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The Ubiquitin Ligase Rsp5p is Required for the Modification and Sorting of Biosynthetic Membrane Proteins into Multivesicular Bodies in *S. cerevisiae*

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Precursor forms of vacuolar proteins with transmembrane domains, such as the carboxypeptidase S Cps1p and the polyphosphatase Phm5p, are selectively sorted in endosomal compartments to vesicles that invaginate, budding into the lumen of the late endosomes, resulting in the formation of multivesicular bodies (MVBs). These proteins are then delivered to the vacuolar lumen following fusion of the MVBs with the vacuole. The sorting of Cps1p and Phm5p to these structures is mediated by ubiquitylation, and in a *doa4* mutant, which has reduced level of free ubiquitin, these proteins are missorted to the vacuolar membrane. A RING-finger ubiquitin ligase Tul1p has been shown to participate to the ubiquitylation of Cps1p and Phm5p. We show here that the HECT-ubiquitin ligase Rsp5p is also required for the ubiquitylation of these proteins, and therefore for their sorting to MVBs. Rsp5p is an essential ubiquitin ligase containing an N-terminal C2 domain followed by three WW domains, and a C-terminal catalytic HECT domain. In cells with low levels of Rsp5p (*npi1* mutant cells), vacuolar hydrolases do not reach the vacuolar lumen and are instead missorted to the vacuolar membrane. The C2 domain and the second WW domain of Rsp5p are important determinants for sorting to MVBs. Removal of the Bul proteins, two components of the Rsp5p ubiquitin ligase complex, also impairs the sorting of these proteins, but to a lesser extent. Ubiquitylation of Cps1p was strongly reduced in the *npi1* mutant strain and ubiquitylation was completely abolished in the *npi1 tul1* double mutant. These data demonstrate that Rsp5p plays a novel and key role in intracellular trafficking, and extend the currently very short list of substrates ubiquitylated *in vivo* by several different ubiquitin ligases acting cooperatively.

Introduction

Endosomes play a crucial role in coordinating vesicular transport between the trans-Golgi network (TGN), the plasma membrane and the lysosome/vacuolar organelles. Endosomes coordinate the transport of newly synthesized lysosomal/vacuolar proteins arriving from the TGN (1). They also separate macromolecules - including lipids and proteins - taken up by endocytosis and destined for degradation in lysosomal/vacuolar organelles from molecules that are either recycled back to the cell surface or routed toward other intracellular destinations. The delivery of biosynthetic and endocytic membrane proteins to the lysosomal/vacuolar lumen requires the prior sorting of these molecules to the multivesicular body (MVB) pathway (2, 3). MVBs arise from endosomes by invagination and budding of the endosomal membrane into the lumen to form internal vesicles. The proteins sorted into the MVBs are ultimately delivered to the lysosomal/vacuolar lumen following the fusion of the MVBs with these organelles. The

proteins that do not enter MVBs are either removed by conventional vesicular transport or become constituents of the vacuolar limiting membrane. Ubiquitin post-translationally appended to proteins may act as a sorting signal at various steps in the endosomal and biosynthetic pathways (4). Ubiquitin is a 76-amino acid protein found in all eukaryotic organisms and cell types. It is conjugated to an internal lysine residue of the protein substrate by means of a three-step cascade mechanism. First, a ubiquitinactivating enzyme, E1, activates ubiquitin in an ATP-dependent reaction. Ubiquitin is then transferred to the active-site cysteine of a ubiquitin-conjugating enzyme (E2). Finally, a ubiquitin protein ligase (E3) catalyzes the transfer of ubiquitin from E2 to the substrate. E3s are proteins or protein complexes that play a key role in protein ubiquitylation because they serve as the specific recognition factors of the system. E3s are heterogeneous, but may be classified into two major groups — HECT domain and RING finger-containing E3s — and several other minor groups (5, 6). Most E3 ubiquitin ligases are RING

finger-containing proteins. RING-finger E3s coordinate the direct transfer of activated ubiquitin from E2 to the E3-bound substrate. With HECT- (homologous to E6-AP COOH terminus) domain E3 enzymes, the ubiquitin is transferred from E2 to the active site of E3 and is then transferred to the ligase-bound substrate.

Ubiquitin was recently shown to be important for MVB sorting, as the entry of several proteins into MVBs depends on their prior ubiquitylation (7-9). Class E VPS (Vacuole Protein Sorting) proteins are required for protein sorting in the MVB pathway. Some of these proteins are organized into three distinct ESCRT (Endosomal Sorting Complex Required for Transport) protein complexes that are required for MVB biogenesis and the sorting of ubiquitylated membrane proteins to the MVBs (10). The Vps27-Hse1 complex recognizes ubiquitylated MVB cargoes and recruits the ESCRT-I complex to the endosomes (11-13). The ESCRT-I complex is involved in the recognition of ubiquitylated proteins targeted into the MVBs. ESCRT-II and -III function downstream from ESCRT-I and play a role in the concentration and sorting of MVB cargoes. The ubiquitin isopeptidase Doa4p is recruited by ESCRT-III for the recycling of ubiquitin molecules from ubiquitin-tagged MVB cargoes after the initial ubiquitin-dependent sorting step is completed. Ubiquitin has been shown to play a direct role in the MVB pathway in studies of two yeast vacuolar hydrolases: the carboxypeptidase S Cps1p and the polyphosphate endophosphatase Phm5p (7, 8). Both proteins are synthesized as a type-II transmembrane precursor with a short cytoplasmic extension. These precursor forms undergo ubiquitylation on leaving the Golgi compartment (7), resulting in their selective sorting to multivesicular bodies (MVBs). They are then delivered, in a membrane-bound form, to the interior of the vacuole, where vacuolar proteases cleave the precursor molecules to release the soluble forms of the active hydrolases. In cells lacking the ubiquitin isopeptidase Doa4p and therefore with low free intracellular ubiquitin levels, poorly ubiquitylated MVB cargoes are not sorted to the internal vesicles of the MVBs. They ultimately reside in the limiting membrane of the vacuole, following the fusion of MVBs with the vacuole. Reggiori and Pelham identified a transmembrane RING-finger E3 ligase, Tul1p, which resides in the Golgi apparatus and is required, together with the E2 enzyme Ubc4p, for the ubiquitylation of proteins with polar TMDs, including vacuolar proteins such as Cps1p and Phm5p. In *tul1* mutant cells, Cps1p and Phm5p are missorted to the

vacuolar limiting membrane (14). The elimination of Tul1p reduced the ubiquitylation of Cps1p by a factor of four to five but did not abolish it completely (14). The authors concluded that at least one other E3 ligase must also be capable of acting on these proteins.

Rsp5p is the only member of the Nedd4 sub-family of HECT E3 ligases in yeast and is essential for cell viability. It has a multitude of functions and acts on many substrates at a number of sites throughout the cell (15, 16). Rsp5p has been shown to play several roles in endocytosis at the plasma membrane (17, 18). It has been shown to be located at multiple sites within the endocytic pathway, suggesting that it may function at multiple steps in the ubiquitin-mediated endocytosis pathway (19). Recent reports have suggested that, depending on nutritional conditions, cell surface transporters are directly sorted from the Golgi to the endosomal system for premature degradation in the vacuole. This pathway requires the prior modification of these transporters by ubiquitylation (20-23). The E3 ligase responsible for this ubiquitylation was shown to be Rsp5p (21-23). Given the importance of Rsp5p-dependent ubiquitylation for the direct routing of transporters to the MVB pathway, we investigated the potential role of Rsp5p in modification of the vacuolar hydrolases Cps1p and Phm5p for correct sorting in the endosomal pathway.

Experimental procedures

Strains, plasmids, media and growth conditions
The S. *cerevisiae* strains used in this study are listed in Table 1. The *doa4* mutant strains used for purification of the His6-tagged ubiquitylated proteins were derived from strain MHY501 (24) or from strain 27061b (25). *npi2* is a specific allele of *DOA4* that carries a point mutation affecting a conserved residue, resulting in the same phenotype as complete *DOA4* deletion (26). Yeast strains from which the *DOA4* or *TUL1* gene had been deleted were obtained by replacing the entire coding region of the gene to be deleted with the geneticin resistance gene *KanMX4* (27). An ORF replacement cassette with long flanking homology regions was amplified by PCR and used to transform yeast, as described elsewhere (28). We used PCR analysis of chromosomal DNA to confirm integration of the *KanMX4* marker into the correct loci in geneticin-resistant cells. We constructed a JM06 strain in which both the *DOA4* and *TUL1* genes were deleted, using a gene disruption cassette designed for repeated use (29). We used pUG6 as a template to generate the *loxP doa4::KanMX4 loxP*, and *loxP tul1::KanMX4 loxP* DNA fragments, which

were then used for the sequential transformation of yeast. *In vivo* excision of the *KanMX4* cassette was achieved by transforming yeast cells with pSH47, which carries the Cre recombinase gene, and inducing expression for 2 h. The *RSP5* gene was replaced by the mutant allele *rsp5 C2* by the Pop-In/Pop-Out replacement method (30), as described below, except that we used 5- FOA rather than ureidosuccinic acid to select against the *URA3* gene for plasmid excision. The *rsp5 C2* ORF was obtained from pRS414-HA-*rsp5 C2* (19) as a *Sac*I-*Cla*I DNA fragment, and inserted into the pRS306 integrative vector (31). The plasmid was linearized by digestion with *Age*I (single site in the C-terminal part of the *RSP5* gene) and integrated into the *RSP5* locus by homologous recombination. This created a duplication containing the wildtype and the mutant that contains the *URA3* selectable marker. Excision of the plasmid was selected using 5-FOA, and the 5-FOA-resistant colonies were screened for the mutant phenotype by colony PCR and immunoblotting.

The centromeric plasmids pRS416, p*TPI1*-*GFP-CPS1*, pRS416, p*TPI1-GFP-PHM5* and pRS416, p*TPI1-UBI-GFP-PHM5* are a gift of H. Pelham (8). The multicopy plasmid Yep96-6His, p*CUP1-6His-Ub, TRP1* was a gift of S. Dupré (our laboratory).

Cells were transformed by the lithium method, as modified by Gietz *et al*. (32). Cells were grown at 30°C in YPD medium (1% yeast extract, 2% peptone, 2% glucose) or in YNB minimal medium containing 0.5% ammonium sulfate, 0.17% yeast nitrogen base (Difco, MI, USA), 2% glucose and supplemented with appropriate nutrients. His6-tagged ubiquitin was overproduced under control of the *CUP1* promoter by growing the cells for three hours in the presence of 0.1 mM CuSO4.

Fluorescence microscopy and vacuole staining

Cells grown to exponential growth phase in YNB or YPD medium were added with 5 μM Cell Tracker Blue CMAC (10 mM stock solution in DMSO, Molecular Probes, Eugene, Oregon, USA) and incubated for 15 min at 30°C. They were then washed with YNB or YPD media, and concentrated by a factor of ten by centrifugation. Cells were viewed immediately, without fixation, under a fluorescence microscope (type BY61, Olympus, Tokyo, japan) and images captured with a digital camera. The results presented are based on observations of >50 cells. To visualize vacuolar membrane, living cells were labeled with the fluorescent lipophilic dye FM4-64 (Molecular Probes, Eugene, Oregon, USA). Labeling was performed as previously described (33) and the cells were observed by fluorescent microscopy.

Protein extracts and Western blotting

Cell extracts were prepared and proteins analyzed by immunoblotting as previously described (34), except that proteins in sample buffer were heated for 10 min at 95°C. Membranes were probed with monoclonal anti-GFP antibody as the primary antibody (Roche diagnostics, Meylan, France), and then with horseradish peroxidase-conjugated anti-mouse immunoglobulin G as the secondary antibody (Sigma, MI, USA). Antibody binding was detected with the enhanced chemiluminescence (ECL) technique.

His6-tagged ubiquitylated protein purification

doa4 cells producing GFP-Cps1p and 6His-Ub under control of the *CUP1* promoter (pJD421) were grown overnight in selective medium to mid-exponential growth phase, then incubated with 0.1 mM CuSO4 for three hours. 6-7 x 108 cells were harvested by centrifugation at 4°C in the presence of 10 mM sodium azide. Cells were washed once with cold water supplemented with 10 mM sodium azide and resuspended in 1.2 ml of cold lysis buffer (50 mM Tris-HCl pH7.4; 150 mM NaCl plus a mixture of EDTA-free protease inhibitors ("Complete" from Roche diagnostics, Meylan, France) and 25 mM freshly prepared Nethylmaleimide (NEM) to prevent artifactual deubiquitylation. Cells were disrupted in a "One Shot" Cell Disrupter (Constant Systems LDT, Daventry, UK) at maximum pressure of 2.7 kbars. The disrupted cells were centrifuged twice (3000 x g, for 3 min, at 4°C) to remove unbroken cells and the resulting lysate was subjected to centrifugation at 13,000 x g for 30 min to generate the supernatant and pellet fractions. The pellet was resuspended in 300 μl of buffer A (lysis buffer supplemented with 5 mM imidazole, 0.1% SDS and 1% Triton X-100). The suspension was incubated on ice for 30 min, then diluted by adding 300 μl of buffer B (lysis buffer supplemented with 5 mM imidazole and 1% Triton X-100) and centrifuged for 10 min at 13,000 x g to remove the remaining non soluble material. The supernatant was then passed through 200 μl Ni-NTA Superflow resin (Qiagen Inc., Hilden, Germany) packed into a disposable polypropylene column (Mini Bio-Spin chromatography columns, BIORAD). The unbound fraction was collected and the resin was washed three times with 200 μl of buffer B. The His6-tagged ubiquitylated proteins were eluted by three passages of 200 μl of elution buffer (50 mM Tris-HCl pH7.4; 150 mM NaCl; 200 mM imidazole) through the column. At each step, a 5 μl aliquot was withdrewed for western blot analysis.

Results

Delivery to the interior of the vacuole is inhibited in *npi1/rsp5* mutant cells

We investigated the potential role of Rsp5p in the sorting of biosynthetic vacuolar enzymes by analyzing the subcellular distribution of previously described fusion proteins in which Cps1p or Phm5p was fused to GFP (8) in an *npi1* mutant strain, *npi1* being a mutant allele of *RSP5* (35). The fully viable *npi1/rsp5* mutant strain displayed low levels of *RSP5* expression, due to a TY1 insertion 500 bp upstream from its open reading frame. Using polyclonal antibodies against mNedd4-2, the mouse homolog of Rsp5p (a gift from O. Staub), we were able to confirm that this mutant produced only small amounts of Rsp5p; *npi1* cells contain less than one tenth the amount of Rsp5p present in wild-type cells ((36) and Fig. 1B). All the other variants of Rsp5p used in this study and affecting specific domains of the protein produced similar amounts of Rsp5p relative to wild-type parental cells, as shown by the intensity of the non-specific band of higher mobility detected by the antibodies (Fig. 1B). GFP-tagged versions of Cps1p and Phm5p were first produced in wild-type cells of various genetic backgrounds. As expected, in cells with the wild-type ubiquitin machinery, GFP-Phm5p was delivered to the lumen of the vacuole, resulting in GFP fluorescence in the vacuole lumen, as shown by the identical vacuolar staining obtained with the dye CMAC (Fig. 2A). Surprisingly, the results were less convincing with GFP-Cps1p and depended on the genetic background. GFP-Cps1p was present within the vacuole in the *npi1* parental strain (Fig. 2A) but was also found partitioned between the lumen and the limiting membrane of the vacuole or even predominantly on the vacuolar membrane in other genetic backgrounds (data not shown). Thus, for reasons of clarity, we will essentially present the results obtained with GFP-Phm5p.

As previously shown, GFP-Phm5p was missorted to the limiting membrane of the vacuole in cells lacking the ubiquitin-conjugating enzyme (E2) Ubc4p and the ubiquitin ligase (E3) Tul1p ((14) and Fig. 2C). In contrast, a protein consisting of a single non-removable ubiquitin fused at the N-terminus of GFP-Phm5p (Ub-GFP-Phm5p) still entered the vacuole in these mutant cells, indicating that Ubc4p and Tul1p play some role in tagging biosynthetic cargoes for entry into MVBs. If GFP-Cps1p or GFP-Phm5p were produced in *npi1* mutant cells, the luminal signals were largely replaced by a strong signal on the edge of the vacuole (Fig. 2A). GFP-Phm5p also strongly labeled patches ajacent to the vacuole, presumably prevacuolar endosomes. This pattern of prevacuolar endosome staining was reminiscent of a VPS class E phenotype, in which MVB formation is blocked, leading to the accumulation of abnormally enlarged prevacuolar compartments, known as the class E compartment (37, 38). Thus, Rsp5p may be required for MVB formation and/or for the ubiquitylation of certain biosynthetic enzymes resulting in the sorting of these enzymes into MVBs. The class E compartment can be visualized directly by FM4-64 staining (39). We observed that no class E comparment could be visualized by FM4-64 staining of *npi1* mutant cells (data not shown). Moreover, Ub-GFP-Phm5p was correctly targeted to the lumen of the vacuole in *npi1* mutant cells, with staining almost entirely disappearing from the prevacuolar compartments (Fig. 2A). Taken together, these data suggest that the low level of production of Rsp5p in *npi1* cells did not disturb MVB formation, but probably limited the tagging of biosynthetic cargoes for entry into them.

The requirements of individual domains of Rsp5p for sorting into MVBs differ

Rsp5p is a modular protein with an N-terminal C2 domain that may interact with membranes (by binding to lipids or membrane proteins), three WW domains involved in protein-protein interactions with proline-rich peptides and a C-terminal catalytic HECT domain (Fig. 1A). We analyzed the contributions of C2 and each of the WW domains to the sorting of vacuolar cargoes. The C2 domain is a major determinant of the location of Rsp5p, not only at the plasma membrane but also within endocytic compartments (19). This made it possible to investigate the role of the C2 domain in the multivesicular sorting of Phm5p chimeras. In contrast to what was observed in wild-type cells, in the majority of *rsp5 C2* mutant cells, the luminal distribution of GFP-Phm5p was replaced by an increase in the signal emanating from the vacuolar surface and the prevacuolar compartments (Fig. 2B), indicating the probable involvement of the C2 domain in the sorting of cargoes to the internal vesicles of MVBs. As observed for *npi1* cells, Ub-GFP-Phm5p was targeted to the interior of the vacuole in *rsp5 C2* mutant cells, indicating that removal of the C2 domain of Rsp5p did not affect MVB formation but did prevent the tagging of biosynthetic cargoes for entry into the MVBs.

There is now a considerable body of data to suggest that the tryptophan-rich WW domains of Rsp5p, or a subset of these domains, are involved, directly or indirectly, in substrate recognition (16, 40, 41, 42, 43, 44). We investigated the role of each of the WW domains of Rsp5p, using *rsp5* null cells expressing either wild-type *RSP5* or *RSP5* with mutations affecting one of the WW domains, from

centromeric plasmids. The *rsp5-w1*, *rsp5- w2*, and *rsp5-w3* mutant alleles correspond to individual mutations of the WW domain that abolish the interaction of Rsp5p WW domains with other proteins by altering two conserved amino acids (41). Strikingly, GFP-Phm5p was found essentially on the vacuolar limiting membrane in cells expressing *rsp5-w2* (Fig. 2B). This was not the case in cells expressing *rsp5-w1* or *rsp5-w3,* in which GFP-Phm5 was sorted to the vacuolar lumen, as in cells producing wild-type Rsp5p. Therefore, only the second WW domain of Rsp5p appears to be important for the sorting of cargoes to the internal vesicles of MVBs. In contrast, Ub-GFPPhm5p entered the vacuole in *rsp5-w2* mutant cells, indicating that the WW2 domain of Rsp5p is also important for the tagging of biosynthetic cargoes for entry into MVBs.

BUL1 and BUL2 requirement for sorting to MVBs

BUL1 and *BUL2* encode two components of the Rsp5p ubiquitin ligase complex (45, 46). The PPXY motif of Bul1p interacts with the WW domains of Rsp5p (46). Bul1p has been shown to assist Rsp5p in several of its many functions in the cell (22, 23, 47-49, 50). Although we know little about their function, it has been suggested that Bul1p and Bul2p are involved in the Golgi to vacuole pathway. We analyzed the effect of the *bul1 bul2* double deletion on the sorting of Phm5p chimeras (Fig. 2C). If GFP-tagged Phm5p was produced in *bul1 bul2* cells, GFP stained both the interior and the limiting membrane of the vacuole. In contrast, GFP stained exclusively the interior of the vacuole if Ub-GFP-Phm5p was produced. Thus, Bul proteins are, at least to some extent, required for the tagging of biosynthetic cargoes for entry into the MVBs. These data exemplify the role played by these adaptor proteins in ubiquitylation and protein trafficking.

Rsp5p is required for the modification of Cps1p with ubiquitin

We analyzed the potential role played by Rsp5p in the ubiquitylation of biosynthetic cargoes by introducing chimeric proteins into cells devoid of Doa4p activity. A lack of Doa4p function results in the depletion of free ubiquitin, interfering with all ubiquitylation processes (51), including the selective sorting of ubiquitin-tagged MVB cargoes. If GFP-tagged versions of Cps1p or Phm5p were produced in *doa4* mutant cells, these proteins were found predominantly on the vacuolar membrane rather than within the vacuole (8), unless the cells were replenished with a new pool of free ubiquitin. We produced His6-tagged ubiquitin from a multicopy plasmid in *doa4* cells, making it possible to detect ubiquitylated proteins specifically based on their

acquisition of the His6 tag. The absence of Doa4p should also result in the stabilization of His6-Ub-cargoes because the function of Doa4p is to remove ubiquitin from cargoes destined for entry into the MVB pathway.

For numerous years the biogenesis of Cps1p is well characterized (3, 52). Cps1p and Phm5p were recently used as model proteins in studies of the role of ubiquitin modification as a sorting signal in the MVB pathway. GFP-Cps1p was shown to be labeled with ubiquitin on one residue, Lys8 in the small cytosolic tail, on its departure from the Golgi (7) whereas the ubiquitylation of GFP-Phm5p involved the four lysine residues in the cytoplasmic extension of this protein (8). To facilitate interpretation, we analyzed the ubiquitylation of GFP-Cps1p. Experiments were carried out in cells lacking Doa4p activity producing recombinant His6- tagged ubiquitin under the control of the CUP1 promoter. Cells were grown to midexponential growth phase and His6-tagged ubiquitin production was induced by adding 100 µM copper for the last three hours before harvest. After lysis, total cell extracts were subjected to electrophoresis and GFP-Cps1p was detected by immunoblotting with anti-GFP antibodies (Fig. 3). The electrophoretic pattern obtained from the extract of wild-type cells showed, in addition to the main GFP-Cps1p band, a set of essentially two bands less mobile than the main GFP-Cps1p signal, with the upper band fainter than the lower band (Fig. 3A). These bands probably corresponded to the chimera conjugated with ubiquitin as they were much fainter in cells in which the *UBC4* and *TUL1* genes had been deleted. These bands were also much fainter in extracts from *npi1* cells. They were not detected at all in lysate from *npi1* cells from which *TUL1* had also been deleted, suggesting that the removal of both ligases abolished the ubiquitylation of Cps1p. The doublets corresponding to the Cps1p and Ub-Cps1p bands, resulting from differential glycosylation of Cps1p in the lumen of the endoplasmic reticulum during its biogenesis (52), were difficult to separate in our conditions, using a GFP-Cps1p fusion protein with a molecular weight of 86 kDa.

To demonstrate directly that *npi1* affects the ubiquitylation status of GFP-Cps1p, protein extracts corresponding to equivalent numbers of wild-type and mutant cells were subjected to cell fractionation; the membrane fractions were solubilized and loaded on a Ni- NTA column. Aliquots from the solubilized pellet (S) from wild-type cells, together with corresponding aliquots of bound (B) and unbound material (NB), were resolved by electrophoresis, subjected to western blotting and the proteins of interest detected with anti-GFP antibodies (Fig. 3B). The electrophoretic pattern obtained with the solubilized

pellet was similar to that shown in Fig. 3A. Unbound material corresponded exclusively to the main, non-ubiquitylated form of GFP-Cps1p. Ubiquitylated bands were specifically retained on nickel columns. Above a faint band corresponding to residual retention of the core GFP-Cps1p, two slower migrating bands were specifically retained, corresponding to GFP-Cps1p modified with ubiquitin moieties. The mobilities of these bands are consistent with the lower band having a single added ubiquitin and the upper band having two added ubiquitins, as the molecular weight of the protein shifts by about 10 kDa in each case. Fig. 3C shows aliquots of bound and unbound fractions corresponding to equivalent amounts of solubilized membrane from *npi1* cells and cells from which *TUL1* or *UBC4* was deleted. We found that *npi1* cells were highly deficient in ubiquitin conjugates because the bands corresponding to these conjugates were barely detectable even if we quadrupled the amount of the bound fraction loaded on the gel. Cells from which *TUL1* or *UBC4* was deleted displayed similar large deficits in ubiquitin conjugates.

Previous work has shown that the ubiquitin-conjugating enzyme E2 required for the ubiquitylation of Cps1p is Ubc4p and that Ubc4p interacts physically with Tul1p (14). It has been suggested that Rsp5p receives ubiquitin from the ubiquitin-conjugating enzymes Ubc4p and Ubc5p enzymes (53, 54). Ubc5p is almost identical in sequence to Ubc4p and is produced following the exposure of cells to environmental stress (55). If Ubc4p and Ubc5p act in concert with Rsp5p to mediate the ubiquitylation of Cps1p, then the absence of Ubc4p should impair most, but not all, Cps1p ubiquitylation. This is indeed what was observed (Fig. 3C). We were unable to test a triple *ubc4 ubc5 doa4* mutant because this strain was not viable (not shown). Nonetheless, in contrast to previous claims (14), our results suggest that Ubc4p is not the only E2 required for the ubiquitylation of Cps1p, although it is probably the principal E2 involved in this process.

Discussion

Recruitment of vacuolar hydrolases to the lumen of the vacuole requires prior ubiquitylation by Rsp5p

We analyzed in detail the mechanism of ubiquitylation of two vacuolar hydrolases Cps1p and Phm5p. Our results suggest that Rsp5p promotes the ubiquitylation and sorting of these two cargoes to MVBs. These proteins were sorted to the vacuolar pathway but did not enter the MVBs in an *npi1* mutant strain, which produces limiting amounts of Rsp5p. The fusion of an ubiquitin moiety in-frame to GFP-Phm5p overcame this sorting defect, suggesting

that Rsp5p is required for the efficient ubiquitylation of Phm5p. Moreover, deletion of the C2 domain of Rsp5p, which has been described as a critical element controlling the targeting of the protein to both the plasma membrane and endosomal compartments (19), also disrupted sorting, suggesting that association with the membrane is required for the efficient ubiquitylation of biosynthetic cargoes. The second WW domain of Rsp5p is also required for the ubiquitylation and sorting of biosynthetic cargoes to MVBs. The WW domains of Rsp5p are type I WW domains and are therefore thought to interact with PPxY motifs. Bul1p, a protein that binds to Rsp5p, interacts physically with Rsp5p via its PPxY motif (45, 46). Our results are consistent with Bul proteins playing some role in the direct sorting of biosynthetic cargoes to the MVB pathway. The role played by Rsp5p in the ubiquitylation and sorting of these hydrolases to the MVBs was demonstrated biochemically by the very low levels of GFP-Cps1p ubiquitylation in *npi1* mutant cells. Of course, we cannot exclude the possibility that Rsp5p also has other effects on the endosomal sorting machinery because some Vps genes in yeast and mammals have been shown to undergo ubiquitin modification and Rsp5p, in particular, is required for the monoubiquitylation of Vps9p, a protein required in the yeast endocytic pathway (56).

A unique function for two ubiquitin-protein ligases

Very low levels of GFP-Cps1p ubiquitylation have also been observed in a mutant strain lacking a newly described E3, Tul1p (14). The Golgi-resident RING-domain ubiquitin ligase, Tul1p has been shown to ubiquitylate proteins with a polar transmembrane domain, and sort them to MVBs. RING-finger ubiquitin ligases are not enzymes as such. They are thought to provide a platform for the simultaneous binding of E2 and substrate, thereby enhancing ubiquitin transfer. The RING-finger domains found in many E3s are essential for the ability of these molecules to "catalyze" substrate ubiquitylation. Pelham's group showed that the Tul1p RING-finger protein is required *in vivo,* because the truncation of Tul1p, removing the RING domain, abolished the ability of the protein to mediate the sorting of Phm5p to the vacuole (14). This domain was also shown biochemically to bind the E2 enzyme Ubc4p.

These data strongly suggested that Tul1p is an E3 ubiquitin ligase for Cps1p and Phm5p. However, it has not yet been directly demonstrated that the Tul1p RING-finger protein has ubiquitin ligase activity *in vitro*. The deletion of *TUL1* and mutations in *RSP5* strongly reduced the ubiquitylation

of Cps1p. Thus, Rsp5p clearly cannot substitute for Tul1p, and *vice versa*. Instead, our data suggest that these two E3 ligases of different classes may cooperate to ubiquitylate vacuolar hydrolases for sorting to MVBs. We suggest that Rsp5p participates in a Ubc4p/Tul1p functional complex, as Cps1p ubiquitylation is abolished in the *npi1/tul1* double mutant. Although RING-finger E3 and HECT E3 have, until now, been considered to function independently, the precise mechanism underlying substrate ubiquitylation remains unclear. The coordinated action of two members of distinct E3 classes may increase the efficiency of the ubiquitylation process. Tul1p may be recognized directly or indirectly by the WW domains of Rsp5p. Alternatively, the two ligases may be in close vicinity as one is a transmembrane protein and the other is associated with the membrane via its C2 domain. We suggest that there is a non-canonical E3-E3 interaction between Tul1p and Rsp5p, both ligases being able to associate with Ubc4p. This association may facilitate the ubiquitylation of vacuolar hydrolases. Alternatively, one of the two E3 may activate the other by ubiquitylation and, indeed, both Tul1p and Rsp5p have been shown to be ubiquitylated *in vivo* (57). Recent reports connecting a RING and a HECT E3 ligase support the notion that several E3 ligases of different structures may interact in numerous signaling pathways. It has been shown that E3 ligases of the RING and HECT families may share a common substrate and could therefore cooperate in its ubiquitylation (58-61). It has been also shown that the ubiquitylation and downregulation of the Tyr-kinase receptor EGFR involve a physical interaction between two E3 ligases: the human AIP4/Itch HECT ligase and the RING-finger ubiquitin ligase Cbl (61). Our data extend the short list of substrates known to be ubiquitylated *in vivo* by several different ubiquitin ligases.

The E3 proteins are thought to be largely responsible for the high degree of specificity in substrate recognition. It is still unclear how Rsp5p ubiquitylates its many substrates, particularly Cps1p and Phm5p, in the absence of PPxY motif. Several other examples of proteins interacting with the E3 members of the Nedd4 sub-family that lack this recognition motif have been discovered in yeast and mammals (17, 61) and references therein). It has been suggested that the tranmembrane part of Tul1p directly recognizes the single polar transmembrane domains of its substrate proteins (14). One interpretation for our results is that Tul1p is required for bridging the interaction between biosynthetic polar transmembrane proteins and the HECT ligase Rsp5p. In other words, Tul1p may act as an adaptor

for Rsp5p and its putative targets, to control ubiquitin-dependent protein sorting and trafficking. Tul1p has not been conserved during evolution, but other transmembrane proteins may display a similar distribution and function in higher eukaryotic cells (62). The process concerned would differ from the recently described process for the ubiquitylation and direct routing of the uracil permease Fur4p to the MVBs in adverse conditions. The membrane-spanning regions of transporters or permeases also contain a few polar or charged amino acids, but they may form charge-neutralizing pairs, thereby stabilizing the folding of the protein in a hydrophobic environment ((63) and ref therein). These proteins would escape Tul1p recognition. Indeed, Fur4p which is directly routed to MVBs was recently shown to be ubiquitylated by Rsp5p, which also ubiquitylates Fur4p at the plasma membrane and Tul1p is not involved in this process (21, 25).

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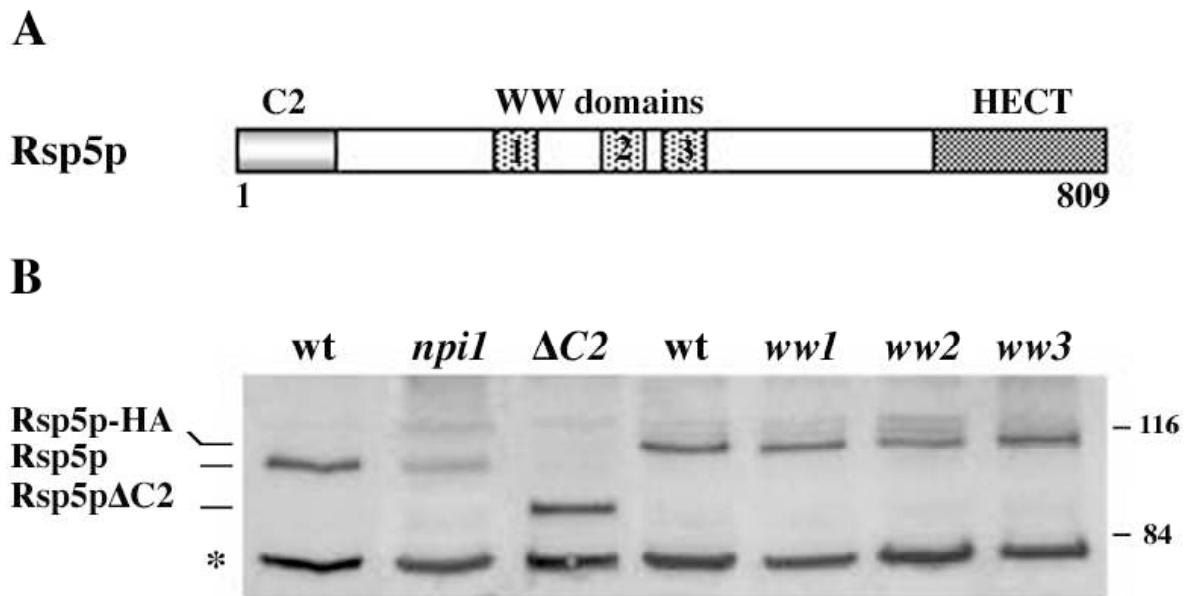
Figure 1.
Western blot analysis to check the levels of mutant versions of Rsp5p produced
A.Schematic representation of Rsp5p.
B.Protein extracts were prepared from cells grown to mid-exponential growth phase in appropriate medium. Proteins were resolved by SDS polyacrylamide gel electrophoresis and analyzed by immunoblotting with anti-Nedd4 antibodies (64). The signal corresponding to an non-specific band is marked with an asterisk. Molecular markers are indicated in kDa.
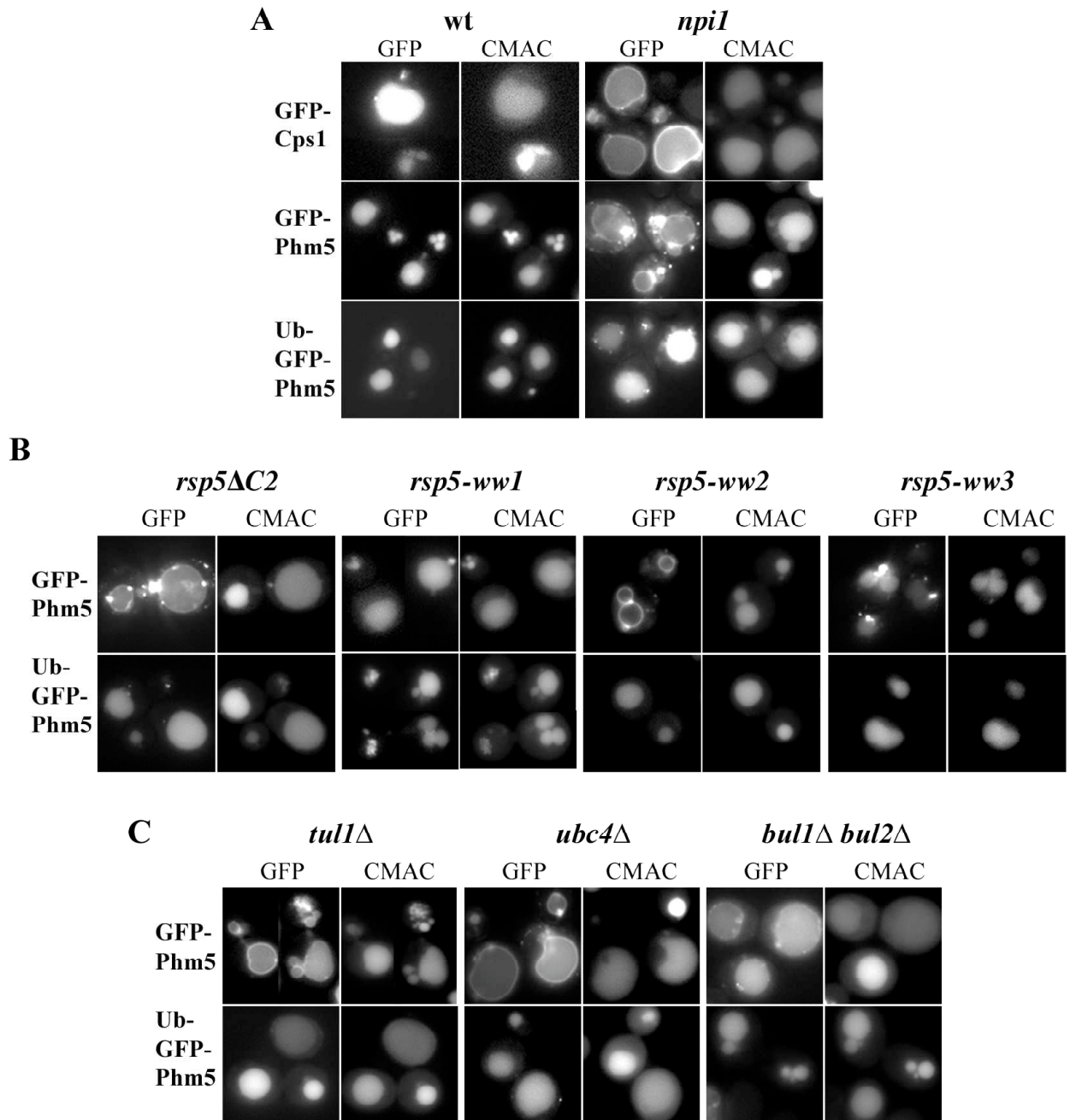
Figure 2.
Microscopy images of GFP-tagged Cps1p and Phm5p in living cells.
Cells producing GFP-Cps1p, GFP-Phm5p or Ub-GFP-Phm5p were grown to mid-exponential ;growth phase and examined by fluorescence microscopy. Only the vacuoles are visible; their identity was confirmed by staining with the dye CMAC as described in Materials and Methods.
A. GFP and CMAC staining in wt and *npi1* mutant cells. **B**. GFP and CMAC staining in cells producing mutant versions of Rsp5p. **C**. GFP and CMAC staining in *tul1*, *ubc4* and *bul1 bul2* mutant cells.
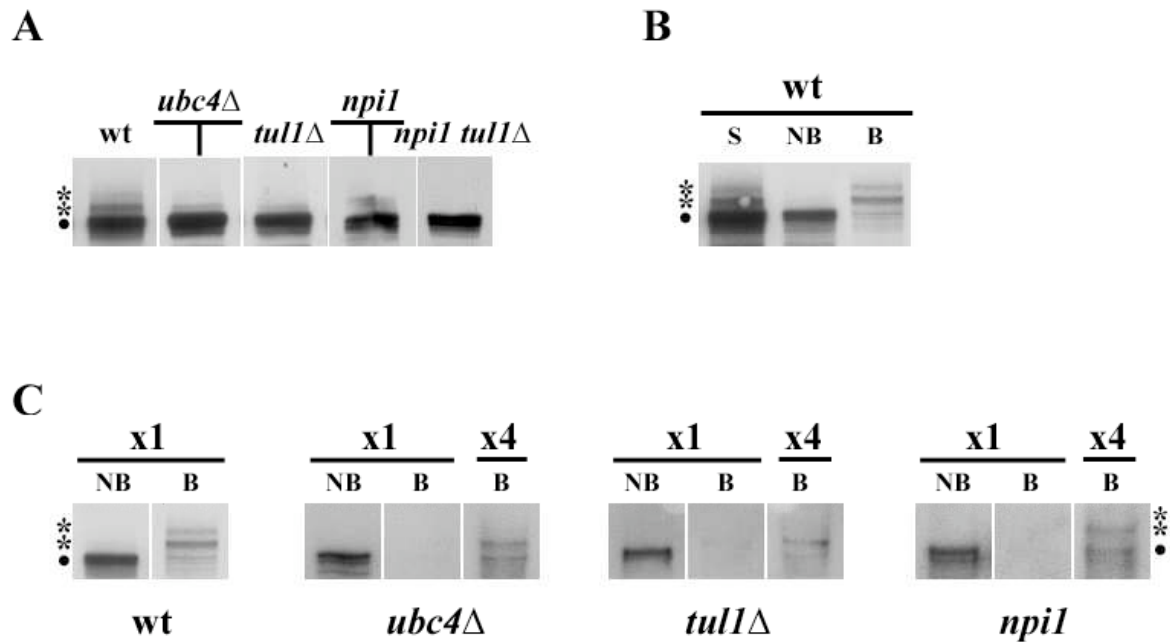
Figure 3.
Ubiquitylation of GFP-Cps1p in various mutants of the ubiquitin machinery

doa4 mutant cells coproducing GFP-Cps1p and copper promoter-driven Ub-6His were grown to mid-exponential growth phase. CuSO4 (100μM) was added and the cells incubated for a further three hours to induce the *CUP1* promoter. Extracts of 6 to 7 x 108 cells were fractionated as described in Materials and Methods. All experiments were conducted in identical conditions of growth and cell fractionation. Solubilized membranes were passed through Ni-NTA columns. The unbound fractions were collected and the His6-tagged ubiquitylated proteins were then eluted by buffer containing 200mM imidazole. At each step, a 5 μl aliquot was withdrawn for western blot analysis. Aliquots of each fraction were resolved by electrophoresis and analyzed by western immunoblotting with anti-GFP antibody.

A. Total protein extracts of wild-type and mutant cells.

B. Solubilized membrane fractions (S) unbound fraction (NB) and purified His6-tagged proteins (B) from wild-type cells.

C. Bound (B) and unbound (NB) fractions of various mutant cells. "x4" indicates that four times more material was loaded on the gel. • : GFP-Cps1p; *: GFP-Cps1p conjugated to His6-tagged ubiquitin.