M. Harbour, et al. (2006). "Comparative proteomics of clathrin-coated vesicles." J Cell Biol **175**(4): 571-8.

Braak, H. and E. Braak (2000). "Pathoanatomy of Parkinson's disease." J Neurol **247 Suppl 2**: II3-10.

Braulke, T. and J. S. Bonifacino (2009). "Sorting of lysosomal proteins." Biochim Biophys Acta **1793**(4): 605-14.

Brett, C. L., R. L. Plemel, et al. (2008). "Efficient termination of vacuolar Rab GTPase signaling requires coordinated action by a GAP and a protein kinase." J Cell Biol **182**(6): 1141-51.

Bright, N. A., M. J. Gratian, et al. (2005). Endocytic delivery to lysosomes mediated by concurrent fusion and kissing events in living cells. Curr Biol. **15:** 360-5.

Brocker, C., S. Engelbrecht-Vandre, et al. (2010). "Multisubunit tethering complexes and their role in membrane fusion." Curr Biol **20**(21): R943-52.

Brunger, A. T. (2005). "Structure and function of SNARE and SNARE-interacting proteins." Q Rev Biophys **38**(1): 1-47.

Bucci, C., O. Bakke, et al. (2010). "Rab7b and receptors trafficking." Commun Integr Biol **3**(5): 401-4.

Bugnicourt, A., M. Froissard, et al. (2004). Antagonistic roles of ESCRT and Vps class C/HOPS complexes in the recycling of yeast membrane proteins. Mol Biol Cell. **15:** 4203-14.

Bull, L. N., V. Mahmoodi, et al. (2006). VPS33B mutation with ichthyosis, cholestasis, and renal dysfunction but without arthrogryposis: incomplete ARC syndrome phenotype. J Pediatr. **148:** 269-71.

Cabrera, M., C. W. Ostrowicz, et al. (2009). Vps41 phosphorylation and the Rab Ypt7 control the targeting of the HOPS complex to endosome-vacuole fusion sites. Mol Biol Cell. **20:** 1937-48.

Cai, H., K. Reinisch, et al. (2007). "Coats, tethers, Rabs, and SNAREs work together to mediate the intracellular destination of a transport vesicle." Dev Cell **12**(5): 671-82.

Caplan, S., L. M. Hartnell, et al. (2001). Human Vam6p promotes lysosome clustering and fusion in vivo. The Journal of Cell Biology. **154:** 109-22.

Chi, C., H. Zhu, et al. (2010). "Disruption of lysosome function promotes tumor growth and metastasis in Drosophila." J Biol Chem **285**(28): 21817-23.

Choi, H.-J., M.-W. Lee, et al. (2005). Ichthyosis associated with ARC syndrome: ARC syndrome is one of the differential diagnoses of ichthyosis. Pediatr Dermatol. **22:** 539-42.

Conner, S. D. and S. L. Schmid (2003). "Regulated portals of entry into the cell." Nature **422**(6927): 37-44.

Cowles, C. R., G. Odorizzi, et al. (1997). "The AP-3 adaptor complex is essential for cargo-selective transport to the yeast vacuole." Cell **91**(1): 109-18.

Cowles, C. R., W. B. Snyder, et al. (1997). Novel Golgi to vacuole delivery pathway in yeast: identification of a sorting determinant and required transport component. EMBO J. **16:** 2769-82.

Craige, B., G. Salazar, et al. (2008). Phosphatidylinositol-4-kinase type II alpha contains an AP-3-sorting motif and a kinase domain that are both required for endosome traffic. Mol Biol Cell. **19:** 1415-26.

Cullinane, A. R., A. Straatman-Iwanowska, et al. (2009). Molecular investigations to improve diagnostic accuracy in patients with ARC syndrome. Hum Mutat. **30:** E330-7.

Cullinane, A. R., A. Straatman-Iwanowska, et al. (2010). "Mutations in VIPAR cause an arthrogryposis, renal dysfunction and cholestasis syndrome phenotype with defects in epithelial polarization." Nat Genet **42**(4): 303-12.

Darsow, T., D. J. Katzmann, et al. (2001). Vps41p function in the alkaline phosphatase pathway requires homo-oligomerization and interaction with AP-3 through two distinct domains. Mol Biol Cell. **12:** 37-51.

de Bivort, B. L., H. F. Guo, et al. (2009). "Notch signaling is required for activity-dependent synaptic plasticity at the Drosophila neuromuscular junction." J Neurogenet **23**(4): 395-404.

Deborde, S., E. Perret, et al. (2008). Clathrin is a key regulator of basolateral polarity. Nature. **452:** 719-723.

Deinhardt, K., A. Reversi, et al. (2007). "Neurotrophins Redirect p75NTR from a clathrin-independent to a clathrin-dependent endocytic pathway coupled to axonal transport." Traffic **8**(12): 1736-49.

Del Conte-Zerial, P., L. Brusch, et al. (2008). "Membrane identity and GTPase cascades regulated by toggle and cut-out switches." Mol Syst Biol **4**: 206.

Delevoye, C., I. Hurbain, et al. (2009). "AP-1 and KIF13A coordinate endosomal sorting and positioning during melanosome biogenesis." J Cell Biol **187**(2): 247-64.

Dell'Angelica, E. C. (2009). "AP-3-dependent trafficking and disease: the first decade." Curr Opin Cell Biol **21**(4): 552-9.

Dell'Angelica, E. C., J. Klumperman, et al. (1998). "Association of the AP-3 adaptor complex with clathrin." Science **280**(5362): 431-4.

Dell'Angelica, E. C., C. Mullins, et al. (2000). "Lysosome-related organelles." FASEB J **14**(10): 1265-78.

Denecke, J., K. P. Zimmer, et al. (2000). "[Arthrogryposis, renal tubular dysfunction, cholestasis (ARC) syndrome: case report and review of the literature]." Klin Padiatr **212**(2): 77-80.

Devos, D., S. Dokudovskaya, et al. (2004). "Components of coated vesicles and nuclear pore complexes share a common molecular architecture." PLoS Biol **2**(12): e380.

Di Paolo, G. and P. De Camilli (2006). "Phosphoinositides in cell regulation and membrane dynamics." Nature **443**(7112): 651-7.

Di Pietro, S. M. and E. C. Dell'Angelica (2005). "The cell biology of Hermansky-Pudlak syndrome: recent advances." Traffic **6**(7): 525-33.

Di Rocco, M., F. Callea, et al. (1995). "Arthrogryposis, renal dysfunction and cholestasis syndrome: report of five patients from three Italian families." Eur J Pediatr **154**(10): 835-9.

Doherty, G. J. and H. T. McMahon (2009). "Mechanisms of endocytosis." Annu Rev Biochem **78**: 857-902.

Dokudovskaya, S., F. Waharte, et al. (2011). "A conserved coatomer-related complex containing Sec13 and Seh1 dynamically associates with the vacuole in Saccharomyces cerevisiae." Mol Cell Proteomics.

Duden, R. (2001). AP-3: what color's your coat? Trends Cell Biol. **11:** 150.

Eastham, K. M., P. J. McKiernan, et al. (2001). "ARC syndrome: an expanding range of phenotypes." Arch Dis Child **85**(5): 415-20.

Eitzen, G., E. Will, et al. (2000). Sequential action of two GTPases to promote vacuole docking and fusion. EMBO J. **19:** 6713-20.

Fasshauer, D. (2003). "Structural insights into the SNARE mechanism." Biochim Biophys Acta **1641**(2-3): 87-97.

Faundez, V., J. T. Horng, et al. (1997). "ADP ribosylation factor 1 is required for synaptic vesicle budding in PC12 cells." J Cell Biol **138**(3): 505-15.

Faundez, V. V. and R. B. Kelly (2000). "The AP-3 complex required for endosomal synaptic vesicle biogenesis is associated with a casein kinase Ialpha-like isoform." Mol Biol Cell **11**(8): 2591-604.

Fotin, A., Y. Cheng, et al. (2004). "Molecular model for a complete clathrin lattice from electron cryomicroscopy." Nature **432**(7017): 573-9.

Gerhardt, B., T. J. Kordas, et al. (1998). The vesicle transport protein Vps33p is an ATP-binding protein that localizes to the cytosol in an energy-dependent manner. J Biol Chem. **273:** 15818-29.

Ghosh, P. and S. Kornfeld (2004). "The GGA proteins: key players in protein sorting at the trans-Golgi network." Eur J Cell Biol **83**(6): 257-62.

Gieselmann, V. (1995). "Lysosomal storage diseases." Biochim Biophys Acta **1270**(2-3): 103-36.

Gillingham, A. K. and S. Munro (2007). The small G proteins of the Arf family and their regulators. Annu Rev Cell Dev Biol. **23:** 579-611.

Gissen, P., C. A. Johnson, et al. (2005). Comparative evolutionary analysis of VPS33 homologues: genetic and functional insights. Hum Mol Genet. **14:** 1261-70.

Gissen, P., C. A. Johnson, et al. (2004). "Mutations in VPS33B, encoding a regulator of SNARE-dependent membrane fusion, cause arthrogryposis-renal dysfunction-cholestasis (ARC) syndrome." Nat Genet **36**(4): 400-4.

Gissen, P., L. Tee, et al. (2006). "Clinical and molecular genetic features of ARC syndrome." Hum Genet **120**(3): 396-409.

Hamamichi, S., R. N. Rivas, et al. (2008). "Hypothesis-based RNAi screening identifies neuroprotective genes in a Parkinson's disease model." Proc Natl Acad Sci U S A **105**(2): 728-33.

Hayes, J. A., W. H. Kahr, et al. (2004). "Liver biopsy complicated by hemorrhage in a patient with ARC syndrome." Paediatr Anaesth **14**(11): 960-3.

Hermansky, F. and P. Pudlak (1959). "Albinism associated with hemorrhagic diathesis and unusual pigmented reticular cells in the bone marrow: report of two cases with histochemical studies." Blood **14**(2): 162-9.

Hershkovitz, D., H. Mandel, et al. (2008). Defective lamellar granule secretion in arthrogryposis, renal dysfunction, and cholestasis syndrome caused by a mutation in VPS33B. Arch Dermatol. **144:** 334-40.

Hickey, C. M., C. Stroupe, et al. (2009). "The major role of the Rab Ypt7p in vacuole fusion is supporting HOPS membrane association." J Biol Chem **284**(24): 16118-25.

Hong, W. (2005). "SNAREs and traffic." Biochim Biophys Acta **1744**(3): 493-517.

Horslen, S. P., O. W. Quarrell, et al. (1994). "Liver histology in the arthrogryposis multiplex congenita, renal dysfunction, and cholestasis (ARC) syndrome: report of three new cases and review." J Med Genet **31**(1): 62-4.

Howells, R. and U. Ramaswami (2002). "ARC syndrome: an expanding range of phenotypes." Arch Dis Child **87**(2): 170-1.

Huizing, M., R. E. Boissy, et al. (2002). "Hermansky-Pudlak syndrome: vesicle formation from yeast to man." Pigment Cell Res **15**(6): 405-19.

Huizing, M., A. Didier, et al. (2001). "Molecular cloning and characterization of human VPS18, VPS 11, VPS16, and VPS33." Gene **264**(2): 241-7.

Huizing, M., A. Helip-Wooley, et al. (2008). "Disorders of lysosome-related organelle biogenesis: clinical and molecular genetics." Annu Rev Genomics Hum Genet **9**: 359-86.

Iwaki, T., F. Osawa, et al. (2003). Characterization of vps33+, a gene required for vacuolar biogenesis and protein sorting in Schizosaccharomyces pombe. Yeast. **20:** 845-55.

Jackson, L. P., B. T. Kelly, et al. (2010). "A large-scale conformational change couples membrane recruitment to cargo binding in the AP2 clathrin adaptor complex." Cell **141**(7): 1220-9.

Jahn, R. and R. H. Scheller (2006). "SNAREs--engines for membrane fusion." Nat Rev Mol Cell Biol **7**(9): 631-43.

Jahn, R. and T. C. Sudhof (1999). "Membrane fusion and exocytosis." Annu Rev Biochem **68**: 863-911.

Jang, J. Y., K. M. Kim, et al. (2009). Clinical characteristics and VPS33B mutations in patients with ARC syndrome. J Pediatr Gastroenterol Nutr. **48:** 348-54.

Jang, W. Y., T. J. Cho, et al. (2011). "Orthopaedic manifestations of arthrogryposis-renal dysfunction-cholestasis syndrome." J Pediatr Orthop **31**(1): 107-12.

Kim, B. (2004). Expression of the mammalian homologue of vacuolar protein sorting 16 (Vps16p) in the mouse and rat brain. Neuroscience Letters. **355:** 217-220.

Kim, B., Y. Sahara, et al. (2006). The interaction of mammalian Class C Vps with nSec-1/Munc18-a and syntaxin 1A regulates pre-synaptic release☆. Biochemical and Biophysical Research Communications. **350:** 691-697.

Kim, B. Y., H. Kramer, et al. (2001). "Molecular characterization of mammalian homologues of class C Vps proteins that interact with syntaxin-7." J Biol Chem **276**(31): 29393-402.

Kim, S., Y. P. Wairkar, et al. (2010). "The novel endosomal membrane protein Ema interacts with the class C Vps-HOPS complex to promote endosomal maturation." J Cell Biol **188**(5): 717-34.

Kim, S. M., H. K. Chang, et al. (2010). "Agranular platelets as a cardinal feature of ARC syndrome." J Pediatr Hematol Oncol **32**(4): 253-8.

Kirchhausen, T. (1999). "Adaptors for clathrin-mediated traffic." Annu Rev Cell Dev Biol **15**: 705-32.

Kirchhausen, T. (2000). "Clathrin." Annu Rev Biochem **69**: 699-727.

Kirchhausen, T., S. C. Harrison, et al. (1987). "Clathrin heavy chain: molecular cloning and complete primary structure." Proc Natl Acad Sci U S A **84**(24): 8805-9.

Kirchhausen, T., S. C. Harrison, et al. (1986). "Configuration of clathrin trimers: evidence from electron microscopy." J Ultrastruct Mol Struct Res **94**(3): 199-208.

L'Hernault, S. W. (2006). "Spermatogenesis." WormBook: 1-14.

L'Hernault, S. W. (2009). "The genetics and cell biology of spermatogenesis in the nematode C. elegans." Mol Cell Endocrinol **306**(1-2): 59-65.

Laage, R. and C. Ungermann (2001). "The N-terminal domain of the t-SNARE Vam3p coordinates priming and docking in yeast vacuole fusion." Mol Biol Cell **12**(11): 3375-85.

LaGrassa, T. J. and C. Ungermann (2005). The vacuolar kinase Yck3 maintains organelle fragmentation by regulating the HOPS tethering complex. The Journal of Cell Biology. **168:** 401-14.

Lai, E. C. (2004). "Notch signaling: control of cell communication and cell fate." Development **131**(5): 965-73.

Li, W., M. E. Rusiniak, et al. (2004). "Murine Hermansky-Pudlak syndrome genes: regulators of lysosome-related organelles." Bioessays **26**(6): 616-28.

Lindmo, K., A. Simonsen, et al. (2006). A dual function for Deep orange in programmed autophagy in the Drosophila melanogaster fat body. Experimental Cell Research. **312:** 2018-27.

Lo, B., L. Li, et al. (2005). Requirement of VPS33B, a member of the Sec1/Munc18 protein family, in megakaryocyte and platelet alpha-granule biogenesis. Blood. **106:** 4159-66.

Lomant, A. J. and G. Fairbanks (1976). "Chemical probes of extended biological structures: synthesis and properties of the cleavable protein cross-linking reagent [35S]dithiobis(succinimidyl propionate)." J Mol Biol **104**(1): 243-61.

Maldonado, E., F. Hernandez, et al. (2006). "The zebrafish mutant vps18 as a model for vesicle-traffic related hypopigmentation diseases." Pigment Cell Res **19**(4): 315-26.

Mari, M., M. V. Bujny, et al. (2008). "SNX1 defines an early endosomal recycling exit for sortilin and mannose 6-phosphate receptors." Traffic **9**(3): 380-93.

Markgraf, D. F., F. Ahnert, et al. (2009). "The CORVET subunit Vps8 cooperates with the Rab5 homolog Vps21 to induce clustering of late endosomal compartments." Mol Biol Cell **20**(24): 5276-89.

Matthews, R. P., N. Plumb-Rudewiez, et al. (2005). Zebrafish vps33b, an ortholog of the gene responsible for human arthrogryposis-renal dysfunction-cholestasis syndrome, regulates biliary development downstream of the onecut transcription factor hnf6. Development. **132:** 5295-306.

Mcvey Ward, D. (2001). hVPS41 Is Expressed in Multiple Isoforms and Can Associate with Vesicles through a RING-H2 Finger Motif. Experimental Cell Research. **267:** 126-134.

Meel, E. and J. Klumperman (2008). Imaging and imagination: understanding the endo-lysosomal system. Histochem Cell Biol. **129:** 253-266.

Moskowitz, H. S., J. Heuser, et al. (2003). Targeted chemical disruption of clathrin function in living cells. Mol Biol Cell. **14:** 4437-47.

Mullins, C., L. M. Hartnell, et al. (2000). "Distinct requirements for the AP-3 adaptor complex in pigment granule and synaptic vesicle biogenesis in Drosophila melanogaster." Mol Gen Genet **263**(6): 1003-14.

Mullins, C., L. M. Hartnell, et al. (1999). "Defective expression of the mu3 subunit of the AP-3 adaptor complex in the Drosophila pigmentation mutant carmine." Mol Gen Genet **262**(3): 401-12.

Nakamura, N., A. Hirata, et al. (1997). "Vam2/Vps41p and Vam6/Vps39p are components of a protein complex on the vacuolar membranes and involved in the vacuolar assembly in the yeast Saccharomyces cerevisiae." J Biol Chem **272**(17): 11344-9.

Nakamura, N., A. Hirata, et al. (1997). Vam2/Vps41p and Vam6/Vps39p are components of a protein complex on the vacuolar membranes and involved in the vacuolar assembly in the yeast Saccharomyces cerevisiae. J Biol Chem. **272:** 11344-9.

Nathke, I. S., J. Heuser, et al. (1992). "Folding and trimerization of clathrin subunits at the triskelion hub." Cell **68**(5): 899-910.

Nickerson, D. P., C. L. Brett, et al. (2009). "Vps-C complexes: gatekeepers of endolysosomal traffic." Curr Opin Cell Biol **21**(4): 543-51.

Nordmann, M., M. Cabrera, et al. (2010). "The Mon1-Ccz1 complex is the GEF of the late endosomal Rab7 homolog Ypt7." Curr Biol **20**(18): 1654-9.

Odorizzi, G., C. R. Cowles, et al. (1998). "The AP-3 complex: a coat of many colours." Trends Cell Biol **8**(7): 282-8.

Ohno, H., R. C. Aguilar, et al. (1998). "The medium subunits of adaptor complexes recognize distinct but overlapping sets of tyrosine-based sorting signals." J Biol Chem **273**(40): 25915-21.

Ohno, H., J. Stewart, et al. (1995). "Interaction of tyrosine-based sorting signals with clathrin-associated proteins." Science **269**(5232): 1872-5.

Ostrowicz, C. W., C. Brocker, et al. (2010). "Defined subunit arrangement and rab interactions are required for functionality of the HOPS tethering complex." Traffic **11**(10): 1334-46.

Ostrowicz, C. W., C. T. Meiringer, et al. (2008). "Yeast vacuole fusion: a model system for eukaryotic endomembrane dynamics." Autophagy **4**(1): 5-19.

Owen, D. J. and P. R. Evans (1998). "A structural explanation for the recognition of tyrosine-based endocytotic signals." Science **282**(5392): 1327-32.

Pagano, A., P. Crottet, et al. (2004). "In vitro formation of recycling vesicles from endosomes requires adaptor protein-1/clathrin and is regulated by rab4 and the connector rabaptin-5." Mol Biol Cell **15**(11): 4990-5000.

Parsch, K. and S. Pietrzak (2007). Arthrogryposis multiplex congenita. Orthopäde. **36:** 281-292.

Peden, A. A., V. Oorschot, et al. (2004). Localization of the AP-3 adaptor complex defines a novel endosomal exit site for lysosomal membrane proteins. The Journal of Cell Biology. **164:** 1065-76.

Peden, A. A., R. E. Rudge, et al. (2002). Assembly and function of AP-3 complexes in cells expressing mutant subunits. The Journal of Cell Biology. **156:** 327-36.

Peplowska, K., D. F. Markgraf, et al. (2007). The CORVET tethering complex interacts with the yeast Rab5 homolog Vps21 and is involved in endo-lysosomal biogenesis. Dev Cell. **12:** 739-50.

Peralta, E. R., B. C. Martin, et al. (2010). "Differential effects of TBC1D15 and mammalian Vps39 on Rab7 activation state, lysosomal morphology, and growth factor dependence." J Biol Chem **285**(22): 16814-21.

Pereira-Leal, J. B. and M. C. Seabra (2001). "Evolution of the Rab family of small GTP-binding proteins." J Mol Biol **313**(4): 889-901.

Pevsner, J., S. C. Hsu, et al. (1996). Mammalian homologues of yeast vacuolar protein sorting (vps) genes implicated in Golgi-to-lysosome trafficking. Gene. **183:** 7-14.

Pfeffer, S. and D. Aivazian (2004). "Targeting Rab GTPases to distinct membrane compartments." Nat Rev Mol Cell Biol **5**(11): 886-96.

Phan, H. L., J. A. Finlay, et al. (1994). "The Saccharomyces cerevisiae APS1 gene encodes a homolog of the small subunit of the mammalian clathrin AP-1 complex: evidence for functional interaction with clathrin at the Golgi complex." EMBO J **13**(7): 1706-17.

Pierson, D. M., D. Ionescu, et al. (2006). "Pulmonary fibrosis in hermansky-pudlak syndrome. a case report and review." Respiration **73**(3): 382-95.

Piper, R. C., N. J. Bryant, et al. (1997). "The membrane protein alkaline phosphatase is delivered to the vacuole by a route that is distinct from the VPS-dependent pathway." J Cell Biol **138**(3): 531-45.

Plemel, R. L., B. T. Lobingier, et al. (2011). "Subunit organization and Rab interactions of Vps-C protein complexes that control endolysosomal membrane traffic." Mol Biol Cell.

Poteryaev, D., S. Datta, et al. (2010). "Identification of the switch in early-to-late endosome transition." Cell **141**(3): 497-508.

Poupon, V., A. Stewart, et al. (2003). "The role of mVps18p in clustering, fusion, and intracellular localization of late endocytic organelles." Mol Biol Cell **14**(10): 4015-27.

Price, A., D. Seals, et al. (2000). The docking stage of yeast vacuole fusion requires the transfer of proteins from a cis-SNARE complex to a Rab/Ypt protein. The Journal of Cell Biology. **148:** 1231-8.

Price, A., W. Wickner, et al. (2000). Proteins needed for vesicle budding from the Golgi complex are also required for the docking step of homotypic vacuole fusion. The Journal of Cell Biology. **148:** 1223-29.

Progida, C., L. Cogli, et al. (2010). "Rab7b controls trafficking from endosomes to the TGN." J Cell Sci **123**(Pt 9): 1480-91.

Pulipparacharuvil, S., M. A. Akbar, et al. (2005). Drosophila Vps16A is required for trafficking to lysosomes and biogenesis of pigment granules. J Cell Sci. **118:** 3663-73.

Radisky, D. C., W. B. Snyder, et al. (1997). Characterization of VPS41, a gene required for vacuolar trafficking and high-affinity iron transport in yeast. Proc Natl Acad Sci USA. **94:** 5662-6.

Raiborg, C., K. G. Bache, et al. (2002). Hrs sorts ubiquitinated proteins into clathrin-coated microdomains of early endosomes. Nat. Cell Biol. **4:** 394-398.

Raiborg, C., J. Wesche, et al. (2006). "Flat clathrin coats on endosomes mediate degradative protein sorting by scaffolding Hrs in dynamic microdomains." J Cell Sci **119**(Pt 12): 2414-24.

Raymond, C. K., I. Howald-Stevenson, et al. (1992). "Morphological classification of the yeast vacuolar protein sorting mutants: evidence for a prevacuolar compartment in class E vps mutants." Mol Biol Cell **3**(12): 1389-402.

Rehling, P., T. Darsow, et al. (1999). Formation of AP-3 transport intermediates requires Vps41 function. Nat. Cell Biol. **1:** 346-53.

Richardson, S. C., S. C. Winistorfer, et al. (2004). "Mammalian late vacuole protein sorting orthologues participate in early endosomal fusion and interact with the cytoskeleton." Mol Biol Cell **15**(3): 1197-210.

Rink, J., E. Ghigo, et al. (2005). Rab conversion as a mechanism of progression from early to late endosomes. Cell. **122:** 735-49.

Robinson, J. S., D. J. Klionsky, et al. (1988). Protein sorting in Saccharomyces cerevisiae: isolation of mutants defective in the delivery and processing of multiple vacuolar hydrolases. Mol Cell Biol. **8:** 4936-48.

Robinson, M. S. (2004). "Adaptable adaptors for coated vesicles." Trends Cell Biol **14**(4): 167-74.

Ronnett, G. V., L. D. Hester, et al. (1990). "Human cortical neuronal cell line: establishment from a patient with unilateral megalencephaly." Science **248**(4955): 603-5.

Ruan, Q., A. J. Harrington, et al. (2009). VPS41, a protein involved in lysosomal trafficking, is protective in Caenorhabditis elegans and mammalian cellular models of Parkinson's disease. Neurobiol Dis.

Sadler, K. C., A. Amsterdam, et al. (2005). "A genetic screen in zebrafish identifies the mutants vps18, nf2 and foie gras as models of liver disease." Development **132**(15): 3561-72.

Salazar, G., S. Zlatic, et al. (2009). Hermansky-Pudlak syndrome protein complexes associate with phosphatidylinositol 4-kinase type II alpha in neuronal and non-neuronal cells. J Biol Chem. **284:** 1790-802.

Sato, T. K., P. Rehling, et al. (2000). "Class C Vps protein complex regulates vacuolar SNARE pairing and is required for vesicle docking/fusion." Mol Cell **6**(3): 661-71.

Schmid, S. L. (1997). "Clathrin-coated vesicle formation and protein sorting: an integrated process." Annu Rev Biochem **66**: 511-48.

Schonthaler, H. B., V. C. Fleisch, et al. (2008). "The zebrafish mutant lbk/vam6 resembles human multisystemic disorders caused by aberrant trafficking of endosomal vesicles." Development **135**(2): 387-99.

Seals, D. F., G. Eitzen, et al. (2000). A Ypt/Rab effector complex containing the Sec1 homolog Vps33p is required for homotypic vacuole fusion. Proc Natl Acad Sci USA. **97:** 9402-7.

Seaman, M. N., C. G. Burd, et al. (1996). "Receptor signalling and the regulation of endocytic membrane transport." Curr Opin Cell Biol **8**(4): 549-56.

Seeger, M. and G. S. Payne (1992). "A role for clathrin in the sorting of vacuolar proteins in the Golgi complex of yeast." EMBO J **11**(8): 2811-8.

Segev, N. (2010). "Coordination of intracellular transport steps by GTPases." Semin Cell Dev Biol **22**(1): 33-8.

Sevrioukov, E. A., J. P. He, et al. (1999). "A role for the deep orange and carnation eye color genes in lysosomal delivery in Drosophila." Mol Cell **4**(4): 479-86.

Shestopal, S. A., I. V. Makunin, et al. (1997). "Molecular characterization of the deep orange (dor) gene of Drosophila melanogaster." Mol Gen Genet **253**(5): 642-8.

Starai, V. J., C. M. Hickey, et al. (2008). HOPS proofreads the trans-SNARE complex for yeast vacuole fusion. Mol Biol Cell. **19:** 2500-8.

Stenmark, H. (2009). "Rab GTPases as coordinators of vesicle traffic." Nat Rev Mol Cell Biol **10**(8): 513-25.

Stepp, J. D., K. Huang, et al. (1997). The yeast adaptor protein complex, AP-3, is essential for the efficient delivery of alkaline phosphatase by the alternate pathway to the vacuole. The Journal of Cell Biology. **139:** 1761-74.

Stepp, J. D., A. Pellicena-Palle, et al. (1995). "A late Golgi sorting function for Saccharomyces cerevisiae Apm1p, but not for Apm2p, a second yeast clathrin AP medium chain-related protein." Mol Biol Cell **6**(1): 41-58.

Stoorvogel, W., V. Oorschot, et al. (1996). "A novel class of clathrin-coated vesicles budding from endosomes." J Cell Biol **132**(1-2): 21-33.

Stoorvogel, W., G. J. Strous, et al. (1991). "Late endosomes derive from early endosomes by maturation." Cell **65**(3): 417-27.

Stroupe, C., K. M. Collins, et al. (2006). Purification of active HOPS complex reveals its affinities for phosphoinositides and the SNARE Vam7p. EMBO J. **25:** 1579-1589.

Suzuki, T., N. Oiso, et al. (2003). The mouse organellar biogenesis mutant buff results from a mutation in Vps33a, a homologue of yeast vps33 and Drosophila carnation. Proc Natl Acad Sci USA. **100:** 1146-50.

Swank, R. T., E. K. Novak, et al. (1998). "Mouse models of Hermansky Pudlak syndrome: a review." Pigment Cell Res **11**(2): 60-80.

Swedlow, J., J. Sedat, et al. (1997). Deconvolution in optical microscopy. San Diego, CA, Academic Press.

Taha, D., A. Khider, et al. (2007). A novel VPS33B mutation in an ARC syndrome patient presenting with osteopenia and fractures at birth. Am J Med Genet A. **143A:** 2835-7.

Tekin, N., S. Durmus-Aydogdu, et al. (2005). "Clinical and pathological aspects of ARC (arthrogryposis, renal dysfunction and cholestasis) syndrome in two siblings." Turk J Pediatr **47**(1): 67-70.

ter Haar, E., A. Musacchio, et al. (1998). "Atomic structure of clathrin: a beta propeller terminal domain joins an alpha zigzag linker." Cell **95**(4): 563-73.

Theos, A. C., D. Tenza, et al. (2005). Functions of adaptor protein (AP)-3 and AP-1 in tyrosinase sorting from endosomes to melanosomes. Mol Biol Cell. **16:** 5356-72.

Venda, L. L., S. J. Cragg, et al. (2010). "alpha-Synuclein and dopamine at the crossroads of Parkinson's disease." Trends Neurosci **33**(12): 559-68.

Volpicelli, L. A., J. J. Lah, et al. (2001). "Rab5-dependent trafficking of the m4 muscarinic acetylcholine receptor to the plasma membrane, early endosomes, and multivesicular bodies." J Biol Chem **276**(50): 47590-8.

Vowels, J. J. and G. S. Payne (1998). "A dileucine-like sorting signal directs transport into an AP-3-dependent, clathrin-independent pathway to the yeast vacuole." EMBO J **17**(9): 2482-93.

Wada, Y. and Y. Anraku (1992). Genes for directing vacuolar morphogenesis in Saccharomyces cerevisiae. II. VAM7, a gene for regulating morphogenic assembly of the vacuoles. J Biol Chem. **267:** 18671-5.

Wang, C.-W. (2003). Yeast homotypic vacuole fusion requires the Ccz1-Mon1 complex during the tethering/docking stage. The Journal of Cell Biology. **163:** 973-985.

Wang, L. (2003). Hierarchy of protein assembly at the vertex ring domain for yeast vacuole docking and fusion. The Journal of Cell Biology. **160:** 365-374.

Wang, L., E. S. Seeley, et al. (2002). Vacuole fusion at a ring of vertex docking sites leaves membrane fragments within the organelle. Cell. **108:** 357-69.

Wang, T., Z. Ming, et al. (2011). "Rab7: role of its protein interaction cascades in endo-lysosomal traffic." Cell Signal **23**(3): 516-21.

Wang, Y., T. Chen, et al. (2007). "Lysosome-associated small Rab GTPase Rab7b negatively regulates TLR4 signaling in macrophages by promoting lysosomal degradation of TLR4." Blood **110**(3): 962-71.

Wang, Y., I. Dulubova, et al. (2001). "Functional analysis of conserved structural elements in yeast syntaxin Vam3p." J Biol Chem **276**(30): 28598-605.

Warner, T. S., D. A. Sinclair, et al. (1998). The light gene of Drosophila melanogaster encodes a homologue of VPS41, a yeast gene involved in cellular-protein trafficking. Genome. **41:** 236-43.

Waters, M. G. and F. M. Hughson (2000). "Membrane tethering and fusion in the secretory and endocytic pathways." Traffic **1**(8): 588-97.

Weaver, T. E., C.-L. Na, et al. (2002). Biogenesis of lamellar bodies, lysosome-related organelles involved in storage and secretion of pulmonary surfactant. Semin Cell Dev Biol. **13:** 263-70.

Weisz, O. A. and E. Rodriguez-Boulan (2009). "Apical trafficking in epithelial cells: signals, clusters and motors." J Cell Sci **122**(Pt 23): 4253-66.

Wickner, W. (2010). "Membrane fusion: five lipids, four SNAREs, three chaperones, two nucleotides, and a Rab, all dancing in a ring on yeast vacuoles." Annu Rev Cell Dev Biol **26**: 115-36.

Wilkin, M., P. Tongngok, et al. (2008). "Drosophila HOPS and AP-3 complex genes are required for a Deltex-regulated activation of notch in the endosomal trafficking pathway." Dev Cell **15**(5): 762-72.

Wurmser, A. E., T. K. Sato, et al. (2000). New component of the vacuolar class C-Vps complex couples nucleotide exchange on the Ypt7 GTPase to SNARE-dependent docking and fusion. The Journal of Cell Biology. **151:** 551-62.

Yang, M., T. Chen, et al. (2004). "Rab7b, a novel lysosome-associated small GTPase, is involved in monocytic differentiation of human acute

promyelocytic leukemia cells." Biochem Biophys Res Commun **318**(3): 792-9.

Yao, M., X. Liu, et al. (2009). "Late endosome/lysosome-localized Rab7b suppresses TLR9-initiated proinflammatory cytokine and type I IFN production in macrophages." J Immunol **183**(3): 1751-8.

Ybe, J. A., F. M. Brodsky, et al. (1999). Clathrin self-assembly is mediated by a tandemly repeated superhelix. Nature. **399:** 371-5.

Ybe, J. A., B. Greene, et al. (1998). "Clathrin self-assembly is regulated by three light-chain residues controlling the formation of critical salt bridges." Embo J **17**(5): 1297-303.

Zhu, G. D. and S. W. L'Hernault (2003). "The Caenorhabditis elegans spe-39 gene is required for intracellular membrane reorganization during spermatogenesis." Genetics **165**(1): 145-57.

Zhu, G. D., G. Salazar, et al. (2009). "SPE-39 family proteins interact with the HOPS complex and function in lysosomal delivery." Mol Biol Cell **20**(4): 1223-40.

Zlatic, S. A., P. V. Ryder, et al. (2010). "Isolation of labile multi-protein complexes by in vivo controlled cellular cross-linking and immuno-magnetic affinity chromatography." J Vis Exp(37).

Zlatic, S. A., K. Tornieri, et al. (2011). "Clathrin-dependent mechanisms modulate the subcellular distribution of class C Vps/HOPS tether subunits in polarized and nonpolarized cells." Mol Biol Cell **22**(10): 1699-715.