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# Ent5p Is Required with Ent3p and Vps27p for Ubiquitin-dependent Protein Sorting into the Multivesicular Body

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**At the late endosomes, cargoes destined for the interior of the vacuole are sorted into invaginating vesicles of the multivesicular body. Both PtdIns(3,5)$P_2$ and ubiquitin are necessary for proper sorting of some of these cargoes. We show that Ent5p, a yeast protein of the epsin family homologous to Ent3p, localizes to endosomes and specifically binds to PtdIns(3,5)$P_2$ via its ENTH domain. In cells lacking Ent3p and Ent5p, ubiquitin-dependent sorting of biosynthetic and endocytic cargo into the multivesicular body is disrupted, whereas other trafficking routes to the vacuole are not affected. Ent3p and Ent5p are associated with Vps27p, a FYVE domain containing protein that interacts with ubiquitinated cargoes and is required for protein sorting into the multivesicular body. Therefore, Ent3p and Ent5p are the first proteins shown to be connectors between PtdIns(3,5)$P_2$- and the Vps27p-ubiquitin–driven sorting machinery at the multivesicular body.**

## INTRODUCTION

Endosomes are crossroads between the biosynthetic and the endocytic pathways. Here, proteins destined for the lysosome/vacuole are segregated away from proteins recycling back to the plasma membrane or to the Golgi apparatus. A critical membrane segregation and protein-sorting event takes place in late endosomes, regions of endosomal membranes invaginate and vesicles bud into the lumen of the organelle, giving rise to the multivesicular body (MVB; Felder *et al.*, 1990). Mature MVB fuses with the vacuole/lysosome releasing internal vesicles into its lumen and thereby exposing them to the activity of hydrolytic enzymes (Futter *et al.*, 1996; Odorizzi *et al.*, 1998). This mechanism allows a subset of endosomal membrane proteins to be sorted to the lumen of the lysosome/vacuole, whereas others are either selectively recycled back to the Golgi or to the plasma membrane, or they end up on the lysosomal/vacuolar membrane (Pelham, 2002). In yeast, the MVB pathway is crucial for the regulated turnover of pheromone receptors and of some permeases (Odorizzi *et al.*, 1998). Further, biosynthetic transmembrane proteins such as carboxypeptidase S (Cps1p) and Phm5p follow this route to reach their final destination, the vacuolar lumen (Odorizzi *et al.*, 1998; Reggiori and Pelham, 2001; Epple *et al.*, 2003).

Ubiquitination has been implicated as an endosomal-sorting signal. Cps1p and Phm5p ubiquitination is essential to target them into the vacuolar lumen after sorting at the MVB (Katzmann *et al.*, 2001; Reggiori and Pelham, 2001). Potential cargo receptors responsible for recognition and recruitment of ubiquitinated cargo into MVB vesicles are class E Vps (vacuolar protein sorting) proteins. Class E mutants accumulate cargo destined for the vacuole in an exaggerated endosomal structure and all (currently identified) class E proteins are required for sorting at the MVB (Conibear and Stevens, 1998). Vps23p, a class E protein of the ESCRT-I complex (endosomal-sorting complex required for transport), carries an ubiquitin-conjugating (UBC)-like domain and interacts directly with ubiquitin in vitro and with ubiquitinated Cps1p in vivo (Katzmann *et al.*, 2001). By binding to ubiquitinated cargo and then activating ESCRT-II, which in turn acts upstream of ESCRT-III, ESCRT-I is the first effector of a coordinated cascade necessary for ubiquitin-dependent protein sorting at the MVB (Babst *et al.*, 2002a, 2002b). Two other class E proteins, Vps27p and Hse1p, contain ubiquitin-interacting motifs (UIMs) and bind ubiquitin in vitro (Bilodeau *et al.*, 2002; Polo *et al.*, 2002; Raiborg *et al.*, 2002; Shih *et al.*, 2002). Mutations in the UIMs of Vps27p and Hse1p cause defects in sorting of Cps1p and Ste2p, suggesting that a Vps27p/Hse1p complex is a sorting receptor for ubiquitinated proteins at the MVB. Vps27p binds PtdIns(3)P via its FYVE domain (Burd and Emr, 1998; Gary *et al.*, 1998; Gaullier *et al.*, 1998; Wishart *et al.*, 2001; Burda *et al.*, 2002). PtdIns(3)P is found on the cytoplasmic

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Abbreviations used: ALP, alkaline phosphatase; CPY, carboxypeptidase Y; ENTH, epsin $NH_2$-terminal homology; ESCRT, endosomal sorting complex required for transport; FYVE domain, Fab1, YGL023, Vps27 and EEA1 domain; MVB, multivesicular body; PC, phosphatidylcholine; PtdIns(3,5)$P_2$, phosphatidylinositol 3,5-bisphosphate; Ub, ubiquitin; UIM, Ub-interacting motif; Vps, vacuolar protein sorting.

**Table 1.** Plasmids used in this study

| Plasmid | Backbone | Yeast ori | Insert | Source |
|---|---|---|---|---|
| pFL644 | pRT21 | 2μ | ENT5-(E-GFP3) | This study |
| pFL647 | pGEX4-T1 | | ENT5 | This study |
| pFL653 | pGEX4-T1 | | *ent5ΔENTH* | This study |
| pFL719 | pGEX4-T1 | | ENTH domain of Ent5p | This study |
| pFL703 | pQE15 | | VPS27 | This study |
| p299 | pGEX2TK | | FYVE of Hrs | Sankaran *et al.* (2001) |
| pGO45 | pRS426 | 2μ | GFP-Cps1 | Odorizzi *et al.* (1998) |
| pGFP-Phm5 | pRS416 | CEN | GFP-Phm5 | Reggiori and Pelham (2001) |
| pGFP-DPAP B | pRS426 | 2μ | GFP-DPAP B | Odorizzi *et al.* (1998) |
| pGFP-Ste2 | pRS426 | 2μ | GFP-Ste2 | Odorizzi *et al.* (1998) |
| pKN32 | | 2μ | UBI4 | Nelson and Lemmon (1993) |
| pEE27-HA | pRS416 | CEN | VPS27-$(HA)_3$ | Shih *et al.* (2002) |

side of endosomes, of internal vesicles of the MVB and of the vacuole (Gillooly *et al.*, 2000). Vps27p binds PtdIns(3)P and ubiquitin and therefore constitutes a direct link between these two sorting signals. Accordingly, recent data showed that Vps27p initiates the MVB sorting reaction and directs the recruitment of ubiquitinated cargo into MVB vesicles in association with ESCRT-I (Bilodeau *et al.*, 2003; Katzmann *et al.*, 2003).

The Ent1 to Ent5 proteins are ENTH (epsin $NH_2$-terminal homology) domain proteins (Kay *et al.*, 1999; Wendland *et al.*, 1999; Duncan *et al.*, 2002). The ENTH domain promotes membrane recruitment by binding phosphoinositides (De Camilli *et al.*, 2002). We have recently reported that Ent3p is a specific PtdIns(3,5)$P_2$ effector involved in protein sorting at the MVB in yeast (Friant *et al.*, 2003). Analysis of yeast mutants unable to synthesize PtdIns(3,5)$P_2$, such as *fab1*, *vac7*, and *vac14*, revealed that this phospholipid is also required for sorting ubiquitinated cargoes into the MVB (Bonangelino *et al.*, 1997; Odorizzi *et al.*, 1998; Reggiori and Pelham, 2001; Dove *et al.*, 2002).

In this study we investigate the role of Ent5p that also specifically binds to PtdIns(3,5)$P_2$. It is associated with the ubiquitin-binding protein Vps27p and together with Ent3p is required for ubiquitin-dependent protein sorting into the MVB.

## MATERIALS AND METHODS

### *Plasmids and Yeast Strains*

Plasmids used in this study are listed in Table 1. Yeast strains used in this study are RH2964 (WT), FLY673 1b (*Mat**a** bar1 ura3 leu2 trp1 ade2 ent3::HIS3*), FLY674 1a (*Mat**a** bar1 ura3 leu2 trp1 ade2 ent4::KanMX),* FLY675 1b (*Mat**a** bar1 ura3 leu2 trp1 ade2 ent5::HIS3*), FLY676 3d (*Mat**a** bar1 ura3 leu2 trp1 ade2 ent4::KanMX ent5::HIS3),* FLY678 2b (*Mat**a** bar1 ura3 leu2 trp1 ade2 ent3::HIS3 ent4::KanMX),* FLY680 16a (*Mat**a** bar1 ura3 leu2 trp1 ade2 ent3::HIS3 ent5::HIS3*), BY4272 (WT), *vps24Δ*, *vsp36Δ*, *vps37Δ*, PLY1020 (WT), PLY2857 (*Matα pep4-3 ura3-52 leu2-3 his4-519 ade6 vps27::KanMX*), PLY2784 (*Matα pep4-3 ura3-52 leu2-3 his4–519 ade6 vps27::KanMX hse1::KanMX*), *vps24Δvps27Δ* (*Mat**a** ura3 leu2 vps24::KanMX vps27::KanMX*), FY833 (WT), and FY833-fab1Δ1 (fab1Δ).

### *Endocytosis Assay and Quinacrine Staining*

Lucifer yellow (LY) uptake and quinacrine assays were performed as previously described (Friant *et al.*, 2003).

### *Lipid Dot Blot Assay*

Lipid solutions containing 100 pmol/μl phospholipids dissolved in $CHCl_3$/MetOH/HCl (50:50:0.1 vol/vol) were spotted on a nitrocellulose membrane and allowed to dry for 1 h. The membrane was blocked in 3% fatty acid free BSA in TBS-Tween for 1 h and incubated overnight with the GST–ENTH domain of Ent5p (at 0.3 μg/ml). After washing, protein binding was revealed with anti-GST.

### *Liposome Recruitment Assay*

Recruitment reactions were performed as previously described (Friant *et al.*, 2003). The FYVE domain was a kind gift of M.A. Lemmon.

### *Subcellular Fractionation, Western Blot, Gel filtration, Coimmunoprecipitation, and Membrane Fractionation*

Subcellular fractionation was carried out as described in Bonangelino *et al.* (1997). Mouse monoclonal anti-GFP (Roche, Nutley, NJ), anti-PGK, and anti-Vph1p antibodies (Molecular Probes, Eugene, OR), anti-HA (Roche Diagnostics Corp.) and anti-GST (Sigma-Aldrich, St. Louis, MO), rabbit anti-Emp47 (kind gift from R. Duden), and anti-Ent3 antibodies (Friant *et al.*, 2003) were used. For detection of $His_6$-tagged proteins the SuperSignal West HisProbe Kit from Pierce (Rockford, IL) was used. For gel filtration, cytosol was prepared by glass bead lysis in PBS containing 0.25 M sorbitol, protease inhibitor cocktail (Sigma), and PMSF, followed by a 10-min spin at 13 000 rpm and a 30-min spin at 100,000 × *g*. Five to 9 mg protein was injected on a Superose 6 column (Pharmacia, Arlington Heights, IL), and 10 μg protein/fraction was analyzed by Western blot. Yeast cytosol or fractions from gel filtration were adjusted to identical protein amounts and immunoprecipitated with Ent3p antibodies and then subjected to anti-HA or anti-GFP Western blotting. Membrane fractionations on sucrose density gradients were carried out as described (Holthuis *et al.*, 1998). Mouse Pep12p and Vps10p antibodies from Molecular Probes were used for detection of endosomal fractions.

### *Pull-down Experiments and Ubiquitin-binding Assays*

GST-pull down experiments were carried out as described (Cosson and Letourneur, 1994), but yeast cells were lysed in PBS, 10 mM EDTA, 0.5% Triton, protease inhibitor cocktail, and PMSF. For binding to ubiquitin-sepharose, 50 μl ubiquitin-sepharose (Sigma) was incubated with 20 μg recombinant protein for 4 h, and washed in TBST as described (Aguilar *et al.*, 2003).

### *Immunoprecipitations and Metabolic Labeling*

Metabolic labeling, pulse-chase analyses, and immunoprecipitations were performed as previously described, by using mouse anti-ALP (Molecular Probes) and rat anti-HA antibodies (Sigma) and protein A-Sepharose (Amersham Biosciences Europe GmbH, Freiburg, Germany; Friant *et al.*, 2003). For immunoprecipitation of ubiquitinated proteins, cells overexpressing ubiquitin (necessary to allow detection of a signal) and expressing GFP-Cps1 were grown to an $OD_{600}$ = 0.5, precipitated in 10% TCA, washed in acetone, resuspended in 200 μl TE, protease inhibitor cocktail, PMSF, and 5 mM NEM, and vortexed for 5 min with glass beads. Thorner buffer, 200 μl, was added and broken cells were incubated at 50°C for 10 min. After centrifugation for 10 min at 13,000 rpm, 100 μl of supernatant was subjected to immunoprecipitation with ant-GFP antibody (Roche). Immunoprecipitated proteins were revealed with anti-ubiquitin antibody (Zymed, San Francisco, CA).

### *Microscopy*

Living cells expressing GFP-Cps1, GFP-Phm5, GFP–DPAP B, or GFP-Ent5 were harvested at an $OD_{600}$ of 0.5 and resuspended in PBS before visualization. In vivo labeling with FM4-64 was done as described (Vida and Emr, 1995). Visualization of cells was performed on a fluorescence Zeiss Axioplan II (Thornwood, NY) or Nikon Optiphot-2 microscope (Melville, NY) equipped with FITC and rhodamine filters, and Nomarski optics. Images were captured with a CoolSnap FX Roper Scientific (Tuscon, AZ) or a Photonic Science digital camera (Turnbridge Wells, UK).

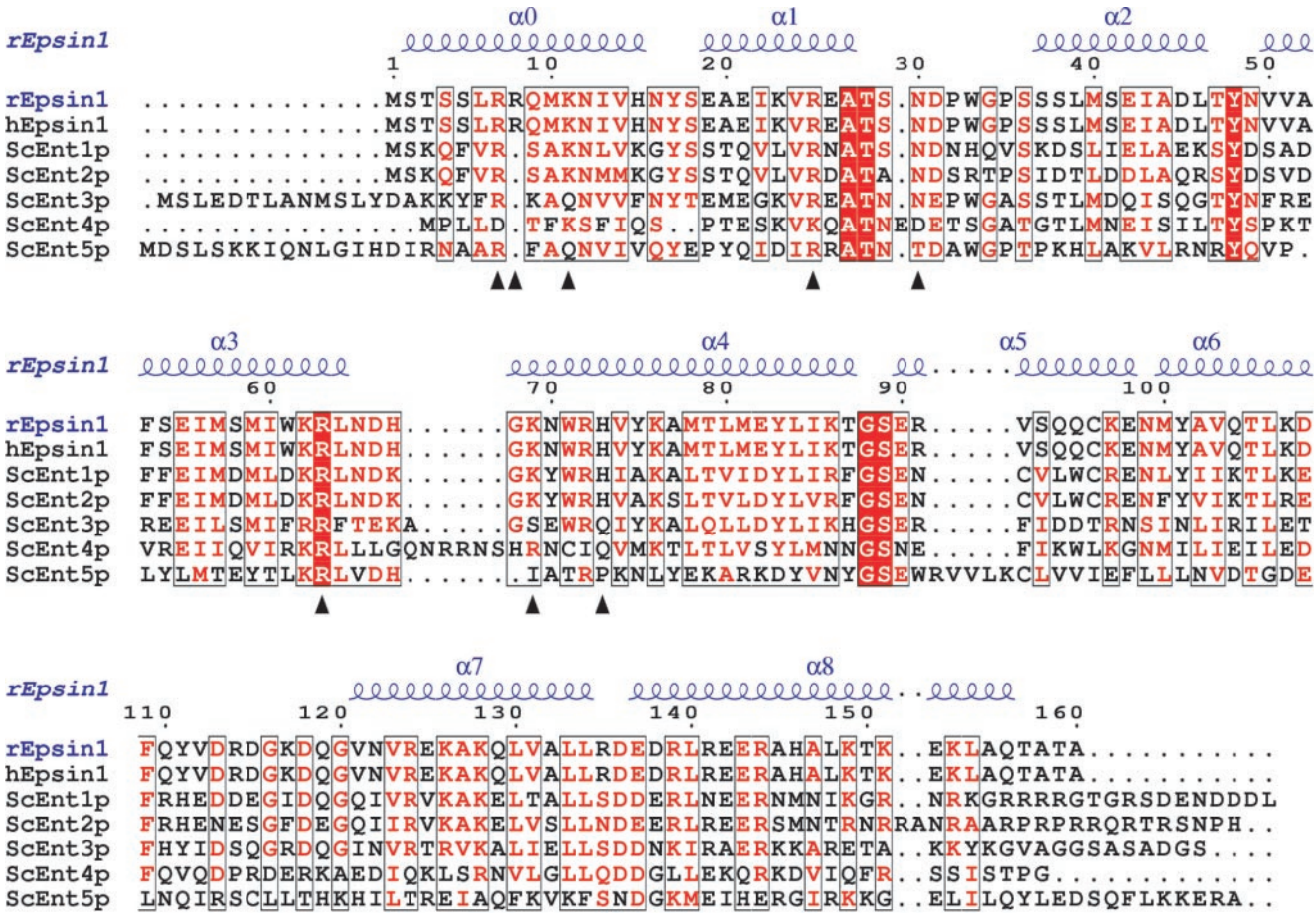


**Figure 1.** Sequence alignment of the ENTH domains of yeast epsins. The sequence of the rat epsin1 was aligned to sequences from yeast Ent1p, Ent2p, Ent3p, Ent4p, and Ent5p proteins. Secondary structure of rat epsin1 is shown above the alignment. Conserved residues are in black boxes; identical residues are indicated by white letters on red background and similar residues are indicated by red letters on white background. The residues in rat epsin1 critical for binding to Ins(1,4,5)$P_3$ are pointed out with an arrowhead. r, *Rattus norvegicus*; h, human; Sc, *S. cerevisiae*.

### *Computer Analysis*

Sequence alignments were done using the ClustalW program (Thompson *et al.*, 1994) and displayed with ESPript (Gouet *et al.*, 1999). Database searches were performed using BLAST and $\psi$ BLAST servers at NIH.

## RESULTS

### *Ent5p Is Redundant with Ent3p and Not Required for the Internalization Step of Endocytosis*

We have recently characterized *ent3-1*, a temperature-sensitive mutant defective in protein sorting at the MVB (Friant *et al.*, 2003). The *ent3*Δ deletion mutant did not display the same phenotype as the *ent3-1* mutant, suggesting that the yeast genome encoded a functional homologue. There are five different yeast ENTH domain-containing proteins named Ent1p to Ent5p (Figure 1). In contrast to Ent3p, Ent1p and Ent2p were shown to be required for endocytosis (Wendland *et al.*, 1999). The function of Ent4p is not known, and Ent5p was recently identified as a functional homologue of Ent3p, involved in clathrin-mediated trafficking at the late Golgi (Duncan *et al.*, 2002). The single *ent3*Δ, *ent4*Δ, and *ent5*Δ and the double *ent3*Δ*ent4*Δ and *ent4*Δ*ent5*Δ deletion strains displayed no obvious phenotype, but the double *ent3*Δ*ent5*Δ mutant was cold sensitive for growth on YPD plates (unpublished data) and showed highly fragmented vacuoles (see also Figure 4, A and D, and 5A). This vacuolar phenotype had already been observed in *ent3-1* mutant cells (Friant *et al.*, 2003). These results suggested that Ent5p and Ent3p shared functions in MVB sorting, in the light of what has been previously shown for clathrin-mediated membrane trafficking (Duncan *et al.*, 2002; Friant *et al.*, 2003). The ENTH domain is a membrane-interacting module found in a number of proteins acting at early stages of endocytosis (De Camilli *et al.*, 2002). We therefore asked whether the presence of Ent5p was required with Ent3p for the internalization step of endocytosis. Uptake of lucifer yellow (LY) was analyzed in *ent3*Δ, *ent5*Δ, and *ent3*Δ*ent5*Δ mutant cells and they accumulated LY in the vacuole like wild-type cells (unpublished data). These data show that Ent5p, like its homologue Ent3p, serves a function different from the "classical" role of epsins in endocytosis.

### *Ent5p-GFP Localizes to Endosomal Structures*

To analyze the intracellular localization of Ent5p, we fused green-fluorescent protein (GFP) to the $NH_2$-terminus of full-length Ent5p. This fusion protein is functional, as it complements the fragmented vacuolar phenotype of *ent3*Δ*ent5*Δ

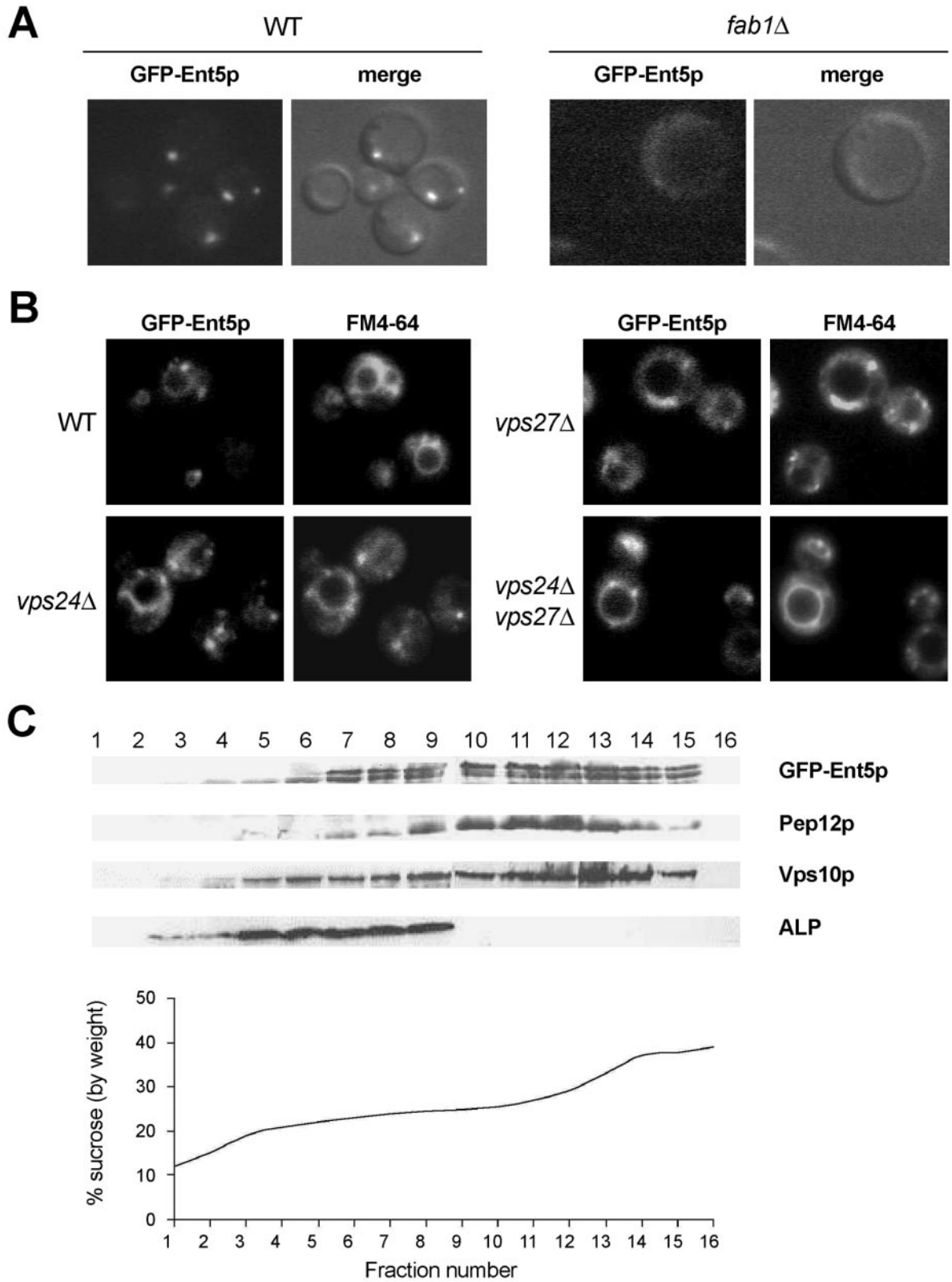


**Figure 2.** GFP-Ent5 localizes to endosomal structures. (A) Wild-type cells (FY833) and *fab1*Δ*1* expressing GFP-Ent5 (pFL644) were observed by fluorescent and Nomarski microscopy. (B) Wild-type cells (BY4741), *vps24*Δ, *vps27*Δ, and *vps24*Δ *vps27*Δ cells expressing GFP-Ent5 (pFL644) cells were labeled with FM4–64 for 20 min to reveal endosomes and vacuolar membranes and observed by fluorescent microscopy. (C) *ent5*Δ cells (FLY675) expressing the GFP-Ent5 fusion construct were subjected to subcellular fractionation on a sucrose density gradient. High-speed membrane pellet (100,000 × *g*) from *ent5*Δ cells (FLY675) expressing the GFP-Ent5 construct was fractionated upon an equilibrium sucrose density gradient. Fractions were assayed by immunoblotting for GFP-Ent5, Pep12p, Vps10p, and ALP. Fractions 1–16 are shown. The immunoblots presented are representative of four separate experiments.

mutant cells. In wild-type cells, GFP-Ent5p localized to the cytoplasm and to punctate structures, indicating association with endosomal compartments (Figure 2A). Some of the dots colocalized with the staining obtained after a short pulse with FM4-64, which visualizes endosomal structures (Figure 2B; Vida and Emr, 1995). In addition, GFP-Ent5p was introduced into class E *vps* mutants that accumulate enlarged prevacuolar/endosomal structures, *vps27*Δ and *vps24*Δ, which affects the ESCRT-III complex (Figure 2B). In these class E *vps* mutants, the majority of GFP-Ent5p was localized to the class E compartment, as seen by its colocalization with FM4-64 (Figure 2B). In the *vps24*Δ*vps27*Δ double mutant, GFP-Ent5p was also localized to the class E compartment (Figure 2B), thus indicating that Vps27p is not responsible the localization of Ent5p to the class E compartment of *vps24*Δ cells. The intracellular localization of GFP-Ent5p was further characterized by a differential sedimentation assay. In wild-type cells, the majority of GFP-Ent5p was membrane-associated in the fraction that pelleted at 13,000 × *g* (P13) that contains large organelles such as vacuoles, ER, nuclei, or structures like the cytoskeleton, and in the high-speed membrane fraction (P100) containing Golgi membranes, endosomes, and vesicles, but also for a minor part in the cytoplasmic pool (S100; Marcusson *et al.*, 1994). A similar fractionation pattern for GFP-Ent5p was observed in the class E mutants *vps23*Δ or *vps27*Δ (unpublished data).

The intracellular distribution of GFP-Ent5p was also analyzed by subcellular fractionation on an equilibrium sucrose density gradient. As shown in Figure 2C, GFP-Ent5p fractionated away from the vacuolar marker ALP and most closely resembled the distribution of Pep12p, the t-SNARE for a late endosomal/prevacuolar compartment and of Vps10p, the early endosomal marker (Figure 2C). All these results suggest that Ent5p is a cytosolic protein able to associate with membranes and is found predominantly on endosomal structures.

### *Ent5p Binds Specifically to PtdIns(3,5)$P_2$*

The ENTH domain is a phospholipid binding module (De Camilli *et al.*, 2002). We have recently shown that the ENTH domain of Ent3p specifically binds PtdIns(3,5)$P_2$ in vivo and in vitro (Friant *et al.*, 2003). To determine the lipid specificity of Ent5p, liposome recruitment assays with recombinant GST-Ent5 fusions were performed. GST-Ent5 or GST-ENTH domain were cosedimented with PtdIns(3,5)$P_2$ containing liposomes but not with liposomes containing PtdIns(3)P, PtdIns(3,4)$P_2$, PtdIns(4,5)$P_2$, or PtdIns(4)P (Figure 3A). In contrast, a truncation of Ent5p lacking the ENTH domain was unable to interact with any of the liposomes tested, thus demonstrating that the ability of Ent5p to bind PtdIns(3,5)$P_2$ was dependent on its ENTH domain (unpublished data). As a control, we tested the lipid specificity of the FYVE domain of Hrs-1 in our liposome recruitment assay (Figure 3A), and the FYVE domain bound specifically to PtdIns(3)P as previously reported (Sankaran *et al.*, 2001). As can be seen in Figures 3A and 6B, recombinant GST-Ent5 consistently appeared as a triplet. To date we are unable to discern the nature of or the modifications on the different species observed. We also performed a protein-lipid overlay assay using the ENTH domain of Ent5p. This assay confirmed the results of the liposome recruitment assay, in that the ENTH domain of Ent5p bound only to PtdIns(3,5)$P_2$ (Figure 3B).

To determine whether lack of PtdIns(3,5)$P_2$ affects the intracellular localization of Ent5p, *fab1*Δ cells that are impaired in PtdIns(3,5)$P_2$ synthesis were transformed with the GFP-Ent5p construct and analyzed by fluorescent microscopy (Figure 2A). GFP-Ent5p was completely diffuse in the cytosol of *fab1*Δ cells. This suggests that Ent5p localizes to endosomes by virtue of its binding to PtdIns(3,5)$P_2$. Taken together, our data demonstrate that Ent5p specifically binds to PtdIns(3,5)$P_2$ via its ENTH domain and show that Ent5p is a new PtdIns(3,5)$P_2$ effector in yeast.

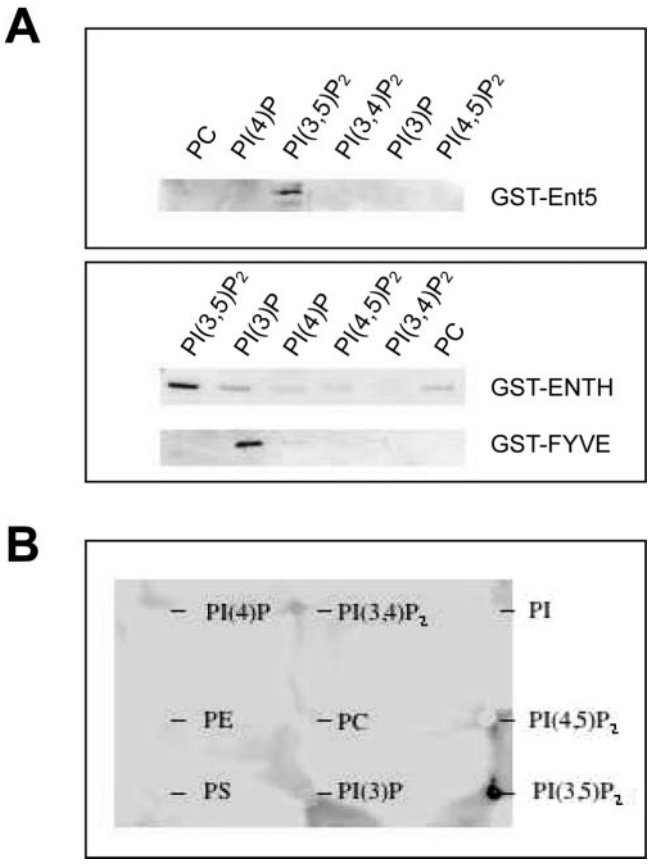


**Figure 3.** Ent5p binds specifically to PtdIns(3,5)$P_2$. (A) GST-Ent5 or GST-ENTH of Ent5p were incubated with either PC (control) or PC-based liposomes containing 5% (mol/mol) PtdIns(3,5)$P_2$, PtdIns(3,4)$P_2$, PtdIns(4,5)$P_2$, PtdIns(3)P, or PtdIns(4)P. After centrifugation, pellets containing liposome-associated proteins were revealed by Western blotting with anti-GST antibodies. The GST-FYVE domain of Hrs (hepatocyte receptor tyrosine kinase), the mammalian homologue of Vps27p, was used as a control for PtdIns(3)P binding. (B) Nitrocellulose-immobilized phospholipids were incubated with GST-ENTH of Ent5p, and bound proteins were visualized by Western blotting with an anti-GST antibody.

### *Ent5p, together with Ent3p, Is Specifically Required for Ubiquitin-dependent Sorting of Biosynthetic and Endocytic Cargoes into the MVB*

It is well established that the lipid kinase Fab1p and its product PtdIns(3,5)$P_2$ are required for the correct sorting of ubiquitinated biosynthetic and endocytic cargo proteins at the MVB (Odorizzi *et al.*, 1998; Reggiori and Pelham, 2001; Shaw *et al.*, 2003). Here we identified Ent5p as a new PtdIns(3,5)$P_2$ effector in yeast, redundant with Ent3p. Both proteins are localized to endosomes, the site of MVB formation (Friant *et al.*, 2003). We therefore asked whether Ent3p and Ent5p were also implicated in ubiquitin-dependent MVB sorting and analyzed the intracellular localization of GFP-Cps1 and GFP-Phm5, two ubiquitinated biosynthetic cargo proteins, in *ent3*Δ, *ent5*Δ, and *ent3*Δ*ent5*Δ mutant cells

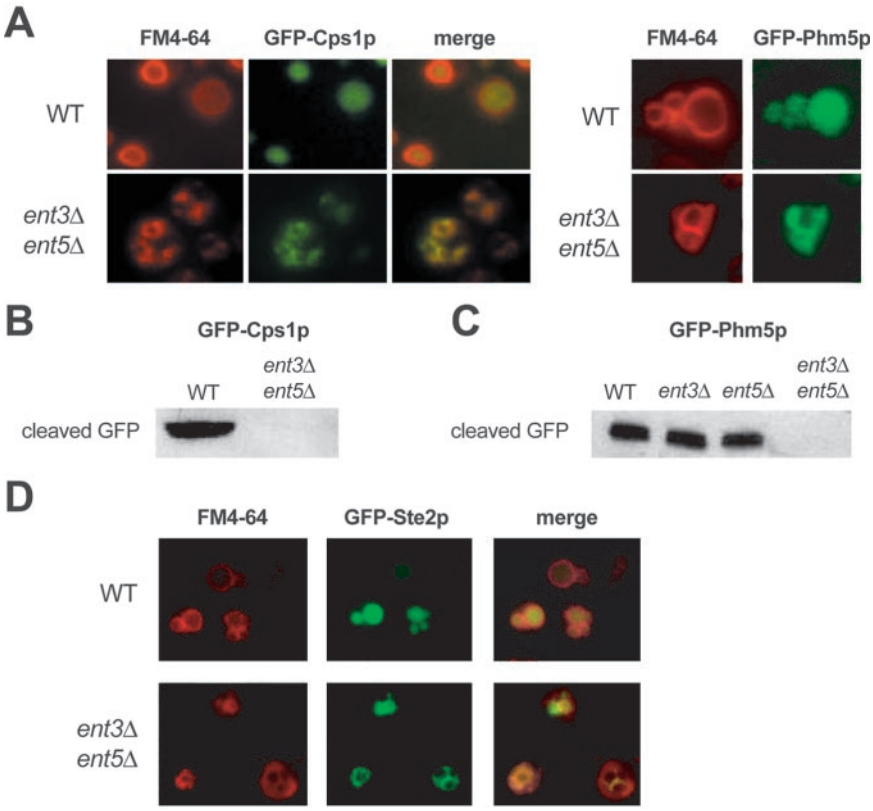


**Figure 4.** Ent3p and Ent5p are required for sorting of ubiquitinated biosynthetic and endocytic proteins to the MVB. (A) Wild-type (RH2964) and *ent3*Δ*ent5*Δ (FLY680 16a) cells were transformed with pGFP-Cps1 or with pGFP-Phm5, labeled with FM4-64 and live cells were observed by fluorescence microscopy. (B) Whole yeast cell extracts of wild-type (RH2964) and *ent3*Δ*ent5*Δ (FLY680 16a) cells carrying pGFP-Cps1 were subjected to Western blot analysis and cleaved GFP was revealed by using a monoclonal anti-GFP antibody. (C) Whole yeast cell extracts of wild-type (RH2964), *ent3*Δ (FLY673 1b), *ent5*Δ (FLY675 1b), and *ent3*Δ*ent5*Δ (FLY680 16a) cells carrying pGFP-Phm5 were subjected to Western blot analysis and cleaved GFP was revealed as in B. (D) Wild-type (RH2964) and *ent3*Δ*ent5*Δ (FLY680 16a) cells expressing pGFP-Ste2 were labeled with FM4-64, and live cells were observed by fluorescence microscopy.

(Figure 4). Precursor forms of Cps1p and Phm5p are synthesized as type II transmembrane proteins. After transit through the secretory pathway to endosomes, they are sorted into invaginating MVB vesicles, resulting in exposure of their short cytoplasmic N-terminal domain to the lumen of the vesicles. Delivery of the vesicles to the vacuolar lumen allows hydrolytic clipping of the precursors to their mature forms: the large C-terminal part is released from the transmembrane domain into the vacuolar lumen where it exerts its activity. In wild-type, *ent3*Δ, and *ent5*Δ cells, expression of GFP-Cps1 or GFP-Phm5 gave rise to a clear staining of the vacuolar lumen (Figure 4A and unpublished data). In contrast, in *ent3*Δ*ent5*Δ mutant cells, GFP-Cps1 and GFP-Phm5 localized to the fragmented vacuolar membrane and not to the vacuolar lumen, as shown by colocalization with the dye FM4-64 (Figure 4A). To confirm that Ent3p and Ent5p were both required for sorting of GFP-Phm5 and GFP-Cps1 at the MVB, total yeast cell extracts were analyzed by Western blotting and probed with an anti-GFP antibody (Figure 4, B and C). In wild-type, *ent3*Δ, and *ent5*Δ cells, free GFP released by vacuolar enzymes after maturation of GFP-Cps1 and GFP-Phm5 were readily detected. In contrast, free GFP was absent from *ent3*Δ*ent5*Δ mutant cells, suggesting that GFP-Phm5 and GFP-Cps1 had not been exposed to the activity of vacuolar proteases and therefore had not been properly sorted into the MVB pathway.

The endocytic cargo Ste2p is also ubiquitinated and this modification is requisite for its sorting into internal vesicles of the MVB as well as for its internalization at the plasma membrane (Katzmann *et al.*, 2001; Bilodeau *et al.*, 2002; Shih *et al.*, 2002). To find out whether Ent3p and Ent5p were also required for the vacuolar targeting of ubiquitin-modified endocytic cargo, the intracellular localization of GFP-Ste2 was analyzed by fluorescence microscopy (Figure 4D). In wild-type cells, GFP-Ste2 was concentrated in the vacuolar lumen. In *ent3*Δ*ent5*Δ mutant cells, GFP-Ste2 was mainly localized to the vacuolar membrane, with some residual staining in the vacuolar lumen, thus suggesting that the correct and efficient sorting of some endocytic cargoes also requires Ent3p and Ent5p.

To ascertain whether the sorting defect observed in *ent3*Δ*ent5*Δ mutant cells was specific for transmembrane proteins sorted into internal MVB vesicles and whether sorting of proteins of the vacuolar membrane was disturbed, the intracellular localization of GFP-DPAP B (Dipeptidyl aminopeptidase B) was analyzed in these cells. DPAP B is a type II transmembrane protein that travels along the CPY pathway to the limiting membrane of the vacuole (Piper *et al.*, 1997). In wild-type as in *ent3*Δ*ent5*Δ mutant cells, GFP-DPAP B was found on vacuolar membranes (Figure 5A), indicating that this pathway functions in the absence of Ent3p and Ent5p.

We also followed the transport/processing of CPY and ALP by pulse-chase analysis. In wild-type cells, after a pulse with Met/Cys $^{35}$S label, the precursor forms of CPY (p1CPY and p2CPY) and ALP (proALP) can be detected and after chase of increasing time length, increasing amounts of mature CPY and ALP appear, resulting from the transport of the precursor forms to the vacuole. In *ent3*Δ, *ent5*Δ, and *ent3*Δ*ent5*Δ cells, ALP and CPY were correctly processed/transported, although *ent3*Δ*ent5*Δ mutant cells displayed a slight delay when compared with wild-type cells (unpublished data).

Fab1p, the PtdIns(3)P 5-kinase is required for proper vacuole acidification (Bonangelino *et al.*, 1997). We therefore tested the acidification of the vacuole in cells lacking Ent3p and/or Ent5p by quinacrine staining. The lumen of the vacuoles of *ent3*Δ, *ent5*Δ, or *ent3*Δ*ent5*Δ mutant cells showed wild-type staining of quinacrine, whereas in *fab1*Δ cells there

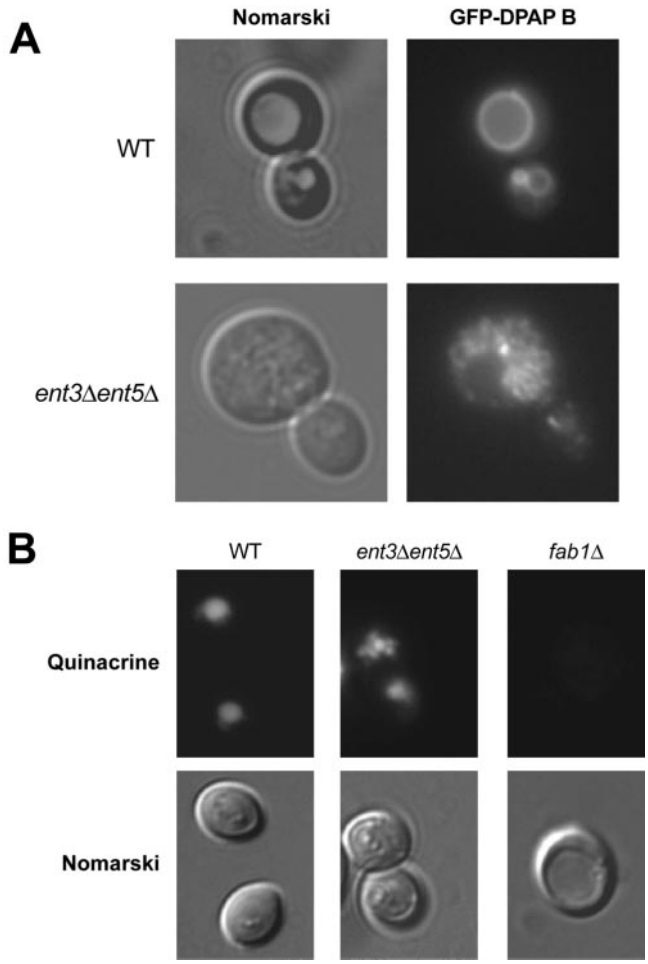


**Figure 5.** Ent3p and Ent5p neither required for sorting of DPAP B to the vacuolar membrane nor for acidification of the vacuole. (A) Wild-type (RH2964) and *ent3*Δ*ent5*Δ (FLY680 16a) cells were transformed with pGFP-DPAP B and cells were observed by fluorescent and Nomarski microscopy. (B) Wild-type (RH2964), *ent3*Δ*ent5*Δ (FLY680 16a) and *fab1*Δ cells were labeled with quinacrine and cells were observed by fluorescent and Nomarski microscopy.

is no quinacrine staining of the vacuolar lumen (unpublished data and Figure 5B).

Taken together, all these data show that the two PtdIns(3,5)$P_2$ effectors, Ent3p and Ent5p, are specifically required for ubiquitin-dependent sorting of endocytic and biosynthetic cargo at the MVB, whereas other routes to the vacuole are not affected, and acidification of the vacuole is normal.

### *Ent3p and Ent5p Do Not Directly Bind to Ubiquitin*

Both Ent3p and Ent5p are required for the correct sorting of ubiquitinated proteins into the MVB. To distinguish whether Ent3p and Ent5p were required for the sorting step per se or rather to mark proteins with ubiquitin for internalization, we investigated whether MVB cargo proteins themselves were ubiquitinated in cells lacking Ent3p and Ent5p. Wild-type and *ent3*Δ*ent5*Δ cells carrying GFP-Cps1 and a high copy number plasmid expressing ubiquitin were lysed, and cell extracts were subjected to immunoprecipitation with an anti-GFP antibody. Western blot analysis of the immunoprecipitated proteins with an anti-ubiquitin antibody revealed the presence of ubiquitinated forms of Cps1p in *ent3*Δ*ent5*Δ cells (Figure 6A), whereas these forms of CPS were difficult to detect in wild-type cells, as already reported previously (Katzmann *et al.*, 2001; Shih *et al.*, 2002). This result indicates that Ent3p and Ent5p function downstream of cargo ubiquitination in the sorting function.

Epsins and Ent1p and Ent2p contain two or three copies of an UIM immediately after the ENTH domain (Polo *et al.*, 2002). The function of these UIMs is to bind ubiquitinated cargo (De Camilli *et al.*, 2002; Shih *et al.*, 2002; Aguilar *et al.*, 2003). Ent3p and Ent5p are required for ubiquitin-dependent sorting at the MVB, but they do not possess obvious ubiquitin-binding domains (UBC-like, UIM, UBA, or CUE; Hofmann and Bucher, 1996; Hofmann and Falquet, 2001; Polo *et al.*, 2002; Shih *et al.*, 2002). To ascertain that in Ent3p and Ent5p there was no other, as yet unidentified ubiquitin-binding motif, we performed ubiquitin-binding assays. GST-Ent3 and GST-Ent5 recombinant proteins were unable to bind ubiquitin-sepharose, whereas recombinant $His_6$-tagged Vps27p, which contains two UIMs, specifically bound to ubiquitin-sepharose (Figure 6B). Taken together, these data suggest that Ent3p and Ent5p, although necessary for sorting ubiquitinated cargo at the MVB, are required neither for ubiquitin addition to cargo proteins nor for the direct binding to ubiquitinated cargoes. Therefore, they might act via interaction with other proteins of the sorting machinery present on endosomes during MVB formation, which in turn directly interact with ubiquitinated cargo.

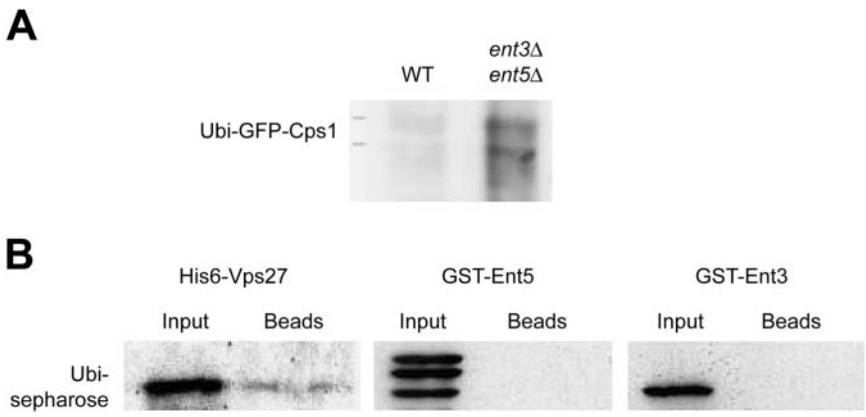


**Figure 6.** Ent3p and Ent5p are not required for cargo ubiquitination and are unable to bind ubiquitin. (A) Wild-type (RH2964) and *ent3*Δ*ent5*Δ (FLY680 16a) cells expressing pGFP-Cps1 and pKN32 (2$\mu$ ubiquitin) were lysed, lysates were subjected to immunoprecipitation with an anti-GFP antibody and immunoprecipitated proteins were analyzed by Western blotting with an antiubiquitin antibody. (B) The purified recombinant proteins GST-Ent3, GST-Ent5 and as a control, $His_6$Vps27 were incubated with ubiquitin-sepharose. Equal amounts of beads and of recombinant proteins were used in each experiment. Purified recombinant proteins (Input) and bound proteins were analyzed by immunoblotting with anti-GST antibodies or with the $His_6$-detection kit from Pierce.

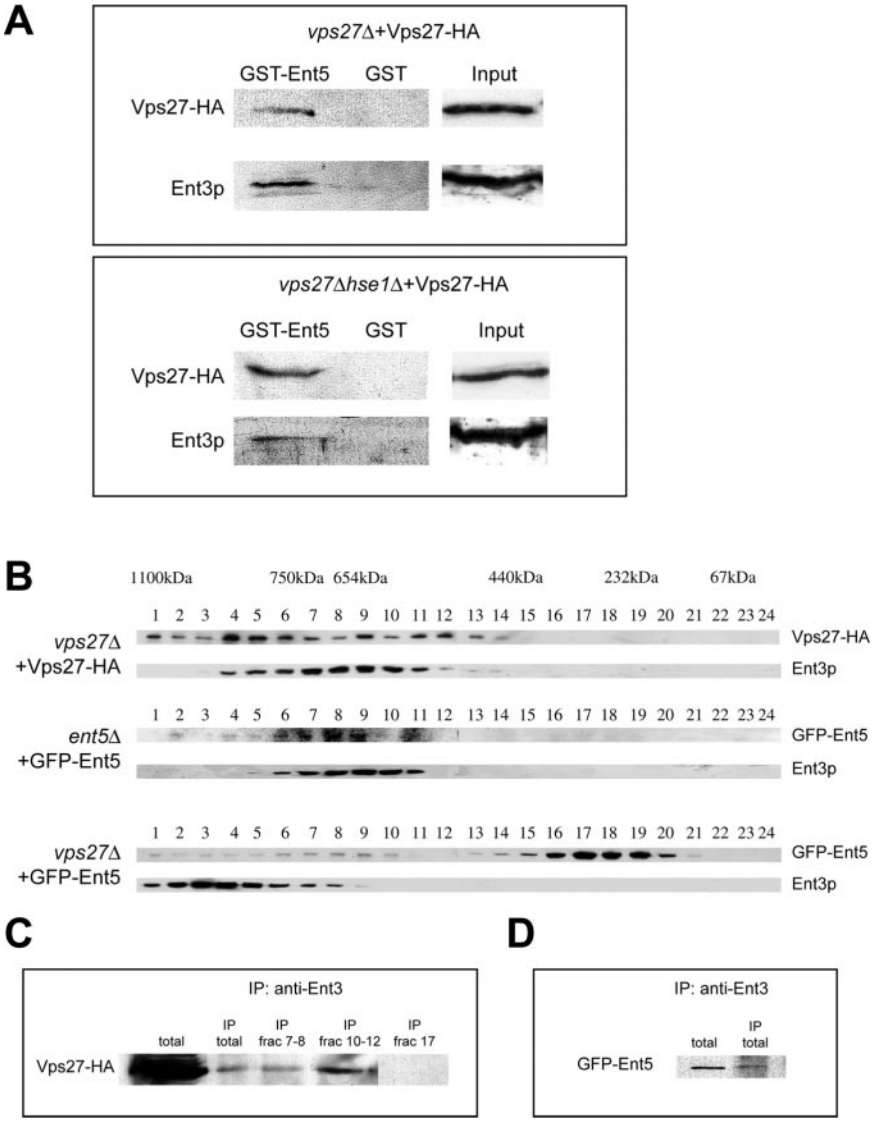


**Figure 7.** Ent3p and Ent5p are associated to the ubiquitin-binding protein Vps27p. (A) Lysates from *vps27*Δ (PLY2857) or *vps27*Δ*hse1*Δ cells (PLY2784) expressing an HA-tagged version of Vps27p were incubated with GST-Ent5 or GST alone coupled to glutathione-sepharose. The same amounts of recombinant proteins and of total cell lysates were used in each experiment. Total lysates (input) and bound proteins were immunoblotted with anti-HA and anti-Ent3 antibodies. (B) Yeast cytosol from either *vps27*Δ (PLY2857) cells expressing an HA-tagged version of Vps27p, or *vps27*Δ (PLY2857) and *ent5*Δ (FLY675 1b) cells expressing GFP-tagged Ent5p were subjected to gel filtration. Note that equal amounts protein for each fraction were analyzed by Western blotting with anti-HA (Vps27p), anti-GFP (Ent5p), or anti-Ent3 antibodies. Fraction numbers and position of markers are indicated: 67 kDa (albumin), 232 kDa (catalase), 440 kDa (ferritin), 654 kDa (thyroglobulin). Higher molecular weights (1100 and 750 kDa) were extrapolated from a calibration curve. (C) Total yeast cytosol from *vps27*Δ (PLY2857) cells expressing an HA-tagged version of Vps27p (IP Total) or total (pooled) fractions from a gel filtration experiment as shown in B. IP fractions 7–8, IP fractions 10–12, and IP fraction 17 were immunoprecipitated with an anti-Ent3 antibody and subjected to Western blot analysis with an anti-HA antibody. (D) Total yeast cytosol from *ent5*Δ (FLY675 1b) cells expressing GFP-tagged Ent5p were immunoprecipitated with an anti-Ent3 antibody and subjected to Western blot analysis with an anti-GFP antibody.

### *Ent3p, Ent5p, and Vps27p Associate*

In yeast, Ent1p/Ent2p UIMs are important for the internalization of receptors at the plasma membrane, and Vps27p/Hse1p UIMs are necessary to sort biosynthetic and endocytic cargo into the MVB (Bilodeau *et al.*, 2002; Shih *et al.*, 2002). In contrast to Vps27p and Ent1p/Ent2p, Ent3p and Ent5p have no known ubiquitin-binding motif. We show that these proteins do not directly interact with ubiquitin, but are required for ubiquitin-dependent sorting at the MVB. Thus there must be (an) additional effector(s) associated with Ent3p/Ent5p to link them to ubiquitin. Since Vps27p contains UIMs and is at the top of the ESCRT cascade, it was the most obvious candidate connector between Ent3p/Ent5p and ubiquitin (Katzmann *et al.*, 2003). To investigate whether Ent3p and Ent5p interacted with Vps27p, we performed pull-down assays using recombinant GST-Ent5 and cytosol from yeast cells expressing epitope-tagged Vps27p (Vps27-HA) as the sole source of Vps27p. Vps27p and Ent3p interacted with GST-Ent5 but not with GST alone (Figure 7A). These interactions were not dependent on the presence of Hse1p, a protein associated in a complex with Vps27p (Bilodeau *et al.*, 2002), as demonstrated by the same experiment using cytosol from a strain lacking Hse1p (Figure 7A). Thus, Vps27p, without its partner Hse1p, is sufficient to promote interaction with Ent3p and Ent5p proteins.

We next wanted to know whether Ent3p, Ent5p, and Vps27p associated in vivo and therefore analyzed yeast cytosol by gel filtration and coimmunoprecipitation assays. Vps27-HA expressed as the sole copy of Vps27p in *vps27*Δ cells eluted in multiple peaks in high-molecular-weight fractions. Four different peaks ranging from 1100 to 500 kDa were observed in fraction 1, fractions 4–6, fraction 9, and fractions 11–12 (Figure 7B). Ent3p eluted in a broad peak (fractions 4–11), with the majority of the protein found in fraction 9 (Figure 7B). Accordingly, in *ent5*Δ cells expressing GFP-Ent5, Ent5p coeluted with Ent3p in corresponding high-molecular-weight fractions (fractions 6–11), both proteins concentrating in a peak in fractions 7–9. To determine whether Ent5p and Vps27p are associated with Ent3p in whole cell extracts, we carried out coimmunoprecipitation assays between Ent3p and epitope-tagged Vps27p (Vps27-HA) or Ent5p (GFP-Ent5). Vps27-HA coprecipitated with Ent3p from total cytosol or from fractions of gel filtration experiments (as shown above; Figure 7C). The greatest

amount of Vps27-HA coimmunoprecipitated with Ent3p from fractions corresponding to shared peaks, for example from fractions 10–12 (Figure 7C) or from fraction 9 (unpublished data), when compared with coimmunoprecipitation from other fractions, as shown here for fractions 7–8. Ent5-GFP was also coprecipitated with Ent3p from *ent5*Δ cytosol (Figure 7D). These experiments show that Ent3p, Ent5p, and Vps27p are associated in vivo.

Further information about Ent3p, Ent5p, and Vps27p association in vivo was obtained by gel filtration of cytosol of yeast cells deleted for Vps27p and carrying GFP-Ent5p. As shown in Figure 7B, GFP-Ent5p was almost completely depleted from the high-molecular-weight peak seen in wild-type extracts. Only a minor fraction of Ent5p remained distributed over fractions 1–10; additionally, a second peak containing the majority of the protein was seen around 230–300 kDa (fractions 16–20). The high-molecular-weight fractions containing Ent3p were shifted to even higher molecular weight fractions (fractions 1–7). This result shows that the deletion of Vps27p had a dramatic effect on the gel filtration elution profile of both Ent3p and Ent5p. On the contrary, deletion of Ent5p affected the fractionation profile of neither Vps27p nor Ent3p (unpublished data), suggesting that in the absence of Ent5p, Ent3p, and Vps27p could still be incorporated into complexes. Taken together, these data suggest that Ent3p, Ent5p, and Vps27p are found together in high-molecular-weight complexes in vivo and suggest that this association may provide a direct link between the ubiquitin- and the PtdIns(3,5)$P_2$-dependent sorting mechanisms into the MVB.

## DISCUSSION

We have recently described Ent3p, the first specific PtdIns(3,5)$P_2$ effector required for protein sorting into the MVB in yeast (Friant *et al.*, 2003). A single *ent3*Δ deletion strain does not display the defects in MVB sorting observed in *ent3-1* mutant cells. This suggested that the point mutation in the ENTH domain of Ent3p found in *ent3-1* mutant cells had a dominant effect on MVB protein sorting and that there could be additional PtdIns(3,5)$P_2$ effectors required for MVB sorting (Friant *et al.*, 2003). In our query for ENTH domain proteins redundant to Ent3p, we identified Ent5p. Ent5p was recently identified as a functional homologue of Ent3p associated with clathrin and Gga2p, required for efficient CPY protein trafficking at the late Golgi, suggesting that these two yeast ENTH proteins function together (Duncan *et al.*, 2002). These results and the results we describe suggest that Ent3p and Ent5p share essential functions for membrane trafficking between the late Golgi and the vacuole.

There are two sequence motifs that constitute the homology between Ent3p and Ent5p: first, the signature of the epsin family proteins, the ENTH domain, and second, the presence of several γ-ear-binding acidic motifs (Duncan *et al.*, 2002). Based on the presence of a lysine-rich ANTH-like (AP180 N-terminal homology) motif in the ENTH domain of Ent5p, but absent from the ENTH domains of Ent3p or epsin1, it has recently been proposed that the ENTH domain of Ent5p rather resembles an ANTH domain (Duncan and Payne, 2003). The authors therefore suggested that the ENTH domains of Ent3p and Ent5p are functionally different. Here, we show that both ENTH domains are specific PtdIns(3,5)$P_2$-binding modules required for protein sorting into the MVB. Despite their sequence divergence the two domains share the same lipid specificity. This could be either due to sequence or structure conservation of the critical PtdIns(3,5)$P_2$ recognition site, or to both. It will be very interesting to gain structural, and hence mechanistic information on the two ENTH domains of Ent3p and Ent5p, and especially to identify the residues responsible for PtdIns(3,5)$P_2$ binding.

Synthesis of PtdIns(3,5)$P_2$ by Fab1p is required for the selective sorting of ubiquitinated cargo into the MVB (Odorizzi *et al.*, 1998; Reggiori and Pelham, 2001; Shaw *et al.*, 2003). Our experiments show that the PtdIns(3,5)$P_2$ effectors Ent3p and Ent5p are also required for the correct sorting of ubiquitinated cargo into the MVB. We show that in the absence of both proteins, biosynthetic and endocytic cargo destined to the vacuolar lumen, like Phm5p, Cps1p, and Ste2p, are mis-sorted to the vacuolar membrane, showing that Ent3p and Ent5p are required for sorting of ubiquitinated cargo into the MVB. Furthermore, we demonstrate that Ent3p and Ent5p are not necessary to mark proteins for ubiquitination and do not directly interact with ubiquitinated proteins to recruit them into vesicles. Collectively, these results strongly suggest that the PtdIns(3,5)$P_2$ effectors Ent3p and Ent5p are required neither for ubiquitination, nor for direct recognition of ubiquitinated cargoes at the late endosome, but rather for a later step of ubiquitin-dependent protein sorting into the MVB.

Here we show that Ent3p, Ent5p, and Vps27p are associated in vivo. Our GST pull-down, gel filtration, and coimmunoprecipitation experiments suggest the three proteins to be in complexes of very high molecular weight. The very large peak observed for all three proteins (ranging from 500 to far >750 kDa) suggests that they are present in different complexes. Furthermore, the presence of the three proteins in complexes eluting in fraction 9 and the pull-down of both Vps27p and Ent3p with GST-Ent5p, suggest that they are associated with each other in this complex or that Ent3p and/or Ent5p may interact indirectly with Vps27p via the ESCRT-I complex (Bilodeau *et al.*, 2003; Katzmann *et al.*, 2003). In the absence of Vps27p, the distribution of Ent5p and Ent3p over the fractionation profile is dramatically altered. Ent5p is mainly found in lower-molecular-weight fractions, in a peak that contains subcomplexes lacking Vps27p, whereas Ent3p is found in higher-molecular-weight fractions. Moreover, the absence of Ent5p affected the fractionation profile of neither Vps27p nor Ent3p (unpublished data), suggesting that in the absence of Ent5p, Ent3p, and Vps27p could still be incorporated into complexes. These results suggest that Ent3p and Vps27p are the key effectors in these high-molecular-weight complexes, and Ent5p might be an accessory protein redundant to Ent3p. This might explain why the *ent3-1* and *ent3-2* mutant strains have a dominant effect on MVB protein sorting and why a double deletion of *ENT3* and *ENT5* is required to obtain the same phenotype as displayed by these mutants. Alternatively, complexes might exist containing either Ent3p or Ent5p together with Vps27p, and different "subcomplexes" might well transiently associate with a larger complex. Therefore, it will be interesting to determine the additional components of complexes containing Vps27p, Ent3p, and/or Ent5p and to determine the domains on Ent3p and Ent5p responsible for interaction with Vps27p.

Vps27p is one of the key effectors required for protein sorting into the MVB pathway, because it binds ubiquitin in vitro and because mutations in the UIMs of Vps27p cause defects in protein sorting into the MVB (Bilodeau *et al.*, 2002; Shih *et al.*, 2002). Furthermore, it was recently shown that Vps27p is directly recruited to PtdIns(3)P-containing endosomes where it binds to ubiquitinated MVB cargoes and subsequently recruits the ESCRT-I complex (Bilodeau *et al.*, 2003; Katzmann *et al.*, 2003). This results in sorting of ubi-

quitinated cargo into the MVB vesicles. Recent studies permitted the unraveling of different steps leading to the recruitment of ubiquitinated cargo to the site of MVB formation. Despite all these new data, some clues are still missing. For example, the mechanism triggering the formation of invaginating vesicles of the MVB compartment is still unknown. Here we identified two new partners of Vps27p: Ent3p and Ent5p, two PtdIns(3,5)$P_2$ effectors required for ubiquitin-dependent protein sorting into the MVB. It has been proposed that a function of the epsin1 ENTH domain at the plasma membrane is to induce membrane curvature (Ford *et al.*, 2002). Once bound to PtdIns(4,5)$P_2$, the 8-helix–folded ENTH domain of epsin 1 rearranges and an additional $\alpha$ helix is formed ($\alpha$0, Figure 1). By inserting $\alpha$0 into the cytoplasmic leaflet of the membrane bilayer, the ENTH domain is capable of pushing aside lipid head groups, thereby inducing curvature of the membrane and facilitating formation of clathrin-coated pits. We envisage that the role of the ENTH domains of Ent3p and Ent5p could be similar to that of epsin1. Ent3p and Ent5p, by inserting their ENTH domains into the endosomal membrane after PtdIns(3,5)$P_2$ binding, could well induce the invagination required for the formation of internal vesicles. This process is topologically reversed when compared with formation of coated pits at the plasma membrane, and it therefore infers that the way the ENTH domains of Ent3p and Ent5p deform membranes might be analogous but inverse. Indeed, neither the lipid specificity nor the residues defining this specificity are conserved between epsin1 and Ent3p or Ent5p ENTH domains (Figure 1). The putative $\alpha$0 helix is exacerbated in length in Ent3p and Ent5p when compared with other members of the epsin family, which could well account for a differing ability to deform membranes. To better understand the mechanism behind this process it will be of invaluable importance to obtain information on the structure of the ENTH domains of Ent3p and/or Ent5p.

Interestingly, the morphology of cells lacking Ent3p and Ent5p differs from both *fab1*Δ and *vps27*Δ cells, *fab1*Δ cells displaying one highly enlarged vacuole and the *vps27*Δ an enlarged endosomal/prevacuolar structure, whereas the *ent3*Δ*ent5*Δ cells showed fragmented and small vacuoles. Additionally, the vacuoles of Fab1p-depleted cells display a defect in acidification, whereas the fragmented vacuoles in *ent3*Δ*ent5*Δ cells are properly acidified. In *fab1*Δ cells the enlarged vacuole is the result of the lack of PtdIns(3,5)$P_2$ synthesis (Bonangelino *et al.*, 2002), whereas it is likely that *ent3*Δ*ent5*Δ cells are not affected in PtdIns(3,5)$P_2$ synthesis, but rather in PtdIns(3,5)$P_2$ binding. The *ent3*Δ*ent5*Δ cells do not display classical class E phenotype, which might be due to their implication in different membrane trafficking pathways to the vacuole (Duncan *et al.*, 2002). Furthermore, Ent3p was recently shown to interact with Vti1p (Chidambaram *et al.*, 2004), a v-SNARE required for biosynthetic membrane trafficking pathways to the vacuole (Fischer von Mollard and Stevens, 1999). Therefore absence of Ent3p and Ent5p could lead to a partial defect in the vesicle fusion with the vacuole, explaining the fragmented vacuolar phenotype and the CPY and ALP maturation delay that was observed in *ent3-1*, *ent3-2* and *ent3*Δ*ent5*Δ mutant strains (Friant *et al.*, 2003).

The intracellular localization of Ent5p to the endosomes is affected in *fab1*Δ cells that are impaired in PtdIns(3,5)$P_2$ synthesis, but not in *vps27*Δ cells. Furthermore, Vps27p is not required for the localization of Ent5p to the class E compartment of *vps24*Δ cells. These results show that the endosomal localization of Ent5p is mediated via its interaction with PtdIns(3,5)$P_2$ and does not require Vps27p.

A picture, illustrating how sorting at the MVB could be regulated, is now emerging from our work and from previous reports. Vps27p, as a key player, binds to PtdIns(3)P rich domains on endosomal membranes, recruits ubiquitinated cargo proteins via its UIM and activates the ESCRT cascade (Bilodeau *et al.*, 2003; Katzmann *et al.*, 2003). These events might trigger an uncharacterized signaling event to Fab1p, which is localized to PtdIns(3)P-rich patches via its FYVE–domain and which, once activated, produces PtdIns(3,5)$P_2$. Ent3p and Ent5p are then recruited to endosomal membranes on PtdIns(3,5)$P_2$-rich patches and might induce membrane curvature and formation of MVB vesicles. By interacting with Vps27p-cargo complexes, Ent3p and Ent5p could ultimately contribute to the recruitment of these cargoes into nascent vesicles. This model for ubiquitinated cargo sorting into the MVB is similar to the one proposed for ubiquitin-dependent protein internalization in the endocytic pathway. The main difference is that the epsins required for endocytosis contain both the ENTH domain responsible for membrane deformations and the UIMs implicated in ubiquitinated receptor binding and in recruitment to the site of plasma membrane invaginations (Bilodeau *et al.*, 2002; Polo *et al.*, 2002; Raiborg *et al.*, 2002; Shih *et al.*, 2002). Here, in the case of the MVB pathway, a second effector, Vps27p, constitutes the link with ubiquinated cargo.

In summary, we identified Ent3p and Ent5p as the first connectors between the PtdIns(3,5)$P_2$- and the Vps27p-ubiquitin–driven sorting machinery at the multivesicular body. Many aspects, such as the downstream effectors of Ent3p and Ent5p, other proteins found in complex with Vps27p, Ent3p, and Ent5p or the mechanism allowing recruitment of cargo into the vesicles remain to be elucidated. Genetic and molecular studies in yeast, structural studies as well as proteomics should provide better understanding of how Ent3p and Ent5p regulate ubiquitin-dependent sorting into the MVB.

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