Regulators of Vps4 ATPase Activity at Endosomes Differentially Influence the Size and Rate of Formation of Intralumenal Vesicles

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Recruitment of endosomal sorting complexes required for transport (ESCRTs) to the cytosolic face of endosomes regulates selective inclusion of transmembrane proteins into the luminal vesicles of multivesicular bodies (MVBs). ESCRT-0, -I, and -II bind directly to ubiquitinated transmembrane cargoes of the MVB pathway, whereas polymerization of ESCRT-III at endosomes is thought to bend the membrane and/or provide the energetic force that drives membrane scission and detachment of vesicles into the endosome lumen. Disassembly of the ESCRT-III polymer and dissociation of its subunits from endosomes requires the Vps4 ATPase, the activity of which is controlled in vivo by regulatory proteins. We identify distinct spatiotemporal roles for Vps4-regulating proteins through examinations of subcellular localization and endosome morphology. Did2 plays a unique role in the regulation of MVB luminal vesicle size, whereas Vta1 and Vps60 promote efficient membrane scission and delivery of membrane to the endosome lumen. These morphological effects probably result from Vps4-mediated manipulations of ESCRT-III, because we show dissociation of ESCRT-0, -I, and -II from endosomes is not directly dependent on Vps4 activity.

INTRODUCTION

Transmembrane proteins ubiquitinated on their cytosolic domains are sorted into the luminal vesicles of multivesicular bodies (MVBs) and are subsequently degraded upon fusion of MVBs with vacuoles/lysosomes. Packaging of ubiquitinated transmembrane protein cargoes into MVB vesicles is mediated by endosomal sorting complexes required for transport (ESCRTs), which are highly conserved and recruited transiently from the cytosol to endosomal membranes (Hurley and Emr, 2006; Williams and Urbe, 2007). ESCRT-0, -I, and -II bind directly to ubiquitinated MVB cargoes (Katzmann et al., 2001; Bilodeau et al., 2002; Alam et al., 2004). In contrast, ESCRT-III consists of four paralogous proteins that assemble on endosomes into a polymer (Babst et al., 2002a) that might deform the membrane toward the lumen (Hanson et al., 2008) and/or execute vesicle scission (Wollert et al., 2009). ESCRT-III also serves as a scaffold for the Bro1-Doa4 deubiquitination machinery that recycles ubiquitin from MVB cargoes before vesicle formation (Luhtala and Odorizzi, 2004).

Dissociation of ESCRTs from endosomes enables repeated rounds of MVB sorting and requires the ATPase Vps4. ATP-bound Vps4 assembles into a double-ring oligomer at endosomes, where it binds directly to ESCRT-III (Babst et al., 1998; Yeo et al., 2003; Nickerson et al., 2006; Obita et al., 2007).

ATP hydrolysis by Vps4 disassembles ESCRT-III and releases its subunits to the cytosol (Babst et al., 2002a). Loss of Vps4 activity not only traps ESCRT-III polymers on endosomes but also prevents dissociation of ESCRT-0, -I, and -II (Katzmann et al., 2001; Babst et al., 2002b; Bilodeau et al., 2002). However, it is unknown whether ESCRT-0, -I, and -II are direct Vps4 substrates, are indirectly dependent on Vps4-mediated disassembly of ESCRT-III, or require another process affected by Vps4.

In vitro studies have characterized five proteins that regulate Vps4 catalytic activity. Vta1 is a positive regulator that binds the catalytic domain of Vps4 (Yeo et al., 2003) to promote oligomerization of Vps4 and ATP hydrolysis (Azmi et al., 2006; Lottridge et al., 2006). Two ESCRT-III paralogues, Did2 and Vps60, are not core subunits of the ESCRT-III polymer but enhance Vta1-mediated stimulation of Vps4 through interactions with the microtubule interaction and transport (MIT) domain of Vta1 (Azmi et al., 2008). Did2 and Vps2 (a core ESCRT-III subunit) also stimulate ATP hydrolysis independently of Vta1 through interaction with the MIT domain of Vps4 (Azmi et al., 2008). Ist1 counterbalances these positive regulators to inhibit assembly of Vps4 into its active oligomeric state (Dimanno et al., 2008).

The functional significance of and coordination among Vps4 regulators in vivo is unknown. To address this issue, we compared ESCRT-III disassembly and endosome morphology in yeast mutants lacking regulators in isolation and in combination. Our results suggest a spatiotemporal separation of Did2 and Vta1-Vps60 functions manifested in distinct phenotypes upon their disruption. ESCRT-III disassembly is more strongly dependent on Did2 than it is on Vta1-Vps60, and the stage at which Did2 promotes Vps4 activity more strongly impacts the size of lumenal vesicles. In contrast, the functions of Vta1 and Vps60 are more closely tied to efficient membrane scission and delivery of vesicles
into the endosome lumen. We further show evidence suggesting that dissociation of ESCRT-0, -I, and -II is not directly mediated by Vps4 activity but instead is dependent upon events leading to normal MVB biogenesis.

MATERIALS AND METHODS

Yeast Strain and Plasmid Construction

Standard protocols were used to construct all yeast strains and plasmids described in Supplemental Table S1 (Longtine et al., 1998).

Microscopy and Imaging

Fluorescence microscopy of cells labeled with N-[3-(triethylammoniumpropyl]-[4-[diethylaminophenylhexatrienyl] pyridinium dibromide (FM 4-64) as well as FM 4-64, was performed as described previously (Luhtala and Odorizzi, 2004). Cryofixation of cells, electron tomography, and three-dimensional modeling has been described previously (Nickerson et al., 2006). Log phase cells were high-pressure frozen and freeze-substituted at ~90°C until fixed (Winey et al., 1995; Giddings, 2003) using a Leica automated freeze-substitution (AFS) system. Standard media for tomography and immunolabel was 0.1% uranyl acetate and 1% glutaraldehyde in 0.1 M cacodylate buffer, 2.5% glutaraldehyde was sometimes used to ensure morphological preservation. In brief, samples were warmed in the Leica AFS system from liquid nitrogen temperatures to ~90°C and incubated 3 d. Samples were washed in additional anhydrous acetone for a day while warmed to ~50°C and then embedded in Lowicryl HM20 over a period of 3 d. Embedded samples were polymerized under UV radiation at ~50°C and slowly warmed to room temperature over 4 d. Plastic blocks were sectioned (150–300 nm) on a Leica ultramicrotome and placed on rhodium-plated copper slot grids with Formvar. Sections were typically assigned contours of the endosomal limiting membrane at the inner leaflet were used to measure the surface periodically every 3.85 nm and calculated using imod software. Digital images were processed using Slidebook (Intelligent Imaging Innovations, Denver, CO) and Photoshop 7.0 (Adobe Systems, Mountain View, CA). Statistical analyses were performed using Prism 4.0 (GraphPad Software, San Diego, CA). Vessel size and lumenal membrane surface area data were analyzed by analysis of variance (ANOVA) (Newman–Keuls multiple comparison) and t tests, respectively. For fluorescence microscopy, electron microscopy, and tomography (EM) analyses of MVB cargo sorting and endosome morphology summarized in Table 1, a minimum of 100 cells were examined for each genetic background.

Endosomal Sorting Assays, Subcellular Fractionation, Immunoprecipitation, and Western Blotting

Colorimetric plate overlay assays for secretion of the carboxypeptidase Y (CPY)-inverterase reporter has been described previously (Daros et al., 2000). Vacular green fluorescent protein (GFP)-cleavage assays were performed by transforming yeast with plasmids encoding GFP-tagged MVB cargo and/or GFP-tagged VPS60 and growing cells to log phase in selective, synthetic media. Cell pellets were washed in chilled water and precipitated using ice-cold 10% trichloroacetic acid. Protein precipitates were washed in acetone and resuspended in Laemmli sample buffer. SDS-polyacrylamide gel electrophoresis resolved 0.5 OD600 unit equivalent of each sample before Western blot analysis. Separation of cell lysates into endosomal membrane pellets and soluble cytosolic fractions has been described previously (Odorizzi et al., 2003). Anti-GFP immunoprecipitations were performed essentially as described previously (Luhtala and Odorizzi, 2004). Antibodies used in Western blotting include polyclonal anti-Vps4 (Babst et al., 1997), polyclonal anti-Snm7 (Babst et al., 1998), polyclonal anti-Vps24 (Babst et al., 1998), monoclonal anti-Port (Invitrogen, Carlsbad, CA), monoclonal anti-3-phosphoglycerate kinase (PGK) (Invitrogen), monoclonal anti-Vph1 (Invitrogen), and monoclonal anti-Vps24 (Roche Diagnostics, Indianapolis, IN). All Western blots were analyzed by chemiluminescence and film exposure except immunoprecipitations of GFP-Did2 and GFP-Vps60 and subcellular fractions in Figure 2A, which were analyzed using a Odyssey fluorescence scanner (LI-COR Biosciences, Lincoln, NE). Quantifications of relative protein abundance in subcellular fractionation Western blots were performed using Odyssey software (LI-COR Biosciences) and statistically examined by ANOVA (Newman–Keuls multiple comparison) using Prism 4.0 (GraphPad Software).

RESULTS

Vta1 Recruits Vps60 to Endosomes of Did2-Ist1

Vta1 and Vps60 form a complex at endosomes (Shiflett et al., 2004), interacting with one another via the N-terminal domain of Vta1 (Xiao et al., 2008) and the C-terminal domain of Vps60 (Azmi et al., 2008). Although Vta1 is recruited to endosomes primarily via its interaction with Vps4 (Azmi et al., 2006), Vps60 is recruited to endosomes by interacting with Vta1 (Azmi et al., 2008). We constructed a GFP-Vps60 fusion protein that fully rescued endosomal sorting defects in cells lacking wild-type Vps60 (Supplemental Figure S1). Vps60 in wild-type cells is both cytosolic and associated with multiple, small endosomal puncta, whereas in mutant cells lacking the VPS4 gene (vps4A), its cytosolic distribution seems unchanged, but its punctate localization coalesces to fewer and larger structures that correspond to class E compartments (Azmi et al., 2008), which are aggregates of endosomes having an aberrant morphology (see below). We observed a striking redistribution of GFP-Vps60 from the cytosol to class E compartments in cells expressing Vps4E233Q (Figure 1A), a catalytically inactive version of Vps4 irrevocably associated with endosomes (Babst et al., 1998). A similar redistribution of Vta1-GFP from the cytosol to class E compartments also occurred in vps4A cells (Figure 1B) but did not in vps4A cells (Azmi et al., 2006). Thus, endosomal recruitment for Vps60 mirrors that of Vta1 and is promoted by Vps4.

Unlike Vta1 and Vps60, Did2 and Ist1 strongly shift from the cytosol to class E compartments in both vps60Δ and vps60A cells (Nickerson et al., 2006; Dimaano et al., 2008). Endosomal recruitment of Did2 is mediated through its interaction with the Vps24 subunit of ESCRT-III (Nickerson et al., 2006) and recruitment of Ist1 occurs through its binding to Did2 (Dimaano et al., 2008; Rue et al., 2008). Although Vta1 is recruited to endosomes primarily through its association with Vps4, the small amount of Vta1 at endosomes in vps4A cells requires Did2, which directly binds Vta1 (Azmi et al., 2008). Deletion of DID2 reduced the residual amount of GFP-Vps60 at endosomes in vps60A cells (data not shown), suggesting recruitment of Vps60 occurs via its interaction with Vta1. Indeed, deletion of VTA1 strongly reduced endosomal localization of GFP-Vps60 in vps60A cells (Figure 1A), whereas deletion of VPS60 had no effect on the strong redistribution of Vta1-GFP to class E compartments in vps60A cells (Figure 1B).

Neither Vta1 nor Vps60 were required for endosomal recruitment of GFP-Did2 (Figure 1, C and D) or Ist1-GFP (Rue et al., 2008). Rather, deletion of VTA1 or VPS60 enhanced localization of GFP-Did2 at endosomes (Figure 1, C and D), presumably due to reduced stimulation of Vps4 in cells lacking functional Vta1-Vps60 (Azmi et al., 2008). Although Vta1 is recruited to endosomes downstream of ESCRT-III–mediated recruitment of Did2-Ist1.

Vps60 Functions Downstream of Did2 to Promote Vps4-mediated ESCRT-III Disassembly

In vitro, Did2 and Vps60 equally enhance Vta1-mediated stimulation of Vps4 ATase activity (Azmi et al., 2008), but recruitment of Vps60 downstream of Did2 (Figure 1) suggests their functions might be spatiotemporally distinct. We analyzed the disassembly of ESCRT-III in vivo by subcellular fractionation and found its Vps24 and Smn7 subunits predominantly in the soluble fraction in wild-type cell lysates, which reflects the steady-state balance between mo-
nomeric cytosolic subunits versus subunits assembled into ESCRT-III at endosomes (Babst et al., 1998). In contrast, both Vps24 and Snf7 are predominantly membrane-associated in vps4Δ cells (Babst et al., 1998) and to a lesser extent in did2Δ cells, indicating Did2 has a major role in promoting Vps4 activity (Nickerson et al., 2006). We found significantly less ESCRT-III to be membrane-associated in vta1Δ, vps60Δ, and vta1Δ vps60Δ cells compared with did2Δ cells (Figure 2A; one-way ANOVA for Snf7, p < 0.001 each). Moreover, significantly less Vps24 and Snf7 remained membrane associated in cells lacking Vta1, Vps60, or both compared with did2Δ vta1Δ (Figure 2A; Snf7, p < 0.001 each; Vps24, p < 0.05 each) and did2Δ vps60Δ cells (Figure 2A; Snf7, p < 0.001 each; Vps24, p < 0.01 each). These results demonstrate that loss of Did2 function is epistatic to the loss of Vta1 and/or Vps60, signifying that disassembly of ESCRT-III relies more strongly on Vps4 stimulation by Did2 than it does on stimulation by Vta1-Vps60. In addition, did2Δ vta1Δ and did2Δ vps60Δ cells accumulated significantly more Snf7 in the membrane-associated fraction compared with did2Δ cells (Figure 2A; p < 0.01 each), consistent with a synthetic defect in which loss of Did2 and either Vta1 or Vps60 more closely phenocopies loss of Vps4 (Dimaano et al., 2008; Rue et al., 2008). That Vps24 does not show a similar sensitivity to the loss of both Did2 and either Vta1 or Vps60 might indicate differing requirements for Vps4 regulators in dissociation of the Vps20-Snf7 and Vps2-Vps24 subcomplexes of ESCRT-III.

Fusion of GFP to the C termini of Vps60 or Did2 results in chimeras with compromised function, evidenced by reduced efficiency in dissociation of Snf7 and Vps24 from endosomes (Figure 2B; data not shown). The C termini of ESCRT-III proteins mediate autoinhibitory intramolecular binding to prevent their spurious polymerization and membrane recruitment, and C-terminal fusion of GFP disrupts this autoinhibition, causing the chimeras to accumulate at endosomes (Lin et al., 2005; Zamborlini et al., 2006; Shim et al., 2007). Although subcellular fractionation indicated the characteristic membrane accumulation of both Vps60-GFP (Figure 2B) and Did2-GFP (Nickerson et al., 2006) in wild-type cells, we were surprised to find that, unlike Did2-GFP (Nickerson et al., 2006), Vps60-GFP shifted to the cytosol in vps4Δ cells (Figure 2B). This result demonstrates that even

Figure 1: Vta1 recruits Vps60 to endosomes. Fluorescence and differential interference contrast (DIC) microscopy of FM 4-64-stained cells expressing GFP-Vps60 (A) or Vta1-GFP (B), or unstained cells expressing GFP-Did2 (C). Closed arrowheads, colocalization of GFP with FM 4-64; open arrowheads, weak or absent colocalization. Bar, 2 μm. (D) Subcellular fractionation and Western blot analysis of yeast cell lysates. PGK and Por1 (mitochondrial porin) were used as cytosolic and membrane-bound controls, respectively. T, total lysate. P13, membrane-associated 13,000 × g pellet fraction. S13, cytosolic 13,000 × g soluble fraction.
when Vps60 is relieved of autoinhibition, its recruitment to endosomes requires Vps4, which underscores the unique status of Vps60 within the ESCRT-III family: whereas Did2 and the core ESCRT-III subunits (Vps20, Snf7, Vps2, and Vps24) localize strongly at endosomes without Vps4 (Babst et al., 1998, 2002a; Nickerson et al., 2006), Vps60 localizes to endosomes downstream of Vps4 recruitment and is not prone to polymerize with other ESCRT-III family members. Immunoprecipitations of functional, N-terminal GFP chimeras of Did2 and Vps60 reinforce this point. ESCRT-III subunits Snf7 and Vps24 readily copurified with immunoprecipitated Did2 (Figure 2C), and this interaction was enhanced in the absence of Vps4, likely due to polymerization of Did2 and ESCRT-III at the endosome. In contrast, Vps60 pulled down little to no ESCRT-III either in the presence or absence of Vps4. In summary, Did2 plays a more central role in dissociation of ESCRT-III than either Vta1 or Vps60 due to a physical interaction with ESCRT-III that Vta1 and Vps60 do not seem to share in vivo. When we further consider that Did2 bridges interactions between ESCRT-III and Vps4 (Nickerson et al., 2006), with the Vps4-stimulator, Vta1 (Lottridge et al., 2006), and with the Vps4-inhibitor, Ist1 (Dimaano et al., 2008; Xiao et al., 2009), the evidence suggests that Did2 occupies a key hub in the management of ESCRT-III dynamics by Vps4.

**Distinct Roles for Did2-Ist1 and Vta1-Vps60 in MVB Biogenesis**

Unlike spherical MVBs in wild-type cells (Figure 3, A and B; Supplemental Video 1), mutants lacking a functional ESCRT machinery have class E compartments, which are flattened endosomes juxtaposed closely against one another (Rieder et al., 1996). Using electron tomography and three-dimensional modeling, we showed previously the absence of luminal vesicles in class E compartments of vps4Δ cells, whereas did2Δ cells have crowded, distended endosomes with luminal vesicles, which we termed “vesicular tubular endosomes,” or VTEs (Nickerson et al., 2006). The persistence of luminal vesicles in didΔ cells indicated that efficient disassembly of ESCRT-III is not strictly required for lumenal vesicle budding, a conclusion supported by subsequent in vitro reconstitution of lumenal vesicle formation (Wollert et al., 2009). We also observed VTEs in cells lacking the other positive Vps4 regulators, Vta1 and Vps60 (Figure 3, C–H, and Supplemental Videos 2–4), whereas cells lacking Ist1 (a negative regulator of Vps4) displayed no defects in general MVB morphology or cargo sorting (Figure 3, I and J, and Supplemental Video 5), suggesting Ist1-mediated inhibition of Vps4 activity is dispensable for MVB function. Nonetheless, deletion of IST1 or DID2 strongly exacerbate the morphological and cargo sorting defects in vta1Δ and vps60Δ cells (Table 1). Simultaneous deletion of both DID2 and VTA1 produce the strongest synthetic phenotypes (Table 2), including formation of class E compartments (Figure 3, K and L, and Supplemental Video 6), which reflects the roles of Did2 in recruiting Ist1 (Dimaano et al., 2008; Rue et al., 2008) and Vta1 in recruiting Vps60 (Figure 1A). In contrast, simultaneous deletion of DID2 and IST1 or of VTA1 and VPS60 produce no synthetic phenotypes, consistent with Did2-Ist1 and Vta1-Vps60 comprising distinct Vps4 regulatory branches (Dimaano et al., 2008; Rue et al., 2008). These results are consistent with genetic relationships derived from a previous EM analysis (Rue et al., 2008), although improvements in sample preservation and fixation methods allow us to detect luminal membrane structures to uncover the following morphological distinctions among Did2-Ist1 and Vta1-Vps60 mutants.
The observation of VTEs in cells lacking individual Vps4 regulators indicate neither Did2-Ist1 nor Vta1-Vps60 are strictly required for the formation of lumenal vesicles, but measurement of vesicle sizes in tomographic models revealed differential misregulations of this process. Although lumenal vesicles in wild-type MVBs had a mean diameter of 24 nm (Figure 3M), we found a modest increase (28 nm) in *vta1Δ vps60Δ* mutants (one-way ANOVA; p < 0.001 for each). In stark contrast, lumenal vesicles in *did2Δ* and *did2Δ ist1Δ* cells were much larger, averaging 36 nm in diameter and frequently ranging above 50 nm (Nickerson et al., 2006). This unique swelling in vesicle size suggests the early stage at which Did2 stimulates Vps4 is critical in regulating the timing of membrane scission potentially executed by the ESCRT-III polymer.

We further measured the surface areas of endosome luminal and limiting membranes to gauge the efficiency of membrane delivery to the endosome lumen. Our selected metric, in which we express luminal membrane surface area as a share of the total, applies a consistent standard across different endosome diameters and volumes while accommodating variations in limiting membrane topology and luminal vesicle size. Wild-type MVBs display an equal distribution of surface area between the limiting and lumenal membranes (Figure 3N), but mean luminal membrane content of endosomes in *vta1Δ vps60Δ* cells fell to 32%, 34% and 27%, respectively (t test; p = 0.0011, 0.0051, and 0.0001). These reductions occur despite the modest increases in mean vesicle size in these mutants (Figure 3M).

Our interpretation of the quantitative tomographic data considered the possibility that the increased ratio of limiting versus luminal membrane content arose from a defect in retrograde trafficking from endosomes. A reliable indicator of endosomal retrograde trafficking is Vps10, a transmem-
brane receptor that transports its soluble ligand, CPY, from the Golgi to endosomes, where Vps10 releases CPY and subsequently recycles to the Golgi (M arcussion et al., 1994; P iper et al., 1995). Disruption of the ESCRT machinery traps Vps10 at endosomes, as evidenced by the strong localization of Vps10-GFP at class E compartments in \( \text{vps10}^- \) cells (Figure 4A). The consequence of Vps10 being unable to recycle from endosomes to the Golgi is the secretion of newly synthesized CPY, which can be detected by expression of a CPY-invertase fusion protein (Figure 4C). In contrast with CPY, which can be detected by expression of a CPY-invertase fusion protein, the secretion of newly synthesized did2/ist1/vta1/vps60 cells exhibited an enhanced degree of cleavage in \( \text{vps60}^- \) cells (Figure 4B) due to retention at endosomes and the inappropriate maturation of luminal vacuolar hydrolases at the class E compartment (Babst et al., 2002a). No enrichment of a Vps10 cleavage product was observed in \( \text{did2}^- \), \( \text{vta1}^- \) or \( \text{vps60}^- \) cells (Figure 4B). Thus, we conclude that the increased ratio of limiting versus luminal membrane content at endosomes of VTEs in cells lacking \( \text{vta1}^- \) or \( \text{vps60}^- \) is derived from a reduction in the budding of luminal vesicles rather than a defect in recycling of limiting membrane away from endosomes.

In contrast with the above-mentioned mutants lacking positive regulators of \( \text{vps4}^- \) activity, deletion of \( \text{ist1}^- \) significantly boosts the luminal membrane content of endosomes relative to that observed in wild-type cells [61% (t test; \( p = 0.0099 \)]. Although the modest increase in \( \text{ist1}^- \) vesicle size (Figure 3M) no doubt contributes to this increased luminal content, \( \text{ist1}^- \) endosomes routinely seem to have achieved their maximal capacity for luminal vesicles. Indeed, \( \text{ist1}^- \) luminal vesicles frequently occur in apposition to each other and the limiting membrane, potentially pressed together due to luminal space constraints. Therefore, although our data do not show an increase in the number of \( \text{ist1}^- \) luminal vesicles compared with wild type, deletion of \( \text{IST1} \) causes accumulation of vesicles that seems to be limited only by the carrying capacity of the endosome. In \( \text{did2}^- \) cells and \( \text{did2}^- \) \( \text{ist1}^- \) endosomes, we observed no difference in luminal membrane delivery, suggesting that, whereas absence of \( \text{Did2} \) directly impairs \( \text{Vps4} \) function, the indirect effect of mislocalizing the negative regulator \( \text{Ist1} \) offsets this impairment.

### Table 1. Endosome sorting phenotypes and morphologies

| Genetic background | CPY-invertase secretion | GFP-CPS localization | Ub-GFP-CPS localization | Morphology |
|--------------------|--------------------------|----------------------|-------------------------|------------|
| Wild-type          | -                        | VL*                  | VL                      | MVBb       |
| \( \text{vps4}^- \) | + +                      | VM c + PVCd          | VM + PVC               | Class E*   |
| \( \text{did2}^- \) | -                        | VM + PVC (VL)        | VL + VM + PVC          | VTEe       |
| \( \text{vta1}^- \) | -                        | VM + PVC (VL)        | VM + PV C              | VTE (Class E) |
| \( \text{vps60}^- \) | +                        | VM + PVC (VL)        | VM + PV C              | VTE (Class E) |
| \( \text{ist1}^- \) | -                        | VL                    | VL                      | MVB        |
| \( \text{did2/vta1}^- \) | + +                      | VM + PVC             | VM + PV C              | Class E    |
| \( \text{did2/vps60}^- \) | + +                      | VM + PVC             | VM + PV C              | VTE        |
| \( \text{vta1/ist1}^- \) | + +                      | VM + PVC (VL)        | VM + PV C              | Class E (VTE) |
| \( \text{vps60/ist1}^- \) | + +                      | VM + PVC             | VM + PV C              | Class E (VTE) |

* Vacuole lumen, determined by fluorescence microscopy.

b Determined by EM.

d Vacuole limiting membrane, determined by fluorescence microscopy.

t Prevacular compartment, determined by fluorescence microscopy.

* Class E compartment, determined by EM.

e Parentheses indicate uncommon or weak phenotype observed in <20% of cells scored either by fluorescence or EM.

* Vesicular tubular endosome, determined by EM.

### Table 2. Localization of putative Vps4 substrate proteins determined by fluorescence microscopy

| Genetic background | Vps23-GFP ESCRT-I | Vps36-GFP ESCRT-II | GFP-Brol1 N.A. | Doa4-GFP N.A. |
|--------------------|-------------------|--------------------|----------------|---------------|
| Wild-type          | Cytosolic         | Cytosolic          | Cytosolic      | Cytosolic     |
| \( \text{vps4}^- \) | Punctate          | Punctate           | Punctate       | Punctate      |
| \( \text{did2}^- \) | Cytosolic         | Cytosolic          | Cytosolic      | Cytosolic     |
| \( \text{vta1}^- \) | Cytosolic         | Cytosolic          | Cytosolic      | Cytosolic     |
| \( \text{vta1/ist1}^- \) | Cytosolic         | Cytosolic          | Cytosolic      | Cytosolic     |
| \( \text{did2/vta1}^- \) | Punctate          | Weak punctate      | Punctate       | Punctate      |
| \( \text{did2/vps60}^- \) | Punctate          | Weak punctate      | Punctate       | Punctate      |

* Predominantly cytosolic GFP signal with occasional weak endosomal puncta.

b More intense punctate GFP signal adjacent to the vacuole compared with wild type with a reduction in cytosolic signal.

c Weak, punctate GFP signal adjacent to the vacuole without reduction in cytosolic signal.
ESCRTs from the endosome (Babst et al., 2002a; Katzmann et al., 2002; Babst, 2005; Hurley and Emr, 2006; Russell et al., 2006; Piper and Katzmann, 2007; Williams and Urbe, 2007). However, Vps4 has only been found to bind ESCRT-III. Reporting previously that dissociation of ESCRT-I and -II from endosomes occurs independently of Did2, we specu-

labeled that these complexes might require alternative adaptor proteins to be coupled to Vps4 (Nickerson et al., 2006). That both Vta1-GFP (Shiflett et al., 2004) and GFP-Vps60 (data not shown) lose their ability to localize to endosomes in the absence of ESCRT-I and -II suggested to us that Vta1-Vps60 might serve as the ESCRT-I/-II adaptor. Contrary to this hypothesis, neither Vps23-GFP (ESCRT-I) nor Vps36-GFP (ESCRT-II) accumulate at endosomes in the absence of Vta1, Vps60, or both (Table 2). However, both ESCRT-I and -II are concentrated at endosomes upon simultaneous disruption of Did2 and Vta1-Vps60 functions, conditions under which class E compartments form (Table 1). We therefore explored whether the accumulation of ESCRT-0, -I, and -II at endosomes correlates not with Vps4 malfunction but, instead, with formation of class E compartments by examining bro1Δ cells, which have class E compartments indistinguishable from those in vps4Δ cells (Richter et al., 2007), even though loss of Bro1 causes no aberrant membrane accumulation of ESCRT-III (Odorizzi et al., 2003). Hse1-GFP (ESCRT-0), Vps23-GFP and Vps36-GFP all are concentrated at class E compartments in bro1Δ cells as strongly as in vps4Δ cells (Figure 5A). Importantly, overexpression of the ubiquitin hydrolase encoded by DOA4 rescues MVB morphology in bro1Δ cells (Luhtala and Odorizzi, 2004) and similarly reduces accumulation of Hse1-GFP, Vps23-GFP, and Vps36-GFP at endosomes (Figure 5A). In contrast, DOA4 overexpression in vps4Δ cells fails to reverse the class E compartment morphology (Luhtala and Odorizzi, 2004) nor does it restore the cytoso-

lamic distributions of Hse1-GFP, Vps23-GFP, and Vps36-GFP (Figure 5A).

Further indication that “early” ESCRTs (0, I, and II) are not Vps4 substrates comes from our observation that Hse1-GFP, Vps23-GFP, and Vps36-GFP accumulate at endosomes in cells overexpressing VPS4 (Figure 5B). This condition disrupts vacuolar protein sorting (Kranz et al., 2001) but has no apparent effect on Vps4-mediated disassembly of ESCRT-III (Figure 5B), demonstrating that impairment in endosomal dissociation of ESCRT-0, -I, and -II can be uncoupled from ESCRT-III disassembly. Moreover, these data argue against the possibility that ESCRT-III couples Vps4 activity to membrane dissociation of ESCRT-0, -I, and -II. Our conclusion that Vps4 does not directly act toward early ESCRTs to catalyze their membrane dissociation is consistent with failures to detect interactions between Vps4 and the early ESCRTs in systematic studies in metazoans (Martín-Serrano et al., 2003; von Schwedler et al., 2003) and yeast (Bowers et al., 2004).

Because biogenesis of class E compartments in vps4Δ, bro1Δ, did2Δ, vta1Δ and did2Δ vps60Δ strains correlates strongly with membrane accumulation of ESCRT-I and -II (Tables 1 and 2), we investigated whether these phenomena share a causative relationship, particularly in light of increasing evidence that early ESCRTs assemble into larger networks on endosome membranes (Hurley, 2008). Class E compartment morphology persists upon simultaneous disruption of ESCRT-I and -II (data not shown), as well as that of ESCRT-0, -I, and -II (Figure 5C). Moreover, class E compart-

ments were also observed upon simultaneous disruption of all four genes encoding the core ESCRT-III subunits (Figure 5D), ruling out the possibility that the ESCRT-III polymer itself drives class E compartment formation. We conclude that accumulation of ESCRTs at endosomes is not directly responsible for the gross morphological defects of class E compartments, rather that class E compartment biogenesis leads to the aberrant endosomal accumulation of ESCRT-0, -I, and -II.

![Figure 4](image-url)
DISCUSSION

Although in vitro studies have described both positive and negative regulators of Vps4 (Azmi et al., 2008; Dimaano et al., 2008), our in vivo findings point to a spatiotemporal separation of their functions. Cells lacking positive regulators (Did2, Vta1, or Vps60) share phenotypes that include accumulation of ESCRT-III at endosomes (Figure 2A) and formation of VTEs rather than MVBs (Figure 3B). However, electron tomography revealed more subtle phenotypic differences in lumenal vesicle sizes (Figure 3M) and the extent to which lumenal vesicles form (Figure 3N). These observations prompt consideration of whether Vps4 activity toward promoting vesicle formation might not be restricted to one event or moment, but that multiple, distinct manipulations of ESCRT-III might be involved. The spatiotemporal separation between Did2 and Vps60, two proteins with partially redundant capacities to stimulate Vps4 activity (Azmi et al., 2008), makes this scenario especially plausible.

A distinct role for Did2 is also evident when comparing endosomal morphologies. Cells lacking Did2 display a unique increase in vesicle size not shared by cells lacking other Vps4 regulators (Figure 3M). The more pronounced defect in ESCRT-III membrane dissociation seen in vta1Δ vps23Δ vps36Δ cells (C) and vps20Δ snf7Δ vps23Δ vps24Δ cells (D). Bar, 200 nm.

Figure 5. Endosome biogenesis determines membrane association of early ESCRTs. (A) Fluorescence and differential interference contrast (DIC) microscopy of cells expressing Hse1-GFP, Vps23-GFP, or Vps36-GFP. 2μ, overexpression of DOA4 or VPS4 from high-copy plasmid. Closed arrowheads indicate localization of GFP to endosomes. Bar, 2 μm. (B) Subcellular fractionation and Western blot analysis of yeast cell lysates. T, total lysate. P13, membrane-associated 13,000 × g pellet fraction. S13, cytosolic 13,000 × g soluble fraction. Thin-section electron micrographs of class E compartments in vps27Δ vps23Δ vps36Δ cells (C) and vps20Δ snf7Δ vps23Δ vps24Δ cells (D). Bar, 200 nm.

Vta1 (Azmi et al., 2008), which agrees with results from in vitro reconstitution of ESCRT-III disassembly by Vps4 (Davies and Katzmann, personal communication). That Did2 is not strictly dependent on Vta1 to promote Vps4 function in vivo is also consistent with its ability to promote ATP hydrolysis through direct binding to the Vps4 MIT domain (Azmi et al., 2008). Did2 might also influence Vps4 indirectly through interaction with the Vps24-Vps2 subcomplex of ESCRT-III (Nickerson et al., 2006), of which the Vps2 subunit also has the ability in vitro to stimulate Vps4 activity independently of Vta1 (Azmi et al., 2008). In contrast to Did2, Vps60 might rely exclusively on Vta1 to promote Vps4 function. Indeed, Vta1 is the only known binding partner for Vps60 in yeast (Shiflett et al., 2004) and metazoans (Ward et al., 2005). Did2, therefore, plays a more central role in management of ESCRT-III dissociation than either Vta1 or Vps60.

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cells compared with vta1Δ and vps60α cells (Figure 2A) suggests the swelled vesicle phenotype results either from misregulated manipulation of ESCRT-III by Vps4 at the moment of vesicle scission or from reduced availability of recycled ESCRT-III subunits capable of assembling to mediate repeated rounds of vesicle budding. We note, however, that ESCRT-III has been recently shown in vitro to perform its membrane scission function in the absence of Vps4 (Wollert et al., 2009). Therefore, considering the capacity of Did2 (and the incapacity of Vps60) to bind the ESCRT-III core complex (Figure 2C), we should also consider the possibility that Did2 might regulate vesicle scission not only through regulation of Vps4, but also through direct participation in the ESCRT-III polymer.

Insight into the relationship between endosome morphology and MVB cargo sorting can be gleaned by comparison of the phenotypes exhibited by vta1Δ cells. Despite having VTEs with reduced luminal membrane content (Figure 3), vta1Δ cells display only very weak MVB cargo sorting defects across a battery of MVB cargo proteins (Table 1 and Supplemental Figure S1). This lack of correlation suggests that the 40% reduction in bulk luminal membrane sorting is insufficient to disrupt MVB cargo sorting and that MVB vesicles typically form without having achieved cargo saturation. Considering the recent discovery that some disruptions of ubiquitin-binding by either ESCRT-I or -II are insufficient to impair MVB cargo sorting (Shields et al., 2009), it also seems that ESCRT-mediated cargo recognition typically operates well short of saturation. These insights are consistent with the proposition that endosome luminal vesicle formation and cargo selection are not strictly interdependent. Indeed, loss of Rsp5 or Doa4 severely disrupts MVB cargo ubiquitination and sorting (Katzmann et al., 2004; Luhtala and Odorizzi, 2004) without disrupting MVB biogenesis (McNatt et al., 2007; Richter et al., 2007). Although an urgent need to silence and degrade cargoes can result in physiological stimulation of vesicle budding (White et al., 2006), it seems that luminal vesicle formation is largely “hard-wired” to occur with or without cargo.

Given that did2Δ and vps60α endosomes suffer no greater impairment in luminal membrane delivery than vta1Δ endosomes (Figure 3), it seems that the more significant MVB cargo sorting defects observed in did2Δ and vps60α cells (Table 1 and Supplemental Figure S1) do not result from insufficient luminal membrane carrying capacity, rather from disruptions to ESCRT-III dynamics that are not shared by vta1Δ. This highlights an ongoing conundrum in understanding the Vta1-Vps60 relationship: how is it that loss of Vps60, the clearest function of which is stimulation of Vps4 through interaction with Vta1 (Azmi et al., 2008), produces consistently stronger cargo missorting phenotypes than does loss of Vta1? At this point, genetic evidence suggests Vps60 performs a secondary, Vta1-independent function, but given the lack of interaction between Vps60 and the other ESCRT-III family proteins (Figure 2), it is unclear what this role is.

Recent studies of ESCRT-III have demonstrated the ability of its core subunits, especially Snf7, to polymerize at membranes in spiral patterns that might serve to induce membrane curvature (Hanson et al., 2008; Lata et al., 2008; Saksea et al., 2009). Observing also the ability of the Vps20-Snf7 subcomplex to protect MVB cargoes from proteolytic cleavage (Babst et al., 2002a) and prevent recycling of MVB cargoes away from endosomes (Teis et al., 2008), current opinion favors a model in which ESCRT-III forms a corral to prevent lateral diffusion of cargoes away from the membrane domain that will form a vesicle. Although most MVB cargoes examined show only partial missorting in did2Δ and vps60α cells (Dimaano et al., 2008; Rue et al., 2008), these mutants both display a complete block in vacuolar trafficking of the a-factor mating receptor Ste3 (Supplemental Figure S1). Given the insufficient membrane dissociation of ESCRT-III in did2Δ and vps60α mutants (Figure 2), this might suggest a failure to corral Ste3 to ensure its luminal targeting. However, we note that MVB trafficking of another endocytic cargo with rapid endosome recycling kinetics, Mup1, is unperturbed in did2Δ cells (Teis et al., 2008), suggesting that defective ESCRT-III assembly kinetics do not provide a sufficient explanation for cargo missorting in did2Δ and vps60α mutants. Interestingly, fusion of a nonhydrolyzable, in-frame ubiquitin to the cytosolic domain of carboxypeptidase S substantially rescues MVB sorting in all single mutants and combinations of did2Δ, vta1Δ, and vps60α capable of forming lumenal vesicles (Table 1), indicating a possible alternative scenario involving misregulation of the Bro1-Doa4 deubiquitination machinery that accumulates at VTEs along with ESCRT-III (Table 2).

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