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

Daniel P. Nickerson,*† Matthew West,* Ryan Henry,* and Greg Odorizzi*

*Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, CO 80309-0347; and †Department of Biochemistry, University of Washington, Seattle, WA 98195-7350

Submitted September 8, 2009; Revised January 7, 2010; Accepted January 13, 2010

Monitoring Editor: Jean E. Grunenberg

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 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 MV B 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.

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., 2006). 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). Is11 counterbalances these positive regulators to inhibit assembly of Vps4 into its active oligomeric state (Dimano 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 luminal vesicles. In contrast, the functions of Vta1 and Vps60 are more closely tied to efficient membrane scission and delivery of vesicles.
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 (Darosow et al., 2000). Vacular green fluorescent protein (GFP)-cleavage assays were performed by transforming yeast with plasmids encoding GFP-tagged MB4 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 OD_{260} unit of equivalent of each sample before Western blotting. 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-SnF7 (Babst et al., 1998), polyclonal anti-Vps24 (Babst et al., 1998), monoclonal anti-3-phosphoglycerate kinase (PGK) (Invitrogen), monoclonal anti-Vps1 in (Invitrogen), and monoclonal anti-Vps4.2 (Rockland Diagnostics, Indianapolis, IN). All Western blots were analyzed by chemiluminescence and film exposure except immunoprecipitations of GFP and Vps60 and subcellular fractionations in Figure 2A, which were analyzed using an 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).
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 vps4/H9004 cells (Babst et al., 1998) and to a lesser extent in did2/H9004 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 vta1/H9004, vps60/H9004, and vta1/H9004 vps60/H9004 cells compared with did2/H9004 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 did2/H9004 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 did2/H9004 cells (Figure 2A; one-way ANOVA for Snf7, p < 0.001 each; Vps24, p < 0.05 each) and did2/H9004 vps60/H9004 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, did2/H9004 vta1/H9004 vps60/H9004 cells accumulated significantly more Snf7 in the membrane-associated fraction compared with did2/H9004 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 vps4/H9004 cells (Figure 2B). This result demonstrates that even...
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 did2Δ cells indicated that efficient disassembly of ESCRT-III is not strictly required for luminal vesicle budding, a conclusion supported by subsequent in vitro reconstitution of luminal vesicle formation (Wollert et al., 2009). We also observed VTEs in cells lacking the other positive Vps4 regulators, Vta1 and/or 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 ist1Δ, vta1Δ vps60Δ mutants (one-way ANOVA; p < 0.001 for each). In stark contrast, lumenal vesicles in did2Δ vta1Δ 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 lumenal 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 lumenal vesicle size. Wild-type MVBs display an equal distribution of surface area between the limiting and lumenal membranes (Figure 3N), but mean lumenal membrane content of endosomes in vta1Δ, vps60Δ, and 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 lumenal membrane content arose from a defect in retrograde trafficking from endosomes. A reliable indicator of endosomal retrograde trafficking is Vps10, a transmem-
Table 1. Endosome sorting phenotypes and morphologies

<table>
<thead>
<tr>
<th>Genetic background</th>
<th>CPY-invertase secretion</th>
<th>GFP-CPS localization</th>
<th>Ub-GFP-CPS localization</th>
<th>Morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>–</td>
<td>VL</td>
<td>MV/V1</td>
<td>Class E</td>
</tr>
<tr>
<td>vps4Δ</td>
<td>+ +</td>
<td>VM + PVC (VL)</td>
<td>VL + VM + PVC</td>
<td>Class E</td>
</tr>
<tr>
<td>did2Δ</td>
<td>–</td>
<td>VM + PVC</td>
<td>VL + VM + PVC</td>
<td>Class E</td>
</tr>
<tr>
<td>vta1Δ</td>
<td>–</td>
<td>VM + PVC (VL)</td>
<td>VL + VM + PVC</td>
<td>Class E</td>
</tr>
<tr>
<td>vps60Δ</td>
<td>+ +</td>
<td>VM + PVC (VL)</td>
<td>VL + VM + PVC</td>
<td>Class E</td>
</tr>
<tr>
<td>ist1Δ</td>
<td>–</td>
<td>VM + PVC (VL)</td>
<td>VL + VM + PVC</td>
<td>Class E</td>
</tr>
<tr>
<td>did2Δvta1Δ</td>
<td>+ +</td>
<td>VM + PVC</td>
<td>VL + VM + PVC</td>
<td>Class E</td>
</tr>
<tr>
<td>did2Δvps60Δ</td>
<td>+ +</td>
<td>VM + PVC (VL)</td>
<td>VL + VM + PVC</td>
<td>Class E</td>
</tr>
<tr>
<td>vta1Δist1Δ</td>
<td>+ +</td>
<td>VM + PVC (VL)</td>
<td>VL + VM + PVC</td>
<td>Class E</td>
</tr>
<tr>
<td>vps60Δist1Δ</td>
<td>+ +</td>
<td>VM + PVC</td>
<td>VL + VM + PVC</td>
<td>Class E</td>
</tr>
</tbody>
</table>

*a* Vacuole lumen, determined by fluorescence microscopy.

*b* Determined by EM.

*c* Vacuole limiting membrane, determined by fluorescence microscopy.

*d* Prevacuolar compartment, determined by fluorescence microscopy.

*e* Class E compartment, determined by EM.

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

*g* Vesicular tubular endosome, determined by EM.

brane receptor that transports its soluble ligand, CPY, from the Golgi to endosomes, where Vps10 releases CPY and subsequently recycles to the Golgi (Marcusson et al., 1994; Piper 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 vps4Δ cells (Figure 3B). 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 vps4Δ cells, we found that cells lacking Did2, Vta1, or Vps60 exhibit a relatively normal distribution of Vps10-GFP (Figure 4A) and secrete little to no CPY-invertase (Figure 4C), whereas Vps10-GFP is strongly concentrated at class E compartments and CPY-invertase is secreted upon simultaneous disruption of Did2 and Vta1, a condition that phenocopies deletion of VPS4 with respect to ESCRT-III disassembly (Figure 2) and endosome morphology (Figure 3). Furthermore, although a small fraction of Vps10-GFP is proteolytically cleaved at its lumenal domain in wild-type cells, Vps10-GFP experienced an enhanced degree of cleavage in vps4Δ cells (Figure 4B) due to retention at endosomes and the inappropriate maturation of lumenal vacuolar hydrodases at the class E compartment (Babst et al., 2002a). No enrichment of a Vps10 cleavage product was observed in did2Δ, vta1Δ or vps60Δ cells (Figure 4B). Thus, we conclude that the increased ratio of limiting versus lumenal membrane content at endosomes of VTEs in cells lacking Vta1 or Vps60 is derived from a reduction in the budding of lumenal vesicles rather than a defect in recycling of limiting membrane away from endosomes.

In contrast with the above-mentioned mutants lacking positive regulators of Vps4 activity, deletion of IST1 significantly boosts the lumenal membrane content of endosomes relative to that observed in wild-type cells [61% (t test; p = 0.0009)]. Although the modest increase in ist1Δ vesicle size (Figure 3M) no doubt contributes to this increased lumenal content, ist1Δ endosomes routinely seem to have achieved their maximal capacity for lumenal vesicles. Indeed, ist1Δ lumenal vesicles frequently occur in apposition to each other and the limiting membrane, potentially pressed together due to lumenal space constraints. Therefore, although our data do not show an increase in the number of ist1Δ lumenal vesicles compared with wild type, deletion of IST1 causes accumulation of vesicles that seems to be limited only by the carrying capacity of the endosome. In did2Δ cells and did2Δ ist1Δ endosomes, we observed no difference in lumenal membrane delivery, suggesting that, whereas absence of Did2 directly impairs Vps4 function, the indirect effect of mislocalizing the negative regulator Ist1 offsets this impairment.

Endosome Morphology Determines Membrane Association of ESCRT-0, -I, and -II

Original studies of ESCRT-0, -I, and -II reported their accumulation, like ESCRT-III, at class E compartments in vps4Δ cells (Katzmann et al., 2001; Babst et al., 2002b; Bilodeau et al., 2002), supporting the broad conclusion that physical manipulation by Vps4 is directly responsible for removal of all
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 speculated 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 adapter. 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 are all 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 cytosolic 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 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.
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. Did2, therefore, plays a more central role in management of ESCRT-III dissociation than either Vta1 or Vps60.

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 did2Δ, 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.

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 did2Δ

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.
cells compared with \(\text{vta}1\Delta\) and \(\text{vps60}\Delta\) 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 available 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 \(\text{vta}1\Delta\) cells. Despite having VTEs with reduced lumenal membrane content (Figure 3), \(\text{vta}1\Delta\) cells display only very weak MVB cargo sorting defects across a battery of MVB cargo proteins (Table 1 and Suplemental Figure S1). This lack of correlation suggests that the 40% reduction in bulk lumenal 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 separate 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 disrupt 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 lumenal vesicle formation is largely “hard-wired” to occur with or without cargo.

Given that \(\text{did}2\Delta\) and \(\text{vps60}\Delta\) endosomes suffer no greater impairment in lumenal membrane delivery than \(\text{vta}1\Delta\) endosomes (Figure 3), it seems that the more significant MVB cargo sorting defects observed in \(\text{did}2\Delta\) and \(\text{vps60}\Delta\) cells (Table 1 and Suplemental Figure S1) do not result from insufficient lumenal membrane carrying capacity, rather from disruptions to ESCRT-III dynamics that are not shared by \(\text{vta}1\Delta\). 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 membrane branches in spiral patterns that might serve to induce membrane curvature (Hanson et al., 2008; Lata et al., 2008; Saksema 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 \(\text{did}2\Delta\) and \(\text{vps60}\Delta\) cells (Dimano 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 inefficient membrane dissociation of ESCRT-III in \(\text{did}2\Delta\) and \(\text{vps60}\Delta\) 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 \(\text{did}2\Delta\) cells (Teis et al., 2008), suggesting that defective ESCRT-III assembly kinetics do not provide a sufficient explanation for cargo missorting in \(\text{did}2\Delta\) and \(\text{vps60}\Delta\) 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 \(\text{did}2\Delta\), \(\text{vta}1\Delta\), and \(\text{vps60}\Delta\) capable of forming luminal vesicles (Table 1), indicating a possible alternative scenario involving misregulation of the Bro1-Dok4 deubiquitination machinery that accumulates at VTEs along with ESCRT-III (Table 2).

**ACKNOWLEDGMENTS**

We thank Caitlin White-Root, Caleb Richter, and Michael Kaempf (University of Colorado–Boulder) for yeast strains; Matthew Russell (University of Colorado–Boulder) for EM assistance; and Andrew Staelin (University of Colorado–Boulder), Ishara Azmi, Brian Davies, and David Katzmann (Mayo Clinic, Rochester, MN) for discussion of unpublished results. This work was funded by National Institutes of Health grant GM-065505.

**REFERENCES**


Vol. 21, March 15, 2010
D. P. Nickerson et al.


