Mvb12 Is a Novel Member of ESCRT-I Involved in Cargo Selection by the Multivesicular Body Pathway[□]

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The multivesicular body (MVB) sorting pathway impacts a variety of cellular functions in eukaryotic cells. Perhaps the best understood role for the MVB pathway is the degradation of transmembrane proteins within the lysosome. Regulation of cargo selection by this pathway is critically important for normal cell physiology, and recent advances in our understanding of this process have highlighted the endosomal sorting complexes required for transport (ESCRTs) as pivotal players in this reaction. To better understand the mechanisms of cargo selection during MVB sorting, we performed a genetic screen to identify novel factors required for cargo-specific selection by this pathway and identified the Mvb12 protein. Loss of Mvb12 function results in differential defects in the selection of MVB cargoes. A variety of analyses indicate that Mvb12 is a stable member of ESCRT-I, a heterologous complex involved in cargo selection by the MVB pathway. Phenotypes displayed upon loss of Mvb12 are distinct from those displayed by the previously described ESCRT-I subunits (vacuolar protein sorting 23, -28, and -37), suggesting a distinct function than these core subunits. These data support a model in which Mvb12 impacts the selection of MVB cargoes by modulating the cargo recognition capabilities of ESCRT-I.

INTRODUCTION

The endosomal system coordinates protein trafficking between various subcellular compartments, including the Golgi, plasma membrane, and lysosome. Cell surface proteins, including activated receptors, that have undergone endocytosis are typically recycled to the plasma membrane or targeted deeper into the endosomal pathway for degradation within the lysosome. Endosomal membrane proteins destined for the lumen of the lysosome undergo an additional sorting reaction during their inclusion into the multivesicular body (MVB) pathway (for review, see Gruenberg and Stenmark, 2004; Babst, 2005). MVBs form when the limiting membrane of the late endosome invaginates and buds into the lumen of the organelle, actively selecting a subset of the proteins from the limiting membrane in the process (Gordon *et al.*, 1978; Haigler *et al.*, 1979). The intraluminal vesicles within the MVB and their contents are exposed to hydrolases within the lysosome as a consequence of heterotypic fusion of these organelles. By contrast, proteins within the limiting membrane of the MVB are deliv-

ered to the limiting membrane of the lysosome/vacuole after heterotypic fusion (Odorizzi *et al.*, 1998).

Sorting of cargo into the MVB pathway and heterotypic fusion with the lysosome/vacuole leads to delivery into the hydrolytic lumen of the organelle, and therefore entry into this pathway must be highly regulated. Ubiquitination is the best-characterized *cis*-acting signal mediating entry into the MVB pathway (for reviews, see Katzmann *et al.*, 2002; Hicke and Dunn, 2003; Raiborg *et al.*, 2003). Studies in organisms ranging from the yeast *Saccharomyces cerevisiae*, to mammalian cells have demonstrated that ubiquitin modification of a variety of MVB cargoes is a requisite for entry into this pathway, and ubiquitin modification of endosomal membrane proteins that are not normally MVB cargoes is sufficient to target them into this pathway (Katzmann *et al.*, 2001; Reggiori and Pelham, 2001; Urbanowski and Piper, 2001; Raiborg *et al.*, 2002). Ubiquitin-independent cargoes of the MVB pathway have also been described, but relevant signals for inclusion have not been precisely defined. In yeast, ubiquitin modification of the Sna3 protein has been reported to be dispensable for Sna3 entry into the MVB pathway (Reggiori and Pelham, 2001). In the case of the mammalian melanosomal protein Pmel17, the relevant sorting information for targeting to intraluminal vesicles resides within the luminal domain of the protein (Theos *et al.*, 2006), suggesting a novel mechanism by which this cargo is selected.

The sorting of all yeast MVB cargoes requires the function of the class E vacuolar protein sorting (Vps) proteins as the *trans*-acting machinery (Odorizzi *et al.*, 1998). This machinery has been conserved through evolution from yeast to humans (for reviews, see Katzmann *et al.*, 2002; Babst, 2005) and has been demonstrated to mediate the budding of retroviruses such as human immunodeficiency virus-1 (Morita and Sundquist, 2004). The class E Vps proteins recognize ubiquitinated MVB cargo proteins and actively sort them into the MVB pathway (for reviews, see Katzmann *et al.*, 2002; Raiborg *et al.*, 2003; Babst, 2005). In class E vps mutants,

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Abbreviations used: CPS, carboxypeptidase S; CPY, carboxypeptidase Y; ESCRT, endosomal sorting complex required for transport; MVB, multivesicular body; ORF, open reading frame; PtdIns(3)P, phosphatidylinositol-3-phosphate; TAP, tandem affinity purification; Ub, ubiquitin; VPS, vacuolar protein sorting.

MVBs fail to form and therefore MVB cargoes fail to reach the vacuolar lumen. (Raymond *et al.*, 1992; Odorizzi *et al.*, 1998). Many of the class E Vps proteins form the endosomal sorting complexes required for transport (ESCRTs). ESCRT-I, -II, and -III, together with additional class E Vps proteins such as Vps27/Hrs and Vps4/SKD1, transiently associate with the endosomal membrane to execute the MVB sorting reaction (Katzmann *et al.*, 2001; Babst *et al.*, 2002a,b; Bache *et al.*, 2003; Bilodeau *et al.*, 2003; Katzmann *et al.*, 2003; von Schwedler *et al.*, 2003; Bowers *et al.*, 2004). The class E proteins Vps23/Tsg101, Vps28, and Vps37 were described previously as subunits of the 350-kDa ESCRT-I complex, which plays a role in the recognition of ubiquitin-modified MVB cargoes through the ubiquitin E2 variant (UEV) domain of Vps23/Tsg101 (Babst *et al.*, 2000; Bishop and Woodman, 2001; Katzmann *et al.*, 2001; Bache *et al.*, 2004; Teo *et al.*, 2004). Recent structural determination of the ESCRT-I core suggests that Vps23, Vps28, and Vps37 may be present in equal molar ratios within this complex (Kostelansky *et al.*, 2006; Teo *et al.*, 2006); however, it is unclear how these subunits of 43, 28, and 25 kDa alone would comprise a complex with an apparent molecular mass of 350 kDa. ESCRT-I recruitment to the endosomal membrane is dependent upon its association with Vps27/Hrs, which seems to dictate site selection for the MVB sorting reaction by virtue of its ability to bind both the endosomally enriched lipid phosphatidylinositol-3-phosphate [PtdIns(3)P] as well as ubiquitin modified MVB cargo through ubiquitin interacting motifs (Bache *et al.*, 2003; Bilodeau *et al.*, 2003; Katzmann *et al.*, 2003).

Cellular components involved in mediating ubiquitin-dependent entry into the MVB pathway have begun to be elucidated. In yeast, deletion of class E *vps* genes results in a dramatic missorting of all MVB cargoes; however, mutants specifically defective for the delivery of ubiquitin-dependent MVB cargoes retain the ability to sort ubiquitin-independent MVB cargoes, including Sna3 (Bilodeau *et al.*, 2002; Katzmann *et al.*, 2004). This suggests additional mechanisms of cargo selection by this pathway exist. To gain insight into this process, we performed a genetic screen for loci affecting the MVB sorting of Sna3, a putative ubiquitin-independent MVB cargo (Reggiori and Pelham, 2001; Katzmann *et al.*, 2004). This screen identified Mvb12 as a critical factor for sorting a subset of MVB cargoes, including Sna3 and Ste3. However, trafficking of other MVB cargoes such as carboxypeptidase S (CPS) and Ste2 is largely unaffected in *mvb12Δ* cells, as is general endosomal function. Several lines of evidence indicate that Mvb12 impacts MVB cargo sorting as a novel subunit of the previously described ESCRT-I. The distinct phenotypes of *mvb12Δ* compared with deletion of other ESCRT-I subunits suggest that Mvb12 is a modulator of the Vps23/28/37 ESCRT-I core machinery's ability to sort specific MVB cargoes. These results identify Mvb12 as a novel subunit of ESCRT-I that modulates the cargo sorting activity of this complex.

MATERIALS AND METHODS

S. cerevisiae strains used in this study are described in Table 1. The collection of viable deletion mutants was obtained from Research Genetics (Huntsville, AL). Methodologies and reagents described previously for the purpose of performing genome-wide synthetic genetic interaction analyses (Tong *et al.*, 2001) were used to introduce Sna3-GFP into the collection of viable deletion mutants (Tong *et al.*, 2001). Double deletion mutants in which both deletions are marked with *HIS3* were generated by performing crosses between opposite mating types containing the relevant deletions, followed by sporulation, tetrad dissection, and verification of genotype.

Plasmids

The *MVB12-GFP* fusion gene was polymerase chain reaction (PCR) amplified from genomic DNA from JPY29, cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA), and subcloned into the pRS415 and pRS414 vectors by using XbaI and SpeI sites. pRS416-Sna3-GFP has been described previously (Katzmann *et al.*, 2004). Sna3^{KallR}-GFP was constructed through mutagenesis of pRS416-Sna3-GFP (GeneTailor; Invitrogen). *GFP-CPS* and *Ste2-GFP* have been described previously (Odorizzi *et al.*, 1998). Ste3-GFP and Ste3-GFP-Ub were graciously provided by Robert Piper (University of Iowa, Iowa City, IA). DsRed FYVE was described previously as an endosomal marker (Katzmann *et al.*, 2003). pMB103 (pRS416-Vps4^{E233Q}) expresses a dominant-negative form of Vps4 that has been described previously (Babst *et al.*, 1997). The *MVB12* open reading frame (ORF) was amplified from yeast genomic DNA and cloned using BamHI and XhoI sites into the pET28b (Novagen, Madison, WI) and pTAP416 vectors digested with SalI and BamHI, for bacterial expression of His₆-Mvb12 and yeast expression of TAP-Mvb12, respectively. To create pGPD414 HA-ubiquitin, pGDP416 HA-ubiquitin (Davies *et al.*, 2003) was digested with SacI and KpnI and subcloned into pRS414. The TAP tag was cloned into the EcoRI site of pGPD416 (Mumberg *et al.*, 1995) to create the pTAP416 vector. All PCR-based plasmids were sequenced to ensure that no aberrant mutations were present.

Microscopy

Fluorescence microscopy was performed on live cells in minimal media, by using a Nikon fluorescence microscope with fluorescein isothiocyanate, rhodamine, green fluorescent protein (GFP), and DsRed filters and a digital camera (Coolsnap HQ; Photometrix, Melbourne, Australia). Images were deconvolved with Delta Vision software (Applied Precision, Seattle, WA). FM4-64 labeling was performed as described in Vida and Emr (1995). Kinetic analysis of FM4-64 uptake was performed by labeling cells on ice for 15 min, followed by chasing at room temperature.

Biochemical Analyses

Gel filtration analyses were performed as described previously (Katzmann *et al.*, 2001). Bacterial expression of His₆-Mvb12 was induced in the HMS174 DE3 strain at 37°C for 3.5 h with 0.5 mM isopropyl β-D-thiogalactoside. Crude lysate subjected to a 100,000 × g clearing spin was used for gel filtration. Tagged proteins were visualized using monoclonal antibodies anti-HA.11 (Covance, Princeton, NJ), monoclonal anti-GFP AV JL-8 (BD Biosciences, San Jose, CA), or penta-His (QIAGEN, Valencia, CA). Pulse-chase analysis of CPS, carboxypeptidase Y (CPY), Sna3-GFP, and Sna3^{KallR}-GFP was performed as described previously (Babst *et al.*, 2002a), and quantitation was performed using phosphorimaging screens and a Storm 840 system (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). Protein A purification using TAP-Mvb12 was performed as described in Azmi *et al.* (2006), with the exception that 15 OD₆₀₀ of cells were lysed in phosphate-buffered saline. Proteins were visualized with polyclonal anti-Vps23 and anti-Vps28 (Katzmann *et al.*, 2001). Ste3 immunoprecipitation was performed essentially as described in Katzmann *et al.* (2001), with all buffers containing 5 mM N-ethylmaleimide and incubations performed at 50°C. Yeast expressing Ste3-GFP and hemagglutinin (HA)-ubiquitin were trichloroacetic acid precipitated, processed, and lysed in urea cracking buffer with glass beads. Immunoprecipitation was performed with monoclonal anti-GFP AV JL-8 (BD Biosciences), and samples were subjected to SDS-PAGE and Western blotting. Ste3 was detected with anti-GFP, and the ubiquitination status was determined with monoclonal anti-HA.11 (Covance) to recognize HA-ubiquitin.

RESULTS

Identification of Mvb12 as an MVB Sorting Factor

Function of the MVB pathway requires the activity of the class E Vps proteins, many of which assemble into the oligomeric ESCRTs. The large number of class E *vps* mutants that yield the same phenotype has made standard genetic approaches to identify new components impractical due to the reisolation of previously known factors. However, screening of the commercially available collection of viable yeast deletion mutants represents a rational method by which to identify additional nonessential genes required for the function of the MVB pathway. Sna3 has been described as a cargo that does not require ubiquitination to be sorted into the MVB pathway (Reggiori and Pelham, 2001); thus, trafficking of Sna3 fused to GFP (Sna3-GFP) was analyzed to identify novel cargo recognition factors and components of the MVB pathway. Methodologies described previously to perform genome-wide synthetic genetic interaction studies

Table 1. Yeast strains used in this study

Descriptive name	Strain	Genotype	Source or reference
WT	SEY6210	<i>MATα, leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9</i>	Robinson <i>et al.</i> (1988)
WT	SEY6210.1	SEY6210; <i>MATα</i>	Babst <i>et al.</i> (1997)
WT	BY4742	<i>MATα, his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0</i>	Research Genetics
WT	BY4741	BY4742; <i>MATα</i>	Research Genetics
<i>vps27Δ</i>	MBY21	SEY6210; <i>vps27Δ::HIS3</i>	Shih <i>et al.</i> (2002)
<i>hse1Δ</i>	MBY44	SEY6210.1; <i>yh1002w::HIS3</i>	Markus Babst (University of Utah)
<i>vps23Δ</i>	EEY6-2	SEY6210; <i>vps23Δ::HIS3</i>	Babst <i>et al.</i> (2000)
<i>vps28Δ</i>	SRY28	SEY6210; <i>vps28Δ::URA3</i>	Babst <i>et al.</i> (2000)
<i>vps37Δ</i>	DKY48	SEY6210; <i>srn2Δ::HIS3</i>	Katzmann <i>et al.</i> (2001)
<i>vps25Δ</i>	BWY101	SEY6210; <i>vps25::HIS3</i>	Babst <i>et al.</i> (2002b)
<i>snf7Δ1</i>	EEY9	SEY6210; <i>snf7::HIS3</i>	Babst <i>et al.</i> (2002a)
<i>vps20Δ1</i>	EEY2-1	SEY6210; <i>vps20::HIS3</i>	Babst <i>et al.</i> (2002a)
<i>vps24Δ1</i>	BWY102	SEY6210; <i>vps24Δ::HIS3</i>	Babst <i>et al.</i> (1998)
<i>vps4Δ</i>	MBY3	SEY6210; <i>vps4Δ::TRP1</i>	Babst <i>et al.</i> (1997)
<i>vps4Δ</i>	MBY4	SEY6210.1; <i>vps4Δ::TRP1</i>	Babst <i>et al.</i> (1997)
<i>bro1Δ</i>	GOY65	SEY6210; <i>bro1Δ::HIS3</i>	Odorizzi <i>et al.</i> (2003)
<i>fti1Δ/did2Δ</i>	EEY17	SEY6210; <i>fti1Δ::HIS3</i>	Markus Babst (University of Utah)
<i>vps60Δ</i>	EEY20	SEY6210; <i>vps60Δ::HIS3</i>	Markus Babst (University of Utah)
<i>mhb12Δ</i>	JPY22	SEY6210; <i>mhb12Δ::HIS3</i>	This study
<i>mhb12Δ</i>	JPY21	SEY6210.1; <i>mhb12Δ::HIS3</i>	This study
<i>mhb12Δ</i>		BY4742; <i>mhb12Δ::KanMX4</i>	Research Genetics
<i>mhb12Δ</i>		BY4741; <i>mhb12Δ::KanMX4</i>	Research Genetics
<i>MVB12-GFP</i>	JPY29	SEY6210; <i>MVB12-GFP::HIS3</i>	This study
<i>MVB12-GFP</i>	JPY28	SEY6210.1; <i>MVB12-GFP::HIS3</i>	This study
<i>MVB12-HA</i>	JPY24	SEY6210; <i>MVB12-HA::HIS3</i>	This study
<i>MVB12-HA</i>	JPY20	SEY6210.1; <i>MVB12-HA::HIS3</i>	This study
<i>vps4Δ mhb12Δ</i>	JPY23	MBY3; <i>mhb12Δ::HIS3</i>	This study
<i>vps4Δ MVB12-HA</i>	JPY25	MBY4; <i>MVB12-HA::HIS3</i> ; <i>MATα</i>	This study
<i>vps27Δ MVB12-GFP</i>	JPY14	MBY21; <i>MVB12-GFP::HIS3</i> ; <i>MATα</i>	This study
<i>hse1Δ MVB12-GFP</i>	JPY10	MBY44; <i>MVB12-GFP::HIS3</i> ; <i>MATα</i>	This study
<i>vps23Δ MVB12-GFP</i>	JPY5	EEY6-2; <i>MVB12-GFP::HIS3</i> ; <i>MATα</i>	This study
<i>vps23Δ MVB12-GFP</i>	JPY11	EEY6-2; <i>MVB12-GFP::HIS3</i> ; <i>MATα</i>	This study
<i>vps28Δ1 MVB12-GFP</i>	JPY51	SRY28; <i>MVB12-GFP::HIS3</i> ; <i>MATα</i>	This study
<i>vps25Δ1 MVB12-GFP</i>	JPY13	BWY101; <i>MVB12-GFP::HIS3</i> ; <i>MATα</i>	This study
<i>vps24Δ1 MVB12-GFP</i>	JPY17	BWY102; <i>MVB12-GFP::HIS3</i> ; <i>MATα</i>	This study
<i>vps24Δ MVB12-GFP</i>	JPY15	BWY102; <i>MVB12-GFP::HIS3</i> ; <i>MATα</i>	This study
<i>snf7Δ MVB12-GFP</i>	JPY19	EEY9; <i>MVB12-GFP::HIS3</i> ; <i>MATα</i>	This study
<i>snf7Δ MVB12-GFP</i>	JPY18	EEY9; <i>MVB12-GFP::HIS3</i> ; <i>MATα</i>	This study
<i>vps4Δ MVB12-GFP</i>	JPY12	MBY3; <i>MVB12-GFP::HIS3</i> ; <i>MATα</i>	This study
<i>bro1Δ MVB12-GFP</i>	JPY9	GOY65; <i>MVB12-GFP::HIS3</i> ; <i>MATα</i>	This study
<i>bro1Δ MVB12-GFP</i>	JPY8	GOY65; <i>MVB12-GFP::HIS3</i> ; <i>MATα</i>	This study
<i>fti1Δ MVB12-GFP</i>	JPY7	EEY17; <i>MVB12-GFP::HIS3</i> ; <i>MATα</i>	This study
<i>fti1Δ MVB12-GFP</i>	JPY6	EEY17; <i>MVB12-GFP::HIS3</i> ; <i>MATα</i>	This study
<i>vps60Δ MVB12-GFP</i>	JPY16	EEY20; <i>MVB12-GFP::HIS3</i> ; <i>MATα</i>	This study

(Tong *et al.*, 2001) were used to introduce Sna3-GFP into the collection of viable yeast deletion strains and fluorescence microscopy was used to analyze subcellular localization of Sna3-GFP in 4570 mutants. Whereas the total number of mutants within the collection is slightly higher than the number analyzed, mutants that failed to come through the selection procedure and were previously known to function in protein trafficking or seemed irrelevant were not pursued. For this reason, as well as essential genes not being included in this collection and the collection itself containing errors, this analysis is not intended to represent an exhaustive survey of all gene products required for the function of the MVB pathway. The majority of strains within the collection displayed wild-type Sna3-GFP distribution, indistinguishable from wild type wherein fluorescence is localized to the lumen of the vacuole (Figure 1; data not shown). However, a large number of previously known *vps* mutants were identified, validating the experimental procedure (Supplemental Figure 1). In addition to previously known traffick-

ing factors, this approach identified two mutants that displayed MVB sorting defects distinct from previously characterized class E *vps* mutants: *vta1 Δ* (Azmi *et al.*, 2006) and deletion of YGR206w (Figure 1 and Supplemental Figure 1). YGR206w encodes a 12-kDa protein that plays a role in MVB sorting and is referred to as *MVB12*.

To allow direct comparison with previously characterized class E *vps* mutants, and to validate the identification of *mhb12 Δ* by using the deletion collection, the *MVB12* gene was deleted in our standard genetic background (SEY6210) and MVB sorting phenotypes were examined in greater detail. Cells were also stained with the fluorescent vacuolar dye FM4-64 to enable visualization of the limiting membrane of the vacuole. As in the BY4742 genetic background, analysis of Sna3-GFP distribution revealed an intermediate MVB sorting phenotype, with a portion mislocalized to perivacuolar structures (indicated by arrows in Figure 2). This Sna3-GFP localization pattern is distinct from both wild-type cells, wherein Sna3-GFP is largely within the vacuole

Figure 1. *MVB12* is required for sorting of a subset of MVB cargoes. Genetic screening identified *mvb12Δ* as defective for the sorting of Sna3-GFP in the BY4742 genetic background. GFP-tagged forms of the MVB cargoes Sna3, Sna3^{KallR}, CPS, and Ste3 were expressed in wild-type and *mvb12Δ* cells and visualized by fluorescence microscopy. Arrows indicate perivacuolar structures. Bar, 5 μ m.

lumen, or a class E *vps* mutant (*vps23Δ*), in which Sna3-GFP localized predominantly to the aberrant class E compartment (Figure 2). Moreover, the colocalization of Sna3-GFP and FM4-64 apparent in *vps23Δ* cells was absent in the *mvb12Δ* and wild-type cells. This suggests that the compartments to which Sna3-GFP localized in an *mvb12Δ* mutant are distinct from those seen in a standard class E *vps* mutant. This intermediate phenotype is consistent with the partial Sna3-GFP sorting defects observed in the *mvb12Δ* strain in the BY4742 genetic background, although less severe, and defects in both strains could be corrected by transforming a *MVB12* plasmid (Figure 3; data not shown).

To more quantitatively address the intermediate phenotype observed in *mvb12Δ* cells, kinetic analysis of Sna3-GFP sorting was performed by pulse-chase analysis (Figure 3A and Supplemental Figure 2A). Sna3-GFP is degraded within the vacuole lumen subsequent to sorting into the MVB pathway; hence, its turnover serves as a measure of its MVB sorting (Oestreich *et al.*, 2007). Consistent with microscopic analysis, loss of Mvb12 resulted in a decreased rate of turnover of Sna3-GFP (Figure 3A and Supplemental Figure 2A). Importantly, the presence of Sna3-GFP in the vacuolar lumen in the *mvb12Δ* cells, as well as the eventual maturation of Sna3-GFP, indicated that Mvb12 is not absolutely required for function of the MVB pathway but that Mvb12 does play a role in Sna3 MVB sorting. This is distinct from the class E *vps* mutant *vps23Δ* wherein Sna3-GFP is not observed within the vacuolar lumen and its turnover is stabilized (Figure 2 and Supplemental Figure 2A).

The role of Mvb12 in MVB sorting was explored by analyzing the trafficking of additional MVB cargoes in the *mvb12Δ* strain. CPS is a well-characterized MVB cargo in yeast (Odorizzi *et al.*, 1998). Ubiquitin modification of CPS by the ubiquitin ligase Rsp5 confers ubiquitin-dependent MVB sorting of CPS in a manner dependent upon ESCRTs (Katzmann *et al.*, 2001, 2004); hence, CPS sorting serves as an indicator of ubiquitin-dependent MVB sorting. Localization

Figure 2. Defects in sorting of MVB cargoes observed upon loss of *MVB12* are distinct from loss of the ESCRT-I subunit *VPS23*. Isogenic wild-type (SEY6210), *mvb12Δ* and *vps23Δ* strains were used to analyze the MVB sorting of GFP-tagged MVB cargoes Sna3, Sna3^{KallR}, and CPS by fluorescence microscopy. FM4-64 was also used to decorate the limiting membrane of the vacuole for easier identification. Arrows indicate peri-vacuolar structures. Bar, 5 μ m.

of a GFP-CPS reporter was examined in wild type, *mvb12Δ*, and the class E mutant *vps23Δ* strains. Whereas the *mvb12Δ* strain exhibited pronounced Sna3-GFP localization to punctate structures, GFP-CPS localization was only subtly per-

Figure 3. Kinetic analysis of vacuolar protein delivery rates using pulse-chase immunoprecipitation. (A) Wild-type, *mvb12Δ*, or *mvb12Δ* cells expressing Mvb12-GFP that were also expressing Sna3-GFP or Sna3^{KallR}-GFP were pulse labeled with [³⁵S]Cys/Met, chased with an excess of Cys/Met, and sample time points were processed for immunoprecipitation with anti-GFP antibody. (B) Wild-type, *vps23Δ*, *mvb12Δ*, or *mvb12Δ* cells expressing Mvb12-GFP were pulse labeled with [³⁵S]Cys/Met, chased with an excess of Cys/Met, and sample time points were processed for immunoprecipitation with anti-CPS antibody. (C) Wild-type and *mvb12Δ* or *mvb12Δ* cells plus Mvb12-GFP were pulse labeled with [³⁵S]Cys/Met, chased with an excess of Cys/Met, and sample time points were processed for immunoprecipitation with anti-CPY antibody. After sample processing, immunoprecipitation, and SDS-PAGE, the amount of recovered proteins was quantitated using a phosphorimager and normalized to the amount recovered at time zero to ascertain processing rates. (Representative gels used for quantitation presented in A, B, and C are provided in Supplemental Figure 2D.) (D) Wild-type, *mvb12Δ*, or *vps23Δ* cells were converted to spheroplasts, pulse labeled with [³⁵S]Cys/Met, and chased with excess Cys/Met for the indicated times. Samples from the indicated time points were separated into intracellular (I) and extracellular (O) fractions and these were used to perform anti-CPY immunoprecipitations, followed by SDS-PAGE and autoradiography.

turbed, displaying minor missorting to the limiting membrane of the vacuole and no obvious class E compartment in both genetic backgrounds (Figures 1 and 2). This can be readily observed in the merged images, wherein the vacuolar limiting membrane looks yellow in both *mvb12Δ* and *vps23Δ* cells (Figure 2). Kinetic analysis of CPS maturation in the *mvb12Δ* strain (JPY22) yielded a consistent result with

only a modest delay evident (Figure 3B). Maturation of the soluble vacuolar protease CPY is also only partially impeded in the *mvb12Δ* strain (Figure 3C). Furthermore, the *mvb12Δ* strain displayed very low levels of secreted p2CPY similar to wild-type cells, in contrast to the large portion secreted in the class E *vps* mutant *vps23Δ* (Figure 3D). The kinetic delay of CPY processing observed for *mvb12Δ* cells in Figure 3C

Figure 4. Loss of Mvb12 displays differential phenotypes on endocytic cargoes. (A) Ste3-GFP displayed a severe missorting phenotype in the *mvb12Δ* background, whereas Ste2-GFP seemed indistinguishable from wild-type cells. A chimera wherein ubiquitin is fused to Ste3-GFP (Ste3-GFP-Ub) suppressed the missorting phenotype seen for Ste3-GFP. Bar, 5 μm. (B) Ubiquitin modification of Ste3 is unaltered in *mvb12Δ* cells. Equivalent numbers of wild-type, *mvb12Δ*, and *vps23Δ* cells expressing Ste3-GFP and HA-ubiquitin were used to generate extracts and Ste3-GFP was immunoprecipitated

can also be observed in Figure 3D wherein p2CPY is present in the intracellular fraction at 20 min, but it has been converted to mature (and not secreted) by 40 min. In contrast, the *vps23Δ* strain exhibited extensive limiting membrane and class E compartment localization for both GFP-CPS and Sna3-GFP (Figure 2) and defects both in CPS and CPY maturation have been observed previously (Figure 3 and Supplemental Figure 2; Babst *et al.*, 2000). As has been observed previously, the class E *vps* mutants processed CPS to an aberrant form that migrates distinctly from wild-type mature (m)CPS. However, *mvb12Δ* cells did not display this misprocessed form; instead, *mvb12Δ* cells displayed mCPS indistinguishable from wild-type cells (Babst *et al.*, 2002a; Supplemental Figure 2D). These results indicate that *mvb12Δ* displays distinct phenotypes from previously characterized class E *vps* mutants.

To further explore these distinctions, endocytic trafficking of the G protein-coupled receptors Ste2 and Ste3 was examined in the *mvb12Δ* strains of the appropriate mating type. Ubiquitin modification of Ste2 has been demonstrated to play a critical role in its internalization from the cell surface and subsequent delivery into the MVB pathway, resulting in delivery to the vacuolar lumen (Terrell *et al.*, 1998). Ste3, by contrast, seems to undergo recycling between the plasma membrane and an endosomal compartment until ubiquitination removes it from the recycling loop by targeting it into the MVB pathway (Chen and Davis, 2002). The intracellular trafficking itineraries of Ste2 and Ste3 seem to be distinct; thus, both cargoes were analyzed in the *mvb12Δ* background. Analysis of Ste2-GFP localization in the *mvb12Δ* strain indicated that Mvb12 does not play a crucial role in this process, because no difference was observed compared with wild-type cells (Figure 4A). By contrast, trafficking of Ste3-GFP to the vacuolar lumen is severely impaired in the *mvb12Δ* strain, displaying vacuolar limiting membrane and perivacuolar punctate structure localization (Figure 4, A and C). Again, whereas *mvb12Δ* cells display Ste3-GFP localization to perivacuolar compartments, these cells do not colocalize with FM4-64 to the same degree observed in the class E *vps23Δ* cells. However, fusion of ubiquitin to Ste3-GFP (Ste3-GFP-Ub) can bypass the requirement for Mvb12 in Ste3 trafficking (Figure 4A). These results suggest that loss of Mvb12 negatively impacts the MVB sorting of Sna3 and Ste3 to a greater degree than sorting of CPS, Ste2, or Ste3-Ub. One explanation for the differential defects on Ste3-GFP and Ste3-GFP-Ub in *mvb12Δ* cells could be a defect in ubiquitin modification of Ste3. This was directly analyzed by immunoprecipitation of Ste3-GFP from wild-type, *mvb12Δ*, and *vps23Δ* cells expressing HA-ubiquitin, followed by Western blotting with either anti-GFP or anti-HA (Figure 4B). Although equivalent numbers of cells were used in for each immunoprecipitation, Western blotting with anti-GFP consistently revealed higher levels of Ste3-GFP in both *mvb12Δ* cells and *vps23Δ* cells compared with wild-type cells, consistent with a defect in its degradation in these backgrounds (Figure 4B). Regardless, *mvb12Δ* cells display an increased level of ubiquitinated Ste3-GFP compared with both wild-type and *vps23Δ* cells (Figure 4B), indicating that *mvb12Δ* cells are not defective for the ubiquitination of Ste3. Whereas the mech-

tated using anti-GFP antibody. Immunoprecipitated material was subjected to SDS-PAGE and Western blotting with anti-GFP to visualize recovered Ste3-GFP or anti-HA to visualize ubiquitinated Ste3-GFP. (C) Wild-type, *mvb12Δ*, and *vps23Δ* cells expressing Ste3-GFP were labeled with FM4-64 and visualized by fluorescence microscopy. Bar, 5 μm.

anism by which Mvb12 functions is not clear from these studies, these results indicate that that some MVB cargoes are more sensitive to Mvb12 function than others. This characteristic is distinct from prototypical class E *vps* mutants and suggests that Mvb12 may be functioning as an accessory factor involved in entry of a subset of cargoes into the MVB pathway.

To gain additional insight into the role of Mvb12 in MVB sorting, trafficking of Sna3 in the *mvb12Δ* strain was examined further. Sna3 sorting has been suggested to occur by a ubiquitin-independent process (Reggiori and Pelham, 2001). However, more recent observations indicate that Sna3 is ubiquitinated (Peng *et al.*, 2003) and that both ubiquitin-dependent and ubiquitin-independent pathways can mediate Sna3 trafficking (Oestreich *et al.*, 2007). Analysis of CPS, Ste2-GFP, and Ste3-GFP-Ub trafficking indicated that Mvb12 does not play an essential role in the general entry of ubiquitin-dependent MVB cargoes into the MVB pathway. Thus, we hypothesized that Mvb12 may contribute more to the sorting process that does not require Sna3 ubiquitination. To examine this possibility, trafficking of Sna3-GFP with all lysines mutated to arginine (Sna3^{KallR}-GFP) was examined in the *mvb12Δ* strain. Whereas Sna3^{KallR}-GFP sorted in a manner indistinguishable from Sna3-GFP in the wild-type strain (SEY6210 and BY4742), mutation of the lysine residues resulted in enhanced Sna3^{KallR}-GFP localization to peri-vacuolar punctate structures upon deletion of *MVB12* compared with Sna3-GFP (Figures 1 and 2) and a delay in Sna3^{KallR}-GFP cleavage (Figure 3, A and B, and Supplemental Figure 2, A and B). These results are consistent with Sna3 trafficking into the MVB via alternative pathways involving either ubiquitination of Sna3 or a process that does not require ubiquitination of Sna3 (Oestreich *et al.*, 2007), with loss of Mvb12 having a greater impact on the latter. The explanation for this observation is not clear at present, but it is interesting to note that Ste3 may also use this pathway. Alternatively, both Mvb12 and Sna3-ubiquitination may affect the kinetics of Sna3 entry into the MVB pathway and thus yield a synthetic phenotype when both are compromised. Although further dissection of Sna3 trafficking and Mvb12 function will be required to resolve these models, these observations are consistent with Mvb12 functioning to facilitate the sorting of a subset of MVB cargoes.

Endosomal Localization of Mvb12 Is Dependent upon the ESCRT-I Subunit Vp s23

Loss of Mvb12 function conferred several phenotypes associated with endosome-to-vacuole protein sorting defects, including partial defects in MVB sorting. Subcellular localization was addressed by integrating the GFP coding sequence in-frame with the chromosomal copy of *MVB12*, resulting in a functional Mvb12-GFP chimera (Figure 3, A–C). Visualization of cells expressing Mvb12-GFP revealed both cytoplasmic and intracellular punctate structures that colocalize with the endosomal marker DsRed-FYVE but not the Golgi marker Sec7-RFP (Figure 5). To further characterize these compartments, kinetic analysis of FM4-64 uptake was analyzed in cells expressing Mvb12-GFP (Figure 6). Colocalization of Mvb12-GFP with FM4-64 structures at early time points (1–3 min) was minimal, but increased from 5 to 7 min (Figure 6). At later time points (15–25 min), the discreet patterns of FM4-64 and Mvb12-GFP indicate that the Mvb12-GFP endocytic structures are largely perivacuolar. Colocalization with endosomes places Mvb12-GFP at a location where the MVB sorting reaction is occurring, consistent with the observed phenotypes upon its deletion. This

Figure 5. Mvb12 localizes to PtdIns(3)P-positive endosomes. Cells expressing Mvb12-GFP from their chromosomal locus were transformed with markers for either the Golgi (Sec7-RFP) or endosomes (DsRed-FYVE) and visualized by fluorescence microscopy. Bar, 5 μ m.

localization is also consistent with results obtained in a previous proteome-wide localization analysis (Huh *et al.*, 2003).

Because MVB sorting defects were observed upon deletion of *MVB12*, an interaction with ESCRT machinery was explored by examining the subcellular localization of Mvb12-GFP in a variety of strains deleted for class E *VPS* genes (Figure 7 and Supplemental Figure 3). This analysis was performed by either crossing the chromosomally integrated *MVB12-GFP* reporter into various class E *vps* mutants or by transforming a plasmid-borne *MVB12-GFP* under the control of its endogenous promoter into the mutants; consistent results were obtained by either method. In *bro1/vps31Δ*, *hse1Δ* and the ESCRT-III-like mutants (*fti1/vps46/did2Δ* and *vps60/mos10Δ*), Mvb12-GFP distribution seemed indistinguishable from wild-type cells (Supplemental Figure 3A). This suggests that these factors play no role in the endosomal association of Mvb12. However, loss of ESCRT-II (*vps25Δ*), ESCRT-III (*vps20Δ*, *vps24Δ*, or *snf7Δ*) or the AAA-ATPase Vps4 (*vps4Δ*) resulted in enhanced endosomal localization of Mvb12-GFP (Figure 7A and Supplemental Figure 3B). These results indicate that these factors are not required for the recruitment of Mvb12 to the endosomal membrane and are consistent with the idea that Mvb12 removal from the endosomal membrane requires the function of ESCRT-II, -III,

Figure 6. Mvb12 localizes with endosomes marked by short time course labeling with FM4-64. Wild-type cells expressing Mvb12-GFP were labeled with FM4-64 on ice, chased for the indicated time, and visualized by fluorescence microscopy. Bar, 5 μ m.

and Vps4. In striking contrast, loss of Vps27 or the ESCRT-I subunits Vps23 and Vps37 resulted in a dramatic reduction in the amount of endosome-associated Mvb12-GFP (Figure 7A and Supplemental Figure 3A). To further address the redistribution of Mvb12 upon loss of the ESCRT-I subunit Vps23, a previously characterized dominant-negative form of Vps4 (Vps4^{E233Q}) (Babst *et al.*, 1997) was expressed in the *vps23 Δ* strain. Expression of Vps4^{E233Q} in SEY6210 resulted in the enhanced accumulation of Mvb12-GFP on endosomes as well as reduced cytoplasmic distribution, consistent with Mvb12 localization in the *vps4 Δ* strain; however, this accumulation was blocked in the *vps23 Δ* strain, suggesting that even transient Mvb12 membrane association is absent without Vps23 (Figure 7B). However, loss of the ESCRT-I subunit Vps28 conferred increased membrane association of Mvb12-GFP, as observed upon loss of ESCRT-II, -III, and Vps4

Figure 7. Mvb12-GFP localization is dependent upon ESCRT-I. (A) Mvb12-GFP localization mimics Vps23 localization. Wild-type, *vps27 Δ* , *vps23 Δ* , and *vps4 Δ* cells expressing chromosomally integrated *MVB12-GFP* were visualized using fluorescence microscopy. (B) Wild-type or *vps23 Δ* cells expressing chromosomally integrated *MVB12-GFP* were transformed with a plasmid expressing a dominant negative form of Vps4 (Vps4^{E233Q}) and visualized by fluorescence microscopy. Bar, 5 μ m.

(Figure 7A and Supplemental Figure 3B). This effect phenocopies the redistribution of Vps23-GFP that has been observed in the *vps28 Δ* strain (Li *et al.*, 1999). These observations are consistent with Mvb12 membrane association requiring the function of Vps27 and the ESCRT-I subunits Vps23 and Vps37. ESCRT-I membrane association is dependent upon Vps27, but Vps27 membrane association is independent of ESCRT-I (Katzmann *et al.*, 2003). Similarly, association of GFP-Vps27 with endosomes marked by DsRed-FYVE is not disrupted in *mbv12 Δ* cells (Supplemental Figure 3C). Thus, Mvb12 behaves in a manner similar to the ESCRT-I subunit Vps23. These observations raised the possibility that Mvb12 is a novel subunit of ESCRT-I that facilitates its recognition of a subset of MVB cargoes.

Mvb12 Is a Component of ESCRT-I

To examine an association of Mvb12 with ESCRT-I, yeast extracts from cells expressing Mvb12-GFP or Mvb12-HA were first analyzed by gel filtration. Regardless of the form of the epitope tag used, Mvb12 behaved as a species of approximately 350 kDa, as has been demonstrated previously for ESCRT-I (Figure 8A; Babst *et al.*, 2000; Katzmann *et al.*, 2001). Monomeric Mvb12 would be predicted to behave as a species smaller than 45 kDa with either epitope and was not detected in yeast extracts (Figure 8A; data not shown), suggesting that it is stably associated with this higher molecular mass complex *in vivo*. Furthermore, bacterially expressed His₆-Mvb12 behaves as a species of approximately 18 kDa when subjected to gel filtration (Figure 8A). Given the separation range of the Sephacryl S-300 gel filtration column, this observation is consistent with bacterially expressed Mvb12 behaving as a monomer. These results sug-

Figure 8. Mvb12 physically associates with ESCRT-I in a stable manner. (A) Sephacryl S-300 gel filtration analyses were performed on extracts prepared from a variety of yeast expressing Mvb12-GFP or bacterial extracts from cells expressing His₆-Mvb12. Fractions generated from sizing runs were subjected to SDS-PAGE and Western blotting with either anti-GFP, anti-HA, or anti-pentaHis antibodies. Immunoreactive species were quantitated using BioImaging Systems and Labworks Image Acquisition and Analysis software (UVP, Upland, CA) and plotted using Microsoft Excel (Microsoft, Redmond, WA) to allow comparison of apparent molecular mass under these varied conditions. (B) S-300 gel filtration analyses were performed on extracts from *mvb12Δ*, *vps28Δ*, and *vps37Δ*. Fractions were subjected to SDS-PAGE and Western blotting with anti-Vps23 antibody, followed by quantitation as was performed in A. (C) The indicated strains were transformed with pTAP416 or pTAP416-Mvb12 and were used to generate native lysates. IgG-Sepharose was used to isolate TAP proteins, and isolated material was resolved by SDS-PAGE by using 10% acrylamide for anti-Vps23 Western blotting or 15% acrylamide for anti-Vps28 Western blotting. The TAP tag contains protein A, allowing detection by anti-Vps28 antisera. Positions of Vps23, Vps28, and TAP proteins are indicated, as is a degradation product of TAP itself (‡).

gest that Mvb12 is stably associated with a higher molecular mass complex in yeast extracts.

The apparent molecular mass of Mvb12-GFP in a variety of class E *vps* deletion backgrounds was analyzed by gel filtration to address their impact on the formation of the higher molecular mass complex. The apparent molecular mass of the Mvb12-GFP containing complex was unaffected in lysates generated from *vps27Δ*, *vps25Δ* (ESCRT-II), *snf7Δ* or *vps24Δ* (ESCRT-III), or *vps4Δ* strains (Figure 8A). However, elimination of the ESCRT-I subunits (*vps23Δ*, *vps28Δ*, and *vps37Δ*) reduced the apparent molecular mass to ~200 kDa in Vps23 or Vps28 and dramatically destabilized the Mvb12-GFP protein to the point that it was undetectable with loss of Vps37 (Figure 8A; data not shown). This result indicated that the formation of the Mvb12-containing complex depends on the function of the ESCRT-I complex. Because ESCRT-I is a 350-kDa complex, the simplest interpretation is that Mvb12 associates with ESCRT-I itself. Moreover, gel filtration analysis of Vps23 and Vps28 in extracts generated from the *mvb12Δ* strain revealed a reduction in the apparent molecular mass of ESCRT-I to less than

200 kDa (Figure 8B). For comparison, loss of Vps28 shifts the apparent molecular mass of Vps23 to approximately 200 kDa, whereas loss of Vps37 has an even greater effect on its apparent mass (Figure 8B). These observations support the conclusion that Mvb12 is a stable subunit of ESCRT-I.

To directly address the association of Mvb12 with ESCRT-I, isolation of a tandem affinity purification (TAP)-tagged form of Mvb12 was performed. TAP-Mvb12 was expressed in wild-type, *vps23Δ*, and *vps37Δ* strains, purified from cleared lysates under native conditions using IgG-Sepharose, and the isolated material was subjected to Western analysis with anti-Vps23 and anti-Vps28 antisera (Figure 8C). The ESCRT-I components Vps23 and Vps28 copurified with TAP-Mvb12 from the wild-type lysate (lane 6), but they did not copurify with the TAP-tag alone (lane 5). This result indicated that Mvb12 is physically associated with ESCRT-I. Elimination of Vps23 did not reduce TAP-Mvb12 isolation of Vps28 (lane 7) nor did loss of Vps28 eliminate TAP-Mvb12 isolation of Vps23 (Supplemental Figure 4). However, isolation of the remaining ESCRT-I components with TAP-Mvb12 was compromised in the *vps37Δ* strain (Figure 8C

and Supplemental Figure 4). Loss of another component of the MVB sorting machinery (*vps4Δ*) did not affect the association between Mvb12 and ESCRT-I (Supplemental Figure 4), consistent with the gel filtration analysis of the Mvb12-containing complex in *vps4Δ* lysates. These results confirmed that Mvb12 is a novel component of ESCRT-I and are consistent with the isolation of Vps23 and Vps37 with Mvb12-TAP in the yeast proteome-wide analysis (Krogan *et al.*, 2006). However, the phenotypes of *mvb12Δ* differ from the phenotypes of *vps23Δ*, *vps28Δ* and *vps37Δ*. These results lead us to propose that Mvb12 is modulating the ability of ESCRT-I to mediate MVB sorting of specific cargoes rather than being a core component of ESCRT-I.

DISCUSSION

The degradative capacity of the MVB pathway dictates that cargo selection must be strictly regulated. Our understanding of this process has been expanded through the identification of ubiquitylation as a posttranslational modification that can target proteins into this pathway, as well as *trans*-acting machinery capable of recognizing this modification and directing entry in this pathway (for reviews, see Katzmann *et al.*, 2002; Hicke and Dunn, 2003; Raiborg *et al.*, 2003; Babst, 2005). ESCRT-I was first identified as a complex that is capable of interacting with ubiquitin-modified MVB cargo via the UEV domain in Vps23 (Katzmann *et al.*, 2001). Subsequent work has revealed the presence of additional members of the class E Vps proteins that contain ubiquitin-interacting domains, including Vps27/Hrs, which participates in recruitment of ESCRT-I (Bilodeau *et al.*, 2002; Shih *et al.*, 2002; Bache *et al.*, 2003; Bilodeau *et al.*, 2003; Katzmann *et al.*, 2003). Together, Vps27 and ESCRT-I seem to play a critical role in the selection of ubiquitin-modified MVB cargoes. The reason behind this apparently complicated mechanism of cargo recognition is not entirely obvious; however, a variety of explanations including site of action, degree/linkage of cargo ubiquitination, and even affinity have merits. Furthermore, cargoes that are capable of entering the MVB pathway independent of ubiquitin modification exist and may require additional mechanisms of recognition by the MVB sorting machinery.

To better understand the mechanisms of cargo selection by the MVB pathway we performed a genetic screen for factors impacting Sna3 sorting, identifying *mvb12Δ* in the process. Deletion of *MVB12* confers cargo-specific defects in MVB sorting, a feature that makes it distinct from previously described class E *vps* deletion mutants that display unilateral defects in MVB cargo sorting. This feature is particularly interesting considering that our data indicate that Mvb12 is a member of the ESCRT-I complex. But given that loss of the core ESCRT-I subunits (Vps23, Vps28, and Vps37) confer significantly more severe phenotypes, Mvb12 is not critical for all functions executed by ESCRT-I. Based on these observations, we suggest that Mvb12 plays a role in assisting ESCRT-I during sorting of a subset of MVB cargoes.

Although further investigation is required to distinguish the mechanism by which this occurs, several possibilities exist that could explain this role in cargo-specific function. One potential model is that Mvb12 facilitates the binding of ESCRT-I to a subset of MVB cargoes, including Ste3 and Sna3, but it is dispensable for binding of ESCRT-I to CPS and Ste2. Although it is attractive to speculate that Mvb12 could directly bind to Ste3 and Sna3 and mediate their interaction with ESCRT-I, it is equally likely that Mvb12 is not involved in directly binding these cargoes but helps to generate the Ste3 and Sna3 binding site(s) within ESCRT-I indirectly.

How would this model explain the suppression of Ste3 trafficking defects in the *mvb12Δ* strain by direct fusion to ubiquitin (Ste3-GFP-Ub)? Although Ste3 has been demonstrated to be a ubiquitin-dependent MVB cargo, there may be ways in which the ubiquitinated form of Ste3 is recognized by the Mvb12-containing ESCRT-I that are distinct from the ESCRT-I recognition of the Ste3-Ub fusion, possibly through differential recognition of ubiquitin itself. Further experimentation will be required to address the model that Mvb12 allows ESCRT-I binding, either directly or indirectly, to a subset of MVB cargoes.

A second model for the role of Mvb12 in the MVB sorting of Ste3 and Sna3 but not CPS or Ste2 involves the trafficking and MVB sorting of distinct cargoes via discrete endosomal compartments. Recent evidence in mammalian cells is consistent with the idea that there are distinct classes of MVBs being formed at distinct sites (White *et al.*, 2006). Similar phenomenon may be occurring in yeast. In this second model, Ste3 and Sna3 would traffic to the vacuole via an endosomal compartment that is distinct from that transited by CPS and Ste2. Although ESCRT-I mediates MVB sorting at both compartments, Mvb12 may be required for ESCRT-I localization or function at the Ste3/Sna3 compartment but not the Ste2/CPS compartment. Direct fusion of ubiquitin to the carboxy terminus of Ste3 (Ste3-Ub) may alter Ste3-Ub trafficking to the Ste2/CPS-sorting compartment, allowing MVB sorting in the absence of Mvb12. This idea seems plausible in that Ste2 is thought to internalize in a ubiquitinated form, whereas Ste3 is thought to recycle between an endosomal compartment and the cell surface until ubiquitination removes it from this recycling loop (Terrell *et al.*, 1998; Chen and Davis, 2002). It is possible that endocytosed cargoes that are ubiquitinated bypass a recycling endosome. At present, characterization of the yeast endosomal system is not sufficient to address the possibility of discrete endosomes for sorting distinct MVB cargoes. Further experimentation will be required to map out the potential subtleties of the yeast endosomal system and cargo-specific MVB sorting.

A third model for the role of Mvb12 in cargo-specific sorting involves some more general form of modulation of ESCRT-I function, perhaps through associations with additional ESCRT machinery. For example, ESCRT-I efficiency could be impacted through Mvb12-modulated interactions with components that act upstream or downstream of ESCRT-I. How might this lead to cargo-specific defects upon loss of Mvb12? Different MVB cargoes, such as Sna3 and Ste3 may be more sensitive to the level of ESCRT-I function, thereby revealing differential sorting phenotypes when ESCRT-I function is compromised by loss of Mvb12. However, in our experience GFP-CPS is the most sensitive cargo that can be used to address function of the MVB pathway (Azmi *et al.*, 2006; our unpublished data). Thus, the observation that *mvb12Δ* has only a subtle impact on the sorting of GFP-CPS seems to cast doubt upon this third model. However, further experimentation will be required to definitively address this role for Mvb12 as a general modulator of ESCRT-I function.

Loss of Mvb12 function confers partial defects in the sorting of cargoes into the MVB pathway. Although the mechanism is unclear, Mvb12 seems to play a role in assisting ESCRT-I to sort a subset of MVB cargoes as a stable component of ESCRT-I. This begs the question as to how this was missed during the initial characterization of ESCRT-I. One possibility is that the small size of the Mvb12 protein (12 kDa) precluded its identification in the isolation procedure by using protein A-tagged Vps23 (Katzmann *et al.*, 2001). Although the reason that Mvb12 was not previously identi-

fied remains unclear, the more intriguing question that arises is whether mammalian ESCRT-I may also contain accessory subunits that have eluded detection to this point. Although Mvb12 has been conserved among fungi, sequence homologues in higher eukaryotes have escaped our detection. However, it seems likely that functional homologues exist that modulate the function of mammalian ESCRT-I with respect to specific MVB cargoes as well. Additional studies will provide a deeper understanding of the role of Mvb12 in cargo-specific ESCRT-I function during the MVB sorting reaction and may provide insight regarding mammalian counterparts as well.

Additional Note: The laboratories of both Markus Babst (University of Utah, Salt Lake City, UT) and Scott Emr (University of California, San Diego, San Diego, CA) have independently identified the gene product of YGR206w as a subunit of the ESCRT-I complex. Because it is a 12-kDa protein involved in MVB sorting, we have agreed on the designation Mvb12 for the gene product of YGR206w.

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REFERENCES

- Azmi, I., Davies, B., Dimaano, C., Payne, J., Eckert, D., Babst, M., and Katzmman, D. J. (2006). Recycling of ESCRTs by the AAA-ATPase Vps4 is regulated by a conserved VSL region in Vta1. *J. Cell Biol.* 172, 705–717.
- Babst, M. (2005). A protein's final ESCRT. *Traffic* 6, 2–9.
- Babst, M., Katzmman, D. J., Estepa-Sabal, E. J., Meerloo, T., and Emr, S. D. (2002a). Escrt-III: an endosome-associated heterooligomeric protein complex required for mvb sorting. *Dev. Cell* 3, 271–282.
- Babst, M., Katzmman, D. J., Snyder, W. B., Wendland, B., and Emr, S. D. (2002b). Endosome-associated complex, ESCRT-II, recruits transport machinery for protein sorting at the multivesicular body. *Dev. Cell* 3, 283–289.
- Babst, M., Odorizzi, G., Estepa, E. J., and Emr, S. D. (2000). Mammalian tumor susceptibility gene 101 (TSG101) and the yeast homologue, Vps23p, both function in late endosomal trafficking. *Traffic* 1, 248–258.
- Babst, M., Sato, T. K., Banta, L. M., and Emr, S. D. (1997). Endosomal transport function in yeast requires a novel AAA-type ATPase, Vps4p. *EMBO. J.* 16, 1820–1831.
- Bache, K. G., Brech, A., Mehlum, A., and Stenmark, H. (2003). Hrs regulates multivesicular body formation via ESCRT recruitment to endosomes. *J. Cell Biol.* 162, 435–442.
- Bache, K. G., Slagsvold, T., Cabezas, A., Rosendal, K. R., Raiborg, C., and Stenmark, H. (2004). The growth-regulatory protein HCRP1/hVps37A is a subunit of mammalian ESCRT-I and mediates receptor down-regulation. *Mol. Biol. Cell* 15, 4337–4346.
- Bilodeau, P. S., Urbanowski, J. L., Winistorfer, S. C., and Piper, R. C. (2002). The Vps27p Hse1p complex binds ubiquitin and mediates endosomal protein sorting. *Nat. Cell Biol.* 4, 534–539.
- Bilodeau, P. S., Winistorfer, S. C., Kearney, W. R., Robertson, A. D., and Piper, R. C. (2003). Vps27-Hse1 and ESCRT-I complexes cooperate to increase efficiency of sorting ubiquitinated proteins at the endosome. *J. Cell Biol.* 163, 237–243.
- Bishop, N., and Woodman, P. (2001). Tsg101/mammalian vps23 and mammalian vps28 interact directly and are recruited to vps4-induced endosomes. *J. Biol. Chem.* 276, 11735–11742.
- Bonangelino, C. J., Chavez, E. M., and Bonifacio, J. S. (2002). Genomic screen for vacuolar protein sorting genes in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* 13, 2486–2501.
- Bowers, K., Lottridge, J., Helliwell, S. B., Goldthwaite, L. M., Luzio, J. P., and Stevens, T. H. (2004). Protein-protein interactions of ESCRT complexes in the yeast *Saccharomyces cerevisiae*. *Traffic* 5, 194–210.
- Chen, L., and Davis, N. G. (2002). Ubiquitin-independent entry into the yeast recycling pathway. *Traffic* 3, 110–123.
- Davies, B. A., Topp, J. D., Sfeir, A. J., Katzmman, D. J., Carney, D. S., Tall, G. G., Friedberg, A. S., Deng, L., Chen, Z., and Horazdovsky, B. F. (2003). Vps9p CUE domain ubiquitin binding is required for efficient endocytic protein traffic. *J. Biol. Chem.* 278, 19826–19833.
- Gorden, P., Carpentier, J. L., Cohen, S., and Orci, L. (1978). Epidermal growth factor: morphological demonstration of binding, internalization, and lysosomal association in human fibroblasts. *Proc. Natl. Acad. Sci. USA* 75, 5025–5029.
- Gruenberg, J., and Stenmark, H. (2004). The biogenesis of multivesicular endosomes. *Nat. Rev. Mol. Cell Biol.* 5, 317–323.
- Haigler, H. T., McKanna, J. A., and Cohen, S. (1979). Direct visualization of the binding and internalization of a ferritin conjugate of epidermal growth factor in human carcinoma cells A-431. *J. Cell Biol.* 81, 382–395.
- Hicke, L., and Dunn, R. (2003). Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins. *Annu. Rev. Cell Dev. Biol.* 19, 141–172.
- Huh, W. K., Falvo, J. V., Gerke, L. C., Carroll, A. S., Howson, R. W., Weissman, J. S., and O'Shea, E. K. (2003). Global analysis of protein localization in budding yeast. *Nature* 425, 686–691.
- Katzmann, D. J., Babst, M., and Emr, S. D. (2001). Ubiquitin-dependent sorting into the multivesicular body pathway requires the function of a conserved endosomal protein sorting complex, ESCRT-I. *Cell* 106, 145–155.
- Katzmann, D. J., Odorizzi, G., and Emr, S. D. (2002). Receptor downregulation and multivesicular-body sorting. *Nat. Rev. Mol. Cell Biol.* 3, 893–905.
- Katzmann, D. J., Sarkar, S., Chu, T., Audhya, A., and Emr, S. D. (2004). Multivesicular body sorting: ubiquitin ligase Rsp5 is required for the modification and sorting of carboxypeptidase S. *Mol. Biol. Cell* 15, 468–480.
- Katzmann, D. J., Stefan, C. J., Babst, M., and Emr, S. D. (2003). Vps27 recruits ESCRT machinery to endosomes during MVB sorting. *J. Cell Biol.* 162, 413–423.
- Kostelansky, M. S., Sun, J., Lee, S., Kim, J., Ghirlando, R., Hierro, A., Emr, S. D., and Hurley, J. H. (2006). Structural and functional organization of the ESCRT-I trafficking complex. *Cell* 125, 113–126.
- Krogan, N. J. *et al.* (2006). Global landscape of protein complexes in the yeast *Saccharomyces cerevisiae*. *Nature* 440, 637–643.
- Li, Y., Kane, T., Tipper, C., Spatrick, P., and Jenness, D. D. (1999). Yeast mutants affecting possible quality control of plasma membrane proteins. *Mol. Cell. Biol.* 19, 3588–3599.
- Morita, E., and Sundquist, W. I. (2004). Retrovirus budding. *Annu. Rev. Cell Dev. Biol.* 20, 395–425.
- Mumberg, D., Muller, R., and Funk, M. (1995). Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. *Gene* 156, 119–122.
- Odorizzi, G., Babst, M., and Emr, S. D. (1998). Fab1p PtdIns(3)P 5-kinase function essential for protein sorting in the multivesicular body. *Cell* 95, 847–858.
- Oestreich, A. J., Aboian, M., Lee, J., Azmi, I., Payne, J., Issaka, R., Davies, B. A., and Katzmman, D. J. (2007). Characterization of multiple multivesicular body sorting determinants within Sna3: a role for the ubiquitin ligase Rsp5. *Mol. Biol. Cell* 18, 707–720.
- Peng, J., Schwartz, D., Elias, J. E., Thoreen, C. C., Cheng, D., Marsischky, G., Roelofs, J., Finley, D., and Gygi, S. P. (2003). A proteomics approach to understanding protein ubiquitination. *Nat. Biotechnol.* 21, 921–926.
- Raiborg, C., Bache, K. G., Gillooly, D. J., Madhus, I. H., Stang, E., and Stenmark, H. (2002). Hrs sorts ubiquitinated proteins into clathrin-coated microdomains of early endosomes. *Nat. Cell Biol.* 4, 394–398.
- Raiborg, C., Rusten, T. E., and Stenmark, H. (2003). Protein sorting into multivesicular endosomes. *Curr. Opin. Cell Biol.* 15, 446–455.
- Raymond, C. K., Howald-Stevenson, I., Vater, C. A., and Stevens, T. H. (1992). Morphological classification of the yeast vacuolar protein sorting mutants: evidence for a prevacuolar compartment in class E *vps* mutants. *Mol. Biol. Cell* 3, 1389–1402.
- Reggiori, F., and Pelham, H. R. (2001). Sorting of proteins into multivesicular bodies: ubiquitin-dependent and -independent targeting. *EMBO J.* 20, 5176–5186.

- Shih, S. C., Katzmann, D. J., Schnell, J. D., Sutanto, M., Emr, S. D., and Hicke, L. (2002). Epsins and Vps27p/Hrs contain ubiquitin-binding domains that function in receptor endocytosis. *Nat. Cell Biol.* 4, 389–393.
- Teo, H., Gill, D. J., Sun, J., Perisic, O., Veprintsev, D. B., Vallis, Y., Emr, S. D., and Williams, R. L. (2006). ESCRT-I core and ESCRT-II GLUE domain structures reveal role for GLUE in linking to ESCRT-I and membranes. *Cell* 125, 99–111.
- Teo, H., Veprintsev, D. B., and Williams, R. L. (2004). Structural insights into endosomal sorting complex required for transport (ESCRT-I) recognition of ubiquitinated proteins. *J. Biol. Chem.* 279, 28689–28696.
- Terrell, J., Shih, S., Dunn, R., and Hicke, L. (1998). A function for monoubiquitination in the internalization of a G protein-coupled receptor. *Mol. Cell* 1, 193–202.
- Theos, A. C., Truschel, S. T., Tenza, D., Hurbain, I., Harper, D. C., Berson, J. F., Thomas, P. C., Raposo, G., and Marks, M. S. (2006). A luminal domain-dependent pathway for sorting to intraluminal vesicles of multivesicular endosomes involved in organelle morphogenesis. *Dev. Cell* 10, 343–354.
- Tong, A. H. *et al.* (2001). Systematic genetic analysis with ordered arrays of yeast deletion mutants. *Science* 294, 2364–2368.
- Urbanowski, J. L., and Piper, R. C. (2001). Ubiquitin sorts proteins into the intraluminal degradative compartment of the late-endosome/vacuole. *Traffic* 2, 622–630.
- Vida, T. A., and Emr, S. D. (1995). A new vital stain for visualizing vacuolar membrane dynamics and endocytosis in yeast. *J. Cell Biol.* 128, 779–792.
- von Schwedler, U. K. *et al.* (2003). The protein network of HIV budding. *Cell* 114, 701–713.
- White, I. J., Bailey, L. M., Aghakhani, M. R., Moss, S. E., and Futter, C. E. (2006). EGF stimulates annexin 1-dependent inward vesiculation in a multivesicular endosome subpopulation. *EMBO J.* 25, 1–12.