Characterization of Multiple Multivesicular Body Sorting Determinants within Sna3: A Role for the Ubiquitin Ligase Rsp5[□]

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A subset of proteins that transit the endosomal system are directed into the intraluminal vesicles of multivesicular bodies (MVBs). MVB formation is critical for a variety of cellular functions including receptor down-regulation, viral budding, antigen presentation, and the generation of lysosome-related organelles. Entry of transmembrane proteins into the intraluminal vesicles of a MVB is a highly regulated process that is positively modulated by covalent modification of cargoes with ubiquitin. To identify additional MVB sorting signals, we examined the previously described ubiquitination-independent MVB cargo Sna3. Although Sna3 ubiquitination is not essential, Sna3 MVB sorting is positively modulated by its ubiquitination. Examination of MVB sorting determinants within a form of Sna3 lacking all lysine residues identified two critical regions: an amino-terminal tyrosine-containing region and a carboxyl-terminal PPAY motif. This PPAY motif interacts with the WW domains of the ubiquitin ligase Rsp5, and mutations in either the WW or, surprisingly, the HECT domains of Rsp5 negatively impacted MVB targeting of lysine-minus Sna3. These data indicate that Rsp5 function is required for MVB targeting of Sna3 in a capacity beyond cargo ubiquitination. These results uncover a series of determinants impacting Sna3 MVB sorting, including unexpected roles for Rsp5.

INTRODUCTION

The endosomal network is dynamic, yet maintains distinct compartments dedicated to coordinating protein traffic between a number of subcellular compartments for function or degradation. Delivery of transmembrane proteins to the limiting membrane of the lysosome exposes luminal domains to potential degradation or clipping; additionally, the formation of a multivesicular body/endosome represents a mechanism by which cytoplasmic and transmembrane domains can also be degraded in the lysosome (Katzmann *et al.*, 2002; Gruenberg and Stenmark, 2004). Multivesicular body (MVB) formation involves the invagination and budding of the limiting membrane of the endosome into its

lumen. Endosomal membrane proteins destined for degradation within the lysosome are actively sorted into the intraluminal vesicles of a MVB, whereas proteins destined for the limiting membrane of the lysosome are retained within the limiting membrane of the MVB. Studies in receptor down-regulation have revealed that ubiquitination of endosomal membrane proteins is sufficient to confer entry into the MVB pathway (Katzmann *et al.*, 2002; Hicke and Dunn, 2003).

A number of ubiquitin ligases have been identified in yeast and animal cells that play a critical role in this process by covalently attaching ubiquitin to cargo proteins, thereby targeting them for inclusion into intraluminal vesicles. In yeast, the HECT ubiquitin ligase Rsp5 has been demonstrated to play a role in targeting a variety of MVB cargoes into this pathway (Soetens *et al.*, 2001; Blondel *et al.*, 2004; Dunn *et al.*, 2004; Hettema *et al.*, 2004; Katzmann *et al.*, 2004; Morvan *et al.*, 2004). It has also been suggested that Rsp5 exerts a role in addition to cargo modification during the endocytic step, possibly modulation of machinery (Dunn and Hicke, 2001b). In animal cells, down-regulation of a variety of cell surface proteins including the epidermal growth factor receptor (EGFR) requires the RING ubiquitin ligase Cbl (Schmidt and Dikic, 2005). Cbl appears to play multiple roles during endocytosis and lysosomal targeting of cell surface proteins. In the case of EGFR, the ubiquitin ligase activity of Cbl is responsible not only for its modification (Joazeiro *et al.*, 1999; Levkowitz *et al.*, 1999; Yokouchi *et al.*, 1999), but also the regulation of associated endocytic machinery (Haglund *et al.*, 2002). In addition to its enzymatic function, Cbl functions as an endocytic sorting adaptor that links cargo to endocytic machinery (Soubeyran *et al.*, 2002). It is therefore possible for ubiquitin ligases to function in a

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The laboratories of both Greg Odorizzi (University of Colorado Boulder; see McNatt *et al.*, 2007) and Rosine Haguenaer-Tsapis (Institut Jacques Monod-CNRS Universites Paris) have also recently uncovered a role for Rsp5 in the sorting of Sna3 into the MVB pathway.

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Abbreviations used: CPS, carboxypeptidase S; DPAP B, dipeptidylaminopeptidase B; ESCRT, endosomal sorting complex required for transport; MVB, multivesicular body; ORF, open reading frame; TAP, tandem affinity purification; VPS, vacuolar protein sorting.

variety of roles during lysosomal targeting of cell surface proteins.

Not all MVB cargoes depend on ubiquitin modification for their entry into MVBs. Although components of the ESCRT machinery required for function of the MVB pathway are also required for the budding of certain viruses from the host cell, the role of ubiquitination in this process is less clear (Morita and Sundquist, 2004). The HIV Gag protein is required for the formation of viral particles and can interact with endosomal sorting complexes required for transport (ESCRTs) during viral assembly through several mechanisms (Strack *et al.*, 2003; von Schwedler *et al.*, 2003). Although ubiquitinated Gag can interact with the ESCRT-I subunit Tsg101 through its UEV domain, this interaction does not appear to be critical for the formation of viral particles. Tsg101 can bind directly to a PTAP sequence within the late domain of Gag (Garrus *et al.*, 2001) and lysine to arginine Gag mutants that are predicted to block ubiquitination are still capable of supporting the formation of particles (Ott *et al.*, 2000). The δ -opioid receptor has been reported to enter the MVB pathway without receiving ubiquitin modification, although it may do so by interacting with other ubiquitinated proteins that have been engaged by the ESCRTs (Hislop *et al.*, 2004). The melanosomal protein Pmel17 transits an MVB intermediate en route to the lysosome-related melanosome, yet neither ubiquitination nor ESCRT function appear to be essential for this (Theos *et al.*, 2006). These data indicate that multiple mechanisms exist for the targeting of cargoes into the MVB pathway.

In yeast, Sna3 has been reported to require ESCRT function to enter the MVB pathway, yet neither its ubiquitination nor the function of Rsp5 have been implicated as critical for this process (Reggiori and Pelham, 2001; Bilodeau *et al.*, 2002; Katzmann *et al.*, 2004). However, ubiquitination of lysine 125 in Sna3 has also been reported (Peng *et al.*, 2003). To further define the role of ubiquitination in Sna3 sorting and to uncover MVB sorting signals independent of cargo ubiquitination, extensive analysis of Sna3 sorting determinants has been performed. We demonstrate that Sna3 is ubiquitinated in a Rsp5-dependent manner. However, Sna3 ubiquitination potentiates but is not essential for Sna3 MVB sorting. Two additional determinants, a tyrosine-containing region and a PPXY motif, were identified that impact Sna3 MVB sorting, and both of these were demonstrated to be sufficient to redirect a vacuolar-limiting membrane protein [green fluorescent protein (GFP)-DPAP B] into the MVB pathway. The PPXY motif responsible for mediating interaction with Rsp5 is required not only for Sna3 ubiquitination, but also the sorting of this cargo regardless of its ubiquitination status. These findings uncover a complicated series of partially redundant signals impacting Sna3 MVB sorting and indicate an unexpected function for Rsp5 in Sna3 trafficking.

MATERIALS AND METHODS

Strains

The following yeast strains were used in this study: SEY6210 (MAT α *leu2-3112 ura3-56 his3- Δ 200 trp1- Δ 901 lys2-801 suc2- Δ 9*; Robinson *et al.*, 1988), MAY3 (SEY6210, *sna3 Δ ::HIS3*; this study), MAY4 (SEY6210, *sna3 Δ ::HIS3, pep12 Δ ::LEU2*; this study), TVY614 (SEY6210, *pep4 Δ ::LEU2 prb1 Δ ::HISG prc1 Δ ::HIS3*; Wurmser and Emr, 1998), MY832 (MAT α , *MDM1, rsp5::HIS3, his3, leu2, ura3*, pRS316-*mdp1-1*; Fisk and Yaffe, 1999), MY833 (MAT α , *MDM1, rsp5::HIS3, his3, leu2, ura3*, pRS316-*mdp1-13*; Fisk and Yaffe, 1999), MY834 (MAT α , *MDM1, rsp5::HIS3, his3, leu2, ura3*, pRS316-*mdp1-14*; Fisk and Yaffe, 1999), MY808 (MAT α , *MDM1, smm1, his3, leu2, ura3*; Fisk and Yaffe, 1999), MY290 (MAT α , *MDM1, his3, leu2, ura3*; Fisk and Yaffe, 1999), DKY83 (MY808, *pep12 Δ ::HIS3*; Katzmann *et al.*, 2004). To generate the WW mutant forms of RSP5, these were subcloned into pDsRed415 and transformed

into a diploid strain in which one copy of RSP5 was deleted with the HIS3 marker (MAY11; SEY6210/SEY6210.1 MAT α / α *rsp5::HIS3*), sporulated and appropriate markers were selected following tetrad dissection. Alternatively, a haploid strain carrying an URA3-marked version of RSP5 on a plasmid was transformed with the LEU-marked WW mutants and subjected to plasmid shuffle to remove the wild-type copy of RSP5. Strains JPY78 (SEY6210, *rsp5 Δ ::HIS3*, pDsRed415-*rsp5^{WT}*), JPY81 (SEY6210, *rsp5 Δ ::HIS3*, pDsRed415-*rsp5^{WW1}*), JPY83 (SEY6210, *rsp5 Δ ::HIS3*, pDsRed415-*rsp5^{WW2}*), JPY79 (SEY6210.1, *rsp5 Δ ::HIS3*, pDsRed415-*rsp5^{WW3}*), and JPY86 (SEY6210, *rsp5 Δ ::HIS3*, pTAP416-*rsp5^{WW3}*) were constructed through tetrad dissection. Strains JPY60 (SEY6210, *rsp5 Δ ::HIS3*, pDsRed415-*rsp5^{WW1,2}*), JPY63 (SEY6210, *rsp5 Δ ::HIS3*, pDsRed415-*rsp5^{WW2,3}*), JPY66 (SEY6210, *rsp5 Δ ::HIS3*, pDsRed415-*rsp5^{WW1,3}*), JPY69 (SEY6210, *rsp5 Δ ::HIS3*, pDsRed415-*rsp5^{WT}*), and JPY74 (SEY6210, *rsp5 Δ ::HIS3*, pDsRed415-*rsp5^{WW1,2,3}*) were constructed through plasmid shuffle.

Plasmids

pRS416-Sna3-GFP has been previously described (Reggiori and Pelham, 2001). Scanning alanine and point mutants of Sna3-GFP were constructed using the GeneTailor Site-Directed Mutagenesis System (Invitrogen, Carlsbad, CA). The TP11 promoter, GFP, and SNA3 were PCR amplified from pRS416-Sna3-GFP or Sna3^{KallR}-GFP and cloned into the XhoI and ClaI, XmaI and BamHI, and BamHI and XbaI sites of pRS416, respectively, to create GFP-Sna3 constructs. pRS315-Sna3-GFP and pRS315-Sna3^{KallR}-GFP were cloned as XhoI and SstI fragments from pRS416-Sna3-GFP and pRS416-Sna3^{KallR}-GFP, respectively, into pRS315. GFP-DPAP B (pGO89) was previously described (Odorizzi *et al.*, 1998). The HindIII fragment from pGO89 was cloned into pGOGFP426 (Odorizzi *et al.*, 1998), and GFP-Sna3^{NTD}-DPAP B, GFP-Sna3^{MSYS}-DPAP B, GFP-Sna3^{MSAS}-DPAP B, GFP-Sna3(AQPPAYDEL)-DPAP B, and GFP-Sna3(AQPPAADEL)-DPAP B chimeras were constructed by cloning phosphorylated oligonucleotides of their respective residues into the BglII and HindIII sites. To make the pET glutathione S-transferase (GST) vector, GST was amplified from pGST parallel 1 (Sheffield *et al.*, 1999), cloned into pBS (Stratagene, La Jolla, CA) at the EcoRV site, and digested with EcoRV and NcoI. The pET28 vector (Novagen, Madison, WI) was digested with NotI, blunted with Klenow fragment, digested with NcoI, and ligated with the GST fragment. CPS-GST and DPAP B-GST were constructed by cloning phosphorylated oligonucleotides corresponding to residues 1-19 and 1-29, respectively, into pET GST. GST-Sna3^{NTD} construct was made by cloning phosphorylated oligonucleotides corresponding to residues 1-21 into BamHI and SalI sites of pGST parallel 1. GST-Sna3^{CTD} construct was made by cloning BamHI and SalI PCR fragments corresponding to Sna3 carboxy terminus (residues 68-133) into pGST parallel 1. GST-Sna3^{ATM} construct was made by PCR of nucleotides corresponding to the amino terminus (residues 1-21) and carboxy terminus (68-133) with overlapping primers and sequential PCR of the products to make 1-21, 68-133 residue PCR product that lacks the portion encoding the transmembrane regions of Sna3, followed by cloning this PCR product into pGST parallel 1 vector via the BamHI and SalI sites. pGPD414HA-Ub was constructed by subcloning into SacI and KpnI sites of pRS414 from pGPD416HA-Ub (Davies *et al.*, 2003). The pRS316-HA-RSP5 vector has been described elsewhere (Gajewska *et al.*, 2001). The RSP5 open reading frame (ORF) was amplified from yeast genomic DNA and cloned into pBC (Stratagene) using BglII and XhoI sites. The following mutants were constructed using GeneTailor (Invitrogen): WW1 (W257A), WW2 (W359A), WW3 (W415A), WW1/2 (W257A, W359A), WW2/3 (W359A, W415A), WW1/3 (W257A, W415A), WW1/2/3 (W257A, W359A, W415A), and G753I. The mutants and wild-type RSP5 were subcloned using BamHI and SalI sites into pET28MBP (Davies *et al.*, 2005), and pTAP416 (Oestreich *et al.*, 2007), and pDsRed415 for bacterial and yeast expression, respectively. To construct pDsRed415, DsRed was amplified from pDsRed-N1 (Clontech, Mountain View, CA) and cloned into pRS416 with the TDH3 promoter and subcloned into pRS415 using SacI and XhoI sites. All PCR-based vectors were sequenced through the coding region to ensure aberrant mutations were not present.

Microscopy

Live cells grown in minimal media were used for fluorescence microscopy. Micrographs were captured using a fluorescence microscope Olympus IX70 (Center Valley, PA) with fluorescein isothiocyanate, GFP, rhodamine, and DsRed filters and a digital camera (Coolsnap HQ; Photometrics, Tucson, AZ) and were deconvolved using Delta Vision software (Applied Precision, Issaquah, WA).

Antibody Production

Sna3CTD polyclonal antibody was generated against GST-Sna3CTD purified from BL21 *Escherichia coli* (Covance, Denver, PA).

Pulse-Chase Analysis

Pulse-chase analysis was performed essentially as described in Babst *et al.* (2002). Briefly, cells with endogenous Sna3 and/or expressing GFP-tagged Sna3 constructs were pulse-labeled with ³⁵S-Cys/Met and chased with an excess of Cys/Met. Samples were precipitated with trichloroacetic acid at the given time points, processed, and immunoprecipitated with either monoclo-

nal anti-GFP AV JL-8 (BD Bioscience, San Jose, CA) or polyclonal anti-Sna3 (this study). After SDS-PAGE, gels were exposed to phosphorimaging screens. Visualization and quantification was performed using a Storm840 system (GE Healthcare, Piscataway, NJ), and the protein amount was normalized to the zero time point. Experiments were performed at least three times, and statistical analysis was performed using one-way ANOVA nonparametric analysis (Prism software package; GraphPad, San Diego, CA).

Protein Expression and Purification

BL21 *E. coli* (Stratagene) expressing His-MBP-Rsp5 were induced with 0.5 mM IPTG at 22°C overnight. The His-MBP-Rsp5 protein was purified using Ni NTA agarose (Qiagen, Valencia, CA), concentrated, and stored at -80°C. BL21 *E. coli* (Stratagene) expressing GST-Sna3^{CTD} and GST-Sna3^{ΔTM} were induced with 0.5 mM IPTG at 37°C for 2 h and lysed by sonication. Lysates were either used directly for GST pull-downs or further purified using glutathione Sepharose beads for antibody production. Glutathione Sepharose (Amersham Biosciences, Piscataway, NJ) was washed with phosphate-buffered saline and incubated with equal amounts of BL21 lysates expressing GST constructs for 1 h at room temperature with rocking, and washed three times in HEPES lysis buffer (20 mM HEPES, pH 6.8, 50 mM KOAc, 2 mM EDTA, 0.5% Triton X-100, 1× Complete Protease Inhibitor without EDTA Cocktail Tablets (Roche, Indianapolis, IN), 32 μg/ml *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), 32 μg/ml *N*-tosyl-L-lysine chloromethyl ketone (TLCK), 3.3 μg/ml leupeptin, and 3.3 μg/ml trypsin inhibitor).

Yeast expressing GFP-Rsp5 or TAP-Rsp5 mutants, 75–100 OD, were lysed under native conditions with HEPES lysis buffer (20 mM HEPES, pH 6.8, 50 mM KOAc, 2 mM EDTA, 0.5% Triton X-100, 1× Complete Protease Inhibitor without EDTA Cocktail Tablets (Roche), 32 μg/ml TPCK, 32 μg/ml TLCK, 3.3 μg/ml leupeptin, and 3.3 μg/ml trypsin inhibitor) using glass bead lysis or douncing and cleared by centrifugation at 100,000 × *g* for 20 min at 4°C.

Equal amounts of yeast lysates or purified His₆-MBP-Rsp5 in HEPES lysis buffer plus 1 mg/ml bovine serum albumin were incubated with glutathione Sepharose for 1 h at 4°C. Bound material was washed two times with HEPES lysis buffer and one time with HEPES lysis buffer without detergent or protease inhibitors and subjected to SDS-PAGE and Western blotting. Bound TAP-Rsp5 or bound GFP-Rsp5 was visualized with polyclonal anti-actin (Sigma, St. Louis, MO) and monoclonal anti-GFP AV JL-8 (BD Bioscience), respectively, and His₆-MBP-Rsp5 was visualized with monoclonal anti-MBP (Sigma). GST inputs were visualized with Coomassie blue stain.

Sna3 Immunoprecipitations

Sna3 immunoprecipitations were performed as in Katzmann *et al.* (2001). Yeast expressing Sna3 constructs (10 OD) with or without hemagglutinin (HA)-ubiquitin were TCA precipitated, processed, and lysed with glass beads. Immunoprecipitation was performed with either monoclonal anti-GFP AV JL-8 (BD Bioscience) or polyclonal anti-Sna3^{CTD} (this study), and samples were subjected to SDS-PAGE and Western blotting. Sna3 was detected with either anti-GFP or anti-Sna3^{CTD}. Ubiquitination status was determined with monoclonal anti-HA.11 (Covance) to recognize HA-ubiquitin or monoclonal anti-ubiquitin (Zymed, South San Francisco, CA).

RESULTS

Ubiquitin Modification of Sna3 Is Not a Requisite for Entry into the MVB Pathway

Sna3 has been characterized as a cargo capable of entering the MVB pathway independent of ubiquitin modification, while retaining ESCRT dependence (Reggiori and Pelham, 2001; Bilodeau *et al.*, 2002; Katzmann *et al.*, 2004). It is predicted to contain two transmembrane regions (residues 22–37 and 47–69), with the amino- and carboxyl-termini residing in the cytoplasm before sorting into the MVB. This predicted topology results in accessibility of lysines at positions 19 and 125 to the ubiquitin conjugation machinery (see Figure 2) and a proteome-wide screen for ubiquitin-modified proteins in yeast identified Sna3 as a protein that receives ubiquitin modification at lysine 125 (Peng *et al.*, 2003). However, conversion of lysine residues 19 and 125 to arginine was found to have no impact on sorting into the MVB pathway (Reggiori and Pelham, 2001). This finding suggested that Sna3 is sorted into the MVB pathway independent of ubiquitination. An alternative explanation could be that other ubiquitination sites, including the amino-terminal primary amine or lysines at positions 38 and 40, were being utilized to direct ubiquitin-dependent MVB sorting. To address this possibility, further analysis of the role of ubiquiti-

nation in Sna3 MVB sorting was performed. An allele was generated with all four lysine codons, encoding residues at positions 19, 38, 40, and 125, mutated to arginine codons (Sna3^{KallR}) to address the potential redundancy of these residues for ubiquitination. This allele and wild-type Sna3 were fused in-frame to the coding region of GFP to generate chimeras in which GFP was fused to the carboxy terminus of Sna3, and the sorting of these reporters was analyzed in the SEY6210 strain lacking endogenous Sna3 (*sna3Δ*). Sna3-GFP and Sna3^{KallR}-GFP displayed localization to the vacuolar lumen (Figure 1A) as well as endosomal structures marked by DsRed-FYVE (see Figure 2E and Supplementary Figure 2), similar to results with Sna3^{K19,125R}-GFP (Reggiori and Pelham, 2001 and data not shown).

Ubiquitination status of Sna3-GFP and Sna3^{KallR}-GFP was addressed by expressing these chimeras in yeast cells lacking the endosomal SNARE Pep12 and endogenous Sna3 (*pep12Δ sna3Δ*), as loss of Pep12 function has been previously shown to stabilize ubiquitin-modified MVB cargoes (Katzmann *et al.*, 2001, 2004). These transformants, also expressing HA-ubiquitin, were used to perform immunoprecipitations with anti-Sna3 polyclonal antibody, followed by anti-GFP and anti-HA Western blotting. Analysis of Sna3-GFP revealed ubiquitinated higher molecular weight species (Figure 1B), consistent with identification of Sna3 as a ubiquitinated protein (Peng *et al.*, 2003). Although Sna3^{KallR}-GFP exhibited decreased modification, residual ubiquitination was still apparent. We do not believe this represents ubiquitination of GFP, as we have observed these higher molecular weight species with alternately tagged forms of Sna3 (data not shown), and the identification of Sna3 as an ubiquitinated protein was via a proteomics approach (utilizing untagged protein; Peng *et al.*, 2003). This result suggested that ubiquitination of the Sna3 amino-terminus was occurring. To address this possibility, Sna3 and Sna3^{KallR} were fused to the carboxyl-terminus of GFP (GFP-Sna3) to block amino-terminal ubiquitination (Bloom *et al.*, 2003; Ciechanover and Ben-Saadon, 2004) and analyzed as described above. Both GFP-Sna3 and GFP-Sna3^{KallR} display localization to the vacuolar lumen in a manner indistinguishable from the carboxyl-terminally tagged chimeras (Figure 1A). However, analysis of GFP-Sna3 ubiquitination indicated that the presence of GFP at the amino-terminus altered Sna3 modification, and the combination of GFP at the amino-terminus and mutation of the four lysine residues (GFP-Sna3^{KallR}) reduced ubiquitination to a level undetectable by this analysis (Figure 1B). This result suggested that ubiquitination of Sna3 via the internal lysines and the amino-terminus can occur but is not required for Sna3 MVB sorting. Although we cannot eliminate the possibility that a very low level of ubiquitination, undetectable in our analysis, allows sorting of GFP-Sna3^{KallR}, the lack of correlation between ubiquitination status and the localization of the Sna3 reporters suggested that ubiquitination of Sna3 is not a requisite for MVB sorting, consistent with previous interpretations (Reggiori and Pelham, 2001; Bilodeau *et al.*, 2002; Katzmann *et al.*, 2004).

To detect more subtle effects on Sna3 trafficking than were apparent in the microscopic analyses of terminal GFP localization, kinetic analyses of Sna3 reporter transport to the vacuole were performed by pulse-chase immunoprecipitation experiments. Yeast expressing the Sna3 reporters were metabolically labeled with [³⁵S]methionine and cysteine and chased for up to 4 h; lysates generated at various time points were subjected to immunoprecipitation with anti-Sna3 polyclonal antisera. In wild-type cells more than 90% of Sna3-GFP was degraded within 60 min (Figure 1C and Supple-

Figure 1. Sna3 ubiquitination is not a requisite for MVB targeting. (A) GFP-tagged forms of Sna3 were expressed in *sna3Δ* cells and visualized by fluorescence microscopy. Sna3^{KallR} denotes a mutant form of Sna3 in which all lysine residues have been mutated to arginines. The position of the GFP tag relative to Sna3 is indicated by their order (e.g., “GFP-Sna3^{KallR}” indicates an amino-terminally tagged form of Sna3 in which all lysines have been mutated to arginines). GFP localization within the vacuole lumen indicates efficient entry into the MVB pathway. Scale bar, 5 μ . (B) Plasmids containing HA-ubiquitin or GFP-tagged forms of Sna3 were transformed into *sna3Δ pep12Δ* cells, and lysates were generated and immunoprecipitated with anti-Sna3 antibody, followed by SDS-PAGE and Western blotting with either anti-GFP or anti-HA. (C) Pulse-chase immunoprecipitation was performed on a variety of GFP-tagged forms of Sna3, as well as endogenous Sna3 using anti-Sna3 polyclonal antisera. Degradation was quantitated using a phosphorimager, and percent remaining relative to time zero was plotted over time. The vacuolar protease deleted strain TVY614 was used to assess vacuolar degradation of endogenous Sna3. Experiments were performed at least three times to allow statistical analyses.

mentary Figure 1). To confirm that Sna3-GFP transport is indicative of endogenous Sna3 transport, a polyclonal antiserum was raised against Sna3. Immunoprecipitation of endogenous Sna3 revealed that more than 90% of Sna3 was degraded within 60 min, consistent with the rate of cleavage of Sna3-GFP. Stabilization of Sna3 was observed in yeast lacking the vacuolar hydrolases proteinase A, proteinase B, and carboxypeptidase Y (TVY614; Figure 1C), indicating that degradation of Sna3 coincided with delivery to the vacuole. In addition, delivery of Sna3-GFP to the vacuole was unaffected by loss of endogenous Sna3 (*sna3Δ*; Figure 1C and Supplementary Figure 1). These results validate Sna3-GFP as an indicator of Sna3 trafficking in vivo. Sna3^{KallR}-GFP, GFP-Sna3, and GFP-Sna3^{KallR} were then expressed in cells lacking endogenous Sna3 (*sna3Δ*), and the kinetics of vacuolar delivery were assessed. Analysis of Sna3^{KallR}-GFP degradation indicated a subtle, yet significant delay in vacuolar delivery of Sna3 upon mutation of lysine residues, with 70% cleaved at 60 min compared with more than 90% Sna3-GFP cleavage ($p < 0.001$, Figure 1C). However, more than 90% of Sna3^{KallR}-GFP was degraded by 180 min, consistent with wild-type terminal phenotype apparent in the microscopic analysis (Figure 1A). Repositioning GFP to the amino-terminus of Sna3 (GFP-Sna3) also resulted in a subtle, yet significant delay in the processing of GFP-Sna3 at 30 min ($p < 0.001$), although the difference was no longer significant at 240 min ($p > 0.05$, Figure 1C), and the lysine mutant form (GFP-Sna3^{KallR}) exacerbated the delay in vac-

uolar delivery further (Figure 1C; $p < 0.001$ at 30 min, $p < 0.001$ at 240 min). Both Sna3^{KallR}-GFP and GFP-Sna3 exhibited partial reductions in ubiquitination and subtle defects in the delivery of Sna3 to the vacuole, although GFP-Sna3^{KallR} exhibited the most severe deficits and delays in ubiquitination and degradation. This correlation suggests that although ubiquitination is not required for Sna3 MVB sorting, ubiquitination does positively modulate the kinetics of Sna3 transport into this pathway.

Alternative Sorting Motifs for Sna3 Entry into the MVB Pathway

The experiments presented above indicate that ubiquitin modification of Sna3 occurs in vivo, but is not a requisite for entry into the MVB pathway. Sna3^{KallR} therefore represents a model MVB cargo with which to characterize alternative signal(s) for entry into the MVB pathway. Scanning alanine mutagenesis was performed on the cytoplasmic tails of Sna3^{KallR}-GFP to identify residues critical for MVB sorting independent of ubiquitination of this reporter (Figure 2A). The resulting mutants were expressed in a *sna3Δ* strain, and localization of the GFP-fusions was visualized by fluorescence microscopy. Cargoes specifically defective for entry into the MVB pathway are localized to the limiting membrane of the vacuole as opposed to its lumen (Odorizzi *et al.*, 1998). Two distinct Sna3 regions (residues 14-17 and 104-108) were identified whose alteration exhibited substantial signal at the limiting membrane of the vacuole, with some

Figure 2. Identification of *cis*-acting sorting determinants in Sna3. (A) Sequence of Sna3 is presented to illustrate the scanning alanine mutagenesis approach, with underlined groups of residues representing mutagenized clusters. Each of the lysine residues is highlighted with an asterisk, and mutagenesis was performed in wild-type and lysine-minus (Sna3^{KallR}) forms of Sna3, and Sna3^{KallR} data are presented. Transmembrane domains are indicated by gray boxes, and motifs that play a role in Sna3 MVB sorting are indicated by yellow boxes. A diagram of Sna3 topology illustrates the locations of the lysine residues with red stars. (B) Residues 14-17 and 104-108 are critical for sorting of Sna3^{KallR} into the MVB pathway. Fluorescence microscopy was used to analyze the mutants generated by scanning alanine mutagenesis. These two mutants displayed a high degree of signal at the limiting membrane of the vacuole indicating a failure to enter the MVB pathway. Scale bar, 5 μ . (C) Mutation of residues surrounding Y15 do not negatively impact Sna3^{KallR} sorting into the MVB pathway to the degree that Y15A does. Site-directed mutagenesis was used to generate the indicated mutant forms of Sna3^{KallR}-GFP, and fluorescence microscopy was used to visualize their localization in *sna3* Δ cells. Scale bar, 5 μ . (D) The presence of an aromatic residue at position 15 in Sna3^{KallR}-GFP is important for delivery into the MVB pathway. Site-directed mutagenesis was used to generate the indicated mutant forms of Sna3^{KallR}-GFP, and fluorescence microscopy was used to visualize their localization in *sna3* Δ cells. Scale bar, 5 μ . (E) Sna3 mutants localize to PtdIns(3)P-positive endosomes in addition to the vacuole. Sna3^{KallR}-GFP, Sna3^{KallR}₁₄AYAA₁₇-GFP, and Sna3^{KallR}₁₂AAAYAA₁₇-GFP were coexpressed with DsRed-FYVE in wild-type cells and visualized by fluorescence microscopy. Scale bar, 5 μ .

additional signal emanating from endosomal structures (Figure 2B).

Critical residues within the sequence between 14 and 17 (SYSI) were examined by mutating each residue singly, revealing that positions 14, 16, and 17 did not block the delivery of Sna3 into the vacuole lumen (Figure 2C); moreover, mutation of residues 14, 16, and 17 together (Sna3^{KallR}_{14AYAA17}-GFP) or even with additional mutations at positions 12 and 13 (Sna3^{KallR}_{12AAAYAA17}-GFP) did not eliminate MVB sorting (Figure 2, C and E). In contrast, mutation of the tyrosine residue at position 15 resulted in mislocalization of Sna3^{KallR}_{Y15A}-GFP to the limiting membrane and endosomal structures in a manner indistinguishable from Sna3^{KallR}₁₄₋₁₇-GFP (Figure 2, B and D, and Supplementary Figure 2). This suggested that the tyrosine residue plays a critical role in the sorting of Sna3 into the MVB pathway, whereas the surrounding residues are less critical but may also impact the kinetics of delivery at a level unappreciable by this assay. Tyrosine can receive a number of posttranslational modifications through its hydroxyl group. Tyrosine 15 was mutated to serine to conserve a reactive hydroxyl group at this position; however, Sna3^{KallR}_{Y15S}-GFP failed to properly enter the MVB pathway, as evidenced by fluorescence emanating from limiting vacuolar membrane (Figure 2D). In contrast, mutation of tyrosine 15 to phenylalanine (Sna3^{KallR}_{Y15F}-GFP), which conserves the aromatic ring, resulted in sorting that was indistinguishable from Sna3-GFP (Figure 2D). Similarly, mutation of tyrosine 15 to tryptophan conferred partial MVB sorting of Sna3^{KallR}_{Y15W}-GFP, suggesting that the presence of an aromatic residue within this region is important for Sna3^{KallR}-GFP MVB sorting.

Alanine scanning mutagenesis also identified residues 104-108 (AQPPA) of Sna3 as critical for sorting of Sna3^{KallR}-GFP into the vacuole lumen (Figure 2B). This mutation included most of a putative PPXY motif (PPAY, residues 106-109), a sequence recognized by WW domains. In support of this idea, mutation of residues 109-113 also resulted in missorting of the resulting chimera to the limiting membrane of the vacuole (data not shown). Specific mutation of the PPAY sequence resulted in Sna3^{KallR}_{PPAYmutant}-GFP mislocalization to the limiting membrane of the vacuole and endosomes (Figure 3C and Supplementary Figure 2), consistent with the phenotype observed for Sna3^{KallR}₁₀₄₋₁₀₈-GFP (Figure 2B). These findings identify two regions that are required for the MVB targeting of Sna3^{KallR}: an amino-terminal region including tyrosine 15, as well as a PPAY motif within the carboxyl-terminus.

To address the sufficiency of these regions to confer MVB sorting, chimeras were generated with Sna3 residues 13-16 (MSYS) or 104-111 (AQPPAYDE) fused to GFP-DPAP B and expressed in the *sna3Δ* strain. GFP-DPAP B is normally delivered to the vacuolar limiting membrane; however, fusion of the CPS MVB sorting determinant has been demonstrated to redirect a GFP-CPS₅₋₁₁-DPAP B chimera into the MVB pathway (Katzmann *et al.*, 2001). Similarly, the GFP-Sna3^{MSYS}-DPAP B chimera localized to the vacuolar lumen (Figure 3A), indicating that the MSYS sequence can confer MVB sorting onto this chimera. Importantly, mutation of the tyrosine residue (MSAS) abrogated MVB sorting of GFP-Sna3^{MSAS}-DPAP B (Figure 3A), supporting the importance of this tyrosine in this Sna3 sorting signal. Similarly, GFP-Sna3^{PPAY}-DPAP B chimera also localized to the vacuolar lumen, and mutation of the tyrosine residue abrogated GFP-Sna3^{PPAA}-DPAP B MVB sorting (Figure 3A). These results indicate that the MSYS and PPAY motifs can confer MVB sorting onto DPAP B chimeras and are consistent with their critical roles in the MVB sorting of Sna3^{KallR}.

Scanning mutagenesis studies identified two *cis*-acting regions required for MVB sorting of Sna3^{KallR}. However, ubiquitination also contributes to the kinetics of Sna3 MVB sorting (Figure 1C). To further address the involvement of the Y15 and PPAY determinants in Sna3 MVB sorting, these motifs were altered in the context of the Sna3 reporter with the lysine residues intact (Sna3-GFP), and these reporters were expressed in the *sna3Δ* strain for microscopic analysis. While Sna3^{KallR}_{Y15A}-GFP exhibited vacuolar-limiting membrane localization (Figures 2D and 3C), Sna3^{KallR}_{Y15A}-GFP was present within the vacuolar lumen (Figure 3C). This result suggested that ubiquitination can suppress the requirement for the Y15-containing motif in Sna3 sorting. In contrast, Sna3^{KallR}_{PPAYmutant}-GFP and Sna3^{PPAYmutant}-GFP appeared indistinguishable. One possible explanation was that the PPAY motif was required for Sna3 ubiquitination in addition to being involved in the apparent ubiquitin-independent sorting of Sna3 into the MVB pathway. To examine this possibility, these Sna3 alleles were transformed into the *pep12Δ sna3Δ* strain expressing HA-ubiquitin and the levels of ubiquitination were determined. Immunoprecipitation with anti-Sna3 antisera was performed, followed by Western blotting with anti-GFP or anti-HA antibodies to detect ubiquitinated species. Mutation of Y15 reduced but did not eliminate ubiquitination of both Sna3^{KallR}_{Y15A}-GFP and Sna3^{KallR}_{Y15A}-GFP (Figure 3D); however, the nominal residual ubiquitination of Sna3^{KallR}_{Y15A}-GFP, presumably at the amino terminus, was not sufficient to permit appreciable amounts of MVB sorting (Figure 3C). In contrast, the PPAY mutation reduced ubiquitination to levels undetectable by this assay in the context of both Sna3^{PPAYmutant}-GFP and Sna3^{KallR}_{PPAYmutant}-GFP (Figure 3D). This result suggested that the PPAY motif is required for Sna3 ubiquitination. To determine if this motif was also sufficient to confer ubiquitination onto the DPAP B chimera (which contains lysine residues within the cytoplasmic tail of DPAP B), ubiquitination of these chimeras was addressed. Sna3^{PPAY}-DPAP B, but not Sna3^{PPAA}-DPAP B, was ubiquitinated (Figure 3B), consistent with results obtained using Sna3 (Figure 3D). In contrast, a chimera containing the amino terminal 20 amino acids of Sna3 (Sna3^{NTD}-DPAP B), which is sorted into the MVB pathway like the Sna3^{MSYS}-DPAP B chimera (data not shown), did not display detectable levels of ubiquitination (Figure 3B). Again, this chimera reproduces the behavior of Sna3 wherein mutation of the MSYS region was only found to confer MVB sorting defects when Sna3 was not ubiquitinated. The MSYS and PPAY motifs within Sna3 therefore contribute MVB sorting via distinct mechanisms. These results indicate that Sna3 ubiquitination is dependent on the PPAY motif, potentially the result of interaction with a WW-containing ubiquitin ligase. However, the PPAY motif also functions in Sna3 sorting independent of Sna3 ubiquitination, as Sna3^{PPAYmutant}-GFP and Sna3^{KallR}_{PPAYmutant}-GFP fail to efficiently enter the MVB pathway, whereas GFP-Sna3^{KallR} does. Together, these data demonstrate that the Y15-containing region, the PPAY motif, and lysine ubiquitination all impact Sna3 sorting into the MVB pathway.

Rsp5 Binds to Sna3 via the PPAY Motif

Sna3 ubiquitination is dependent on the PPAY motif, and the PPAY motif is a consensus WW domain target. The HECT domain ubiquitin ligase Rsp5 contains three WW domains, and its involvement in MVB sorting has been documented, both via ubiquitination of cargo proteins as well as trafficking machinery (Dunn and Hicke, 2001b; Soetens *et al.*, 2001; Blondel *et al.*, 2004; Dunn *et al.*, 2004; Hettema *et al.*, 2004; Katzmann *et al.*, 2004; Morvan *et al.*, 2004; Stamenova *et al.*, 2004). This raised the

Figure 3. Sna3 sorting determinants and ubiquitination. (A) Sna3 sorting determinants are sufficient to confer MVB sorting. Chimeras were generated between the amino- and carboxyl-terminal sorting determinants of Sna3 and the non-MVB cargo GFP-DPAP B and analyzed by fluorescence microscopy. Scale bar, 5 μ . (B) *pep12 Δ* cells were transformed with plasmids expressing the indicated GFP-tagged protein, as well as HA-ubiquitin, and used to perform immunoprecipitations with anti-GFP antibody. Immunoprecipitated material was subjected to SDS-PAGE and Western blotting with either anti-GFP, to visualize the indicated protein or anti-HA to visualize ubiquitinated forms. (C) GFP-tagged Sna3 mutants were visualized in *sna3 Δ* cells using fluorescent microscopy. Scale bar, 5 μ . (D) Sna3-GFP mutants and HA-tagged ubiquitin were expressed in *sna3 Δ pep12 Δ* cells and used to make lysates that were subjected to immunoprecipitation with anti-Sna3 antisera. Immunoprecipitated material was subjected to SDS-PAGE and Western blotting with either anti-GFP or anti-HA.

possibility that Rsp5 binds Sna3 and is involved in Sna3 MVB sorting. We have previously analyzed loss of function *rsp5* HECT alleles for defects in the sorting of MVB cargoes, revealing that CPS ubiquitination was defective, resulting in missorting of

CPS, whereas Sna3 MVB sorting was unperturbed (Katzmann *et al.*, 2004). This suggested that these alleles were specifically defective for ubiquitin modification of a subset of MVB cargoes. However, given the complexity of Sna3 sorting

Figure 4. Sna3-Rsp5 interaction requires the PPAY motif within Sna3 and the WW domains of Rsp5. (A) Diagram of GST constructs used to test interactions with Rsp5. (B and C) Interaction between Sna3 Rsp5 was addressed in vitro using immobilized GST constructs and Rsp5 that was expressed in yeast (B) or bacteria (C). (D) TAP-tagged Rsp5 or WW mutant forms thereof were expressed in wild-type yeast cells and used to make lysates. Equivalent amounts of the indicated extract were incubated with GST-Sna3^{CTD} to test the relative contributions of the WW domains. Bound material was washed, subjected to SDS-PAGE and either Coomassie stained or Western blotted with anti-actin to visualize the TAP tag, quantitated, and normalized to wild-type Rsp5.

determinants uncovered in our analyses, the involvement of Rsp5 in Sna3 trafficking was reexamined.

Rsp5 contains 3 WW motifs that could interact with the PPAY motif in the carboxyl-terminus of Sna3. The ability of Rsp5 to bind Sna3 was analyzed using in vitro GST pull-down assays. GST fusions containing the amino-terminal tail of Sna3 (residues 1–21, GST-Sna3^{NTD}), the carboxyl-terminal tail (residues 68–163, GST-Sna3^{CTD}), or both the amino- and carboxyl-terminal tails without the transmembrane and luminal domains (GST-Sna3^{ΔTM}) were heterologously expressed in bacteria (Figure 4A). Additionally, GST fusions with the cytoplasmic domains of the known Rsp5-interacting MVB cargo CPS (CPS-GST) and the non-MVB cargo DPAP B (DPAP B-GST) were generated. GST or GST fusion proteins were purified using glutathione Sepharose and incubated with cleared yeast lysate made from cells expressing GFP-Rsp5, and the amount of GFP-Rsp5 bound following extensive washing was determined by Western blotting with

anti-GFP antibody. Although glutathione Sepharose beads alone or GST did not yield detectable amounts of GFP-Rsp5, CPS-GST was able to bind GFP-Rsp5 (Figure 4B), validating the experimental procedure. Analysis of the Sna3-GST fusions revealed that GST-Sna3^{ΔTM} and GST-Sna3^{CTD} could also interact with GFP-Rsp5, whereas GST-Sna3^{NTD} could not. These results suggested that Rsp5 associated with the carboxyl-terminal region of Sna3, which included the PPAY motif (residues 106–109). To evaluate the requirement for the PPAY motif in the Sna3-Rsp5 interaction, a GST-Sna3^{CTD} fusion was generated with the PPAY mutated to alanines (GST-Sna3^{CTD} PPAYmutant). This mutation abrogated MVB sorting and Sna3 ubiquitination in vivo (Figure 3) and reduced the interaction with GFP-Rsp5 to a level undetectable by this procedure (Figure 4B). This result suggested that Rsp5 associates with Sna3 via the PPAY motif.

To directly test the ability of Rsp5 to bind to Sna3, GST pull-down assays were performed with purified Rsp5 rather

Figure 5. Rsp5 ligase activity is required for the sorting of Sna3^{KallR} into the MVB pathway. (A) GFP-CPS and forms of GFP-tagged Sna3 were expressed in an *rsp5*^{G753I} mutant and visualized by fluorescence microscopy. Scale bar, 5 μ . (B) Sna3-GFP and Sna3^{KallR}-GFP were coexpressed with DsRed-FYVE in *rsp5*^{G753I} cells and visualized by fluorescence microscopy. Scale bar, 5 μ . (C) Sna3-GFP and Sna3^{KallR}-GFP were expressed in *pep12Δ* or *rsp5*^{G753I} *pep12Δ* cells, and lysates were subjected to immunoprecipitation with anti-GFP antibody. Immunoprecipitated material was subjected to SDS-PAGE and Western blotting with anti-GFP or anti-ubiquitin antibodies.

than yeast extracts containing GFP-Rsp5. Full-length Rsp5 was expressed as a His₆-MBP fusion in bacteria and purified by Ni²⁺-affinity chromatography. This purified His₆-MBP-Rsp5 was used to perform binding studies, followed by Western blotting with anti-MBP antibody. Although the glutathione beads alone, GST, or DPAP B-GST did not yield appreciable His₆-MBP-Rsp5 binding, CPS-GST was able to interact with His₆-MBP-Rsp5 (Figure 4C). Analysis of the Sna3 GST fusions revealed that GST-Sna3^{ATM} and GST-Sna3^{CTD} could also interact with purified His₆-MBP-Rsp5, whereas GST-Sna3^{NTD} or GST-Sna3^{CTD} PPAYmutant could not (Figure 4C). These results are consistent with the analysis using GFP-Rsp5 yeast lysates (Figure 4B) and indicate that Rsp5 can directly bind Sna3 in a PPAY-dependent manner.

Given that the PPAY motif represents a consensus WW domain target, and Rsp5 contains three WW domains, their individual contributions to this interaction were addressed. The conserved WXXP sequence in each domain was mutated to AXXP, because previous analysis has indicated that these mutations compromise function of the WW domains

without destabilizing Rsp5 (Dunn and Hicke, 2001a; Supplementary Figure 3A). These single WW mutant forms of Rsp5 (Rsp5^{WW1}, Rsp5^{WW2}, Rsp5^{WW3}) as well as the double (Rsp5^{WW1,2}, Rsp5^{WW1,3}, Rsp5^{WW2,3}) and triple (Rsp5^{WW1,2,3}) combination mutants were expressed as tandem affinity purification (TAP)-tagged fusions in wild-type yeast. GST-Sna3^{CTD} or GST-Sna3^{CTD} PPAYmutant were used to perform binding studies with equivalent amounts of cleared yeast lysates containing wild-type or mutant TAP-Rsp5, and the amount of TAP-Rsp5 bound was determined by Western blotting with an anti-actin antibody to detect the protein A portion of the TAP tag. The amount of bound material was quantitated and normalized to the amount of wild-type Rsp5 recovered, and the results presented in Figure 4D represent the average of two experiments. Consistent with analysis using purified His₆-MBP-Rsp5 and GFP-Rsp5 lysates, wild-type TAP-Rsp5 was isolated using GST-Sna3^{CTD} but not with GST-Sna3^{CTD} PPAYmutant (Figure 4D). Mutation of any of the WW domains compromised the ability of Rsp5 to bind to GST-Sna3^{CTD}, with the triple WW mutant display-

ing the greatest defect in binding. This finding is consistent with pulldown experiments using either GFP-Rsp5 or His₆MBP-Rsp5 and GST-Sna3^{CTD^{PPAYmutant}}. These results suggest that the WW domains of Rsp5 mediate the interaction with Sna3 via its PPAY motif.

Rsp5 Function Is Required for the Sorting of Sna3^{KallR} into the MVB Pathway

Rsp5 binds the Sna3 PPAY motif via its WW domains in vitro, and the PPAY motif is one determinant for Sna3 MVB sorting. These observations suggested that Sna3 MVB sorting is dependent on Rsp5; however, previous analyses using a HECT-domain mutant form of Rsp5 that displayed decreased ubiquitination of the MVB cargo CPS suggested that Sna3 MVB sorting is not dependent on Rsp5 (Katzmann *et al.*, 2004). To resolve this apparent contradiction, further analyses of Sna3 MVB sorting in *rsp5* mutants was undertaken. The *rsp5*^{G753I} allele has previously been shown to display defects in the ubiquitination of CPS, resulting in its missorting to the limiting membrane of the vacuole (Katzmann *et al.*, 2004). As expected, GFP-CPS failed to be delivered to the lumen of the vacuole, whereas GFP-Sna3 delivery into the vacuolar lumen appears to be intact (Figure 5A). These observations were consistent with previous reports. Our analyses have revealed that multiple determinants (the Y15-containing region, the PPAY motif, and ubiquitination) contribute to Sna3 MVB sorting. We therefore examined localization of GFP-Sna3^{KallR} and Sna3^{Y15A} in the *rsp5*^{G753I} strain. Surprisingly, GFP-Sna3^{KallR}-GFP displayed a dramatic phenotype of localizing to both endosomal compartments and the limiting membrane of the vacuole (Figure 5, A and B). Increased endosomal localization of both forms of Sna3 can be seen in the *rsp5*^{G753I} background, suggestive of a kinetic delay at the endosome (Figure 5B). Similar results were obtained using additional HECT mutant forms of Rsp5 (Figure 6), although some variation in the sorting defect was observed, with Sna3-GFP displaying a partial signal from the vacuolar limiting membrane in the *rsp5*^{P784T} and *rsp5*^{G707D} backgrounds. This variability is likely due to differing severities of the mutations, with all retaining some level of activity as the catalytically inert form is inviable (Wang *et al.*, 1999). These results are consistent with Rsp5 impacting Sna3 MVB sorting in a manner unappreciated in the previous analysis with Sna3 in the *rsp5*^{G555D} background. Although ubiquitination of GFP-Sna3^{KallR} is undetectable even in wild-type (*RSP5*) cells, MVB sorting of GFP-Sna3^{KallR} is not perturbed in wild-type cells, but is perturbed in *rsp5* HECT mutant cells. Surprisingly, it would therefore appear that the ubiquitin ligase activity of Rsp5 is required for targeting of Sna3 into the MVB pathway independent of Sna3 ubiquitination. The simplest interpretation would be that Rsp5 is modulating some component of the sorting machinery involved in the MVB targeting of Sna3^{KallR}.

The role of Rsp5 in Sna3^{Y15A}-GFP MVB sorting was also examined. Although the presence of lysine residues (Sna3^{Y15A}) can bypass the requirement for the tyrosine-containing motif in Sna3^{KallR}^{Y15A}-GFP sorting in wild-type cells (Figure 3B), the Sna3^{Y15A}-GFP construct (with lysines intact) displayed a similar phenotype of localizing to the limiting membrane of the vacuole and increased endosomal localization in the *rsp5*^{G753I} background. This observation suggested that the *rsp5*^{G753I} strain was defective for the ubiquitination of Sna3. Analysis of the ubiquitination status of Sna3 was performed by immunoprecipitation of Sna3-GFP and Sna3^{KallR}-GFP from *pep12Δ* or *rsp5*^{G753I} *pep12Δ* cells, followed by Western blotting with either anti-ubiquitin or anti-GFP antibodies (Figure 5C). This analysis revealed that ubiquitination of Sna3-GFP is dramatically reduced in the

Figure 6. Sna3^{KallR}-GFP is mislocalized in *rsp5* HECT mutants. Sna3-GFP and Sna3^{KallR}-GFP were expressed in *rsp5*^{P784T} (MY832), *rsp5*^{G707D} (MY833), and *rsp5*^{G747E} (MY834) cells and visualized by fluorescence microscopy. Scale bar, 5 μ .

uitination of Sna3-GFP is dramatically reduced in the *rsp5*^{G753I} background, with the Sna3^{KallR}-GFP ubiquitination being reduced to undetectable levels. These results demonstrate that robust ubiquitination of Sna3 in vivo depends on the ubiquitin ligase activity of Rsp5. This conclusion is supported by the finding that the PPAY motif is required for both Rsp5 binding (Figure 4) and Sna3 ubiquitination (Figure 3). Therefore, a somewhat surprising finding was that the ubiquitin ligase activity of Rsp5 played a role in the

Figure 7. WW domains of Rsp5 are required for sorting of Sna3^{KallR} into the MVB pathway. The *rsp5*^{G753I} allele was transformed with the indicated plasmids expressing WW mutant forms of Rsp5, as well as Sna3^{KallR}-GFP, and visualized by fluorescence microscopy. Scale bar, 5 μ .

sorting of Sna3^{KallR} into the MVB pathway independent of cargo modification.

The role of the WW domains of Rsp5 in Sna3 sorting was addressed by two independent manners. First, the WW mutants were expressed in the *rsp5*^{G753I} background and complementation of the sorting defect was assessed microscopically. Although the *rsp5*^{G753I} allele displays a dramatic missorting of Sna3^{KallR}-GFP, this defect is complemented by wild-type RSP5 (Figure 7). Mutants in either WW1 or WW2 also largely complemented the Sna3^{KallR}-GFP sorting defects with only a subtle exaggeration of endosomal localization evident. Combination of the WW1 and WW2 mutations (WW1, 2) resulted in less complementation as indicated by increased limiting membrane Sna3^{KallR}-GFP localization (Figure 7). The WW3 mutant was less effective at complementing *rsp5*^{G753I} Sna3^{KallR}-GFP sorting defects than the WW1 or WW2 mutants, with the WW3 single mutant displaying a phenotype that is similar to the WW1, 2 double mutant. Combining the WW3 mutation with the WW1 or WW2 mutations further diminished the complementation of *rsp5*^{G753I} Sna3^{KallR}-GFP sorting defects, and the triple WW mutant is indistinguishable from the *rsp5*^{G753I} allele alone. These results indicate that functional WW domains of Rsp5 are required to mediate Sna3^{KallR}-GFP MVB sorting, although some level of redundancy exists between the WW domains. A similar analysis was conducted in the SEY6210 *rsp5* Δ genetic background using plasmid shuffle to introduce these WW mutants as the sole form of Rsp5, again revealing a role for the WW domains in this process (Supplementary Figure 3B). These results are largely consistent with the WW domain redundancy observed in the *rsp5*^{G753I} complementation analyses. This redundancy is also supported by the GST-Sna3^{CTD} pulldown experiments wherein TAP-Rsp5^{WW1,2,3} exhibited less binding than the TAP-Rsp5^{WW1}, TAP-Rsp5^{WW2}, and TAP-Rsp5^{WW3} mutants. Together with

the physical association of Rsp5 with Sna3 via a PPAY motif in Sna3 and the sorting defect observed for the Sna3 PPAY mutant, these results support the model that the interaction between Rsp5's WW domains and Sna3's PPAY motif is critical for Sna3 MVB sorting in vivo.

DISCUSSION

The regulated sorting of proteins into the MVB pathway is critical for maintaining the proper cellular repertoire of transmembrane proteins, and thereby impacts a number of cellular functions (Katzmann *et al.*, 2002; Gruenberg and Stenmark, 2004). To date, the only characterized signal capable of mediating entry into this pathway is ubiquitination of the cargo. Multiple ubiquitin-binding components of the MVB sorting machinery then recognize and sort the ubiquitinated cargo into intraluminal vesicles (Katzmann *et al.*, 2001; Bilodeau *et al.*, 2002; Shih *et al.*, 2002; Katzmann *et al.*, 2003). However, a series of ubiquitin-independent MVB cargoes have also been described, including the δ -opioid receptor and Pmel17, and potentially the HIV Gag protein (Ott *et al.*, 2000; Hislop *et al.*, 2004; Theos *et al.*, 2006). In yeast, Sna3 has also been reported to sort into the MVB in an ubiquitination-independent manner that depends on ESCRT function (Reggiori and Pelham, 2001; Bilodeau *et al.*, 2002; Katzmann *et al.*, 2004) and represented a model substrate with which to identify novel sorting determinants. We initiated studies regarding the signals and factors mediating ubiquitin-independent MVB sorting utilizing Sna3 and uncovered a complex series of determinants that impact Sna3 trafficking. Although ubiquitination is not required for Sna3 sorting, mutation of the lysine acceptor residues (Sna3^{KallR}-GFP and GFP-Sna3^{KallR}) reduces the kinetics of Sna3 transport to the vacuole. Alanine-scanning mutagenesis of Sna3^{KallR}-GFP uncovered two additional determinants im-

Figure 8. Model for the function of Rsp5 in the sorting of MVB cargoes via ubiquitin-dependent and alternative pathways. Rsp5 is responsible for the ubiquitination of a number of MVB cargoes, subsequently recognized by the ESCRTs to direct entry into the MVB pathway. Rsp5 can interact with Sna3 via WW-PPAY interactions, and this can lead to ubiquitination of Sna3 and ubiquitin-dependent recognition by ESCRTs. The interaction of Sna3 with Rsp5 appears to play an additional role in sorting via an alternate pathway that depends on Y15, presumably through the ubiquitination of an unknown sorting factor.

packing Sna3 sorting: the Y15-containing region and the PPAY motif. Both of these motifs can permit MVB sorting in the context of a GFP-DPAP B chimera that is not normally a cargo of the MVB pathway. Further analysis revealed that the requirement for the Y15-containing region can be bypassed by Rsp5-dependent ubiquitination of Sna3, which is dependent on the PPAY-motif itself. We have also uncovered an unexpected role for Rsp5 in Sna3 sorting. In addition to resulting in the ubiquitination of Sna3, this interaction appears to play a role in the sorting of Sna3^{KallR} into the MVB pathway. HECT domain mutants of Rsp5 are also defective for proper sorting of Sna3^{KallR}, suggesting that Rsp5 impacts the sorting of Sna3 through multiple mechanisms: ubiquitinating Sna3, functioning as a sorting adaptor, and/or regulating MVB sorting machinery. Together, these results uncover a complicated series of determinants impacting Sna3 inclusion into the MVB pathway.

The model presented in Figure 8 summarizes our current understanding of Sna3 MVB sorting. Rsp5 has been shown to play a major role in the ubiquitin modification of a number of MVB cargoes (Soetens *et al.*, 2001; Blondel *et al.*, 2004; Dunn *et al.*, 2004; Hettema *et al.*, 2004; Katzmann *et al.*, 2004; Morvan *et al.*, 2004), leading to recognition by the ESCRTs and sorting into the MVB pathway (Katzmann *et al.*, 2001; Bilodeau *et al.*, 2002; Shih *et al.*, 2002; Katzmann *et al.*, 2003). Characterization of sorting determinants in Sna3 uncovered an interaction between Sna3 and Rsp5 that can lead to ubiquitin-dependent entry into the MVB pathway (Figures 3 and 4). However, ubiquitination of Sna3 is not required for its trafficking, as evidenced by the MVB sorting of GFP-Sna3^{KallR} (Figure 1). Surprisingly, the PPAY motif within Sna3 responsible for interacting with Rsp5 is required for targeting Sna3^{KallR} into the MVB pathway. Furthermore, the ubiquitin ligase activity of Rsp5 is required for the delivery of Sna3^{KallR} into the MVB pathway. It would therefore appear that the association of Sna3 and Rsp5 has multiple consequences that are relevant to the targeting of the cargo into the MVB pathway. When Sna3 is not ubiquitinated (Sna3^{KallR}), entry into the MVB pathway depends on a tyrosine-containing region within the amino terminus of Sna3 as well as the interaction with Rsp5 (Figures 2 and 3). One possibility is that Rsp5 is functioning as an adaptor to mediate Sna3 sorting independent of Sna3 ubiquitination, similar to the cargo sorting adaptor role that has been as-

cribed to the ubiquitin ligase Cbl (Soubeyran *et al.*, 2002). Mammalian homologues of Rsp5 belonging to the Nedd4 family also play a role in the targeting of cargoes into the MVB pathway, and this can involve an interaction between WW domains and PPXY motifs within the cargo (Staub *et al.*, 2000; Marchese *et al.*, 2003). Furthermore Nedd4 can interact with both Gag and the ESCRT-I subunit Tsg101 to drive the release of viral particles, suggesting an additional mechanism by which Gag can interface with ESCRTs (Martin-Serrano *et al.*, 2005; Medina *et al.*, 2005). Although no interaction has been reported between Rsp5 and Vps23 (the yeast homolog of Tsg101), it is possible that a similar interaction is occurring during the sorting of Sna3 into the MVB pathway. However, the activity of the HECT domain was required for targeting of lysine-minus Sna3 into the MVB pathway (Figure 5), suggesting that Rsp5 is acting as more than an adaptor. The enzymatic activity of Rsp5 could be responsible for an additional regulatory mechanism at the level of machinery responsible for identification of nonubiquitin MVB signals such as those in Sna3^{KallR}. Moreover, there is no reason that these mechanisms would be mutually exclusive.

Our data are also consistent with the WW domains, in particular WW3, of Rsp5 playing a role in the sorting of Sna3 through this PPAY motif. GST pulldown experiments revealed that all three WW domains contribute to Sna3 binding *in vitro*. *In vivo* analysis of Sna3^{KallR} sorting in various *rsp5* WW mutants demonstrated that mutation of all three WW domains (Rsp5^{WW1,2,3}) was more defective than the individual mutants (Figure 7 and Supplementary Figure 3B). These observations indicated that some level of Rsp5 WW domain functional redundancy exists with respect to Sna3^{KallR} sorting, with WW3 contributing the predominant interaction domain. However, this interpretation is complicated by the known roles of the WW2 and WW3 domains in a variety of activities that could impact MVB sorting (Dunn and Hicke, 2001a; Gajewska *et al.*, 2001; Kaminska *et al.*, 2002), as well as demonstration that the ubiquitin-dependent MVB cargoes CPS and Phm5 fail to properly enter the MVB pathway in these mutants (Katzmann *et al.*, 2004; Morvan *et al.*, 2004). However, the *rsp5* WW mutants examined do not display the hallmark class E compartment observed upon deletion of class E *VPS* (vacuolar protein sorting) genes. Clearly the PPAY motif within Sna3 is critical for its sorting into the MVB pathway. Although we cannot eliminate the possibility that additional factors are capable of interacting with Sna3 via this PPAY motif, the simplest interpretation of the data would be that its role is to associate with Rsp5.

In addition to the PPAY motif bound by Rsp5, MVB sorting of Sna3^{KallR} required a tyrosine-containing region within its amino terminus. Amino acid substitutions flanking this residue did not disrupt MVB sorting to the degree of the Sna3^{Y15A} mutant, suggesting that these positions are not as critical. An alternative explanation is that the mutation of the flanking residues to alanine was conservative enough to not perturb the secondary structure and/or affect the presentation of Y15. Conservative mutations in which the tyrosine was changed to another aromatic residue retained varying degrees of MVB sorting (Figure 2D). Furthermore, this motif was sufficient to target DPAP B into the MVB pathway (Figure 3A). We do not believe that the Sna3^{MSYS}-DPAP B chimera is merely being ubiquitinated, because none was detected, and the dependence on this tyrosine was suppressed by ubiquitination of Sna3 (Figure 3); yet Sna3^{MSAS}-DPAP B is missorted to the limiting membrane, recapitulating the behavior observed in Sna3^{KallR}_{Y15A}. It therefore seems possible that this region is responsible for providing an interaction surface that facilitates its targeting

into the MVB pathway. However, this Y15-containing region is not in itself sufficient for MVB sorting of Sna3 because the PPAY motif is also required. It is not clear why the MSYS sequence is sufficient in the context of the chimera with DPAP B but not Sna3. Perhaps in the case of Sna3^{KallR} Rsp5 binding to the PPAY motif positively modulates the machinery responsible for interaction with the Y15-region during MVB sorting. Alternatively, Rsp5 binding to the PPAY motif may be required to expose the Y15-containing interaction surface in the context of Sna3 but not in the Sna3^{MSYS}-DPAP B chimera. Further experimentation will be required to resolve this issue.

Although Sna3 had previously been described as an ubiquitin-independent cargo, our analyses demonstrate that Sna3 ubiquitination does impact Sna3 trafficking. Reduced ubiquitination of Sna3 delays the kinetic delivery of Sna3 into the MVB pathway (Figure 1). Although our results are consistent with Sna3-ubiquitination-independent sorting, it is formally possible that residual ubiquitination below the level of detection is facilitating entry into the MVB pathway. It is, however, unclear how this modification would occur. Our results support the model that targeting of Sna3 into the MVB pathway can occur via Sna3 ubiquitination or via ubiquitination-independent signals within Sna3. The necessity for redundant Sna3 sorting pathways is unclear as is the function for Sna3 itself. The PPAY motif of Sna3 interacting with Rsp5 via its WW domains raises an interesting parallel to the interaction of Bsd2 and Rsp5. Bsd2 interaction with Rsp5 also requires a PPXY motif within Bsd2, resulting in ubiquitination and MVB targeting of Bsd2 (Hettema *et al.*, 2004). However, one distinction appears to be that loss of Bsd2 confers defects in Rsp5-dependent MVB sorting of other cargoes (Hettema *et al.*, 2004), whereas loss of Sna3 does not (Reggiori and Pelham, 2001 and data not shown). Sna3 belongs to a family of closely related proteins (Sna1-4), suggesting that functional redundancy among the family may exist. Alternatively, Sna3 may be required for trafficking of only a subset of MVB cargoes not identified to date. Further experimentation is required to resolve these issues.

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