The guanine nucleotide exchange factors Vps9 and Mon1-Ccz1 coordinate endosome maturation in *Saccharomyces cerevisiae*

By

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The guanine nucleotide exchange factors Vps9 and Mon1-Ccz1 coordinate endosome maturation in *Saccharomyces Cerevisia*e

Thesis directed by Professor Greg Odorizzi

Transmembrane proteins at the plasma membrane that are targeted for degradation in the lysosome are sorted through the endosomal pathway. At endosomes they are sorted into inward budding intralumenal vesicles (ILVs) through the concerted action of the endosomal sorting complexes required for transport (ESCRTs). Deletion of ESCRT genes in *S. cerevisiae* disrupts endosomal morphology leading to aberrant flattened stacks of endosomal membranes known as class E compartments. Recently we have shown that class E compartments form due to hyperactivation of the Rab5 homolog, Vps21, as well as failure to activate the Rab7 homolog, Ypt7. Here we show that class E compartment formation requires the guanine nucleotide exchange factor (GEF) Vps9. Disruption of ubiquitin binding by Vps9 severely diminishes localization of Vps9 at class E compartments but does not suppress the formation of class E compartments. In addition we find the removal of Vps9 from endosomes requires the putative endosomal conversion factor Mon1-Ccz1 as well as endosome-to-vacuole fusion. Mon1-Ccz1 fails to localize to class E compartments but loss of Mon1-Ccz1 does not disrupt MVB biogenesis indicating ESCRT activity precedes Rab conversion. Our data suggest a model where endosomal Rab conversion occurs after completion of ESCRT-mediated sorting and involves multiple mechanisms including removal of ubiquitin by ESCRTs and recruitment of Mon1-Ccz1.

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Table of Contents

Chapter 5. Summary and future directions

Figures

Tables

Abbreviations

AAA	ATPase associated with a variety of cellular activities
ATP	Adenosine triphosphate
CCZ	Calcium Caffeine Zinc sensitivity
CORVET	Class C core vacuole/endosome tethering complex
CPS	Carboxy peptidase S
CPY	Carboxy peptidase Y
CUE	Coupling of ubiquitin conjugation to ER degradation
DOA	Degradation of alpha factor
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EM	Electron microscopy
ER	Endoplasmic reticulum
ESCRT	Endosomal sorting complex required for transport
GAP	GTPase activating protein
GDP	Guanine diphosphate
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GTP	Guanine triphosphate
HOPS	Homotypic fusion and vacuole protein sorting
ILV	Intralumenal vesicle
LDL	Low density lipoprotein
LDLR	Low density lipoprotein receptor
MIU	Motif interacting with ubiquitin
MON	Monesin sensitivity

Abbreviations

MVB	Multivesicular body
PI(3)P	Phosphatidyl inositol-3-phosphate
RAB	Ras in brain
RSP	Reverses Spt phenotype
SNARE	SNAP Receptor
VPS	Vacuolar protein sorting
YPT	Yeast protein two

Chapter 1. Introduction

Overview of the endosomal pathway

The endosomal system consists of a series of compartments regulating transport between the plasma membrane and the degradative lysosome (Figure 1.1). Extra cellular fluid and transmembrane proteins are brought into cells via inward budding of the plasma membrane. This transport can occur through a variety of mechanisms, including clathrin-mediated endocytosis, caveolin-mediated endocytosis, phagocytosis and pinocytosis (Mellman 1996). Clathrin-mediated endocytosis has been studied in the greatest detail and is the major mechanism for internalization of transmembrane proteins. Upon internalization clathrin-coated vesicles first fuse with early endosomes. The majority of internalized cargo is recycled back to the plasma membrane (Huotari & Helenius 2011). This recycled material includes nutrients receptors such as the LDL, receptor which, after release of the LDL particle in the lower pH of the early endosome, returns to the cell surface to engage in further rounds of LDL particle internalization. Cargo destined for the lysosome is retained on early endosomes and is ultimately transported to late endosomes. This includes many cell surface receptors, such as the EGF receptor, that are targeted to the lysosome as a means of turning off signaling. These transmembrane proteins are sorted into intralumenal vesicles that bud into the lumen of the endosome, which gives late endosomes their multivesicular body (MVB) morphology. These vesicle containing late endosomes, or MVBs ultimately fuse with lysosomes where the vesicles and proteins can be degraded.

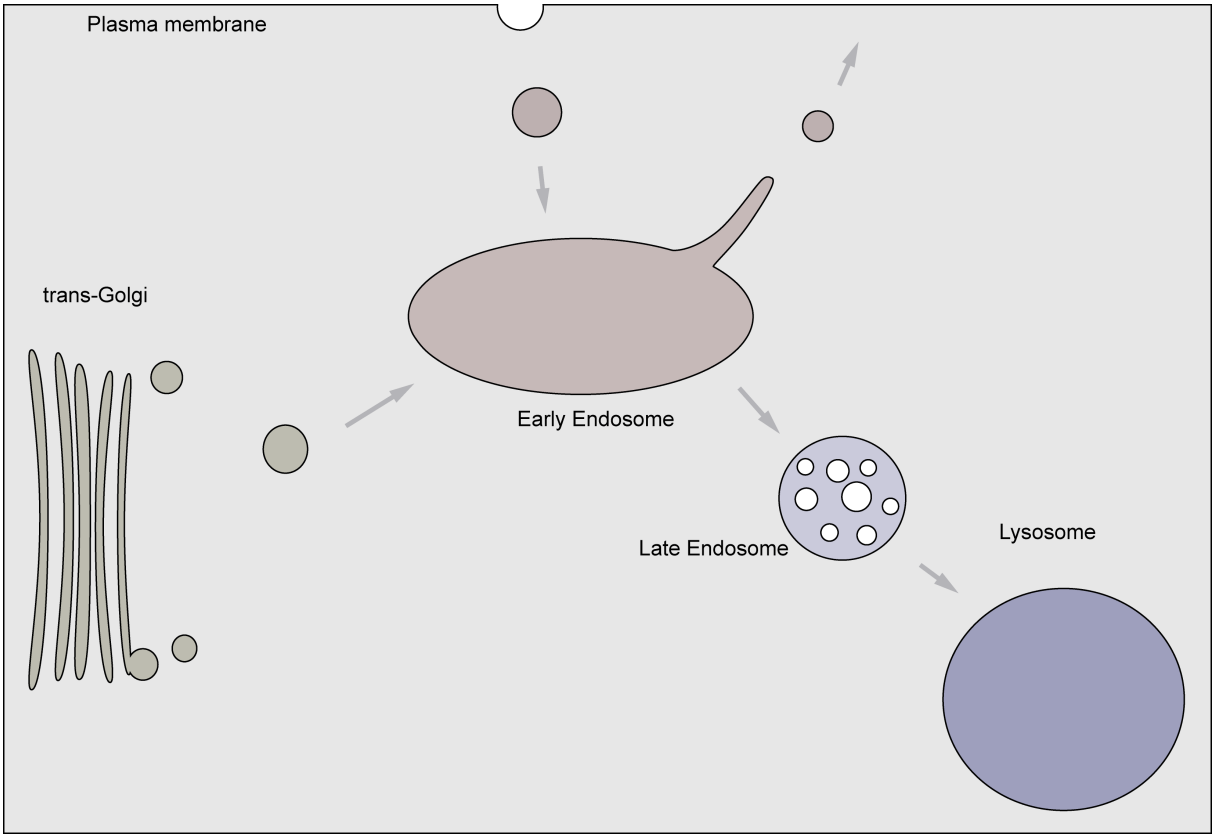

Figure 1.1 Endosomal transport. Extracellular fluid and plasma membrane are brought in to the cell via endocytosis. Endocytic vesicles then fuse with early endosomes. At the early endosome contents can either be recycled back to the plasma membrane or retained and transported to late endosomes. Late endosomes are marked by the accumulation of numerous intralumenal vesicles (ILVs) that contain transmembrane proteins destined for degradation. Late endosomes ultimately fuse with with lysosomes where ILVs and their contents are degraded.

Studies in mammalian cells have established that transport from the plasma membrane to the lysosome traverses sequentially through early and then late endosomes. In *Saccharomyces cerevisiae* the ease of genetic manipulation and the ability to perform large scale screens has led to the discovery of many of the genes required for transport to the vacuole, the equivalent of the mammalian lysosome. Two of the early screens took advantage of sorting of CPY, which is a soluble vacuolar hydrolase that is sorted from the Golgi to vacuole (Rothman & Stevens 1986, Robinson et al. 1988). These screens selected for mutants with disrupted sorting to the vacuole leading to secretion of CPY via the secretory pathway. These mutants displayed disruptions in vacuole morphology that could be used to organize them into six distinct groups. These six groups are summarized here in Table1 and a subset of these will be described in more detail later in this chapter where it is relevant.

Table 1.1 Morphological classes of *vps* mutants. Adapted from Raymond et. al. 1992	
Class	Morphology
Class A	Wild-type vacuolar morphology. Vacuoles frequently appear as 3-10 subcompartments that cluster to one region of the cytoplasm.
Class B	Mutants have highly "fragmented" vacuoles exhibiting > 20 vacuole-like compartments per cell. In some mutants, these sub-compartments are clustered, whereas in others they are distributed randomly throughout the cell.
Class C	Mutant cells do not have an organelle readily identifiable as a vacuole.
Class D	These mutants are defective for vacuolar segregation, meaning mother cells have a large, single, spherical vacuolar compartment that fails to extend into developing buds, and newly formed daughter cells often lack coherent vacuoles and assume the appearance of class C mutants.
Class E	V-ATPase antibodies label a prominent organelle distinct from the vacuole. Significant portions of soluble vacuolar hydrolases (CPY and PrA) and Golgi membrane proteins (DPAPA and A-ALP) also localize to the novel organelle. These data indicate that class E mutants may possess an exaggerated form of a prevacuolar endosome-like compartment.
Class F	These mutants often have a large central vacuole surrounded by a number of class B-like fragmented vacuolar structures.

This collection of vacuolar protein sorting (*vps*) genes includes many of the genes required for transport to early endosomes, sorting into MVBs and fusion with the vacuole. These studies also indicated that many of the hydrolases that function at the vacuole are transported there via endosomes. In addition yeast and mammalian studies have shown that many of the genes are functionally conserved in the two systems.

Rab GTPases regulate fusion in the Endocytic pathway

Overview of Rabs in membrane transport

Transport between membrane compartments throughout the eukaryotic cell generally occurs by budding from one compartment, transport along the cytoskeleton and fusion with the target membrane. Each of these steps is regulated by Rab GTPases, which bind membranes via the addition of hydrophobic geranylgeranyl groups to their C-terminus. Rabs localize to distinct cellular compartments and are considered master regulators of organelle function and identity. Rab GTPases are molecular switches whose activity is controlled by binding of guanine nucleotides (Figure 1.2). Interaction with a guanine nucleotide exchange factor (GEF) promotes exchange of GDP for GTP, which induces a conformational change in the Rab switch I and switch II regions. This change to the active, GTP-bound conformation promotes recruitment and activity of effector proteins that carry out different functions of membrane transport, including cargo selection, formation of transport vesicles, adaptors for binding to cytoskeletal motors as well as tethering to SNAREs for membrane fusion. Rabs have low intrinsic GTPase activity and are brought into their inactive, GDP-bound state by GTPase-activating proteins (GAPs) which stimulate hydrolysis of GTP to GDP.

In the GDP-bound state, Rabs are subject to extraction from the membrane by guanine nucleotide dissociation inhibitor (GDIs). GEFs, GAPs and GDI act to maintain the spatiotemporal control of Rabs (Stenmark 2009).

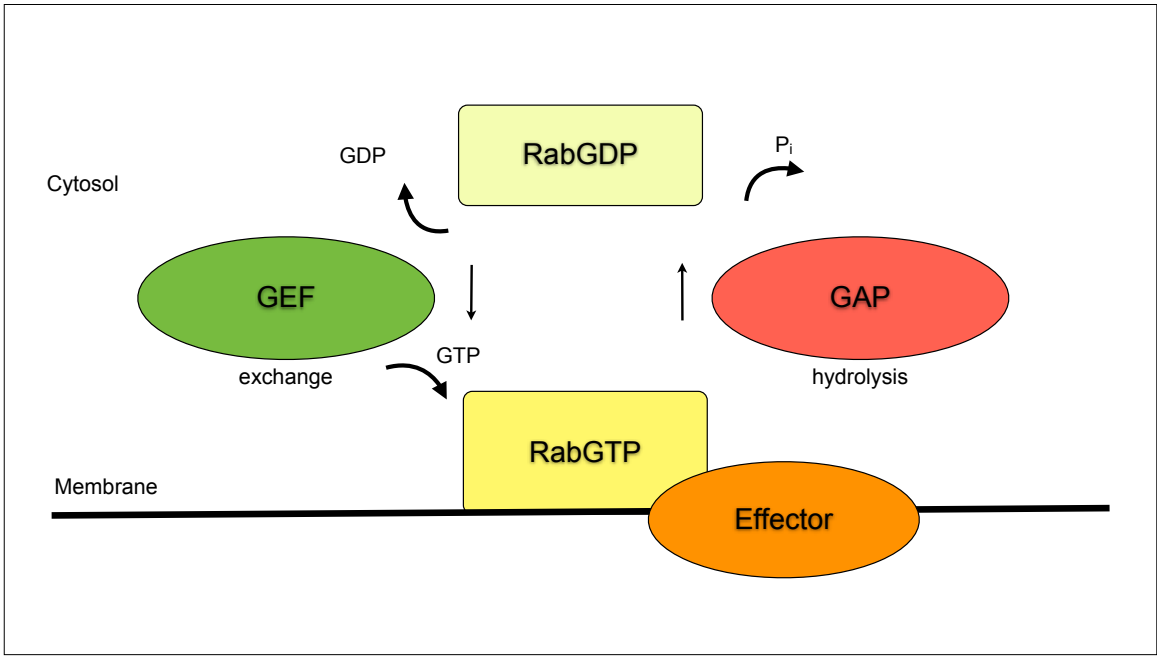

Figure 1.2 Rab nucleotide cycle. Rabs are in their inactive, GDP-bound form in the cytosol. Interaction with a GEF promotes nucleotide exchange and membrane association. At the target membrane the active Rab interacts with effectors. Interaction with a GAP promotes hydrolysis by the RAb and removal of the Rab from the membrane.

Rab5 regulates fusion with early endosomes

Rab5 was among the first mammalian Rabs identified and was shown to localize to early endosomes (Chavrier et al. 1990). Subsequent *in vitro* and *in vivo* studies have shown that Rab5 promotes fusion of endocytic vesicles with early endosomes as well as homotypic fusion between early endosomes (Bucci et al. 1995, Gorvel et al. 1991). Two similar Rab proteins were later discovered and were also shown to function in early endosome fusion. These proteins were named Rab5b and Rab5c, and the original Rab5 was renamed Rab5a (Bucci et al. 1995). Although the precise role of each Rab5 family member is not known, genetic knock down of the expression of all three Rabs simultaneously results in a complete loss of endosomes, indicating Rab5 proteins are required for biogenesis of early endosomes (Zeigerer et al. 2012). The yeast gene *VPS21* was identified in the original *vps* screens and was placed in the class D category of genes whose deletion results in a single large vacuole and defects in vacuole inheritance (Raymond et al. 1992). The Vps21 protein sequence was found to be highly similar to Rab5a, and deletion of *VPS21* caused severe defects in delivery of endocytic and Golgi vesicles to early endosomes (Horazdovsky et al. 1996). *S. cerevisiae* also has three Rab5 family members, Ypt52 and Ypt53, in addition to Vps21. Although all three Rab5 proteins in yeast function in the endocytic pathway, the precise function of each has yet to be clearly defined. Deletion of *VPS21* causes the most severe disruption in sorting and endosome biogenesis and is, therefore, thought to be the primary Rab5 family member regulating transport to early endosomes (Nickerson et al. 2012, Paulsel et al. 2013).

The mammalian protein Rabex-5 was isolated from cell extracts through binding to Rab5 and was found to have GEF activity towards Rab5 (Horiuchi et al. 1997). Deletion of the yeast gene *VPS9*, like deletion of *VPS21,* also leads to class D vacuole morphology and was therefore, thought to function at a similar stage of the endocytic pathway. The Vps9 protein sequence is similar to that of mammalian Rabex-5 and was shown to promote nucleotide exchange on Vps21 (Hama et al. 1999). Rabex-5 and Vps9 contain a conserved, central “Vps9 domain” that promotes nucleotide exchange on Rab5 proteins. A crystal structure of the Vps9 domain bound to a human Rab5-related protein, Rab21, revealed that a conserved aspartic acid (position 251 in yeast Vps9) acts to stabilize the nucleotide-free state, thereby promoting GDP association (Delprato & Lambright 2007). Additional residues in Rabex-5 have been shown to be important for nucleotide exchange activity but these are within the binding interface and likely promote binding of the Vps9 domain to Rab5 rather than nucleotide exchange per se (Delprato et al. 2004).

In addition to their Vps9 domains, both yeast Vps9 and mammalian Rabex5 contain ubiquitin-binding domains. The addition of ubiquitin to transmembrane proteins at the plasma membrane acts as a signal for internalization and transport to early endosomes (Piper & Lehner 2011). The ubiquitin-binding domains of Vps9 and Rabex-5 likely bind ubiquitin to direct delivery of endocytic vesicles to Rab5 endosomes. The Vps9 domain alone has higher nucleotide exchange activity than full length Rabex-5 *in vitro*, indicating potential auto-inhibition by the ubiquitin binding domain (Delprato et al. 2004). In this model, binding ubiquitin at the endosomal membrane would both recruit

Vps9 to the appropriate domain and increase its GEF activity by relieving auto-inhibition.

Rab7 regulates fusion with the late endosome

Rab7 binds late endosomes and lysosomes and is required for fusion of late endosomes with lysosomes as well has homotypic lysosome fusion (Chavrier et al. 1990, Bucci et al. 2000). Ypt7, the yeast Rab7 homolog, is similarly required for fusion of late endosomes with vacuoles (Schimmöller & Riezman 1993). Although Ypt7 was not isolated in the original *vps* screens, *ypt7*Δ cells have fragmented, class B vacuoles indicative of a defect in vacuole fusion. The ability to purify vacuoles from yeast led to the development of *in vitro* homotypic vacuole fusion assays which provided evidence that Ypt7 was involved in homotypic vacuole fusion (Haas et al. 1995). In addition, these studies showed fusion requires Ypt7 on both membranes.

The identification of the GEF for Ypt7 has been somewhat controversial. It was initially identified as Vps39, which is also part of the Ypt7 effector complex HOPS, discussed below (Wurmser et al. 2000). Soon after, it was discovered that mutations in the Ypt7 nucleotide-binding site suppressed the vacuole transport defects of cells lacking the protein Ccz1 (Kucharczyk et al. 2001). This, in combination with evidence showing Ccz1 and Ypt7 physically interact, indicated that Ccz1 functions with Ypt7 in vacuole fusion, although its precise role was not clear (Kucharczyk et al. 2001). Later, Ccz1, along with its binding partner Mon1, was shown to have nucleotide exchange activity toward Ypt7 (Nordmann et al. 2010). This study used Mon1-Ccz1 purified from bacteria in an *in vitro* exchange assay, whereas the previous work used Vps39 purified

from yeast, which might have contained residual Mon1-Ccz1. Mon1-Ccz1 is therefore considered the GEF for Ypt7.

HOPS and CORVET endosomal tethering complexes

Tethering complexes play an important role in numerous membrane fusion events throughout the cell and are thought to provide the initial contacts between two membranes. Tethering complexes also regulate the trans-SNARE pairing that drives membrane fusion (Stenmark 2009). Two related complexes, HOPS and CORVET, act as tethers in the yeast endosomal pathway. The HOPS complex was first isolated from yeast through its association with Ypt7 and was found to be composed of six subunits, Vps11, Vps16, Vps18, Vps33, Vps39 and Vps41 (Seals et al. 2000). HOPS can tether vacuole membranes, and it has been shown to bind vacuole SNARES and guide assembly of the correct trans-SNARE complex. HOPS likely functions in a similar way in heterotypic endosome-vacuole fusion.

Deletion of the genes encoding HOPS subunits Vps39 and Vps41 results in cells with a *vps* class B phenotype of highly fragmented vacuoles, which is consistent with their role in mediating vacuole fusion (Raymond et al. 1992). Deletion of the genes encoding the other four subunits of the HOPS complex, Vps11, Vps16, Vps18 and Vps33 causes a *vps* class C phenotype whose mutant cells have no discernible vacuole. These class C genes were shown to have functions more upstream in the endosomal pathway (Srivastava et al. 2000). It was later discovered that the four proteins encoded by the class C genes are also part of an additional complex, along with Vps8 and Vps3, named CORVET (Peplowska et al. 2007). The CORVET complex

is a Vps21 effector (Peplowska et al. 2007) that is likely an early endosome tethering factor. Overexpression of Vps8 alone can stimulate clustering of endosomes *in vivo* (Markgraf et al. 2009) and suprisingly, Vps21 has been shown to promote tethering of membranes *in vitro,* independent of CORVET. It is unclear if this tethering capability of Vps21 is relevant *in vivo,* but it is GTP dependent and can be stimulated by the GEF Vps9 (Lo et al. 2012). Given the potential tethering functions of Vps8 and Vps21, it is unclear if tethering by CORVET is required for early endosome fusion.

Less is known about endosomal tethers in other organisms. Homologs of all the CORVET and HOPS subunits except Vps3 have been found in worms, flies, mice, and humans (Solinger & Spang 2013). It is not yet clear whether these complexes perform the same function in metazoans. Recent work has indicated that human Vps39 and Vps41 are required for fusion of late endosomes with lysosomes (Pols et al. 2013). Whether the HOPS and CORVET components in metazoans assemble into the same two complexes as they do in yeast is unknown. Even in yeast, hybrid CORVET/HOPS complexes have been detected *in vivo,* the function of which is unknown (Peplowska et al. 2007). There may be modularity to complex assembly in all species, allowing more diversity of function. In addition, interconversion between these complexes has been proposed to regulate endosomal transport in yeast (Nickerson et al. 2009).

Directional Transport in the endocytic pathway

One of the important questions in the study of the endosomal system has been the mechanism of transport from early to late endosomes. There is evidence for a vesicular transport model as well as a compartment maturation model. In the vesicular

transport model, vesicles containing cargo destined for late endosomes and lysosomes bud off an early endosome and fuse with late endosomes. This model is supported by experiments that followed the transport of semliki forrest virus by live-cell imaging (Vonderheit & Helenius 2005). The virus is taken up by cells and transported to Rab5-positive early endosomes and then to hybrid compartments marked by Rab5 and Rab7. From these hybrid compartments, Rab7 domains containing virus detach and are transported away from the Rab5 domains. In the second model, transport occurs through maturation, where an early endosome compartment exists transiently and eventually loses properties of an early endosome as it acquires characteristics of a late endosome. This model is supported by work using live-cell imaging to follow transport of LDL from early to late endosomes (Rink et al. 2005). In these experiments, individual compartments containing LDL were initially marked by Rab5. As endosomal compartments migrated to the center of the cell, Rab5 was rapidly replaced by Rab7 on the same compartment (see model in Figure 1.3). More recently this Rab5-Rab7 conversion was shown to occur on endosomes in *C. elegans* coelomocytes indicating, this is a conserved mechanism of endosome transport (Poteryaev et al. 2010).

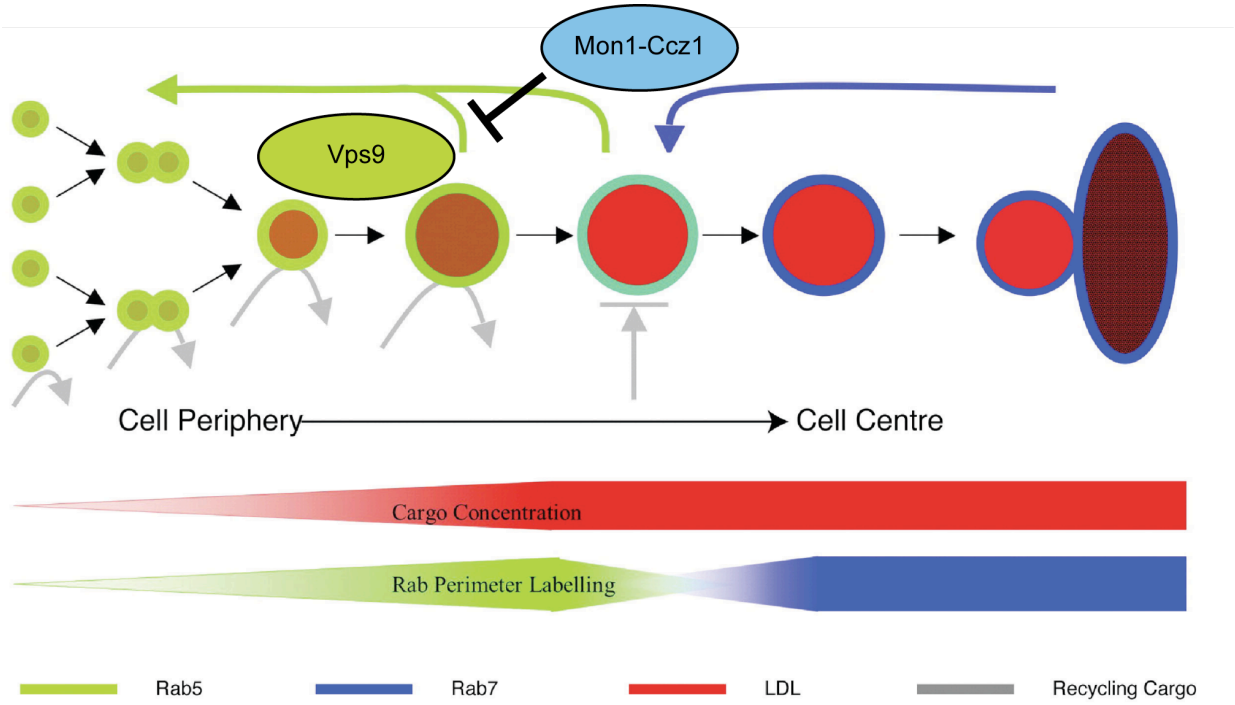

Figure 1.3 Mammalian endosomal Rab conversion model. Rab5 associates with early endosomes and directs fusion of incoming vesicles. The GEF Vps9 recruits and activates Rab5 at early endosomes. As an endosomal compartment matures cargoes, such as LDL, become more concentrated and at a certain point Rab5 is rapidly replaced by Rab7. This transition is thought to be triggered by Mon1-Ccz1, which displaces Vps9, leading to displacement of Rab5. Mon1-Ccz1 is also a GEF for Rab7 and promotes Rab7 recruitment and activation at late endosomes. Adapted from Rink et. al. 2005

In the vesicle transport model, vesicles or larger membrane domains could bud off an early endosome. Rabs have been shown to play a role in vesicle budding by recruiting coat proteins, and endosomal transport could occur through a similar mechanism. The compartment maturation model requires removal of the early Rab, followed by recruitment of the downstream Rab. Removal of the upstream Rab could occur through recruitment of that Rab's GAP, which would catalyze GTP hydrolysis and promote removal by GDI. There is evidence for this mechanism in Golgi transport where the Rab Ypt31 recruits Gyp1 which is a GAP for the upstream Rab, Ypt1 (Rivera-Molina & Novick 2009). Removal of an upstream Rab in such a Rab cascade could also occur through displacement of that Rab's GEF. In *C. elegans,* SAND-1 promotes endosomal Rab conversion by displacing RABEX-5, leading to displacement of Rab5 (Poteryaev et al. 2010). In addition, SAND-1 triggers endosome maturation by promoting recruitment of Rab7 (Poteryaev et al. 2007). The yeast homolog of SAND-1, Mon1, binds Ccz1 and is the GEF for Ypt7. The role of Mon1-Ccz1 in terminating Vps21 activity or displacing Vps9 in yeast is largely unaddressed.

Sorting into multivesicular bodies

One function of the endocytic pathway is the degradation of transmembrane proteins endocytosed from the plasma membrane. Many cell surface receptors, such as the EGF receptor in mammalian cells, must be degraded to terminate signaling. In addition, resident transmembrane proteins on the cell surface can accumulate damage and need to be removed and degraded. Simply transporting these proteins to the lysosome would result in their delivery to the limiting membrane of the compartment, which is protected from degradation. Work on the EGF receptor indicated that eukaryotic cells have circumvented this problem by directing these proteins at late endosome into intralumenal vesicles (ILVs) that arise from inward budding of the limiting endosomal membrane (Felder et al. 1990, Figure 1.4). The resulting late endosomes that are filled with ILVs are referred to as multivesicular bodies (MVBs) and fuse with lysosomes to allow degradation of ILVs and their transmembrane protein cargo (Futter et al. 1996).

A subset of the original *VPS* genes identified in *S. cerevisiae* accumulated vacuole cargo at an aberrant perivacuolar compartment termed the “class E compartment” (Raymond et al. 1992). Later work showed that both endocytic and biosynthetic cargo accumulated at this compartment, which electron microscopy revealed is composed of flattened stacks of cisternae (Rieder & Emr 1997). CPS is a vacuole protease that is synthesized as a transmembrane protein but then solubilized by protease cleavage after delivery to the vacuole. Deletion of class E genes causes CPS to accumulate at the class E compartment as well the vacuole perimeter, indicating

that these genes are required for sorting transmembrane proteins into the ILVs of MVBs (Odorizzi et al. 1998).

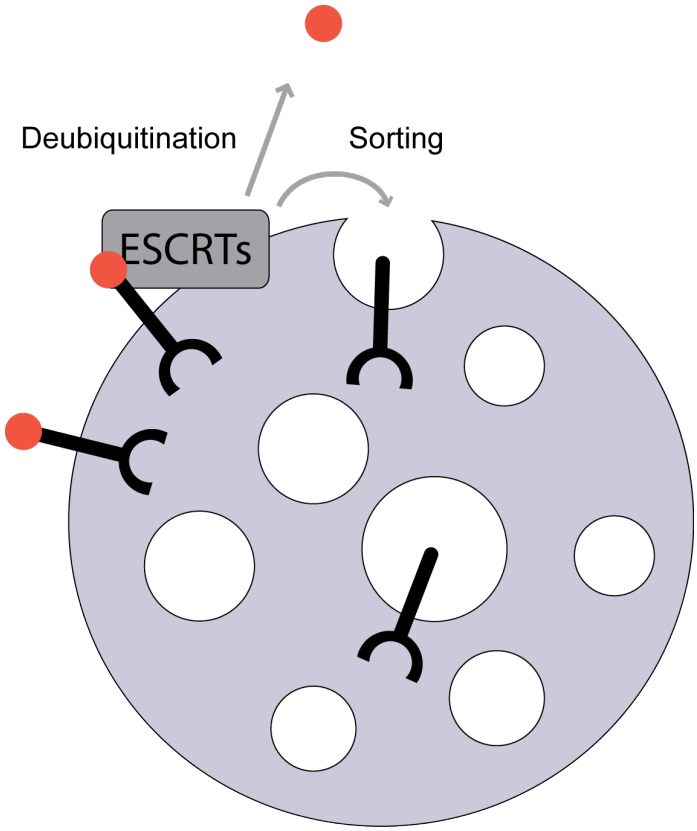

Figure 1.4 ESCRT-mediated sorting. At the endosomal membrane, ESCRT complexes sort ubiquitinated transmembrane proteins into ILVs. Ubiquitin acts as a sorting signal and is removed prior to incorporation into ILVs.

Further genetic and biochemical analysis has shown the proteins encoded by the class E genes are organized into a series of complexes termed Endosomal Sorting Complexes Required for Transport (ESCRTs) (Hanson & Cashikar 2012). These complexes, which are conserved throughout eukaryotes, act to sort cargo as well as generate the ILVs. ESCRTs 0, I and II form stable complexes that act to sequester cargo. More recent work has indicated that they might also play a role in initiating inward bending of the endosomal membrane (Wollert & Hurley 2010). ESCRT III is thought to form a transient high molecular weight polymer that generates the inward budding of membrane and pinches it away from the limiting membrane (Wollert et al. 2009, Saksena et al. 2009). The AAA ATPase, Vps4, oligomerizes into a dodecameric ring that disassembles the ESCRT III polymer to dissociate its subunits from the endosomal membrane (Babst et al. 1998). It is unclear why the ESCRT III polymer needs to be disassembled but loss of Vps4 activity inhibits ILV formation and causes the formation of class E compartments. None of the ESCRTs are incorporated into the ILVs, so the mechanism of budding and scission is still not clear.

Proteins are directed into ILVs by the reversible covalent attachment of ubiquitin to their cytosolic side (Katzmann et al. 2001). In yeast, the E3 ligase, Rsp5, ubiquitinates ILV cargoes to target them for incorporation into ILVs (Katzmann et al. 2004). Cargoes are then deubiquitinated by the deubiquitinating enzyme, Doa4, prior to their incorporation into ILVs, (Dupré & Haguenauer-Tsapis 2001) which seems to be important for maintenance of cellular ubiquitin because loss of Doa4 catalytic activity leads to reduction in total levels of cellular ubiquitin (Swaminathan et al. 1999).

Focus of this work: Coordination of Transport and Sorting

The processes of MVB sorting and endosome maturation must be coordinated to ensure cargoes efficiently reach the proper destination. For example, a transmembrane protein cargo must be sorted into at MVBs into ILVs prior to fusion of MVBs with the vacuole to ensure the cargo is not directed to the vacuole perimeter.

Chapter 2 we shows that deletion of ESCRT genes leads to a failure in endosome Rab conversion, where Vps21 is chronically active and Ypt7 localizes to endosomes but is not activated. Mon1-Ccz1 fails to localize to endosomes in ESCRT mutant cells indicating a potential mechanism for this disruption in endosome maturation. In Chapter 3, I present evidence indicating Vps9 GEF activity is driving Vps21 hyperactivation in ESCRT-mutant cells, and the failure to sort and deubiquitinate cargo in ESCRT mutants leads to an accumulation of ubiquitin on the endosomal membrane. I propose a model where this ubiquitin promotes prolonged binding of Vps9 at endosomes and relieves its auto-inhibition leading to Vps21 hyperactivation.

The focus of Chapter 4 is to understand if Mon1-Ccz1 has a direct role in promoting endosome maturation by displacing Vps9 from endosomes, as it does in *C. elegans*. In support of this hypothesis I find that loss of Mon1 causes an accumulation of Vps9 and Vps21 on endosomes as well as endosome enlargement. Surprisingly, I find that loss of other vacuole fusion factors, including the HOPS complex, also leads to accumulation of Vps9 and enlargement of endosomes. The data suggest that there is likely a direct role for Mon1 in displacement of Vps9 but that other downstream factors also contribute.

Chapter 2. Class E compartments form in response to ESCRT dysfunction in yeast due to hyperactivity of the Vps21 Rab GTPase

This chapter contains a manuscript that was published in the Journal of Cell Science in November 1, 2012. This work shows that the aberrant flattened stacked membranes that form when ESCRT genes are deleted are the result of a failure of endosomal conversion. The Rab5 homolog, Vps21 is chronically hyperactive and there is a failure to activate the Rab7 homolog, Ypt7. My primary contribution to this work is Figure 6E showing that the endosome conversion factor Mon1-Ccz1 is not recruited to endosomes. Mon1-Ccz1 is believed to promote endosome maturation by displacing Vps9, the GEF for Vps21, and activating Ypt7. Therefore this result provides a potential mechanistic basis for the failure of rab conversion seen upon deletion of ESCRT genes.

As referenced in the discussion, I conducted additional experiments to test this model, including overexpression of hyperactive Ypt7 (*ypt7*^{Q68L}), but these results were not included in the final manuscript. In addition I contributed to discussions and manuscript preparation.

Class E compartments form in response to ESCRT dysfunction in yeast due to hyperactivity of the Vps21 Rab GTPase

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Summary

The endosomal sorting complexes required for transport (ESCRTs) mediate the budding of intralumenal vesicles (ILVs) at late endosomes. ESCRT dysfunction causes drastic changes in endosome morphology, which are manifested in *Saccharomyces cerevisiae* by the formation of aberrant endosomes known as class E compartments. Except for the absence of ILVs, the mechanistic basis for class E compartment biogenesis is unknown. We used electron microscopy to examine endosomal morphology in response to transient ESCRT inactivation and recovery in yeast expressing the temperature-sensitive mutant *vps4^{ts}* allele. Our results show class E compartments accumulate fourfold the amount of membrane normally present at multivesicular bodies and that multivesicular bodies can form directly from class E compartments upon recovery of ESCRT function. We found class E compartment formation requires Vps21, which is orthologous to the Rab5A GTPase in metazoans that promotes fusion of endocytic vesicles with early endosomes and homotypic fusion of early endosomes with one another. We also determined that class E compartments accumulate GTP-bound Vps21 and its effector, the class C core vacuole/endosome tethering (CORVET). Ypt7, the yeast ortholog of Rab7 that in metazoans promotes fusion of late endosomes with lysosomes, also accumulates at class E compartments but without its effector, the homotypic fusion and protein sorting (HOPS), signifying that Ypt7 at class E compartments is dysfunctional. These results suggest that failure to complete Rab5–Rab7 conversion is a consequence of ESCRT dysfunction, which results in Vps21 hyperactivity that drives the class E compartment morphology. Indeed, genetic disruption of Rab conversion without ESCRT dysfunction autonomously drives the class E compartment morphology without blocking ILV budding.

Key words: ESCRT, Endosome, Rab

Introduction

As early endosomes mature into late endosomes, transmembrane proteins targeted for lysosomal degradation are sorted into intralumenal vesicles (ILVs) by the endosomal sorting complexes required for transport (ESCRTs). ILVs are delivered into the hydrolytic interior of the lysosome upon endolysosomal fusion (Piper and Katzmann, 2007). In *Saccharomyces cerevisiae*, ESCRT dysfunction not only blocks ILV budding but also drastically alters endosomal morphology to result in class E compartments (Raymond et al., 1992; Rieder et al., 1996). These aberrant stacks of flattened endosomal cisternae are uniformly observed in yeast in response to ESCRT dysfunction, but the mechanistic basis for their formation has been a mystery. Determining the underlying cause of class E compartment biogenesis might reveal cellular activities not previously known to be functionally linked to ESCRTs.

siRNA-mediated knockdown of most ESCRTs in human cells causes enlarged swollen endosomes, although class E compartments like those in yeast have been observed (Bache et al., 2003; Doyotte et al., 2005; Razi and Futter, 2006). Swollen endosomes are similarly induced upon overexpression of a dominant-active Rab5A GTPase (Stenmark et al., 1994; Wegner et al., 2010). Rab5A regulates early endosomal fusion activity (Gorvel et al., 1991; Rubino et al., 2000) and is replaced during the course of endosomal maturation by Rab7 (Rink et al., 2005), which regulates fusion of late endosomes with lysosomes (Bucci et al., 2000; Vanlandingham and Ceresa, 2009). Rab5–Rab7 conversion is thought to guide endosomal maturation (Rink et al., 2005; Poteryaev et al., 2010), but its mechanistic basis is poorly understood. In *Caenorhabditis elegans*, Rab conversion requires SAND-1, which promotes endosomal dissociation of the guanonucleotide exchange factor (GEF) that activates Rab5A (Poteryaev et al., 2010). The yeast SAND-1 ortholog, Mon1, is also thought to promote Rab conversion by associating with Ccz1 to form a GEF complex that activates the Rab7 ortholog, Ypt7 (Nordmann et al., 2010).

We show that class E compartments accumulate chronically active Vps21, the yeast Rab5A ortholog, and dysfunctional Ypt7, the yeast Rab7 ortholog. ESCRT dysfunction, therefore, inhibits the completion of Rab5–Rab7 conversion. Hyperactive Vps21 resulting from ESCRT dysfunction causes unregulated endosomal membrane accumulation, which drives the class E compartment morphology. Chronic endosomal localization of Vps9, the GEF that activates Vps21, coupled with impaired endosomal localization of the Mon1–Ccz1 GEF complex that activates Ypt7

Journal of Cell Science

(Nordmann et al., 2010), suggests a mechanistic basis for defective Rab conversion in ESCRT-mutant cells. We propose completion of Rab5–Rab7 conversion at endosomes requires ESCRT activity to ensure transmembrane proteins targeted for degradation are sequestered away from the perimeter endosomal membrane before endolysosomal fusion occurs.

Results

Membrane accumulation at class E compartments

Vps4 ATPase activity sustains ESCRT function by catalyzing disassembly of ESCRT-III (Wollert et al., 2009). In yeast, prolonged inactivation of a temperature-sensitive mutant *vps4* allele (*vps4^{ts}*) results in class E compartments (Babst et al., 1997), but intermediate stages of class E compartment formation in *vps4^{ts}* cells have not been investigated. Therefore, we explored class E compartment biogenesis by electron microscopy (EM) during a time-course analysis of *vps4^{ts}* cells shifted between permissive (26℃) and non-permissive (38℃) temperatures (Fig. 1A).

Endosomes in *vps4^{ts}* cells maintained continuously at 26℃ (Fig. 1B) had a normal multivesicular body (MVB) morphology indistinguishable from wild-type yeast (Nickerson et al., 2006). However, the number of ILVs sharply decreased within 10 minutes at 38℃, coincident with a steep rise in non-spherical/flattened endosomes (Fig. 1C; Fig. 2A,B). More extensive endosomal flattening and stacking was observed later, resulting in a typical class E compartment morphology after 70 minutes of *vps4^{ts}* inactivation (Fig. 1D,E; Fig. 2B). Within 10 minutes of returning *vps4^{ts}* cells from 38℃ to 26℃, ILV biogenesis had resumed, and MVBs were abundant after 30 minutes (Fig. 1F,G; Fig. 2C). After 70 minutes, MVBs with normal appearance predominated (Fig. 2C,D; and data not shown). Significantly, ILVs were seen both in stacked cisternae and unstacked flattened endosomes (Fig. 1F,G, white arrowheads). The ILV-containing portions of cisternae were enlarged (Fig. 1F,G, black arrowheads), and cisternae were accompanied by loosely associated MVBs (Fig. 1G), suggesting that many of the MVBs observed during the recovery period derived directly from class E compartments that had formed during *vps4^{ts}* inactivation. Indeed, class E compartments are probably a source of MVBs for a significant period after *vps4^{ts}* reactivation because many stacked cisternae and loosely associated endosomes remained at 30 minutes (Fig. 1G; Fig. 2D), with some even observed at 70 minutes (Fig. 1H; Fig. 2D).

We also examined *vps4^{ts}* cells subjected to a similar temperature-shift protocol by fluorescence microscopy to quantify class E compartment puncta at which the lipid marker, FM 4–64, colocalizes with GFP fused to Sna3, an ILV cargo (Fig. 2F–I). Sna3–GFP is transported from the Golgi to endosomes and subsequently delivered into the lumen of the vacuole (lysosome) in wild-type cells, but it exclusively localizes

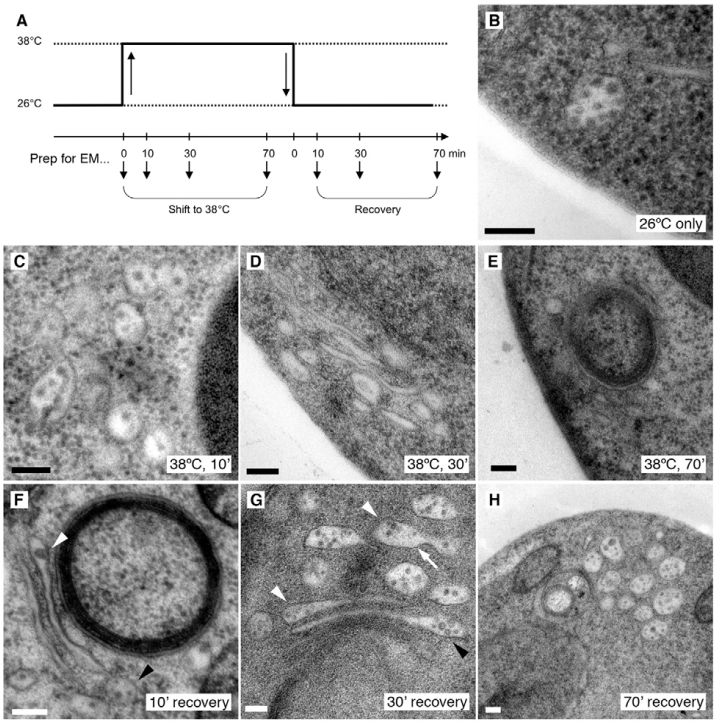


Fig. 1. Formation of class E compartments and restoration of MVBs in temperature-shifted *vps4^{ts}* cells. (**A**) Temperature shift protocol. (**B–H**) Electron micrographs of *vps4^{ts}* cells that were maintained at 26℃ (B), shifted from 26℃ to 38℃ for the indicated times (C–E), or shifted from 26℃ to 38℃ for 70 minutes and returned to 26℃ for the indicated times (F–H). In F and G white arrowheads indicate ILV budding in flattened stacked class E compartment cisternae and unstacked flattened endosomes; black arrowheads indicate enlarged ILV-containing regions of class E compartment cisternae. In G the dumbbell-shaped MVB profile is indicated by an arrow. Scale bars: 100 nm.

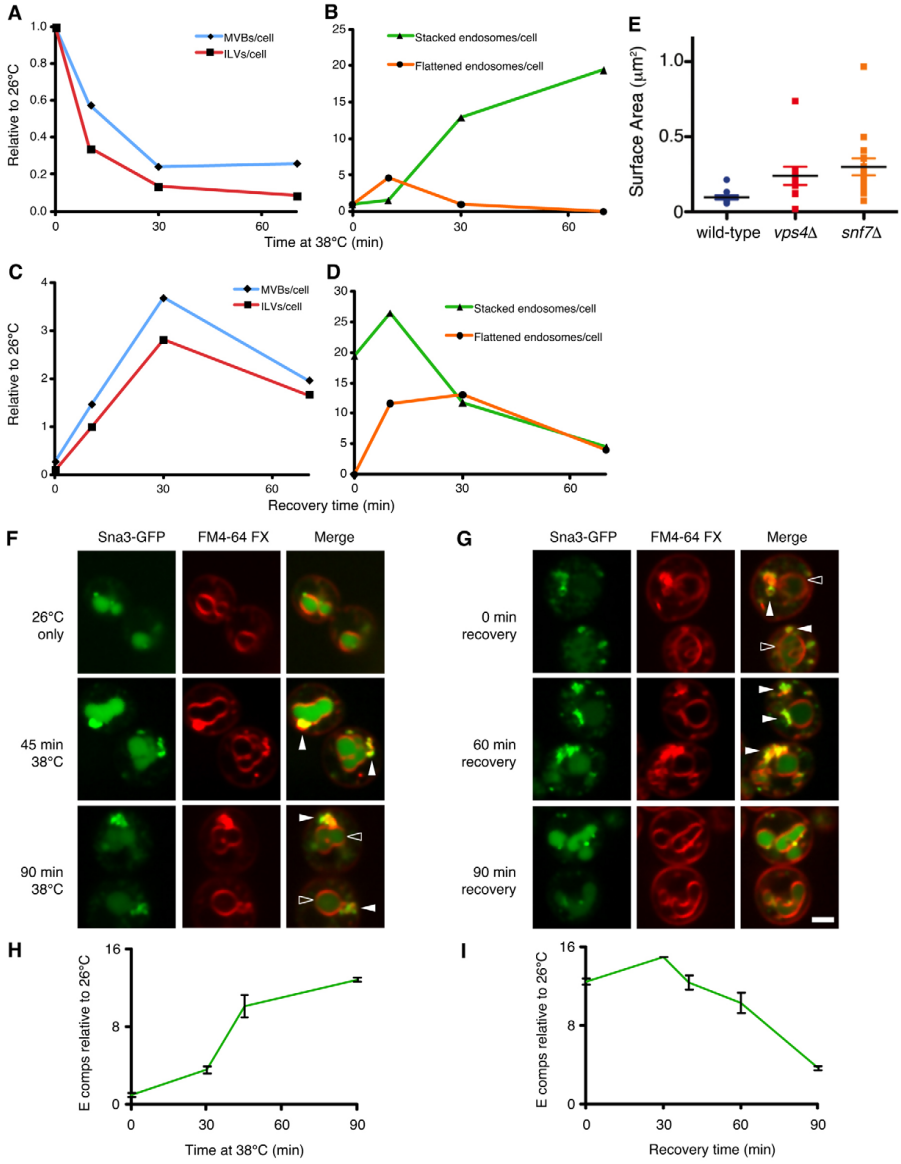


Fig. 2. Membrane accumulation at class E compartments. (**A–D**) Quantification of endosome morphologies in the temperature-shifted *vps4*^{ts} cells from Fig. 1 (frequency relative to *vps4*^{ts} cells maintained at 26℃, n=50–300 cell profiles). (**E**) Mean membrane surface areas of MVBs (including ILVs) relative to individual class E compartment cisternae in electron tomograms of wild-type cells (0.096±0.013 μm^2, N=11), *vps4Δ* cells (0.240±0.061 μm^2, N=10, P<0.05) and *snf7Δ* cells (0.300±0.0561 μm^2, N=15, P<0.01). (**F–I**) Quantification of class E compartment biogenesis using confocal fluorescence microscopy. Class E compartments were labeled with FM 4-64FX, a fixable FM 4-64 analog. (F,G) Fluorescence micrographs of Sna3–GFP and FM 4-64FX in temperature-shifted *vps4*^{ts} cells. The indicated times include a 30-minute fixation step. Closed arrowheads, class E compartments marked by both fluorophores; open arrowheads, vacuoles depleted of Sna3–GFP. Scale bar: 2 μm. Images are representative of two independent experiments. (F) Cells shifted to 38℃ for the indicated times. (G) Cells shifted to 38℃ for 70 minutes and returned to 26℃ for the indicated times. (H,I) Quantification of class E compartments marked by both Sna3–GFP and FM 4-64FX in the experiment shown in F and G [mean frequency (± s.e.m.) relative to cells maintained at 26℃, n=140–220 cells over two independent experiments].

to class E compartments in response to ESCRT dysfunction (Reggiori and Pelham, 2001). The results from this time-course fluorescence analysis support the kinetics of class E compartment formation and recovery of MVB biogenesis that we determined by EM (Fig. 2A–D) and are consistent with previous biochemical analyses showing that endosomal cargoes trapped at class E compartments are delivered to vacuoles after a delay upon reactivation of *vps4*^{ts} (Babst et al., 1997).

Our EM observation that ILVs and MVBs are formed immediately after reactivation of *vps4*^{ts} indicates that the resumption in vacuolar cargo delivery is likely due to class E compartments recovering normal endosomal function and morphology prior to late endosome–vacuole fusion rather than direct fusion of class E compartments with vacuoles. Indeed, no structures suggestive of class E compartment–vacuole fusion intermediates were observed by EM after reactivation of *vps4*^{ts}. Surprisingly, 30 minutes of *vps4*^{ts} recovery yielded a transient threefold increase in the number of ILVs and a fourfold increase in the abundance of MVBs relative to 26℃ (Fig. 2C), suggesting that a significant amount of membrane accumulates at class E compartments. Membrane quantification by electron tomography revealed each class E compartment cisterna in *vps4Δ* cells averaged ~2.5-fold more membrane than the sum of ILVs and perimeter membrane at a typical wild-type MVB (Fig. 2E). Similar results were found in *snf7Δ*, an ESCRT-III-mutant strain (Fig. 2E). A class E compartment stack typically has three to six cisternae, but ESCRT-mutant cells have fewer class E compartment stacks compared to the number of MVBs in wild-type yeast (Fig. 3F). Nonetheless, class E compartment cisternae in *vps4Δ* cells still outnumbered MVBs in wild-type cells by 1.5 fold (0.56 versus 0.36 per cell section, respectively), which, when multiplied by the 2.5-fold membrane excess at each cisterna, indicates the combined membrane content of class E compartments in an ESCRT-mutant cell is ~4-fold the total membrane represented by all MVBs in a wild-type cell.

The Vps21 Rab5 GTPase is required for biogenesis of class E compartments but not MVBs

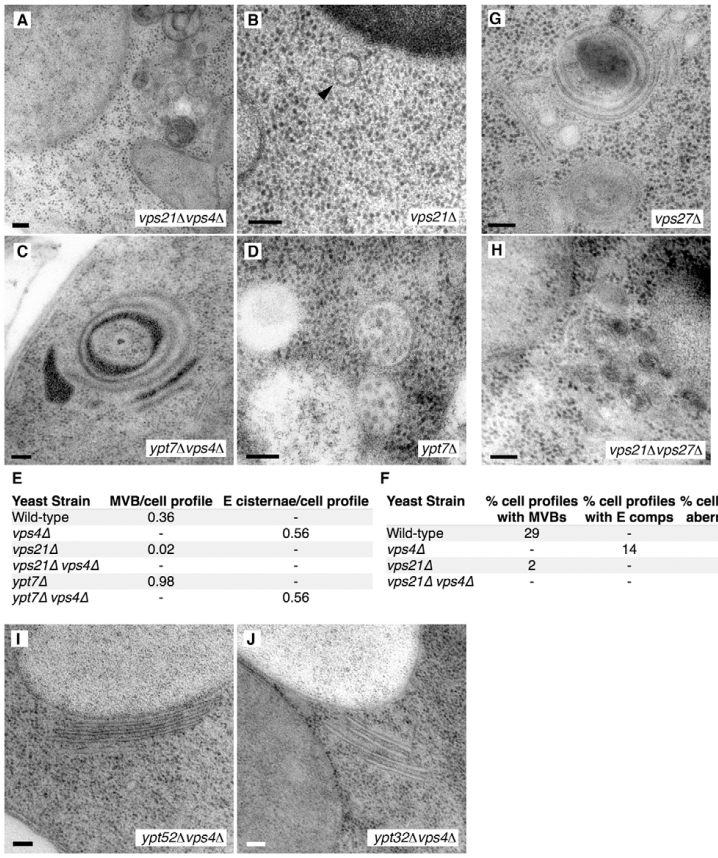
The fourfold membrane excess at class E compartments cannot solely result from the lack of ILVs, which account for only 50% of total MVB membrane (Wemmer et al., 2011). Some accumulation might stem from defective membrane retrieval (Raymond et al., 1992; Piper et al., 1995), but the burst of MVB biogenesis directly from class E compartments upon *vps4*^{ts} recovery indicated much of the accumulated membrane is en route to vacuoles. Indeed, the amount of excess membrane at class E compartment cisternae (Fig. 2E) implies each can give rise to two or more MVBs, as suggested by the dumbbell-shaped profiles of non-spherical MVBs seen upon *vps4*^{ts} recovery (Fig. 1G, arrow). Therefore, we investigated if unregulated membrane fusion at endosomes has a role in class E compartment biogenesis.

Membrane fusion is regulated by Rab GTPases, which promote membrane tethering and subsequent assembly of trans-SNARE complexes (reviewed by Stenmark, 2009). In animals, Rab5A promotes fusion of endocytic vesicles with early endosomes and homotypic fusion of early endosomes with one another (Gorvel et al., 1991; Rubino et al., 2000), while Rab7 promotes fusion of late endosomes with lysosomes (Bucci et al., 2000; Vanlandingham and Ceresa, 2009). Among three Rab5 paralogs in yeast, Vps21 is the functional ortholog of Rab5A (Singer-Krüger et al., 1995), whereas Ypt7 is the only yeast Rab7 ortholog.

The flattened stacks of closely apposed endosomal cisternae characteristic of class E compartments no longer formed upon deletion of *VPS21* in either *vps4Δ* cells (Fig. 3A,E) or in *vps27Δ*, an ESCRT-0-mutant strain (Fig. 3G,H). Suppression of class E compartment biogenesis was specific to the loss of Vps21 Rab5 activity because class E compartments still occurred in *vps4Δ* cells despite deletion of *YPT52* (Fig. 3I), the only other Rab5 paralog expressed in vegetative yeast (MacKay et al., 2004). Class E compartment biogenesis also was unaffected by deleting *YPT32* (Fig. 3J), a Rab that regulates endosome-to-Golgi trafficking (Sciorra et al., 2005; Buvelot Frei et al., 2006). Thus, the enlargement of endosomes into flattened, closely apposed cisternae is dependent on Vps21. However, aberrant endosomal morphology remains in ESCRT mutants lacking Vps21 because class E compartments were replaced by loose clusters of abnormal vesicles ~100 nm in diameter that lacked ILVs (Fig. 3A,H,F).

Although deletion of *VPS21* suppressed class E compartment formation in *vps4Δ* and *vps27Δ* cells, it did not restore MVB biogenesis (Fig. 3A,H; see below). Previous studies suggested Vps21 regulates fusion of Golgi-derived transport vesicles with early endosomes (Horazdovsky et al., 1994; Peterson et al., 1999; Tall et al., 1999), and a block in this fusion step could thus indirectly suppress class E compartment formation. However, we still observed MVBs in *vps21Δ* cells at low frequency (Fig. 3B,E), suggesting that the loss of Vps21 function either reduced MVB biogenesis or accelerated membrane flux through the late endosome-to-vacuole pathway in some manner. That endosomes remain functional with respect to MVB biogenesis in the absence of Vps21 was further confirmed by our observation that GFP–Cps1 was correctly delivered into the vacuole lumen in *vps21Δ* cells. Like Sna3, Cps1 is transported from the Golgi to endosomes where it is sorted into ILVs, but unlike Sna3, Cps1 is mislocalized to both the class E compartments and the vacuole membrane upon ESCRT dysfunction (Odorizzi et al., 1998a). The sorting of GFP–Cps1 in *vps21Δ* cells was ESCRT-dependent because it was mislocalized to the vacuole perimeter membrane in *vps21Δ vps4Δ* cells, similar to its vacuolar localization in *vps4Δ* cells (Fig. 4). However, in *vps21Δ vps4Δ* cells, GFP–Cps1 no longer colocalized with FM 4-64 at large puncta characteristic of class E compartments (Fig. 4), consistent with loss of the characteristic class E compartment morphology (Fig. 3A,E). *VPS21* deletion did not cause GFP–Cps1 to bypass endosomes and reach vacuoles directly from the Golgi via the AP3-dependent pathway (Cowles et al., 1997) because deletion of the *APM3* gene required for AP3 function did not block vacuolar delivery of GFP–Cps1 either in *vps21Δ* cells or in *vps21Δ vps4Δ* cells (Fig. 4). Intact vacuoles in these cells support the view that delivery of membrane to this organelle does not absolutely depend on Vps21. However, when *VPS4* is deleted in addition to *VPS21*, endosomes are unable to support ILV biogenesis even though class E compartments do not form (Fig. 3A).

Unlike deletion of *VPS21*, deletion of *YPT7* in *vps4Δ* cells did not suppress class E compartment biogenesis [Fig. 3C,E; confirmation of class E compartments in this strain by fluorescence microscopy was not possible because of the severe fragmentation of vacuoles caused by *YPT7* deletion (Wichmann et al., 1992)]. The only obvious difference was that the lumen of class E compartment cisternae in *ypt7Δ vps4Δ* cells



E

Yeast Strain	MVB/cell profile	E cisternae/cell profile
Wild-type	0.36	-
vps4Δ	-	0.56
vps21Δ	0.02	-
vps21Δ vps4Δ	-	-
ypt7Δ	0.98	-
ypt7Δ vps4Δ	-	0.56

F

Yeast Strain	% cell profiles with MVBs	% cell profiles with E comps	% cell profiles with aberrant clusters
Wild-type	29	-	-
vps4Δ	-	14	-
vps21Δ	2	-	-
vps21Δ vps4Δ	-	-	14

Fig. 3. Vps21 is required for biogenesis of class E compartments but not MVBs. (**A–D**,**G–J**) Electron micrographs of the indicated strains. Scale bars: 100 nm. Arrowhead in B, MVB. (**E**) Quantification of MVBs and class E compartment cisternae represented as frequency of structure per cell profile, $n=50$. (**F**) Quantification of cell profiles containing MVBs, class E compartments, or aberrant vesicle clusters; $n=50–100$.

was occasionally stained more darkly for unknown reasons. Deletion of *YPT7* alone caused a threefold increase in the number of MVBs seen in wild-type yeast (Fig. 3D,E), consistent with Ypt7 mediating late endosome-vacuole fusion. Notably, MVBs in *ypt7Δ* cells were enlarged (mean diameter in wild type=131 nm, *ypt7Δ*=171 nm; $n=40$ for both), suggesting persistent membrane delivery through Vps21 function and/or reduced activity of retromer, which mediates recycling to the Golgi and requires Ypt7 to function (Balderhaar et al., 2010; Liu et al., 2012).

Vps21 is concentrated at class E compartments in its active GTP-bound state

Because of the requirement for Vps21 but not Ypt7 in class E compartment biogenesis, we compared the localization of GFP–Vps21 versus GFP–Ypt7. As seen previously in wild-type yeast, GFP–Vps21 displayed a punctate distribution, while GFP–Ypt7 localized predominantly to vacuole membranes; however, both Rabs were concentrated together with FM 4–64 at class E compartments in *vps4Δ* cells (Fig. 5A,B) and in *vps23Δ*, an ESCRT-I-mutant strain (data not shown). In contrast, GFP was not concentrated at class E compartments when fused to Ypt32 (Fig. 5C), the Rab that regulates retrograde transport from endosomes (Sciorra et al., 2005; Buvelot Frei et al., 2006).

We anticipated Vps21 would be concentrated at class E compartments based on its requirement for the formation of these aberrant structures. Although a similar concentration of Ypt7 had been observed previously (Balderhaar et al., 2010), our EM analysis indicated its activity was not required for class E compartment biogenesis (Fig. 3). To evaluate whether the concentration of Vps21 and Ypt7 at class E compartments correlated with each Rab being in the active GTP-bound state, we tested their sensitivity to recombinant GDP dissociation inhibitor (GDI). Upon hydrolyzing their bound GTP, membrane-associated Rabs return to the inactive GDP-bound state. GDI preferentially

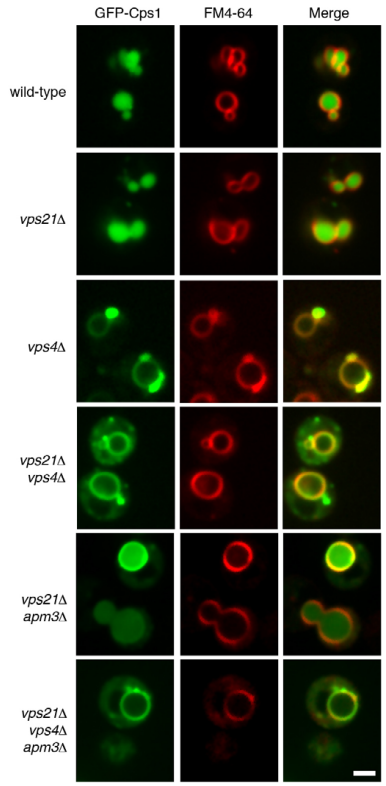


Fig. 4. Vps21 activity is not required for endosomal membrane trafficking. Confocal fluorescence micrographs of FM 4-64-stained cells expressing GFP–Cps1. Scale bar: 2 µm.

binds GDP-bound Rabs and extracts them from membranes, solubilizing Rabs and recycling them to the cytosol so that they can engage in subsequent rounds of membrane targeting and function (reviewed by Seabra and Wasmeier, 2004).

Compared to wild-type cells, *vps4Δ* cells contained more membrane-associated Vps21 (Fig. 5D, 12% and 20% of the total fraction recovered in the pellet fraction in wild-type and *vps4Δ* cells, respectively), and membrane-associated Vps21 in *vps4Δ* cells was more resistant to GDI extraction (Fig. 5E, 27% compared to 9% extracted in lanes 2 and 5, respectively). Importantly, the increased GDI resistance of Vps21 in response to *VPS4* deletion was not due to an indirect consequence of the Rab somehow being inaccessible to the addition of recombinant protein because the vast majority of Vps21 could be solubilized by GDI if membranes from *vps4Δ* cells were first incubated with the recombinant catalytic domain of Gyp1 ($Gyp1^{TBC}$), a GTPase-activating protein (GAP) shown *in vitro* to stimulate GTP hydrolysis by both Vps21 and Ypt7 (Albert et al., 1999) (Fig. 5D). The enhanced GDI resistance of Vps21 in *vps4Δ* cells mirrors results recently observed upon disruption of Msb3, a GAP specific for Vps21 (Nickerson et al., 2012), and signifies that Vps21 is predominantly at class E compartments in its active GTP-bound state upon ESCRT disruption.

Unlike Vps21, the proportion of Ypt7 that was susceptible to extraction by GDI was similar in wild-type and *vps4Δ* cells, even if membranes were first incubated with $Gyp1^{TBC}$ (Fig. 5E, 25% compared to 21% extracted in lanes 2 and 5, respectively). Thus, we did not detect a change in the nucleotide-binding state of Ypt7 upon *VPS4* deletion. However, this assay could not discriminate between Ypt7 localized to class E compartments versus the residual amount detected by fluorescence microscopy at vacuole membranes (Fig. 5B).

ESCRT disruption inhibits the completion of Rab5–Rab7 conversion

The results described above indicated that Vps21 is concentrated at class E compartments in its active GTP-bound state, but the activation status of Ypt7 concentrated at these aberrant structures was unclear. Therefore, we evaluated the activity of both Rabs by examining the localization of their corresponding effector proteins. Rab effectors bind selectively to active GTP-bound Rabs but not to inactive GDP-bound or nucleotide-free Rabs (reviewed by Stenmark, 2009). Vps21 and Ypt7 have multi-protein effector complexes known, respectively, as the class C core vacuole/endosome tethering (CORVET) (Peplowska et al., 2007) and the homotypic fusion and protein sorting (HOPS) (Seals et al., 2000) complexes. CORVET and HOPS share a common set of four core subunits (the Vps-C proteins: Vps11, Vps16, Vps18 and Vps33) that associate with two additional subunits specific either for Vps21 or Ypt7 (Fig. 6H). Like GFP–Vps21, a CORVET-specific effector, Vps8–GFP (Fig. 6A), was concentrated at class E compartments, as was GFP–Vps33, a core subunit shared by CORVET and HOPS (Fig. 6C). However, GFP–Vps41, a HOPS-specific effector (Fig. 6B), failed to localize to these aberrant structures, signifying that Ypt7 concentrated at this site is nonfunctional. These observations, therefore, suggest that ESCRT dysfunction inhibits the completion of Rab5–Rab7 conversion. Consistent with the suppression of class E compartment morphology by *VPS21* deletion (Fig. 3), Vps8–GFP no longer accumulated in *vps21Δ vps4Δ* cells (Fig. 6I).

Rabs are activated by GEFs, which trigger the release of GDP so that Rabs can bind GTP (Stenmark, 2009). Based on the concentration of GTP-bound Vps21 and CORVET at class E compartments, we anticipated that the Vps21 GEF, Vps9, would similarly be localized at these aberrant structures, where it could drive Vps21 activation. Indeed, Vps9–GFP was aberrantly concentrated at class E compartments in *vps4Δ* cells (Fig. 6D). In contrast, we found that ESCRT dysfunction caused the opposite response for GFP fused to Ccz1, which functions together with Mon1 as the GEF complex that activates Ypt7 (Nordmann et al., 2010). In wild-type yeast, Ccz1–GFP localized to puncta adjacent to vacuoles (Fig. 6E) (Nordmann et al., 2010), but it was not concentrated at class E compartments in *vps4Δ* cells (Fig. 6E). The lack of Ccz1–GFP at class E compartments was not due to defective Mon1–Ccz1 complex formation (Fig. 6F), nor was it due to aberrant proteolytic cleavage of the GFP moiety because western blot analysis of total cell extracts showed no difference in the abundance of Ccz1–GFP between wild-type and *vps4Δ* cells (Fig. 6G). However, Ccz1–GFP exhibited markedly lower expression in wild-type and *vps4Δ*

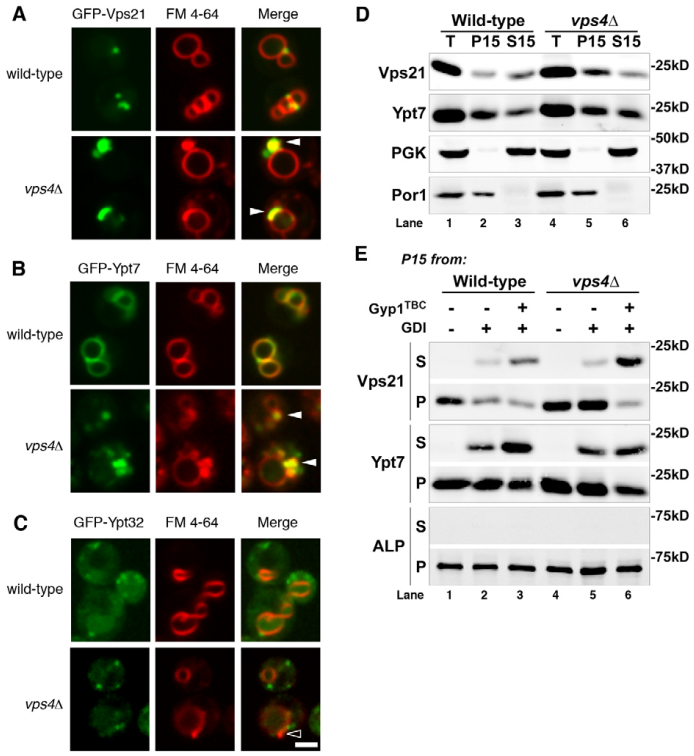


Fig. 5. Vps21 is concentrated at class E compartments in its active GTP-bound state. (**A–C**) Confocal fluorescence micrographs of FM 4-64-stained cells expressing the indicated GFP fusion proteins. Scale bar: 2 μm. Arrowheads, colocalization with FM 4-64-stained class E compartments. (**D**) Subcellular fractionation of wild-type and *vps4Δ* cells. PGK1 and mitoporin (Por1) were used as fiducial markers for cytosol and membrane fractions. T, total lysate after 1000 ***g*** spin; P15, membrane-associated 15,000 ***g*** pellet fraction; S15, cytosolic 15,000 ***g*** soluble fraction. (**E**) Extraction of Rab GTPases Vps21 and Ypt7 by RabGDI. P15 membrane pellets prepared from wild-type or *vps4Δ* cells were resuspended in lysis buffer or buffer supplemented with 3 μM $Gyp1^{TBC}$, and incubated for 15 minutes on ice. Indicated samples received 9 μM recombinant GDI immediately prior to another 15,000 ***g*** spin in which all samples were again separated to membrane-bound (P) and soluble fractions (S). Alkaline phosphatase (ALP) served as a fiducial marker for sedimentation of membranes.

cells compared to Vps9–GFP (Fig. 6G, expression of each GFP fusion was driven by the endogenous promoters for *CCZ1* and *VPS9*, respectively), which presumably explains why redistribution of Ccz1–GFP elsewhere within *vps4Δ* cells is not noticeable (Fig. 6E).

Genetic disruption of Rab conversion can autonomously induce class E compartment biogenesis without ESCRT dysfunction

Having found that class E compartment biogenesis requires Vps21 and that Vps21 is concentrated in its active GTP-bound state along with its CORVET effector at class E compartments, we investigated whether genetically driving chronic Vps21 activity could induce class E compartment biogenesis without mutation of ESCRT genes. The $vps21^{Q66L}$ allele encodes a mutant version of Vps21 that has crippled nucleotide hydrolysis activity and is consequently locked in its active GTP-bound state (Tall et al., 1999). However, overexpression of $vps21^{Q66L}$ in wild-type yeast resulted in clusters of enlarged MVBs (Fig. 7A) but not class E compartments, suggesting that impaired Ypt7 function might also be required for the formation of class E compartments. Indeed, we found deletion of *YPT7* enabled $vps21^{Q66L}$ overexpression to induce flattened, stacked endosomes (Fig. 7B) like those seen during transient inactivation and recovery of $vps4^{ts}$ (Fig. 1). The morphological identification of these class E compartment-like structures as being endosomal in origin was unequivocal because they contained ILVs, and their similarity to bona fide class E compartments in ESCRT-mutant strains was further evident in serial sections, which showed that the stacked endosomal membranes are flattened cisternae, not tubules (Fig. 7B–D). The presence of ILVs in the class E compartment-like structures also demonstrated that ESCRTs are functional in this context with respect to ILV budding. While unbiased quantification showed that class E compartment-like structures were infrequent (Fig. 7E), they were also observed in an independent strain in which the chromosomal *VPS21* locus had been deleted (data not shown). The ability of $vps21^{Q66L}$ overexpression in *ypt7Δ* cells to achieve 14% penetrance of the class E compartment phenotype seen upon ESCRT dysfunction (Fig. 7E) (Rieder et al., 1996) must be considered significant because the effects of a functional ESCRT machinery would need to be overcome.

Discussion

Deletions of ESCRT genes in yeast have long been known to cause the formation of class E compartments (Raymond et al., 1992; Rieder et al., 1996). However, the mechanistic basis for class E compartment biogenesis was unknown. Our results show that the formation of class E compartments is driven by Vps21 hyperactivity coupled with dysfunctional Ypt7 at endosomes.

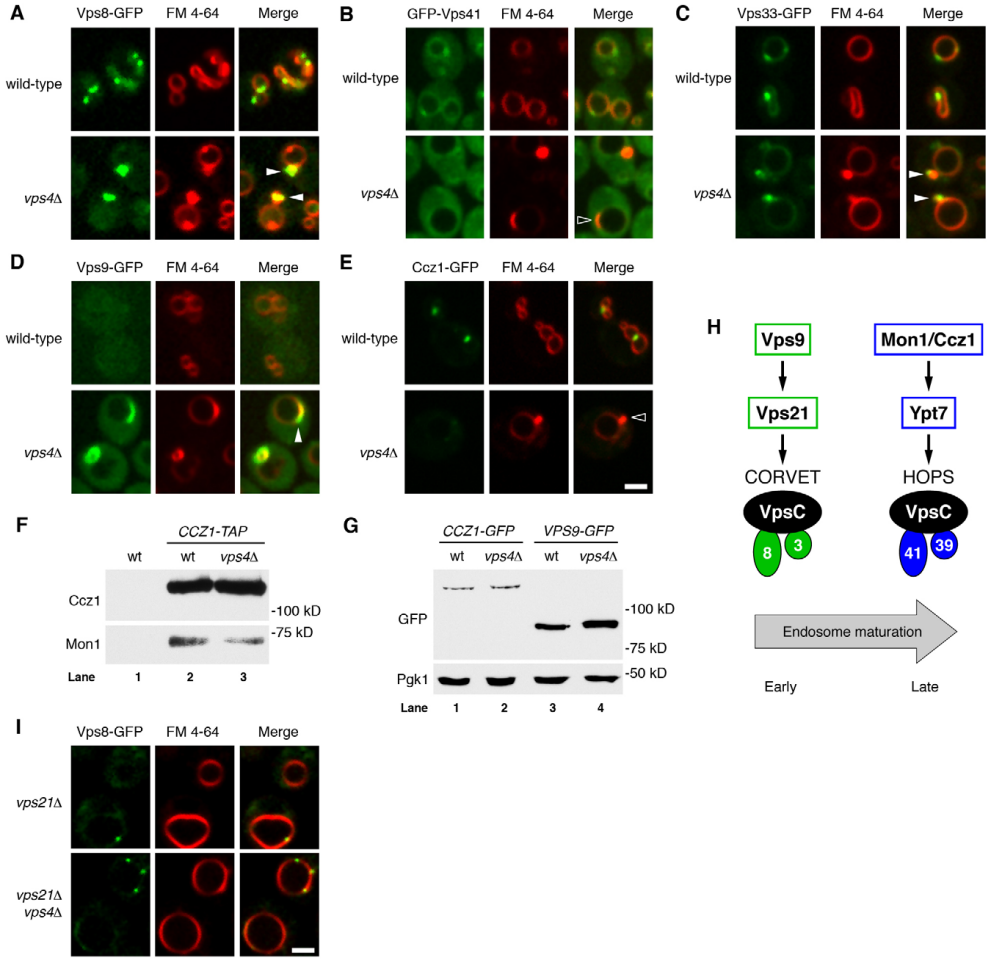


Fig. 6. ESCRT disruption inhibits the completion of Rab5–Rab7 conversion at endosomes. (**A–E**,**I**) Confocal fluorescence micrographs of FM 4-64-stained cells expressing the indicated GFP fusion proteins. Scale bars: 2 µm. Closed arrowheads, colocalization with FM 4-64-stained class E compartments; open arrowheads, FM 4-64-stained class E compartment without GFP colocalization. In E, identical exposure settings were used for GFP in wild-type and *vps4Δ*. Exposure settings for I were identical to those for A, and strains used for these panels all contained chromosomal integrations of Vps8–GFP controlled by the endogenous *VPS8* promoter. (**F**) Western blot using antibodies against Ccz1 or Mon1 to detect proteins from total lysates that were bound to IgG Sepharose. The *PEP4* gene was deleted in each strain to inactivate vacuolar proteases prior to lysis. (**G**) Western blot analysis of total cell extracts from strains used for D and E. PGK1 was used as loading control. (**H**) Diagram of Vps21 and Ypt7 GEFs and effector complexes.

Vps21 is the yeast ortholog of Rab5A in metazoans (Singer-Krüger et al., 1995), which regulates early endosome fusion events (Gorvel et al., 1991; Bucci et al., 1992; Rubino et al., 2000). Ypt7 is the yeast ortholog of Rab7 in metazoans (Bucci et al., 2000; Vanlandingham and Ceresa, 2009), which replaces Rab5A at endosomal membranes, thereby marking the maturation of early endosomes into late endosomes that can consequently fuse with lysosomes (Rink et al., 2005; Poteryaev et al., 2010). We propose the completion of Rab5–Rab7 conversion requires ESCRT activity to ensure transmembrane proteins targeted for degradation are sequestered away from the perimeter endosomal membrane before endolysosomal fusion occurs.

Chronic Vps21 activity resulting from ESCRT dysfunction was indicated by the aberrant concentration at class E compartments of Vps21 in its active GTP-bound state together with its CORVET effector. Although we could not detect a change in the nucleotide-binding state of Ypt7 in response to

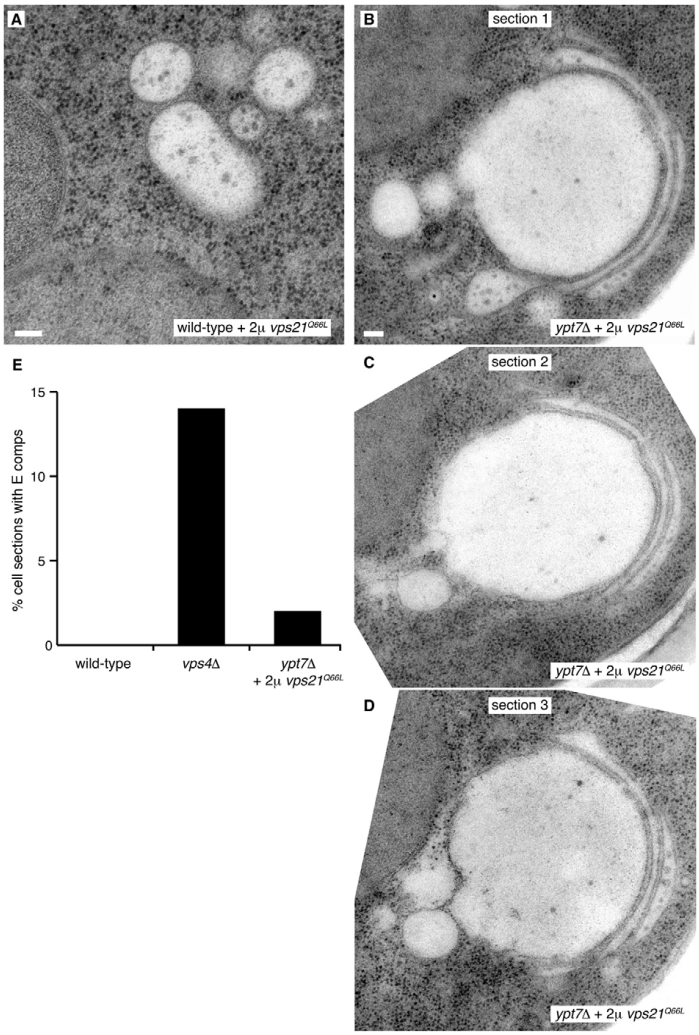


Fig. 7. Class E compartment biogenesis without ESCRT dysfunction. (**A–D**) Electron micrographs of the indicated strains transformed with high-copy (2μ) *vps21*^{Q66L}. (B–D) Consecutive 75-nm serial sections. Scale bars: 100 nm. (**E**) Percentage of cell profiles containing class E compartments; n=100–300 cells.

ESCRT disruption, the absence of its HOPS effector at class E compartments signifies Ypt7 concentrated at these structures was dysfunctional. Vps21 hyperactivity together with the loss of Ypt7 function is a driving force in class E compartment biogenesis because this signature morphological aberration in ESCRT-mutant cells was suppressed by disruption of Vps21 whereas overexpression of dominant-active Vps21 coupled with disruption of Ypt7 induced the class E compartment morphology independently of ESCRT dysfunction. Consistent with our findings are results showing that growth factor stimulation of animal cells activates Rab5A (Barbieri et al., 2000) and enhances the class E compartment morphology caused by ESCRT-I depletion (Razi and Futter, 2006). The relatively low frequency of class E compartment-like structures induced by genetic disruption of Rab conversion in yeast expressing wild-type ESCRT genes (Fig. 7) suggests how challenging it is to form these structures in the context of ESCRT function. The aberrant accumulation of ubiquitin at endosomes, which occurs in response to ESCRT dysfunction (Ren et al., 2008), might also be involved in class E compartment biogenesis because yeast lacking endosomal deubiquitylation activity do not form class E compartments unless cellular ubiquitin levels are maintained (Richter et al., 2007). Evidence that ubiquitylated cargoes inhibit endosomal maturation is the enhanced class E compartment morphology seen upon stimulation of epidermal growth factor receptor endocytosis after ESCRT-I depletion in human cells

(Razi and Futter, 2006). These observations warrant future investigation into whether the ubiquitin-binding capability of Vps9 (Prag et al., 2003), the Vps21 GEF, senses ubiquitylated cargoes accumulated at class E compartments.

The apparent failure to complete Rab5–Rab7 conversion upon ESCRT disruption in yeast correlates with impaired localization of Mon1–Ccz1 at endomembranes. Mon1–Ccz1 is regarded as a key regulator of endosomal Rab conversion (Cabrera and Ungermann, 2010) because the *C. elegans* ortholog of Mon1 inhibits endosomal localization of RABX-5, the GEF that activates Rab5A (Poteryaev et al., 2010). A similar mechanism might exist in yeast because the loss of Ccz1–GFP localization at endosomes coincided with endosomal accumulation of Vps9, the Vps21 GEF (Fig. 6D,E). Because Mon1–Ccz1 also regulates Rab conversion through its function as the Ypt7 GEF (Nordmann et al., 2010), defective endosomal recruitment of Mon1–Ccz1 in ESCRT-mutant cells might account for both Vps21 hyperactivity and the apparent lack of Ypt7 function at class E compartments.

The concentration of Vps9 at class E compartments explains the accompanying accumulation of GTP-bound Vps21 and CORVET, but it is unclear why Ypt7 is also concentrated at class E compartments in the absence of Mon1–Ccz1. That Ypt7 is dysfunctional at these structures without its HOPS effectors is consistent with the severely impaired delivery of endosomal cargoes to vacuoles/lysosomes upon ESCRT disruption (Babst et al., 1997; Doyotte et al., 2005). The accumulation of aberrant vesicle clusters instead of class E compartments in *vps21Δ vps4Δ* cells suggests Ypt7 might also be dysfunctional in this circumstance. While we detected no reduction in GTP-bound Ypt7, impaired Rab7 function in animal cells in response to protein kinase C delta inhibition has similarly been shown to occur without a reduction in GTP-bound Rab7 (Romero Rosales et al., 2009). The mechanistic basis for Ypt7 dysfunction resulting from ESCRT disruption remains to be determined, but it is possible that the persistence of Vps21 and CORVET at endosomes interferes with the ability of Ypt7 to activate HOPS assembly due to CORVET and HOPS sharing a common set of core subunits (Peplowska et al., 2007) (Fig. 6H). Notably, overexpression of the constitutively active GTP-locked $ypt7^{Q68L}$ allele was unable to suppress class E compartment formation in *vps4Δ* cells (T.S. and G.O., unpublished results), demonstrating that activation of Ypt7 is insufficient to override the aberrant Vps21 activity that drives class E compartment biogenesis.

Despite the reduction in Ypt7 and HOPS localization at vacuoles upon ESCRT disruption (Fig. 5B; Fig. 6B) (Balderhaar et al., 2010), neither homotypic vacuole fusion nor vacuolar fusion with other types of transport vesicles is impaired (Odorizzi et al., 1998b; Balderhaar et al., 2010). The trace amounts of Ypt7 evident at vacuolar membranes upon ESCRT dysfunction might sustain vacuolar membrane fusion activity. Alternatively, ESCRT dysfunction might suppress the effects of Yck3 protein kinase activity, the loss of which reduces dependence on Ypt7 for vacuole membrane fusion (LaGrassa and Ungermann, 2005; Brett et al., 2008).

Notably, ILV budding was not impaired by $vps21^{Q66L}$ overexpression in yeast (Fig. 7B–D) or by overexpression of a similar dominant-active Rab5A in human cells (Wegner et al., 2010). ILV biogenesis was similarly unaffected by disruption of Ypt7/Rab7 function (Fig. 3D) (Vanlandingham and Ceresa, 2009). These observations are consistent with Rab conversion occurring downstream of ESCRT-driven ILV budding. The biogenesis of MVBs has been proposed to be essential during endosomal maturation so that transmembrane proteins targeted for degradation are sequestered before late endosomes fuse with vacuoles/lysosomes (Mellman, 1996). Indeed, when $vps4^{ts}$ function is restored by shifting to the permissive temperature, class E compartments resume ILV budding before fusing with the vacuole (Fig. 1), suggesting that removal of ubiquitylated cargoes by the ESCRT machinery is required for effective activation of the endolysosomal fusion machinery.

Vps21 was originally thought to be essential for membrane trafficking from the Golgi to early endosomes (Cowles et al., 1994; Horazdovsky et al., 1994; Becherer et al., 1996; Burd et al., 1996). However, we found that GFP–Cps1 was delivered to the vacuole lumen via ESCRT-mediated sorting despite Vps21 disruption. Cps1 is a transmembrane protein transported from the Golgi to early endosomes, where it is subsequently sorted by the ESCRT machinery into ILVs (Odorizzi et al., 1998a). Therefore, despite a reduction in both the normal frequency and size of MVBs, endosome biogenesis in the absence of Vps21 remains sufficient for ESCRT-mediated protein sorting into the MVB pathway. This observation indicates that Vps21 is essential for class E compartment formation not because of an *ab initio* requirement in endosome biogenesis but, instead, because Vps21 activity is necessary to provide sufficient delivery of membrane for the accumulation at class E compartment cisternae. Partial redundancy with the Ypt52 Rab5 paralog likely explains the continued biogenesis of endosomes without Vps21 function because simultaneous disruption of both Vps21 and Ypt52 abrogates both MVB cargo sorting and ILV biogenesis (Nickerson et al., 2012). However, we found no evidence to support a role for Ypt52 in the formation of class E compartments, which signifies the specificity of Vps21 in class E compartment biogenesis and suggests the effectors of Vps21 and Ypt52 are not identical.

The timing of class E compartment biogenesis relative to endocytic trafficking was previously unknown because almost all studies had described these abnormal structures at steady state in yeast containing deletions of ESCRT genes. Our results confirm class E compartments and MVBs share a common biogenesis, as indicated by the block in ILV formation coupled with the progressive flattening and stacking of endosomes upon $vps4^{ts}$ inactivation, followed by the recovery of ILV budding within flattened endosomal cisternae upon $vps4^{ts}$ reactivation. Vps4 is the ATPase that catalyzes disassembly of ESCRT-III, which is the final step executed in the ESCRT pathway and is, therefore, essential for sustaining the activity of this machinery (Wollert et al., 2009). Coupled with the disappearance of class E compartments upon $vps4^{ts}$ reactivation, our results indicate these aberrant endosomes are not static dead-end structures, which explains the resumption of vacuolar protein sorting seen upon shifting $vps4^{ts}$ cells back to permissive temperature (Fig. 2) (Babst et al., 1997).

Unlike yeast, animal cells do not uniformly respond to ESCRT dysfunction by forming class E compartments but, instead, often form swollen, spherical endosomes lacking ILVs (Doyotte et al., 2005; Razi and Futter, 2006). As in yeast, the disruption in ILV budding alone cannot account for all membrane accumulated at these enlarged endosomes (Razi and Futter, 2006), pointing to their formation being driven by sustained membrane fusion. Whether Rab5A in metazoans, like Vps21 in yeast, drives this membrane accumulation merits investigation.

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Chapter 3. Vps9 regulates endosomal maturation by binding ubiquitin

Introduction

Cell surface proteins marked for degradation are transported to the lysosome via the endocytic pathway. This includes activated cell surface receptors that are degraded to regulate their activity and resident proteins that are damaged. These proteins are directed into endocytic vesicles that first fuse with early endosomes. Fusion events in the endosomal system, like fusion events throughout the eukaryotic cell, are regulated by Rab GTPases. Rab5 coordinates heterotypic fusion of endocytic vesicles with early endosomes as well as homotypic fusion between early endosomes (Gorvel et al. 1991, Bucci et al. 1992; Rubino et al. 2000). Transport from early to late endosomes is thought to occur through a process of compartment maturation, where Rab5 is replaced by Rab7 on the same endosome (Rink et al. 2005, Poteryaev et al. 2010). Rab7 then regulates fusion of late endosomes with lysosomes where lumenal contents are degraded in the hydrolytic interior (Vanlandingham & Ceresa 2009).

As endosomes mature, transmembrane proteins marked for lysosomal degradation are sorted into intralumenal vesicles (ILVs) generating what is known as a multivesicular body (MVB). Upon fusion of late endosome/MVBs with lysosomes the intralumenal vesicles and the transmembrane proteins are degraded in the lysosome lumen. Cargo sorting and ILV formation requires the conserved Endosomal Sorting Complexes Required for Transport (ESCRT) (Hanson & Cashikar 2012). Ubiquitin serves as a signal for sorting into ILVs (Katzmann et al. 2001). In *S. cerevisiae* disruption of any of the ESCRT genes results in a loss of ILVs and an accumulation of

flattened stacks of endosomal membrane known as class E compartments (Raymond et al. 1992, Rieder et al. 1996). The processes of transmembrane protein sorting into ILVs and fusion of endosomes with lysosomes are likely coordinated to ensure proper sorting into ILVs prior to fusion. How this regulation is achieved is unknown but recently we have shown that class E compartments result from a failure of endosomal Rab conversion where the Rab5 homolog, Vps21, is chronically active and there is a failure to activate the Rab7 homolog, Ypt7 (Russell et al. 2012). It is unclear why ESCRT disruption would lead to failure in endosomal rab conversion but elucidation of this mechanism should provide insights into how transport and sorting fidelity are maintained.

Rabs coordinate fusion by recruiting and regulating tethers and SNARE complexes which ultimately drive membrane fusion (Stenmark 2009). Rabs are brought into their active GTP-bound state through interaction with a guanine nucleotide exchange factor (GEF) which promotes dissociation of GDP allowing binding of GTP. Nucleotide exchange on Rab5 family GTPases is stimulated by proteins containing a conserved Vps9 domain (Carney et al. 2006). In *S. cerevisiae,* Vps9 promotes nucleotide exchange on Vps21 (Hama et al. 1999). Loss of Vps9 causes severe missorting of vacuolar proteins and is therefore an important regulator of endosomal transport (Burd et al. 1996). The C-terminus of Vps9 contains a ubiquitin binding CUE domain which is involved in vacuolar protein sorting (Davies et al. 2003). A second Vps9 domain containing protein, Muk1, has recently been shown to have nucleotide exchange activity for Vps21 (Cabrera et al. 2012, Paulsel et al. 2013). The precise roles of these two GEFs in the endosomal system is not fully understood.

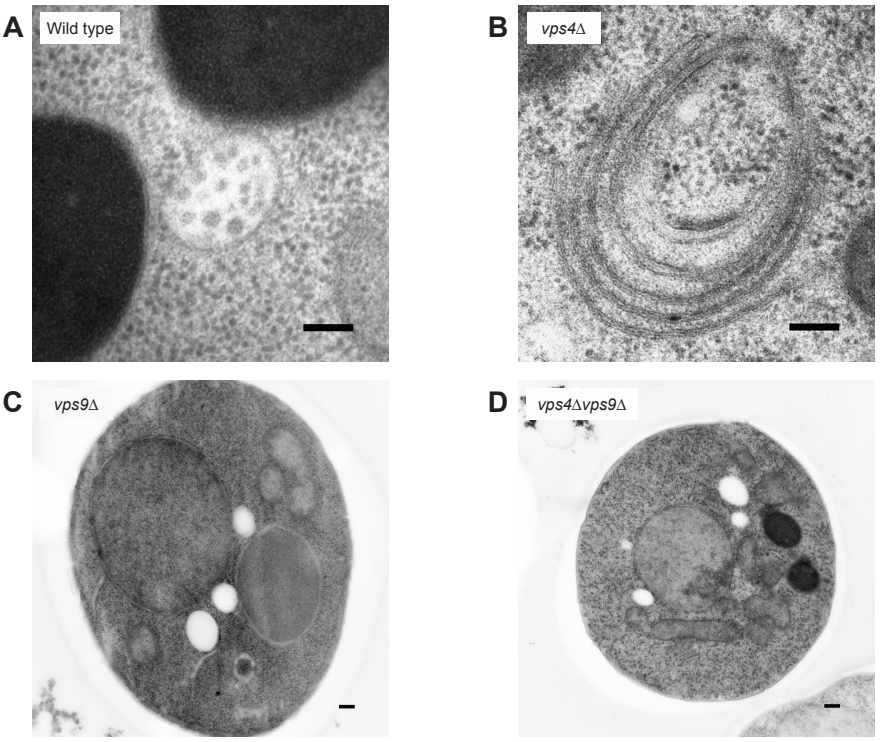
Here we show that Vps9 is the GEF maintaining hyperactivation of Vps21 upon ESCRT deletion. Vps9 is enriched at E compartments through its CUE domain binding ubiquitin. We propose that removal of ubiquitin acts as a signal for endosome maturation to ensure proper sorting prior to fusion with the vacuole.

Results

Class-E compartments form due to higher than normal Vps9 activity.

Endosome maturation is marked by the accumulation of numerous intralumenal vesicles (ILVs) containing transmembrane proteins targeted for degradation in the lysosome or yeast vacuole. The intralumenal vesicles of MVBs are formed through the action of ESCRT complexes. In yeast, disruption of ESCRT activity causes the formation of aberrant, flattened stacked endosomal cisternea, known as class E-compartments (Raymond et al. 1992, Rieder et al. 1996). Disruption of ESCRT-mediated sorting leads to increased Vps21 activity and an accumulation of endosomal membrane (Russell et al. 2012). Vps21 can be activated by two Vps9 domain containing proteins, Vps9 and Muk1 (Hama et al. 1999, Cabrera et al. 2012). Muk1, like Vps9, functions in the endocytic, MVB and Golgi to endosome pathways. Recent work has shown that although these two GEFs are partially redundant they do have distinct functions (Paulsel et al. 2013). Vps9, unlike Muk1, localizes to class E-compartments therefore we speculated that it is the GEF driving Vps21 hyperactivation in ESCRT mutants (Russell et al. 2012, Paulsel et al. 2013). By thin-section electron microscopy we see that loss of VPS9, like loss of VPS21 (Russell et al. 2012), in *vps4*Δ cells completely suppresses the formation E-compartments (Figure 3.1D, F). We occasionally

see clusters of membranes (not shown) similar to those seen in *vps4*Δ *vps21*Δ cells but not the flattened stacked cisternae typical of *vps4*Δ cells (Figure 3.1B). Loss of MUK1 does not suppress the formation E-compartments (Figure 3.1F) therefore Vps9 is the GEF required for hyperactivation of Vps21 upon ESCRT disruption. Vps9 residues D251 and E288 in the Vps9 domain have been shown to be important for GEF activity towards Vps21 *in vitro* (Delprato et al. 2004, Keren-Kaplan et al. 2012). Single GEF mutants still support the formation of class E-compartments but in cells expressing the double down GEF Vps9 mutant, *vps9*$^{D251A\ E288A}$, which has more reduced GEF activity *in vitro* compared to single mutants, E-compartments do not form (Figure 3.1F). Therefore the formation of E-compartments requires a high level of Vps9 exchange activity.



E

	MVBs in 50 cells
wild type	16
vps9	0
vps9^{D251A}	7
vps9^{E288A}	12
vps9$^{D251A\ E228A}$	5
*muk1*Δ	12

F

	E-compartments in 50 cells
wild type	8
vps9	0
vps9^{D251A}	5
vps9^{E288A}	11
vps9$^{D251A\ E228A}$	0
*muk1*Δ	7

Figure 3.1 Vps9 GEF activity is required for E compartments but not MVBs

Electron micrographs of (A) a wild type MVB, (B) a *vps4Δ* E compartment, (C) *vps9Δ* whole cell and (D) *vps4Δ vps9* whole cell. Quantification of the number of (E) MVBs in 50 ESCRT wild type cell profiles with Vps9 mutants or (F) E-compartments in 50 *vps4Δ* cell profiles with Vps9 mutants. Scale bars are 100 nm in all.

The failure to form E-compartments in *vps4*Δ cells with Vps9 deleted or with crippled GEF activity is likely not due to a general loss of delivery to the endosome. It has been well established that Vps9 plays an important role in the vacuole sorting pathway in *S. cerevisiae* by promoting fusion of endocytic and post Golgi vesicles with endosomes (Burd et al. 1996, Davies et al. 2003). But recent work has shown that MVB sorting and delivery to the vacuole are still partially functional in the absence of either Vps9 or Vps21 (Russell et al. 2012, Paulsel et al. 2013). Although *vps9*Δ cells have severe trafficking defects recent work has shown that MUK1 is partially redundant with VPS9 (Paulsel et al. 2013). We see that *vps9*Δ cells lack multivesicular bodies (Figure 3.1C). We occasionally see structures with irregular intralumenal membrane but these are rare (not shown). Deletion of *MUK1* does not cause any disruption in MVB biogenesis (Figure 3.1E).

Cells expressing single GEF mutants or the double down GEF mutant still have a number of MVBs similar to wild type cells (Figure 3.1E). Perhaps these Vps9 mutants can still support a basal level of Vps21 nucleotide exchange *in vivo*. The lack of class E-compartments in the double down Vps9 mutant indicates the membrane accumulation represents excessive Vps9 activity beyond what is normally involved in late endosome biogenesis.

Ubiquitin binding by the Vps9 CUE domain enriches Vps9 at endosomes

We wondered what is causing excess Vps9 activity when ESCRT-mediated sorting is disrupted. Vps9 contains the ubiquitin binding CUE domain which is thought to

recruit Vps9 to early endosomal membranes enriched with ubiquitnated cargo (Carney et al. 2006). We speculated that the CUE domain of Vps9 might also be involved in regulating endosomal maturation by maintaining Vps9, and therefore, Vps21 activity at endosomes until ubiquitinated cargoes have been removed through ESCRT-mediated sorting.

We first asked whether the CUE domain of Vps9 is important for endosomal localization in yeast. In wild type cells very few GFP-Vps9 puncta are detected perhaps because Vps9 is cycling on and off membranes as endosomes mature (Figure 3.2B). We and others have previously shown that Vps9 dramatically accumulates at endosomes in *vps4*Δ cells (Figure 3.2C, Russell et al. 2012, Paulsel et al. 2013). We expressed GFP-tagged Vps9 truncations in cells with endogenous wild type Vps9 to ensure that the Vps9 mutants did not affect endosomal morphology. GFP-Vps9 lacking the CUE domain (Figure 3.2E) or with a mutation in the ubiquitin binding interface (M419D, Figure 3.2D) causes Vps9 to dramatically shift to the cytosol in *vps4*Δ cells indicating that ubiquitin binding by the CUE domain enriches Vps9 at endosomes in *vps4*Δ cells. The Vps9 CUE domain alone (Figure 3.2G) can localizes to endosomes but the CUE domain ubiquitin binding mutant (M419D, Figure 3.2H) no longer localizes to endosomes indicating the CUE domain is only associated with endosomes through ubiquitin binding. Further truncation of the central Vps9 Domain, leaving only the associated helical bundle no longer localizes to the endosomes (Figure 3.2F), indicating the Vps9 domain can bind to endosomal membranes, although to a lesser extent than full length Vps9 or the CUE domain alone. We conclude that the Vps9 domain has

endosomal localization determinants but when ESCRT activity is disrupted Vps9 is enriched at endosomes by binding to ubiquitin.

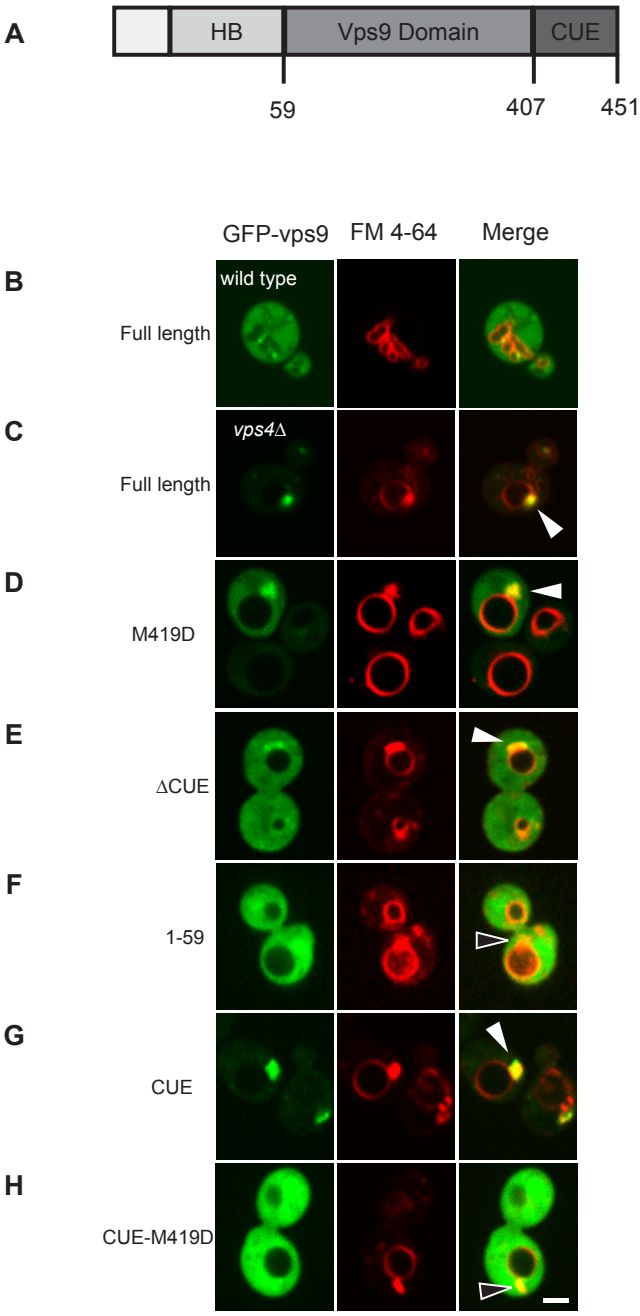


Figure 3.2 Vps9 is concentrated at E compartments by its CUE domain binding ubiquitin (A) Schematic diagram of Vps9. (B-E) Confocal fluorescence micrographs of FM 4-64 stained cells expressing GFP-tagged versions of Vps9 from a 2*μ* plasmid in (B) wild type and (C-H) *vps4Δ* cells where Vps9 truncations do (filled arrow heads) or do not empty arrow heads localize to E compartments. Scale: 2*μ*.

Loss of Ubiquitin binding does not disrupt Vps9 activity

Vps9 has been proposed to be important for delivery of ubiquitnated transmembrane proteins to early endosomes (Donaldson et al. 2003, Davies et al. 2003). Ubiquitin binding by Vps9 and the human Rabex-5 has been proposed to regulate association with endosomes, thereby directing fusion of endocytic vesicles with ubiquitinated cargos to endosomes (Carney et al. 2006, Mattera & Bonifacino 2008). We wondered whether the CUE domain might be also be regulating later stages of endosomal maturation by acting as a sensor for endosomal ubiquitin. Under normal circumstances Vps9 likely only transiently associates with endosomes and is turned over as the endosome matures. Perhaps the CUE domain maintains Vps9 at endosomes until proteins are sorted into ILVs and deubiquitinated. Disruption of ESCRT activity causes an accumulation of ubiquitinated cargoes at endosomes (Ren et al. 2008). This might cause prolonged association of Vps9 with endosomes and therefore, hyperactivation of Vps21. Based on this model we speculated that disruption of ubiquitin binding by Vps9 might relieve the aberrant accumulation of Vps9 at endosomes and suppress the formation of E-compartments. We see however that in cells expressing only Vps9 lacking the CUE domain or *vps9*^{M419D} there is no suppression of E-compartments (Figure 3.3C and D). This was surprising because the localization of these mutants to endosomes was dramatically reduced (Figure 3.2). In addition, ubiquitin binding by Vps9 CUE domain is not required for normal MVB biogenesis (Figure 3.3A and B). These Vps9 ubiquitin binding mutants have previously been shown to have slightly reduced vacuole protein sorting *in vivo* (Davies et al. 2003, Donaldson et al. 2003). The CUE domain has been proposed to regulate Vps9 activity

allosterically, through cis-ubiquitin binding, in addition to regulating its localization to early endosomes (Carney et al. 2006). Vps9 lacking the CUE domain or the *vps9*^{M419D} mutant has increased GEF activity toward Vps21 *in vitro* (Keren-Kaplan et al. 2012). Perhaps these Vps9 mutants also have more GEF activity *in vivo*, compensating for the reduction in endosomal localization.

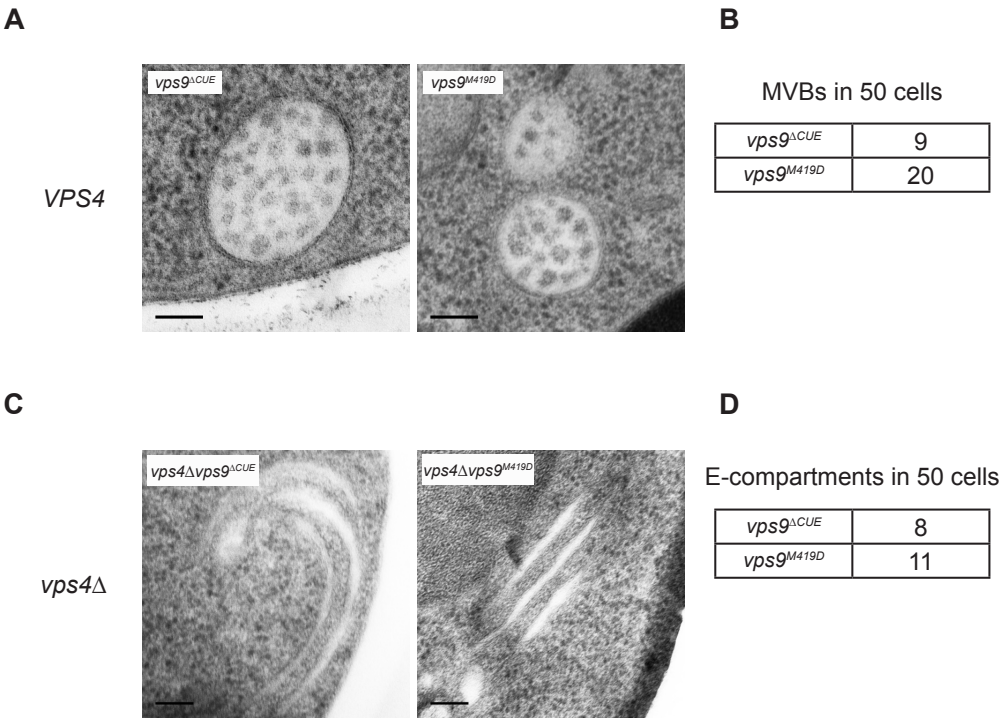


Figure 3.3 Disruption of ubiquitin binding by Vps9 does not suppress E compartments. Electron micrographs of (A) *vps9Δ* and (C) *vps9Δ vps4Δ* cells transformed with with CEN plasmids expressing the indicated *vps9* mutants. Quantification of (B) MVBs and (D) E-compartments in 50 cell profiles from (A) and (C), respectively. Scale bars are 100 nm.

Discussion

Here we present evidence that Vps9 is the GEF required for Vps21 hyperactivation in ESCRT deleted cells. Vps9 is enriched at E-compartments due to its CUE domain binding ubiquitin. Although disruption of ubiquitin binding causes a dramatic loss of endosomal localization it does not suppress the formation of class E-compartments consistent with work showing CUE domain also negatively regulates Vps9 GEF activity (Donaldson et al. 2003). We present a model where removal of ubiquitin by the ESCRT complexes is a signal for normal maturation of endosomes. Moreover, Vps9 is likely a key regulator by binding to endosomal ubiquitin until MVB sorting and deubiquitination are complete.

Two GEFs are known to activate Vps21: Vps9 and Muk1 (Hama et al. 1999, Cabrera et al. 2012, Paulsel et al. 2013). Loss of Vps9 causes severe vacuole sorting defects and is therefore considered the major GEF for directing fusion with and between early endosomes (Burd et al. 1996, Paulsel et al. 2013). Through CPS sorting and the newly developed LUCID assay, Paulsel et. al. showed that sorting in the MVB pathway is still partially functional *in vivo* in vps9Δ cells due to activity of Muk1. Our finding that Vps9 is the GEF leading to Vps21 hyperactivity is consistent with these findings that Vps9 is the primary GEF for delivery to early endosomes. Loss of Vps9, like loss of Vps21, causes a dramatic reduction but not a complete loss of endosome biogenesis (Russell et al. 2012; Paulsel et al. 2013). CPY secretion, Sna3 and Ste3 sorting by LUCID and CPS sorting by light microscopy showed similar defects in *vps9*Δ and *vps21*Δ cells (Nickerson et al. 2012, Cabrera et al. 2012, Paulsel et al. 2013). However, sorting in the MVB pathway is still partially functional in these cells. Perhaps we did not

see any MVBs in *vps9*Δ cells because they are very rare and transient. This is surprising because a small number MVBs have been reported in *vps21*Δ cells (Russell et al. 2012, Nickerson et al. 2012). This supports our conclusion that failure to form E-compartments in *vps4*Δ *vps9*Δ cells, as in *vps4*Δ *vps21*Δ cells, is not simply due to a complete loss of endosomes biogenesis.

Mutation of the two critical Vps9 GEF residues has been shown to cause a reduction in GEF activity *in vitro* and combining the two mutations causes almost complete loss of activity (Keren-Kaplan et al. 2012). The highly conserved aspartate at position 251 has been shown through structural studies with the mammalian Rabex-5 and *Arabidopsis* Vps9a to be directly stimulating nucleotide exchange by promoting GDP dissociation (Delprato et al. 2004, Delprato et al. 2004, Uejima et al. 2010). The glutamate at position 288 is thought to be required for binding to Vps21 (Delprato et al. 2004). Distinct functions of these two residues is consistent with our findings that there is a more dramatic loss of activity when the two mutations are combined in the context of *vps4*Δ cells. Loss of nucleotide exchange activity or Vps21 binding is still sufficient to maintain Vps21 hyperactivation but loss of both functions causes a more dramatic failure to activate Vps21. It is surprising that the double GEF Vps9 mutant did not cause a reduction of MVBs by EM. Perhaps there is still a very low level of nucleotide exchange on Vps21 with this mutant or some structural feature of Vps9 is the minimal requirement for MVB biogenesis. The suppression of E-compartments in *vps4*Δ cells expressing the Vps9 double GEF mutant while there is no MVB biogenesis defect in wild type cells expressing the same mutant indicates that E-compartments represent higher GEF activity than what is required for normal MVB biogenesis. This is consistent

with our previous finding that deletion of ESCRT genes leads to an increase in active, GTP-bound, Vps21 (Russell et al. 2012).

Our finding that the ubiquitin binding domain of Vps9 is an important localization determinant is consistent with findings from mammalian Rabex-5. The zinc finger and MIU (motif interacting with ubiquitin) of Rabex-5 were shown to be necessary for localization to endosomes (Mattera et al. 2006). Therefore, the importance of ubiquitin binding for endosomal localization is conserved in both mammalian cells and budding yeast. Rabex-5 has additional localization determinants within the C-terminus, likely through interaction with Rabaptin-5 (Mattera & Bonifacino 2008). We find that Vps9 also has additional localization determinants in the Vps9 domain. This is consistent with expression of the *C. elegans* RABX-5 Vps9 domain in yeast which was sufficient to localize to yeast endosomes. (Poteryaev et al. 2010). These Rab5 GEFs therefore have similar but distinct localization determinants within these systems. GEFs have been postulated to be important localization determinants for their cognate RabGTPases (Blümer et al. 2012). Rabex-5 and Vps9 were recently shown to be important for localization of Rab5 in mammals and Vps21 in yeast, respectively (Blümer et al. 2013, Cabrera & Ungermann 2013). Exploration of how the Vps9 domain localizes to endosomes may provide insights into Vps9 function and regulation of endosomal delivery and maturation. Our findings indicate that although the Vps9 domain contains localization determinants, ubiquitin binding is causing the aberrant accumulation on endosomes in ESCRT mutants. Therefore, ubiquitin levels on the endosomal membrane are likely playing a role in regulation of Vps21 activity during endosome maturation.

The Vps9 CUE domain has been proposed to regulate Vps9 GEF activity, in addition to regulating its localization (Donaldson et al. 2003, Carney et al. 2006). Vps9$^{\Delta CUE}$ can suppress the severe sorting defects of cargo fused to mutant ubiquitin I44A indicating that the CUE domain negatively regulates Vps9 activity (Donaldson et al. 2003). Vps9 and Rabex-5 are both ubiquitinated (Shih et al. 2003, Davies et al. 2003, Mattera et al. 2006). The ubiquitinated form of Rabex-5 is found primarily in the cytosol leading to a model where cis-ubiquitin binding is auto-inhibitory (Mattera & Bonifacino 2008). Trans-binding to ubiquitin on the endosomal membrane could both promote recruitment and relieve auto-inhibition. This model is supported by evidence that the Vps9 domain alone is more active *in vitro* (Delprato et al. 2004). Relief of inhibition of Rabex-5, however, was shown to occur through interaction of the C-terminus with Rabaptin-5 (Lippé et al. 2001). Recently, it has been shown that ubiquitinated Vps9 is no less active in stimulating exchange on Vps21 *in vitro* than unmodified Vps9 (Keren-Kaplan et al. 2012). Therefore, inhibition by the CUE domain may be occurring through interaction with some other factor *in vivo*. Our data are consistent with an auto-inhibitory role of the CUE domain because the dramatic loss in localization results in no change E-compartments which requires Vps9 GEF activity.

Ubiquitin has multiple roles in the endosomal system. It serves as a signal for removal of transmembrane proteins from the plasma membrane into endocytic vesicles . It also serves as a signal for inclusion of proteins destined for lysosomal degradation into intralumenal vesicles at endosomes (Katzmann et al. 2001). Here we present a model for an additional role as a marker for the completion of MVB sorting and therefore endosome maturation. We have previously shown that overexpression of

Doa4, which also reduces ubiquitination of MVB cargo, suppresses E-compartments in *bro1*Δ cells (Luhtala & Odorizzi 2004). In addition restoration of ubiquitin levels in *doa4*Δ cells was previously shown to drive the formation of E-compartments (Richter et al. 2007). Together, these data support a model where higher levels of endosomal ubiquitin correspond to more severe E-compartment morphology and similarly reduction in endosomal ubiquitin suppresses Class E-compartments. The Vps9 CUE domain has higher affinity for ubiquitin than most other ubiquitin binding domains making it likely to be a key factor in responding to endosomal ubiquitin levels (Prag et al. 2003). This might also explain why loss of ESCRT activity in yeast leads to more severe disruption of endosomal morphology than it does in mammalian cells (Doyotte et al. 2005).

Although Vps9 is a likely candidate for responding to endosomal ubiquitin levels it is possible that other ubiquitin binding factors are regulating endosome maturation. A number of proteins that function throughout the endosomal system are known to bind ubiquitin. ESCRTs-0, I and II components contain ubiquitin binding domains that are required for directing cargo into intralumenal vesicles (Henne et al. 2011). A ubiquitin binding domain in Bro1 was recently discovered that was shown to function, along with ESCRT-0, in directing cargo into ILVs (Pashkova et al. 2013). In addition the GGAs proteins, which are believed to be important for sorting from the trans Golgi to endosomes, bind ubiquitin (Scott et al. 2004). Ubiquitin binding by these proteins may have additional roles in the endosomal system. New endosomal ubiquitin binding proteins and ubiquitin binding capability of known proteins are still likely to be discovered.

Our recent work has shown that class E-compartments form due to hyperactivation of Vps21 as well as a failure to activate the Rab7 homolog Ypt7, which regulates fusion of late endosomes/MVBs with vacuoles (Russell et al. 2012, Wichmann et al. 1992). Overexpression of hyperactive Vps21 alone was not sufficient to drive the formation of class E compartments in cells with functional ESCRTS (Russell et al. 2012). We speculate that there is a mechanism for inhibiting fusion of endosomes with vacuoles prior to completion of ESCRT-mediated sorting to prevent severe mislocalization of MVB cargo to the vacuole perimeter. Endosome vacuole fusion requires the Mon1-Ccz1 complex which is the GEF for Ypt7 and promotes Rab conversion in *C. elegans* (Nordmann et al. 2010, Poteryaev et al. 2010). We previously saw a failure to recruit Mon1-Ccz1 to endosomes in ESCRT mutant cells (Russell et al. 2012). SAND-1, the *C. elegans* homolog of Mon1, is thought to be recruited endosomes by PI(3)P (Poteryaev et al. 2010) however GFP-FYVE accumulates on E compartments indicating there are high levels of PI(3)P (Nickerson et al. 2012). Mon1 may be recruited to endosomes differently in yeast or ESCRT dsyfunction may be interfering with its recruitment in some way. Perhaps removal of endosomal ubiquitin is required for recruitment of Mon1-Ccz1. The continued presence of ubiquitin could also inhibit other aspects of endosome maturation such as HOPS recruitment or activation. Data presented here suggest that excess ubiquitin is contributing to chronic activation of Vps21 seen in ESCRT mutants through enrichment and activation of Vps9. It will be interesting to see if removal of ubiquitin also contributes to activation of Ypt7.

Materials and Methods

Yeast strains and plasmid construction

Standard procedures were used for manipulating S. cerevisiae (Guthrie and Fink, 2002) and for DNA manipulations using Escherichia coli (Sambrook and Russell, 2001). Plasmids were confirmed by DNA sequence analysis. Gene deletions and chromosomal integrations in yeast were constructed by homologous recombination using site-specific cassettes amplified by PCR (Longtine et al., 1998; Gueldener et al., 2002).

Fluorescence Microscopy

Yeast were grown to logarithmic phase at 30ºC and stained with FM 4-64 (Invitrogen), using a pulse-chase procedure as previously described (Odorizzi et al., 2003). Cells were labeled in 50 uL of YPD plus 30 nM FM 4-64 for 20 min at 30ºC then washed with 1 mL YPD and chased in 5 mL YPD alone. Labeled cells were washed and resuspended in YNB, then placed on slides for viewing. Confocal fluorescence microscopy was performed using an inverted fluorescence microscope (TE2000-U; Nikon) equipped with an electron-multiplying charge-coupled device camera (Cascade II; Photometrics) and a Yokogawa spinning disc confocal system (CSU-Xm2; Nikon). Images were taken with a 1006 NA 1.4 oil objective, acquired using MetaMorph (version 7.0; MDS Analytical Technologies), and processed using Adobe Photoshop CS3 software (Adobe Systems, San Jose, CA).

Electron Microscopy

Yeast cells were grown to logarithmic phase at 30ºC transferred to aluminum planchettes and frozen in a Wohlwend Compact 02 High Pressure Freezer, Planchettes

were transferred to vials containing freeze substitution solution (0.1% uranyl acetate, 2% glutaraldehyde in anhydrous acetone). Vials were transferred to a freeze substitution machine (Leica EM AFS2, Vienna, Austria) at -150℃. Samples were warmed to -80℃ over 24 hours, then the cells were removed from the planchettes, transferred to chilled tubes, and the freeze substitution solution replaced. After 48 hours, samples were warmed to -60℃. Over the next 96 hours, samples were washed three times with acetone, then 1:3, 1:1 and 3:1 acetone/Lowicryl HM20 (Polysciences, Warrington, PA), and six times with HM20. The HM20 was UV-polymerized during warming to 20℃ over 48 hours. A Leica Ultra-microtome was used to cut 90 nm thin sections and 250 nm thick sections which were collected onto 1% formvar films adhered to rhodium-plated copper grids (Electron Microscopy Sciences). 90 nm thin sections were post-stained in 2% uranyl acetate for 10 minutes and Reynold's lead citrate for 20 minutes and imaged using a Philips CM10 transmission electron microscope at 80 kV. Images were processed using Adobe CS3 software (Adobe Systems, San Jose, CA). For quantification MVBs were defined as spherical structures surrounded by a discernible bi-layer and containing at least 2 intralumenal vesicles.

EM quantification

For quantification of MVBs per cell profile 50 random cell profiles were chosen. MVBs were defined as spherical structures surrounded by a discernible bi-layer and containing at least 2 intralumenal vesicles with discernible bi-layer. E compartments were defined as two or more cisternae where each cisternae is at least twice as long as it is wide and the cisternae are separated by a ribosome excluded region.

Chapter 4. Displacement of Vps9 from endosomes requires Mon1-Ccz1 and additional vacuole fusion factors.

Introduction

Material is transported from the plasma membrane to the lysosome through the endocytic pathway. Transmembrane proteins and extracellular fluid are brought into the cell in endocytic vesicles which then fuse with early endosomes. Those cargoes that are destined for degradation in the lysosome are transported to late endosomes where they are sorted into intralumenal vesicles (ILVs) that bud into the lumen of the endosome. Sorting into and formation of intralumenal vesicles requires the conserved endosomal sorting complexes required for transport, ESCRTs. These late endosomes containing intralumenal vesicles, also called multivesicular bodies (MVBs), fuse with lysosomes releasing the intralumenal vesicles to the interior of the lysosome (Huotari & Helenius 2011) .

Like most membrane transport in the eukaryotic cells, endosomal transport is regulated by Rab GTPases. Rabs regulate fusion by recruiting tethers which bring membranes together and engages SNAREs that drive membrane fusion (Stenmark et al. 1994). In yeast the Rab5 homolog, Vps21, regulates fusion with early endosomes by recruiting the early endosome multi-subunit tethering complex, CORVET. Fusion with the vacuole (the functional equivalent of the lysosome in yeast) requires the Rab7 homolog, Ypt7. Ypt7 recruits the HOPS complex which shares 4 core subunits with CORVET. Interplay between HOPS and CORVET is thought to have a role in regulating endosomal transport (Nickerson et al. 2012).

Rab GTPases act as molecular switches that are regulated by their nucleotide binding state. They are brought into their active, GTP-bound state by guanine nucleotide exchange factors (GEFs) which stimulate exchange of GDP for GTP. GTP binding causes a conformational change that promotes interaction with effectors such as tethering complexes (Stenmark et al. 1994). Exchange on Rab5 family proteins is stimulated by GEFs that contain a conserved Vps9 domain (Carney 2007). The yeast protein Vps9 stimulates exchange on Vps21 and is required for endosomal transport (Hama et al. 1999). Fusion with the vacuole requires the Ypt7 GEF complex Mon1-Ccz1 (Nordmann et al. 2010). These two GEFs therefore coordinate endosomal transport by directing fusion at the appropriate location.

Transport from early endosomes to lysosomes is believed to occur through a process of maturation where an early endosome compartment transitions into a late endosome. Live-cell tracking of endosomes in mammalian cells showed that as endosomes mature Rab5 is replaced by Rab7 (Rink et al. 2005). This was also shown to occur in *C. elegans* where the transition is believed to be triggered by SAND-1 (Poteryaev et al. 2010). Recruitment of SAND-1 to endosomes promotes Rab conversion by displacing RABX-5, the homolog of Vps9, (Poteryaev et al. 2010). The mechanism of RABX-5 displacement by SAND-1 is unknown. In yeast the SAND-1 homolog is Mon1, which, as mentioned above, functions in a complex with Ccz1 as a GEF for Ypt7.

We wondered whether Mon1 in yeast also promotes endosome maturation by displacing Vps9. In *mon1*Δ cells we see endosome enlargement and membrane accumulation of Vps9 and Vps21 consistent with Mon1 playing a role in terminating

Vps21-mediated fusion. Unlike overexpression of SAND-1 in *C. elegans*, however, overexpression of Mon1 in yeast fails to displace Vps9 from endomembranes. Surprisingly, we find that deletion of factors downstream of Ypt7 that are required for endosome to vacuole fusion, also have enlarged endosomes and show Vps9 membrane accumulation. We conclude that displacement of Vps9 and cessation of Vps21-mediated fusion is stimulated by Mon1 directly as well as by an unknown mechanism that requires vacuole fusion factors.

Results

Deletion of *MON1* causes an increase in Vps9 and Vps21 positive puncta

Mon1 is thought to trigger endosome Rab conversion by displacing the early endosomal Rab5 GEF (Poteryaev et al. 2010). Loss of Mon1 in mammals or SAND-1 in *C. elegans* leads to an aberrant accumulation of both Rab5 and its GEF Rabex5 and RABX-5, respectively. We wondered if loss of *MON1* would have the same effect in *S. cerevisiae*. We see that Vps9 in wild type cells appears mostly cytosolic with occasional small, faint puncta (Figure 4.1A). Deletion of *MON1* causes Vps9 to be concentrated on larger cytosolic puncta which is consistent with Mon1 stimulating Vps9 displacement from endosomes. The Rab5 homolog, Vps21, normally localizes to a small number of distinct perivacuolar puncta that are likely endosomes because they stain with the lipophilic dye FM 4-64((Peplowska et al. 2007), Figure 4.1B). Loss of *MON1* leads to an increase in GFP-Vps21 puncta although the intensity of these is reduced (Figure 4.1B). Mon1 in yeast is also required for homotypic vacuole fusion and *mon1*Δ cells have fragmented vacuoles (Wang et al. 2002). It is therefore difficult to determine the location

of these puncta relative to vacuoles. Loss of Mon1 may cause Vps21 to have prolonged association with endosomes or there may be more endosomes to which Vps21 localizes.

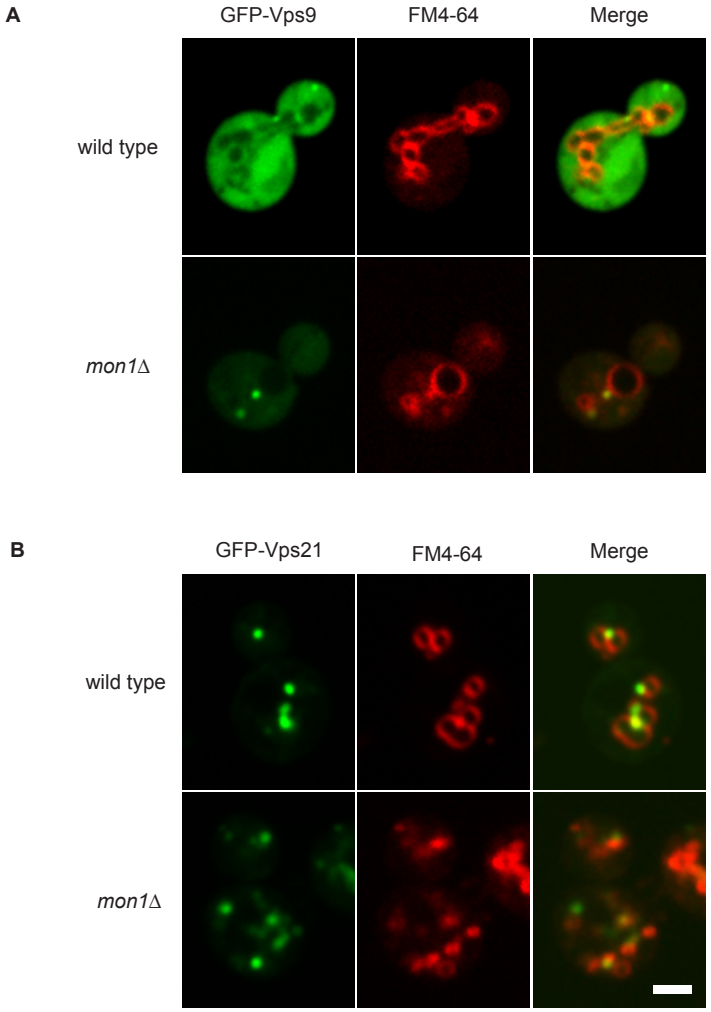
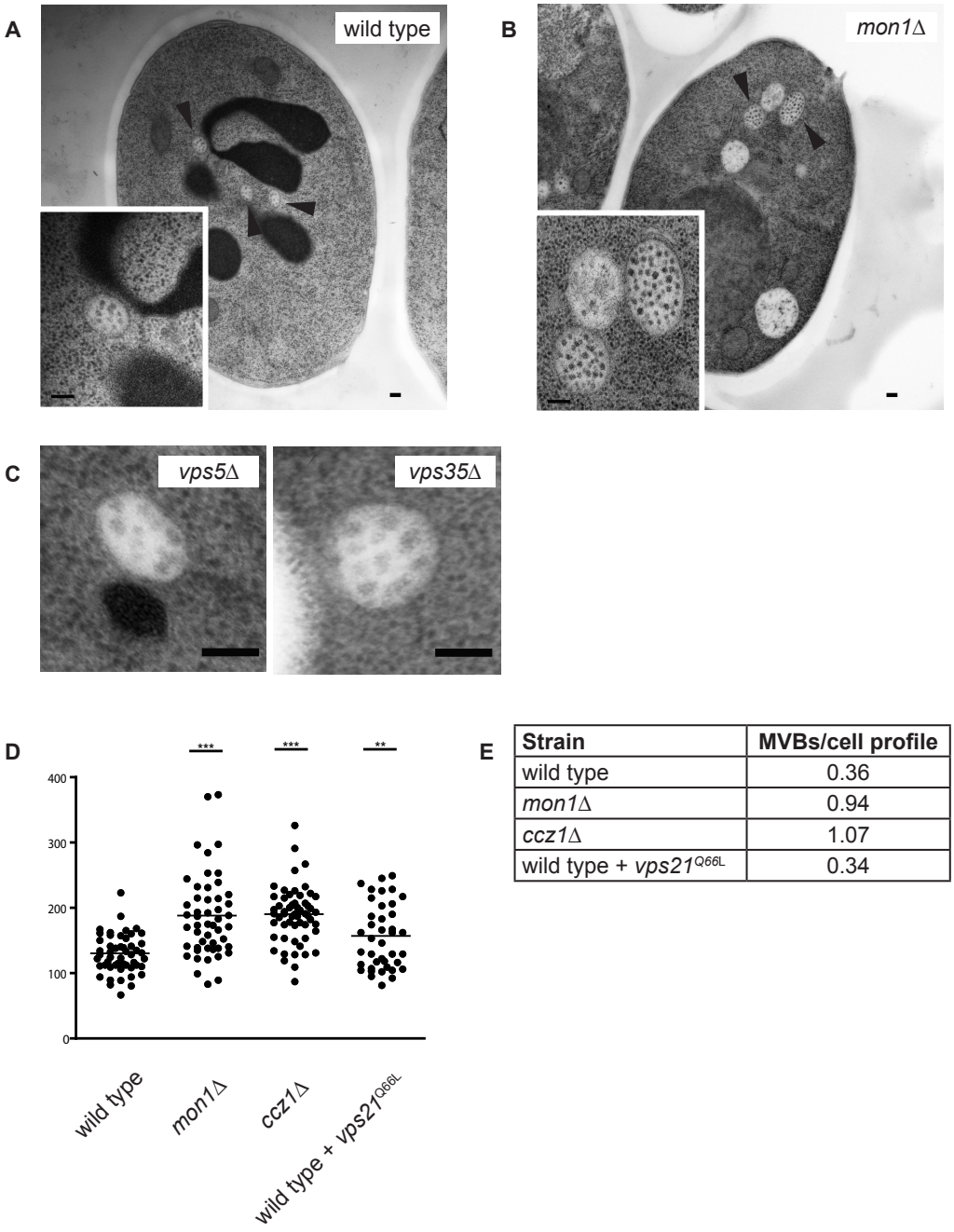


Figure 4.1 Loss of MON1 changes Vps9 and Vps21 localization. Confocal microscopy of (A) GFP-Vps9 expressed from a plasmid and (B) genomic integration GFP-Vps21 in wild type and *mon1*Δ cells. Vacuoles are marked by FM4-64. Scale bar: 2μ.

MVBs in *mon1*∆ cells are enlarged due to increased endosome fusion

The increased membrane association of Vps9 and Vps21 suggests the possibility of increased endosomal fusion. In *C. elegans* and mammalian cells loss of SAND-1/ Mon1 resulted in enlargement of Rab5 positive endosomes (Poteryaev et al. 2010). In these systems the enlargement of early endosomes is believed to be due to delayed displacement of the GEF RABX-5, leading to hyperactivation of Rab5. We wondered if the same were true in *S. cerevisiae*. As endosomes mature they accumulate numerous intralumenal vesicles giving rise to a structure known as a multivesicular body (MVB) (Felder et al. 1990) Figure 4.2A. By electron microscopy we see that loss of Mon1 causes an enlargement of MVBs. In wild type cells MVBs have a mean diameter of about 131 nm whereas *mon1*∆ cells have a mean diameter of about 189 nm. In yeast Mon1 is in a complex with Ccz1 that acts as a GEF for the Rab7 homolog Ypt7 (Nordmann et al. 2010) but it is not known if Ccz1 acts with Mon1 in stimulating displacement of RABX-5 from endosomes in *C. elegans*. We see a similar increase in diameter of MVBs in *ccz1*∆ cells, with a mean of about 191 nm, consistent with Ccz1 and Mon1 acting together in terminating Vps21-mediated fusion.



E

Strain	MVBs/cell profile
wild type	0.36
*mon1*Δ	0.94
*ccz1*Δ	1.07
wild type + *vps21*^{Q66L}	0.34

Figure 4.2 Endosomes are enlarged in *mon1*Δ cells due to Vps21 mediated fusion. (A-C) Electron micrographs of indicated strains. All scales are 100nm. (A, B) Whole cells with zoom of MVBs inset. (C) Representative zoom of MVBs in indicated strains. (D) Quantification of endosome diameters. Distribution of endosome diameters where the mean (central bar) is compared to wild type by unpaired t test. P values are *** < 0.0001 or ** = 0.0021. (E) Quantification of the frequency of MVBs represented as number per cell profile n ≥ 50.

The enlargement of MVBs is likely due to increased endosome fusion but it could also result from disruption of retrograde transport from endosomes to the trans-Golgi. Recruitment of the retromer complex, which regulates retrograde transport, has been show to require Ypt7 in yeast and Rab7 in mammalian cells and therefore might also require Mon1-Ccz1 GEF activity (Liu et al. 2012, Seaman et al. 2009). Loss of Vps35, which is part of the cargo recognition retromer sub-complex (Seaman 2012) did not cause any noticeable enlargement of MVBs, nor did deletion of Vps5, which is thought to be involved in generating tubes or vesicles budding from the endosome(Figure 4.2C). Therefore loss of retrograde transport does not result in enlarged MVBs indicating that the enlargement of MVBs we seen in *mon1*Δ cells is a result of increased endosome fusion. Consistent with this we see overexpression of hyperactive, GTP-locked, Vps21 (*vps21*^{Q66L}) also results in an increase in the diameter of MVBs, with a mean diameter of about 157 nm. Loss of Mon1 and Ccz1 also causes an increase in the number of MVBs per cell whereas overexpression of GTP locked Vps21 does not (Figure 4.2D). Loss of Mon1 and Ccz1 also disrupts endosome to vacuole fusion whereas overexpression of GTP locked Vps21 does not (Lachmann et al. 2012). Therefore the increase in the number of MVBs in *mon1*Δ and *ccz1*Δ cells is likely due to a failure of MVBs to fuse with vacuoles and the enlargement of MVBs is due to continued fusion between endosomes.

MON1 is not required for ESCRT activity

Intralumenal vesicles of MVBs are generated through the action of ESCRTs which sort ubiquitinated transmembrane proteins destined for degradation in the vacuole (Hanson & Cashikar 2012). The timing of ESCRT activity during endosome

maturation is not precisely known although our recent work indicates that ESCRT activity is required for proper activation of Ypt7 (Russell et al. 2012). It is not clear if ESCRT-mediated sorting and Ypt7 activation occur sequentially or concurrently. Transition to late endosomes might be required for efficient ESCRT activity. We wondered if ESCRT activity was affected by loss of Mon1-Ccz1. We used electron microscopy and 3D tomographic modeling to asses the morphology of MVBs in *mon1*Δ and *ccz1*Δ cells. We see that the MVBs in *mon1*Δ and *ccz1*Δ cells are filled with intralumenal vesicles similar to MVBs in wild type cells (Figure 4.3 A) . The surface area of the ILVs in wild type MVBs accounts for about 52 percent of the combined endosomal surface area. In *ccz1*Δ cells the surface area of the ILVs accounts for 63 percent of the total endosomal membrane indicating there is no defect in generation of ILVs in these cells. Although the MVBs in *ccz1*Δ and *mon1*Δ cells are larger than the MVBs in wild type cells, ILV budding and scission are functioning normally.

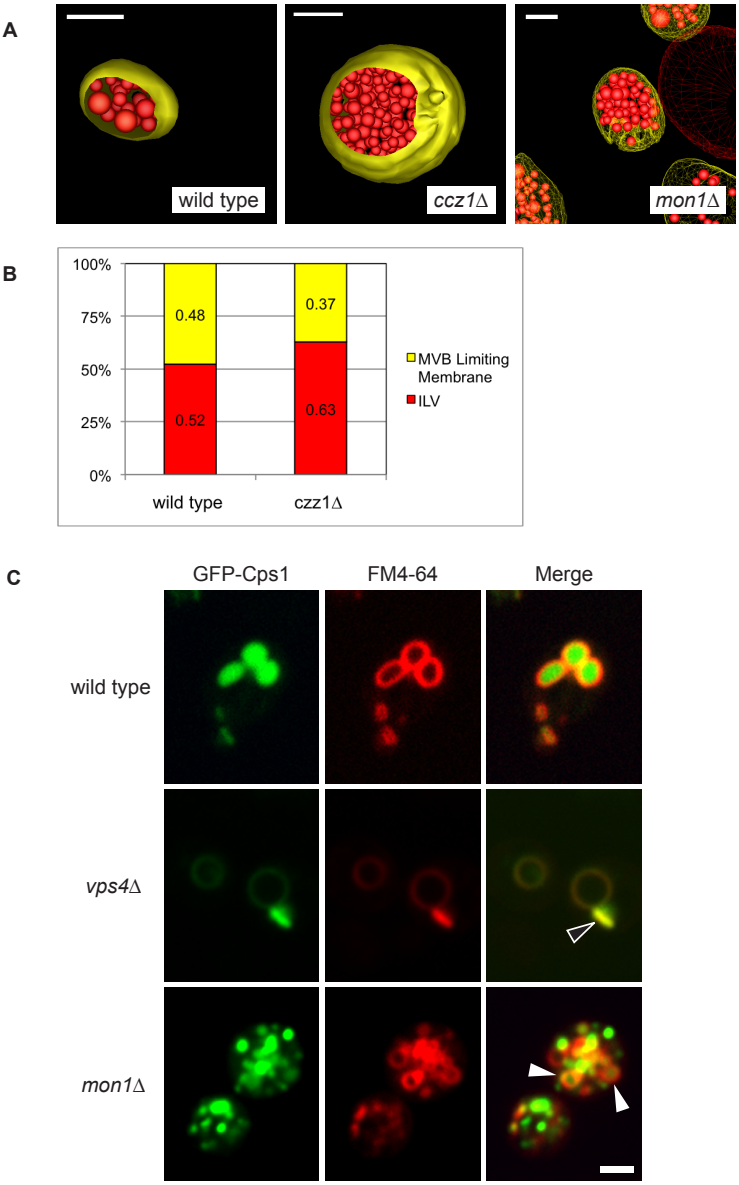


Figure 4.3 ESCRTs are functional in *mon1Δ* and *ccz1Δ* cells. (A) Electron tomographic models MVBs from the indicated strains cells. Scale: 100nm. (B) Quantification of the relative percentages of membrane surface areas of ILVs and limiting membrane for wild type and *ccz1Δ* MVBs. Confocal micrographs of cells expressing GFP-Cps1. Vacuoles and E-compartments (open arrow head) are labeled with FM 4-64. Closed arrowheads shows vacuoles with lumenal GFP-Cps1. Scale bar: 2*μ*. Tomography by Matt West

Generation of ILVs does not guarantee proper sorting of cargo into intralumenal vesicles as deletion of the ESCRT associated deubiquitinating enzyme, DOA4, leads to missorting even though ILVs are generated (Richter et al. 2007). Loss of Mon1 or Ccz1 is known to disrupt transport of MVB cargoes to the vacuole (Wang et al. 2002) but we speculated that this may simply be a result of failure of the late endosome to fuse with the vacuole. Cps1 is a peptidase that is sorted to the vacuole lumen via the MVB pathway (Odorizzi et al. 1998, Figure 4.3C). Disruption of ESCRTs causes Cps1 mislocalization to the vacuole perimeter as well as an aberrant endosomal membrane known as the class E compartment (Odorizzi et al. 1998, Figure 4.3C). In *mon1*Δ cells, the vacuoles are fragmented making them more difficult to distinguish by light microscopy. Much of the Cps1 localizes to small FM4-64 labeled puncta which likely correspond to endosomes or small fragmented vacuoles. However, when the vacuoles are large enough to distinguish we see Cps1 in the lumen not on the perimeter (Figure 4.3C). Taken together this indicates that MVB cargos are sorted into ILVs at endosomes but endosomes do not efficiently fuse with vacuoles.

Mon1 does not displace Vps9 from endosomes

The Mon1 homolog, SAND-1, is thought to promote endosome maturation by displacing the Rab5 GEF, RABX-5, from endosomes in *C. elegans* (Poteryaev et al. 2010). The mammalian homolog, Rabex-5, likely has the same function indicating this activity is conserved throughout eukaryotes (Poteryaev et al. 2010). We wanted to test whether Mon1 alone or whether the Mon1-Ccz1 complex can displace Vps9 from

endosomes in *S. cerevisiae*. Very few puncta are detected in wild type cells expressing genomically tagged Vps9 but we have previously shown that deletion of ESCRT genes such as *VPS4* leads to endosomal accumulation of Vps9 (Russell et al. 2012, Paulsel et al. 2013). We see that neither over expression of Mon1 alone nor co-overexpression of Mon1 and Ccz1 changed the distribution of Vps9 in wild type cells (Figure 4.4A) or *vps4*Δ cells (Figure 4.4B). We recently showed that endosomal localization of Mon1-Ccz1 is lost in *vps4*Δ cells (Russell et al. 2012). Therefore overexpression of Mon1 may not be sufficient to displace Vps9 in *vps4*Δ cells because there is not enough recruited to endosomes. Alternatively, Mon1 may not displace Vps9 from endosomes in yeast cells.

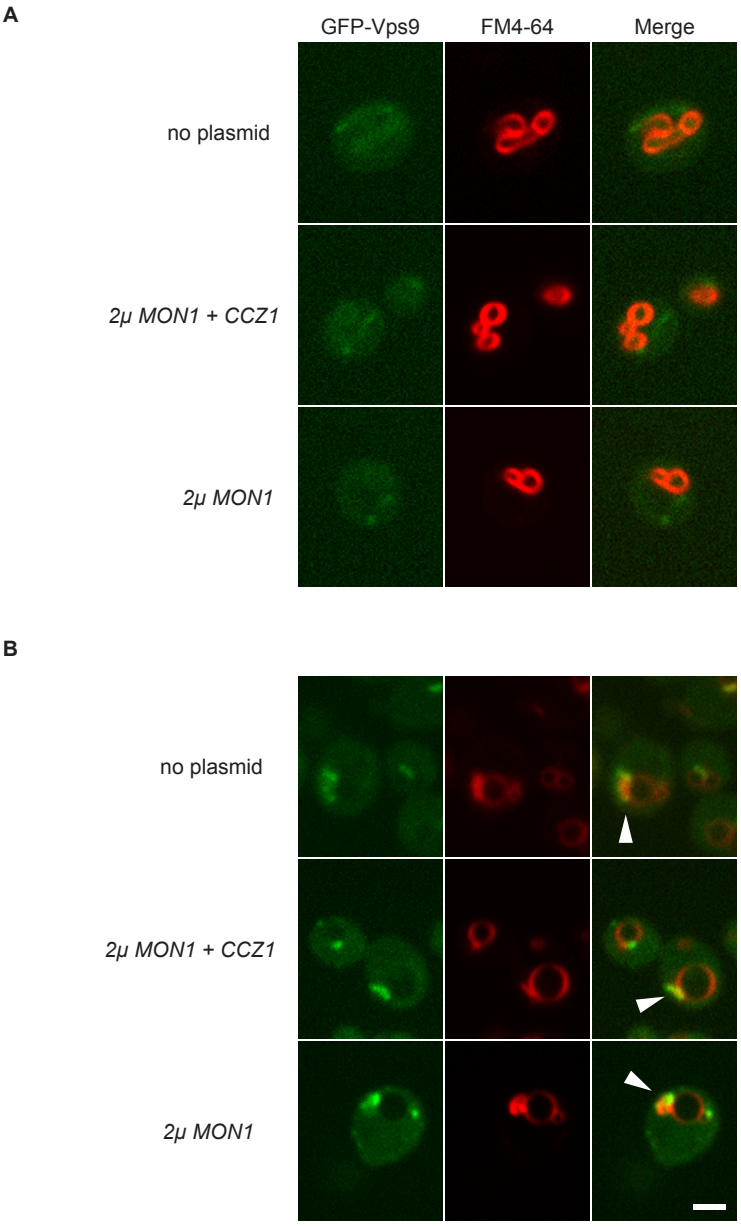


Figure 4.4 Overexpression of Mon1 does not displace Vps9 from endosomes. Genomic integration of Vps9-GFP in (A) wild type and (B) *vps4Δ* cells. Vacuoles are labeled with FM 4-64. Arrow heads show E compartments. Scale bar: 2*μ*

Overexpression of Mon1 does not suppress E compartments

Recently we have shown that class E compartments are the result of increased endosomal fusion driven by Vps21 hyperactivity (Russell et al. 2012). In addition evidence presented in Chapter 3 of this work indicates that Vps9 is the GEF driving Vps21 hyperactivation in ESCRT∆ cells. We speculated that if Mon1 is involved in displacement of Vps9 from endosomes and therefore termination of Vps21 activity then over-expression of Mon1-Ccz1 might suppress class E compartment morphology. As seen in Figure 4.5 neither over expression of Mon1 alone nor Mon1 and Ccz1 suppressed class E-compartments in *vps4*∆ cells. It is not clear whether this is because Mon1 does not displace Vps9 in yeast or simply because Mon1 can not efficiently localize to endosomes in ESCRT-mutants(Russell et al. 2012).

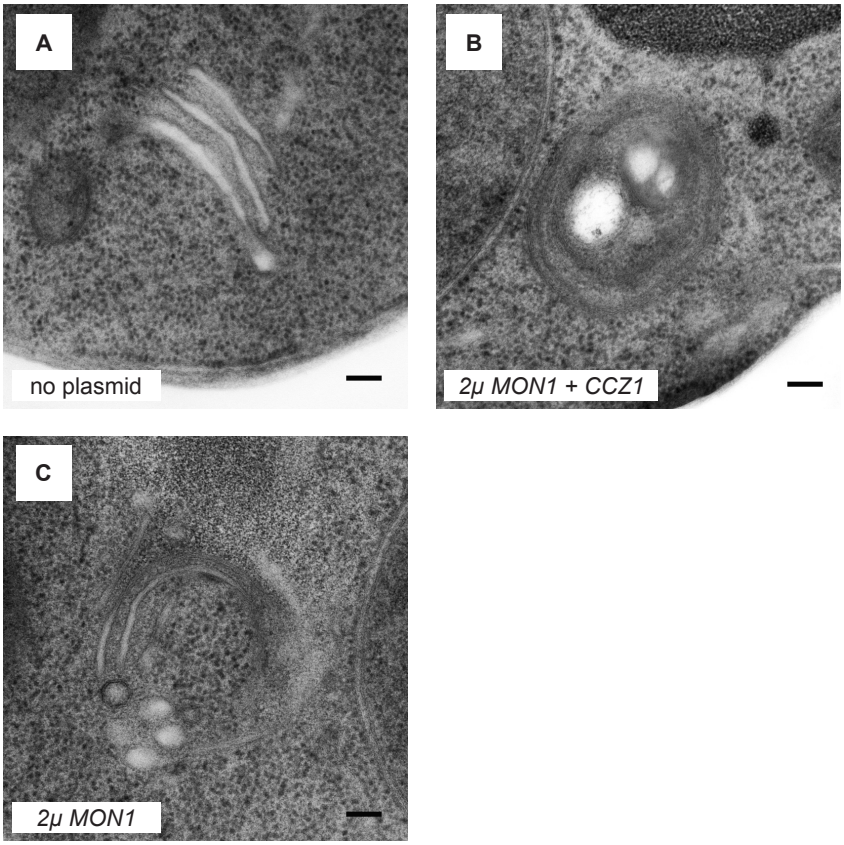


Figure 4.5 Overexpression of Mon1 does not suppress E compartments. Electron micrographs of *vps4Δ* cells with (A) no plasmid (B) MON1 and CCZ1 co-overexpressed from a single 2*μ* plasmid or (C) MON1 expressed from a 2*μ* plasmid. Scale bar: 100 nm

Vps9 accumulates in other vacuole fusion mutants

Based on data presented here it is still unclear whether Mon1 stimulates displacement of Vps9 from endosomes in *S. cerevisiae*. Mon1-Ccz1 is also the GEF for Ypt7 and is, therefore required for heterotypic endosome-to-vacuole fusion as well as homotypic vacuole fusion. We wondered if Vps9 accumulation on endosomes and endosome enlargement could be an indirect effect of failure of endosomes to fuse with vacuoles. Vps41 and Vps39 are components of the HOPS tethering complex required for endosome-to-vacuole fusion (Seals et al. 2000). We see that deletion of either Vps39 or Vps41 causes an accumulation of GFP-Vps9 similar to what is seen in *mon1*Δ cells (Figure 4.6A, 1A). Vam3 is a SNARE protein required for endosome-to-vacuole fusion (Darsow et al. 1997) and we also see an accumulation of GFP-Vps9 puncta in *vam3*Δ cells (Figure 6.6A). This indicates that Vps9 accumulation is a consequence of disrupted endosome-vacuole fusion and not specifically loss of Mon1. In addition deletion of *VAM3* also leads to an increase in MVB diameter, with a mean of 183 nm, similar to *mon1*Δ cells (Figure 4.6B). This indicates that termination of Vps21 activity requires endosome-vacuole fusion.

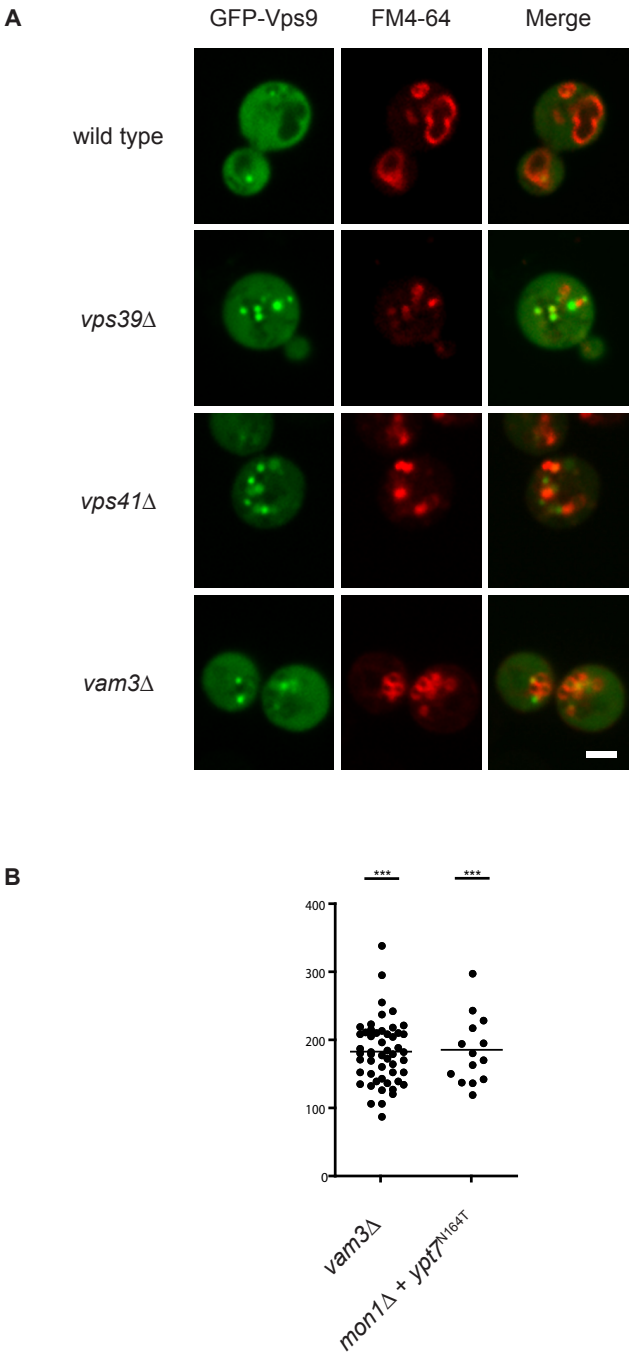


Figure 6. Deletion of other vacuole fusion factors causes endosome enlargement. (A) Confocal microscopy of GFP-Vps9 expressed in indicated strains. Vacuoles are labeled with FM 4-64. Scale bar: 2μ. (B) Quantification of MVB diameters. Means compared to wild type as in Figure 2. *** p value < 0.0001.

We wondered whether Mon1-Ccz1 has a direct role in terminating Vps21 activity in *S. cerevisiae*, separate from its role in endosome vacuole fuison. Results thus far have made it difficult to discern whether disruptions seen in *mon1*Δ cells are direct or simply due to failure to activate Ypt7. Specific mutations in the nucleotide binding site of Ypt7 allow its nucleotide exchange independent of a GEF (McCray et al. 2010). These Ypt7 mutants can support vacuole fusion in the absence of Mon1. In *mon1*Δ cells expressing mutant *ypt7*^{N164T} MVBs still have a larger diameter (mean = 186) indicating this enlargement is due to loss of Mon1 activity toward Vps9 not disruption of endosome to vacuole fusion. Taken together we support a model where termination of Vps21 mediated fusion occurs after ESCRTs mediated sorting and requires Mon1 as well as additional factors downstream of or concurrent with vacuole fusion.

Discussion

Here, we show that loss of *MON1* leads to an accumulation of Vps21 and its GEF Vps9 on cytosolic puncta, as well as an enlargement of endosomes suggesting that Mon1 plays a role in termination of Vps21 activity during endosome maturation. We show that loss of Mon1 does not cause any disruption in ESCRT-mediated vesicle formation or sorting of Cps1 indicating that the transition from Vps21 to Ypt7 occurs after ESCRT-mediated sorting. Overexpression of Mon1 or Mon1 and Ccz1 does not displace Vps9 and does not suppress class E compartments, which are the result of hyperactive Vps21 (Russell et al. 2012). This indicates that Mon1 might not directly displace Vps9 in yeast as has been proposed in *C. elegans*. Surprisingly, deletion of

other endosome-to-vacuole fusion factors, including components of the HOPS tethering complex leads to punctate accumulation of Vps9 similar to what is seen in *mon1*Δ cells. In addition we see that MVBs are also enlarged in *vam3*Δ cells. Taken together this indicates that Vps9 is displaced from endosomes by multiple mechanisms including a potentially novel one involving endosome to vacuole fusion.

Vps21 mediates MVB enlargment

GEFs regulate activation of specific Rabs at the appropriate cellular location. In addition there is evidence that Vps9 helps to recruit Vps21 to endosomes (Blümer et al. 2012, Cabrera & Ungermann 2013). Therefore the timing of Vps9 localization on endosome membranes is critical for ensuring Vps21 is active at the appropriate place and time. In *C. elegans* loss of the Mon1 homolog, SAND-1, leads to increased endosome association of the Vps9 homolog, RABX-5 which results in endosome enlargement and disruption of transport to lysosomes (Poteryaev et al. 2010, Poteryaev et al. 2007). In agreement with this we see that loss of Mon1 leads to increased membrane association of Vps9 and Vps21 as well as an increase in the diameter of MVBs. The increase in MVB diameter in *mon1*Δ and *ccz1*Δ cells is likely due to increased endosome fusion rather than disruption of retrograde transport because deletion of retromer components does not cause endosome enlargement. In addition this increased endosome fusion in *mon1*Δ cells is likely mediated by Vps21, as we see a similar increase in MVB diameter upon overexpression of hyperactive, GTP-locked Vps21. This is similar to what has been reported in mammalian cells where

overexpression of either wild type or GTP-locked Rab5 causes an enlargement of early endosomes and MVBs (Bucci et al. 1995, Wegner et al. 2010).

The enlargement of MVBs in *mon1*Δ and *ccz1*Δ cells was somewhat greater than what is seen by overexpression of hyperactive GTP-locked Vps21. However, overexpression of GTP-locked Vps21 does not prevent fusion of endosomes with vacuoles (Lachmann et al. 2012). In these cells MVBs are likely consumed at the vacuole which may limit the extent of their enlargement. Loss of *MON1*, however, also disrupts endosome-to-vacuole fusion due to its role in activating Ypt7 (Wang et al. 2002). In agreement with this, the number of MVBs in cells overexpressing GTP-locked Vps21 is similar to the number seen in wild type. Loss of *MON1* or *CCZ1* causes an increase in size and number of MVBs indicative of an increase in endosome fusion and a failure of endosome vacuole fusion.

ESCRT activity precedes Rab Conversion

Sorting of transmembrane cargo into intralumenal vesicles of endosomes ensures they are properly targeted to the lumen of the vacuole for degradation. The timing of ESCRT activity during endosome maturation is not precisely known. Deletion of ESCRT genes causes a flattening and stacking of endosomal membranes known as class E compartments (Rieder et al. 1996) and we have shown that this is a result of chronic activation of Vps21 as well as a failure to activate Ypt7 (Russell et al. 2012). In addition we saw that Mon1-Ccz1 fails to localize to endosomes indicating that ESCRT-mediated sorting precedes Rab conversion. In agreement with this we see that loss of Mon1 does not disrupt the formation of intralumenal vesicles or the sorting of Cps1 into

ILVs. Taken together this establishes that ESCRT activity precedes endosomal Rab conversion. The mechanism by which this timing is regulated is unknown but it might serve to prevent fusion with the vacuole until sorting at endosomes is complete. Data presented here and in our previous work indicates that recruitment of Mon1 to endosomes is likely a key factor (Russell et al. 2012). Recruitment of Mon1 is believed to require Vps21 (Nordmann et al. 2010). In addition localization of the *C. elegans* homolog, SAND-1, is thought to require PI(3)P (Poteryaev et al. 2010). Both Vps21 (Russell et al. 2012) and PI(3)P (Nickerson et al. 2012) are enriched at endosomes in ESCRT mutants so there may be other factors required for Mon1 recruitment or there may be factors inhibiting Mon1 binding to endosomes in ESCRTΔ cells. Exploration of how Mon1 binds to endosome will likely provide insights into how Mon1 and ESCRTs are coordinating endosome maturation.

Mechanism of displacement by Mon1.

The Mon1 homolog, SAND-1, is thought to promote endosome maturation by displacing the Rab5 GEF, RABX-5, from endosomes(Poteryaev et al. 2010). We were unable to address whether Mon1 can displace the Vps21 GEF, Vps9, in wild type yeast cells due to the primarily cytosolic localization and low fluorescence intensity of GFP-Vps9. The overexpression of Mon1 or Mon1-Ccz1 did not displace Vps9 from class E compartments in *vps4*Δ cells. In yeast, overexpression of Mon1 has been shown to cause a redistribution of Vps21 to the cytosol consistent with Mon1 being able to displace yeast Vps9 (Nordmann et al. 2010). This difference may be due to expression level as we used a 2μ plasmid and others have used galactose or methionine inducible

systems (Nordmann et al. 2010, Poteryaev et al. 2010). In addition we are looking in the context of *vps4*Δ cells where, as mentioned above, Mon1-Ccz1 fails to localize to endosomes (Russell et al. 2012). In addition we see that overexpression of Mon1 or Mon1-Ccz1 does not suppress the formation of class E-compartments, which form due to hyperactivation of Vps21 (Russell et al. 2012). The failure to displace Vps9 and suppress class E compartments points to three possibilities 1) Mon1 does not directly displace Vps9, 2) overexpression of Mon1 is not sufficient to drive its localization to class E-compartments or 3) Mon1 is blocked from interacting with Vps9 at E compartments. Continued enlargement of MVBs in *mon1*Δ cells upon expression of the GEF independent Ypt7 allele indicates Mon1 has some function in terminating Vps21 mediated fusion at endosomes that is separate from its GEF activity towards Ypt7. The mechanism of RABX-5 displacement by SAND-1 is unknown but previous work suggests it involves the helical bundle of RABX-5 which stabilizes the Vps9 domain and is conserved in all know Vps9 homologs (Poteryaev et al. 2010, Delprato et al. 2004). Therefore the ability of Mon1 to displace Vps9 through a similar mechanism might also be conserved.

Displacement by vacuole fusion

We were surprised to see that the loss of other endosome-to-vacuole fusion factors also results in accumulation of Vps9 puncta. This indicates that vacuolar fusion may be required for displacement of Vps9 and termination of Vps21-mediated fusion activity at endosomes. In support of this MVBs in *vam3*Δ cells are enlarged similar to those in *mon1*Δ cells. Similarily, knockdown of human Vps39 in mammalian cells causes

an enlargement of Rab5-positive endosomes (Rink et al. 2005) indicating that this mechanism might be conserved in other systems. We propose a model where Vps9 displacement occurs just prior to (or concurrent with) vacuole fusion, but how this fusion event might affect Vps9 association with endosomes is unknown. One possibility is that there might be a factor at the vacuole that displaces Vps9 upon endosome fusion. Alternatively initiation of endosome-to-vacuole fusion might somehow cause displacement of Vps9. Fusion in the endocytic pathway is regulated by the CORVET and HOPS tethering complexes which share core subunits between one another (Nickerson et al. 2012). It has been proposed that these complexes transition through an intermediate where the CORVET components dissociate from the complex as endosomes mature and then HOPS specific components associate prior to vacuole fusion (Peplowska et al. 2007). It is intriguing to speculate that there may be a feedback mechanism where transition from CORVET to HOPS signals displacement of Vps9 from endosomes. In this model deletions of HOPS components may favor prolonged assembly of CORVET which could feedback to prolonged Vps9 association and therefore increased endosome fusion.

Endosome maturation involves termination of Vps21 activity which requires cessation of further activation of Vps21 by Vps9 as well as recruitment of the Vps21 GAP, Gyp3 (Lachmann et al. 2012, Nickerson et al. 2012). Recently the BLOC-1 complex in yeast was proposed to inhibit Vps21 by recruiting Gyp3. This, in addition to data presented here, indicates that termination of Vps21 activity and the process of Rab conversion is a complex process that requires coordination of numerous factors including ESCRT-mediated sorting, Mon1-Ccz1 and the HOPS tethering complexes.

Materials and Methods

Yeast strains and plasmid construction

For information see chapter 3

Fluorescence Microscopy

Cells were labeled and imaged as in chapter 3. For each figure identical exposure settings and intensity adjustments were used for each strain or plasmid transformation within that figure.

Electron Microscopy

Samples were prepared as described in chapter3. For MVB diameter measurements structures with less than 3 ILVs per 10,000 nm^2 were excluded. Diameters were measured from the outer leaflet to outer leaflet of the MVB limiting membrane and analyzed by unpaired t-test using Prism5 software (La Jolla, CA).

EM tomography

For tomography, 250 nm thick sections were labeled on both sides with fiduciary 15- nm colloidal gold (British Biocell International). Typically, Z-shrinkage of semi-thick sections was 20 percent volume and corrected in final models and measurements. Dual-axis tilt series were collected from 660° with 1° increments at 200 kV using a Tecnai F20 (FEI-Company, Eindhoven, the Netherlands and Hillsboro, OR) at a magnification of 29,0006 using SerialEM (Mastronarde, 2005). 26 binning on the recording 4K 6 4K CCD camera (Gatan, Inc., Abingdon, UK) creates a 2K 6 2K image with a pixel size of 0.764 nm. Dual-axis electron tomograms (Mastronarde, 1997) of

endosomes and ILVs required the IMOD package (Kremer et al., 1996) for tomogram construction and modeling (3DMOD 4.0.11). Manually assigned contours of the endosomal limiting membrane at the inner leaflet were used to measure the surface of the bilayers periodically every 3.85 nm and calculated using imodmesh. Best-fit sphere models were used to measure the diameters of nearly spherical lumenal vesicles from the outer leaflet of the membrane bilayers (O'Toole et al., 2002). IMODINFO provided surface area and volume data of contour models. Data were sorted, analyzed, and graphed using Microsoft Excel and Prism 5.

Chapter 5. Summary and future directions

Summary

Transmembrane proteins that are degraded in the lysosome, or yeast vacuole, are transported through the MVB pathway. The ESCRT complexes act at the endosomal membrane to direct these proteins into the intralumenal vesicles of MVBs. This ensures that upon fusion with the vacuole these transmembrane proteins are efficiently degraded by the hydrolases in the vacuole lumen. Regulation of fusion by two endosomal Rabs, Vps21 and Ypt7 ensures the directionality of transport, such that endocytic vesicles fuse with early endosomes and late endosomes fuse with lysosomes. How are these processes coordinated to ensure that ESCRT-mediated sorting is complete, prior to fusion with the vacuole? Our recent work shows that disruption of ESCRT-mediated sorting leads to a failure of Rab conversion where Vps21 is chronically active and there is a failure to activate Ypt7 (Russell et al. 2012), thereby preventing fusion with the vacuole until ESCRT mediated sorting is complete.

Evidence presented here indicates endosomal Rab conversion is regulated by two GEFs, Vps9, which activates Vps21 and Mon1-Ccz1, which activates Ypt7 (Figure 5.1) . I find that the class E compartments that form upon ESCRT deletion are the result of increased Vps9 GEF activity. Loss of ESCRT activity leads to an accumulation of ubiquitnated cargo at the endosomal surface (Ren et al. 2008). Here, I present evidence that this increase in ubiquitin at endosomal membranes causes increased recruitment of Vps9 to endosomes by the Vps9 ubiquitin binding, CUE domain. The CUE domain has been proposed to regulate Vps9 activity through autoinhibition

(Donaldson et al. 2003). Data presented here indicates the increased endosomal ubiquitin may promote increased Vps9 GEF activity by relieving inhibition by the CUE domain. Therefore, removal of endosomal ubiquitin, through ESCRT-mediated sorting, may act as a signal for endosome maturation, by promoting removal of Vps9 and therefore, termination of Vps21 activity.

I find that removal of Vps9 from endosomes in *S. cerevisiae* also requires the Mon1-Ccz1 complex, which is consistent with what has been shown in mammalian cells and *C. elegans* coelomocytes (Poteryaev et al. 2010). Mon1-Ccz1, therefore, plays a dual role in promoting endosome maturation by displacing Vps9, and activating Ypt7. I find that Mon1-Ccz1 fails to localize to endosomes when ESCRT activity is disrupted. This suggests that completion of sorting by the ESCRT complexes can signal endosome maturation through recruitment of Mon1-Ccz1, as well as removal of ubiquitin. These mechanisms, by which ESCRT regulates endosome maturation, could serve to ensure transmembrane proteins destined for degradation in the lysosome, or yeast vacuole, are sorted prior to fusion with the vacuole.

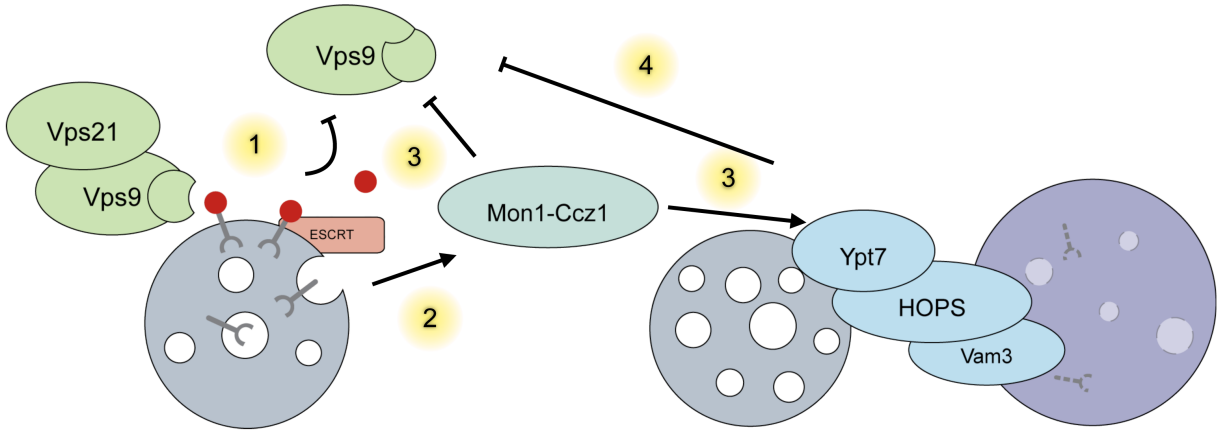


Figure 5.1 Multiple mechanisms promote endosomal maturation. 1. Removal of ubiquitin by ESCRTs leads to dissociation of Vps9. 2. Completion of ESCRT-mediated sorting promotes recruitment of Mon1-Ccz1. 3. Mon1-Ccz1 has a dual role in promoting endosome maturation by promoting displacement of Vps9 as well as activation of Ypt7. 4. Endosome to vacuole fusion may also promotes removal of Vps9 from endosomes.

Vps9 recruitment to endosomes

Recently it has been shown that Vps9 helps recruit Vps21 to endosomes. We have demonstrated here that ubiquitin binding by the CUE domain is important for Vps9 binding to endosomes but Vps9 that lacks the CUE domain can also localize to endosomes. This recruitment is sufficient to generate MVBs and E compartments, which requires Vps9 and Vps21. An understanding of how the Vps9 domain binds endosomes could provide insights into regulation of Vps9 and Vps21 activity. We show that a truncated form of Vps9 containing the Vps9 domain and its associated helical bundle can bind endosomes. We still need to address whether the helical bundle, which is required GEF activity, is required for the Vps9 domain to bind endosomes but this should be relatively straightforward. To address what this minimal localization domain is binding to *in vivo* we could immunoprecipitate this domain from yeast and perform mass spectrometry to determine what proteins it is bound to. It is possible that the Vps9 domain binds lipids at the endosomes rather than protein. This could be addressed using an *in vitro* liposome binding assay where recombinant Vps9 truncations are added to liposomes with varying lipid composition.

How is Vps9 activity regulated?

The data presented here suggest that the CUE domain is inhibiting Vps9 activity. The rationale for this is that *vps9*$^{\Delta CUE}$ and the Vps9 ubiquitin binding mutant (*vps9*^{M419D}) can still support the formation of E compartments despite their reduced localization to endosomes. This is consistent with *in vitro* studies showing the the Vps9 domain alone

has higher GEF activity towards Rab5 (Delprato et al. 2004). However, Keran-Kaplan et al. reported that inhibition does not occur by cis-ubiquitin binding of the CUE domain in an *in vitro* exchange assay. There is still not a good assay for determining Vps9 activity *in vivo*. Increased Vps9 activity would result in higher levels of GTP-bound Vps21. We have previously used a GDI extraction assay to asses the levels of Vps21-GTP at membranes. In this assay the extent to which purified GDI can extract Vps21 from membranes provides an indication of the amount of Vps21-GDP. This assay is not quantitative and might not detect minor difference in the amount of Vps21 extracted by GDI. Another read-out for Vps21 activity is binding to the CORVET complex. The CORVET subunit, Vps8, binds specifically to Vps21-GTP (Markgraf et al. 2009). We are currently working on an assay to purify tagged Vps8 from yeast to detect Vps21 binding. This assay could then be used to detect Vps21-Vps8 interaction in cells expressing Vps9 mutants to determine how they affect Vps21 activity.

Does Mon1 directly regulate Vps9?

It is still unclear whether Mon1-Ccz1 promotes dissociation of Vps9 by directly displacing it from membranes. Poteryaev et al. showed that recombinant human Mon1 could displace Rabex-5 from endosomes that were purified from HeLa cells. In this assay Mon1 could be displacing Rabex-5 from endosomes through an indirect mechanism involving additional endosomal factors. For example, Mon1 might be promoting assembly of HOPS through increased activity of Rab7. In addition, there still has not been any demonstration that yeast Mon1-Ccz1 can displace Vps9 from membranes. An *in vitro* liposome binding assay could address this question. This

system would required purification of recombinant Vps9 and Mon1-Ccz1 which has been achieved elsewhere (Keren-Kaplan et al. 2012, Poteryaev et al. 2010).

How is Mon1-Ccz1 recruited to endosomes?

Recruitment of Mon1-Ccz1 to endosomes promotes endosome maturation by displacing Vps9 and by recruiting and activating Ypt7 (Poteryaev et al. 2007, Cabrera & Ungermann 2013). The timing and mechanism of Mon1-Ccz1 endosome binding is not precisely known. In *C. elegans* SAND-1 was shown to bind PI(3)P whereas recruitment of Mon1-Ccz1 in yeast requires Vps21. Both Vps21 and PI(3)P are markers of early endosomes. In addition we showed that Mon1-Ccz1 is not recruited to endosomes in ESCRT mutant cells, where both Vps21 and PI(3)P are enriched on endosomes. Therefore, there is likely an additional factor that regulates Mon1-Ccz1 recruitment after ESCRTs have completed sorting of ubiquitinated cargo. Mon1 and Ccz1 contain N-terminal longin domains which are required for Mon1 and Ccz1 to bind one another. The structure and function of the C-terminus of these proteins is unknown. It would be useful to determine if either of the longin domains or C-terminal domains can localize to endosomes independent of the Mon1-Ccz1 complex. If such a minimal binding domain could be isolated that domain could be used in a liposome binding assay to determine if it can bind lipids. In addition the minimal binding domain might interact with a protein. This could be tested by immunoprecipitation and mass spectrometry or yeast two hybrid against an array of endosomal proteins.

Vps9 displacement by vacuole fusion

The finding that Vps9 accumulates at endosomes in other vacuole fusion mutants suggests that either initiation or completion of vacuole fusion is required for displacement of Vps9 and therefore termination of Vps21 mediated fusion. One possibility is that the docking stage MVB-vacuole fusion provides a signal for displacement of Vps9. Mutants of the vacuole SNARE Vam7 allow partial zippering of the trans-SNARE complex which does not inhibit vacuole docking *in vitro* but it does inhibit membrane mixing (Schwartz & Merz 2009). If MVB to vacuole docking is sufficient for displacement of Vps9 expression of this mutant Vam7 should not result in punctate accumulation of Vps9 and enlargement of MVBs.

Appendix

Table A.1 Yeast strains used in this work		
Strain	**Genotype**	**Reference**
SEY6210	MATa *leu2-3,112 ura3-52 his3200 trp1901 lys2801 suc29*	(Robinson et al., 1988)
BHY10	SEY6210; *leu2-3,112*::pBHY11 (*CPY-INV::LEU2*)	(Horazdovsky et al., 1994)
MBY3	SEY6210; *vps4::TRP1*	(Babst et al., 1997)
GOY223	BHY10; *vps9::HIS3*	This study
GOY224	GOY223; *vps4Δ::TRP1*	This study
GOY100	BHY10; *doa4^{C571S}*	(Richter et al., 2007)
GOY65	SEY6210; *bro1Δ::HIS3*	(Luhtala and Odorizzi, 2004)
CRY195	SEY6210; *bro1Δ::HIS3; rsp5^{G555D}*	This study
DNY516	BHY10; *muk1Δ::KANMX6*	(Paulsel et al. 2013)
TSY23	DNY516; *vps4::TRP1*	This study
GOY243	BHY10; *mon1Δ::HIS3*	This study
DNY223	SEY6210; *GFP-VPS21::URA3 CPY-INV::LEU2*	(Russell et al., 2012)
TSY12	DNY223; *mon1Δ::KAN*	This study
TSY16	SEY6210; *ccz1Δ::KAN*	This study
BHY152	SEY6210; *vps5Δ::HIS3*	Matt Seaman
DNY137	SEY6210: *CPY-Inv::LEU, vps35Δ::KAN*	This study
GOY274	BHY10; *VPS9-GFP::HIS3*	(Russell et al., 2012)
GOY275	MBY3; *VPS9-GFP::HIS3*	(Russell et al., 2012)
	SEY6210; *vps39Δ*	Scott Emr
WSY41	SEY6210; *vps41Δ::LEU2*	Scott Emr
TDY2	SEY6210; *vam3Δ::LEU2*	Scott Emr

Table 3.2 Plasmids used in this work		
Plasmid	**Description**	**Reference**
pBD1	*URA3* Ap^R (pRS416) *VPS9*	(Davies, 2003)
pBD3	*URA3* Ap^R (pRS416) $vps9^{\Delta CUE}$	(Davies, 2003)
pBD4	*URA3* Ap^R (pRS416) $vps9^{M419D}$	(Davies, 2003)
pGO656	*URA3* Ap^R (pRS416) $vps9^{D251A}$	This study
pGO741	*URA3* Ap^R (pRS416) $VPS9^{E288A}$	This study
pGO763	*URA3* Ap^R (pRS416) $VPS9^{D251A\ E288A}$	This study
pCB243	*URA3* Ap^R (pRS426) *GFP-VPS9*	Chris Burd
pGO730	*URA3* Ap^R (pRS426) *GFP-*$vps9^{M419D}$	This study
pGO678	*URA3* Ap^R (pRS426) *GFP-*$vps9^{\Delta CUE}$	This study
pGO737	*URA3* Ap^R (pRS426) *GFP-vps9-1-167*	This study
pGO723	*URA3* Ap^R (pRS426) *GFP-vps9-CUE*	This study
pGO729	*URA3* Ap^R (pRS426) *GFP-vps9-*CUE^{M419D}	This study
YEp96	*TRP1* Ap^R (pYSK102) Ubiquitin	Ecker et al., 1987
pCR135	*TRP1* Ap^R (pYSK102) $Ubiquitin^{I44A}$	This study
pDN144	*URA3* Ap^R (pRS426) *vps21Q66L*	(Russel et al., 2012)
pGO45	*URA3* Ap^R (pRS426) *GFP-CPS1*	(Odorizzi et al., 1998)
pTS14	*URA3* Ap^R (pRS426) *MON1*	This study
pGO654	*URA3* Ap^R (pRS426) *MON1 + CCZ1*	This study
pGO653	*URA3* Ap^R (pRS426) $ypt7^{N164T}$	This study

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