Multivesicular Body Sorting: Ubiquitin Ligase Rsp5 Is Required for the Modification and Sorting of Carboxypeptidase S

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The multivesicular body (MVB) sorting pathway provides a mechanism for delivering transmembrane proteins into the lumen of the lysosome/vacuole. Recent studies demonstrated that ubiquitin modification acts in *cis* as a signal for the sorting of cargoes into this pathway. Here, we present results from a genetic selection designed to identify mutants that mis-sort MVB cargoes. This selection identified a point mutation in ubiquitin ligase Rsp5 (Rsp5-326). At the permissive temperature, this mutant is specifically defective for ubiquitination and sorting of the ubiquitin-dependent MVB cargo precursor carboxypeptidase S (pCPS), but not ligand-induced ubiquitination of Ste2. A previous study implicated Tul1 as the ubiquitin ligase responsible for MVB sorting of pCPS. However, we detected no defect in either the sorting or ubiquitination of pCPS in *tul1* mutants. We had previously shown that Fab1 phosphatidylinositol 3-phosphate 5-kinase is also required for MVB sorting of pCPS, but not Ste2. However, our analyses reveal that *fab1* mutants do not exhibit a defect in ubiquitination of pCPS. Thus, both Rsp5 and Fab1 play distinct and essential roles in the targeting of biosynthetic MVB cargoes. However, whereas Rsp5 seems to be responsible for cargo ubiquitination, the precise role for Fab1 remains to be elucidated.

INTRODUCTION

Ubiquitin is a 76-amino acid polypeptide that is covalently attached to substrate proteins via an isopeptide bond. This modification requires the action of a cascade of enzymes referred to as a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3) (for review, see Hershko and Ciechanover, 1998). Within this reaction cascade, the E3 seems to play the primary role in determining substrate specificity (for review, see Pickart, 2001). E3s can be grouped into two major categories based on the motif used to transfer ubiquitin to a substrate: those containing a RING domain and those containing a HECT domain. The major distinction between the two groups is that RING E3s coordinate the transfer of ubiquitin to the substrate by bringing the E2-ubiquitin complex to the substrate, whereas HECT E3s form a thiol-ester bond with ubiquitin before transferring it directly to a substrate (for review, see Joazeiro and Weissman, 2000; Pickart, 2001). The mech-

anisms governing substrate recognition by E3s are not well understood, but in some cases, they are regulated by the phosphorylation status of the substrate (for review, see Laney and Hochstrasser, 1999).

Ubiquitin modification has been shown to regulate a broad range of cellular pathways and processes, ranging from selective protein degradation to modulation of transcription, translation, and protein localization (for review, see Hicke, 2001). The function of ubiquitin ligases, as they impact upon a variety of cellular functions, is therefore of considerable interest. Our interest has been in the role ubiquitin modification plays in protein sorting. Ubiquitination has been shown to control down-regulation of a number of plasma membrane proteins, including receptors, via directing them into the multivesicular body (MVB) pathway, ultimately resulting in delivery of the proteins to the lumen of the hydrolytic lysosome/vacuole (for review, see Hicke, 1997; Katzmann *et al.*, 2002). Ubiquitin serves as a sorting determinant both at the plasma membrane, where it has been shown to function in driving endocytic internalization of certain proteins (for review, see Hicke, 1999; Shaw *et al.*, 2001), and at Golgi/endosomal compartments, where it directs sorting into the degradative MVB pathway (Dupre and Hagenauer-Tsapir, 2001; Helliwell *et al.*, 2001; Katzmann *et al.*, 2001; Losko *et al.*, 2001; Reggiori and Pelham, 2001; Urbanowski and Piper, 2001; Chen and Davis, 2002). Ubiquitin serves not only as a sorting determinant when covalently attached to transmembrane cargo proteins but also it seems to regulate components of the endocytic machinery, such as Eps15, Hrs, and β -arrestin, which have been shown to be ubiquitinated (van Delft *et al.*, 1997; Shenoy *et al.*, 2001; Polo *et al.*, 2002).

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Abbreviations used: ESCRT, endosomal complex required for transport; FYVE domain, for Fab1, YGL023, Vps27, and EEA1; EGFR, epidermal growth factor receptor; MVB, multivesicular body; pCPS, precursor carboxypeptidase S; PI(3)P, phosphatidylinositol 3-phosphate; ts, temperature sensitive; Ub, ubiquitin.

Table 1. *S. cerevisiae* strains used in this study

Strain	Genotype	Reference or Source
SEY6210	<i>MATα leu2-3, 112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9</i>	Robinson <i>et al.</i> , 1988
SEY6211	<i>MATα leu2-3, 112 ura3-52 his3-Δ200 trp1-Δ901 ade2-101 suc2-Δ9</i>	Lab strain
<i>muv326</i>	<i>MATα leu2-3, 112 ura3-52 his3-Δ200 trp1-Δ901 ade2-101 suc2-Δ9 rsp5-326</i>	This study
SSY22	<i>MATα leu2-3, 112 ura3-52 his3-Δ200 trp1-Δ901 ade2-101 suc2-Δ9 rsp5-326</i>	This study
CBY16	<i>SEY6210; pep12Δ::LEU2</i>	Burd <i>et al.</i> , 1997
SSY25	<i>rsp5-326 pep12Δ::LEU2</i>	This study
MBY3	<i>SEY6210; vps4Δ::TRP1</i>	Babst <i>et al.</i> , 1997
GOY19	<i>SEY6210; cps1::ΔLEU2</i>	Lab strain
BY4742	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3 Δ0</i>	Research Genetics
14833	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3 Δ0 tul1Δ::<i>Kan</i></i>	Research Genetics
SSY11	<i>SEY6210; tul1 Δ::HIS3</i>	This study
SSY16	<i>CBY16; tul1Δ::HIS3</i>	This study
LH21	<i>ubc4::TRP1 ubc5::LEU2 ade2 his4 ura3 trp1 leu2 his3 lys2 bar1</i>	Hicke and Riezman, 1996
LH291	<i>MATα his3 trp1 lys2 ura3 leu2 bar1</i>	Dunn and Hicke, 2001
LH23	<i>MATα rsp5-1 ura3 leu2 trp1 bar1</i>	Dunn and Hicke, 2001
LH183	<i>ubc1::HIS3 ubc4::TRP1 ura3 leu2 his3 trp1 lys2 bar1</i>	Hicke and Riezman, 1996
EMY119	<i>MATα fab1-2 leu2-3, 2-112 ura3-52</i>	Gary <i>et al.</i> , 1998
<i>fab1Δ1</i>	<i>SEY6210 fab1 Δ::LEU2</i>	Gary <i>et al.</i> , 1998
MYY290	<i>MATα his3 leu2 ura3</i>	Smith and Yaffe 1991
MYY833	<i>MATα rsp5::HIS3 his3 leu2 ura3 pRS316-mdp1-13</i>	Fisk and Yaffe, 1999
DKY83	<i>MATα smm1 his3 leu2 ura3 pep12::HIS3</i>	This study
TCY81	<i>SEY6210; rsp5Δ::HIS3 HA-rsp5-w3</i>	This study
TCY84	<i>SEY6210; rsp5Δ::HIS3 HA-rsp5-w1</i>	This study
TCY86	<i>SEY6210; rsp5Δ::HIS3 HA-rsp5-w2</i>	This study
TCY124	<i>SEY6210; rsp5Δ::HIS3 rsp5-ΔC2</i>	This study

The sorting of cell surface receptors into the degradative MVB pathway is highly regulated. Binding of extracellular agonist by cell surface receptors induces intracellular signaling cascades that elicit the appropriate cellular response. However, this activated state also results in receptor binding to components of the endocytic machinery that targets the receptor for internalization and degradation. In mammalian cells phospho-tyrosine residues within the activated epidermal growth factor receptor (EGFR) recruit the RING-domain containing Cbl ubiquitin ligase via its SH2 domain (Levkowitz *et al.*, 1998; Joazeiro *et al.*, 1999; Levkowitz *et al.*, 1999; Waterman *et al.*, 1999; Yokouchi *et al.*, 1999). Ubiquitination of EGFR by Cbl results in the targeting of EGFR into the MVB pathway. Failure to target activated EGFRs for degradation, in cells expressing a dominant negative Cbl protein, results in prolonged signaling through downstream effectors, contributing to deregulated growth control (Blake *et al.*, 1991). Likewise, in yeast, G protein-coupled receptors such as the pheromone receptors Ste2 and Ste3 are hyperphosphorylated upon ligand binding, and this phosphorylation leads to their ubiquitination and degradation via the MVB pathway (for review, see Hicke, 1999; Shaw *et al.*, 2001). The targeting of membrane proteins into the MVB pathway, therefore, seems to be regulated at least in part by ubiquitin ligases.

We have previously characterized the green fluorescent protein (GFP)-tagged version of the plasma membrane receptor Ste2 and the precursor form of vacuolar hydrolase carboxypeptidase S (pCPS) as cargoes of the MVB pathway in yeast (Odorizzi *et al.*, 1998). Ubiquitination of pCPS and Ste2 result in the sorting of these proteins into the MVB pathway (Shih *et al.*, 2000; Katzmman *et al.*, 2001). A genetic selection based on the MVB cargo pCPS revealed a crucial role for the class E *VPS* genes in the function of the MVB sorting pathway (Odorizzi *et al.*, 2003). Loss of class E *Vps* function results in a block in the MVB pathway (for review, see Katzmman *et al.*, 2002). Thus, cargo destined for delivery

to the lumen of the vacuole is missorted to the limiting membrane of the vacuole, as well as the exaggerated endosomal compartment seen in these mutants (the "class E compartment") (Raymond *et al.*, 1992; Odorizzi *et al.*, 1998). Because our previous selection was biased against essential genes, we modified the selection strategy to identify essential genes that are involved in MVB sorting. We have characterized a new mutant, obtained from this modified selection, which displays a defect only in the sorting of a subset of MVB cargoes. Further analysis revealed that this mutant contains an allele of the Rsp5 ubiquitin ligase that is defective for modifying biosynthetic cargoes, such as pCPS, with ubiquitin. These results suggest a conserved role for Rsp5 in the targeting of ubiquitin-dependent MVB cargoes.

MATERIALS AND METHODS

Yeast Strains, Media, and DNA Manipulations

The *Saccharomyces cerevisiae* strains used in this study are described in Table 1. SSY11 and SSY16 were constructed by transforming SEY6210 and CBY16 with DNA fragment containing the *HIS3* open reading frame (ORF), flanked by 41 base pairs specific for the upstream and downstream regions of the *TUL1* ORF. The deletion was confirmed by polymerase chain reaction (PCR) analysis of the chromosomal DNA. SSY25 was constructed by crossing SSY22 with CBY16.

Yeast strains were grown in standard yeast extract-peptone-dextrose (YPD) or synthetic medium supplemented with essential amino acids as required for maintenance of plasmids (YNB). Luria-Bertani (LB) medium was used for growth of *Escherichia coli* cells.

Recombinant DNA manipulations were performed using standard protocols (Sambrook *et al.*, 1989). To fuse ubiquitin to GFP-CPS, the ubiquitin ORF was PCR-amplified using primers containing *Nar* I sites. The resulting PCR product was inserted in-frame at the N terminus of GFP, in pGOGFP426 (Cowles *et al.*, 1997a), at a unique *Nar* I site that had been engineered by the PCR method of gene splicing by overlap extension (gene SOE) (Yon and Fried, 1989). The *CPS1*-containing *Kpn* I fragment from pGOGFPCPS (Odorizzi *et al.*, 1998) was inserted in-frame at the C terminus of Ub-GFP to generate Ub-GFP-CPS. GFP-Rsp5 fusion was constructed in two steps. First, the *RSP5* ORF was cloned by PCR from genomic DNA with *Bgl* II and *Sal* I restriction sites introduced with primers. Next, the resulting PCR product was digested with *Bgl* II and *Sal* I and ligated into pGO36, a *CEN*-based vector

(Odorizzi *et al.*, 1998), digested with *Bgl*II and *Sal*I. To delete the C2 domain (1–140) of *Rsp5*, the portion of the *RSP5* ORF, beginning at codon 141, was PCR amplified using primers that introduced a *Not*I at the N terminus. The resulting PCR product was digested with *Not*I and *Bst*E II and ligated into YCpHA-Rsp5 (Gajewska *et al.*, 2001), which has been digested with *Not*I and *Bst*E II, resulting in Δ C2-Rsp5. The *rsp5-326* allele was cloned from genomic DNA isolated from the *mob326* mutant using homologous recombination. A portion of the *RSP5* ORF was removed from YCpHA-RSP5 by digestion with *Bst*EII and *Bse* RI. The resulting gapped plasmid was transformed into the *mob326* mutant. Gap-repaired plasmids were isolated from the transformants growing on uracil-free plates and sequenced.

Microscopy

For visualizing DsRed and GFP-tagged proteins, cells were grown to an OD₆₀₀ of 0.4 and resuspended in water for visualization. An Axiovert S1002TV microscope (Carl Zeiss, Thornwood, NY), equipped with fluorescein isothiocyanate and rhodamine filters, was used to visualize cells. Images were captured with a Photometrix camera and deconvolved using Delta Vision software (Applied Precision, Issaquah, WA). Image processing was done using Adobe Photoshop (Adobe Systems, Mountain View, CA). For N-[3-triethylammoniumpropyl]-4-[p-diethylaminophenylhexatrienyl] pyridinium dibromide (FM4-64) labeling of yeast vacuoles, ~1 OD₆₀₀ of yeast cells was harvested at an OD₆₀₀ of 0.4–0.6. Harvested cells were labeled with the vital vacuolar dye FM4-64 as described previously (Vida and Emr, 1995). FM4-64 was obtained from Molecular Probes (Eugene, OR).

Western Blot analysis of Ste2-HA

SEY6211 and SSY22 cells, transformed with plasmid pRS415-Ste2-HA (a gift from Chris Stefan and Ken Blumer, Washington University St. Louis, MO), were grown to an OD of 0.4. Two ODs of cells were harvested for each time point and resuspended in 1 ml of media lacking leucine. Some cultures were incubated at 37°C for 15 min before addition of α factor. Where indicated, α factor was added at a concentration of 5 μ M. After 8 min, the cells were transferred into tubes containing NaF-Na₃N₃. Cells were collected by centrifugation and resuspended in 100 μ l of cracking buffer (8 M urea, 50 mM Tris pH 6.8, 1 mM EDTA, 1% β -mercaptoethanol, and 5 mM N-ethylmaleimide). Glass beads (75 μ l) were added, and the cells were mechanically lysed by vortexing for 5 min, followed by heating at 37°C for 5 min. The lysate (50 μ l) was removed from the glass beads after centrifugation at 13,000 rpm for 5 min and added to 25 μ l of sample buffer containing 8 M urea. Samples were heated at 37°C, and 12 μ l of each sample was loaded onto 10% SDS-polyacrylamide gels. Anti-hemagglutinin (HA) antibody from Roche Diagnostics (Indianapolis, IN) was used for immunodetection of Ste2-HA.

Cell Labeling and Immunoprecipitations

Pulse-chase analysis for CPS was performed as described previously (Cowles *et al.*, 1997a), except 4 OD₆₀₀ units of cells were labeled with 40 μ Ci of Trans³⁵S label (PerkinElmer Life Sciences, Boston, MA) per time point. Immunoprecipitation of CPS, followed by Western blotting with anti-ubiquitin antibody was performed as described previously (Katzmann *et al.*, 2001). Carboxypeptidase Y (CPY) immunoprecipitation was also performed as described previously (Cowles *et al.*, 1997b).

RESULTS

Genetic Selection for MVB Sorting Pathway Mutants Reveals a Mutant Defective for Ubiquitination of pCPS

We have described previously a genetic selection used to identify mutants in the MVB sorting pathway (Odorizzi *et al.*, 2003). Briefly, this selection used a fusion between the histidine biosynthesis enzyme His3 and the transmembrane MVB cargo CPS. Wild-type cells rapidly sequester the His3-CPS chimera into the MVB pathway and away from its cytoplasmic substrate, whereas MVB sorting mutants that misdirect this chimera to the limiting membrane of the vacuole allowed the His3 moiety to remain in the cytoplasm and interact with its substrate, resulting in normal histidine biosynthesis. Mutant cells defective for the function of the MVB pathway are, therefore, histidine prototrophs, whereas wild-type cells are histidine auxotrophs. Such a selection should uncover gene products that function in several stages of the MVB sorting pathway, including cargo recognition, cargo sorting, and MVB vesicle formation. Originally, we simply selected for His⁺ mutants that grew well on media lacking histidine. This resulted in the identification of most of the class E *VPS* genes, which, when deleted, give rise to

viable cells with gross defects in the MVB pathway (Odorizzi *et al.*, 1998). The large number of nonessential class E genes biased our selection against essential genes that may function in the MVB pathway. To extend our genetic approach and identify candidate essential genes that function in the MVB sorting pathway, we chose mutants that were not only His⁺ but also temperature sensitive (ts) for growth. We initially selected His⁺ mutants by using the His3-CPS chimera at 23°C and then replica plated all the His⁺ mutants and incubated them at 37°C. We screened ~400 independent His⁺ mutants and found one mutant, mutant 326 (henceforth referred to as *mob326*), which did not grow at 37°C. Staining of *mob326* with the vital dye FM4-64, which stains the vacuolar membrane (Vida and Emr, 1995), revealed an absence of any obvious “class E compartment” at both permissive (Figure 1A) and nonpermissive temperatures (our unpublished data). The class E compartment is a characteristic of the class E *vps* mutants such as *vps4 Δ* (Figure 1A) (Raymond *et al.*, 1992; Babst *et al.*, 1997). However, like the class E *vps* mutants, *mob326* also missorted the chimeric MVB cargo GFP-CPS to the limiting membrane of the vacuole (Figure 1B). Genetic analysis revealed that the mutation in *mob326* was recessive and segregated as a single locus mutation (our unpublished data), suggesting that the mutated gene encodes a protein required for the efficient sorting of pCPS into the MVB pathway.

Missorting of the MVB cargo GFP-CPS could be the result of a defect at a number of steps in the pathway, ranging from a defect in the recognition of cargoes (which could be the result of a defect in pCPS ubiquitination or an inability of the sorting machinery to interact with ubiquitinated MVB cargoes) to a defect in the formation and fission of MVB vesicles. However, given that this mutant did not contain an obvious class E compartment, it would seem that the MVB pathway is still functioning in this mutant. To better understand the nature of the GFP-CPS sorting defect observed in *mob326*, we analyzed the sorting of two additional MVB cargoes: *SnaIII*-GFP (a biosynthetic MVB cargo that does not seem to require ubiquitin modification) (Reggiori and Pelham, 2001) and a fusion protein in which ubiquitin has been added to GFP-CPS, “Ub-GFP-CPS” (thereby obviating its need for ubiquitin modification). In striking contrast to GFP-CPS, both of these MVB cargoes were sorted to the lumen of the vacuole in the *mob326* mutant (Figure 1B). This result indicated that although GFP-CPS fails to be sorted into MVBs, the MVB pathway itself is still functional in *mob326*. Given that the Ub-GFP-CPS chimera was efficiently sorted into the MVB pathway, as was *SnaIII*-GFP, the simplest interpretation of this result is that *mob326* displays a defect in the ubiquitination of biosynthetic MVB cargoes such as pCPS.

We have previously demonstrated that efficient sorting of pCPS into the MVB pathway requires ubiquitination at a lysine within its cytoplasmic tail (Katzmann *et al.*, 2001). The ubiquitination status of pCPS was analyzed to directly address whether *mob326* displayed a defect in the ubiquitination of this biosynthetic MVB cargo. The ubiquitinated form of pCPS is transient and thus difficult to detect in wild-type cells, whereas deletion of the *PEP12* gene (encoding an endosomal soluble N-ethylmaleimide-sensitive factor attachment protein receptor) results in stabilization of this intermediate (Katzmann *et al.*, 2001). To optimize detection of ubiquitinated pCPS, it was immunoprecipitated from *pep12 Δ* or *mob326 pep12 Δ* double mutant cells at the permissive temperature of 26°C and the nonpermissive temperature of 37°C. Anti-ubiquitin Western blotting of the immunoprecipitated material revealed that ubiquitinated pCPS

Figure 1. *mvb326* cells fail to exhibit a class E compartment but specifically mislocalize the MVB cargo GFP-CPS. (A) Vacuole morphology of *mvb326* and MBY3 (*vps4Δ*) cells was visualized by either staining with FM 4-64 (left) or by using Nomarski optics (right). Cells were grown to midlog phase at 26°C, stained with FM 4-64 for 20 min, and chased with YPD for 1 h. (B) Localization of GFP-CPS, Ub-GFP-CPS, and SnaIII-GFP in SEY6210 (wild-type) and *mvb326* at 26°C.

was readily detectable in *pep12Δ* cells, but it was not detected in the *mvb326 pep12Δ* double mutant at both the permissive (Figure 2A) and nonpermissive temperatures (our unpublished data). The data indicate that even at the permissive temperature of 26°C *mvb326* cells do not display any detectable level of ubiquitinated pCPS. This could be the result of either a defect in ubiquitination of pCPS or an increase in the rate of its deubiquitination. To distinguish between these possibilities, the ubiquitination status of pCPS in *mvb326* was also analyzed by immunoprecipitation from ³⁵S-labeled cells. A small, transient pool of Ub-pCPS can be observed in wild-type cells after a brief pulse label (10 min), but this pool was absent from *mvb326* cells (Figure 2B). The data indicate that *mvb326* is defective for the ubiquitination of the biosynthetic MVB cargo pCPS.

Failure to ubiquitinate a mutant form of CPS, GFP-CPS^{KSR}, results in its mislocalization to the limiting membrane of the vacuole (Katzmann *et al.*, 2001). Mislocalization of endogenous pCPS to the vacuole membrane also results in a delay in its maturation. To address whether there was a kinetic delay in pCPS maturation in the *mvb326* mutant cells, we assayed the maturation kinetics of endogenous pCPS by pulse-chase radiolabeling and immunoprecipitation of CPS from wild-type and *mvb326* cells (Figure 3A). Although wild-type cells converted 95% of pCPS to mature CPS after a 10-min pulse and 35-min chase, *mvb326* cells processed only 55% of pCPS during this same period of time (Figure 3A). Consistent with the results obtained using GFP-CPS (Figure 1B), nonubiquitinated endogenous pCPS would also seem to be missorted to the limiting membrane of the vacuole in the *mvb326* cells, resulting in a delay in its maturation.

Because *mvb326* had a defect in pCPS sorting, we wanted to test whether it had a *vps* phenotype as well. The transport of luminal cargoes that transit through the endosome en route from the Golgi to the vacuole (e.g., CPY) is partially defective in class E *vps* mutants (Raymond *et al.*, 1992; Cereghino *et al.*, 1995; Piper *et al.*, 1995). CPY sorting and processing can, therefore, serve as a useful indicator of gen-

eral endosomal function. Although wild-type cells transport p2CPY (the form that has received glycosylation within the Golgi) to the vacuole efficiently, class E *vps* mutants, such as *vps4*, secrete ~40% of their p2CPY (Figure 3B) (Babst *et al.*, 1997). Unlike the *vps4* class E mutant, *mvb326* efficiently matured p2CPY and did not secrete any detectable p2CPY, suggesting that endosomal function is not dramatically perturbed. Together, the data presented in Figures 1–3 indicate that *mvb326* is not blocked in the function of the MVB sorting pathway (other MVB cargoes are still delivered into this pathway), nor does it exhibit any obvious defect in Golgi-to-vacuole sorting (CPY processing is normal). However, GFP-CPS largely fails to enter the MVB pathway, as indicated by its missorting to the limiting membrane of the vacuole. Direct analysis of the ubiquitination status of pCPS suggested that this defect is the result of a failure to ubiquitinate endogenous pCPS. Therefore, selective cargo recognition and ubiquitin modification, rather than MVB formation, seem to be blocked in the *mvb326* mutant.

mvb326 Is Not Allelic to *TUL1*, *UBC4*, or *FAB1*

The GFP-CPS missorting defect seen in *mvb326* and the defect in ubiquitination of pCPS suggest that *mvb326* is defective in a specific ubiquitin ligase or adapter. An obvious candidate for such a mutant is the putative E3 ubiquitin ligase Tul1, previously shown to have a defect in the sorting of biosynthetic MVB cargoes (Reggiori and Pelham, 2002). Similar to *mvb326*, *tul1Δ* mutants were shown to be defective for the trafficking of biosynthetic MVB cargoes that require ubiquitination for their efficient sorting into this pathway, whereas *SnaIII* and a translational fusion between ubiquitin and a ubiquitin-dependent MVB cargo were sorted normally (Reggiori and Pelham, 2002). To address whether *mvb326* was allelic to *TUL1*, we generated a *tul1Δ* strain in our background, which could then be mated to *mvb326* to test for complementation. Unexpectedly, in contrast to previously published data, we observed efficient MVB sorting of GFP-CPS in the *tul1Δ* single mutant, as indicated by its efficient delivery to the vacuole lumen (Figure 4). To further inves-

Figure 2. *mvb326* cells exhibit a defect in ubiquitination of pCPS. (A) CPS was immunoprecipitated from either CBY16 (*pep12Δ*) or SSY25 (*mvb326 pep12Δ*) cells, grown at 26°C, followed by Western blot analysis by using anti-ubiquitin antibody. (B) SEY6210 (wild-type), GOY19 (*cps1Δ*), and *mvb326* cells were labeled with Trans ³⁵S-label for 10 min at 26°C. Labeling was terminated by addition of unlabeled methionine and cysteine, and CPS was immunoprecipitated from the extracts. Before SDS-PAGE analysis, extracts were deglycosylated by treatment with endoglycosidase H, which resulted in only a single band for Ub-pCPS. An asterisk highlights a nonspecific band.

tigate a role for Tul1 in MVB sorting, we used a *tul1Δ* in the same strain background, BY4742, as was published previously. However, interpretation of a sorting defect in BY4742 *tul1Δ* cells was complicated by the fact that the wild-type BY4742 parent strain already displayed a partial defect in GFP-CPS sorting (Figure 4). It can be seen that BY4742 *tul1Δ* cells display a subtle missorting phenotype for GFP-CPS, but it is not clear that this is significantly different from wild-type BY4742 cells. *SnaIII*-GFP sorted normally in all strains tested, with the exception of the class E mutant *vps4Δ*, which fails to deliver detectable amounts of either cargo to the lumen of the vacuole (Figure 4; Reggiori and Pelham, 2001). These results indicate that *mvb326* cannot be allelic to *TUL1*, because Tul1 is not required for MVB sorting in our strain background, but it may play a role in others.

We were puzzled by the lack of any detectable phenotype for the *tul1Δ* in our strain background. One explanation is that ubiquitination of pCPS is kinetically delayed in the absence of Tul1 function but not enough to perturb its entry into the MVB pathway. To examine this possibility, we directly analyzed ubiquitinated pCPS in both wild-type and

Figure 3. *mvb326* cells exhibit a specific defect in pCPS processing. (A) SEY6210 (wild-type) and *mvb326* cells were labeled with Trans ³⁵S-label for 10 min at 26°C and chased with unlabeled methionine and cysteine for 35 min. Extracts were processed as in Figure 2. An asterisk highlights a nonspecific band as described in Figure 2. (B) SEY6210 (wild-type), MBY3 (*vps4Δ*), and *mvb326* cells were grown at 26°C and converted to spheroplasts. The spheroplasts were labeled with Trans ³⁵S-label for 10 min at 26°C and then chased for 30 min by using unlabeled methionine and cysteine. Spheroplasts were then harvested and separated into intracellular (I) or extracellular (E) fractions, and CPY was immunoprecipitated from each fraction.

tul1Δ cells in the context of the *pep12Δ* mutant, which stabilizes ubiquitinated pCPS. Anti-CPS immunoprecipitations, followed by anti-ubiquitin Western blotting, revealed no defect in the steady-state level of ubiquitinated pCPS (Figure 5A). Consistent with the results in Figures 4 and 5A, pulse-chase radiolabeling and immunoprecipitation of CPS from *tul1Δ* cells indicated no defect in the appearance of the ubiquitinated intermediate after a short pulse labeling (10 min) (Figure 5B), nor any delay in the maturation kinetics of pCPS (our unpublished data). These results are in striking contrast to those seen for *mvb326*, where there are clear defects in the ubiquitination, sorting, and maturation of pCPS (Figures 1B, 2, A and B, and 3A). Together, these data indicate that a role for Tul1 in the sorting of pCPS into the MVB pathway is minimal under the assay conditions used.

In addition to identifying Tul1 as an ubiquitin ligase involved in the ubiquitination of biosynthetic MVB cargoes, a previous study identified Ubc4 as the requisite E2 for this modification (Reggiori and Pelham, 2002). For this reason, we addressed whether *mvb326* had a mutation in *UBC4*. Surprisingly, GFP-CPS localization was unaffected not only in cells containing a *UBC4* deletion (our unpublished data)

Figure 4. Entry into the MVB pathway is not affected in *tul1Δ* or *ubc4Δ ubc5Δ* cells. Localization of GFP-CPS (top) and SnaIII-GFP (bottom) in BY4742 (wild-type), 14833 (*tul1Δ* in BY4742), SEY6210 (wild-type), SSY11 (*tul1Δ* in SEY6210), LH21 (*ubc4Δ ubc5Δ*), and MBY3 (*vps4Δ*) cells.

but also in cells where both *UBC4* and *UBC5* (encoding another E2 that functionally overlaps with Ubc4) were deleted (Figure 4). The GFP-CPS localization pattern in the wild-type parent of these cells was identical to that observed for SEY6210 (our unpublished data). Another E2 enzyme, which has functional overlap with Ubc4 and Ubc5, is Ubc1. To see whether deletion of *UBC1* alone, or in conjunction with *UBC4*, affected pCPS ubiquitination we performed pulse-chase analysis using either *ubc1Δ* or *ubc1Δ ubc4Δ* cells. We observed that pCPS was ubiquitinated in the *ubc1Δ* (our unpublished data), as well as the *ubc1Δ ubc4Δ* cells (Figure 5B). There was also no reduction in the level of ubiquitination in these cells compared with their wild-type counterparts (our unpublished data). The lack of any missorting phenotype in *ubc4Δ ubc5Δ* cells and normal pCPS ubiquitination in *ubc1Δ ubc4Δ*, in conjunction with several reports in the literature documenting overlapping functions of Ubc1, Ubc4, and Ubc5 (Seufert *et al.*, 1990; Arnason and Ellison, 1994; Hicke and Riezman, 1996; Gitan and Eide, 2000; Horak and Wolf, 2001), and the fact that *mvb326* harbors a single mutation made it unlikely that the mutation in *mvb326* is in the gene encoding any of these E2 enzymes.

Another mutant that has been demonstrated to missort biosynthetic MVB cargo is the *fab1* mutant, which is defective for phosphatidylinositol 3-phosphate [PI(3)P] 5-kinase activity (Odorizzi *et al.*, 1998). *fab1* mutants display normal MVB sorting of endocytic cargoes such as Ste2-GFP; however, GFP-CPS is missorted to the limiting membrane of the vacuole (Odorizzi *et al.*, 1998). Sorting is restored when ubiquitin is fused to biosynthetic MVB cargo (Reggiori and Pelham, 2002). Because the sorting phenotypes of the *fab1* mutant were similar to the phenotypes, we observed in *mvb326*, we tested whether ubiquitination of pCPS was altered in cells lacking the Fab1 kinase activity. Surprisingly,

pulse-chase radiolabeling and immunoprecipitation of CPS from wild-type and *fab1^{ts}* cells revealed that, at the restrictive temperature of 38°C, there is no apparent decrease in the amount of ubiquitinated pCPS in *fab1^{ts}* cells compared with wild-type cells (Figure 5B). Pulse-chase analysis using *fab1Δ* cells also showed no defect in pCPS ubiquitination (our unpublished data). Given that there is no apparent defect in the appearance of ubiquitinated pCPS in *fab1* strains, an increased deubiquitination rate could explain the missorting defect seen for GFP-CPS. For this reason, we analyzed steady-state levels of ubiquitinated pCPS in wild-type and *fab1Δ* cells by immunoprecipitation of CPS, followed by anti-ubiquitin Western blotting. Not only is pCPS ubiquitinated in *fab1Δ* cells but also there seems to be a slight accumulation of the ubiquitinated species compared with wild-type cells (Figure 5C), indicating that *fab1Δ* cells do not deubiquitinate MVB cargoes more rapidly than wild-type cells. We chose not to perform this experiment in a *fab1Δ pep12Δ* strain because these cells are very sick, and we were therefore reluctant to draw any conclusions from them. Therefore, *mvb326* did not seem to have a mutation in *FAB1*, because Fab1 kinase activity does not seem to be required for ubiquitination of pCPS. This indicates that the defect in ubiquitin-dependent MVB cargo sorting, observed in *fab1* cells, results from a defect that is distinct from cargo ubiquitination.

A Role for Rsp5 in the Ubiquitination of pCPS

Given that *mvb326* did not correspond to the *TUL1*, *UBC4*, or *FAB1* genes, we opted to test other known ubiquitin ligases that play a role in protein trafficking. The HECT domain-containing ubiquitin ligase Rsp5 has been shown to ubiquitinate a number of cell surface proteins (for review, see Rotin

Figure 5. pCPS is ubiquitinated in *tul1Δ*, *ubc1Δ ubc4Δ*, and *fab1* cells. (A) CPS was immunoprecipitated from either CBY16 (*pep12Δ*) or SSY16 (*tul1Δ pep12Δ*) cells, grown at 26°C, followed by Western blot analysis by using anti-ubiquitin antibody. (B) SEY6210 (wild-type), SSY11 (*tul1Δ*), and LH183 (*ubc1Δ ubc4Δ*) cells were labeled with Trans ³⁵S-label for 10 min at 26°C and chased with unlabeled methionine and cysteine. Extracts are prepared and immunoprecipitated for CPS as described in Figure 2. EMY119 (*fab1^{ts}*) cells were treated similarly except for a brief shift to 38°C (15 min) before labeling. An asterisk highlights a nonspecific band as described in Figure 2. (C) CPS was immunoprecipitated from either SEY6210 (wild-type) or *fab1Δ* (*fab1Δ*) cells, grown at 26°C, followed by Western blot analysis by using anti-ubiquitin antibody.

et al., 2000). In addition, *RSP5* is an essential gene (Huibregtse *et al.*, 1995), and our genetic selection was designed to identify essential genes that may be required for MVB sorting. To test the possibility that *mbv326* was allelic to *RSP5*, a previously published complementing clone of Rsp5 tagged with HA was transformed into the *mbv326* mutant and growth at 37°C was tested (Gajewska *et al.*, 2001). The *RSP5* gene complemented the ts growth defect of *mbv326* (our unpublished data). We next analyzed GFP-CPS sorting and found that plasmidborne *RSP5* suppressed the missorting defect seen in *mbv326* (Figure 6A). Furthermore, the *RSP5* plasmid restored ubiquitination of pCPS and the normal maturation kinetics for pCPS as measured by pulse-

chase immunoprecipitation (our unpublished data). This suggested that the *mbv326* contained a mutation in the *RSP5* gene. To test this directly, the *RSP5* gene from *mbv326* cells was cloned by gap-repair. Sequencing of the ORF revealed a single point mutation that changed glycine 555 to an aspartic acid residue within the HECT domain. To verify that this was the cause of the defect seen in *mbv326* (henceforth referred to as *rsp5-326*), the plasmidborne *rsp5-326* (containing a *URA3* marker) was transformed into heterozygous diploid cells deleted for one copy of *RSP5*. The transformed diploids were sporulated and screened for cells that carried the *rsp5-326* plasmid as well as the deletion of chromosomal *RSP5*. These cells were temperature sensitive for growth and they exhibited the same GFP-CPS missorting phenotype as the original *mbv326* mutant (Figure 6A). This demonstrated that the phenotype seen in *mbv326* is the result of a mutation in *RSP5*.

Another target of Rsp5 is the plasma membrane localized α factor receptor Ste2 (Dunn and Hicke, 2001a). On binding of its ligand, α factor, Ste2 undergoes hyperphosphorylation, followed by ubiquitination at multiple sites in the cytoplasmic portion of the receptor (Hicke and Riezman, 1996). To determine whether *rsp5-326* cells also have a defect in the ubiquitination of Ste2, we assayed for Ste2 ubiquitination by Western blot analysis. This approach has been used previously to demonstrate that Ste2 is hyperphosphorylated and ubiquitinated within 8 min of α factor addition, and this ubiquitination is blocked in the *rsp5-2* allele at the nonpermissive temperature (Dunn and Hicke, 2001a). We observed that in both wild-type and *rsp5-326* cells, Ste2 was ubiquitinated within 8 min of α factor addition at 26°C (Figure 6B), a temperature at which there is a clear defect in pCPS ubiquitination (Figure 2, A and B). After a 15-min shift to 37°C, *rsp5-326* cells were defective in Ste2 ubiquitination (compared with wild-type cells), indicating that at this temperature the ability of Rsp5-326 to ubiquitinate other substrates is also affected, which probably contributes to the loss of viability of these cells at the elevated temperature. Thus, at 26°C, the *rsp5-326* allele has a specific defect in ubiquitination of pCPS but not that of Ste2. This indicates that at the temperature permissive for growth, this allele of *RSP5* is selectively impaired in the ubiquitination of a biosynthetic cargo.

The fact that the mutation in *rsp5-326* is located within the HECT domain was somewhat surprising, given the selective cargo ubiquitination defect observed in this mutant (Ste2 is ubiquitinated properly at 26°C, but pCPS is not). To address whether this was a HECT domain-specific phenotype, three other known Rsp5 mutants that map to the HECT domain were tested. Besides *rsp5-326*, only one other allele of *RSP5* (*smm1*) (Fisk and Yaffe, 1999) failed to sort GFP-CPS to the lumen of the vacuole at 26°C (our unpublished data). Similar to the *rsp5-326* allele, ubiquitinated pCPS was virtually undetectable in the *smm1* allele by anti-CPS immunoprecipitation followed by anti-ubiquitin Western blotting (Figure 6C). Two other ts alleles of *RSP5*, *rsp5-1* (Dunn and Hicke, 2001a) and *mdp1-13* (Zoladek *et al.*, 1997), displayed no defect in the ubiquitination of pCPS at the permissive temperature of 26°C (our unpublished data) and the nonpermissive temperature of 37°C compared with the corresponding wild-type strains (Figure 6D). These results were unexpected, because the *rsp5-1* allele has been shown to display a defect in the ubiquitination and down-regulation of a number of cell surface proteins (Dunn and Hicke, 2001a, b; Helliwell *et al.*, 2001; Wang *et al.*, 2001), yet displays no defect in pCPS ubiquitination (Figure 6D). One explanation would be that the defect seen in endocytic cargo but not biosynthetic cargo

Figure 6. Specific alleles of *RSP5* differentially affect pCPS entry into the MVB pathway and ubiquitination of Ste2. (A) Localization of GFP-CPS in *mvb326* cells, harboring a plasmid encoding HA-Rsp5 (top), or *rsp5*Δ cells harboring a plasmid encoding Rsp5-326 (bottom), at 26°C. (B) SEY6211 (wild-type) and SSY22 (*mvb326*) cells expressing Ste2-HA were treated with alpha factor for 8 min at the indicated temperatures and lysates were prepared and probed with anti-HA antibody. Unmodified Ste2, phosphorylated Ste2, and ubiquitinated Ste2 are indicated by brackets. (C) CPS was immunoprecipitated from either CBY16 (*pep12*Δ) or DKY83 (*smm1 pep12*Δ) cells, grown at 26°C, followed by Western blot analysis by using anti-ubiquitin antibody. (D) LH291 (wild-type), LH23 (*rsp5-1*) MY290 (wild-type) and MY833 (*mdp1-13*) cells were incubated at 37°C for 10 min, before labeling with Trans ³⁵S-label for 10 min at 37°C. Cells were treated as described in Figure 2. An asterisk marks a nonspecific band as described in Figure 2.

(or vice versa) is the result of differential defect in the targeting of the different mutant forms of Rsp5 to one subcellular compartment, but not the other.

The Role of C2 and WW Domains of Rsp5 in Ubiquitin Modification and Sorting of pCPS

The data presented above indicate that the HECT domain plays a critical role in the ubiquitination of pCPS. To examine whether other domains of Rsp5, namely, the C2 (mediating protein-lipid interaction) and WW (mediating protein-protein interaction) domains, play any role in the ubiquitin modification and sorting of pCPS, we tested mutant forms of Rsp5 that either lack the C2 domain (Δ C2) or contain point mutations in the conserved residues (WXXP $\rightarrow$ FXXA) within each of the three WW domains (*ww1*, *ww2*, and *ww3*) (Figure 7A). These WW mutations have previously been demonstrated to confer defects in endocytosis of Fur4 (Gajewska *et al.*, 2001). Other point mutations within each of the WW domains displayed defects in alpha factor-induced ubiquitination and internalization of Ste2 (Dunn and Hicke, 2001a). GFP-CPS trafficking to the vacuolar lumen was not affected by mutations in the first WW domain (Figure 7B). However, GFP-CPS mislocalized to the limiting membrane of the vacuole in a C2 deletion mutant and in mutants of WW2 and WW3 domains (Figure 7B). Consistent with the GFP-CPS sorting, pulse-chase analysis indicated that ubiquitination of pCPS was largely unaffected in a *ww1* mutant, but was defective in the Δ C2 mutant and in the *ww2* and *ww3* mutants (Figure 7C). Interestingly, the sorting of GFP-CPS into the vacuole lumen was not completely blocked in the Δ C2 mutant (Figure 7B); consistent with this, upon longer exposure, we have observed a faint band for ubiquitinated pCPS in pulse-chase radiolabeling experiments (our unpublished data). Therefore, it seems that ubiquitination of pCPS is not completely blocked by a C2 domain deletion. These data indicate that in addition to the HECT domain, the C2 and WW domains of Rsp5 are also required

for the ubiquitination and sorting of pCPS, and suggest that recruitment/activation of Rsp5 function within the MVB sorting pathway may depend on interactions between these domains and other components of the MVB sorting apparatus.

Subcellular Localization of Rsp5

Rsp5 function has been implicated at almost every subcellular compartment, including the endoplasmic reticulum, Golgi, mitochondria, endosomes, and the plasma membrane (Fisk and Yaffe, 1999; Hoppe *et al.*, 2000; Gajewska *et al.*, 2001; Helliwell *et al.*, 2001; Wang *et al.*, 2001). A previously published report has demonstrated that GFP-Rsp5, expressed under the control of a *GAL1* promoter, localizes to the plasma membrane as well as to intracellular punctate structures; also, the same report described Rsp5 colocalizing with endosomal proteins by immunogold electron microscopy (Wang *et al.*, 2001). Immunofluorescence studies have also shown that Rsp5 localizes to punctate structures in the cytoplasm (Gajewska *et al.*, 2001). However, there is some discrepancy in both the localization and the fractionation patterns of Rsp5 published by different groups (Dunn and Hicke, 2001a; Gajewska *et al.*, 2001; Wang *et al.*, 2001). Thus, to examine the localization of Rsp5 in live cells, we constructed a GFP-Rsp5 chimera expressed under the control of the CPY promoter. This promoter is considerably weaker in strength compared with the *GAL1* promoter used in the previous study (Ichikawa *et al.*, 1993). Therefore, the level of GFP-Rsp5 is closer to endogenous Rsp5 levels. Transformants, wherein GFP-Rsp5 was the only source of Rsp5, were viable and their growth was similar to the wild-type control cells (our unpublished data), indicating that the chimera is functional. The GFP-Rsp5 chimera revealed a fluorescence pattern that was largely cytoplasmic but that also was clearly enriched at the plasma membrane and a number of intracellular puncta (Figure 8). To determine the nature of these intracellular puncta, we performed colocalization ex-

Figure 7. The C2 and WW domains of Rsp5 also play a role in the ubiquitination and sorting of pCPS. (A) Schematic of Rsp5 domain structure with mutations mapped to each domain. (B) Localization of GFP-CPS in SEY6210 (wild-type), TCY124 (Δ C2), TCY84 (*ww1*), TCY86 (*ww2*), and TCY81 (*ww3*) mutants. (C) SEY6210 (wild-type), TCY124 (Δ C2), TCY84 (*ww1*), TCY86 (*ww2*), and TCY81 (*ww3*) cells were labeled with Trans 35 S-label for 10 min at 26°C and chased with unlabeled methionine and cysteine. Extracts were processed as in Figure 2. An asterisk highlights a nonspecific band as described in Figure 2.

periments by using known markers for both the Golgi (Sec7 fused to Ds-Red) (Calero *et al.*, 2003) and endosomes (chimeras of DsRed with the FYVE domain that binds to PI3P on endosomes) (Katzmann *et al.*, 2003). Interestingly, GFP-Rsp5

colocalized with both Sec7-DsRed and DsRed-FYVE in wild-type cells, indicating that Rsp5 is present at the plasma membrane, Golgi, and endosomal membranes (Figure 8). Rsp5-326 was also fused to GFP, resulting in a localization

Figure 8. Localization of GFP-Rsp5. SEY6210 cells expressing either GFP-Rsp5 and Sec7-DsRed (top) or GFP-Rsp5 and DsRed-FYVE (bottom) were grown at 26°C, and the GFP and DsRed labeled proteins were visualized by fluorescence microscopy.

pattern indistinguishable from wild-type pattern shown in Figure 8 (our unpublished data). Together, these data indicate that Rsp5 is present on intracellular structures, including endosomes, the apparent site of pCPS ubiquitination, and are therefore consistent with our genetic studies, which show that Rsp5 is required for ubiquitination of pCPS. However, the ubiquitination defect seen in Rsp5-326 apparently is not due to a defect in localization of the mutant protein.

DISCUSSION

MVB sorting occurs at an endosomal compartment where a subset of proteins in the limiting membrane is recognized and actively sorted into vesicles that bud into the lumen of the organelle (for review, see Piper and Luzio, 2001; Katzmann *et al.*, 2002). Entry of cargo into the MVB pathway is a highly regulated event, requiring both *cis*- and *trans*-acting factors. Ubiquitination of endosomal membrane proteins serves as a positive sorting signal for inclusion in the intraluminal MVB vesicle (Katzmann *et al.*, 2001; Reggiori and Pelham, 2001; Urbanowski and Piper, 2001). At the endosomal compartment, a subset of the more than 15 class E Vps proteins function in the recognition of the ubiquitin signal on MVB cargoes (for review, see Katzmann *et al.*, 2002). Several of these class E Vps proteins assemble into the endosomal sorting complex required for transport I, II, and III (ESCRT) complexes, the sequential activities of all of which are required for the completion of the MVB sorting process (Katzmann *et al.*, 2001; Babst *et al.*, 2002a,b). Therefore, an obvious mechanism by which to control entry into the MVB pathway is at the level of ubiquitination. Understanding the machinery responsible for the ubiquitination of MVB cargoes is of critical importance for understanding how the MVB pathway is regulated and how MVB cargo selection is determined.

The class E *vps* mutants seem to display a complete lack of function of the MVB sorting pathway, because all cargoes fail to be properly sorted into the lumen of the vacuole (Odorizzi *et al.*, 1998; Reggiori and Pelham, 2001). Although our previous work has begun to dissect the functions of the class E Vps proteins, it seems that deletion of any of the class E *VPS* genes results in a dramatic shutdown of the pathway. However, cargo ubiquitination is not blocked in class E *vps* mutants. As previously mentioned, the class E Vps proteins function as components of the ESCRT complexes. However, none of the class E *VPS* genes have been found to be essential for yeast growth under normal laboratory conditions. For this reason, the identification of mutants with defects in essential genes that might also function in the MVB sorting pathway was of particular interest. We identified a mutant that displays a missorting phenotype for the ubiquitin-dependent biosynthetic MVB cargo pCPS. This mutant is temperature sensitive for growth, yet displays an MVB sorting defect at the permissive growth temperature. Characterization of this mutant revealed that it seems to be selectively defective for ubiquitin addition to the biosynthetic cargo pCPS at permissive temperature, because the endocytic cargo Ste2 is still ubiquitinated in response to alpha factor treatment. Complementation revealed that this mutant corresponds to a new allele of *RSP5*, *rsp5-326*. *RSP5* encodes a HECT-domain containing ligase that has previously been shown to play a role in the ubiquitination of a number of cell surface proteins that are degraded subsequent to their entry into the MVB pathway (for review, see Rotin *et al.*, 2000). Unlike the class E *vps* mutants, which accumulate an aberrant endosomal compartment, the *rsp5-326* mutant did not display the "class E compartment."

Rsp5 has been demonstrated to function as an ubiquitin ligase at multiple locations within the cell, including the plasma membrane, endoplasmic reticulum, Golgi, endosome, and mitochondria (Fisk and Yaffe, 1999; Hoppe *et al.*, 2000; Gajewska *et al.*, 2001; Helliwell *et al.*, 2001; Wang *et al.*, 2001). Therefore, Rsp5 must be selectively recruited to/activated at diverse membrane compartments within the cell. This recruitment/activation of Rsp5 is likely to be highly regulated and dependent on both protein-protein and protein-lipid interactions. Indeed, Rsp5 contains motifs responsible for driving interactions with both proteins and lipids (Wang *et al.*, 1999). In addition to the C-terminal HECT domain, the N-terminal portion of Rsp5 contains a C2 domain that binds charged lipids (Hofmann and Bucher, 1995) and three WW protein-interaction domains capable of binding proline-rich sequences (Wang *et al.*, 1999).

The C2 domain has previously been implicated in binding negatively charged lipids (Rizo and Sudhof, 1998). One previous report indicated that deletion of the C2 domain of Rsp5 causes a redistribution of the protein to the cytoplasm (Wang *et al.*, 2001), and this would suggest that the C2 domain plays a relatively nonspecific role in recruitment of Rsp5 onto membranes. However, C2 mutants are not defective for the internalization of the ubiquitin-dependent endocytic cargo Ste2, indicating that recruitment to membranes may not be completely defective (Dunn and Hicke, 2001a). Similarly, C2 mutants are not defective in their ability to ubiquitinate Fur4 and Gap1 at the cell surface, yet the turnover of these cell surface proteins is delayed (Springael *et al.*, 1999; Wang *et al.*, 2001). These reports suggest a role for the C2 domain in the recruitment of Rsp5 to intracellular membranes. Consistent with these reports, we have shown that the C2 domain of Rsp5 is required for the efficient ubiquitination and sorting of pCPS into the MVB pathway. However, the ubiquitination and sorting of pCPS is not completely blocked by a C2 domain deletion, suggesting that the C2 domain plays a limited role in the recruitment of Rsp5 to endosomes and/or the stabilization of the Rsp5 protein on membranes, where it would act to ubiquitinate MVB cargoes such as pCPS.

Unlike the C2 domain, the WW domains on Rsp5 have been shown to mediate protein-protein interactions and to have been shown to preferentially bind to proline-rich motifs (Chen *et al.*, 1997). Nedd4, one potential mammalian homolog of Rsp5, plays a critical role in regulating the subcellular localization of epithelial sodium channel (ENaC) (Staub *et al.*, 2000). Subcellular localization of ENaC regulates its ability to control sodium homeostasis, and Nedd4 targets the channel for internalization (Kamynina *et al.*, 2001). The interaction between Nedd4 and ENaC depends upon one of the WW domains within Nedd4, and ENaC mutants that cannot interact with Nedd4 lead to an inherited form of hypertension (Liddle's syndrome) (Gormley *et al.*, 2003). Rsp5 has three WW domains that seem to perform nonredundant functions, suggesting distinct interactions (Hein *et al.*, 1995; Chang *et al.*, 2000; Gajewska *et al.*, 2001). In the case of Ste2 internalization, mutation of any of the WW domains causes a decrease in alpha factor internalization, with the most severe defect seen in the triple mutant (Dunn and Hicke, 2001a). In the case of a *ww2* mutant, Rsp5 localization is not perturbed, nor is fluid phase uptake, but Fur4 internalization from the plasma membrane is delayed (Gajewska *et al.*, 2001). Likewise, in the present study, we found that both the WW2 and WW3 domains of Rsp5 are required for the ubiquitination and sorting of pCPS into the MVB pathway, suggesting a conserved role for these domains in the ubiquitination of MVB cargoes. Therefore, it would seem

that the WW domains play a role in cargo selection and localization of both the Nedd4 and Rsp5 ubiquitin ligases. However, given that the cytoplasmic tail of pCPS contains no proline-rich elements, it seems unlikely that the WW domains of Rsp5 function in the direct recognition of this MVB cargo. Rather, the WW domains may bind other membrane-associated proteins that help target Rsp5 to the appropriate membrane. The class E Vps proteins are obvious candidates for this role, however class E *vps* mutants do not block ubiquitination of pCPS (e.g., *vps27*, *vps23*, and *vps4*) (Katzmann *et al.*, 2001; Shih *et al.*, 2002), indicating that the class E Vps proteins are not essential for Rsp5 localization. This is consistent with the assignment of Rsp5 function upstream of MVB sorting.

The HECT domain of Rsp5 catalyzes ubiquitin transfer to its substrates (Huibregtse *et al.*, 1995). This domain is also thought to play a role in substrate recognition. The HECT domain can be divided into two sections, the C-terminal region that interacts with E2s and provides the enzymatic activity responsible for transfer of ubiquitin to substrate (Nuber *et al.*, 1996), and the amino terminal portion that provides substrate specificity (Huibregtse *et al.*, 1993; Huang *et al.*, 1999). Two alleles of *RSP5*, *rsp5-326* and *smm1*, both of which map to the HECT domain, displayed a specific sorting defect for the ubiquitin-dependent biosynthetic MVB cargo pCPS, without an obvious defect in the general function of the MVB pathway. Conversely, another HECT domain mutant, *rsp5-1*, which has previously been shown to display a defect in ubiquitination/internalization of Ste2 (Dunn and Hicke, 2001a), displays no defect in pCPS ubiquitination. The selective sorting defect observed in these *rsp5* alleles is consistent with a role for the HECT domain in target protein recognition (e.g., pCPS).

We analyzed the subcellular localization of Rsp5 as a GFP-tagged functional chimera. This analysis revealed that Rsp5 localized to a variety of subcellular puncta, as well as the plasma membrane. The identity of these intracellular puncta was addressed by colocalization with known marker proteins, revealing both Golgi and endosome-associated pools of Rsp5. Localization of the mutant form of Rsp5 (Rsp5-326) failed to reveal an obvious change in the distribution of Rsp5 (our unpublished data). Present data are therefore most consistent with the Rsp5-326 mutant having a defect not in its subcellular localization, but rather in the selective recognition/modification of specific MVB cargo (pCPS). A possible role for adaptors and Ubc proteins in this specificity cannot be ruled out. Indeed, Rsp5 has been shown to interact with a number of adapter proteins (e.g., Bul1 and Bul2) (Yashiroda *et al.*, 1996).

Besides localizing to the appropriate membrane compartment, Rsp5 must selectively recognize appropriate target proteins for ubiquitination. This is clearly demonstrated at the endosome where the MVB cargoes such as pCPS are efficiently ubiquitinated, whereas others, such as the transmembrane protein DPAP B, which contains multiple lysines in its cytoplasmic tail, fail to receive this modification (and do not become MVB cargoes) (Katzmann *et al.*, 2001). It would, therefore, seem that the activity of Rsp5 is dictated only in part by its subcellular localization. It may also be that there is some conserved motif within Rsp5 substrates (e.g., phosphorylated peptide motif) that is directly recognized by Rsp5.

Previous work also implicated Tul1 as the E3 responsible for ubiquitination of biosynthetic MVB cargoes (Reggiori and Pelham, 2002). However, the assignment of E3 function to Tul1 was based on sequence homology to known RING domain-containing ligases, and a formal demonstration of

this activity is lacking. Furthermore, deletion of *TUL1* resulted in only a partial defect in ubiquitination of biosynthetic MVB cargo. We have further tested the role of Tul1 in the ubiquitination of pCPS and were unable to detect any defect in either MVB sorting of GFP-CPS or the ubiquitination of pCPS. It may be that the growth conditions used in the previous study partially inhibited ubiquitination and sorting of biosynthetic MVB cargoes. Together with the dramatic MVB sorting defect and defect in pCPS ubiquitination reported here, we believe Rsp5 represents the predominant ubiquitin ligase functioning in the MVB sorting pathway. Prior reports of the involvement of Rsp5 in diverting newly synthesized Tat2 and Gap1 from the Golgi to vacuole, via the MVB pathway, supports this idea (Beck *et al.*, 1999; Helliwell *et al.*, 2001; Soetens *et al.*, 2001; Umebayashi and Nakano, 2003).

We previously reported another mutant, *fab1*, which displays sorting phenotypes similar to those observed in *rsp5-326* (Odorizzi *et al.*, 1998). Fab1 encodes a PI(3)P 5-kinase that when mutated results in a defect in sorting of GFP-CPS into the MVB pathway, whereas Ste2-GFP is sorted normally (Odorizzi *et al.*, 1998). These results are consistent with a role for phosphatidylinositol 3,5-bisphosphate (PI[3,5]P₂) in some aspect of the regulation of MVB sorting, possibly controlling the ubiquitination of MVB cargoes such as pCPS. This could be mediated by the recruitment of Rsp5 to membranes enriched in PI(3,5)P₂. However, we did not detect any defect in the ubiquitination of pCPS in *fab1* mutants. Fab1 seems to play an important role in the context of sorting biosynthetic MVB cargoes, but this is apparently not via ubiquitin addition. Based on these results we speculate that Fab1 may be required for the efficient recognition of ubiquitinated pCPS by some component of the ESCRT machinery. Identification of mammalian Vps24 as a potential PI(3,5)P₂ effector lends support for this idea (Whitley *et al.*, 2003). However, it is possible that even though pCPS is ubiquitinated in *fab1* cells, this ubiquitination occurs at a location different from that in wild-type cells making it difficult for the ESCRT machinery to recognize it as a MVB cargo. Limitations of the present assay techniques make it difficult for us to determine the precise cellular location of pCPS ubiquitination. Therefore, we were unable to test this hypothesis. The precise role of Fab1 and its product PI(3,5)P₂ in MVB sorting remains to be elucidated.

We have screened a relatively small number of mutants in our genetic study. It is reasonable to expect that extending this genetic screen will help to identify other essential gene products required for MVB sorting, as well as additional genes that regulate Rsp5 localization and cargo recognition.

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REFERENCES

Arnason, T., and Ellison, M.J. (1994). Stress resistance in *Saccharomyces cerevisiae* is strongly correlated with assembly of a novel type of multiubiquitin chain. *Mol. Cell Biol.* 14, 7876–7883.

- Babst, M., Katzmann, 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., Katzmann, 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., 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.
- Beck, T., Schmidt, A., and Hall, M.N. (1999). Starvation induces vacuolar targeting and degradation of the tryptophan permease in yeast. *J. Cell Biol.* 146, 1227–1238.
- Blake, T.J., Shapiro, M., Morse, H.C., 3rd, and Langdon, W.Y. (1991). The sequences of the human and mouse c-bcl proto-oncogenes show v-cbl was generated by a large truncation encompassing a proline-rich domain and a leucine zipper-like motif. *Oncogene* 6, 653–657.
- Calero, M., Chen, C.Z., Zhu, W., Winand, N., Havas, K.A., Gilbert, P.M., Burd, C.G., and Collins, R.N. (2003). Dual prenylation is required for rab protein localization and function. *Mol. Biol. Cell* 14, 1852–1867.
- Cereghino, J.L., Marcusson, E.G., and Emr, S.D. (1995). The cytoplasmic tail domain of the vacuolar sorting receptor Vps10p and a subset of VPS gene products regulate receptor stability, function and localization. *Mol. Biol. Cell* 6, 1089–1102.
- Chang, A., Cheang, S., Espanel, X., and Sudol, M. (2000). Rsp5 WW domains interact directly with the carboxyl-terminal domain of RNA polymerase II. *J. Biol. Chem.* 275, 20562–20571.
- Chen, H.I., Einbond, A., Kwak, S.J., Linn, H., Koepf, E., Peterson, S., Kelly, J.W., and Sudol, M. (1997). Characterization of the WW domain of human yes-associated protein and its polyproline-containing ligands. *J. Biol. Chem.* 272, 17070–17077.
- Chen, L., and Davis, N.G. (2002). Ubiquitin-independent entry into the yeast recycling pathway. *Traffic* 3, 110–123.
- Cowles, C.R., Odorizzi, G., Payne, G.S., and Emr, S.D. (1997a). The AP-3 adaptor complex is essential for cargo-selective transport to the yeast vacuole. *Cell* 91, 109–118.
- Cowles, C.R., Snyder, W.B., Burd, C.G., and Emr, S.D. (1997b). An alternative Golgi to vacuole delivery pathway in yeast: identification of a sorting determinant and required transport component. *EMBO J.* 16, 2769–2782.
- Dunn, R., and Hicke, L. (2001a). Domains of the Rsp5 ubiquitin-protein ligase required for receptor-mediated and fluid-phase endocytosis. *Mol. Biol. Cell* 12, 421–435.
- Dunn, R., and Hicke, L. (2001b). Multiple roles for Rsp5p-dependent ubiquitination at the internalization step of endocytosis. *J. Biol. Chem.* 276, 25974–25981.
- Dupre, S., and Haguenauer-Tsapis, R. (2001). Deubiquitination step in the endocytic pathway of yeast plasma membrane proteins: crucial role of Doa4p ubiquitin isopeptidase. *Mol. Cell Biol.* 21, 4482–4494.
- Fisk, H.A., and Yaffe, M.P. (1999). A role for ubiquitination in mitochondrial inheritance in *Saccharomyces cerevisiae*. *J. Cell Biol.* 145, 1199–1208.
- Gajewska, B., Kaminska, J., Jesionowska, A., Martin, N.C., Hopper, A.K., and Zoladek, T. (2001). WW domains of Rsp5p define different functions: determination of roles in fluid phase and uracil permease endocytosis in *Saccharomyces cerevisiae*. *Genetics* 157, 91–101.
- Gitan, R.S., and Eide, D.J. (2000). Zinc-regulated ubiquitin conjugation signals endocytosis of the yeast ZRT1 zinc transporter. *Biochem. J.* 346, 329–336.
- Gormley, K., Dong, Y., and Sagnella, G.A. (2003). Regulation of the epithelial sodium channel by accessory proteins. *Biochem. J.* 371, 1–14.
- Hein, C., Springael, J.Y., Volland, C., Haguenauer-Tsapis, R., and Andre, B. (1995). NPI1, an essential yeast gene involved in induced degradation of Gap1 and Fur4 permeases, encodes the Rsp5 ubiquitin-protein ligase. *Mol. Microbiol.* 18, 77–87.
- Helliwell, S.B., Losko, S., and Kaiser, C.A. (2001). Components of a ubiquitin ligase complex specify polyubiquitination and intracellular trafficking of the general amino acid permease. *J. Cell Biol.* 153, 649–662.
- Hershko, A., and Ciechanover, A. (1998). The ubiquitin system. *Annu. Rev. Biochem.* 67, 425–479.
- Hicke, L. (1997). Ubiquitin-dependent internalization and down-regulation of plasma membrane proteins. *FASEB J.* 11, 1215–1226.
- Hicke, L. (1999). Gettin' down with ubiquitin: turning off cell-surface receptors, transporters and channels. *Trends Cell Biol.* 9, 107–112.
- Hicke, L. (2001). Protein regulation by monoubiquitin. *Nat. Rev. Mol. Cell Biol.* 2, 195–201.
- Hicke, L., and Riezman, H. (1996). Ubiquitination of a yeast plasma membrane receptor signals its ligand-stimulated endocytosis. *Cell* 84, 277–287.
- Hofmann, K., and Bucher, P. (1995). The rsp5-domain is shared by proteins of diverse functions. *FEBS Lett.* 358, 153–157.
- Hoppe, T., Matuschewski, K., Rape, M., Schlenker, S., Ulrich, H.D., and Jentsch, S. (2000). Activation of a membrane-bound transcription factor by regulated ubiquitin/proteasome-dependent processing. *Cell* 102, 577–586.
- Horak, J., and Wolf, D.H. (2001). Glucose-induced monoubiquitination of the *Saccharomyces cerevisiae* galactose transporter is sufficient to signal its internalization. *J. Bacteriol.* 183, 3083–3088.
- Huang, L., Kinnucan, E., Wang, G., Beaudenon, S., Howley, P.M., Huibregtse, J.M., and Pavletich, N.P. (1999). Structure of an E6AP-UbcH7 complex: insights into ubiquitination by the E2-E3 enzyme cascade. *Science* 286, 1321–1326.
- Huibregtse, J.M., Scheffner, M., Beaudenon, S., and Howley, P.M. (1995). A family of proteins structurally and functionally related to the E6-AP ubiquitin-protein ligase. *Proc. Natl. Acad. Sci. USA* 92, 5249.
- Huibregtse, J.M., Scheffner, M., and Howley, P.M. (1993). Localization of the E6-AP regions that direct human papillomavirus E6 binding, association with p53, and ubiquitination of associated proteins. *Mol. Cell Biol.* 13, 4918–4927.
- Ichikawa, K., Shiba, Y., Jigami, Y., and Serizawa, N. (1993). Secretion and overproduction of carboxypeptidase Y by a *Saccharomyces cerevisiae* ssl1 mutant strain. *Biosci. Biotechnol. Biochem.* 57, 1686–1690.
- Joazeiro, C.A., and Weissman, A.M. (2000). RING finger proteins: mediators of ubiquitin ligase activity. *Cell* 102, 549–552.
- Joazeiro, C.A., Wing, S.S., Huang, H., Leverson, J.D., Hunter, T., and Liu, Y.C. (1999). The tyrosine kinase negative regulator c-Cbl as a RING-type, E2-dependent ubiquitin-protein ligase. *Science* 286, 309–312.
- Kamynina, E., Debonneville, C., Bens, M., Vandewalle, A., and Staub, O. (2001). A novel mouse Nedd4 protein suppresses the activity of the epithelial Na⁺ channel. *FASEB J.* 15, 204–214.
- 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., 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.
- Laney, J.D., and Hochstrasser, M. (1999). Substrate targeting in the ubiquitin system. *Cell* 97, 427–430.
- Levkowitz, G., et al. (1999). Ubiquitin ligase activity and tyrosine phosphorylation underlie suppression of growth factor signaling by c-Cbl/Sli-1. *Mol. Cell* 4, 1029–1040.
- Levkowitz, G., Waterman, H., Zamir, E., Kam, Z., Oved, S., Langdon, W.Y., Beguinot, L., Geiger, B., and Yarden, Y. (1998). c-Cbl/Sli-1 regulates endocytic sorting and ubiquitination of the epidermal growth factor receptor. *Genes Dev.* 12, 3663–3674.
- Losko, S., Kopp, F., Kranz, A., and Kolling, R. (2001). Uptake of the ATP-binding cassette (ABC) transporter Ste6 into the yeast vacuole is blocked in the doa4 mutant. *Mol. Biol. Cell* 12, 1047–1059.
- Nuber, U., Schwarz, S., Kaiser, P., Schneider, R., and Scheffner, M. (1996). Cloning of human ubiquitin-conjugating enzymes UbcH6 and UbcH7 (E2-F1) and characterization of their interaction with E6-AP and RSP5. *J. Biol. Chem.* 271, 2795–2800.
- 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.
- Odorizzi, G., Katzmann, D.J., Babst, M., Audhya, A., and Emr, S.D. (2003). Bro1 is an endosome-associated protein that functions in the MVB pathway in *Saccharomyces cerevisiae*. *J. Cell Sci.* 116, 1893–1903.
- Pickart, C.M. (2001). Mechanisms underlying ubiquitination. *Annu. Rev. Biochem.* 70, 503–533.
- Piper, R.C., Cooper, A.A., Yang, H., and Stevens, T.H. (1995). VPS27 controls vacuolar and endocytic traffic through a prevacuolar compartment in *Saccharomyces cerevisiae*. *J. Cell Biol.* 131, 603–618.
- Piper, R.C., and Luzio, J.P. (2001). Late endosomes: sorting and partitioning in multivesicular bodies. *Traffic* 2, 612–621.

- Polo, S., Sigismund, S., Faretta, M., Guidi, M., Capua, M.R., Bossi, G., Chen, H., De Camilli, P., and Di Fiore, P.P. (2002). A single motif responsible for ubiquitin recognition and monoubiquitination in endocytic proteins. *Nature* 416, 451–455.
- 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.
- Reggiori, F., and Pelham, H.R. (2002). A transmembrane ubiquitin ligase required to sort membrane proteins into multivesicular bodies. *Nat. Cell Biol.* 4, 117–123.
- Rizo, J., and Sudhof, T.C. (1998). C2-domains, structure and function of a universal Ca²⁺-binding domain. *J. Biol. Chem.* 273, 15879–15882.
- Rotin, D., Staub, O., and Haguenauer-Tsapis, R. (2000). Ubiquitination and endocytosis of plasma membrane proteins: role of Nedd4/Rsp5p family of ubiquitin-protein ligases. *J. Membr. Biol.* 176, 1–17.
- Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.
- Seufert, W., McGrath, J.P., and Jentsch, S. (1990). UBC1 encodes a novel member of an essential subfamily of yeast ubiquitin-conjugating enzymes involved in protein degradation. *EMBO J.* 9, 4535–4541.
- Shaw, J.D., Cummings, K.B., Hoyer, G., Michaelis, S., and Wendland, B. (2001). Yeast as a model system for studying endocytosis. *Exp. Cell Res.* 271, 1–9.
- Shenoy, S.K., McDonald, P.H., Kohout, T.A., and Lefkowitz, R.J. (2001). Regulation of receptor fate by ubiquitination of activated beta 2-adrenergic receptor and beta-arrestin. *Science* 294, 1307–1313.
- Shih, S.C., Katzmann, D.J., Schnell, J.D., Sutanto, M., Emr, S.D., and Hicke, L. (2002). Epsins and Vps27/Hrs contain ubiquitin-binding domains that function in receptor endocytosis and downregulation. *Nat. Cell Biol.* 4, 389–393.
- Shih, S.C., Sloper-Mould, K.E., and Hicke, L. (2000). Monoubiquitin carries a novel internalization signal that is appended to activated receptors. *EMBO J.* 19, 187–198.
- Soetens, O., De Craene, J.O., and Andre, B. (2001). Ubiquitin is required for sorting to the vacuole of the yeast general amino acid permease, Gap1. *J. Biol. Chem.* 276, 43949–43957.
- Springael, J.Y., De Craene, J.O., and Andre, B. (1999). The yeast Npi1/Rsp5 ubiquitin ligase lacking its N-terminal C2 domain is competent for ubiquitination but not for subsequent endocytosis of the gap1 permease. *Biochem. Biophys. Res. Commun.* 257, 561–566.
- Staub, O., Abriel, H., Plant, P., Ishikawa, T., Kanelis, V., Saleki, R., Horisberger, J.D., Schild, L., and Rotin, D. (2000). Regulation of the epithelial Na⁺ channel by Nedd4 and ubiquitination. *Kidney Int.* 57, 809–815.
- Umebayashi, K., and Nakano, A. (2003). Ergosterol is required for targeting of tryptophan permease to the yeast plasma membrane. *J. Cell Biol.* 161, 1117–1131.
- 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.
- van Delft, S., Govers, R., Strous, G.J., Verkleij, A.J., and van Bergen en Henegouwen, P.M. (1997). Epidermal growth factor induces ubiquitination of Eps15. *J. Biol. Chem.* 272, 14013–14016.
- 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.
- Wang, G., McCaffery, J.M., Wendland, B., Dupre, S., Haguenauer-Tsapis, R., and Huibregtse, J.M. (2001). Localization of the Rsp5p ubiquitin-protein ligase at multiple sites within the endocytic pathway. *Mol. Cell Biol.* 21, 3564–3575.
- Wang, G., Yang, J., and Huibregtse, J.M. (1999). Functional domains of the Rsp5 ubiquitin-protein ligase. *Mol. Cell Biol.* 19, 342–352.
- Waterman, H., Levkowitz, G., Alroy, I., and Yarden, Y. (1999). The RING finger of c-Cbl mediates desensitization of the epidermal growth factor receptor. *J. Biol. Chem.* 274, 22151–22154.
- Whitley, P., Reaves, B.J., Hashimoto, M., Riley, A.M., Potter, B.V., and Holman, G.D. (2003). Identification of mammalian Vps24p as an effector of phosphatidylinositol 3, 5-bisphosphate-dependent endosome compartmentalization. *J. Biol. Chem.* 278, 38786–38795.
- Yashiroda, H., Oguchi, T., Yasuda, Y., Toh, E.A., and Kikuchi, Y. (1996). Bul1, a new protein that binds to the Rsp5 ubiquitin ligase in *Saccharomyces cerevisiae*. *Mol. Cell Biol.* 16, 3255–3263.
- Yokouchi, M., Kondo, T., Houghton, A., Bartkiewicz, M., Horne, W.C., Zhang, H., Yoshimura, A., and Baron, R. (1999). Ligand-induced ubiquitination of the epidermal growth factor receptor involves the interaction of the c-Cbl RING finger and UbcH7. *J. Biol. Chem.* 274, 31707–31712.
- Yon, J., and Fried, M. (1989). Precise gene fusion by PCR. *Nucleic Acids Res.* 17, 4895.
- Zoladek, T., Tobiasz, A., Vaduva, G., Boguta, M., Martin, N.C., and Hopper, A.K. (1997). MDP1, a *Saccharomyces cerevisiae* gene involved in mitochondrial/cytoplasmic protein distribution, is identical to the ubiquitin-protein ligase gene RSP5. *Genetics* 145, 595–603.