ESCRT ubiquitin-binding domains function cooperatively during MVB cargo sorting

S. Brookhart Shields,1 Andrea J. Oestreich,2 Stanley Winistorfer,1 Doris Nguyen,2 Johanna A. Payne,2 David J. Katzmann,2 and Robert Piper1

1Department of Molecular Physiology and Biophysics, University of Iowa, Iowa City, IA 52240
2Department of Biochemistry and Molecular Biology, Mayo Clinic College of Medicine, Rochester, MN 55905

Ubiqutin (Ub) sorting receptors facilitate the targeting of ubiquitinated membrane proteins into multivesicular bodies (MVBs). Ub-binding domains (UBDs) have been described in several endosomal sorting complexes required for transport (ESCRT). Using available structural information, we have investigated the role of the multiple UBDs within ESCRTs during MVB cargo selection. We found a novel UBD within ESCRT-I and show that it contributes to MVB sorting in concert with the known UBDs within the ESCRT complexes. These experiments reveal an unexpected level of coordination among the ESCRT UBDs, suggesting that they collectively recognize a diverse set of cargo rather than act sequentially at discrete steps.

Introduction

Many integral membrane proteins targeted for lysosomal degradation are ubiquitinated and sorted into vesicles that bud from the limiting membrane into the lumen of endosomes during the biogenesis of multivesicular bodies (MVBs; Piper and Katzmann, 2007). Cargo selection during MVB sorting is dependent on several endosomal protein complexes known as endosomal sorting complexes required for transport (ESCRT). Several ESCRT components contain ubiquitin (Ub)-binding domains (UBDs) that may act as receptors for ubiquitinated membrane proteins. For instance, ESCRT-0, comprised of yeast Vps27 and Hse1 or mammalian Hrs and STAM (signal transducing adaptor molecule), contains Ub-interacting motifs (UIMs) required for MVB sorting of ubiquitinated cargo (Bilodeau et al., 2002; Shih et al., 2002; Piper and Katzmann, 2007). The Ub E2 variant (UEV) domain in Vps23/TSG101, an ESCRT-I component in yeast/mammalian cells, and the GLUE (GRAM-like Ub binding in EAP45) or Npl4 zinc finger (NZF) domains of Vps36, an ESCRT-II component, also contain UBDs that have been implicated in MVB cargo selection (Katzmann et al., 2001; Alam et al., 2004; Slagsvold et al., 2005). This had led to a model in which Ub cargo is passed sequentially from ESCRT-0 to ESCRT-I and to ESCRT-II for final deposit into the forming lumenal vesicles (Katzmann et al., 2002; Gruenberg and Stenmark, 2004; Hurley, 2008). Previous studies have indicated the importance of these UBDs during MVB sorting; however, recent high resolution structures that detail Ub interactions have enabled a more precise approach to dissect the contributions of these UBDs to MVB sorting (Alam et al., 2004, 2006; Sundquist et al., 2004; Teo et al., 2004; Hirano et al., 2006). Surprisingly, we found that mutant forms of Vps23 and Vps36 unable to bind Ub had no defects in MVB sorting, even in combination. This led us to search for additional UBDs within ESCRT-I and resulted in the discovery of a novel UBD within Mvb12. The UBD of Mvb12 works in concert with the UBDs of the ESCRTs to promote cargo recognition and MVB sorting.

Results and discussion

Mutants of Vps23 and Vps36 defective for Ub binding

Cocrystal structures of Ub with the UEV domains of Vps23 and TSG101 were used to guide mutagenesis experiments rendering Vps23 unable to bind Ub (Sundquist et al., 2004; Teo et al., 2004). The structures show that the UEV domains bind to Ub via a β hairpin tongue and a lip (Fig. 1 A). The structure of the
Figure 1. Generation of Vps23 and Vps36 mutants incapable of binding Ub. (A) Model of the Vps23 UEV domain in complex with Ub (Protein Data Bank accession no. 1UZX). The residues mutated to create the vps23UB alleles are shown in red. (bottom) A schematic of the position of the UEV domain and the core region of Vps23 involved in complex formation with other ESCRT-I subunits is shown. (B) Recombinant V5 epitope–tagged wild-type and mutant Vps23 UEV domains were used for binding experiments with GST, Ub-GST, or GST-Vps27 C terminus. Bound proteins were immunoblotted with 1 or 10% of the input lysates. (C) Cell lysates from strains (PLY335, PLY3529, and PLY3530) bearing the wild-type (WT) or mutant alleles of VPS23 were immunoblotted with α-Vps23 and α-PGK.

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[Figure and text continued as needed]
tongue interface is highly conserved and includes residues F52-G57 of Vps23 and F14-G17 of Tsg101. The lip of Vps23 contacts Ub with residues Q117 and W125, which is different from Tsg101 (Porinillos et al., 2002a,b). Two triple-mutant alleles of VPS23 were generated: vps23ΔUb1 (F52A, Q117A, and W125A) and vps23ΔUb2 (F52A, M105A, and W125A). The corresponding UEV domains were expressed as V5 epitope-tagged proteins in bacteria and used for binding assays with GST-Ub. The wild-type UEV domain of Vps23 bound Ub, whereas no binding was observed for the mutant UEV domains (Fig. 1 B). However, all UEV domains bound to the C terminus of Vps27, which contains two PSDP motifs (P44SDP and P52SDP) that directly interact with Vps23 (Bilodeau et al., 2003). Tsg101 has a similar interaction with PTAP motifs of viral gag proteins and Hrs (Porinillos et al., 2002a,b). Although the structural basis of the TSG101-PTAP interaction is known, it is not known for the Vps23–UEV–PSDP interaction, and the PTAP-binding pocket of Tsg101 is not conserved in Vps23. Regardless, this demonstrated that the Vps23 UEV mutants are specifically defective for Ub binding and did not compromise expression of corresponding full-length Vps23 in vivo (Fig. 1 C).

Mutagenesis of the ESCRT-II subunit Vps36 was also performed. The Ub-binding activity of Eap45 (mammalian Vps36) is found within the N-terminal GLUE domain (Alam et al., 2006; Hirano et al., 2006). However, the GLUE domain of yeast Vps36 lacks these key elements of Ub binding. Instead, the yeast Vps36 GLUE domain has an insertion of two NZF domains, the second lacks these key elements of Ub binding. Instead, the yeast Vps36 GLUE domain has an insertion of two NZF domains, the second lacks these key elements of Ub binding. The GLUE domain of yeast Vps36 is found within the N-terminal GLUE domain (Alam et al., 2006; Alam et al., 2004). Vps23 and Vps36 interact with Vps23 (Bilodeau et al., 2003). Tsg101 has a similar interaction with PTAP motifs of viral gag proteins and Hrs (Porinillos et al., 2002a,b). Although the structural basis of the TSG101-PTAP interaction is known, it is not known for the Vps23–UEV–PSDP interaction, and the PTAP-binding pocket of Tsg101 is not conserved in Vps23. Regardless, this demonstrated that the Vps23 UEV mutants are specifically defective for Ub binding and did not compromise expression of corresponding full-length Vps23 in vivo (Fig. 1 C).

Effect of inactivation of Vps23 and Vps36 Ub binding

To assess the role of Ub binding by Vps23 and Vps36, yeast strains were constructed in which the mutant alleles were integrated in place of the endogenous genes. We found that the inability of Vps23 or Vps36 to bind Ub had no effect on the sorting of GFP-Cps1, a model MVB cargo (Odorizzi et al., 1998), into the vacuolar lumen (Fig. 2 A). No difference was observed between the vps23ΔUb1 or vps36ΔUb alleles (unpublished data). Analysis of additional mutant vps23 alleles with mutations in other residues that line the Ub interface (S96, G77, F105, and N123) also did not display any sorting defects (Fig. S1). Likewise, a double mutant containing vps23ΔUb1 and vps36ΔUb showed normal sorting of GFP-Cps1 (Fig. 2 B). These results were confirmed using vps23Δ and vps36Δ-null mutants transformed with wild-type and ΔUb alleles of VPS23 and VPS36 housed on low copy plasmids (unpublished data). Lastly, sorting of other MVB cargoes, including Ste3-GFP (Urbanowski and Piper, 2001), Fur4-GFP (Blondel et al., 2004), and Sna3-GFP (Reggiori and Pelham, 2001) was normal in the vps23ΔUb1 and vps36ΔUb alleles (unpublished data).

These findings differ from previous studies that found severe sorting defects upon mutation of the ESCRT-I UEV domain and the ESCRT-II NZF domain (Katzmann et al., 2001; Alam et al., 2004). Prior analysis of the UEV domain centered on a single mutant (M63T) identified by a loss of function mutagenesis screen. M63 faces the interior of Vps23 and not the Ub-binding interface (Fig. 1 A). Thus, mutation of this residue may indirectly impact the ability of the UEV domain to bind to Ub or other factors. The NZF mutation used in this study was also distinct from previously described mutations that displayed MVB sorting defects (Alam et al., 2004). In both cases, mutations used in this study may maintain the overall integrity of Vps23 and Vps36 (based on in vivo function), although specifically ablation Ub binding (based on in vitro assays). Additionally, our functional analysis was performed with untagged, integrated alleles of VPS23 and VPS36. The ability of the vps23ΔUb1 vps36ΔUb double mutant to successfully sort cargo into the MVB interior indicated that there might be additional UBDs within the ESCRT machinery.

Mvb12 binds Ub

A potential candidate for a protein with a novel UBD was Mvb12, a recently identified subunit of ESCRT-I. Previous studies

α-Vps23 and α-Pgk (3-phosphoglycerate kinase) antibodies. (J) Schematic of Vps36 showing the GLUE domain with an insertion of two NZF domains, the second of which binds Ub. The core region of Vps36 interacts with the rest of ESCRT-I. (K) Summary of HsQC NMR experiments with 15N-labeled Ub, but the Vps36 GLUE domain did not. The ΔUb mutation was then placed into the context of full-length Vps36 to create vps36ΔUb. The mutations in Vps36 did not appear to have a deleterious effect on expression level because HA epitope–tagged alleles of VPS36 and vps36ΔUb expressed from the endogenous VPS36 promoter showed similar levels (Fig. 1 H).
The genetic interactions between Mvb12 and ESCRT-I and -II UBDs prompted us to determine whether Mvb12 could bind Ub. Recombinant V5 epitope–tagged full-length Mvb12 from *Saccharomyces cerevisiae* and the related budding yeast *Saccharomyces kluyveri* were used in binding assays with Ub-GST. Both proteins bound specifically to Ub-GST (Fig. 3 A). Deletion analysis of Mvb12 mapped the Ub binding to the C terminus (W50-S101; unpublished data). This interaction was confirmed by NMR HSQC experiments of 15N-labeled Ub combined with the C terminus of Mvb12. Mvb12 induced chemical shift perturbations in the spectrum of 15N-labeled Ub that mapped to a surface of Ub that included residues R42, V70, and L8 (Fig. 3 E). This overlaps with the surface engaged by the Vps23 UEV domain, the Vps36 NZF domain, and the UIM domains of Vps27 and Hse1, indicating that simultaneous binding of these components to a single Ub molecule is unlikely (Bilodeau et al., 2003; Alam et al., 2004; Teo et al., 2004).

Figure 2. Loss of Mvb12 reveals sorting defect of vps23ΔUb1 and vps36ΔUb1 mutants. (A) GFP-Cps1 localization was assessed in vps23ΔUb1 [PLY3528] and vps36ΔUb1 [PLY395] cells. GFP fluorescence images along with matching DIC images are shown. (B) Sorting of GFP-Cps1, Ste3-GFP–Ub, and Ste3-GFP–Ub in vps23ΔUb1 vps36ΔUb1 [PLY3522], mvb12Δ-null [JPY21], vps23ΔUb1 vps36ΔUb1 mvb12Δ [PLY3623], and vps23ΔUb1 mvb12Δ [PLY3624] cells. (C) GFP-Cps1 from wild-type (WT) and vps23ΔUb1 vps36ΔUb1 mvb12Δ cells was immunoprecipitated with anti-GFP antibodies and immunoblotted with anti-Ub and anti-GFP antibodies. Black line indicates that intervening lanes have been spliced out. IP, immunoprecipitation. Bars, 5 µm.

showed that deletion of *MVB12* caused differential defects in MVB sorting; Ste3-GFP and Sna3-GFP display sorting defects, whereas GFP-Cps1, Ste2-GFP, and Ste3-GFP–Ub (in which Ub is fused in frame to the C terminus) sorting is largely unaffected (Chu et al., 2006; Curtiss et al., 2007; Oestreich et al., 2007). This indicates that Mvb12 plays a specific role in recognizing a subset of MVB cargoes. A role for Mvb12 cargo recognition was tested with strains that combined vps23ΔUb1, vps36ΔUb1, and mvb12Δ to probe for synthetic defects in MVB sorting. Combining these mutations caused dramatic sorting defects in GFP-Cps1 and Ste3-GFP (Fig. 2 B). The defect in sorting of GFP-Cps1 is not the result of a detectable loss of Cps1 ubiquitination (Fig. 2 C) but rather indicates a defect in recognition of Ub cargos. This was supported by examining the sorting of Ste3-GFP–Ub, which was normal in mvb12Δ-null and vps23ΔUb1 vps36ΔUb1 double-mutant cells but defective in vps23ΔUb1 vps36ΔUb1 mvb12Δ triple-mutant cells (Fig. 2 B).

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Figure 3. Binding of Mvb12 to Ub. (A) Recombinant V5 epitope–tagged Mvb12 for S. cerevisiae and S. kluveri were assayed for Ub binding with Ub-GST. (B) V5 epitope–tagged fusions of the C terminus of either wild-type [WT] Mvb12 or the indicated mutants were expressed in bacteria and assayed for Ub binding with Ub-GST. (C) A sequence of Mvb12 with the Ub-binding region shown in red. Regions that make contact with Vps37 in the ESCRT-I crystal structure (Protein Data Bank accession no. 2P22) are underlined. The asterisk indicates the end of the protein sequence. (D) Cell lysates prepared from mvb12∆ cells transformed with low copy plasmids expressing Mvb12-HA (pPL3713), Mvb12∆Ub1-HA (pPL3709), or Mvb12∆Ub2-HA (pPL3711) were immunoblotted with anti-HA and anti-PGK antibodies. (E) Summary of NMR HSQC experiments with 15N-labeled Ub (50 µM) with the C-terminal fragment of Mvb12 (100 µM). (left) Part of the spectra of 15N-labeled Ub in the presence (red) and absence (green) of Mvb12 is shown. Chemical shift differences were quantified and plotted on the linear sequence of Ub (middle) or onto the surface of Ub (right). Red indicates (0.2δN2 + δH2)1/2 ≥ 0.02. Black residues were not observed. ppm, parts per million.
The C-terminal fragment of Mvb12 was then mutated to produce a series of recombinant proteins that were subjected to Ub-GST–binding experiments. Mutations that disrupted Ub binding were localized to residues F171–G182 as shown by two mutants with residues 71–76 and 79–84 replaced with alanines (Fig. 3 B). A recent crystal structure has shown that two patches of Mvb12, F171–D172, and W237Y–S238 pack against a hydrophobic segment of Vps37 (Kostelansky et al., 2007). However, these residues did not seem critical for Ub binding, as mutation of W237Y to R237S, and F171 to S171N did not block binding to Ub-GST (Fig. 3 B). In contrast, mutation of residues C terminal to this region abolished Ub binding. Interestingly, in the context of the entire ESCRT-I, the UBD of Mvb12 would be oriented near the UEV domain of Vps23, suggesting that both of these UBDs are close in native ESCRT-I.

**Contribution of Mvb12 Ub binding to MVB sorting**

We initially assessed the function of wild-type and the two Mvb12 Ub-binding mutants as HA epitope–tagged proteins expressed from low copy plasmids containing the native MVB12 promoter. All three proteins expressed at comparable levels (Fig. 3 D), and all coprecipitated with Vps23 and Vps28, demonstrating that they properly assembled with ESCRT-I (Fig. 4 A). Gel filtration showed that tandem affinity purification (TAP)–tagged Mvb12ΔUB1 (Y79–G85>A79AAAAA) supported assembly into a large complex with an apparent molecular mass of 350 kD, as has been previously observed for ESCRT-I (Fig. S2). Furthermore, GFP-tagged Mvb12ΔUB1 localized to endosomal compartments similar to GFP-tagged wild-type Mvb12 (Fig. S2). These data indicate that these Mvb12 mutants were able to assemble into the ESCRT-I complex. This allowed us to ascribe phenotypes to a deficiency in Ub binding of Mvb12 rather than loss of ESCRT-I association.

Cells expressing mvb12ΔUB1 or mvb12ΔUB2 (K524PPP<->D524DDGT) as their sole source of MVB12 showed no sorting defects for Ste3-GFP or Sna3-GFP (Fig. 4 B). Agreeing with previous studies, Ste3-GFP and to a large extent Sna3-GFP showed defective sorting in mvb12Δ-null cells (Fig. 4 C; Chu et al., 2006; Curtiss et al., 2007; Oestreich et al., 2007). Other MVB cargo proteins such as GFP-Cps1 and Ste3-GFP–Ub were normal (unpublished data). The same results were obtained with wild-type or chromosomally integrated mvb12ΔUB1 mutants (unpublished data).

Together, these data indicate that the mutations used to ablate Ub binding did not indiscriminately compromise Mvb12 function. They also implied that the function of Ub binding by Mvb12 alone is not critical for MVB sorting. We next assessed the role of the Mvb12 UBD in cells lacking the UBDs of Vps23 and Vps36 (Fig. 4 C). The vps23ΔUB1 vps36ΔUB1 mutant was cotransformed with low copy plasmids expressing wild-type MVB12, mvb12ΔUB1, or mvb12ΔUB2 and GFP-tagged MVB cargos. We found that wild-type MVB12 conferred proper sorting of all cargos, whereas both mvb12ΔUB1 alleles showed defective sorting of GFP-Cps1 when combined with vps23ΔUB1 and vps36ΔUB1. These data show that the UBDs of Mvb12, Vps23, and Vps36 work jointly to efficiently sort MVB cargo because sorting was defective only when mutations were combined (Fig. 2 B and Fig. 4 C).

In contrast to the sorting of GFP-Cps1, other MVB cargos were sorted normally in the vps23ΔUB1 vps36ΔUB1 mvb12ΔUB triple mutants. These cargos included Sna3-GFP and Ste3-GFP (Fig. 4 C). This demonstrated that some degree of Ub-dependent MVB sorting was still intact and that there might be other ESCRT components that contribute to MVB cargo recognition; one candidate is Vps27–Hse1 (ESCRT-0). The Vps27–Hse1 complex contains three UIMs and binds the Vps23 UEV domain via its two PSDP motifs within the C terminus (P94SADP and P95SDP). Although deletion of Vps27 altogether blocks ESCRT-I localization to endosomes (Katzmann et al., 2003), little phenotype is observed when the protein–protein interface between ESCRT-0 and ESCRT-I is disrupted by mutation of the two PSDP motifs in Vps27 (Vps27ΔVps23, AA94ADP and P95AAA; Bilodeau et al., 2003). Localization of Vps23-GFP to endosomes was unperturbed as was recruitment of mCherry-tagged ESCRT-III subunit Vps20 (Fig. S1). To investigate whether the interaction with ESCRT-0 helped maintain sorting of MVB cargos in the vps23ΔUB1 vps36ΔUB1 mvb12ΔUB strain, these mutations were combined with the vps27ΔVps23 mutation. The resulting quadruple mutant displayed very significant sorting defects (Fig. 5 A). Sorting of all MVB cargoes was defective in vps23ΔUB1 vps36ΔUB1 vps27ΔVps23 mvb12ΔUB mutant cells. This included Ste-GFP, Ste3-GFP–Ub, and Sna3-GFP, which otherwise sorted normally in vps23ΔUB1 vps36ΔUB1 mvb12ΔUB mutant cells (Fig. 4 C) or vps23ΔUB1 vps36ΔUB1 vps27ΔVps23 alleles (Fig. 5 A). These experiments clearly showed a critical contribution of the Mvb12 UBD because dramatic sorting defects were observed when the mvb12ΔUB1 vps23ΔUB1 vps36ΔUB1 and vps27ΔVps23 alleles were combined. In spite of mis-sorting MVB cargoes, the quadruple mutant cells were still capable of a residual level of sorting lipids into the interior lumenal membranes, indicated by sorting of the fluorescent lipid marker NBD-PC into the vacuole lumen. Unlike ESCRT-null mutants in which NBP-PC accumulates exclusively in endosomes (Bilodeau et al., 2002), NDB-PC accumulated within the vacuoles of vps23ΔUB1 vps36ΔUB1 mvb12ΔUB mutant cells (Fig. 5 B). Disruption of the Vps27–Vps23 interaction combined with mutation of the UBDs within ESCRT-I and -II revealed a separation of cargo sorting and MVB biogenesis.

**Model for Ub-sorting receptors at the endosome**

Our data show that Ub binding by Mvb12 is functionally important and contributes to the overall process of protein sorting into MVBs. Moreover, models proposed previously that require ubiquitinated cargo to be sequentially recognized by the UIMs of ESCRT-0, the Vps23 UEV domain of ESCRT-I, and the Vps36 component of ESCRT-II need to be amended. The available data support several functional models (Fig. 5 C) that can account for the multiple UBDs with the ESCRT network (Fig. 5 D). The one we favor is that ESCRTs function together as a large supercomplex that contains multiple UBDs, each of which can recognize and sort partially overlapping sets of ubiquitinated cargo. Multiple UBDs within this complex would enable recognition of Ub attached to a wide variety of membrane proteins or allow for
and -II may perform other functions besides cargo recognition. In particular, coupled monoubiquitination of the ESCRT machinery itself, which has been observed in animal cells but not yet yeast, could cause UBDs to mediate a series of intra- and intermolecular interactions that could regulate assembly or efficiency of MVB sorting (Haglund and Stenmark, 2006; Hoeller et al., 2006). Indeed, the loss of UBDs in combination with the synthetic mutations that led to profound MVB-sorting defects also caused defects in vacuolar protein sorting (e.g., carboxypeptidase Y multiple cargo molecules per supercomplex. If this model were correct, then the observation that sorting of some cargo still occurs in the absence of Vps23, Vps36, and Mvb12 UBDs implies that there remain other UBDs within the ESCRT machinery. One possibility is that remaining UBDs are provided by components such as Vps27–Hse1 because disruption of the Vps27–Vps23 interface (the vps27ΔVps23 mutation) caused a dramatic synthetic phenotype when combined with UBD mutations in Vps23, Vps36, and Mvb12. Alternatively, the UBDs of ESCRT-I and -II may perform other functions besides cargo recognition. In particular, coupled monoubiquitination of the ESCRT machinery itself, which has been observed in animal cells but not yet yeast, could cause UBDs to mediate a series of intra- and intermolecular interactions that could regulate assembly or efficiency of MVB sorting (Haglund and Stenmark, 2006; Hoeller et al., 2006). Indeed, the loss of UBDs in combination with the synthetic mutations that led to profound MVB-sorting defects also caused defects in vacuolar protein sorting (e.g., carboxypeptidase Y
Figure 5. Synthetic defects revealed by uncoupling ESCRT-0 from ESCRT-I. (A) Strains (PLY3734, PLY3779, PLY3777, and PLY3781) of the indicated genotype were assessed for sorting of GFP-Cps1, Ste3-GFP, Ste3-GFP–Ub, and Sna3-GFP. All alleles were stably integrated into the genome as nonepitope
(CPY) secretion; Fig. S2), suggesting that UBDs may contribute to the general functions of the ESCRTs in a way that remains to be determined.

Materials and methods

Materials, yeast strains, and plasmids

Chemicals, antibodies, and, growth methods were used as previously described (Bilodeau et al., 2003; Ren et al., 2007). S. cerevisiae strains used in this study are listed in Table I. Plasmids used in this study are listed in Supplementary Table S1.

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Table I. Yeast strains used in this study

| Strain      | Genotype                                      | Source               |
|-------------|-----------------------------------------------|----------------------|
| SEY6210     | MATa leu2-3,112 ura3-52 his3-D200 trp1-d901 lys2-801 suc2-Δ9 mel | Robinson et al., 1988 |
| PLY3392     | vps36Δ::HIS3                                   | This study           |
| PLY3394     | PVS36::TRP1::vps36Δ::HIS3                      | This study           |
| PLY3395     | vps36Δ::Ub1 TRP1::vps36Δ::HIS3                 | This study           |
| PLY3405     | vps36Δ::Ub1 TRP1::vps36Δ::HIS3                 | This study           |
| PLY3407     | VPS36::TRP1::vps36Δ::HIS3 vps27Δ::Kan1         | This study           |
| PLY3409     | vps36Δ::Ub2 TRP1::vps36Δ::HIS3                 | This study           |
| PLY3410     | VPS36::TRP1::vps36Δ::HIS3 vps27Δ::Kan1         | This study           |
| PLY3522     | vps36Δ::Ub1 TRP1::vps36Δ::HIS3                 | This study           |
| PLY3528     | VPS36::TRP1::vps36Δ::HIS3 vps27Δ::Kan1         | This study           |
| PLY3623     | vps36Δ::Ub2 TRP1::vps36Δ::HIS3                 | This study           |
| PLY3624     | VPS36::TRP1::vps36Δ::HIS3 vps27Δ::Kan1         | This study           |
| PLY3725     | vps36Δ::Ub2 TRP1::vps36Δ::HIS3                 | This study           |
| PLY3734     | vps36Δ::Ub2 TRP1::vps36Δ::HIS3                 | This study           |
| PLY3744     | vps36Δ::Ub2 TRP1::vps36Δ::HIS3                 | This study           |
| PLY3776     | vps36Δ::Ub2 TRP1::vps36Δ::HIS3                 | This study           |
| PLY3777     | vps36Δ::Ub2 TRP1::vps36Δ::HIS3                 | This study           |
| PLY3779     | vps36Δ::Ub2 TRP1::vps36Δ::HIS3                 | This study           |
| PLY3777     | vps36Δ::Ub2 TRP1::vps36Δ::HIS3                 | This study           |
| JPY21       | vps36Δ::HIS3                                   | Oestreich et al., 2007 |

alleles under the control of their endogenous promoter. The GFP fluorescence images and corresponding DIC images are shown. (B) The same strains as in A were analyzed for sorting of the lipid marker NBD-PC. (C) Model for function of UBDs in the ESCRT machinery. Model 1 depicts the UBDs of ESCRT-I and -II acting together to present multiple binding sites for cargo. This model predicts that other UBDs are present in the ESCRT complexes. Model 2 shows that although UBDs of ESCRT-I bind Ub cargo, the UBDs of ESCRT-I and -II may interact with Ub in other proteins and may regulate the assembly or activity of the MVB sorting apparatus. (D) Schematic of the various UBDs within the ESCRTs and the connections that tie the ESCRTs together in a supercomplex. Bars, 5 µm.
Table II.  **Plasmids used in this study**

| Plasmid | Description | Source |
|---------|-------------|--------|
| pPL2612 | vps36\(^{Glu}126\) (T187F G187A) | This study |
| pPL2613 | VPS36 [−309–1,698 VPS36 and 840 bp of the PHO8 3’UT] | This study |
| pPL2612 | vps36\(^{Glu}126\) (T187F/G187A) | This study |
| pPL2645 | vps36\(^{Glu}126\) (T187F/G187A) as a Noll-Acc61 fragment | This study |
| pPL2646 | VPS36 as a Noll-Acc61 fragment | This study |
| pPL2940 | vps36\(^{Glu}126\) in pS306 [F52AG117AW125A] | This study |
| pPL2180 | VPS23 [−598–1,298] as an XbaI–SacI fragment | This study |
| pPL2645 | vps36\(^{Glu}126\) (T187F/G187A) as a NotI–Acc65I fragment from pPL2612 in pRS304 | This study |
| pPL2646 | VPS36 as a NotI–Acc65I fragment from pPL2613 in pRS304 | This study |
| pPL2447 | vps23\(^{Glu}141\) with UEV mutation F52A in pPL2180 | This study |
| pPL2448 | vps23\(^{Glu}141\) with UEV mutation N123A in pPL2180 | This study |
| pPL2449 | vps23\(^{Glu}141\) with UEV mutation W125A in pPL2180 | This study |
| pPL2465 | vps23\(^{Glu}141\) with UEV mutation S55AD56A in pPL2180 | This study |
| pPL2466 | vps23\(^{Glu}141\) with UEV mutation F52AS55AD56A in pPL2180 | This study |
| pPL2467 | vps23\(^{Glu}141\) with UEV mutation S55A in pPL2180 | This study |
| pPL2468 | vps23\(^{Glu}141\) with UEV mutation S55AF105A in pPL2180 | This study |
| pPL2469 | vps23\(^{Glu}141\) with UEV mutation F105AW125A in pPL2180 | This study |
| pPL2470 | vps23\(^{Glu}141\) with UEV mutation F52AF105A in pPL2180 | This study |
| pPL2567 | vps23\(^{Glu}141\) with UEV mutation F52AQ117AW125A in pPL2180 | This study |
| pPL2568 | vps23\(^{Glu}141\) with UEV mutation F52AM107AW125A in pPL2180 | This study |
| pPL2569 | vps23\(^{Glu}141\) with UEV mutation F52AL102AW125A in pPL2180 | This study |
| pPL2570 | vps23\(^{Glu}141\) with UEV mutation Q117A in pPL2180 | This study |
| pPL2571 | vps23\(^{Glu}141\) with UEV mutation F52AF105A in pPL2180 | This study |
| pPL2487 | vps23 with UE mutation F52A in pPL2180 | This study |
| pPL2488 | vps23 with UE mutation N123A in pPL2180 | This study |
| pPL2499 | vps23 with UE mutation W125A in pPL2180 | This study |
| pPL2501 | vps23 with UE mutation S55A in pPL2180 | This study |
| pPL2502 | vps23 with UE mutation S55AF105A in pPL2180 | This study |
| pPL2503 | vps23 with UE mutation F105AW125A in pPL2180 | This study |
| pPL2504 | vps23 with UE mutation F52AF105A in pPL2180 | This study |
| pPL2251 | vps27\(^{Glu}123\) in pRS306 PSD1 and PSD2 motifs mutated (AA448ADP and P523AAA) | This study |
| pPL3230 | MVB12 (aa 1–101) | This study |
| pPL3355 | GST-Vps36 GLUE domain (residues 1–289) | This study |
| pPL3358 | GST-Vps36\(^{Glu}126\) GLUE domain (residues 1–289) | This study |
| pPL3353 | VPS36 GLUE domain (residues 1–289) in pET151 | This study |
| pPL3356 | vps36\(^{Glu}126\) (residues 1–289) in pET151 | This study |
| pPL2251 | vps36\(^{Glu}126\) from pPL2652 in pRS306 | This study |
| pPL2941 | vps36\(^{Glu}126\) from pPL2653 in pRS306 | This study |
| pPL3543 | MV12 (aa 1–102) | This study |
| pPL3545 | MV12 residues 56–101 in pET151 | This study |
| pPL3541 | MV12 residues 56–101 F17Q WWYK-A101AAA in pET151 | This study |
| pPL3542 | MV12 residues 56–101 L26Q WWQ in pET151 | This study |
| pPL3544 | MV12 residues 56–101 W32WW35 in pET151 | This study |
| pPL3545 | MV12 residues 56–101 K82KPPG-D82DGGT in pET151 | This study |
| pPL3551 | MV12 residues 56–101 Y79LSKKPP-A79AAAA in pET151 | This study |
| pPL3552 | MV12 residues 56–101 F71DS71N in pET151 | This study |
| pPL3709 | mvb1\(^{Glu}126\) Y79LSKKPP-A79AAAA with single HA tag in pRS316 | This study |
| pPL3711 | mvb1\(^{Glu}126\) K82KPPG-D82DGGT with single HA tag in pRS316 | This study |
| pPL3713 | MV12 with single HA tag in pRS316 | This study |
| pPL3780 | mvb1\(^{Glu}126\) K82KPPG-D82DGGT in pRS306 | This study |
| pPL3781 | mvb1\(^{Glu}126\) Y79LSKKPP-A79AAAA in pRS306 | This study |
| pPL3784 | MV12 in pRS306 | This study |
| NA | MV12-TAP [TAP-tagged MV12 driven by PRC1 promoter] | Oestreich et al., 2007 |
| pAO94 | mvb1\(^{Glu}126\) Y79LSKKPP-A79AAAA -TAP [TAP-tagged mvb1 mutant driven by PRC1 promoter] | This study |
| NA | MV12-GFP (GFP-tagged MV12 driven by PRC1 promoter) | Oestreich et al., 2007 |
| pAO104 | mvb1\(^{Glu}126\) Y79LSKKPP-A79AAAA -GFP (GFP-tagged mvb1 mutant driven by PRC1 promoter) | This study |
| NA | Ub-GST; expression plasmid for Ub fused to GST | Katzmann et al., 2001 |
| pPL1945 | GST-Vps27; expression plasmid for residues 353–610 of Vps27 fused to GST | Bilodeau et al., 2003 |
| pGEX3x | GST expression plasmid Smith and Johnson, 1988 |
| NA | VPS23-GFP (GFP-tagged Vps23 driven by the endogenous promoter in pRS416) | Nickerson et al., 2006 |
| pPL3982 | Vps23\(^{Glu}126\)-GFP (GFP-tagged Vps23 driven by the endogenous promoter) | This study |
| pPL3979 | Vps20-mCherry (mCherry-tagged Vps20 under the control of the endogenous promoter in pRS316) | This study |

NA, not applicable.
[ORCA; Hamamatsu Photonics] with IPLABS software [BD] as previously described [Urbanowski and Piper, 1999]. Image processing was performed in Photoshop [CS; Adobe], where the original 12-bit images were converted to 8-bit images and the brightest pixel was set to 255. NBD-PC (1-myristoyl-2-[6(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl)-sn-glycero-3-phosphocholine; Avanti Polar Lipids, Inc.) was used as previously described [Bilodeau et al., 2002].

NMR analysis
Recombinant proteins mixed with 15N-labeled Ub were prepared as previously described [Bilodeau et al., 2004] or purchased from Spectra Stable isotopes. [15N]Ub HSQC spectra were collected on a 500-MHz spectrometer [Avance II 52; Bruker] and analyzed with SPARKY [T. Goddard and D. Kneller, SPARKY 3, University of California, San Francisco, San Francisco, CA] as previously described [Bilodeau et al., 2004]. Chemical shift differences were calculated using the formula \( \Delta \delta = \sqrt{\Delta \delta^2 + \Delta \delta^2} \). Binding experiments with Vps36 were performed in 50 mM imidazole, pH 7.2; binding differences were calculated using the formula \( \Delta \delta = \sqrt{\Delta \delta^2 + \Delta \delta^2} \). Binding experiments with Mvb12 were performed in 50 mM imidazole and D.G. Kneller, SPARKY 3, University of California, San Francisco, San Francisco, CA) as previously described [Bilodeau et al., 2004].

Other methods
Metabolic [35S]methionine labeling and immunoprecipitation of CPY were performed as described previously [Cooper and Stevens, 1996]. Preparation of yeast lysates for gel filtration, immunoblotting, and CPY pulse-chase analysis were performed as described previously [Oestreich et al., 2007]. Protein models were drawn with the PyMOL molecular graphics system (http:// pymol.sourceforge.net). A model for the Vps36 NZF domain was computed using the Wurst protein server (Torda et al., 2004).

Online supplemental materials
Fig. S1 shows the localization of GFP-Cps1 in strains with a range of mutations in Vps23, the structure of Vps23-UB complex in domain with Ub highlighting mutated residues, the localization of Vps23-GFP, and Vps20-mCherry in strains containing vps20Δ+ and vps20Δ mutations. Fig. S2 shows the localization of Mvb12-GFP, gel filtration experiments performed on wild-type and mutant Mvb12, and the results of CPY pulse-chase analysis of all of the strains used in this study. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.20081130/D1C1.

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