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Hse1, a Component of the Yeast Hrs-STAM Ubiquitin-sorting Complex, Associates with Ubiquitin Peptidases and a Ligase to Control Sorting Efficiency into Multivesicular Bodies[D]

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Ubiquitinated integral membrane proteins are delivered to the interior of the lysosome/vacuole for degradation. This process relies on specific ubiquitination of potential cargo and recognition of that Ub-cargo by sorting receptors at multiple compartments. We show that the endosomal Hse1-Vps27 sorting receptor binds to ubiquitin peptidases and the ubiquitin ligase Rsp5. Hse1 is linked to Rsp5 directly via a PY element within its C-terminus and through a novel protein Hua1, which recruits a complex of Rsp5, Rup1, and Ubp2. The SH3 domain of Hse1 also binds to the deubiquitinating protein Ubp7. Functional analysis shows that when both modes of Rsp5 association with Hse1 are altered, sorting of cargo that requires efficient ubiquitination for entry into the MVB is blocked, whereas sorting of cargo containing an in-frame addition of ubiquitin is normal. Further deletion of Ubp7 restores sorting of cargo when the Rsp5:Hse1 interaction is compromised suggesting that both ubiquitin ligases and peptidases associate with the Hse1-Vps27 sorting complex to control the ubiquitination status and sorting efficiency of cargo proteins. Additionally, we find that disruption of *UBP2* and *RUP1* inhibits MVB sorting of some cargos suggesting that Rsp5 requires association with Ubp2 to properly ubiquitinate cargo for efficient MVB sorting.

INTRODUCTION

Integral membrane proteins are sorted into intralumenal vesicles of endosomes before delivery and degradation in lysosomes (Gruenberg and Stenmark, 2004). One of the sorting signals for sending proteins into the endosomal lumen is ubiquitin (Ub; Katzmann *et al.*, 2002; Hicke and Dunn, 2003). Many studies have demonstrated that monoubiquitination of cargo is sufficient for efficient sorting into the interior of multivesiculated endosomes/bodies (MVB). Ubiquitination works as an MVB-sorting signal for a variety of proteins, including those internalized from the cell surface as well as those delivered to endosomes from the Golgi apparatus via the biosynthetic pathway. Ub also serves as a sorting signal at other post-Golgi compartments with the cumulative effect of sending cargo toward the lysosome. Ub can serve as a signal for internalization, thus hastening the delivery of cell surface proteins to endosomes, and Ub addition can also sort membrane proteins at the *trans*-Golgi network (TGN), diverting them to endosomes and preventing their delivery to the cell surface (Staub and Rotin, 2006).

Ubiquitin works as a sorting signal by binding Ub-sorting receptors that recognize ubiquitinated cargo (Ub-cargo) and incorporate it into various transport intermediates (Staub and Rotin, 2006). At the MVB, proteins such as the Hrs-STAM (hepatocyte receptor substrate-signal transducing adaptor molecule) complex and the yeast Hse1-Vps27 complex bind Ub-cargo using multiple UIMs (ubiquitin interaction motifs) and help sort cargo into lumenal vesicles. At the TGN, the monomeric clathrin-associated GGA (Golgi-localized, gamma-ear containing, Arf-binding) proteins bind Ub via a GAT (GGA and TOM1) domain to facilitate cargo incorporation into transport vesicles targeted to endosomes. At the cell surface, Ub-binding proteins such as Epsin and Eps15, which also contain UIM motifs, participate in Ub-mediated internalization and may play a critical role in recognizing Ub-cargo for internalization (Hicke and Dunn, 2003).

The kinetics of lysosomal degradation of a particular membrane protein is tightly correlated with the extent of its ubiquitination (Hicke and Dunn, 2003). However, in some cases where a large proportion of a particular receptor undergoes lysosomal degradation, only a small proportion of the total receptor is ubiquitinated at any one time during its journey to the lysosome. One example is the degradation of the epidermal growth factor receptor (EGFR) via the ubiquitin ligase c-Cbl (Levkowitz *et al.*, 1998; Lill *et al.*, 2000). Accordingly, sustained ubiquitination coupled with the translocation of c-Cbl as it follows receptor from the cell surface to endosomes appears to be required for the efficient EGFR down-regulation (de Melker *et al.*, 2001; Longva *et al.*, 2002; Alwan *et al.*, 2003). Together, these observations imply that although ubiquitination can initiate a sorting pathway to the lysosome at a variety of compartments, multiple rounds of ubiquitination must be sustained to efficiently

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commit a given cargo protein to the MVB/lysosomal degradation pathway. This model predicts that the competing actions of Ub peptidases and ligases would have ample opportunity to reverse or reinforce the decision to degrade a particular cargo protein as it progresses toward lysosomes (Urbe, 2005).

In yeast, the HECT-type Ub E3 Rsp5 is a broad specificity ligase required for ubiquitination of a variety of membrane protein cargo at multiple compartments. Loss of Rsp5 impairs ubiquitination of cell surface proteins and inhibits their internalization (Hicke and Dunn, 2003; Staub and Rotin, 2006). Rsp5 is also required for delivering proteins such as Cps1 to the vacuole interior via the MVB-sorting pathway and is required for sorting proteins such as Fur4 and Gap1 directly from the TGN to endosomes (Helliwell *et al.*, 2001; Blondel *et al.*, 2004; Dunn *et al.*, 2004; Katzmann *et al.*, 2004). Rsp5 belongs to the family of Nedd4-related E3 ligases that contain a number of domains that help adapt Rsp5 to a variety of ubiquitination roles. Rsp5 contains 3 WW domains, which can target Rsp5 to specific substrates or cofactors that contain a PY motif (Yashiroda *et al.*, 1998; Wang *et al.*, 1999; Hettema *et al.*, 2004; Shcherbik *et al.*, 2004). Rsp5 also contains an N-terminal C2 domain, which is required for proper ubiquitination of MVB cargo and likely facilitates association with Golgi and endosomal membranes that are enriched in phosphorylated phosphatidylinositols (Dunn *et al.*, 2004). In addition, Rsp5 associates with other factors such as Bsd2 and Bul1/2 to facilitate ubiquitination of specific cargo proteins such as Cps1 or Gap1 (Helliwell *et al.*, 2001; Soetens *et al.*, 2001; Hettema *et al.*, 2004). Recently, Rsp5 has been shown to interact with the Hse1-Vps27 complex and the mammalian Rsp5-related Itch ligase has been shown to interact with Hrs (Marchese *et al.*, 2003; Bowers *et al.*, 2004). The inferred function of these interactions is that they either foster ubiquitination of cargo or the Ub-recognition/sorting machinery, thus regulating its activity.

De-ubiquitinating peptidases (DUbs) have also been implicated in the process of Ub-dependent sorting of membrane proteins (Urbe, 2005; Millard and Wood, 2006). Specifically, Dubs have been found to associate with the Hrs-STAM Ub-sorting receptor complex, although the exact function of these associations remains to be fully deciphered. STAM associates with two DUbs via its SH3 domain: the UBP-family cysteine protease UBPY/USP8 and the JAMM-(JAB1/MPN/Mov34 metalloenzyme) domain metallo-protease AMSH (associated molecule with the SH3 domain of STAM; Tanaka *et al.*, 1999; Kato *et al.*, 2000). AMSH is activated upon binding the SH3 domain of STAM and shows preference for disassembling K63-linked Ub chains over K48-linked chains (McCullough *et al.*, 2006). Depletion of AMSH accelerates degradation of EGFR, whereas overexpression of enzymatically inactive AMSH causes accumulation of ubiquitinated proteins on endosomes and the accumulation of ubiquitinated forms of STAM (McCullough *et al.*, 2004). Depletion of UBPY also leads to accumulation of ubiquitinated proteins on endosomes as well as other changes to endosome morphology (Mizuno *et al.*, 2005, 2006; Row *et al.*, 2006). However, depletion of UBPY alters the trafficking of internalized EGFR and blocks its degradation, suggesting a potentially complex role for associated DUbs (Row *et al.*, 2006; Mizuno *et al.*, 2006).

In the present study, we find that Hse1, the yeast ortholog of STAM, associates with the E3 ligase Rsp5. Disrupting the interaction between Hse1 and Rsp5 impairs sorting of MVB cargo such as Cps1 to the vacuole interior without affecting the global function of Hse1. In contrast, Hse1 also associates with the counteracting DUb Ubp7, whose deletion increases the efficiency of Cps1 sorting. These data imply that the association of Hse1 with ligases and Dubs helps modify Ub-cargo in order to regulate its final disposition with respect to MVB sorting into the degradative pathway.

MATERIALS AND METHODS

Materials, Yeast Strains, and Plasmids

Synthetic dextrose (SD) media was made using yeast nitrogen base containing ammonia and 2% glucose. Yeast nitrogen base was purchased from RPI Research Products International (Mt. Prospect, IL). Amino acid supplements were purchased from Bio101 (La Jolla, CA). Glutathione-agarose beads and calmodulin beads were purchased from Amersham Biosciences (Uppsala, Sweden). L-azetidine-2-carboxylic acid (ADCB), bathocuprione sulfonate (BCS), and cycloheximide was purchased from Sigma (St. Louis, MO). Zymolyase 100T was purchased from Seikagaku (East Falmouth, MA). Protease inhibitor mixture (complete) was purchased from Roche Molecular Biochemicals (Mannheim, Germany). The pCR2.1, pYES2.1, and pET151 TOPO cloning kits were purchased from Invitrogen (Carlsbad, CA). Ubiquitin derivatized with 7-amino-4-methylcoumarin (Ub-AMC) was purchased from Boston Biochemicals (Cambridge, MA). Anti-HA antibody was purchased from Covance Research Product (Berkeley, CA), anti-V5 from Invitrogen, and anti-6xHIS from Berkeley Antibody Company (Berkeley, CA), anti-green fluorescent protein (GFP) antibody from Clontech (Mountain View, CA), and HRP-linked secondary antibodies from Amersham Biosciences (Buckinghamshire, United Kingdom). Polyclonal antisera to Cps1 was kindly provided by D. Katzmann (Mayo Clinic, Rochester, MN). L-^{35}S-methionine was purchased from PerkinElmer Life and Analytical Science (Boston, MA). The *Saccharomyces cerevisiae* Tandem Affinity Purification (TAP)-tagged and GFP-tagged collection (Ghaemmaghami *et al.*, 2003) was obtained from Open Biosystems (Huntsville, AL).

S. cerevisiae strains used in this study are listed in Table 1. Gene disruptions were performed by replacing the entire open reading frame with the indicated selectable marker. For disruptions using the Kanr marker, the disruption cassette was amplified from the genomic DNA of the relevant strain from the yeast gene deletion project (Giaever *et al.*, 2002). For disruptions using the *URA3* marker, a disruption cassette was made by amplifying the *URA3* gene from *Kluyveromyces lactis* using pUG72 (Gueldener *et al.*, 2002) with oligos containing 50 base pairs of flanking DNA homologous to the insertion site. For disruptions using the *his5+* marker, which is derived from *Schizosaccharomyces pombe* and complements the *S. cerevisiae HIS3* gene, a disruption cassette was made by amplifying the *His5* gene from *K. lactis* using pUG27 (Gueldener *et al.*, 2002).

The *hse1-ΔPY* mutation was made by deleting the Hse1-C terminus Rsp5 binding motif PPPGYEQ within the *HSE1* genomic locus. A *HIS3*-containing cassette was amplified from the plasmid pFA6a-GFP(S65T)-HIS3MX6 (Longtine *et al.*, 1998) with oligos: ATACCCCACAATACGGTTATGATCTTG-GATATTCAGTTGTTAGCCAATGAGGCGCGCCACTTCTAAA; CTTCGTA-AAAATTAAAGATATGTAAAGGTGCTATATAAGTTGAAGGGGAATTCG-AGCTCGTTTAAAC. The genomic DNA from transformants were then analyzed by PCR and sequencing to verify correct integration.

Plasmids used in this study are listed in Table 2. Plasmids expressing glutathione *S*-transferase (GST) fusion proteins of fragments of Hua1, Hse1, or Pex13 were made by subcloning an EcoRI-restricted PCR fragment into pGEX6p-1. For expression from the *GAL1* promoter, PCR fragments of the open reading frames from Hua1, Ubp7, and Rup1 were subcloned into pYES2.1 TOPO (Invitrogen), which resulted in the introduction of a C-terminal V5 epitope. For expression in bacteria, gene fragments were subcloned into PET151D (Invitrogen) behind the T7 promoter and an N-terminal V5 epitope. The HA-Ub-GFP-Cps1 is composed of the *RPL40B* promoter followed by an HA epitope, Ub (residues 1–74) followed by the *GFP-CPS1* fusion from pGO45.

Deubiquitinating Enzyme Activity Assay by a Fluorogenic Substrate Ub-AMC

Bacterial lysate containing equal amount of recombinant Ubp7 fragments was diluted with reaction buffer (50 mM HEPES, 0.5 mM EDTA, pH 7.5, 0.1 mg/ml ovalbumin, and 1 mM DTT) and Ub-AMC (0.5 μM) was added to initiate the reaction. The enzyme activity was monitored as increased fluorescence at 460 nm using 380-nm excitation using a Hitachi 2000 Fluorescence Spectrophotometer (San Jose, CA).

Glutathione-Agarose Affinity Chromatography

GST-fusion proteins were isolated from bacteria using glutathione-Sepharose beads as previously described (Smith *et al.*, 1988). Isolated GST fusion proteins, 250 μg of each, was bound to 50 μl of glutathione-Sepharose in phosphate-buffered saline (PBS) by rotation for 30 min at 25°C. Bound GST or GST fusion proteins were then pelleted and washed three times with PBS. Cell lysate was added to each protein/bead complex and incubated for 2 h at 4°C. Unbound proteins were removed from beads using four washes of IC buffer

Table 1. Yeast strains used in this study

Strain	Genotype	Reference
SF838-9D	*MATα leu2-3,112 ura3-52 his4-519 ade6 pep4-3*	Raymond *et al.* (1992)
BY 4742	*MATα his3 leu2 lys2 ura3*	Brachmann *et al.* (1998)
PLY 2498	*MATα hse1Δ::Kan^r his3, leu2, lys2, ura3*	Yeast Gene Deletion Project
PLY 3173	MATα *hse1-ΔPY::HIS3 leu2 lys2, ura3*	This study
PLY 3453	*MATα rup1Δ::Kan^r his3 leu2 lys2 ura3*	This study
PLY 3555	*MATα hse1Δ::Kan^r rup1Δ::LEU2 ura3 his4 ade6*	This study
PLY 3490	*MATα hua1Δ::Kan^r leu2 lys2 ura3*	This study
PLY 3492	MATα hua1Δ::*Kan^r hse1-ΔPY::HIS3 leu2, lys2, ura3*	This study
PLY 3545	*MATα hua1Δ::Kan^r hse1-ΔPY::HIS3 ubp7Δ::URA3 leu2 lys2*	This study
PLY 3557	*MATα end3Δ::HIS3 leu2 lys2 ura3*	This study
PLY 3132	*MATα ubp2Δ::Kan^r his3 leu2 lys2 ura3*	
PLY 3559	*MATα end3Δ::HIS3 ubp2Δ::Kan^r leu2 lys2 ura3*	This study
PLY 3543	*MATα ubp2Δ::Kan^r ubp7Δ::HIS3 leu2 lys2 ura3*	This study
PLY 3137	*MATα ubp7Δ::Kan^r his3 leu2 lys2 ura3*	This study
PLY 3258	*MATα bsd2Δ::Kan^r his3 leu2 lys2 ura3*	Yeast Gene Deletion Project
PLY 3610	*MATα doa4Δ::Kan^r his3 leu2 lys2 ura3*	Yeast Gene Deletion Project
PLY 3620	*MATα doa4Δ::Kan^r ubp7Δ::URA3 his3 leu2 lys2*	This study
Ubp14-GFP	*MATA leu2, ura3, his3, met1, ade6*	Ghaemmaghami *et al.* (2003)
Ubp6-GFP	*MATA leu2, ura3, his3, met1, ade6*	Ghaemmaghami *et al.* (2003)
Doa4-GFP	*MATA leu2, ura3, his3, met1, ade6*	Ghaemmaghami *et al.* (2003)
Ubp2-GFP	*MATA leu2, ura3, his3, met1, ade6*	Ghaemmaghami *et al.* (2003)
Ubp1-GFP	*MATA leu2, ura3, his3, met1, ade6*	Ghaemmaghami *et al.* (2003)
Rup1-GFP	*MATA leu2, ura3, his3, met1, ade6*	Ghaemmaghami *et al.* (2003)
Hua1-GFP	*MATA leu2, ura3, his3, met1, ade6*	Ghaemmaghami *et al.* (2003)
Ubp2-TAP	*MATA leu2, ura3, his3, met1, ade6*	Ghaemmaghami *et al.* (2003)
Hse1-TAP	*MATA leu2, ura3, his3, met1, ade6*	Ghaemmaghami *et al.* (2003)

(100 mM KAc, 50 mM KCl, 200 mM sorbitol, 20 mM PIPES, pH 6.8), and the bound bead fraction was analyzed by SDS-PAGE and immunoblotting.

Calmodulin-Agarose Affinity Chromatography

Cells expressing TAP-tagged forms of Ubp2 or Hse1 were transformed with plasmids expressing epitope-tagged Hua1, Hse1, or Ubp7. Sorting defects were not evident in the Ubp2-Tap and Hse1-TAP strains, indicating that these tagged proteins were functional (data not shown). Nondenatured yeast lysates made from spheroplasts were with 50 μl of calmodulin beads in the presence of 2 mM $CaCl_2$. Beads were washed four times in IC buffer and analyzed by immunoblot analysis.

Fluorescence Microscopy

Cells containing GFP-expressing plasmids were grown in SD media to midlog phase. Cells, 1 OD_{600}, were pelleted and resuspended in 100 μl of KILL buffer (1 M Tris, pH 8.0, 5% sodium azide). Cells were viewed using an Olympus BX-60 microscope (Melville, NY) equipped with FITC filters and Nomarski optics. Images were captured with a Hamamatsu ORCA CCD camera (Bridgewater, NJ) as previously described (Urbanowski and Piper, 1999).

ADCB Sensitivity Assay

Yeast were first grown in SD media (ammonia) overnight, serially (1:5) diluted, and plated onto an SD plate containing 75 μg/ml ADCB as described previously (Scott *et al.,* 2004).

Cell Labeling and Immunoprecipitation of Carboxypeptidase Y

Metabolic ^{35}S-Met labeling and immunoprecipitation of carboxypeptidase Y (CPY) was performed as described previously (Cooper and Stevens, 1996).

Kinetics of Ste3-GFP Degradation

Cells were cultured to midlog phase and then adjusted to 100 μg/ml cycloheximide. Aliquots (4 OD_{600}) were taken at various times after cycloheximide addition and stored on ice for up to 40 min in the presence of 0.2% NaAzide, 0.2% NaFluoride, 100 mM Tris, pH. 7.0. Proteins were then precipitated with in 10% TCA and analyzed by SDS-PAGE and immunoblotting with anti-GFP monoclonal antibodies.

Cell Labeling and Immunoprecipitation of Cps1

Yeast cells containing GFP-Cps1 were grown to midlog phase and labeled with [^{35}S]methionine for 10 min. Denaturing immunoprecipitations were carried out as outlined previously (Katzmann *et al.,* 2001). After labeling, yeast cultures were adjusted to 10 mM NEM and then precipitated by the addition of TCA (10% final). Protein pellets were resuspended in 6 M urea, 1% SDS, 50 mM Tris, pH 7.5, and 1 mM EDTA. Lysate was then diluted to a final composition of 0.6 M urea, 0.1% SDS, 1% Triton X-100, 50 mM Tris, pH 7.5, and 0.1 mM EDTA. Immunoprecipitation was performed as above for CPY except using anti-Cps1 polyclonal antibody. Immunoprecipitates were treated with endoglycosidase H before analysis by SDS-PAGE and autoradiography. Labeled proteins were detected on phosphorimaging screens (Packard Instruments, Meriden, CT).

Yeast Lysate Preparation

Yeast culture, 200 ml, was pelleted, washed in water, and resuspended in 5 ml of 0.1 M Tris-HCl, pH 9.4, 10 mM DTT for 5 min. Cells were pelleted and resuspended in 5 ml of spheroplast buffer (0.1× YPD, 1.0 M sorbitol, 25 mM Tris-HCl, pH 7.5, 250 μg of zymolyase 100T) for 30 min at 30°C. Spheroplasts were then layered onto a sucrose cushion (1.2 M sucrose, 20 mM HEPES, pH 7.2, 2 mM EDTA) and collected as a pellet after centrifugation at 7000 × *g* for 20 min. Cells were lysed in IC buffer with protease inhibitors and spun at 15,000 rpm for 15 min to collect the supernatant.

Bacterial Lysate Preparation

Bacteria cultures [500 ml; BL21(DE3)] were grown to exponential phase and induced with 1 mM IPTG for 4 h. Cells were pelleted and resuspended in 5 ml of PBS, pH 8.0, with protease inhibitors. Cells were then lysed by French Press lysis and followed by spinning down at 11,000 rpm for 20 min to remove the cell debris.

Ubiquitin-Vinyl-Methyl Ester Labeling

HA-tagged ubiquitin-vinyl-methyl-ester (HA-UbVME) was a kind gift from Hidde Ploegh (Whitehead Institute, Cambridge, MA). Lysates were made from yeast spheroplasts as above and labeled with HA-UbVME (2 μg/ml) for 30 min at 30°C and processed as described previously (Borodovsky *et al.,* 2001, 2002).

RESULTS

Interactions of Hse1 with Peptidases and a Ubiquitin Ligase

The Hse1-Vps27 complex binds Ub and is required for sorting of ubiquitinated proteins into MVBs (Bilodeau *et al.,* 2002). To better understand how this complex operates, we

Table 2. Plasmids used in this study

Plasmid	Description	Reference
pPL2998	GST-Hua1. The *HUA1* open reading frame encoding residues 1-198 subcloned into the EcoRI site of pGEX-6P-1	This study
pPL2710	GST-Hse1-SH3. The region encoding the SH3 domain of Hse1 (residues 200-300) was subcloned into the EcoRI site of pGEX-6P-1	This study
pPL2831	GST-Pex13-SH3. The region encoding the SH3 domain of Pex13 (residues 310-370) was subcloned into the EcoRI site of pGEX-6P-1	This study
pPL3164	GST-Hse1-SH3*. GST-Hse1-SH3 plasmid in which the codons encoding W254 and W255 within the Hse1 SH3 domain were altered to Alanine	This study
pPL3170	*GAL1-HUA1*-V5. The *HUA1* open reading fram was subcloned behind the *GAL1* promoter of pYES2.1 and flanked with a C-terminal V5 epitope	This study
pPL3165	*GAL1-UBP7*-V5. The *UBP7* open reading frame was subcloned behind the *GAL1* promoter of pYES2.1 and flanked with a C-terminal V5 epitope	This study
pPL3169	*GAL1-RUP1*-V5. The *RUP1* open reading frame was subcloned behind the *GAL1* promoter of pYES2.1 and flanked with a C-terminal V5 epitope	This study
pPL3168	pET151 containing the Hse1-SH3 domain encoding region (residues 200-300)	This study
pPL3166	pET151 with *UBP7* fragment encoding residues 488-1072	This study
pPL3167	pET151 with *UBP7* fragment encoding residues 600-1072	This study
pPL2995	pET151 with *UBP7* fragment encoding residues 600-847	This study
pPL3171	GST-Hse1-C. The region encoding the C-terminus of Hse1 (residues 275-452) was subcloned into the EcoRI site of pGEX-6P-1	This study
pPL3172	GST-Hse1-C*. The region encoding the C-terminus of Hse1 (residues 275-452 with 446PPP448 mutated to 446AAA448) subcloned into the EcoRI site of pGEX-6P-1	This study
pPL3267	*HA-Ub-GFP-CPS1* in pRS316	This study
pGO45	pRS426 carrying *GFP-CPS1*	Odorizzi *et al.* (1998)
pJE59	pRS426 carrying *GFP-CPS1*^{K8,12R}	Katzmann *et al.* (2001)
pPL1640	*FTH1-GFP-Ub*	Urbanowski and Piper (2001)
pPL967	*STE3-GFP*	Urbanowski and Piper (2001)
pPL2334	*CUP1-GAP1*-GFP	Scott *et al.* (2004)
pUG72	Plasmid containing gene disruption cassettes with *URA3* (*K. lactis*) as selection marker	Gueldener *et al.* (2002)
pUG27	Plasmid containing gene disruption cassettes with *his5+* (*S. pombe*) as selection marker	Gueldener *et al.* (2002)
YCpHA-Rsp5	HA-tagged *RSP5* in YCplac33	Gajewska *et al.* (2001)
pPL1808	HA-tagged *HSE1* in pRS316	This study
pPL1124	*VPS4* dominant-negative in pRS426	This study
pGEX-6P-1	GST expression vector	Kaelin *et al.* (1992)
pYES2.1	High copy (2μ) *URA3*-containing *GAL1*-expression plasmid	Invitrogen
pET151	T7-promoter based bacterial expression vector	Invitrogen

investigated the role of possible interactions between Hse1 and other proteins. One potential protein interaction site is the SH3 domain of Hse1, which is predicted to bind proteins with proline-rich motifs typical of SH3 interaction modules (Tong *et al.*, 2002). We found that replacing the SH3 domain of Hse1 with that of Pex13 or mutation of the tryptophan residues that define the predicted hydrophobic binding face of the Hse1 SH3 domain (Yu *et al.*, 1994) resulted in a complete loss of function (data not shown). Because these data indicated the SH3 domain performed a significant function, we sought to identify proteins that associate with Hse1. Previous large-scale protein interaction studies provided a list of candidate interacting proteins, among which were both Ub E3 ligases and DUbs (Tong *et al.*, 2002; Bowers *et al.*, 2004). These candidates were intriguing because their association with the Hse1-Vps27 complex indicated a potential mechanism to regulate the ubiquitination status of cargo. Using a series of protein-binding studies, we deduced a model for protein interactions shown in Figure 1a.

Hse1 Interacts with Ubp7

Because the mammalian Hse1 homolog STAM binds to two DUbs via its SH3 domain, we screened a subset of TAP-tagged DUb proteins for interaction with the Hse1 SH3 domain using GST pulldown assays (Rigaut *et al.*, 1999). We found that Ubp7-TAP bound the Hse1 SH3 domain; however, Ubp7 is expressed at very low levels (Ghaemmaghami *et al.*, 2003). Other DUbs such as Ubp6, Ubp1, and Doa4, which is localized with the class E Vps machinery, were not associated with Hse1 in our GST pulldown assays or immunoprecipitation assays (data not shown). To confirm the interaction between Ubp7 and the Hse1 SH3 domain, extracts from yeast expressing an epitope-tagged (V5) allele of *UBP7* expressed via the *GAL1* promoter were used in GST-SH3 pulldown experiments. Figure 1b shows that Ubp7-V5 specifically bound the SH3 domain of Hse1 but did not bind the SH3 domain of Pex13 nor the Hse1 SH3 domain containing mutations within the predicted ligand binding site (SH3*; Yu *et al.*, 1994). To confirm that this interaction could occur in yeast lysates with full-length proteins, TAP-tagged Hse1 was isolated under nondenaturing conditions from lysates prepared from cells expressing Ubp7-V5. Figure 2 shows that Ubp7-V5 could specifically associate with Hse1-TAP, supporting the idea that these proteins associate in vivo.

Using a series of recombinant protein fragments derived from Ubp7, we found that the Hse1-SH3 domain can bind directly to Ubp7 and requires a region from 487 to 600 that

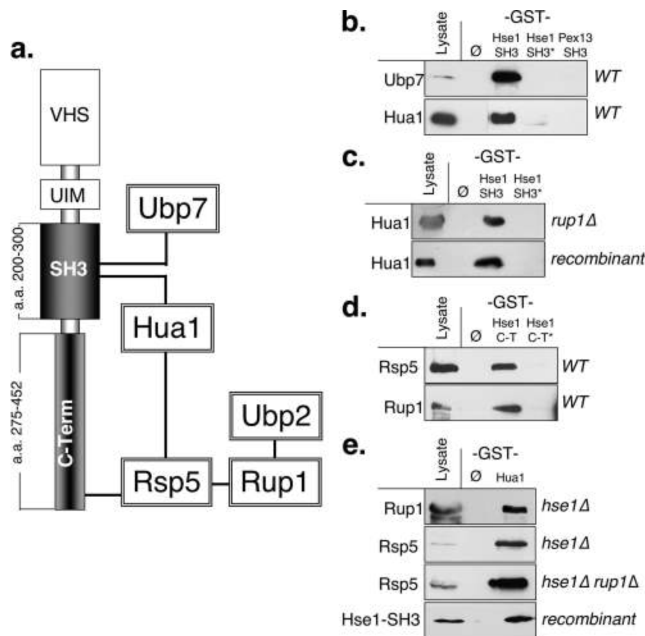

Figure 1. Interaction map of Hse1 with a ubiquitin ligase and ubiquitin peptidases. (a) Summary of interactions between Hse1, Ubp7, Hua1, and the Rsp5-Rup1-Ubp2 complex. (b) The SH3 domain of Hse1 binds Hua1 and Ubp7 in GST pulldown assays. GST fusion protein alone (∅) or containing the wild-type Hse1 SH3 domain, a mutant (*) SH3 domain, or the SH3 domain from Pex13, were bound to GSH agarose and incubated with lysates from wild-type yeast (WT) expressing V5-epitope–tagged Ubp7 or V5-epitope–tagged Hua1. Bound fractions together with 10% of the input (lysate) were analyzed by immunoblotting. (c) Association of Hua1 with the Hse1-SH3 domain is direct and does not rely on the presence of Rup1. Hua1 from lysates of yeast lacking Rup1 (*rup1*Δ) or bacterial lysates expressing recombinant Hua1 was allowed to bind GST alone (∅), GST-Hse1-SH3 domain, or GST fused to a mutant Hse1 SH3 domain. Bound fractions together with 10% of the input (lysate) were analyzed by immunoblotting. (d) Rsp5 and Rup1 interact with the C-terminal tail of Hse1 via a PY motif. A GST fusion protein containing the C-terminus of Hse1 (a.a. 275–452) or a truncated C-terminal fragment (*) lacking a the distal PPPGYEN (PY) motif (a.a. 275–445) were bound to GSH beads and incubated with lysates from wild-type (WT) yeast expressing HA-epitope–tagged Rsp5 or V5 epitope–tagged Rup1. Bound fractions together with 10% of the input lysate were analyzed by immunoblotting. (e) Interactions of Hua1. GST alone or GST fused to Hua1 were used in GST pulldown assays with lysates from yeast lacking Hse1 (*hse1*Δ) or both Hse1 and Rup1 (*hse1*Δ *rup1*Δ*)*. Rup1 specifically bound GST-Hua1 independent of Hse1 while Rsp5 bound GST-Hua1 independent of Rup1 or Hse1. A recombinant Hse1 SH3 domain from bacterial lysates also bound to GST-Hua1. Shown is an immunoblot of bound fractions together with 10% of the input (lysate).

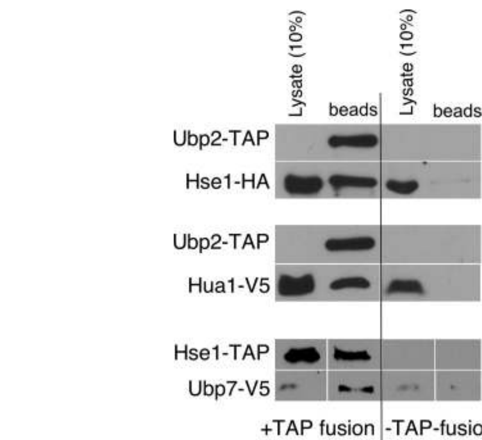

Figure 2. Full-length Hse1 interacts with Ubp2 and Ubp7. Yeast strains expressing the indicated TAP-tag fusion proteins were transformed with plasmids expressing epitope-tagged Hse1, Hua1, or Ubp7 as indicated. Yeast lysates were made from spheroplasts and were passed over calmodulin Sepharose beads in the presence of 2 mM $CaCl_2$. As a control for specificity, wild-type yeast that did not express a TAP-tagged fusion protein were used. Bound fractions were eluted and immunoblotted as indicated along with 10% equivalent of input lysate.

encompasses the putative SH3 ligand, K_{512}RPPPPPPVS (Figure 3a). These results confirm previous predictions made from phage display and bioinformatics studies on all the SH3 domains in the yeast genome, which identified K_{512}RPPPPPPVS as a likely binding peptide for the Hse1 SH3 domain (Tong *et al.,* 2002). We also analyzed Ubp7 activity by covalently labeling yeast extracts with HA-UbVME, a chemical derivative of Ub that reacts with the active cysteine residue within papain-like Ub peptidases (Borodovsky *et al.,* 2002). Previous experiments using this method have shown that the major DUbs labeled by this method are Ubp1, Ubp2, Ubp6, Ubp12, and Ubp15, and this labeling correlates with the estimated expression level of these DUbs (Borodovsky *et al.,* 2001). In wild-type extracts, Ubp7, a ~125-kDa protein, is barely detectable, consistent with its estimated low level of expression (Ghaemmaghami *et al.,*

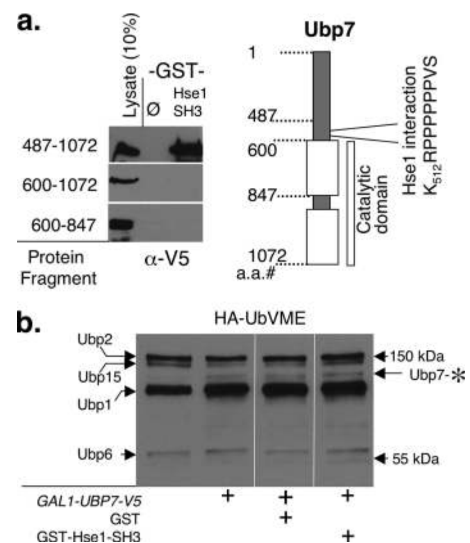

Figure 3. Direct binding of Ubp7 to the Hse1 SH3 domain does not alter activity. (a) V5-epitope tagged protein fragments of Ubp7 encompassing the designated residues made in *E. coli* were passed over GST fusion protein alone (∅) or containing the wild-type Hse1 SH3 domain. Bound fractions were immunoblotted with anti-V5 along with a 10% equivalent of input lysate. At right is shown a schematic of Ubp7. The region that shares high homology to the catalytic domain of other Ubps is shown in white boxes. The putative SH3 ligand K_{512}RPPPPPPVS is shown. (b) Ubp7 binds HA-UbVME. Yeast carrying vector plasmid or plasmid carrying *UBP7* driven by the *GAL1* promoter were grown in galactose and lysed. Lysates were preincubated with no additions or 100 μg/ml GST or GST-Hse1-SH3 fusion protein before labeling with HA-UbVME. Labeled extracts were analyzed by anti-HA immunoblotting. Arrows pointing to the positions of known DUbs are designated. A new band (*) corresponds to the predicted MW of Ubp7.

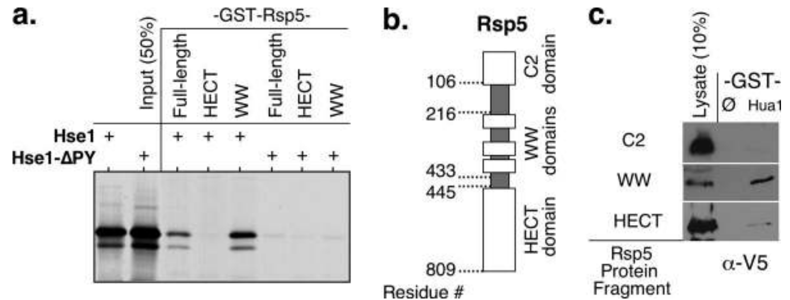

Figure 4. Hse1 interacts with Rsp5. (a) Binding of in vitro–translated Hse1 to GST-Rsp5 proteins. ^{35}S-labeled Hse1 and Hse1 lacking the C-terminal Rsp5 binding PY element (Hse1-ΔPY) were translated in vitro using reticulocyte lysate and incubated with GST fusion proteins containing full-length Rsp5, a region containing the WW domains, or containing the HECT domain. Bound fractions were analyzed by SDS-PAGE and autoradiography together with 50% of the input reaction. (b) A schematic of the domain organization of Rsp5 and the regions corresponding to the recombinant protein fragments used. (c) V5-epitope tagged protein fragments of Rsp5 encompassing the designated residues made in *E. coli* were passed over GST fusion protein alone (∅) or GST fused to Hua1. Bound fractions were immunoblotted with anti-V5 along with a 10% equivalent of input lysate.

2003). However, when Ubp7-V5 is overexpressed from the *GAL1* promoter, a new band corresponding to Ubp7 was found labeled with HA-UbVME (Figure 3b). Inclusion of GST or the GST-Hse1-SH3 domain did not increase reactivity of Ubp7 to HA-UbVME, indicating that Ubp7 activity is not altered upon association with Hse1. The lack of enzyme activation differs to what is observed for the Hse1 homolog STAM and the AMSH Ub peptidase. In the latter case, AMSH is significantly activated once it is bound to the STAM SH3 domain (McCullough *et al.,* 2006).

Hse1 Interacts with Rsp5

Previous interaction studies focused on the proteins involved in MVB sorting predicted that Hse1 associates with the Ub E3 ligase, Rsp5 (Bowers *et al.,* 2004). Rsp5 contains an N-terminal lipid-binding C2 domain, three WW domains, and a C-terminal HECT ligase domain (Wang *et al.,* 1999). We determined that there were two modes by which Hse1 could associate with Rsp5: first, by binding directly to a PY element within the Hse1 C-terminus, and second by binding to the novel Hua1 protein via the Hse1-SH3 domain (Figure 1a). Evidence that Rsp5 could bind directly to Hse1 came from attempts to identify yeast proteins that could interact with the WW domains of Rsp5 (J. Huibregste, unpublished data). A peptide library made of protein fragments encoded within the yeast genome that contained putative PY elements was screened for binding to a region of Rsp5 that encompassed its WW domains. This search identified a PY element within the extreme C-terminus of Hse1 (P_{446}PPGYEQ) that could potentially interact with the Rsp5 WW domains. To confirm this interaction, extracts from cells expressing HA-epitope tagged Rsp5 were used in GST pull-down assays using a GST fusion to the C-terminal 179 residues (residues 275–453) of Hse1. Figure 1d shows that Rsp5-HA bound specifically to the C-terminus of Hse1 but not to the same fragment containing a deletion of the last seven residues that encode the PY element identified by the library screen. Figure 4a shows that a recombinant fragment encompassing the WW domains of Rsp5 was sufficient to bind the Hse1 C-terminus directly, whereas the HECT domain only bound very weakly.

Large-scale proteomic studies also indicated that Hse1 could interact with the Hua1 protein (Ito *et al.,* 2001). *HUA1* encodes an 22-kDa protein that contains a C-terminal Zn finger domain belonging to a subclass found within DnaJ proteins and that is thought to promote protein–protein interactions (Burri *et al.,* 2004). Although there are no apparent Hua1 homologues in animal cells, Hua1-like genes are present in plants. Proteomic studies also indicated that Hua1 might interact with Rsp5 and Ubp2 (Ito *et al.,* 2001). These proteins have recently been described in a novel complex that also contains Rup1, which mediates binding between Rsp5 and Ubp2 (Kee *et al.,* 2005). Thus, we tested whether Hse1 could indeed associate with the Rsp5-Rup1-Ubp2 complex through Hua1. Using a series of Hse1 protein fragments, we found that Hua1 within yeast lysates readily bound the SH3 domain of Hse1 but not a mutant Hse1 SH3 domain with an alteration in the interaction surface (Figure 1b). Hua1 still bound the Hse1-SH3 domain in yeast lysates devoid Rup1, and we also found that recombinant Hua1 bound the Hse1-SH3 domain, indicating a direct interaction between these two components (Figure 1, d and e). A GST-Hua1 protein was able to bind Rsp5 from yeast lysates in the absence of Hse1 and Rup1, indicating that Hua1 associated with the Rsp5-Rup1-Ubp2 complex through binding Rsp5. Also, the C-terminal PY element of Hse1 not only bound Rsp5 but also retained Rup1 (Figure 1d). Both full-length Hua1 and Hse1 also associated with TAP-tagged Ubp2, indicating that these associations were relevant in vivo (Figure 2). We also found that GFP tagged Hua1, Rup1, and to some extent Ubp2 could accumulate on enlarged endosomal structures induced by dominant-negative *VPS4,* further supporting a role for these proteins on endosomes (Supplementary Data). We then confirmed a direct interaction of Hua1 with the region of Rsp5 containing the WW domains (residues 232-420; Figure 4c). These combined data showed that Hse1 has two interaction sites that are each independently capable of recruiting the Rsp5-Rup1-Ubp2 complex.

Function of the Hse1-Rsp5 Interaction

We hypothesized two potential roles for the interaction of Hse1 with the Rsp5 E3 ligase. One possibility is that this association helps modify ubiquitinated cargo, possibly reubiquitinating it to ensure efficient delivery of cargo into the degradative MVB-sorting pathway. This model predicts that perturbation of the Hse1-Rsp5 interaction will decrease the sorting efficiency of proteins that require ubiquitination into the MVB pathway but will not affect proteins that carry their own ubiquitination signal as an in-frame fusion or proteins that are very efficiently ubiquitinated and sorted to the vacuole interior. Alternatively, Rsp5 might regulate some general aspect of the Hse1-Vps27 sorting machinery, which could be required to sort a variety of Ub-dependent cargo proteins and possibly also be required for Hse1-Vps27 to mediate sorting of vacuolar hydrolases along the *VPS* (vacuolar protein sorting) pathway.

To functionally characterize the interaction of Hse1 with Rsp5, we first examined the sorting of GFP-Cps1 (Figure 5). GFP-Cps1 is transported along the biosynthetic pathway to the vacuolar lumen via the MVB-sorting pathway (Odorizzi *et al.,* 1998). Sorting of GFP-Cps1 depends on proper ubiquitination of the lysine residues within its cytosolic N-ter-

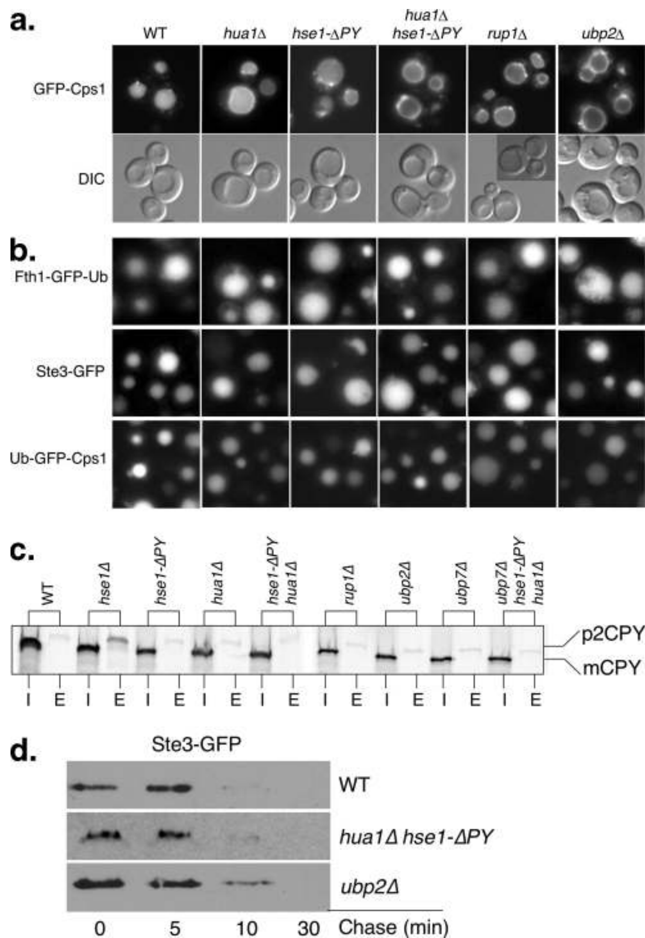

Figure 5. Effect of disrupting association of Hse1 with Rsp5. (a) The extent of sorting of GFP-Cps1 to the vacuole interior was visualized in wild-type (WT) cells and the indicated mutant cells grown in SD media to midlog phase. Also shown is the corresponding DIC image. (b) Sorting of Fth1-GFP-Ub, Ste3-GFP, and Ub-GFP-Cps1 to the vacuole is normal in wild-type and mutant cells. Cells were transformed with Ste3-GFP– or Ub-GFP-Cps1–producing plasmid and grown in SD or Fth1-GFP-Ub–producing plasmid grown in SD media containing 100 μM BPS to midlog phase before photography. (c) Neither disrupting association of Hse1 with Rsp5 nor deletion of *UBP2* or *RUP1* results in secretion of vacuolar proteases. Wild-type and the indicated mutant cells were pulse labeled with ^{35}S-Met for 10 min and chased with cold Met for 60 min. CPY was immunoprecipitated from intracellular (I) and extracellular/secreted (E) fractions and analyzed by SDS-PAGE and autoradiography. A CPY secretion phenotype was observed only for *hse1*Δ; all the other mutants analyzed sorted CPY normally. (d) Ste3-GFP degradation is delayed in *ubp2*Δ cells but not in *hua1*Δ*hse1*-Δ*PY cells*. Wild-type and *ubp2*Δ and *hua1*Δ *hse1*-Δ*PY* cells expressing Ste3-GFP were grown at 30°C to midlog phase in SD media. Cells were harvested at 0, 5, 10, and 30 min after cycloheximide addition. Samples were examined by SDS-PAGE and Western blotting with anti-GFP antibodies.

minal tail by Rsp5 (Katzmann *et al.,* 2001, 2004). Sorting of GFP-Cps1 also depends on proper recognition and processing of that Ub-sorting signal by a host of proteins termed the class E Vps proteins, which are also required for the delivery of soluble hydrolases to the vacuole (Odorizzi *et al.,* 1998). In cells that fail to properly ubiquitinate Cps1 or that lack proper class E Vps protein function, GFP-Cps1 accumulates on the limiting vacuolar membrane rather than within the vacuolar lumen. We found that cells containing an allele of *HSE1* lacking the Rsp5-binding PY element (*hse1*-Δ*PY*) had only very minor defects in sorting GFP-Cps1. Likewise, disruption of the *HUA1* gene (*hua1*Δ) also resulted in only minor defects in the sorting of GFP-Cps1. In contrast, GFP-Cps1 sorting was clearly defective when these mutations were combined (*hua1*Δ *hse1*-Δ*PY* double mutant), indicating that both modes of Hse1 interaction with Rsp5 contribute to proper sorting of Cps1 (Figure 5a).

In contrast, MVB cargo that is sorted by virtue of an in-frame fusion of Ub, which does not rely on ligase-mediated ubiquitination for MVB sorting, was sorted correctly in *hua1*Δ *hse1*-Δ*PY* double mutant cells. Figure 5b shows that the Fth1-GFP-Ub fusion reporter, which is sorted into the vacuolar lumen via a C-terminal Ub fusion (Urbanowski and Piper, 2001), was found entirely within the vacuolar lumen. Ub-GFP-Cps1, in which Ub is fused to the N-terminus of GFP-Cps1, was also found entirely within the vacuolar lumen (Figure 5b). We also found that unlike *hse1*Δ cells, the *hua1*Δ *hse1*-Δ*PY* cells were able to properly sort CPY to the vacuole (Figure 5c). Finally, *hua1*Δ *hse1*-Δ*PY* cells did not accumulate an exaggerated "class E" endosomal compartment, which is observed in class E *vps* mutant strains including *hse1*Δ (Raymond *et al.,* 1992; Bilodeau *et al.,* 2002). Together, these data suggest that the defect incurred by the *hua1*Δ *hse1*-Δ*PY* mutant affected only the efficiency of GFP-Cps1 ubiquitination but not the downstream processes of recognizing and sorting Ub-cargo.

We also found that Ste3 (Ste3-GFP), a G-protein–coupled receptor that undergoes efficient internalization from the cell surface and delivery to the vacuole lumen (Davis *et al.,* 1993), was also delivered to the vacuole lumen in *hua1*Δ *hse1*-Δ*PY* cells (Figure 5b). Even though vacuolar degradation of GPCRs Ste3 and Ste2 depend on proper Rsp5 function (Roth and Davis, 2000; Dunn and Hicke, 2001), the accumulation of Ste3-GFP in the vacuole that we observe in these mutants likely reflects a very high overall efficiency of Ste3 ubiquitination and MVB sorting, making their steady state accumulation in the vacuole lumen less sensitive to perturbations in the ubiquitination machinery or the Ub sorting machinery. Previous experiments have shown that although mutations in Rsp5 can result in the accumulation of Cps1 at the vacuole surface, those same mutations do not block accumulation of Ste2 (Dunn and Hicke, 2001; Dunn *et al.,* 2004) or Ste3 (D. Katzmann, personal communication) in the vacuole. Likewise, mutations that partially block Ub-recognition by the Hse1-Vps27 complex or that compromise interactions among the class E Vps proteins can affect sorting of Cps1 and Fth1-GFP-Ub without being severe enough to alter the steady state distribution of Ste3 in the vacuole (Bilodeau *et al.,* 2002, 2003). This could be due to fact that Ste3 is multiply ubiquitinated, which could carry it past a further requirement for additional ubiquitination at the endosomal Hse1-Vps27 sorting complex. In addition, Ste3 has been shown to recycle from endosomes, perhaps allowing underubiquitinated Ste3 to have repeated contact with the endosomal MVB machinery, whereas proteins such as Cps1 and Fth1-GFP-Ub are merely delivered to the vacuolar surface without the ability to be resurveyed by the MVB-sorting machinery.

We also found that sorting of GFP-Cps1 was affected in *rup1*Δ and *ubp2*Δ mutants, which would disrupt the function of the Rsp5-Rup1-Ubp2 complex recruited to Hse1 (Figure 5a). Although the steady state distribution of Ste3-GFP looked normal in *ubp2*Δ mutants, we did find that the kinetics of its delivery and degradation in the vacuole were slightly delayed (Figure 5d). This was measured using a cycloheximide chase to inhibit new synthesis of Ste3-GFP while measuring the depletion of existing Ste3-GFP over 15

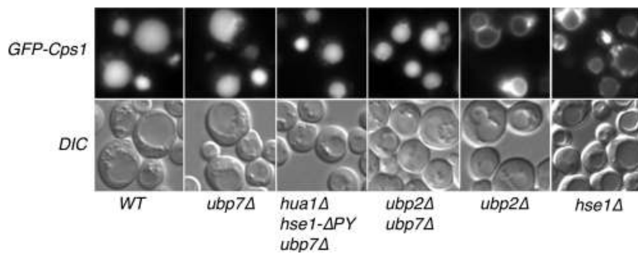

Figure 6. Loss of Ubp7 increases efficiency of sorting GFP-Cps1 into the vacuole interior. Wild-type cells and the indicated mutants expressing GFP-Cps1 were grown to midlog phase in SD media and visualized for the extent of GFP-Cps1 sorting to the vacuole interior.

min. In contrast, proteins such as Fth1-GFP-Ub and Ub-GFP-Cps1, which do not require cellular ubiquitination, still accumulated within the vacuolar lumen (Figure 5b). Furthermore, sorting of newly synthesized CPY to the vacuole measured by pulse/chase analysis was unaffected, again indicating that the Hse1-Vps27 machinery was functioning normally but that certain cargos such as Cps1 failed to be temporally or spatially ubiquitinated properly (Figure 5c). This was surprising in view of previous data suggesting that Ubp2 antagonized various aspects of Rsp5 activity (Kee *et al.*, 2005). In particular, loss of Ubp2 enhanced the ability of Rsp5 to ubiquitinate and process the transcription factor Spt23. Our data indicated that loss of Ubp2 or Rup1 compromised Ub-dependent vacuolar sorting in a manner that mimicked loss of Rsp5 activity.

Ubp7 Counteracts Rsp5

The above data indicated that Rsp5 associates with Hse1 to help ubiquitinate cargo and thus increase the efficiency by which particular cargo proteins would undergo MVB sorting. Likewise, we wanted to determine whether Ubp7 acted in an opposing way, to perhaps deubiquitinate cargo, making sorting less efficient.

Disrupting *UBP7* alone had no affect on the sorting of GFP-Cps1, Ste3-GFP, Fth1-GFP-Ub, or Ub-GFP-Cps1 (Figure 6 and data not shown). However, because these reporter proteins are already sorted efficiently in wild-type cells, any increase in sorting efficiency afforded by loss of Ubp7 would be undetected. Therefore, we analyzed the effect of Ubp7 in cells where the efficiency of GFP-Cps1 sorting had been compromised by mutations affecting the ligase machinery. Figure 6 shows that although *ubp2*Δ mutants are defective in sorting GFP-Cps1 to the vacuole interior, additional loss of Ubp7 (*ubp2*Δ *ubp7*Δ) restored efficient sorting. Furthermore, although the *hua1*Δ *hse1*-Δ*PY* mutant showed defects in GFP-Cps1 sorting, proper sorting of GFP-Cps1 was restored upon further deletion of *UBP7*.

A Broader Role for Ubp2 in Ub-dependent Sorting

Experiments in Figures 5 and 6 showed the surprising result that Ubp2, which can antagonize some functions of Rsp5, actually boosts the efficiency of Cps1 sorting into the MVB. These results indicated that Ubp2 is somehow required for Rsp5 to be effective at ubiquitinating proteins for efficient entry into the MVB-sorting pathway. We next wanted to determine whether the effects of *UBP2* deletion or the effects of perturbing the interaction of Rsp5 with Hse1 were specific for MVB sorting or whether there were consequences for other Ub-dependent sorting steps. One such step is the Ub-dependent sorting pathway from the Golgi to the endosome taken by ubiquitinated proteins such as Gap1, Fur4, Tat2, and mutant forms of Pma1 (Beck *et al.*, 1999; Helliwell *et al.*, 2001; Blondel *et al.*, 2004; Pizzirusso and Chang, 2004). In poor nitrogen conditions, newly synthesized Gap1 is sorted to the plasma membrane where it stably resides to effect amino acid transport. In nitrogen-rich conditions, Gap1 is ubiquitinated and moves directly from the Golgi to endosomes where it is subsequently sorted into the MVB pathway (Roberg *et al.*, 1997). The Ub-dependent Golgi-to-endosome routing of Gap1 depends on the GGA coat proteins, which are thought to recognize ubiquitinated cargo proteins at the TGN and package them into clathrin-coated vesicles targeted to endosomes (Bilodeau *et al.*, 2004; Scott *et al.*, 2004). To test whether the Ub-dependent targeting of Gap1 to endosomes was compromised, we first measured sensitivity to the toxic proline-like compound ADCB, a crude measure of how much Gap1 is on the cell surface (Figure 7a). ADCB is transported by Gap1 and sensitivity to ADCB correlates with the level of Gap1 at the cell surface

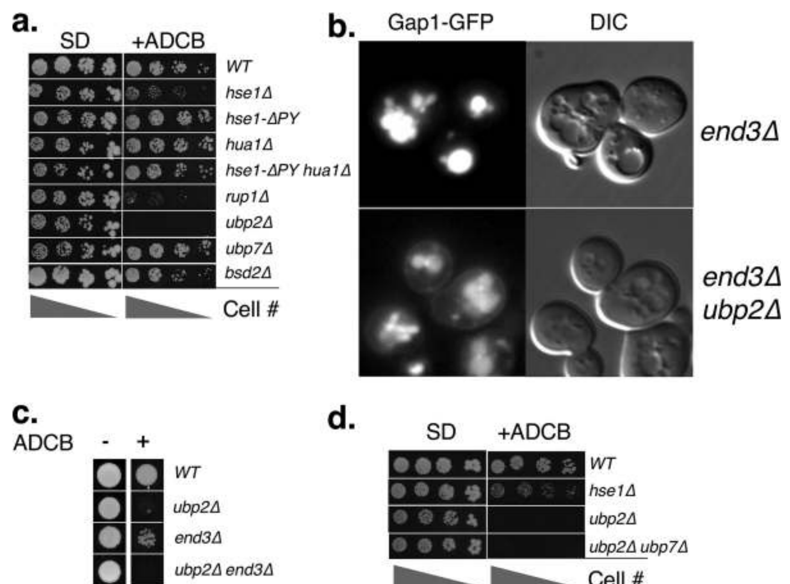

Figure 7. Rup1 and Ubp2 contribute to the ubiquitin-dependent trafficking of amino acid permeases. (a) Loss of Ubp2 or Rup1 renders cells sensitive to the proline analog ADCB. Wild-type (WT) and the indicated mutant cells were serially diluted and grown on plates containing SD alone or also containing 75 μg/ml ADCB. (b) Loss of *UBP2* results in some rerouting of Gap1 from the Golgi to the cell surface in nitrogen-replete conditions. The indicated mutant cells were transformed with a plasmid expressing Gap1-GFP under the control of the *CUP1* promoter. Cells were grown in YPD and induced for Gap1-GFP expression for 8 h with the addition of 100 μM $CuSO_4$. The *end3*Δ *ubp2*Δ cells showed a fraction of the Gap1-GFP at the cell surface, whereas *end3*Δ mutants did not. (c) Mutant *ubp2*Δ cells are more sensitive to ADCB than *end3*Δ mutant cells. The indicated strains were grown on SD plates with or without 75 μg/ml ADCB. (d) Loss of Ubp7 does not suppress the ADCB sensitivity of *ubp2*Δ mutants. The indicated strains were grown on plates containing SD alone or also containing 75 μg/ml ADCB.

(Hoshikawa *et al.*, 2003; Andreasson *et al.*, 2004). We found that that there was no difference in ADCB toxicity between wild-type cells and *hse1-ΔPY hua1Δ* double mutant cells, indicating that complex formation between Hse1 and Rsp5 was not required to prevent Gap1 sorting to the plasma membrane. These data indicate that the Hse1-Rsp5 interaction has a function restricted to endosomes, consistent with the localization of the Hse1-Vps27 complex and corresponding STAM-Hrs complex to endosomal compartments (Komada *et al.*, 1997; Bache *et al.*, 2003). We did observe a small increase in ADCB sensitivity in *hse1Δ* cells, a result expected because full disruption of *HSE1* leads to a "class E" phenotype, which can foster recycling of transporters such as Gap1 and Fur4 back to the cell surface (Nikko *et al.*, 2003; Bugnicourt *et al.*, 2004). We also found that loss of Bsd2, which interacts with Rsp5 and helps direct ubiquitination of a variety of ubiquitinated cargo (Hettema *et al.*, 2004; Stimpson *et al.*, 2006), also was slightly sensitive to ADCB. In contrast, we saw marked sensitivity to ADCB in strains lacking Rup1 or Ubp2. The ADCB sensitivity in *rup1Δ* and *ubp2Δ* strains could be explained by a loss in Ub-dependent Golgi-to-endosome sorting of Gap1, resulting in more initial deposition of Gap1 at the cell surface. Alternatively, ADCB sensitivity could result from stabilization of the fraction of Gap1 transporters that are transported to the cell surface when cells are grown in SD media.

To determine whether the Golgi-to-endosome route was affected, we analyzed the distribution of Gap1-GFP in *end3Δ* cells, in which internalization from the cell surface is blocked, and *end3Δ ubp2Δ* double mutant cells (Figure 7b). Cells were grown in nitrogen-rich YPD media to maximize ubiquitination and delivery of Gap1-GFP to endosomes. Under these conditions, *end3Δ* cells showed low to undetectable levels of Gap1-GFP fluorescence at the cell surface, consistent with previous results (Bilodeau *et al.*, 2004; Scott *et al.*, 2004). Instead, all the Gap1-GFP was delivered to the vacuole lumen. However, in *end3Δ ubp2Δ* cells Gap1-GFP was clearly observed at the cell surface in addition to the intravacuolar compartment, indicating that a fraction of Gap1 was misrouted to the cell surface in the absence of Ubp2. As further evidence, we compared the ADCB sensitivity of wild-type and *end3Δ* and *end3Δ ubp2Δ* cells. If increased ADCB sensitivity from loss of Ubp2 was caused only by inhibiting internalization of Gap1 from the cell surface, then loss of Ubp2 should not increase sensitivity beyond that of an *end3Δ* single mutant where internalization is essentially blocked (Raths *et al.*, 1993). Although the *end3Δ* mutation alone conferred some sensitivity to ADCB, both *ubp2Δ* and *end3Δ ubp2Δ* double mutants were far more sensitive to ADCB (Figure 7c). Thus, although collectively these results cannot eliminate the possibility that Ub-dependent internalization from the cell surface is also compromised by loss of Ubp2, they do show that at some level the Ub-dependent trafficking step from the Golgi to endosomes is less efficient in *ubp2Δ* and *rup1Δ* cells.

We also examined the effect of deleting *UBP7* on ADCB sensitivity. We found that loss of Ubp7 did not increase resistance to ADCB in either cells that were otherwise wild-type or *ubp2Δ* (Figure 7d) or *rup1Δ* cells (data not shown). These results suggested that although Ubp2 functions broadly in the post-Golgi/endocytic system at a different Ub-dependent sorting steps, Ubp7 plays a much more restricted role perhaps limited only to the endosomal MVB-sorting step.

DISCUSSION

One of the key steps in sorting ubiquitinated cargo proteins into the MVB lumen for degradation is their recognition by Ub-sorting receptors (Raiborg *et al.*, 2003; Gruenberg and Stenmark, 2004). One such receptor complex is the Hse1-Vps27 complex, whose mammalian equivalent is the Hrs-STAM complex. Both complexes localize to endosomes and interact with clathrin and other "class E" Vps proteins to effect MVB formation and protein sorting (Komada and Kitamura, 2005). Our data indicate that the Hse1 subunit can associate with both a Ub ligase and Ub peptidases and that this association is primarily directed at regulating the ubiquitination of cargo. Importantly, none of the alterations we made in the ubiquitination/deubiquitination machinery affected the general properties of the sorting machinery with regard to its ability to sort "constitutively" ubiquitinated cargo or other functions required for correct sorting of CPY (vacuolar protein sorting). Thus, although ubiquitination of the sorting machinery could still exist as a form of regulation or as part of a cycle of reactions that drives MVB sorting as has been proposed by various models (Polo *et al.*, 2002; Hicke and Dunn, 2003), we found no evidence that the ligase and DUb interactions described here contribute in such a manner. Rather, the association of the Hse1-Vps27 complex is important for a subset of cargo that requires ubiquitination and may serve to alter the ubiquitination status of cargo right at the site where it is recognized by the sorting receptor. This role for the DUbs studied here contrasts with that for Doa4, a endosome-associated deubiquitinating enzyme required to strip ubiquitin off of cargo after it has been recognized by Ub-sorting receptors and after it is committed to incorporation into the MVB lumen (Amerik *et al.*, 2000). Our data are consistent with a broader notion that a single ubiquitination event is not sufficient to carry a substrate protein all the way through a series of Ub-dependent trafficking steps to the lysosome interior (Urbe, 2005). Rather, a particular cargo protein may have to be repeatedly ubiquitinated at multiple compartments in order to complete its designated itinerary to the lysosomal/vacuolar interior. Indeed, our attempt to measure the extent of ubiquitination of GFP-Cps1 using published methods did not show any differences in the level of ubiquitinated GFP-Cps1 in our mutant strains (Supplementary Data). However, these assays can only examine the ubiquitination of the bulk of newly synthesized GFP-Cps1, which can be generated well before Cps1 arrives at the endosome. Currently, the tools to specifically examine the smaller but functionally relevant pool of cargo that is undergoing sorting at the endosome surface remain to be developed. Thus, the total level of ubiquitinated protein measured may not reflect the active pool that is undergoing the sorting process and that would be acutely modified by ligases and DUbs under the model we propose here. Such a model also invokes that there would be counteracting Ub-peptidases at multiple compartments that would compete with ligase activity over the final ubiquitination status and thus targeting of cargo proteins.

The association of Hse1 with Rsp5 is mediated in part by a PY element in the C-terminus of Hse1 and by the novel protein Hua1, which can bind directly and simultaneously to the WW domains of Rsp5 and the SH3 domain of Hse1. Elimination of both connections to Rsp5 was required to see a significant sorting defect for the MVB cargo protein GFP-Cps1 but not the Ub-fusion reporter Fth1-GFP-Ub of Ub-GFP-Cps1. Proper sorting of GFP-Cps1 is sensitive to loss of function of either the machinery responsible for ubiquitinating GFP-Cps1 as well as the sorting machinery itself that

recognizes Ub-cargo (Katzmann *et al.,* 2001, 2004). In contrast, Fth1-GFP-Ub, which contains an in-frame fusion of ubiquitin is sensitive only to compromised sorting machinery and not loss of ubiquitination machinery (Bilodeau *et al.,* 2002). The differential sensitivity of these two markers suggests that the association of Rsp5 with Hse1 helps promote ubiquitination of cargo at the correct time and place in the cell to increase the sorting efficiency of cargo into the MVB. These data imply that there is tight coupling between regulation of substrate ubiquitination and the recognition of ubiquitinated substrates, perhaps providing a failsafe mechanism for properly designating cargo for degradation.

Interestingly, Rsp5 is found heavily associated with clathrin (Kee *et al.*, 2005). Rsp5 has also been shown to localize to endosomal compartments in wild-type cells and exaggerated endosomes that accumulate in class E *vps* mutants (Wang *et al.*, 2001). We have found that the localization of Rsp5 to endosomes in class E mutants persists even in the absence of Hse1-Vps27 complex (data not shown). Thus, it may be that Rsp5 can associate with other endosomal proteins to affect specific ubiquitination events or be coupled directly with other clathrin-mediated Ub-dependent sorting events such as the GGA/clathrin-mediated sorting of Ub-cargo to endosomes directly from the TGN.

The association of Rsp5 with Hse1 may be functionally analogous to the association of other Nedd4-family ligases with the Hrs-STAM complex in animal cells (Marchese *et al.*, 2003). Here, the E3 ligase Itch associates with Hrs on early endosomes and modifies both cargo, namely CXCR4, and also the sorting machinery such as Hrs. Conceivably, the latter action could regulate the activity of Hrs by promoting intra- or intermolecular interactions, as has been proposed by others (Polo *et al.,* 2002; Hoeller *et al.,* 2006). With respect to the Rsp5-Hse1 interaction, our data indicate that the general sorting functions of the Hse1-Vps27 complex is intact and capable of properly sorting proteins that are properly ubiquitinated. Furthermore, we have not been able to detect ubiquitinated forms of Hse1 or Vps27 in wild-type or *ubp7*Δ mutant strains (data not shown). Thus, the function Rsp5 association with Hse1 may be restricted to only modifying cargo.

The Hrs-STAM complex can also associate with two DUbs via the SH3 domain of STAM, AMSH, and UBPY/USP8 (Urbe, 2005). AMSH is activated when bound to the STAM SH3 domain, and loss of AMSH increases lysosomal degradation of EGFR by allowing c-Cbl to sustain higher levels of ubiquitinated EGFR. Ubp7 appears to function analogously by associating with the SH3 domain of Hse1 to antagonize MVB sorting of Cps1. Disruption of Ubp7 could increase Cps1 sorting to the vacuole interior when Rsp5 activity was otherwise compromised. Other functions such as Gap1 sorting as inferred from ADCB sensitivity and vacuolar protein sorting were unaltered by loss of Ubp7, suggesting that Ubp7 plays a restricted role at endosomes to modify ubiquitinated cargo before sorting. It may also be possible that loss of Ubp7 fosters the association of Rsp5 with some other DUb that would partly suppress loss of Ubp2 or loss of Hse1 association. STAM also associates with UBPY, yet the role of this DUb is complex because its loss destabilizes the Hrs-STAM sorting complex (Row *et al.*, 2006; Mizuno *et al.*, 2006). A similar role for Ubp7 is not supported by our data.

Together these results suggest that Rsp5 associates with the Hse1-Vps27 sorting complex in order to "reubiquitinate" cargo and increase its efficiency of sorting. This provides a potential mechanism whereby the degradation of specific cargos could be regulated or the overall sorting efficiency of the complex is controlled by modulating association of Ubp7 or Rsp5. Indeed, our data suggest that the DUb Ubp7- and Rsp5-associated Hua1 proteins bind to the same SH3 domain interface, suggesting these two competing activities can also compete for occupancy on Hse1. Determining how the balance between ubiquitination versus deubiquitination might be controlled and whether this balance influences the general throughput of the MVB-sorting process or is largely specific to particular cargo has yet to be resolved. Altered expression levels of either the DUbs (McCullough *et al.*, 2004; Mizuno *et al.*, 2005) or ligases (Dunn *et al.*, 2004; Katzmann *et al.*, 2004) do affect MVB sorting and could represent a bona fide mode of regulating the complex. Alternatively, Hrs and STAM are known to interact with various signaling pathways that results in their phosphorylation (Row *et al.*, 2005) or association with Gαs (Zheng *et al.*, 2004), which could in turn affect their association with E3 ligases or DUbs.

Interestingly, we found that the complex between Rsp5, Rup1, and Ubp2 is required for efficient Ub-dependent trafficking of cargo not only at the MVB-sorting step but also at the Golgi. Because the sorting defects associated with loss of Rup1 or Ubp2 did not include a loss of sorting "constitutively" ubiquitinated cargo like Fth1-GFP-Ub or loss of vacuolar protein sorting, these results indicate that Ubp2 and Rup1 are required to properly ubiquitinate cargo before sorting. Thus, in contrast to its previously described role in antagonizing Rsp5 activity with regard to Ub-dependent processing of the Spt23 transcription factor (Kee *et al.*, 2005), physical linkage of Ubp2 to Rsp5 accentuates Rsp5's ability to effect Ub-dependent trafficking steps throughout the TGN/Endocytic pathway.

How Ubp2 accentuates Rsp5 activity specifically with regard to sorting and not other events is unclear. Other complexes between Ub ligases and DUbs have been noted, some of which may function to destabilize the DUb or to rescue the ligase from the effects of autoubiqitination. These explanations, however, are neither unlikely to apply to the function of the Rsp5-Rup1-Ubp2 complex in sorting. Rsp5 levels are not affected by loss of Ubp2 nor is the stability of Rsp5 altered when its potential for self ubiquitination is removed by mutation of its active site (Wang *et al.,* 1999; Kee *et al.,* 2005). Another possibility is that loss of Ubp2 might also shift more of the Ub pool into conjugates that might then deplete free ubiquitin levels. Still, these scenarios would not explain the opposite effects of *UBP2* deletion on Rsp5-dependent Spt23 processing and proteins sorting to the lysosome. We envisage three possibilities. One possibility would be that Rup1 and Ubp2 help recruit Rsp5 to a particular compartment, to a particular set of potential substrates, or to a particular sorting step. This model is similar to what has been proposed for other Rsp5-binding proteins such as Bsd2 complex, Bsd2-related proteins, and Tul1 (Reggiori and Pelham, 2002; Hettema *et al.,* 2004). A second possibility is that proteins such as Bsd2 or Bul1/2, which specify Rsp5 activity, may require Ubp2 for stabilization against Rsp5-mediated ubiquitination that could alter their effectiveness or stability. Interestingly, Rsp5-interacting proteins such as the Tre1-Bsd2 complex are ubiquitinated and degraded through their association with Rsp5 (Stimpson *et al.*, 2006). A third possibility may be that Ubp2 could specify how Rsp5 modifies its substrates. A similar model has been proposed for the Bul1/2 proteins that associate with Rsp5 to perhaps promote formation of polyubiquitin chains (Helliwell *et al.,* 2001). Rsp5 and Ubp2 show specificity for polymerization and depolymerization of K63 polyUb chains in vitro (Kee *et al.*, 2005). Furthermore, K63 linked ubiquitin chains have been found on a variety of ubiquitinated membrane proteins that undergo lysosomal degradation (Hicke and Dunn, 2003).

Perhaps, Ubp2 might help trim K63-linked polyubiquitin chains on Ub-cargo to facilitate binding and processing by the Ub-recognition machinery at various sorting sites within the post-Golgi/endocytic pathway. Indeed, recent experiments have shown that loss of UBP2 results in aberrant accumulation of K63 polyubiquitin chains, which appear detrimental for proper sorting of Gap1 (Kee *et al*., 2006).

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