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Multiple Interactions Drive Adaptor-Mediated Recruitment of the Ubiquitin Ligase Rsp5 to Membrane Proteins In Vivo and In Vitro

James A. Sullivan, Michael J. Lewis, Elina Nikko, and Hugh R.B. Pelham

Medical Research Council Laboratory of Molecular Biology, Cambridge CB2 2QH, United Kingdom

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Recognition of membrane proteins by the Nedd4/Rsp5 ubiquitin ligase family is a critical step in their targeting to the multivesicular body pathway. Some substrates contain "PY" motifs (PPxY), which bind to WW domains in the ligase. Others lack PY motifs and instead rely on adaptors that recruit the ligase to them. To investigate the mechanism of adaptor-mediated ubiquitination, we have characterized the interactions between the adaptor Bsd2, the ubiquitin ligase Rsp5, and the membrane proteins Cps1, Tre1, and Smf1 from *Saccharomyces cerevisiae*. We have reconstituted adaptor-mediated modification of Cps1 and Tre1 in vitro, and we show that two PY motifs in Bsd2 and two WW domains (WW2 and WW3) in Rsp5 are crucial for this. The binding of a weak noncanonical DMAPSY motif in Bsd2 to WW3 is an absolute requirement for Bsd2 adaptor function. We show that sorting of the manganese transporter Smf1, which requires both Bsd2 and Tre1, depends upon two PY motifs in Bsd2 and one motif in Tre1 but only two WW domains in Rsp5. We suggest that sequential assembly of first a Bsd2/Rsp5 complex, then a Tre1/Bsd2/Rsp5 complex followed by a rearrangement of PY–WW interactions is required for the ubiquitination of Smf1.

INTRODUCTION

The sorting of membrane proteins into the multivesicular body (MVB) pathway and subsequent transport to and degradation in the yeast vacuole (or the mammalian lysosome) almost invariably involves their ubiquitination (Hicke and Dunn, 2003). The addition of a single ubiquitin moiety to one or several lysine residues in the target membrane protein can serve as a signal for endocytosis from the plasma membrane (Hicke and Riezman, 1996), for sorting from the Golgi to endosomes (Reggiori and Pelham, 2002), and for sorting into the MVB (Katzmann *et al*., 2001; Reggiori and Pelham, 2001). Membrane proteins destined for the MVB are recognized by ubiquitin receptors, notably through the multiple UIM motifs in the Vps27/Hse1 complex; the UEV domain found in Vps23, which forms part of the ESCRT I complex; and the NZF domain in Vps36 found in the ESCRT II complex (Hurley and Emr, 2006). The recognition of ubiquitinated membrane proteins allows the subsequent assembly of the ESCRT machinery required for formation of the MVB, thus ensuring the incorporation of the target protein into the internal vesicles of the MVB (Hurley and Emr, 2006). Internalization into MVBs is a fate shared by vacuolar enzymes (Katzmann *et al*., 2001), endocytosed cell surface receptors and transporters (Galan *et al*., 1996; Springael and Andre, 1998; Liu and Culotta, 1999; Dunn and Hicke, 2001; Nikko *et al*., 2003; Blondel *et al*., 2004), and misfolded mutant proteins (Luo and Chang, 1997; Reggiori and Pelham, 2002).

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Address correspondence to: Hugh R.B. Pelham (hp@mrc-lmb.cam.ac.uk).

A critical step in the sorting of membrane proteins is their recognition as substrates for ubiquitin ligases. Several ligases have been shown to play a role in sorting including yeast Tul1, implicated in the sorting of both vacuolar enzymes and mutant proteins (Reggiori and Pelham, 2002), and the mammalian ligase c-Cbl, which initiates down-regulation of the epidermal growth factor receptor (Levkowitz *et al*., 1998). In a number of cases, the ligase responsible is a member of the Nedd4/Rsp5 family of HECT domain-containing ubiquitin ligases (Springael and Andre, 1998; Dunn and Hicke, 2001; Blondel *et al*., 2004; Hettema *et al*., 2004; Rougier *et al*., 2005). The Nedd4/Rsp5 family is widely conserved from humans to the yeast *Saccharomyces cerevisiae* where a single family member, Rsp5, is found (Shearwin-Whyatt *et al*., 2006). These ligases are characterized by the presence of three distinct domains: an N-terminal phospholipid-binding C2 domain, implicated in membrane association, a variable number (1–4) of WW domains, and a C-terminal HECT domain that contains the active site for ubiquitin ligation (Shearwin-Whyatt *et al*., 2006). The WW domains found in Nedd4/Rsp5 are the type that bind short "PY" motifs typically with the sequence PPxY (Sudol, 1996; Harty *et al*., 2000), and it has been shown that the addition of a PY motif can make a protein a substrate for Rsp5 in vitro (Saeki *et al*., 2005). One of the best characterized substrates of the Nedd4/Rsp5 family is the epithelial sodium channel (ENaC). Under high sodium concentrations, Nedd4 binds to a PY motif in ENaC to mediate its degradation in the lysosome (Rotin *et al*., 2001). The PY motif in ENaC is critical for down-regulation, and it is interesting to note that patients with Liddle's syndrome, a type of familial hypertension, have mutations in the ENaC PY motif, thereby preventing Nedd4-mediated degradation of ENaC in high sodium conditions (Snyder, 2005).

Many Nedd4/Rsp5 substrates, however, lack PY motifs, and they must rely on other interactions to be recognized. In

two cases, clear candidates for membrane proteins that serve as adaptors have been identified. In *Drosophila*, axon growth away from the midline is mediated by the repellent protein Slit and its receptor at the cell surface, Roundabout (Robo). In turn, levels of Robo are controlled by a second protein, Commissureless (Comm), which contains a double PY motif capable of binding to *Drosophila* Nedd4 (dNedd4) (Myat *et al*., 2002). Comm allows ubiquitination of Robo by dNedd4 and its removal from the cell surface into intracellular vesicles (Myat *et al*., 2002). In yeast, Bsd2 contains a PY motif and is required for the targeting of several proteins into the MVB pathway, notably the vacuolar proteins Cps1 and Phm5, the manganese transporters Smf1 and Smf2, certain mutant forms of the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) Tlg1, and the plasma membrane ATPase Pma1 (Luo and Chang, 1997; Liu and Culotta, 1999; Hettema *et al*., 2004; Valdez-Taubas and Pelham, 2005). Bsd2 acts by recruiting Rsp5, via the PY motif, to substrates such as Cps1 and Phm5, with which it presumably interacts only transiently. Its action on Smf1 is even more complex, in that an additional protein, Tre1 (or its relative Tre2), is required to bridge the gap between Bsd2 and Smf1 (Stimpson *et al*., 2006). Curiously, the Tre proteins each have a PY motif, yet are unable to down-regulate Smf1 efficiently without the assistance of Bsd2 (Stimpson *et al*., 2006). Although Tre1 has no functional counterpart in mammalian cells, Bsd2 homologues NDFIP1 and NDFIP2 do exist (Jolliffe *et al*., 2000; Shearwin-Whyatt *et al*., 2004). Recently, NDFIP1 has been implicated in the protection of neural cells from injury (Sang *et al*., 2006), and it has been shown to promote the function of the Itch ubiquitin ligase (a Nedd4-family member) to prevent T-cell activation (Oliver *et al*., 2006).

Many uncertainties remain about the crucial process of recruitment of Rsp5 and its homologues to their substrates. It is not clear what is the role of the multiple WW domains in Nedd4/Rsp5, nor what distinguishes a PY-containing substrate from an adaptor, nor whether the specificity of Rsp5 action requires additional proteins. To address such questions, we have examined in detail the properties of Bsd2, focusing on its interaction with Cps1 and Tre1, and the regulation of Smf1. We first defined the critical regions of Bsd2 required for its functions in vivo. Then, to allow more precise dissection of their properties, we developed an in vitro ubiquitination assay that reconstitutes Bsd2-dependent modification of Cps1 and Tre1 with purified proteins. Our results show that the adaptor function of Bsd2 requires precise multiple interactions with the WW domains of Rsp5.

MATERIALS AND METHODS

Plasmids and Strains

Yeast strains were derived from the Euroscarf strain BY4742 (*MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*). Coprecipitation experiments were performed in a Euroscarf strain deleted for *bsd2* with the *pep4* gene knocked out by homologous recombination with a fragment containing the *Schizosaccharomyces pombe HIS5* gene.

Strains deleted for *rsp5* were made by using the heterozygous Euroscarf deletion strain (*MATa/MATα; his3Δ1/his3Δ1; leu2Δ0/leu2Δ0; met15Δ0/MET15; LYS2/lys2Δ0; ura3Δ0/ura3Δ0 RSP5/rsp5Δ0*). The diploid strain was transformed with a *URA3*-bearing plasmid expressing a genomic fragment of *RSP5* lacking the C2 domain, but which supports growth; it was sporulated; and tetrads were selected. Viable spores were transformed with appropriate *rsp5* WW domain mutants on pRS313-based plasmids, and the original supporting plasmid was selected against on plates containing 5-fluoroorotic acid.

Strains bearing the *rsp5* deletion were made *tul1* by replacing the *TUL1* gene with *S. pombe HIS5* by using homologous recombination. Resulting strains were transformed with CRE recombinase to remove the *HIS* marker (Sauer, 1994) and then transformed with appropriate WW domain mutants and selected as described above on 5-fluoroorotic acid.

Both full-length and truncated (amino acids [aa] 110–809, lacking the N-terminal C2 domain) *Rsp5* open reading frames (ORFs) were obtained from yeast genomic DNA by using polymerase chain reaction (PCR), and they were inserted into the vector pGEX6P-2 (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) via EcoRI/XhoI restriction sites. QuikChange site-directed mutagenesis (Stratagene, Amsterdam, The Netherlands) was then used to introduce W225A (ΔWW1), W359A (ΔWW 2) and W415A (ΔWW 3) point mutations in the *RSP5* ORF (Dunn and Hicke, 2001). *RSP5* and its WW domain variants used in gene-shuffling experiments were cloned from the bacterial expression plasmids and expressed from the *RSP5* promoter in pRS313. The protein A-tagged versions of *RSP5* were amplified by PCR from the bacterial expression plasmids and cloned into a *TPI1* promoter-driven N-terminal tagging plasmid in pRS315.

An N-terminal cytoplasmic fragment of *BSD2* (aa 1–171) was inserted into the vector pET30a (Novagen, Darmstadt, Germany) via BamHI/XhoI sites (introduced using PCR) to produce an N-terminal His6/S-tag construct or via NdeI/XhoI sites to produce a C-terminal His6-tagged construct. All mutations in the *BSD2* open reading frame were introduced via PCR except the PY1PY1 construct, which was produced by inserting a DNA fragment 5′-GGCTGACATACCACCTACATACGACGAAGCTGCTGGAATGGATTTGA-ACAATTCAGAT-3′ between an introduced silent SacII site and a unique EcoRV site in the *Bsd2* ORF. The PY2PY1 mutant was produced by replacing an XbaI/SacII fragment in the PY1PY1 construct with a PCR fragment in which the IPPTYD sequence in PY1 was replaced with the sequence MAPSYY. The C-terminal truncations of the *Tre1* ORF *Tre1*Δlumen and *Tre1*PPAGΔlumen (Stimpson *et al*., 2006) and full-length *SsoI* were inserted into the vector pET30a to produce N-terminal His6/S-tag constructs. A yeast expression vector expressing an N-terminal fusion between green fluorescent protein (GFP) and Cps1 (Reggiori and Pelham, 2001) was used as a template to produce a DNA fragment encoding GFP and aa 1–77 of Cps1, which was inserted via introduced NcoI/XhoI sites into pTrcHisA (Invitrogen, Paisley, United Kingdom) to produce a C-terminal His6-tagged construct. A full-length *UBC1* ORF was obtained using yeast genomic DNA as a template via PCR and inserted into pET15b (Novagen) via introduced NdeI/BamHI sites producing an N-terminally tagged His6 construct. An N-terminal fragment of Matα 2 (aa 1–66) containing the Deg1 degron (Swanson *et al*., 2001) was isolated from yeast genomic DNA and inserted with GFP via introduced NcoI/XhoI sites into a modified pTrcHisA (Invitrogen) to produce a C-terminal His6-tagged construct.

Yeast expression plasmids for Bsd2 were based on the YCpLac series of plasmids, (Gietz and Sugino, 1988) using either the *TPI1* promoter driving GFP or 3XHA-tagged *BSD2*, or *BSD2* promoter-driven untagged constructs (Hettema *et al*., 2004).

The Q190L, T197A, S210A, T217A S224A STS186-8A, and STS210/17/24 mutations were made in Bluescript by using QuikChange (Stratagene) mutagenesis and transplanted into yeast expression vectors.

The GVG170/173/174AAA mutant and N175A, N178A, and N182A mutants were made by PCR of upstream and downstream fragments by using mutagenic oligonucleotides overlapping the endogenous Nsi1 site in *BSD2* and cloned into appropriate vectors.

Constructs containing the 113-8A mutant were made by PCR of upstream and downstream fragments, introducing a silent Not1 site at nucleotide 347 together with the mutations, and cloning into appropriate vectors. The alanine scanning point mutants (146–152) were made by PCR of upstream and downstream fragments, joined at a silent SacII site introduced at nucleotide 431. The double mutant combining the P149A and the upstream PPTA mutation were made using a similar strategy using a silent Pst1 site in the PPTA mutant (Hettema *et al*., 2004). The Tre1 yeast expression plasmids used are described in Stimpson *et al*. (2006). GFP-tagged Cps1 was as described in Reggiori and Pelham (2001), and the protein A-tagged version was generated by subcloning from this plasmid into a *TPI1*-protein A Tobacco Etch Virus protease cleavage site construct in pRS415. Smf1 and the N-terminal truncation of Smf1 were N-terminally GFP-tagged by amplification using PCR and subcloning into appropriate YCpLac series vectors driven by the *TPI1* promoter. PCR generated constructs were verified by DNA sequencing.

Growth of Yeast in Metal-depleted Media

Growth in metal-depleted minimal defined medium was performed as described previously (Dancis *et al*., 1990; Liu and Culotta, 1999). Briefly, the medium was incubated twice for 24 h with 50 g/l chelex 100 resin to remove all divalent metal ions, and salts were added back to the following concentrations: 2.4 mM $MgSO_4$, 30 mM KCl, 2.0 mM $CaCl_2$, and 0.86 mM NaCl.

Production of Recombinant Proteins

All recombinant protein procedures were performed using the bacterial strain BL21(DE3). To increase the solubility of recombinant Rsp5 and transmembrane domain-containing proteins, incubation at 16°C for 18 h was performed after induction with 1 mM isopropyl β-D-thiogalactoside for other proteins cultures were incubated at 30°C for 5 h. Bacterial pellets were resuspended in lysis buffer (50 mM Na_2HPO_4, 300 mM NaCl, 10 mM imidazole [His-tagged proteins only], 2% [vol/vol] Triton X-100, 1 mg/ml lysozyme), lysed using a high-pressure microfluidiser (Avestin, Mannheim, Germany) at 12,000 kPa,

and a cleared lysate produced by centrifugation at 15,000 × *g* for 15 min. Recombinant proteins were purified from lysates using either glutathione-Sepharose (GE Healthcare) or nickel-nitrilotriacetic acid resin (QIAGEN, Crawley, United Kingdom) according to standard manufacturer's protocols. After elution, recombinant proteins were desalted using NAP-10 columns (GE Healthcare) into storage buffer (20 mM Tris, pH 7.4, and 10% [vol/vol] glycerol) before being concentrated using Amicon Ultra centrifugation filter devices (Millipore, Billerica, MA) and stored in aliquots at −80°C until required. The purity of recombinant proteins was confirmed using Coomassie brilliant blue staining after SDS-polyacrylamide gel electrophoresis (PAGE), and protein concentrations were adjusted to ensure equal quantities were used in subsequent assays.

Production of Bsd2 Antibodies

The His6/S-tag fragment of Bsd2 described above was used as an antigen for injection into rabbits (Eurogentec, Southampton, United Kingdom). Bsd2-specific polyclonal antibodies were then partially purified from rabbit serum by using recombinant Bsd2.

Tandem Affinity Purification

A shuttle vector based on pYCplac111 containing an N-terminal TAP-tagged *BSD2* ORF under the control of the *TPI1* promoter was introduced into a Δ*bsd2* yeast strain. The cell pellet from a 4-l mid-log phase culture of the Tap-*BSD2* strain was resuspended in lysis buffer (100 mM Tris, pH 7.4, 150 mM KCl, 5 mM $MgCl_2$, and 2% [vol/vol] Triton X-100) and lysed using a high-pressure microfluidiser (Avestin) at 14,000 kPa. A cleared lysate was produced by centrifugation at 15,000 × *g* for 15 min, and tandem affinity purification of Bsd2 was performed essentially as described previously (Puig *et al*., 2001; Kee *et al*., 2005). Purified Bsd2 was desalted using NAP-10 columns (GE Healthcare) into storage buffer (20 mM Tris, pH 7.4, and 10% [vol/vol] glycerol) before being concentrated using Amicon Ultra centrifugation filter devices (Millipore) and stored in aliquots at −80°C until required. Polyclonal Anti-calmodulin binding protein (CBP) antibodies used to detect Tap-purified proteins were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

In Vitro Ubiquitination Assays

In vitro ubiquitination assays were performed in a total volume of 30 μl consisting of 50 mM Tris, pH 7.4, 10 mM $MgCl_2$, 10 mM ATP, 125 ng of human E1 (BIOMOL International UK, Exeter, United Kingdom), 500 ng of Ubc1, and 1 μg of methylated ubiquitin (BIOMOL International UK) or where indicated 1 μg of hemagglutinin (HA)-tagged ubiquitin (BIOMOL International UK). In addition assays were performed with 300 ng of glutathione *S*-transferase (GST)-Rsp5 (or derivatives), 500 ng of the target protein, and ~200 ng of Tap-purified Bsd2 (or derivatives). Reactions were incubated for 20 min at 30°C before being terminated by the addition of 30 μl of SDS-sample buffer. Polyclonal anti-ubiquitin antibodies were obtained from BIOMOL International UK.

Coprecipitation Experiments

Protein A coprecipitations were performed essentially as described by Siniossoglou *et al*. (2000). Cells were grown to OD 0.5–1.00 and spheroplasted. Washed spheroplasts from 50 OD of cells were lysed in 1.8 ml of Triton X-100–containing buffer (150 mM KCl, 5 mM $MgCl_2$, 1% Triton X-100, and 20 mM Tris-HCl, pH 8.0) containing Complete protease inhibitors (Roche, Welwyn Garden City, United Kingdom). The lysate was cleared by centrifugation at 13,000 × *g* for 10 min and subsequently incubated at 4°C for 2 h with 25 μl of immunoglobulin G (IgG)-Sepharose beads (GE Healthcare). The beads were washed twice with 10 ml of lysis buffer (and once with lysis buffer containing 1.5 M KCl for protein A pull-downs with Rsp5), and bound material was eluted with pH3.4 NH_4OAc before being lyophilized. Blots of SDS-PAGE separations of total protein from early log phase cells were made using the protocol of Volland *et al*. (1994). SDS-PAGE separations were Western blotted and probed with 12CA5 (anti-HA) anti-GFP monoclonal clones 7.1 and 13.1 (Roche) or with peroxidase-coupled anti-peroxidase for protein A fusions (Dako Denmark A/S, Glostrup, Denmark).

Cadmium Sensitivity Tests

Tests of cadmium sensitivity were performed as described in Stimpson *et al*. (2006) by using the indicated concentrations of $CdCl_2$. Typically, serial two-fold dilutions of cells were spotted on cadmium-containing plates.

Fluorescence Imaging

GFP-labeled cells in early log phase were imaged either in medium or in water on a Bio-Rad (Hemel Hempstead, United Kingdom) Radiance confocal microscope. Images were adjusted for contrast and brightness, and in some cases, they were blurred to filter noise, by using Adobe Photoshop (Adobe Systems, Mountain View, CA). They are shown inverted for clarity. Some images, notably those in Figure 1A, were obtained using a MicroMax charge-coupled device camera (Princeton Instruments, Lurgan, United Kingdom) on a conventional microscope, and they were processed similarly. Except for the indicated panels in Figure 1A, all images shown are of cells grown in normal (metal-replete) medium.

RESULTS

Role of Bsd2 in Smf1 Regulation

Smf1 is a high-affinity metal transporter whose substrates include manganese and cadmium (Liu and Culotta, 1999). Normal growth medium is metal replete, and under these conditions Smf1 is down-regulated. A failure to down-regulate Smf1, as in a *bsd2* mutant, results in increased surface expression of Smf1 and leads to cadmium sensitivity and cell death due to increased uptake of the toxic heavy metal. Two levels of regulation have been reported previously (Liu and Culotta, 1999; Eguez *et al*., 2004; Hettema *et al*., 2004; Stimpson *et al*., 2006). One level affects the distribution of Smf1 between endosomes and the plasma membrane; the other level results in its transport directly from the Golgi to the vacuole for degradation. Bsd2 is required for degradation but apparently not for transport to endosomes, raising the possibility of two different regulatory mechanisms capable of responding to metal levels.

We were able to clarify this using a deletion of the N-terminal 68 residues of Smf1, a region that is absent from the related protein Smf2 and whose removal increases the activity of Smf1 expressed in *Xenopus* oocytes (Sacher *et al*., 2001). As shown in Figure 1A, a GFP-tagged version of this mutant (Smf1ΔN) under normal growth conditions is found in the vacuole lumen. However, metal depletion of the growth medium caused the protein to accumulate on the cell surface. Mutation of *Bsd2* also resulted in surface expression of Smf1ΔN, even in the presence of metals. Full-length Smf1 accumulated in the vacuole in metal-depleted wild-type cells, but in Δ*bsd2* cells it was in punctate structures presumably corresponding to endosomes (Figure 1A). Thus, the ΔN mutant retains the ability to be sorted from the Golgi to the vacuole in a manner that is dependent on the presence of both metals and Bsd2 but that differs from wild-type Smf1 in that it preferentially accumulates on the plasma membrane when this regulation does not occur. This difference seems to be due to defective endocytosis of the ΔN mutant. We found that full-length Smf1 could be observed at the cell surface when Δ*bsd2* cells were imaged immediately after mounting on slides, but that during incubation under the coverslip the protein was internalized. In contrast, the ΔN mutant protein remained at the cell surface even after prolonged incubation (Figure 1A, right). These results imply that Smf1 endocytosis depends on the N terminus, is independent of Bsd2, and may be induced by hypoxia or other stress associated with incubation on slides rather than being a specific response to metals.

We conclude that the primary metal- and Bsd2-dependent down-regulation of Smf1 occurs within the cell rather than at the level of endocytosis. Because the ΔN mutant eliminates variability due to endocytosis, we used this mutant for subsequent studies of the requirements for Bsd2-dependent Smf1 sorting.

Role of TMD Sequences in Bsd2

Degradation of Smf1 requires the single transmembrane domain (TMD) protein Tre1 (or Tre2) in addition to Bsd2. The cytoplasmic region of Tre1 is required to recognize Smf1, whereas the Tre1 TMD is in turn recognized by Bsd2 (Stimpson *et al*., 2006). TMD sequences are also implicated in the recognition of the vacuolar protease Cps1, and we have proposed that recognition by Bsd2 involves polar residues within the TMDs of the substrates (Hettema *et al*., 2004). This

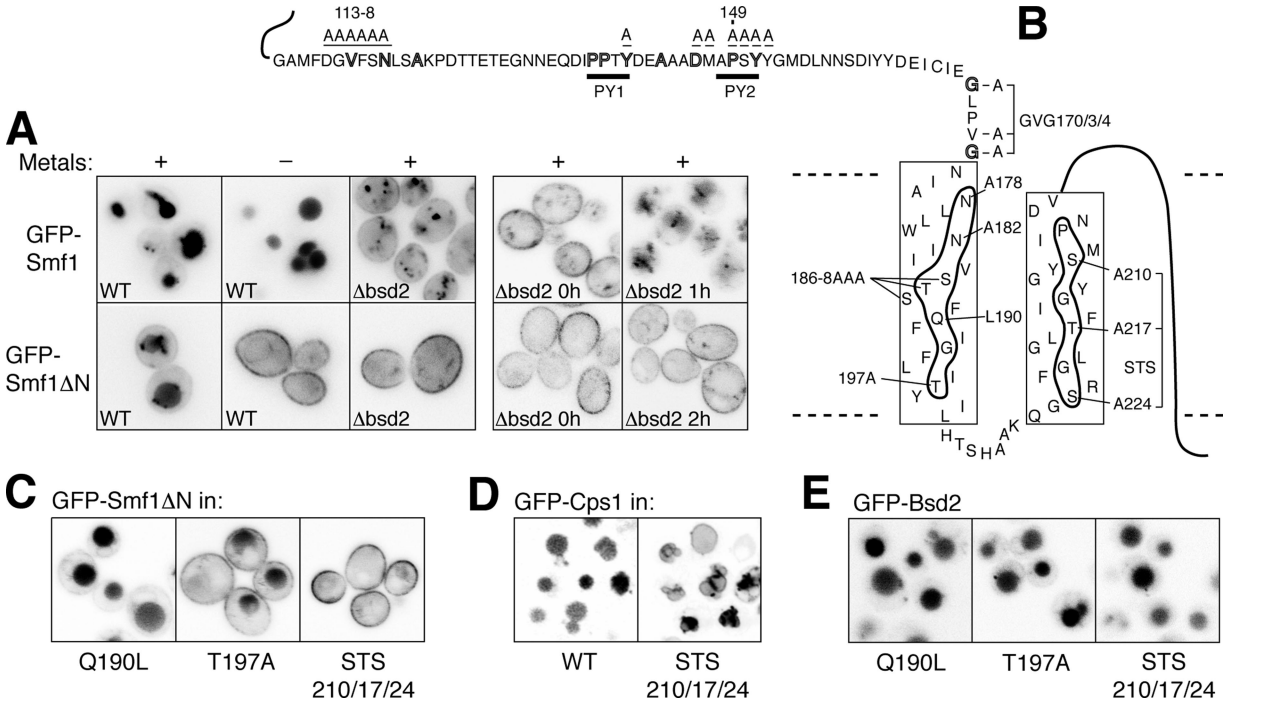

Figure 1. Sorting of Smf1 is regulated by sequences within the transmembrane domains of Bsd2. (A) Localization of GFP fused to Smf1 (GFP-Smf1) or to an N-terminal 68 amino acid deletion of Smf1 (GFP-Smf1ΔN) in wild-type (WT) or *bsd2* null mutant yeast (Δ*bsd2*), grown in metal replete (+) or metal depleted (−) media as indicated. The right-hand four panels show an experiment in which cells were mounted under coverslips and imaged either immediately (0 h) or after 1 or 2 h. (B) Schematic representation showing position of mutations in the cytoplasmic and first two transmembrane domains of Bsd2. Substituted residues (mostly changed to alanine) are indicated. Larger letters in the cytoplasmic domain indicate residues that are completely invariant in all currently available fungal sequences. The polar faces of the TMDs are encircled on the helical maps. The portions of Bsd2 not shown as a sequence are indicated by lines (not to scale). (C) Localization of GFP-Smf1ΔN in Δ*bsd2* yeast complemented with plasmid containing Q190L, T197A, or STS210/17/24 Bsd2 variants expressed from a *BSD2* promoter. (D) Localization of GFP-Cps1 in Δ*bsd2*Δ*tul1* yeast complemented with either wild-type or STS210/17/24 Bsd2. (E) Localization in a Δ*bsd2*Δ*tul1* yeast strain of GFP-Bsd2 with Q190L, T197A, or STS210/17/24 mutations.

predicts that Bsd2, which has three TMDs, will itself have polar residues that act as hydrophilic binding sites within the lipid bilayer.

Examination of the Bsd2 sequence shows that the first two TMDs, which form the most widely conserved part of the protein, each have polar residues aligned along one face (Figure 1B). We made a series of mutations within and adjacent to this region (as indicated in Figure 1B), and we assayed them for sorting of Smf1. An initial screen for cadmium sensitivity showed little effect of most of the single mutants (at positions 178, 182, 190, 210, 217, and 224) or of the triple mutant 186–188, but the T197A mutation did show increased cadmium sensitivity, and this correlated with partial missorting of GFP-Smf1ΔN to the cell surface (Figure 1C). A stronger effect was obtained by combining three mutations (STS210/17/24) that lie along the hydrophilic face of the second transmembrane domain, namely, S210A, T217A, and S224A (Figure 1C). Strikingly, the triple STS210/17/24 mutant also affected sorting of GFP-Cps1, resulting in more of the protein being on the vacuolar membrane rather than inside the vacuole (Figure 1D). However, neither this nor the single mutants affected the abundance or sorting of Bsd2 itself (Figure 1E and unpublished observations). Thus, polar residues within the TMDs of Bsd2 clearly contribute to its adaptor function.

A more dramatic effect was seen with a triple GVG mutation that affected conserved sequences just upstream of the predicted TMD, G170A/V173A/G174A. This mutant caused Cps1 to accumulate on the vacuolar membrane and Tre1 to occur in punctate structures, rather than inside the vacuole (Figure 2A). In the absence of Bsd2 function, Tre1 is known to accumulate in endosomes, as indicated by colocalization with endocytosed FM4-64 dye (Stimpson *et al*., 2006). The GVG mutation also induced cadmium sensitivity (see below; Figure 3A), indicating missorting of Smf1. We also tested a form of the SNARE Tlg1 in which two cysteines at the cytoplasmic end of the TMD, which are normally palmitoylated, had been mutated to serine (Valdez-Taubas and Pelham, 2005). We have previously shown that this protein is a substrate for the Bsd2 system, as indicated by the appearance of free GFP generated by vacuolar proteolysis (Figure 2B). This, too, was blocked by the GVG mutation. It seems unlikely that this region of Bsd2 is a critical contact point for all these proteins. Rather, it may indirectly control the structure of the transmembrane region, perhaps affecting the rotational position of the helices and thus the exposure of their polar faces.

To test the prediction that the GVG mutation affects substrate binding, rather than some other function such as Rsp5 recruitment, we used coimmunoprecipitation of tagged versions of Tre1 and Bsd2 expressed in yeast (Figure 2C). The GVG mutant showed greatly reduced interaction with Tre1, whereas mutations affecting a conserved upstream region (residues 113–118) or the Bsd2 PY motifs, which affect Rsp5 binding (see below), did not. Thus, the GVG region is of particular importance for Tre1 binding. Although we would predict that it is similarly important for recognition of Cps1, we were unable to test this directly because Bsd2 and Cps1

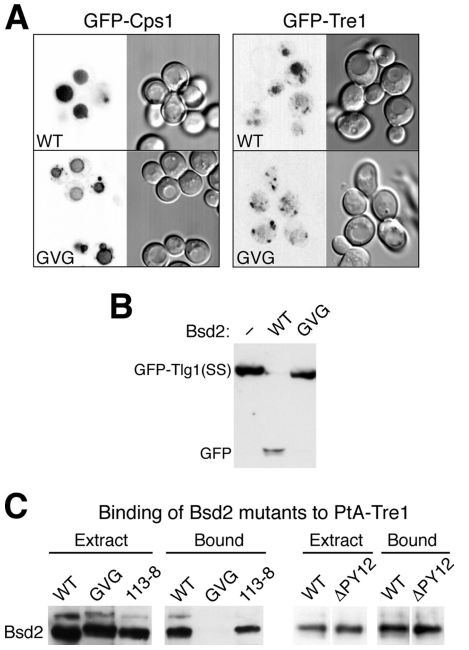

Figure 2. Sequences in Bsd2 adjacent to the membrane are involved in substrate recognition. (A) Localization of GFP-Cps1 and GFP-Tre1, with corresponding differential interference contrast images, in a Δ*bsd2* yeast strain complemented with either wild-type (WT) or *Bsd2* carrying G170A/V173A/G174A mutations (GVG). (B) Western blot of total protein extract from Δ*bsd2*Δ*tul1* cells expressing GFP-tagged Tlg1 CC205/6SS [GFP-Tlg1(SS)] in the presence of either a control plasmid (−), wild-type *Bsd2* (WT), or *Bsd2* G170A/V173A/G174A (GVG) driven from the *BSD2* promoter. The blot was probed with an anti-GFP monoclonal antibody. (C) Protein A pull-down by tagged Tre1 (Δlumenal domain) of wild-type (WT), G170A/V173A/G174A (GVG), 113-8A (113-8), and ΔPY1 ΔPY2 (ΔPY12) triple HA-tagged Bsd2 from Δ*bsd2*Δ*pep4* cells. Eluates from IgG-Sepharose beads are enriched over the extracts by a factor of 80.

did not form a complex stable enough to be detected by coprecipitation (unpublished observation). This suggests that Bsd2 binds more tightly to Tre1, which has a specialized function in Smf1 regulation, than to Cps1, a transient substrate.

Recruitment of Rsp5

The above-mentioned results indicate that substrate recognition involves the transmembrane portion of Bsd2, whereas previous work has implicated the N-terminal cytoplasmic domain in the recruitment of Rsp5. We set out to map the cytoplasmic regions critical for this. There is a PPTY sequence at position 137 that can bind in vitro to all three isolated WW domains from Rsp5, and that is required for ubiquitination of substrates (Hettema *et al*., 2004). However, this cannot be the only important feature of the cytoplasmic domain, because the *bsd2* point mutation originally identified as having a defect in Smf1 sorting was of a proline residue not in the PPTY motif, but in the nearby sequence DMAPSYY (residues 146–152) (Liu and Culotta, 1994). We performed alanine-scanning mutagenesis of this sequence, using both cadmium resistance and visualization of N-terminally truncated Smf1 as assays. As shown in Figure 3, A and B, the D146 makes some contribution to activity, but P149 and Y151 are most critical for the sorting of Smf1, suggesting that the DMAPSY sequence functions as a second, variant PY motif. We then tested the same mutants for their ability to sort Cps1 to the vacuole, and found exactly the same pattern, with D146 and particularly P149 and Y151 being crucial for activity (Figure 3C). For simplicity, we refer below to the PPTY sequence as PY1, and the variant DMAPSY sequence as PY2.

One other region of the cytoplasmic domain (around residues 113–118; Figure 1B) stood out as being well conserved among fungal species, and we tested the effects of mutating the highly conserved region DGVFSN to a run of six ala-

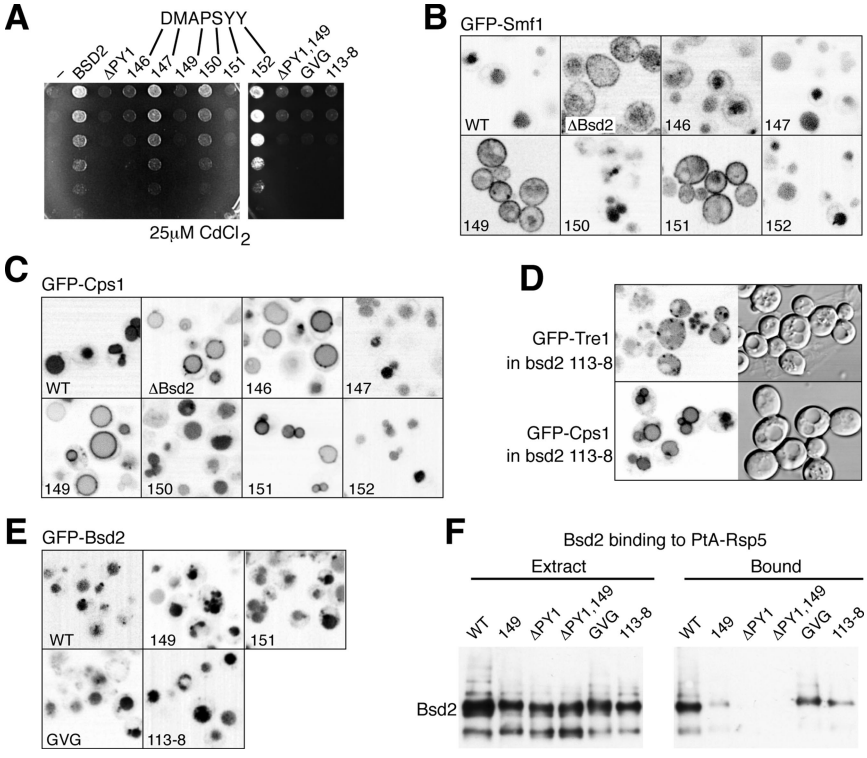

Figure 3. Mutation of sequences within the cytoplasmic domain of Bsd2. (A) Growth of a Δ*bsd2* strain in the presence of 25 μM $CdCl_2$, complemented with either control vector (−), wild-type *BSD2* (*BSD2*), Y140A (ΔPY1), D146A (146), M147A (147), P149A (149), S150A (150), Y151A (151), Y152A (152), Y140A/P149A (ΔPY1,149), G170A/V173A/G174A (GVG), or D113A/G114A/V115A/F116A/S117A/N118A (113-8) *BSD2* mutants. (B) Localization of GFP-Smf1ΔN and (C) GFP-Cps1 in a Δ*bsd2* yeast strain complemented with wild-type (WT), control plasmid (ΔBsd2), or plasmids expressing D146A (146), M147A (147), P149A (149), S150A (150), Y151A (151), or Y152A (152) forms of Bsd2. (D) Localization of GFP-Tre1 and GFP-Cps1 in a Δ*bsd2* strain expressing 113-8 Bsd2. (E) Localization in a Δ*bsd2*Δ*tul1* strain of GFP tagged wild-type Bsd2 (WT), P149A (149), Y151A (151) G170A/V173A/G174A (GVG), or D113A/G114A/V115A/F116A/S117A/N118A (113-8) Bsd2 mutants. (F) Immunoprecipitation of Triple HA-tagged Bsd2 mutant proteins with protein-A tagged Rsp5 from Δ*bsd2*Δ*pep4* cells. IgG-Sepharose eluates were enriched over the extracts by a factor of 20.

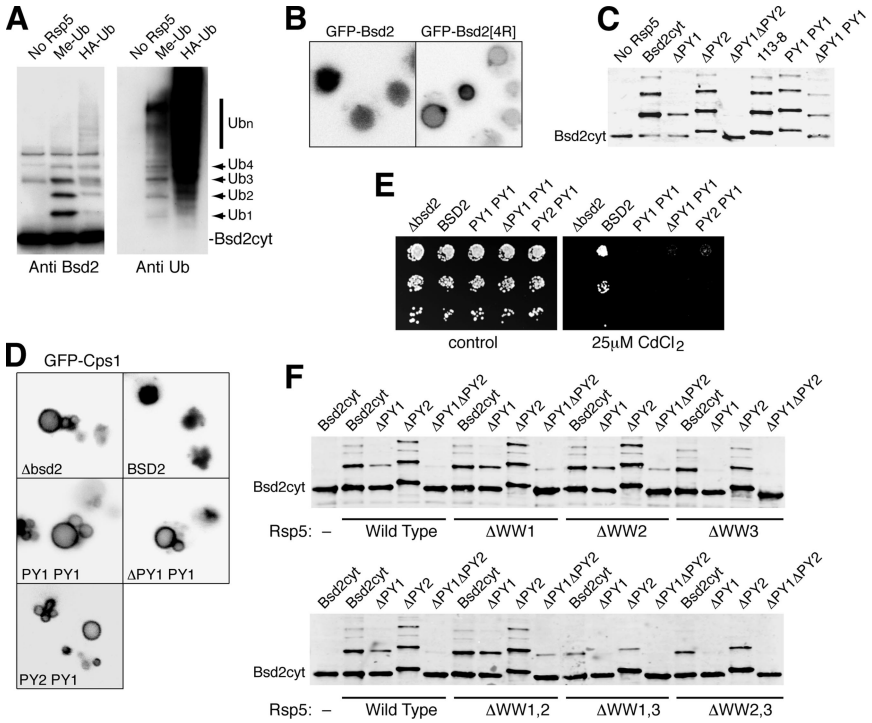

Figure 4. PY-dependent ubiquitination of the Bsd2 cytoplasmic domain. (A) In vitro ubiquitination by Rsp5 of cytoplasmic fragment of Bsd2 with nonextendable methylated ubiquitin (Me-Ub) or extendable HA-tagged ubiquitin (HA-Ub). Western blots were probed with either anti-Bsd2 (left) or anti-ubiquitin (right). Addition of up to four ubiquitins to Bsd2 can be seen (Ub1-4) as well as a smear of polyubiquitinated protein (Ubn). (B) Localization in a Δ*bsd2*Δ*tul1* strain of GFP-tagged wild-type Bsd2 and Bsd2 in which all four lysines in the cytoplasmic domain have been mutated to arginine (Bsd2[4R]). (C) Western blot, probed with anti Bsd2 antibody, showing in vitro ubiquitination by recombinant Rsp5 of a cytoplasmic fragment of Bsd2 (Bsd2cyt) or fragments containing Y140A (ΔPY1), P149A (ΔPY2), Y140A/P149A (ΔPY1ΔPY2), D113A/G114A/V115A/F116A/S117A/N118A (113-8), a duplicated PY1 region in place of PY2 (PY1PY1) or the PY1PY1 construct with a Y140A mutation (ΔPY1PY1). (D) Localization of GFP-Cps1 in a Δ*bsd2*Δ*tul1*yeast complemented with control plasmid (Δbsd2), or with plasmids expressing wild-type Bsd2 (BSD2), Bsd2 with a duplicated PY1 region (PY1PY1), with duplicated PY1 but also carrying the Y140A mutation (ΔPY1PY1), or with the PY1 and PY2 sequences interchanged (PY2PY1). (E) The strains used in D tested for growth in the presence or absence of cadmium as indicated. (F) Western blot probed with anti Bsd2 showing in vitro ubiquitination of Bsd2cyt, ΔPY1, ΔPY2, and ΔPY1ΔPY2 Bsd2 fragments described in C with either wild-type Rsp5, or Rsp5 carrying W225A (ΔWW1), W359A (ΔWW2), W415A (ΔWW3), W225A/W359A (ΔWW1,2), W225A/W415A (ΔWW1,3), or W359A/W415A (ΔWW2,3) mutations. Note that the P149A mutation (ΔPY2) did not completely abolish recognition of PY2 by WW3, leaving a weak WW3-dependent signal in this experiment (ΔPY1ΔPY2 samples); a more complete PY2 block was obtained with the Y151A mutation (unpublished observation).

nines. Sorting of both Tre1 and Cps1 was affected by this mutation, both being largely excluded from the interior of the vacuole (Figure 3D), and in addition, cells also became sensitive to cadmium (Figure 3A).

These mutations did not greatly affect the levels or sorting of Bsd2 itself. To check this, we used a strain lacking endogenous Bsd2 and Tul1, both of which have the ability to mediate ubiquitination of Bsd2 (Hettema *et al*., 2004). The P149A, Y151A, and 113–118 mutants all showed the same level of vacuolar GFP fluorescence as wild-type GFP-Bsd2 (Figure 3E), indicating that even when PY2 or the 113–118 region is mutated Bsd2 has a residual ability to direct its own ubiquitination by Rsp5 and be trafficked normally to the vacuole, a step for which PY1 is necessary (Hettema *et al*., 2004) and probably sufficient. This fits with a simple model in which modification of Bsd2 requires only a fleeting interaction with Rsp5, whereas modification of other substrates requires a more stable Rsp5/Bsd2 complex to form, which can then transiently interact with proteins such as Cps1.

To examine more directly the recruitment of Rsp5, we tested the ability of the various Bsd2 mutants to bind coexpressed protein A-tagged Rsp5. As shown in Figure 3F, binding of protein A-Rsp5 to wild-type Bsd2 could readily be detected. The GVG mutation, which abolished Tre1 binding, had little effect on Rsp5 binding. In contrast, the P149A mutation in PY2 noticeably weakened binding, though not as severely as loss of PY1. The 113–118 mutation also somewhat reduced Rsp5 binding, but it did not abolish it. Together, these results indicate that both the PY1 and PY2 motifs, and possibly the 113–118 region, contribute to Rsp5 binding. In general, of the mutations we tested that show a phenotype, those upstream of position 170 affected Rsp5 binding, whereas those downstream of this point affected substrate recognition.

In Vitro Ubiquitination of Bsd2 by Rsp5

With the main functional features of Bsd2 defined, we set out to test our understanding of its action by reconstituting the ubiquitination process with purified proteins in vitro. Our first goal was to characterize the Rsp5–Bsd2 interaction in more detail and to confirm the function of the second PY motif. The importance of this sequence in vivo was surprising, because previous work had shown that binding of isolated WW domains to the Bsd2 cytoplasmic domain in vitro was essentially eliminated by mutation of PY1, implying that any binding to PY2 is weak.

We first developed an in vitro ubiquitination assay using recombinant GST-Rsp5 purified from *Escherichia coli*, recombinant E1 and E2, ubiquitin, and a histidine-tagged fusion protein containing only the N-terminal cytoplasmic domain of Bsd2. After incubation, ubiquitinated forms of the Bsd2 fragment were readily detectable. Using methylated ubiquitin, with all the lysine residues blocked and thus unavailable for chain formation, resulted in up to four ubiquitins being added (Figure 4A), whereas with HA-tagged ubiquitin a smear of polyubiquitinated Bsd2 occurred. The autoubiquitination of Rsp5 in the in vitro assay was also observed with both methylated and HA-tagged ubiquitin, and results in the high molecular smear detectable with anti-ubiquitin antibodies (Figure 4A). The Bsd2 N terminal sequence contains four lysine residues, and mutation of all four of these to arginine abolished the modification, implying that these were the major sites of ubiquitin addition, not lysines within the epitope tag (unpublished observations). Loss of these same four lysines, but not fewer, in full-length

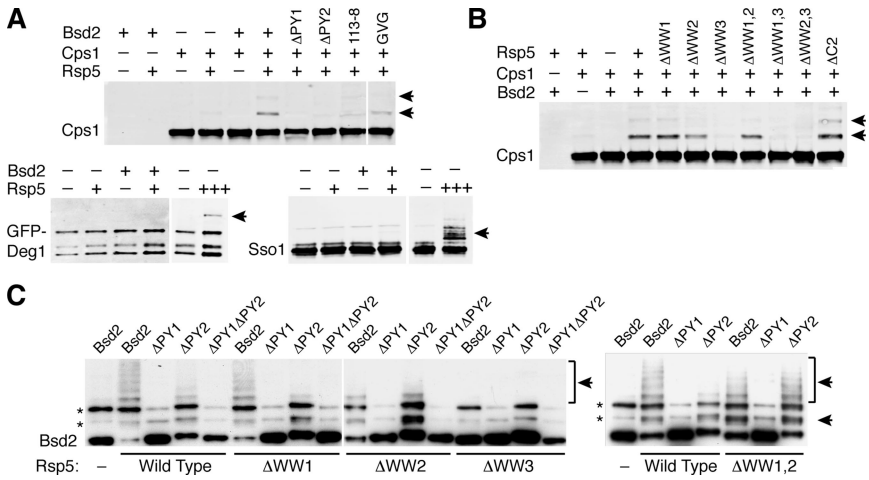

Figure 5. Requirements for in vitro ubiquitination of Cps1 mediated by Bsd2. (A) Top, Western blot, probed with anti-GFP, of in vitro ubiquitination of recombinant GFP-tagged Cps1 by Rsp5 and Tap-purified wild-type Bsd2, or Bsd2 carrying the ΔPY1, ΔPY2, 113-8, or GVG mutations described above. Arrows indicate two distinct ubiquitinated forms of GFP-CPS. Bottom, control ubiquitination assays using GFP-Deg1 (probed with anti GFP antibodies) and S-tagged Sso1 (probed with anti-S-tag antibodies) with wild-type Rsp5 and Tap-purified Bsd2. The right-hand two lanes in each case show overnight incubations without Rsp5 or with a 60-fold higher amount of Rsp5 than normal, which demonstrates that these substrates are intrinsically capable of ubiquitination (arrows). (B) Western blot, probed with anti-GFP, of in vitro ubiquitination of recombinant GFP-tagged Cps1 mediated by Tap-purified Bsd2 and either wild-type Rsp5 or Rsp5 carrying ΔWW1, ΔWW2, ΔWW3, ΔWW1,2, ΔWW1,3, ΔWW2,3, ΔC2 mutations described above. Arrows indicate two distinct ubiquitinated forms of GFP-CPS. (C) Western blot, probed with anti CBP antibody, showing in vitro ubiquitination of full-length Tap-purified Bsd2 carrying ΔPY1, ΔPY2, or ΔPY1ΔPY2 mutations described above. Left, Bsd2 ubiquitination mediated by wild-type recombinant Rsp5 or with mutations in single WW domains. Right, Bsd2 ubiquitination mediated by Rsp5 carrying ΔWW1,2 mutation. Arrow indicates multimonoubiquitinated forms of Bsd2, asterisks indicate nonspecific cross-reacting bands. Irrelevant lanes in A and C have been excised, as indicated by the gaps, but exposures are identical for all lanes.

Bsd2 was also sufficient to block sorting into the vacuole interior, indicating that these are the major sites of modification in vivo (Figure 4B; unpublished observations).

Mutation of the PY1 motif to PPTA reduced, but did not abolish, in vitro ubiquitination (Figure 4C, ΔPY1). In contrast, mutation of the downstream PY2 motif or the upstream 113–118 sequence had little effect (Figure 4C, ΔPY2, 113-8). However, the residual modification of the PPTA mutant could largely be abolished by combining it with the PY2 mutation P149A, indicating that PY2 was responsible for some recognition by Rsp5 (Figure 4C, ΔPY1ΔPY2). Similar experiments showed that mutation of Y151 also abolished the activity of PY2 (unpublished observation).

The weak activity of PY2 correlated with its noncanonical sequence. When a second copy of the PY1 sequence IPPTYDEAA was inserted in place of the PY2 sequence MAPSYY (construct PY1PY1), and then the original PY1 was mutated, enhanced modification was observed relative to that obtained with PY2 alone (compare ΔPY1PY1 with ΔPY1 in Figure 4C). This raises the question of why PY2 has not evolved into a better binding site in any of the fungi for which the Bsd2 sequence is known. We therefore introduced the changed PY2 sequence into full-length Bsd2 and tested its activity in vivo. Surprisingly, Bsd2 with two copies of the PY1 sequence was unable to rescue Cps1 sorting (Figure 4D), or Smf1 sorting as judged by resistance to growth on cadmium (Figure 4E). It seems that interaction with this duplicated sequence is unproductive in vivo. The unusual sequence of PY2 is thus crucial for function. The order of the two PY sequences also proved crucial for activity in vivo: a version of Bsd2 in which the two sequences were exchanged could neither sort Cps1 correctly nor confer resistance to cadmium (Figure 4, D and E).

The existence of two functional PY motifs suggested that more than one WW domain in Rsp5 might contribute to Bsd2 modification. We therefore repeated the in vitro assay using variants of Rsp5 in which either one or two of the three WW domains were mutated. These assays revealed first that any two WW domains were sufficient for full in vitro activity on the wild-type Bsd2 cytoplasmic domain (Bsd2cyt in Figure 4F, top). Moreover, when PY1 was intact, even if PY2 was mutated, any one of the three WW domains alone was sufficient for modification, although noticeably lower activity was observed when WW3 was mutated (Bsd2cyt in Figure 4F, bottom). In contrast, the residual activity of PY2 observed in the absence of PY1 (ΔPY1) absolutely required WW3, because no modification of the ΔPY1 Bsd2 construct was observed when WW3 was missing from Rsp5 (Figure 4F, top). The WW1 and WW2 domains did not contribute as mutating these domains had little effect on the modification of ΔPY1 (Figure 4F). We conclude that PY1 can bind any of the Rsp5 WW domains, whereas PY2 seems weaker and is recognized only by WW3.

Because the PPTY and APSY motifs are very close in the Bsd2 sequence, as are WW2 and WW3 in Rsp5, it seems likely that WW2 can bind PY1 at the same time as WW3 binds PY2. The unique properties of PY2, which allow it to be recognized only by WW3, may serve to ensure that this is the preferred stable complex. However, it is clear that in vitro ubiquitination of the Bsd2 cytoplasmic domain does not require any such precisely defined complex, and it has much less stringent sequence requirements than those required for ubiquitination of substrates such as Cps1 in vivo. We therefore sought to develop an in vitro assay that would more accurately reflect the in vivo function of Bsd2.

Bsd2-dependent Ubiquitination of Cps1 In Vitro

Initial attempts to express in *E. coli* full-length Bsd2 with its three transmembrane domains were unsuccessful, so instead we purified TAP-tagged protein from detergent-solubilized yeast extracts. To this we added a bacterially expressed construct consisting of GFP fused to the cytoplasmic and transmembrane domains of Cps1, together with Rsp5 and the other components of the ubiquitination assay.

Figure 5A shows that under these conditions we could detect ubiquitination of the Cps1 construct. This was specific, in that a comparable GFP fusion bearing the Deg1 sequence, an amphipathic peptide that is recognized by the Doa10 ubiquitin ligase, was not modified, and nor was the TMD-containing SNARE Sso1 (Figure 5A, bottom). These

substrates were intrinsically capable of receiving ubiquitin, but this required extreme conditions (overnight incubation with a 60-fold higher amount of Rsp5, shown in the right-hand lanes). Cps1 modification in the standard assay was dependent on the presence of Bsd2, and importantly it was abolished not only by the ΔPY1 PPTA mutation but also by the ΔPY2 P149A mutation. It was also reduced by the 113–118, and, to a lesser extent, by the GVG mutations. Thus, this soluble assay mimics key characteristics of Bsd2 function in vivo.

Because this assay showed a dependence on PY2, which is recognized only by Rsp5 WW3, we next tested the various WW mutants. Figure 5B shows that WW3 was indeed essential for modification of Cps1. A WW2 mutant reduced activity, but it did not abolish activity. In contrast, mutation of WW1 had no detectable effect. Thus, as shown previously in vivo (Blondel *et al*., 2004; Katzmann *et al*., 2004), Cps1 modification in vitro depends on WW2 and WW3. This occurs in concert with PY1 and PY2. In vivo, the N-terminal C2 domain of Rsp5 is also required (Katzmann *et al*., 2004; Morvan *et al*., 2004), but this was dispensable in vitro (Figure 5B). This is consistent with the presumed role of the C2 domain in binding to membrane lipids—the in vitro assay contains only detergent micelles and thus would not be expected to require such a function.

Because these requirements were quite different from those defined above for ubiquitination of the cytoplasmic domain of Bsd2, we also examined the modification of full-length Bsd2 in the assay. As shown in Figure 5C, multiple ubiquitins were added, probably to the TAP tag sequences as well as to the lysines in Bsd2, and the efficiency of ubiquitination was closely followed that of Cps1. In particular, it required WW3 and was substantially reduced by the ΔPY2 mutation as well as by the ΔPY1 mutation. These results are strikingly different from the behavior of the Bsd2 cytoplasmic domain, whose modification was not greatly affected by loss of either WW3 or PY2 (see the ΔPY2, ΔWW3 sample in Figure 4F, top). Modification of full-length Bsd2 was more dependent on WW3 than on PY2; when WW3 was the only functional WW domain present it acted through PY1, and PY2 was dispensable (Figure 5C, right).

It seems that the conformation or physical state of full-length Bsd2 imposes a stringent requirement for Rsp5 to interact in a particular way before ubiquitination of either Bsd2 or Cps1 is optimal. Greatest efficiency is achieved with the double interaction WW2/PY1 and WW3/PY2. It is likely that this provides a particularly stable Rsp5-Bsd2 complex, but it is also possible that engagement of WW3, which is immediately adjacent to the catalytic HECT domain of Rsp5, helps to bring the active site into a position where it can efficiently reach the target lysine residues.

Sorting of Tre1 and Smf1

Tre1 has a highly specialized role, acting not merely as a substrate for the Bsd2 system, but also as a coadaptor for the ubiquitination of Smf1. It was therefore of interest to compare its properties with those of Cps1.

Unlike Cps1, Tre1 has a PPVY motif of its own. However, in the absence of Bsd2, Tre1 is poorly ubiquitinated in vivo, and it is not efficiently sorted to the vacuole (Stimpson *et al*., 2006). In agreement with this, we were able to detect only a very small amount of a Tre1–Rsp5 complex when both were expressed in yeast, although this was specific in that it was dependent on the Tre1 PY motif (Figure 6A; compare with Bsd2 binding, Figure 3F). However, a recombinant form of Tre1 consisting of its cytoplasmic and transmembrane domains was ubiquitinated efficiently by Rsp5 in vitro, with any one of the WW domains being sufficient, although WW3 was the most effective (Figure 6B). Evidently the in vivo modification of Tre1 is under more stringent regulation than is evident from these simple in vitro assays.

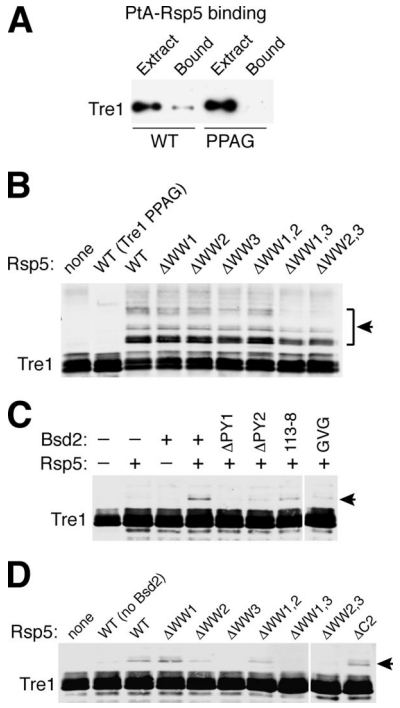

Figure 6. Requirements for ubiquitination of Tre1. (A) Immunoprecipitation of GFP-tagged Tre1Δlumenal or Tre1 Δlumenal PPAG with protein A-tagged Rsp5 from Δ*pep4* cells. Eluates from IgG-Sepharose beads enriched by a factor of 20. (B) Western blot, probed with anti-S-tag, of in vitro ubiquitination of recombinant Tre1Δlumenal (and Tre1 Δlumenal PPAG) by wild-type recombinant Rsp5 (WT) or Rsp5 carrying ΔWW1, ΔWW2, ΔWW3, ΔWW1,2, ΔWW1,3, or ΔWW2,3 mutations described above. (C) Western blot, probed with anti-S-tag, of in vitro ubiquitination of recombinant S-tagged TreΔlumenal PPAG by Rsp5 and Tap-purified wild-type Bsd2 or Tap-purified Bsd2 carrying ΔPY1, ΔPY2, 113-8, or GVG mutations described above. Arrows indicate mono-ubiquitinated forms of TreΔlumenal PPAG. (D) Western blot, probed with anti-S-tag, of in vitro ubiquitination of recombinant S-tagged TreΔlumenal PPAG mediated by Tap-purified Bsd2 and either wild-type Rsp5 or Rsp5 carrying ΔWW1, ΔWW2, ΔWW3, ΔWW1,2, ΔWW1,3, ΔWW2,3, ΔC2 mutations described above. Arrows indicate monoubiquitinated forms of TreΔlumenal PPAG. Irrelevant lanes in C and D have been excised, as indicated by the gaps, but exposures are identical for all lanes.

To circumvent direct recognition by Rsp5 and test Bsd2-dependent modification in vitro, we used a version of recombinant Tre1 in which the PPVY motif was mutated to PPAG. This was assayed with full-length Bsd2 in the same way as the Cps1 construct. Figure 6, C and D, shows that the results were very similar to those observed with Cps1. Modification of Tre1 was dependent on Bsd2, was substantially reduced by the GVG mutation and to a lesser extent by the 113–118 mutation, depended on both PY1 and PY2 of Bsd2, and was eliminated by mutation of WW3. ΔWW2 had a lesser but noticeable effect, whereas ΔWW1 had no apparent effect. Removal of the C2 domain (Figure 6D, ΔC2) also had no effect on Tre1 modification suggesting that, in vitro, the Rsp5 C2 domain was not required. Thus, in this assay Tre1 seems indistinguishable from Cps1.

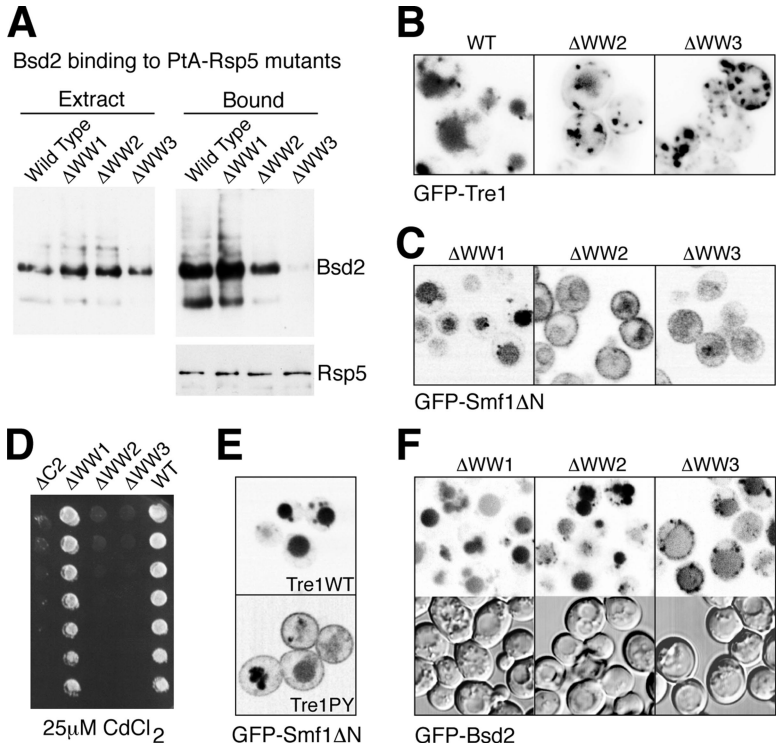

Figure 7. Role of Rsp5 WW domains in vivo. (A) Immunoprecipitations of triple HA-tagged Bsd2 with protein A fusions of Rsp5 WW domain mutants. The blot was probed with the monoclonal anti-HA antibody 12CA5. Eluates were enriched over extracts by a factor of 50. Rsp5 was visualized in the eluates by peroxidase-coupled anti-peroxidase, and the eluate was diluted by a factor of 50 for this. (B) Localization of GFP-Tre1 in wild-type cells (WT) or cells bearing *rsp5* point mutations in WW2 or WW3. (C) Localization of GFP-tagged SmfΔN in Δ*rsp5* cells expressing Rsp5 with the WW1, 2, and 3 mutations. (D) Cadmium sensitivity of Δ*rsp5* cells carrying *Rsp5* with either a deletion of the C2 domain, or mutations in WW domains 1, 2, or 3. (E) Localization of GFP-tagged SmfΔN in a Δ*tre1* Δ*tre2* strain complemented with a plasmid expressing either wild type Tre1 (Tre1WT) or a mutant in which the PPxY motif was changed to PPAG (Tre1PY). (F) Localization of GFP-tagged Bsd2 expressed in Δ*rsp5*Δ*tul1* cells complemented by Rsp5 carrying mutations in single WW domains.

Requirements for Rsp5 WW Domains In Vivo

The results from the cell-free system imply a particular importance for the Rsp5 WW3 domain, which is most easily explained if this domain is important for the formation of a stable Rsp5–Bsd2 complex. To test this prediction, we examined the coprecipitation of Rsp5 WW mutants with Bsd2 in vivo. As shown in Figure 7A, loss of functional WW3 was sufficient to prevent binding to Bsd2, whereas mutation of WW2 reduced binding, but it did not eliminate binding. Together with the previous demonstration that PY2 contributes significantly to Rsp5 binding (Figure 3F), these results emphasize the importance of WW3 and PY2, and they demonstrate a good correlation between the in vivo binding of Rsp5 to Bsd2 and the in vitro ubiquitination assay.

From the in vitro results, we could also predict that Tre1 ubiquitination and hence sorting should require both the Rsp5 WW2 and WW3 domains in vivo. Figure 7B shows that this was the case, GFP-Tre1 occurring in punctate endosomal structures rather than inside vacuoles in the ΔWW2 and especially in the ΔWW3 *rsp5* mutants.

Because Smf1 sorting requires the simultaneous interaction of Rsp5, Tre1 and Smf1 so that Rsp5 can modify Smf1, we can further predict that WW2 and WW3 should be required for Smf1 sorting. This was confirmed by assaying both cadmium resistance and the localization of GFP-Smf1ΔN: the corresponding mutants express Smf1 on the cell surface and consequently were sensitive to cadmium (Figure 7, C and D). In this in vivo situation the C2 domain of Rsp5 is also required for cadmium resistance, presumably to help recruit Rsp5 to the membranes containing Smf1, Bsd2, and Tre1.

Smf1 sorting requires not only PY1 and PY2 on Bsd2, but also the PY motif of Tre1 (Stimpson *et al.*, 2006). One obvious possibility was that this third PY element provides a binding site for WW1. However, Figure 7, C and D, clearly show that WW1 is not required for sorting of Smf1 to the vacuole or for cadmium resistance. In contrast, disrupting the PY element of Tre1 had a significant effect on Smf1 sorting (Figure 7E). Hence, there is the curious situation that three binding sites are required but only two WW domains. Possible explanations for this are discussed below.

Finally, despite the evidence that two WW domains can interact with Bsd2 in vivo, results in Figure 3E indicated that sorting of Bsd2 itself did not require PY2. In agreement with this, we found that entry of GFP-Bsd2 into the vacuole is not only unaffected in a ΔWW1 mutant but also largely unaffected in a ΔWW2 mutant, although it is much more severely blocked in a ΔWW3 mutant (Figure 7F). These results mirror the in vitro ubiquitination of Bsd2 (Figure 5C), and they suggest that a WW3/PY1 interaction is sufficient to mediate enough modification of Bsd2 to ensure its sorting. The reduced requirement for this as compared with the sorting of substrates such as Tre1 or Smf1 can be explained by the need for only a transient interaction for Rsp5 to modify Bsd2, whereas more stable binding is required if an Rsp5–Bsd2 complex is to form and then modify a third protein.

DISCUSSION

Previous work has shown that recognition of a substrate protein by Rsp5 can be induced by the insertion of a single PY motif, which binds one or more of the Rsp5 WW domains (Saeki *et al.*, 2005). In vivo, however, the recognition and modification of substrates is a much more complex process. In this article, we have analyzed the interaction of Rsp5 with the membrane protein Bsd2, and with proteins that in turn interact with Bsd2, and shown that the principal features of substrate modification can be reconstituted in vitro with purified proteins. We have demonstrated that recognition of other proteins by Bsd2 involves polar residues within the membrane, and that this can be reconstituted in detergent extract.

Our results confirm that a single WW/PY interaction can be sufficient for ubiquitination in vitro, but this is not always reflected in vivo. Notably, the single PY motif on Tre1 is well recognized in vitro, yet leads to only minimal modification of Tre1 in cells lacking Bsd2. Evidently competition from other PY proteins, the presence of deubiquitinating enzymes, or steric constraints limit the process. Efficient modification seems to require optimal interactions, and/or simultaneous engagement of two WW domains with their binding sites. The simplest explanation for this is that the stability of Rsp5 binding is thereby enhanced, prolonging the opportunity for modification.

For Bsd2, a very precise double PY motif is required for adaptor activity, although some additional protein sequences also contribute to activity. Notably, the conserved DGVFSN motif at position 113-8 has a clear role in Rsp5 binding in vivo, although it shows no ability to bind directly to Rsp5 in vitro. Thus, the DGVFSN motif may act to increase the "exposure" of the PY motifs to Rsp5 in vivo, either by acting as a structural motif or conceivably as a binding site for a nonessential cofactor that enhances the interaction of Bsd2 with Rsp5. The double PY motif is very well conserved in fungi, including the variant PY2 sequence APSY, and our evidence suggests that it is recognized by the WW2–WW3 combination in Rsp5, whose close spacing is also a conserved feature.

The evolutionary retention of the PY2 sequence seems curious, because it is weak binding site. However, replacing it with a functional copy of PY1 did not allow Bsd2 to mediate Cps1 or Smf1 modification in vivo. The key feature of PY2 may be that it is recognized only by WW3, and thus serves to orient Rsp5 on Bsd2. In agreement with this, we found that interchanging PY1 and PY2 prevented Bsd2 from functioning in vivo, even though both sequences were present. Precise positioning of the ligase may be crucial for its proper function, or for additional stabilizing interactions with Bsd2. Interestingly, in our cell free system quite efficient modification of Bsd2 can be induced by binding of WW3 to PY1, when PY2 is mutated. This is actually slightly improved by the deletion of WW1 and WW2, suggesting that WW3 occupancy is critical and that the other WW domains can interfere by competing for the single binding site (Figure 5C, right). Whether the importance of WW3 is simply due to its close proximity to the catalytic HECT domain, or whether engagement of this domain actively stimulates Rsp5 activity remains to be seen.

Comparison of the in vitro results with those obtained in cells suggests a simple hierarchy of binding stability that can account for the data (Figure 8). For ubiquitination and hence sorting of Bsd2 itself, only a transient interaction with Rsp5 is required (Figure 8A), and this is reflected in the fact that mutation of PY2, or WW2, does not prevent this despite greatly reducing the yield of stable Rsp5–Bsd2 complexes. For Bsd2 to mediate Cps1 sorting, there is a clear requirement for the joint interaction of WW2 and WW3 with PY1 and PY2, leading to a stable Rsp5–Bsd2 complex (Figure 8Bi). This complex presumably has a sufficient lifetime to encounter and modify Cps1 molecules, which probably only interact fleetingly since we were unable to detect stable Cps1-Bsd2 complexes.

The most indirect effect, and the effect with the most stringent requirements, is the sorting of the manganese transporter Smf1. This requires Tre1, which binds Bsd2 readily, as well as WW2 and WW3 and both the Bsd2 PY domains. It seems likely that a stable complex containing Rsp5, Bsd2, and Tre1 has to form, before interacting with Smf1 (Figure 8, Bii and C). By using a form of Smf1 that is endocytosed inefficiently, we have shown that recognition occurs within the exocytic pathway and is metal dependent, suggesting that it is the metal-bound conformation of Smf1 that is recognized. However, when endocytosis is permitted, Smf1 enters the vacuole in a Bsd2-dependent manner even when cells are starved of metals (Figure 1A), suggesting that under these conditions cells cannot completely prevent ubiquitination of Smf1.

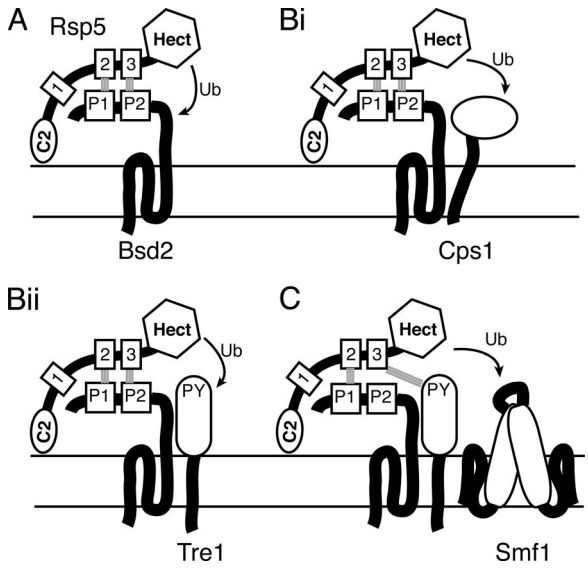

Figure 8. Model for Bsd2-dependent ubiquitination by Rsp5. (A) Self-modification of Bsd2 requires the formation of a complex with Rsp5 mediated by the PY1-WW2 and PY2-WW3 interactions (indicated by gray bars). The subsequent association of either Cps1 (Bi) or Tre1 (Bii) allows their ubiquitination, although this is still mediated by PY1-WW2 and PY2-WW3. (C) Modification of Smf1 requires first the association of Rsp5/Bsd2 with Tre1 and a subsequent rearrangement that allows the PY motif of Tre1 to interact with WW3 displacing PY2 in Bsd2. Cps1 and Tre1 are depicted highly schematically, with the luminal domains omitted.

The most striking finding is that Smf1 sorting requires three PY motifs—two motifs on Bsd2 as well as the Tre1 PY motif. All of these are able to bind WW domains, and one motif obvious possibility would be that all three WW domains of Rsp5 are engaged. However, WW1 is not required for Smf1 sorting. A possible explanation for this apparent paradox is that assembly of the complexes has an obligatory order: Rsp5 must first stably interact with PY1 and PY2 on Bsd2, then when Tre1 is bound there is a rearrangement of the WW domains so that they bind both Bsd2 and Tre1, presumably releasing PY2 (Figure 8, Bii and C). Simultaneous binding of Rsp5 to both Tre1 and Bsd2 might help to stabilize the complex. Alternatively, a precise orientation of Rsp5 may be required to access appropriate lysine residues on Smf1.

The requirement for precise orientation and a stable multipoint interaction with the ubiquitin ligase may be a common feature of adaptors. *Drosophila* Commissureless, the only other well-characterized membrane protein adaptor, has like Bsd2 a double PY motif with the second having a variant sequence (LPSY). Furthermore, this sequence is bound by only one of the WW domains of dNedd4, whereas the upstream PPXY sequence is bound by another (Kanelis *et al*., 2006), suggesting that the binding site has evolved to ensure correct orientation of the ligase. The specificity of recognition in Commissureless is achieved through interac-

tions with the extended sequence TGLPSYDEALH. This may be analogous to the requirement for the Asp residue in the Bsd2 PY2 sequence DMAPSY. The two mammalian homologues of Bsd2 have three PY elements, two of which are very similar to the double element of Commissureless, including the sequence T(S/T)LPSYDEAE(R/K), suggesting that this region interacts with Nedd4 in a similar manner.

Clearly, the ubiquitination of membrane proteins is a highly controlled and precise process, as befits one that could easily lead to erroneous degradation. Our development of a biochemical assay for Rsp5 recruitment to Bsd2 substrates has shown that the key features of this can be explained by the properties of the purified proteins and that no essential components are missing. This physiologically relevant assay system provides a starting point to investigate the roles of regulatory proteins such as Bul1, Ubp2, and Rup1 (Yashiroda *et al*., 1996; Kee *et al*., 2005; Kee *et al*., 2006) that have been reported to control ubiquitin chain length, and of other potential regulators of membrane protein ubiquitination.

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REFERENCES

Blondel, M. O., Morvan, J., Dupre, S., Urban-Grimal, D., Haguenauer-Tsapis, R., and Volland, C. (2004). Direct sorting of the yeast uracil permease to the endosomal system is controlled by uracil binding and Rsp5p-dependent ubiquitylation. Mol. Biol. Cell *15*, 883–895.

Dancis, A., Klausner, R. D., Hinnebusch, A. G., and Barriocanal, J. G. (1990). Genetic evidence that ferric reductase is required for iron uptake in *Saccharomyces cerevisiae*. Mol. Cell Biol. *10*, 2294–2301.

Dunn, R., and Hicke, L. (2001). Domains of the Rsp5 ubiquitin-protein ligase required for receptor-mediated and fluid-phase endocytosis. Mol. Biol. Cell *12*, 421–435.

Eguez, L., Chung, Y. S., Kuchibhatla, A., Paidhungat, M., and Garrett, S. (2004). Yeast Mn2+ transporter, Smf1p, is regulated by ubiquitin-dependent vacuolar protein sorting. Genetics *167*, 107–117.

Galan, J. M., Moreau, V., Andre, B., Volland, C., and Haguenauer-Tsapis, R. (1996). Ubiquitination mediated by the Npi1p/Rsp5p ubiquitin-protein ligase is required for endocytosis of the yeast uracil permease. J. Biol. Chem. *271*, 10946–10952.

Gietz, R. D., and Sugino, A. (1988). New yeast-*Escherichia coli* shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. Gene *74*, 527–534.

Harty, R. N., Brown, M. E., Wang, G., Huibregtse, J., and Hayes, F. P. (2000). A PPxY motif within the VP40 protein of Ebola virus interacts physically and functionally with a ubiquitin ligase: implications for filovirus budding. Proc. Natl. Acad. Sci. USA *97*, 13871–13876.

Hettema, E. H., Valdez-Taubas, J., and Pelham, H. R. (2004). Bsd2 binds the ubiquitin ligase Rsp5 and mediates the ubiquitination of transmembrane proteins. EMBO J. *23*, 1279–1288.

Hicke, L., and Dunn, R. (2003). Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins. Annu. Rev. Cell Dev. Biol. *19*, 141–172.

Hicke, L., and Riezman, H. (1996). Ubiquitination of a yeast plasma membrane receptor signals its ligand-stimulated endocytosis. Cell *84*, 277–287.

Hurley, J. H., and Emr, S. D. (2006). The ESCRT complexes: structure and mechanism of a membrane-trafficking network. Annu. Rev. Biophys. Biomol. Struct. *35*, 277–298.

Jolliffe, C. N., Harvey, K. F., Haines, B. P., Parasivam, G., and Kumar, S. (2000). Identification of multiple proteins expressed in murine embryos as binding partners for the WW domains of the ubiquitin-protein ligase Nedd4. Biochem. J. *351*, 557–565.

Kanelis, V., Bruce, M. C., Skrynnikov, N. R., Rotin, D., and Forman-Kay, J. D. (2006). Structural determinants for high-affinity binding in a Nedd4 WW3* domain-Comm PY motif complex. Structure *14*, 543–553.

Katzmann, D. J., Babst, M., and Emr, S. D. (2001). Ubiquitin-dependent sorting into the multivesicular body pathway requires the function of a conserved endosomal protein sorting complex, ESCRT-I. Cell *106*, 145–155.

Katzmann, D. J., Sarkar, S., Chu, T., Audhya, A., and Emr, S. D. (2004). Multivesicular body sorting: ubiquitin ligase Rsp5 is required for the modification and sorting of carboxypeptidase S. Mol. Biol. Cell *15*, 468–480.

Kee, Y., Lyon, N., and Huibregtse, J. M. (2005). The Rsp5 ubiquitin ligase is coupled to and antagonized by the Ubp2 deubiquitinating enzyme. EMBO J. *24*, 2414–2424.

Kee, Y., Munoz, W., Lyon, N., and Huibregtse, J. M. (2006). The deubiquitinating enzyme Ubp2 modulates Rsp5-dependent Lys63-linked polyubiquitin conjugates in *Saccharomyces cerevisiae*. J. Biol. Chem. *281*, 36724–36731.

Levkowitz, G., Waterman, H., Zamir, E., Kam, Z., Oved, S., Langdon, W. Y., Beguinot, L., Geiger, B., and Yarden, Y. (1998). c-Cbl/Sli-1 regulates endocytic sorting and ubiquitination of the epidermal growth factor receptor. Genes Dev. *12*, 3663–3674.

Liu, X. F., and Culotta, V. C. (1994). The requirement for yeast superoxide dismutase is bypassed through mutations in BSD2, a novel metal homeostasis gene. Mol. Cell Biol. *14*, 7037–7045.

Liu, X. F., and Culotta, V. C. (1999). Post-translation control of Nramp metal transport in yeast. Role of metal ions and the BSD2 gene. J. Biol. Chem. *274*, 4863–4868.

Luo, W., and Chang, A. (1997). Novel genes involved in endosomal traffic in yeast revealed by suppression of a targeting-defective plasma membrane ATPase mutant. J. Cell Biol. *138*, 731–746.

Morvan, J., Froissard, M., Haguenauer-Tsapis, R., and Urban-Grimal, D. (2004). The ubiquitin ligase Rsp5p is required for modification and sorting of membrane proteins into multivesicular bodies. Traffic *5*, 383–392.

Myat, A., Henry, P., McCabe, V., Flintoft, L., Rotin, D., and Tear, G. (2002). Drosophila Nedd4, a ubiquitin ligase, is recruited by Commissureless to control cell surface levels of the roundabout receptor. Neuron *35*, 447–459.

Nikko, E., Marini, A. M., and Andre, B. (2003). Permease recycling and ubiquitination status reveal a particular role for Bro1 in the multivesicular body pathway. J. Biol. Chem. *278*, 50732–50743.

Oliver, P. M. *et al*. (2006). Ndfip1 protein promotes the function of Itch ubiquitin ligase to prevent T cell activation and T helper 2 cell-mediated inflammation. Immunity *25*, 929–940.

Puig, O., Caspary, F., Rigaut, G., Rutz, B., Bouveret, E., Bragado-Nilsson, E., Wilm, M., and Seraphin, B. (2001). The tandem affinity purification (TAP) method: a general procedure of protein complex purification. Methods *24*, 218–229.

Reggiori, F., and Pelham, H. R. (2001). Sorting of proteins into multivesicular bodies: ubiquitin-dependent and -independent targeting. EMBO J. *20*, 5176–5186.

Reggiori, F., and Pelham, H. R. (2002). A transmembrane ubiquitin ligase required to sort membrane proteins into multivesicular bodies. Nat. Cell Biol. *4*, 117–123.

Rotin, D., Kanelis, V., and Schild, L. (2001). Trafficking and cell surface stability of ENaC. Am. J. Physiol. *281*, 391–399.

Rougier, J. S., van Bemmelen, M. X., Bruce, M. C., Jespersen, T., Gavillet, B., Apotheloz, F., Cordonier, S., Staub, O., Rotin, D., and Abriel, H. (2005). Molecular determinants of voltage-gated sodium channel regulation by the Nedd4/Nedd4-like proteins. Am. J. Physiol. *288*, 692–701.

Sacher, A., Cohen, A., and Nelson, N. (2001). Properties of the mammalian and yeast metal-ion transporters DCT1 and Smf1p expressed in *Xenopus laevis* oocytes. J. Exp. Biol. *204*, 1053–1061.

Saeki, Y., Isono, E., and Toh, E. A. (2005). Preparation of ubiquitinated substrates by the PY motif-insertion method for monitoring 26S proteasome activity. Methods Enzymol. *399*, 215–227.

Sang, Q. *et al*. (2006). Nedd4-WW domain-binding protein 5 (Ndfip1) is associated with neuronal survival after acute cortical brain injury. J. Neurosci. *26*, 7234–7244.

Sauer, B. (1994). Recycling selectable markers in yeast. Biotechniques *16*, 1086–1088.

Shearwin-Whyatt, L., Dalton, H. E., Foot, N., and Kumar, S. (2006). Regulation of functional diversity within the Nedd4 family by accessory and adaptor proteins. Bioessays *28*, 617–628.

Shearwin-Whyatt, L. M., Brown, D. L., Wylie, F. G., Stow, J. L., and Kumar, S. (2004). N4WBP5A (Ndfip2), a Nedd4-interacting protein, localizes to multivesicular bodies and the Golgi, and has a potential role in protein trafficking. J. Cell Sci. *117*, 3679–3689.

Siniossoglou, S., Peak-Chew, S. Y., and Pelham, H. R. (2000). Ric1p and Rgp1p form a complex that catalyses nucleotide exchange on Ypt6p. EMBO J. *19*, 4885–4894.

Snyder, P. M. (2005). Regulation of epithelial Na+ channel trafficking. Endocrinology *146*, 5079–5085.

Springael, J. Y., and Andre, B. (1998). Nitrogen-regulated ubiquitination of the Gap1 permease of *Saccharomyces cerevisiae*. Mol. Biol. Cell *9*, 1253–1263.

Stimpson, H. E., Lewis, M. J., and Pelham, H. R. (2006). Transferrin receptor-like proteins control the degradation of a yeast metal transporter. EMBO J. *25*, 662–672.

Sudol, M. (1996). Structure and function of the WW domain. Prog. Biophys. Mol. Biol. *65*, 113–132.

Swanson, R., Locher, M., and Hochstrasser, M. (2001). A conserved ubiquitin ligase of the nuclear envelope/endoplasmic reticulum that functions in both ER-associated and Matalpha2 repressor degradation. Genes Dev. *15*, 2660–2674.

Valdez-Taubas, J., and Pelham, H. (2005). Swf1-dependent palmitoylation of the SNARE Tlg1 prevents its ubiquitination and degradation. EMBO J. *24*, 2524–2532.

Volland, C., Urban-Grimal, D., Geraud, G., and Haguenauer-Tsapis, R. (1994). Endocytosis and degradation of the yeast uracil permease under adverse conditions. J. Biol. Chem. *269*, 9833–9841.

Yashiroda, H., Oguchi, T., Yasuda, Y., Toh, E. A., and Kikuchi, Y. (1996). Bul1, a new protein that binds to the Rsp5 ubiquitin ligase in *Saccharomyces cerevisiae*. Mol. Cell Biol. *16*, 3255–3263.