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Key Steps in ERAD of Luminal ER Proteins Reconstituted with Purified Components

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SUMMARY

Misfolded proteins of the endoplasmic reticulum (ER) are retrotranslocated into the cytosol, polyubiquitinated, and degraded by the proteasome, a process called ER-associated protein degradation (ERAD). Here, we use purified components from *Saccharomyces cerevisiae* to analyze the mechanism of retrotranslocation of luminal substrates (ERAD-L), recapitulating key steps in a basic process in which the ubiquitin ligase Hrd1p is the only required membrane protein. We show that Hrd1p interacts with substrate through its membrane-spanning domain and discriminates misfolded from folded polypeptides. Both Hrd1p and substrate are polyubiquitinated, resulting in the binding of Cdc48p ATPase complex. Subsequently, ATP hydrolysis by Cdc48p releases substrate from Hrd1p. Finally, ubiquitin chains are trimmed by the deubiquitinating enzyme Otu1p, which is recruited and activated by the Cdc48p complex. Cdc48p-dependent membrane extraction of polyubiquitinated proteins can be reproduced with reconstituted proteoliposomes. Our results suggest a model for retrotranslocation in which Hrd1p forms a membrane conduit for misfolded proteins.

INTRODUCTION

Protein homeostasis in the endoplasmic reticulum (ER) is maintained by a quality control system. When a protein misfolds, it is retained in the ER and ultimately retrotranslocated into the cytosol, polyubiquitinated, and degraded by the proteasome. This pathway is referred to as ER-associated protein degradation (ERAD) (for review, see Bagola et al., 2011; Brodsky, 2012). It alleviates cytotoxic stress imposed by protein misfolding and is implicated in numerous diseases (Guerriero and Brodsky, 2012). ERAD is found in all eukaryotic cells but is best understood in *Saccharomyces cerevisiae*. Here, substrates use three ERAD pathways (ERAD-L, -M, or -C), depending on whether the misfolded domain is localized in the ER lumen, inside the ER membrane, or at the cytosolic side of the ER membrane (Carvalho et al., 2006; Huyer et al., 2004; Vashist and Ng, 2004). The pathways use distinct ubiquitin ligase complexes. ERAD-L requires a heterotetrameric membrane protein complex, the Hrd1p complex, comprised of the ubiquitin ligase Hrd1p and three additional membrane proteins (Hrd3p, Usa1p, and Der1p). ERAD-M also requires Hrd1p but only a subset of the other components, and ERAD-C uses the ubiquitin ligase Doa10p. On the cytosolic side of the ER membrane, all pathways require an ATPase complex, which includes the ATPase Cdc48p and the cofactors Ufd1p and Npl4p.

Among the ERAD pathways, ERAD-L is arguably most complex, as polypeptides have to be inserted into and moved across the ER membrane. ERAD-L begins with the recognition of a misfolded protein in the ER lumen, which is best understood for misfolded glycoproteins (for review, see Xie and Ng, 2010). The N-linked glycan of these proteins is trimmed to generate a terminal α-1,6-mannose residue, which is recognized by the luminal protein Yos9p. Yos9p binds to the luminal domain of Hrd3p, which also binds a misfolded segment around the glycan-attachment site of the substrate. Once a segment of the substrate emerges on the cytoplasmic side of the ER membrane, it is polyubiquitinated by the RING finger domain of Hrd1p (Bays et al., 2001a; Bordallo et al., 1998). The major ubiquitin-conjugating enzyme participating in this reaction is Ubc7p, which also requires the activator Cue1p (Bays et al., 2001a; Biederer et al., 1997). The recruitment of the Cdc48p complex to the Hrd1p complex involves recognition of a polyubiquitin chain by the cofactor Ufd1p/Npl4p (Meyer et al., 2002). However, it is unclear whether the recruitment requires the ubiquitin chain to be attached to the substrate, to Hrd1p, or to an unknown component. The binding of Cdc48p to the membrane might be facilitated by the adaptor protein Ubx2p (Neuber et al., 2005; Schuberth and Buchberger, 2005). Cdc48p is then thought to pull on the polyubiquitinated polypeptide substrate to move it into the cytosol (Bays et al., 2001b; Jarosch et al., 2002; Rabinovich et al., 2002; Ye et al., 2001). Recent experiments have also implicated deubiquitinating enzymes (DUBs) in ERAD (for review, see Liu and Ye, 2012). Several DUBs associate with Cdc48p or its mammalian homolog p97, and the overexpression of dominant-negative forms blocks ERAD in mammalian cells. However, it remains unclear how DUBs participate in ERAD.

The events of ERAD-L inside the ER membrane are less well understood. Hrd1p seems to be a central component, as its overexpression bypasses the need for the other membrane components, as well as for the luminal protein Yos9p (Carvalho et al., 2010; Denic et al., 2006; Garza et al., 2009; Plemper et al., 1999). Under these conditions, glycan trimming is not required and both glycosylated and nonglycosylated proteins are degraded. All downstream cytosolic components, including the Cdc48p complex, are still required. Hrd1p overexpression makes Hrd1p unstable and slows, but does not abolish, cell growth. These results suggest that Hrd1p is the only membrane component required for a basic ERAD-L process. The minimal components required for retrotranslocation therefore comprise Hrd1p, Ubc7p, Cue1p, and the Cdc48p complex. Because Hrd1p is a multispanning membrane protein, it is a good candidate to be part of a retrotranslocation channel. In fact, cross-linking experiments show that a retrotranslocating substrate interacts with Hrd1p (Carvalho et al., 2010). However, the exact function of Hrd1p during retrotranslocation remains unclear.

Our knowledge on ERAD-L comes from genetics and biochemical experiments in intact cells. In vitro reconstitution of ERAD-L using purified components is critical to demonstrate that all ERAD components have been identified. Even more importantly, reconstitution experiments are instrumental in addressing the molecular mechanism of ERAD. Here, we have recapitulated crucial reactions of a basic ERAD-L process with purified *S. cerevisiae* components in both detergent and reconstituted proteoliposomes. Our results suggest a mechanistic model for how misfolded luminal ER proteins associate with Hrd1p inside the ER membrane and how they are extracted by Cdc48p on the cytoplasmic side of the membrane.

RESULTS

Direct Interaction of ERAD-L Substrates with Hrd1p

In vivo experiments suggested that Hrd1p is the only membrane protein required for a basic ERAD-L process (Carvalho et al., 2010). We therefore tested whether purified Hrd1p directly interacts with purified misfolded proteins. We used a well-characterized ERAD-L substrate, misfolded procarboxypeptidase Y (CPY*), which differs from the native protein by a single-point mutation (Finger et al., 1993). His-tagged CPY* was purified in urea after expression in *S. cerevisiae*, but it remained soluble after removal of urea (Figure S1A available online). To facilitate detection, CPY* was labeled with a fluorescent dye (Figure S1B).

Hrd1p with a C-terminal streptavidin-binding peptide (SBP) tag (Hrd1p-SBP) was purified from *S. cerevisiae* in the detergent decyl maltose neopentyl glycol (DMNG). Although Hrd1p appeared pure by SDS-PAGE (Figure S1C), it was heterogeneous in size by gel filtration (Figure S1D), consistent with it forming homo-oligomers in vivo (Carvalho et al., 2010).

For binding experiments, we mixed labeled CPY* with increasing concentrations of immobilized Hrd1p. Quantification of the bound and nonbound fractions gave an apparent affinity of ~30 nM (Figures 1A and 1B). Wild-type pro-CPY (CPY) did not bind to Hrd1p (Figure 1B; see Figures S1A and S1B for purified and labeled protein, respectively). Even when CPY was treated like CPY*, i.e., denatured in urea followed by removal of urea, only a small fraction was able to bind (Figure 1B). This suggests that Hrd1p can discriminate misfolded from folded polypeptides.

Hrd1p also bound other misfolded CPY variants, such as a C-terminal fragment of CPY* (sCPY*), which is an ERAD-L substrate in vivo (Carvalho et al., 2010). sCPY* was purified as a mixture of glycosylated and nonglycosylated species (as shown by treatment with peptide N-glycosidase F; Figure S1A) and was labeled with a fluorescent dye at a single C-terminal Cys residue (Figure S1B). sCPY* bound to Hrd1p with significantly lower affinity than did CPY* (Figures 1C and 1D; ~300 nM). Glycosylated and nonglycosylated sCPY* bound equally well to Hrd1p, consistent with the fact that other ERAD components are required for their discrimination and with both species being substrates in Hrd1p-overexpressing cells (Denic et al., 2006). A fusion of sCPY* with dihydrofolate reductase (DHFR) bound more tightly than did sCPY*, whereas a fusion with GFP (sCPY*-GFP) bound more weakly (Figure 1E; purity shown in Figures S1A and S1B). These differences correlate with the tendency of these proteins to aggregate; the concentration of urea required to keep these proteins in solution was lowest for sCPY*-GFP, intermediate for sCPY*, and highest for sCPY*-DHFR (data not shown). Purified GFP or DHFR alone, or bovine serum albumin, did not bind (Figure 1E; data not shown; purified DHFR and GFP are shown in Figures S1 and S1B, respectively). Thus, Hrd1p binds selectively to unfolded polypeptides.

The membrane-embedded domain of Hrd1p is necessary and sufficient for substrate interaction, as the C-terminal cytoplasmic domain (Hrd1p-c) did not bind sCPY* (Figure 1F) and a fusion of the membrane-embedded domain of Hrd1p with GFP (Hrd1p-TM-GFP) bound substrate with the same affinity as wild-type Hrd1p (Figure 1F; purity of the proteins shown in Figure S1C). A folded state of the transmembrane segments (TMs) seems to be required for substrate interaction, as full-length Hrd1p was unstable in detergents other than DMNG, and this correlated with the loss of substrate binding (data not shown).

We used the high-affinity substrate sCPY*-DHFR to test whether substrate binds equally well to different oligomeric states of Hrd1p. Labeled sCPY*-DHFR alone behaved in gel filtration as a homogeneous, low-molecular weight species (Figure 1G), as the low concentration and the presence of detergent prevented its aggregation. When labeled sCPY*-DHFR was mixed with a 100-fold excess of Hrd1p, most substrate molecules migrated at very high molecular weight, where few Hrd1p molecules were found, and vice versa, the smallest-sized Hrd1p species did not contain bound substrate. Because sCPY*-DHFR is much smaller than are the Hrd1p oligomers (~40 kDa versus >250 kDa), the size shift of the substrate indicates that it preferentially binds to high-molecular weight Hrd1p oligomers.

Substrate Polyubiquitination by Hrd1p

Next, we tested whether Hrd1p polyubiquitinates bound substrate. To this end, we purified the ubiquitin-conjugating enzyme Ubc7p and its activator Cue1p (Figure S1C). Cue1p is a single-spanning membrane protein (Biederer et al., 1997), but in our experiments the full-length protein and a truncated version

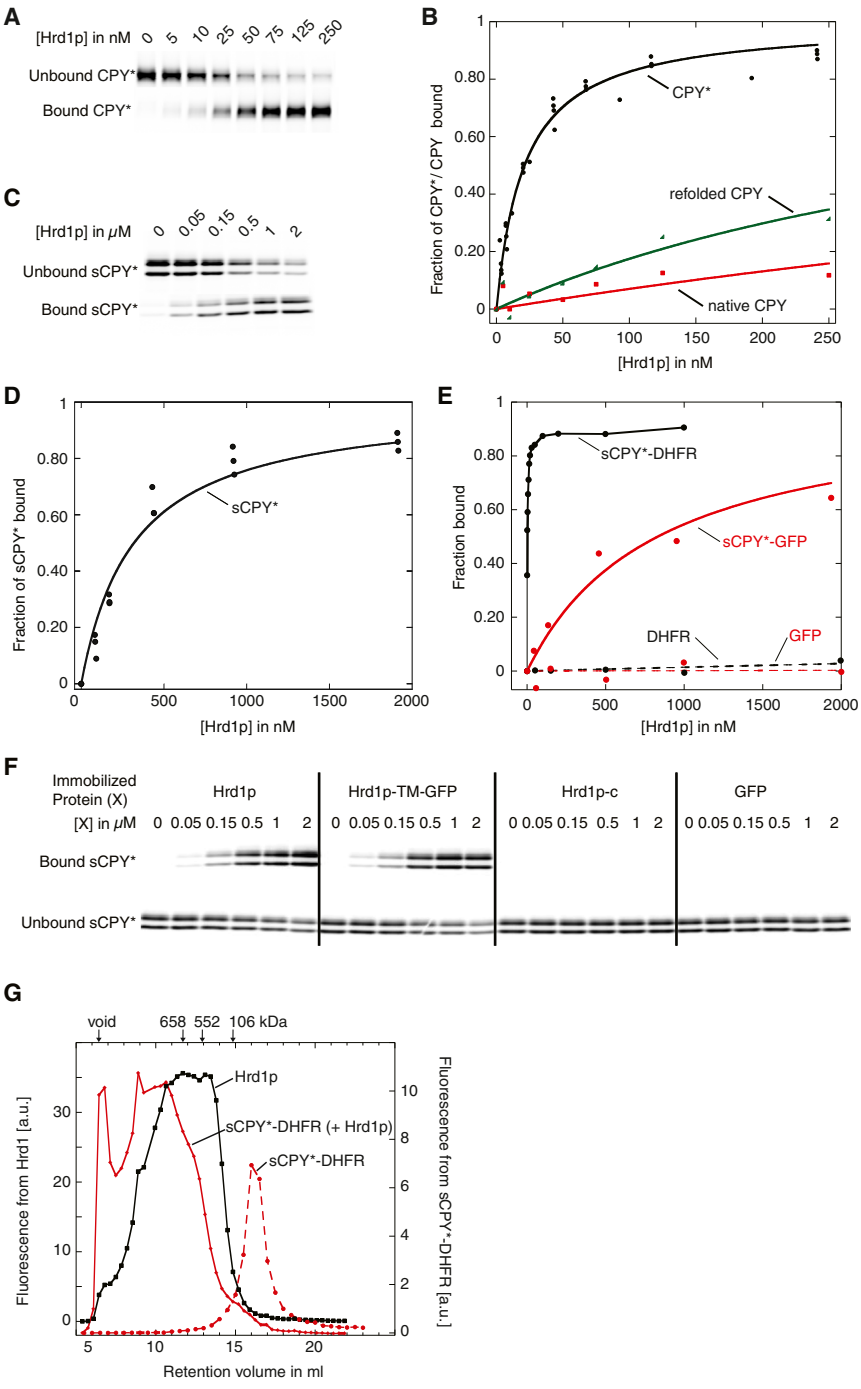

Figure 1. Substrate Interaction with Hrd1p

(A) Fluorescently labeled CPY* (10 nM) was incubated with increasing concentrations of bead-immobilized SBP-tagged Hrd1p (Hrd1p). The bound and unbound fractions were analyzed by SDS-PAGE and fluorescence scanning.

(B) Quantification of four different experiments as in (A). Fitting of the data points gives an apparent dissociation constant of 30 nM. Also shown are experiments with wild-type CPY, either purified as a native protein or after unfolding and refolding, as done with CPY*.

(C) As in (A) but with sCPY* (100 nM).

(D) Quantification of three different experiments as in (C). The apparent dissociation constant is ~300 nM.

(E) Quantification of binding experiments of wild-type Hrd1p with fluorescently labeled sCPY*-DHFR (10 nM), sCPY*-GFP (100 nM), DHFR (100 nM), or GFP (100 nM).

(F) As in (C), but sCPY* was incubated with wild-type Hrd1p, a fusion of the TMs of Hrd1p with GFP (Hrd1p-TM-GFP), the cytoplasmic domain of Hrd1p (Hrd1p-c), or GFP.

(G) sCPY*-DHFR (200 nM) labeled with DyLight800 was incubated with a mixture of unlabeled Hrd1p (20 μM) and Hrd1p (200 nM) labeled with DyLight680. The sample was subjected to gel filtration in a buffer containing 120 μM DMNG, and fractions were analyzed in two fluorescence channels. A control was performed with labeled sCPY*-DHFR alone. The arrows indicate the void volume, and the retention volume of size standards.

See also Figure S1.

containing only the cytosolic domain (Cue1p-c) behaved identically (data not shown). When Hrd1p was incubated with fluorescently labeled CPY*, Ubc7p, Cue1p-c, purified Uba1p (Figure S1C), ubiquitin, and ATP, polyubiquitinated CPY* was generated (Figure 2A). The reaction rate depended mostly on the concentrations of Hrd1p and Ubc7p and was complete within 60 min. No polyubiquitination was observed if ATP, Uba1p, Ubc7p, or Hrd1p were omitted or if an inactive Hrd1p mutant (Hrd1p C399S) was used (Bordallo and Wolf, 1999), and little modification was seen in the absence of Cue1p-c (Figure 2A; for purity of Hrd1p C399S, see Figure S1C). Efficient polyubiquitination was also observed with sCPY* and sCPY*-DHFR (shown for sCPY* in Figure S2A). The experiments with sCPY* showed that the glycosylated and nonglycosylated species were equally modified. Most of the polyubiquitin chains on sCPY* and CPY* are linked through Lys48 in ubiquitin, as indicated by the much shorter chains generated with a Lys48Arg ubiquitin mutant (Figure S2B). When ubiquitin was replaced with methylated ubiquitin, which permits the attachment of a single ubiquitin molecule but prevents the synthesis of ubiquitin chains, several modified bands appeared, indicating that the substrates were modified at several different Lys-residues (Figure S2B).

A purified cytoplasmic fragment of Hrd1p (Hrd1p-c) did not ubiquitinate CPY* (Figure 2A, lane 14) or sCPY* (Figure S2A, lane 12), even when added at 10-fold higher concentrations, although it could generate polyubiquitin chains (Figure S2C). Thus, substrate needs to bind to the membrane-embedded domain of Hrd1p to become polyubiquitinated.

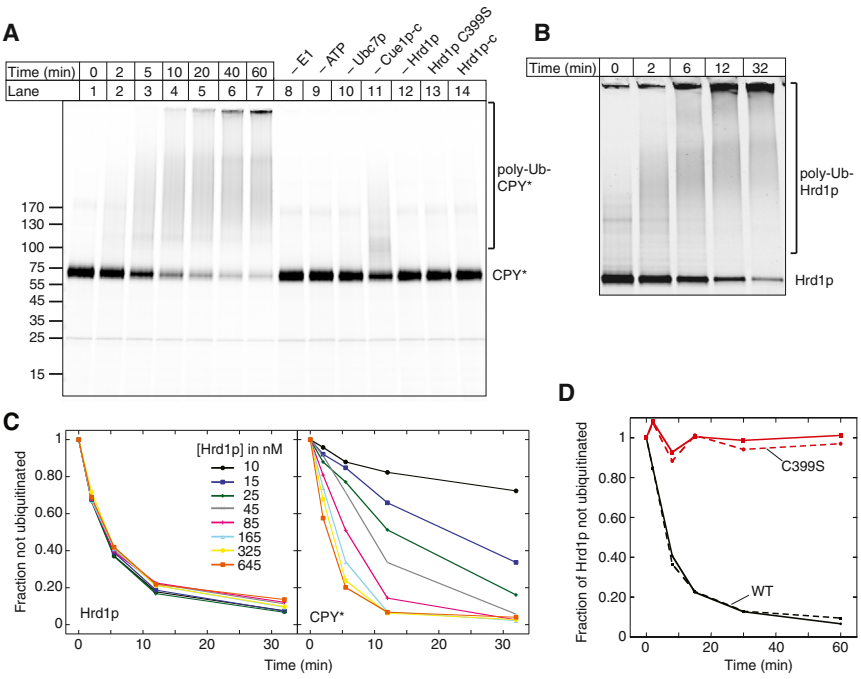

Figure 2. Polyubiquitination by Hrd1p

(A) Time course of ubiquitination of CPY* labeled with DyLight800. Some reactions were analyzed after 60 min with the indicated components omitted. Where indicated, wild-type Hrd1p (100 nM) was replaced with 100 nM of an inactive Hrd1p mutant (C399S) or 1 μM of the cytoplasmic domain of Hrd1p (Hrd1p-c).

(B) Time course of autoubiquitination of Hrd1p labeled with DyLight680.

(C) The time course of auto- and substrate-ubiquitination was determined in parallel. The concentration of labeled Hrd1p was kept constant, whereas that of unlabeled Hrd1p was varied.

(D) The time course of autoubiquitination was determined with 50 nM of labeled wild-type (WT) Hrd1p or C399S mutant in the absence or presence of a 10-fold excess of unlabeled WT Hrd1p (solid and broken curves, respectively).

See also Figure S2.

Autoubiquitination of Hrd1p

Hrd1p itself was also polyubiquitinated. To study Hrd1p autoubiquitination in more detail, we attached, by sortase labeling, a fluorescent dye to the C terminus of Hrd1p, allowing the simultaneous detection of Hrd1p and substrate. Hrd1p polyubiquitination was very efficient (Figure 2B). Experiments with methylated ubiquitin showed that modification of Hrd1p occurs at several different Lys residues and that Hrd1p molecules often contain two ubiquitin chains (Figure S2C, last lane). Mass spectrometry confirmed the modification of several Lys residues (positions 126, 143, 282, 325, 387, 407, 511, 518, 539, 540, and 546), but replacement of single Lys residues with Arg did not drastically reduce autoubiquitination (data not shown). Because a Hrd1p mutant in which all 27 Lys residues were changed to Arg did not express, we generated mutants, in which Lys residues were replaced in the transmembrane domain (residues 1–301), the RING finger domain (302–407), or the C-terminal tail (408–551) (RKK, KRK, and KKR mutants, respectively). The RKK had significantly reduced levels of both auto- and substrate-ubiquitination, whereas the KKR mutant was specifically affected in autoubiquitination (Figure S2D). The KRK mutant showed an intermediate phenotype. In none of the mutants was auto-ubiquitination completely abolished. The cytoplasmic fragment of Hrd1p (Hrd1p-c) showed little or no autoubiquitination; much of Hrd1p-c remained unmodified, and no modified protein was seen with methylated ubiquitin (Figure S2C). Thus, the transmembrane domain is required for efficient autoubiquitination.

Hrd1p seems to modify itself by an intramolecular reaction, because the rate of Hrd1p polyubiquitination was independent of the Hrd1p concentration, in contrast to substrate polyubiquitination (Figure 2C). Furthermore, when wild-type Hrd1p was mixed with an inactive Hrd1p mutant (Hrd1p C399S), only the wild-type, and not the mutant, was modified (Figure 2D), even though the two proteins bind each other (Figure S2E).

We found no conditions in which only substrate ubiquitination was observed. For example, reducing the concentrations of Ubc7p or Cue1p-c did not favor substrate- over automodification (Figure S2F). Autoubiquitination was observed even under conditions in which Hrd1p was saturated with substrate, although the rate was somewhat reduced (Figure S2G). Thus, autoubiquitination of Hrd1p appears to be an integral part of the polyubiquitination reaction.

Polyubiquitinated Hrd1p Recruits the Cdc48p ATPase Complex

Next, we tested whether Hrd1p polyubiquitination leads to recruitment of the Cdc48p ATPase complex. We first separately purified hexameric Cdc48p and a complex of the heterodimeric cofactor Ufd1p/Npl4p from *Escherichia coli* (Figure S1C). The individual components assembled into the Cdc48p complex, as shown by gel filtration (Figure S3A). Next, we treated beads containing polyubiquitinated Hrd1p with Cdc48p in the presence or absence of Ufd1p/Npl4p. Binding of Cdc48p was observed in the presence of the cofactor at low or physiological salt concentrations (Figure 3A, lane 4 versus 1 and lane 9 versus 6), but not at high-salt concentrations (lane 14). Ufd1p/Npl4p alone also bound to ubiquitinated Hrd1p (lanes 2 and 7), indicating that the cofactor is responsible for the recruitment of the Cdc48p complex. When Hrd1p was not preincubated with the ubiquitination machinery, Cdc48p binding was drastically reduced (Figure 3C); the residual binding is likely due to purified Hrd1p carrying some ubiquitin chains (shown by mass spectroscopy).

We also tested the role of Ubx2p, a protein that interacts with Cdc48p and ubiquitin and is involved in ERAD (Neuber et al., 2005; Schuberth and Buchberger, 2005). Full-length Ubx2p with an N-terminal His-tag was purified in the detergent DMNG by Ni-NTA chromatography, followed by gel filtration (Figure S1C). Ubx2p bound to ubiquitinated Hrd1p (Figure 3A, lanes

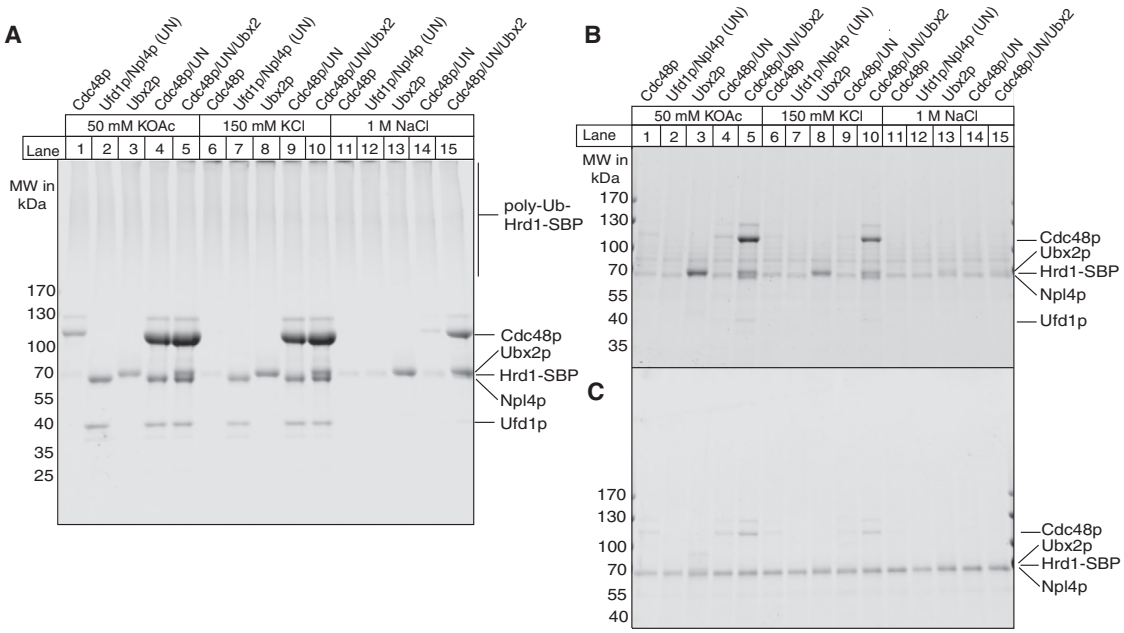

Figure 3. Recruitment of Cdc48p to Ubiquitinated Hrd1p

(A) Bead-immobilized Hrd1p (1 μM) was ubiquitinated and incubated at different salt conditions with 2 μM of the indicated components (UN, Ufd1p/Npl4p) in the presence of 250 μM ATPγS. The bound material was analyzed by SDS-PAGE and stained with IRDye Blue.

(B) As in (A), but bead-immobilized Hrd1p was modified with methylated ubiquitin.

(C) As in (A), but with nonubiquitinated Hrd1p.

See also Figure S3.

3, 8, and 13), but not to unmodified Hrd1p (Figure 3C). Ubx2p increased the binding affinity of the Cdc48p complex for polyubiquitinated Hrd1p, as indicated by the resistance of the interaction to high-salt concentrations (Figure 3A, lane 15). In contrast to Cdc48p complex alone, a complex with Ubx2p also bound to monoubiquitinated Hrd1p generated with methylated ubiquitin (Figure 3B, lanes 5 and 10). Even in the presence of Ubx2p, the Ufd1p/Npl4p complex was essential for the recruitment of Cdc48p (data not shown), suggesting that Ubx2p interacts only weakly with Cdc48p itself. Indeed, gel filtration showed an increased association of Ubx2p's UBX domain (Ubx2p-c) with Cdc48p in the presence of Ufd1p/Npl4p (Figures S3A and S3B; see Figure S1C for purity of the protein). A similar hierarchy has been observed with another UBX domain containing protein (Hänzelmann et al., 2011). Taken together, our results show that polyubiquitinated Hrd1p recruits the Cdc48p complex. Ubx2p stimulates or stabilizes the association but is not essential, consistent with in vivo data (Neuber et al., 2005; Schuberth and Buchberger, 2005).

Cdc48p-Dependent Substrate Release from Hrd1p

Next, we tested whether Cdc48p can release substrate bound to Hrd1p. We first formed a complex between bead-immobilized SBP-tagged Hrd1p and fluorescently labeled CPY* and then incubated the beads with the ubiquitination machinery. After removal of the ubiquitination machinery, the beads were incubated with Cdc48p in the presence of Ufd1p/Npl4p and ATP. About 50% of polyubiquitinated CPY* was released from the beads (Figure 4A, lane 6; quantification in Figure 4B). No release above background was seen if ATP was depleted with hexokinase/glucose (Hk/G) (Figure 4A, lane 7) or if Cdc48p or Ufd1/Npl4p were omitted (lanes 4 and 5). Similar results were obtained with sCPY* (Figures S4A and S4B).

We noticed that nonubiquitinated CPY* and sCPY* were also released from Hrd1p by Cdc48p activity (Figures 4A and S4A). This was confirmed in experiments in which CPY* was added after the ubiquitination reaction, so that only Hrd1p was modified (Figure 4C; quantification in Figure 4D). Some CPY* was released from the beads without Cdc48p action, likely because ubiquitinated Hrd1p is partially dissociating from the beads. No release was seen for substrate bound to nonubiquitinated Hrd1p (Figures 4C and 4D). Similar results were obtained with sCPY* and sCPY*-DHFR (Figures S4C and S4D), although more spontaneous release was seen with sCPY*, consistent with its reduced binding affinity for Hrd1p. Together, these data indicate that the Cdc48p ATPase complex is first recruited to polyubiquitinated Hrd1p and then uses ATP hydrolysis to release both polyubiquitinated and nonubiquitinated substrate from Hrd1p.

To test whether autoubiquitination at a specific site is required for the release of nonubiquitinated substrate, we employed Lys mutants of Hrd1p (Figure 4E). The release was significantly reduced with the RKK and KRK mutants but was even stimulated with the KKR mutant. Thus, ubiquitin chains attached to either one of the N-terminal regions, but not the C-terminal tail, are required for efficient release of nonmodified substrate from Hrd1p.

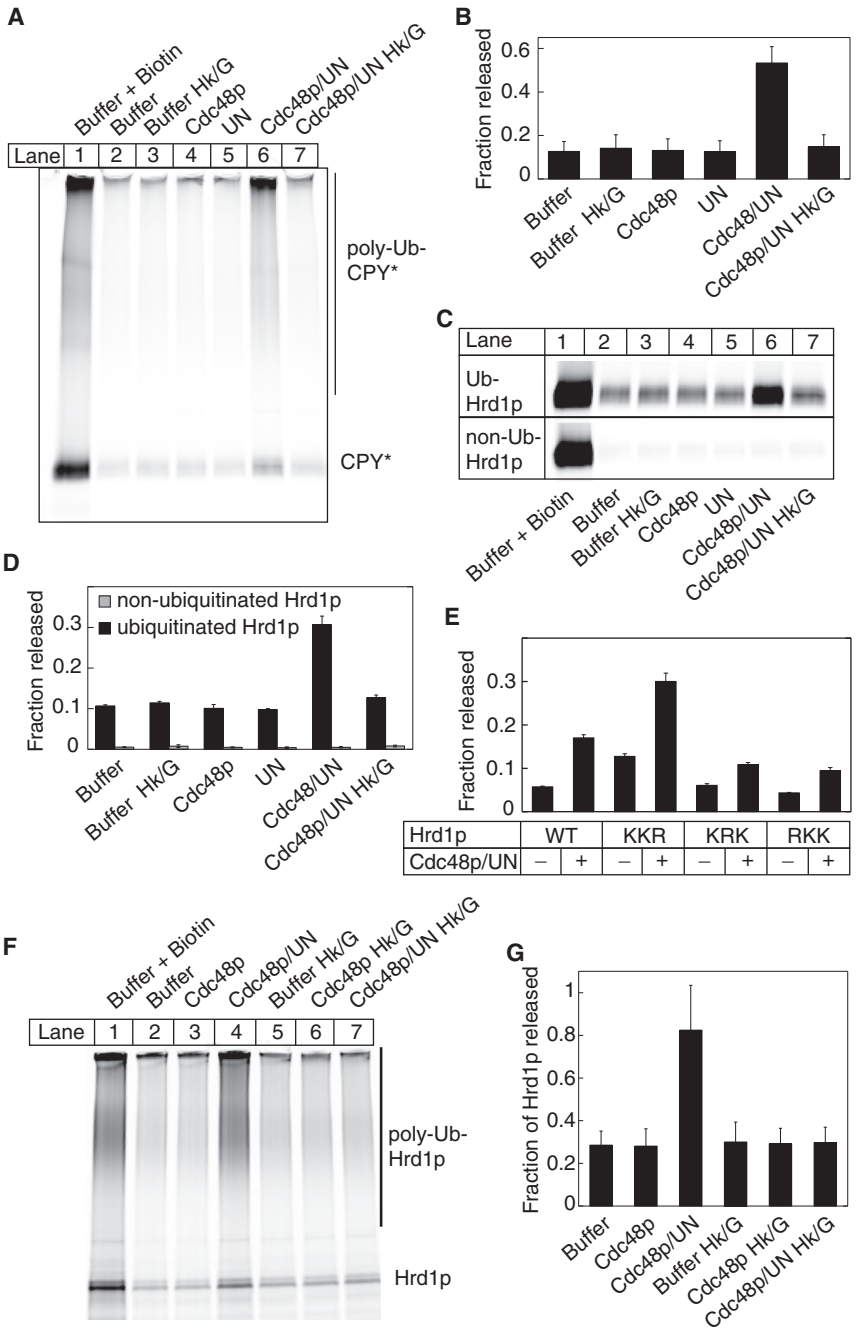

Figure 4. Substrate Release from Hrd1p by Cdc48p ATPase

(A) A bead-immobilized complex of 500 nM SBP-tagged Hrd1p and 100 nM fluorescently labeled CPY* was incubated with the ubiquitination machinery. The beads were washed and incubated with 100 nM Cdc48p and ATP in the absence or presence of 100 nM Ufd1/Npl4p (UN) complex or with UN alone. Where indicated, ATP was depleted with hexokinase/glucose (Hk/G). The released material was analyzed by SDS-PAGE and fluorescence scanning. The total releasable amount of CPY* was determined by incubating the beads with biotin.

(B) Quantification (means and SD) of released polyubiquitinated CPY* determined from four experiments as shown in (A). The released fraction is expressed relative to the total releasable material.

(C) Bead-immobilized SBP-tagged Hrd1p was ubiquitinated (Ub-Hrd1p) for 1 hr. The beads were washed, incubated with fluorescently labeled CPY*, and treated as in (A). The lower panel shows the same experiment with nonubiquitinated Hrd1p (non-Ub-Hrd1p).

(D) Quantification (means and SD) of released nonubiquitinated CPY* determined from four experiments as shown in (C) (released from Ub-Hrd1p, black columns; released from non-Ub-Hrd1p, gray columns).

(E) Immobilized Hrd1p mutants with Lys-to-Arg mutations in three different regions were ubiquitinated for 30 min, and Cdc48p-dependent release of unmodified CPY* was tested as in (C). Shown are means and SD of three experiments.

(F) Bead-immobilized Hrd1p was incubated with untagged, fluorescently labeled Hrd1p. After ubiquitination and washing, the beads were incubated with the indicated components. The material released from the beads was analyzed by SDS-PAGE and fluorescence scanning.

(G) Quantification of three experiments performed as in (E) (means and SD).

See also Figure S4.

Next, we tested whether substrate release from Hrd1p is mediated by the dissociation of Hrd1p oligomers. Streptavidin beads were incubated with a mixture of Hrd1p-SBP and fluorescently labeled Hrd1p, so that labeled Hrd1p was bound to the beads through Hrd1p-SBP. After polyubiquitination, the addition of Cdc48p, Ufd1p/Npl4p, and ATP led to the dissociation of ~80% of labeled polyubiquitinated Hrd1p (Figure 4F, lane 4; Figure 4G). Significantly less Hrd1p was released when ATP was depleted with Hk/G or in the absence of Ufd1/Npl4p (Figures 4F, lanes 7 and 3; Figure 4G). These results indicate that the Cdc48p ATPase causes the dissociation of polyubiquitinated Hrd1p oligomers. As monomers bind substrate more weakly (Figure 1G), this may explain the release of substrate from Hrd1p, particularly the release of nonubiquitinated substrate, which itself cannot interact with the Cdc48p complex. Cdc48p complex added directly to the ubiquitination reaction reduced substrate modification (Figure S4E), consistent with increased substrate release. Autoubiquitination was not affected (Figure S4E), as expected from an intramolecular reaction (Figure 2D).

Involvement of the DUB Enzyme Otu1p in ERAD

Because the DUB enzyme Yod1p had been implicated in ERAD-L (Ernst et al., 2009; Rumpf and Jentsch, 2006), we next investigated the role of its yeast homolog Otu1p. Expression of an inactive Otu1p mutant (Otu1p C120S), but not wild-type Otu1p,

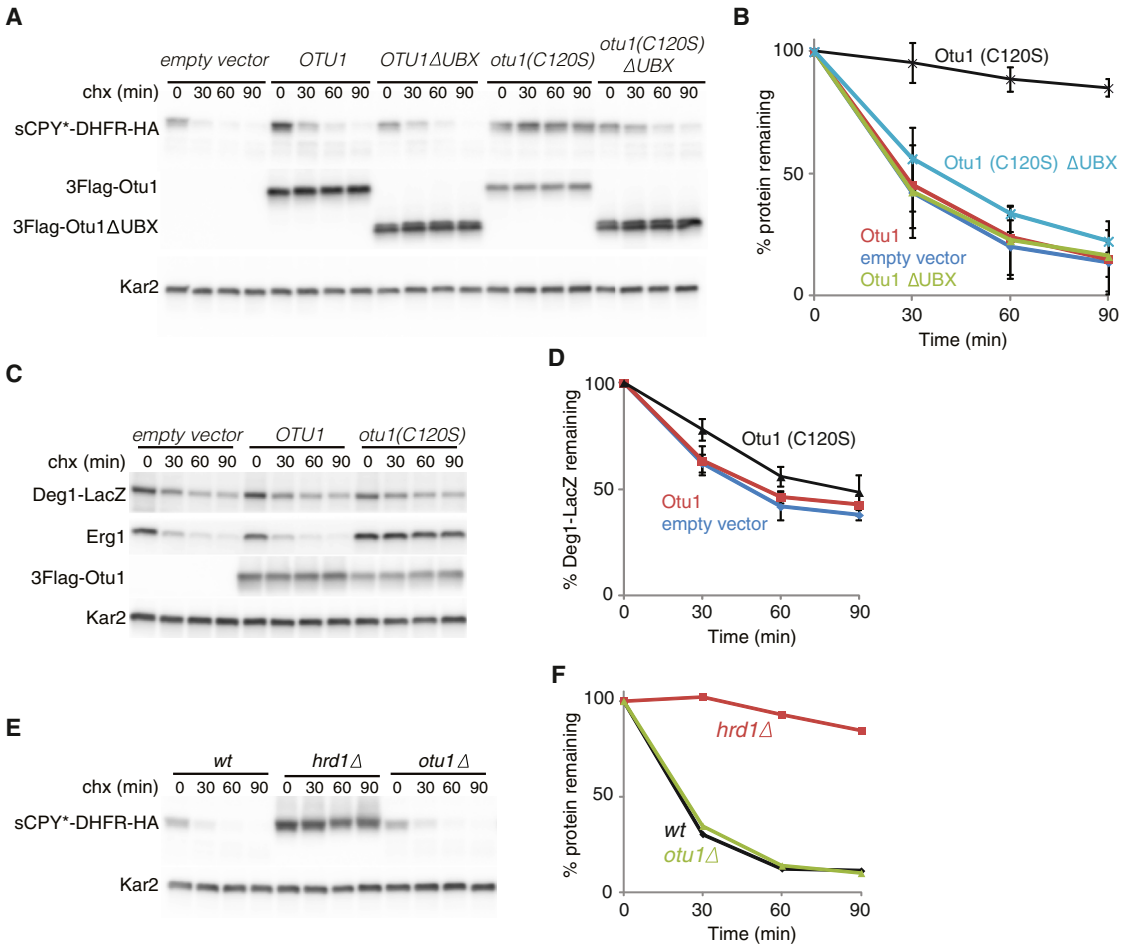

Figure 5. Cdc48p-Dependent Function of Otu1p In Vivo

(A) The degradation of a fusion of sCPY* with DHFR and a hemagglutinin (HA) tag (sCPY*-DHFR-HA) was tested in *S. cerevisiae*. The cells were transformed with an empty vector or plasmids expressing FLAG-tagged wild-type or mutant Otu1p (Otu1p [C120S]) from a Gal1 promoter. Where indicated, Otu1p variants lacking their UBX domains were expressed instead. The samples were analyzed at different time points after addition of cycloheximide (chx) by SDS-PAGE and immunoblotting with anti-HA and anti-FLAG antibodies. Loading controls were performed with Kar2p antibodies.

(B) Quantification of two experiments as in (A) (means and SD).

(C) As in (A) but following simultaneously the degradation of Erg1p and Deg1-LacZ with antibodies to the endogenous protein and to LacZ, respectively.

(D) Quantification of three experiments as in (C) (means and SD).

(E) The degradation of sCPY*-DHFR-HA was analyzed in cells lacking Otu1p and WT cells. Cells lacking Hrd1p were analyzed in parallel.

(F) Quantification of the experiment in (E).

See also Figure S5.

strongly inhibited the degradation of the ERAD-L substrate sCPY*-DHFR in *S. cerevisiae* cells (Figures 5A and 5B). The Hrd1p levels were not greatly affected (Figure S5A). The degradation of the ERAD-C substrate Erg1p was also inhibited (Figures 5C and 5D). Although Otu1p C120S expression inhibited cell growth (Figure S5B), the effect on ERAD occurred in viable cells, as demonstrated by the degradation of the cytosolic proteasome substrate Deg1-LacZ (Figures 5C and 5D; data not shown). As Deg1-LacZ degradation does not depend on Cdc48p (Ravid et al., 2006), the Otu1p mutant seems to affect only Cdc48p-dependent substrates. Indeed, deletion of the Cdc48p-interacting UBX domain from Otu1p C120S greatly reduced the inhibition (Figures 5A and 5B). Otu1p does not seem to be the only DUB involved in ERAD, as a yeast strain lacking Otu1p did not show ERAD-L defects (Figures 5E and 5F).

To analyze Otu1p in vitro, we expressed a His-tagged version and purified it by Ni-affinity chromatography and gel filtration (Figure S1C). Otu1p deubiquitinated modified fluorescently labeled Hrd1p or CPY* efficiently only when Cdc48p and Ufd1/Npl4p were present (Figures 6A–6C). Much less deubiquitination was observed with Otu1p and Cdc48p alone, even though they interact with one another, consistent with the slow reaction previously observed (Rumpf and Jentsch, 2006). Addition of Ufd1p/Npl4p alone significantly accelerated Otu1p action (Figures 6A and 6B), in contrast to another ubiquitin-binding protein (Rad23p) (data not shown). As expected, the

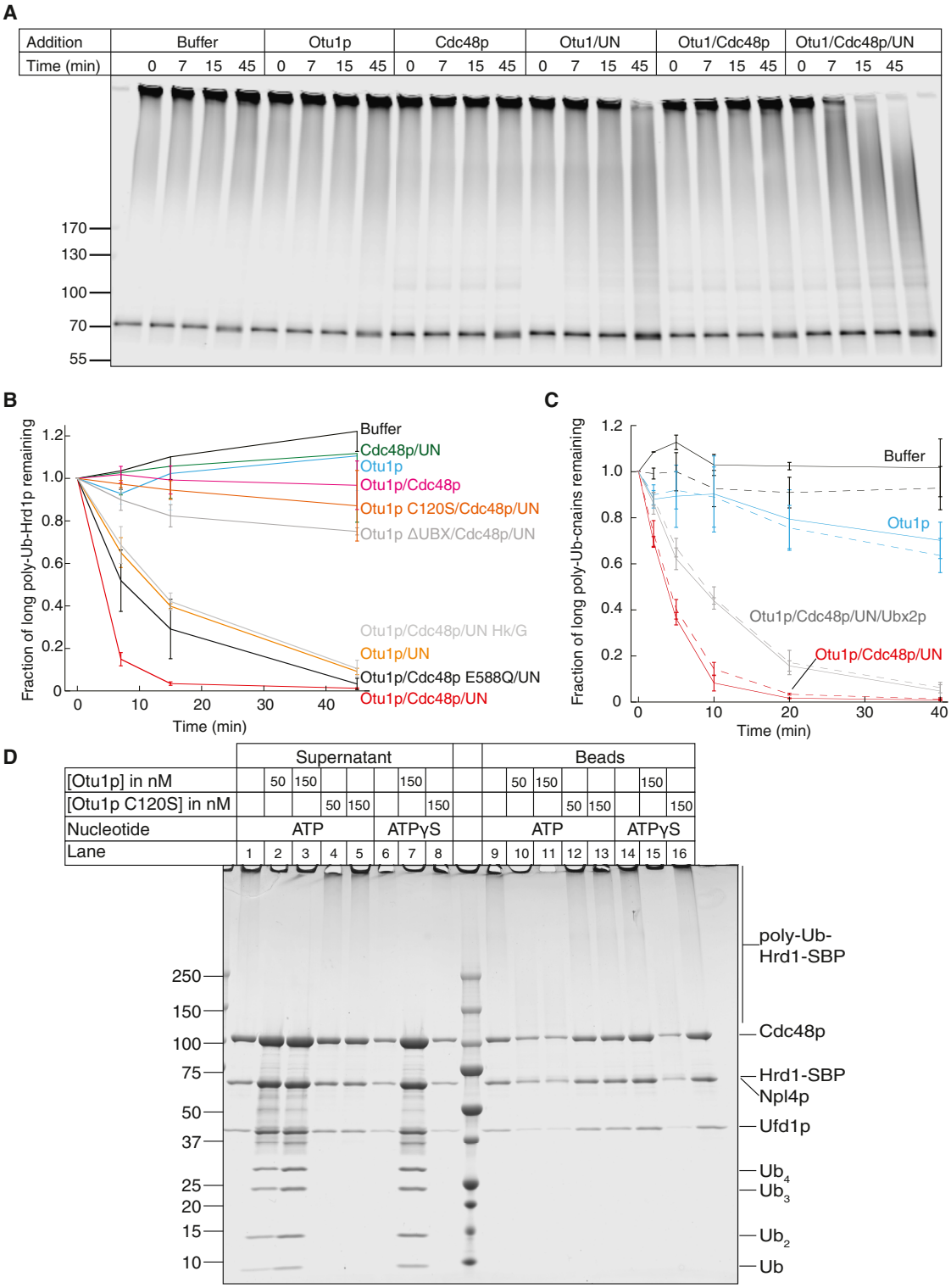

Figure 6. Cdc48p-Dependent In Vitro Deubiquitination by Otu1p

(A) Bead-immobilized fluorescently labeled Hrd1p-SBP was incubated with the ubiquitination machinery. After washing, Hrd1p was eluted from the beads with biotin and incubated with the indicated components (Ufd1/Npl4p; UN) for different time periods in the presence of ATP. Hrd1p was in a 30-fold excess over Otu1p, whereas all other components were about equimolar to Hrd1p. The samples were analyzed by SDS-PAGE and fluorescence scanning.

(legend continued on next page)

Otu1p C120S mutant was inactive (Figure 6B). ATP hydrolysis by Cdc48p stimulated deubiquitination by Otu1p, as shown with an ATPase-deficient Cdc48p mutant (E588Q mutation) and by ATP depletion with Hk/G (Figure 6B). Otu1p needs to bind to the Cdc48p complex, as deletion of the UBX domain drastically reduced DUB activity (Figure 6B). Similar results were obtained with polyubiquitinated Hrd1p containing fluorescently labeled ubiquitin, instead of labeled Hrd1p (Figures S6A and S6B). Otu1p removes short ubiquitin chains, rather than individual ubiquitin molecules, and it does not completely deubiquitinate Hrd1p.

We found that Otu1p inhibited Cdc48p-dependent substrate release by only ~50%, even at high concentrations (data not shown). Thus, Cdc48p often functions before Otu1p has a chance to make the ubiquitin chains too short. On the other hand, experiments with bead-immobilized polyubiquitinated Hrd1p showed that extended incubation with Otu1p causes most of the Cdc48p complex to eventually dissociate, both in the absence or presence of ATP hydrolysis (Figure 6D; see supernatants in lanes 7 and 3 versus those in lanes 6 and 1). The inactive Otu1p C120S mutant had no effect. These data suggest that Cdc48p function generally precedes Otu1p-mediated trimming of the ubiquitin chains. Interestingly, Ubx2p inhibited Cdc48p-dependent deubiquitination (Figure 6C), suggesting that in vivo it could help to prevent the premature function of Otu1p at the membrane.

Cdc48p-Dependent Protein Extraction from Reconstituted Proteoliposomes

Finally, we tested whether the ERAD-L reactions would also occur with Hrd1p reconstituted into proteoliposomes. Proteoliposomes were generated by detergent removal from a mixture of purified Hrd1p and synthetic phospholipids, both in DMNG. We also added a fluorescently labeled lipid (Texas Red phosphatidyl ethanolamine; TR-PE) prior to detergent removal. The reconstituted vesicles, including labeled Hrd1p and lipid, floated in a Nycodenz-step gradient (Figure 7A). The majority of Hrd1p was found in the second fraction from the top, whereas much of the lipid floated all the way to the top, indicating that some vesicles contain no protein and others Hrd1p oligomers. Most Hrd1p molecules have their cytoplasmic domain exposed to the outside of the vesicles, as demonstrated by the accessibility of a C-terminal TEV cleavage site to the TEV protease (Figure 7B). When labeled Hrd1p-containing proteoliposomes or protein-free liposomes were mixed with fluorescently labeled CPY*, only ~10% of substrate floated with the proteoliposomes (Figure 7C, blue and black columns). Thus, substrate binds only weakly to the cytoplasmic side of membrane-incorporated Hrd1p. Next, we coreconstituted labeled CPY* and Hrd1p into vesicles. Flotation experiments showed that essentially all substrate comigrated with the reconstituted vesicles (Figure 7C; red columns). The efficiency of coflotation correlated with the binding affinity of substrate for Hrd1p in detergent; ~40% were found for sCPY* (data not shown), whereas wild-type CPY did not float at all (Figure 7C). Addition of trypsin to the proteoliposomes showed that labeled CPY* was not protected by the lipid bilayer (Figure 7D), indicating that the substrate is bound to the transmembrane domain of Hrd1p with segment(s) exposed to the outside of the vesicles.

Next, we incubated proteoliposomes containing labeled Hrd1p and substrate with the ubiquitination machinery. About 80% of both Hrd1p and CPY* were polyubiquitinated (Figures 7E and 7F). Wild-type CPY remained largely unmodifed. About 90% of polyubquitinated Hrd1p and CPY* floated with the vesicles in a Nycodenz gradient (Figure 7G; lanes 1–6), indicating that polyubiquitination alone does not extract proteins from the membrane. Ubiquitination increased the density of the vesicles. Unmodified Hrd1p and CPY* also shifted their position in the gradient, consistent with the observation that the vesicles contain multiple Hrd1p molecules, some of which become polyubiquitinated.

Finally, we tested whether polyubiquitinated Hrd1p and CPY* molecules are extracted from the membrane by the Cdc48p complex. Proteoliposomes containing fluorescently labeled Hrd1p and CPY* were incubated with the ubiquitination machinery, followed by the addition of Cdc48p and Ufd1p/Npl4p. The sample was then subjected to flotation in a Nycodenz gradient. Approximately 20%–25% of CPY* and up to 35% of Hrd1p were found in the bottom fractions (Figures 7G, lanes 7–12; quantification in Figure 7H), indicating that they were no longer associated with the liposomes. Longer incubation times or higher Cdc48p complex concentrations did not increase the efficiency of membrane extraction (Figures S7A and S7B). No membrane extraction was observed in the absence of ubiquitination, when Cdc48p or Ufd1p/Npl4p were omitted, when an ATPase-defective Cdc48p mutant was used, or when ATP was depleted with Hk/G (Figure 7H). The extracted Hrd1p and CPY* proteins carried relatively short ubiquitin chains (Figure 7G); most of the longer chains remained in the floated fractions, suggesting that they are poor substrates for the Cdc48p complex.

Otu1p addition inhibited the membrane extraction of polyubiquitinated proteins to a maximum of ~50% (Figures S7C and S7D), similar to the effect of Otu1p on substrate release from Hrd1p in detergent (data not shown). The inactive Otu1p C120S mutant had no effect on membrane extraction, even when added in a 10-fold excess over Cdc48p (Figure S7D), suggesting that its effect in vivo (Figures 5A and 5B) is caused at a step following substrate release into the cytosol.

(B) Quantification of experiments performed as in (A). The disappearance of the longest ubiquitin chains was quantified under different conditions (means and SD of three experiments). ATP was depleted with hexokinase/glucose (Hk/G). Where indicated, an ATPase-defective Cdc48p mutant (Cdc48p E588Q) or an Otu1p mutant lacking the UBX domain was used.

(C) Bead-immobilized complexes of Hrd1p-SBP and CPY*, labeled with different fluorescent dyes, were treated as in (A). The deubiquitination of modified Hrd1p and CPY* was followed in parallel (solid and broken lines, respectively). Shown are the means and SD of three experiments.

(D) Bead-immobilized ubiquitinated Hrd1-SBP was incubated with Cdc48p complex in the presence of ATPγS. After washing, the beads were incubated for 1 hr with the indicated components. Supernatants and beads were analyzed by SDS-PAGE and Coomassie staining.

See also Figure S6.

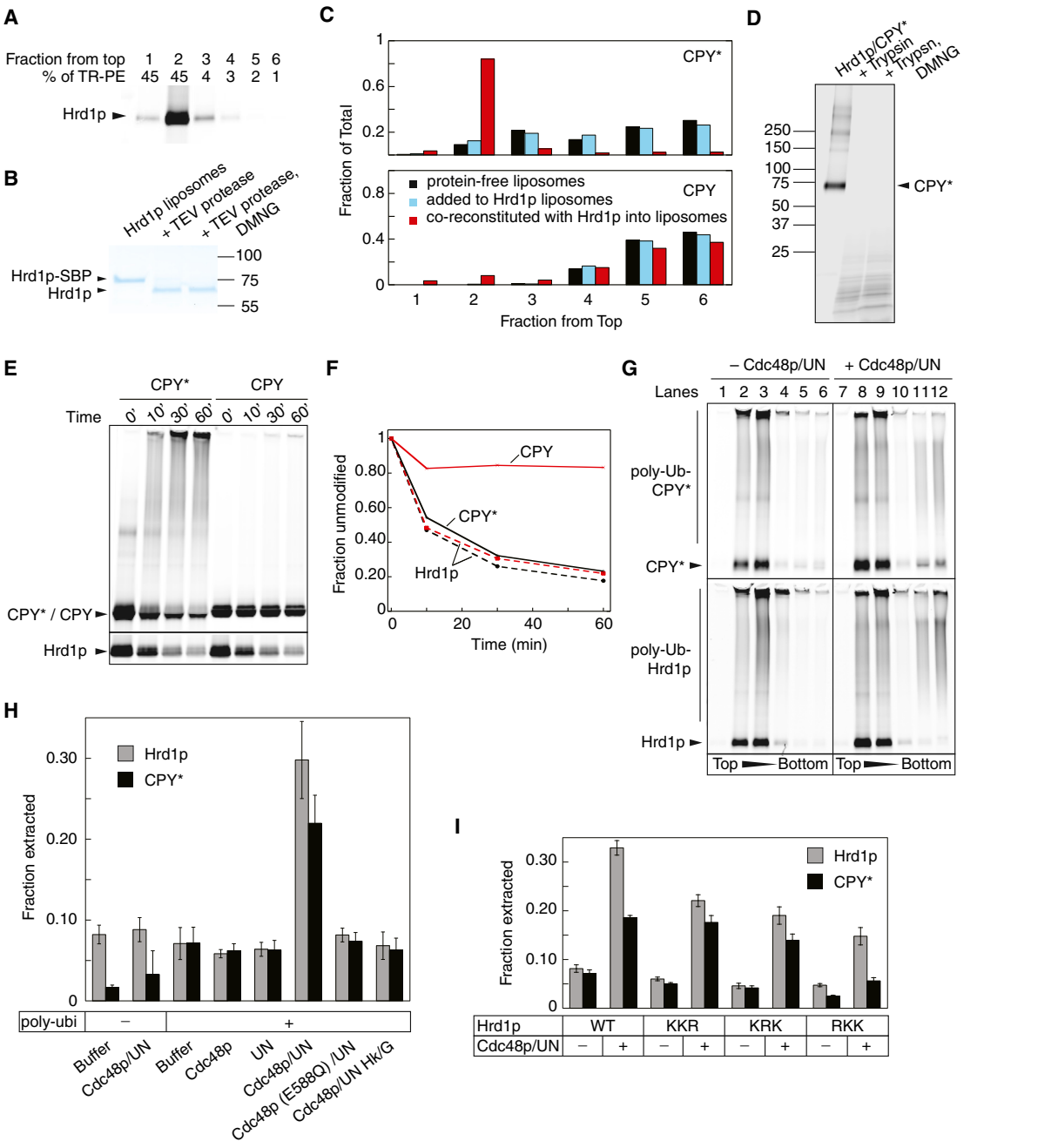

Figure 7. Membrane Extraction of Polyubiquitinated Proteins by Cdc48p

(A) Proteoliposomes containing fluorescently labeled Hrd1p-SBP and Texas red-labeled phosphatidyl ethanolamine (TR-PE) were subjected to flotation in a Nycodenz gradient. Fractions were collected from the top and analyzed by SDS-PAGE and fluorescence scanning of the gel. The lipid content of the fractions was determined by absorbance at 590 nm.

(B) Proteoliposomes containing Hrd1p-SBP with a TEV cleavage site at the C terminus were treated with TEV protease in the absence or presence of DMNG. The samples were analyzed by SDS-PAGE and staining with Coomassie blue.

(C) Fluorescently labeled CPY* was incubated with either protein-free liposomes or proteoliposomes containing Hrd1p, or it was coreconstituted with Hrd1p into vesicles (black, light blue, and red bars, respectively). The samples were subjected to flotation in a Nycodenz gradient, and fractions were analyzed by SDS-PAGE and fluorescence scanning. Wild-type CPY was used as a control.

(D) Proteoliposomes containing Hrd1p and labeled CPY* were incubated with trypsin in the absence or presence of DMNG. The samples were analyzed by SDS-PAGE and fluorescence scanning.

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All three Hrd1p mutants with Lys mutations showed reduced extraction of polyubiquitinated Hrd1p from the membrane (Figure 7I), consistent with their lowered level of modification (Figure S2D). The site of modification does not seem to be important, in contrast to the release of unmodified substrate from polyubiquitinated Hrd1 in detergent (Figure 4E). Although less pronounced, the site of modification also affected the extraction of polyubiquitinated substrate from Hrd1p-containing proteoliposomes, as the RKK mutant had a significantly stronger effect on substrate extraction than on Hrd1p extraction (Figure 7I). The KKR mutant was not affected in membrane extraction of the substrate (Figure 7I), indicating that modification of the C-terminal tail is not required for substrate release from Hrd1p and suggesting that substrate is not obligatorily extracted as a complex with Hrd1p.

DISCUSSION

We have reproduced key steps of ERAD-L with purified protein components, both in detergent and in reconstituted proteoliposomes. Our minimal in vitro system mimics essential aspects of ERAD-L in vivo when Hrd1p is overexpressed (basic ERAD-L). In both systems, glycosylated and nonglycosylated misfolded proteins serve as substrates for Hrd1p, and Hrd1p itself is polyubiquitinated and extracted from the membrane. Because Hrd1p appears to be the only membrane component required for a basic ERAD-L reaction in vivo, and because Hrd1p is sufficient for the binding and membrane extraction of misfolded proteins in vitro, it is likely to form a channel for the transport of misfolded proteins through the ER membrane.

Our in vitro system allowed us to break down the basic ERAD-L process into individual steps. First, Hrd1p binds unfolded polypeptides through its membrane-spanning domain. Next, both Hrd1p and substrate are polyubiquitinated, resulting in the recruitment of the Cdc48p ATPase complex, a process mediated by the cofactor Ufd1p/Npl4p and facilitated by the adaptor protein Ubx2p. Then the polyubiquitinated proteins are extracted from the membrane by the Cdc48p complex in an ATP hydrolysis-dependent reaction. Finally, Otu1p trims the polyubiquitin chains in a Cdc48p complex-dependent manner, resulting in the dissociation of the Cdc48p complex from substrate.

In basic ERAD-L, substrates are exclusively selected by Hrd1p. We show that Hrd1p in detergent discriminates folded from unfolded polypeptides. Hrd1p uses its hydrophobic TM segments to bind unfolded polypeptide segments. Oligomeric Hrd1p may provide more TMs for substrate interaction than does monomeric Hrd1p, explaining why it has a higher affinity. Hrd1p does not interact with all hydrophobic polypeptide segments, because it does not bind Ubx2p (Figure S3C) or a fragment of Usa1p lacking the N-terminal cytoplasmic interaction domain (data not shown). Perhaps, a loosely folded polypeptide structure is also required for substrate recognition. It seems likely that several TMs in Hrd1p interact with an unfolded polypeptide chain because mutations scattered throughout the membrane-embedded domain of Hrd1p affect different substrates to varying degrees (Sato et al., 2009). Although some substrate interaction may be caused simply by the hydrophobicity of the TMs, the membrane-embedded domain of Hrd1p must have unique properties, as it needs to be folded, and only little substrate interaction is seen with purified Usa1p containing two TM segments (data not shown).

Insertion of a polypeptide loop into the Hrd1p channel could be the first step in the actual retrotranslocation process. Such a model would be analogous to loop insertion of a signal sequence-containing polypeptide into the Sec61/SecY channel during forward translocation (Park and Rapoport, 2012). In both cases, a substrate segment would reach the other side of the membrane and the binding of a hydrophobic region to the channel would provide the driving force for polypeptide chain insertion. Our experiments show that a misfolded substrate coreconstituted with Hrd1p indeed exposes a segment to the outside of vesicles. As expected, substrate does not bind to Hrd1p from the cytoplasmic side when added after reconstitution. However, it remains to be shown that reconstituted Hrd1p can bind substrate on the luminal side of the membrane, as suggested by experiments in intact yeast cells overexpressing Hrd1p (Carvalho et al., 2010). A system in which the starting proteoliposomes contain only Hrd1p or substrate and are subsequently fused will also address whether actual retrotranslocation can be reproduced with the purified components.

Similar to forward translocation, a polypeptide chain may be able to slide back and forth in a Hrd1p channel, but there has to be energy input to achieve net movement into the cytosol. One possibility is that the attachment of polyubiquitin chains to the substrate would bias polypeptide sliding, providing a ratcheting mechanism for translocation. However, polyubiquitination alone is insufficient to completely move a polypeptide chain from the ER membrane into the cytosol, as shown both in vivo (Flierman et al., 2003) and by our in vitro experiments; this requires the function of the Cdc48p complex as well. In fact, our data indicate that the Cdc48p complex is sufficient to move polyubiquitinated proteins into the cytosol, although other cytosolic

(E) Proteoliposomes were generated by coreconstitution of Hrd1p with either CPY* or wild-type CPY. Hrd1p and substrate were labeled with different fluorophores. The vesicles were incubated with the ubiquitination machinery for different time periods, and samples were analyzed by SDS-PAGE and fluorescence scanning. For Hrd1p, the gel was cropped to only show the disappearance of unmodified protein.

(F) The disappearance of unmodified protein in (D) was quantitated. Solid and broken lines show the modification of substrate and Hrd1p, respectively.

(G) Fluorescently labeled Hrd1p and CPY* were coreconstituted into proteoliposomes. The vesicles were incubated with the ubiquitination machinery, followed by incubation in the absence or presence of the Cdc48p complex (Cdc48p/UN). The vesicles were floated in a Nycodenz gradient, and fractions were analyzed by SDS-PAGE and fluorescence scanning.

(H) Experiments as in (G) were quantified by determining the total fluorescence in the bottom two fractions (material released from the vesicles) as a fraction of the total fluorescence in the gradient (mean and SD of at least three experiments). Where indicated, ATP was depleted with hexokinase/glucose (Hk/G) or an ATPase-deficient Cdc48p mutant (Cdc48p E588Q) was used.

(I) As in (G) but with Hrd1p mutants carrying Lys to Arg mutations in three different regions. Quantification of three experiments was done as in (H).

See also Figure S7.

factors could be stimulatory. Current models assume that the Cdc48p-binding polyubiquitin chains need to be attached to the substrate, but our data raise the possibility that the crucial modification is on Hrd1p itself. We show that Cdc48p releases unmodified substrate from polyubiquitinated Hrd1p in detergent, probably by disassembling Hrd1p oligomers into smaller assemblies that have a lower affinity for substrate. Indeed, for Cdc48p's segregase activity, it is not necessary that the ubiquitin chains are attached to the protein extracted from the membrane, as shown for the generation of the transcription factors Mga2p and Spt23p. Here, Cdc48p action releases an unmodified polypeptide (the p90 fragment) into the cytosol by acting on polyubiquitin chains attached to an associated membrane-anchored protein (the p120 precursor) (Shcherbik and Haines, 2007). Autoubiquitination of Hrd1p followed by Cdc48p function could provide the driving force for retrotranslocation by allowing substrate segments to move through the membrane by multiple rounds of binding to and release from Hrd1p. Such a model does not exclude a role for substrate ubiquitination, which could determine directionality of polypeptide movement by a ratcheting mechanism or by interaction with the Cdc48p complex.

A model in which autoubiquitination of Hrd1p is a crucial modification event would be consistent with studies showing that substrates, in which all Lys residues are removed, continue to be degraded; in these cases, the ubiquitination machinery was still required, suggesting that a protein other than the substrate is ubiquitinated (Hassink et al., 2006; Wang et al., 2013; Yu and Kopito, 1999). There are also ERAD-L substrates, such as pre-pro-α-factor and cholera toxin, which are never ubiquitinated and whose retrotranslocation can be blocked with a dominant-negative DUB (Bernardi et al., 2013), again indicating ubiquitination of another component. Protease-protection studies showed that ubiquitination is required to expose a substrate segment to the cytosol (Jarosch et al., 2002), and photocrosslinking demonstrated that the ubiquitination activity of Hrd1p and Cdc48p action are needed for an early Hrd1p-substrate interaction on the luminal side of the membrane (Carvalho et al., 2010). These results suggest that ubiquitination by Hrd1p of a component other than substrate is crucial for ERAD-L.

Although a role for autoubiquitination of Hrd1p is attractive, it is difficult to exclude that at least some Hrd1p modification is the result of a nonspecific side reaction that has been observed with other ligases. Indeed, we found that ubiquitination of the C-terminal tail of Hrd1p has no effect on substrate modification and release. There is also little evidence that Hrd1p is polyubiquitinated in wild-type yeast cells. It is therefore possible that Hrd1p modification and Cdc48p-dependent extraction serve to regulate Hrd1p levels in the membrane. However, because autoubiquitination occurs in an intramolecular reaction, our results argue that Hrd1p molecules do not recognize each other as unfolded ERAD-M substrates. Regardless of whether autoubiquitination is required for ERAD or as a regulatory mechanism, it is minimized in wild-type cells.

Our in vitro results faithfully recapitulate what is observed in vivo in Hrd1p-overexpressing yeast cells. However, they do not recapitulate substrate selection as seen in wild-type cells, where the additional ERAD components Yos9p, Hrd3p, and Der1p ensure that only genuine substrates are degraded (Denic et al., 2006; Gauss et al., 2006; Mehnert et al., 2014; Xie and Ng, 2010). Our results indicate that recognition of an unfolded polypeptide by Hrd1p is the last checkpoint before substrate is committed to retrotranslocation and degradation. This may explain why bypassing all upstream steps in Hrd1p-overexpressing cells slows, but does not prevent, cell growth. In addition, Hrd1p may provide the main checkpoint for nonglycosylated ERAD substrates. The additional ERAD components may also minimize excessive autoubiquitination and membrane extraction of Hrd1p. Hrd3p has a particularly important role, as in its absence, Hrd1p is poly-ubiquitinated and degraded (Gardner et al., 2000; Plemper et al., 1999). Perhaps, Hrd3p is regulating autoubiquitination of Hrd1p in response to substrate binding.

Our results support the idea that DUBs, specifically Otu1p, play a role in ERAD. We show that Otu1p is only activated after recruitment by the Cdc48p complex. This ensures that polyubiquitination, Cdc48p recruitment, and deubiquitination occur in a sequential manner. Our data suggest that Cdc48p-mediated membrane extraction precedes Otu1p function, so that most substrate deubiquitination occurs in the cytosol. Ubx2p could help to prevent premature deubiquitination at the membrane by competing with Otu1p for Cdc48p binding. The kinetic delay would guarantee that Otu1p does not interfere with Cdc48p's function as a segregase. The sequential action of Cdc48p and Otu1p would be further enhanced by the ubiquitin ligase Hrd1p counteracting deubiquitination at the membrane, but not in the cytosol. A function of Otu1p downstream of Cdc48p's segregase activity explains why overexpression of wild-type Otu1p does not affect ERAD in vivo. The inactive Otu1p mutant would inhibit by interacting with polyubiquitin chains bound to the Cdc48p complex, preventing the dissociation of Ufd1p/Npl4p and blocking the access of other DUBs. Indeed, expression of the equivalent Yod1p mutant in mammalian cells leads to the accumulation of ubiquitinated substrate bound to the ATPase complex (Ernst et al., 2009). The inactive Otu1p mutant does not inhibit membrane extraction in vitro, because there is probably no need for the Cdc48p complex to be recycled. Otu1p/Yod1p probably function not only in ERAD but also in other processes involving the Cdc48p/Ufd1p/Npl4p complex.

Finally, our results suggest that ERAD-L proceeds through an intermediate in which the substrate is bound to the membrane-embedded domain of Hrd1p, a situation that resembles the recognition of an ERAD-M substrate. Thus, ERAD-M substrates may enter the same process at a later stage, explaining why the same ubiquitin ligase (Hrd1p) is involved and why ERAD-M requires only a subset of the ERAD-L components. Many other ubiquitin ligases, such as Doa10p involved in ERAD-C, are also multispanning membrane proteins. As proposed previously for Doa10p (Swanson et al., 2001), the membrane-spanning domains may serve as conduits for polypeptides through the membrane.

EXPERIMENTAL PROCEDURES

Details of protein purifications and experimental procedures are described in the Extended Experimental Procedures.

Protein Expression and Purification

All proteins are from *S. cerevisiae*. Uba1p and Ubx2p and the substrates CPY*, sCPY*, sCPY*-DHFR, and CPY, as well as Hrd1p and its variants, were expressed in *S. cerevisiae* under the Gal1 promoter. All other proteins were expressed in *E. coli* strain BL21 DE3 RIPL. The membrane proteins Ubx2p and Hrd1p and its variants were solubilized in DMNG. ERAD substrates were extracted from a crude membrane fraction with urea. Hrd1p and its variants were first purified by streptavidin affinity chromatography utilizing a C-terminal streptavidin-binding peptide (SBP) tag, followed by gel filtration. All other proteins were purified as His-tagged variants by Ni-affinity chromatography, followed by ion-exchange chromatography and/or gel filtration. Unless noted otherwise, the tags were proteolytically removed.

Labeling with Fluorescent Dyes

The substrates sCPY*, sCPY*-DHFR, sCPY*-GFP, DHFR, and GFP were labeled at a C terminally attached Cys residue with DyLight 800 maleimide. CPY* and CPY were labeled with a N-hydroxy-succinimidyl ester of DyLight 800. Hrd1p was labeled using the sortase technique.

Ubiquitination Assays

All ubiquitination assays were performed at 30°C. Unless indicated otherwise, the concentrations of the components of the ubiquitination machinery were 100 nM Uba1p, 1 μM Ubc7p, 1 μM Cue1p-c, 100 nM Hrd1p, 100 nM substrate, 100 μM ubiquitin, and 2.5 mM ATP.

Binding and Release Experiments

All binding experiments were performed at room temperature with Hrd1p immobilized on magnetic streptavidin beads via its C-terminal SBP-tag. Unbound and bound fluorescent material was analyzed by SDS-PAGE and fluorescence scanning of the gel in an Odyssey scanner (Li-COR). Binding of substrates or Cdc48p to ubiquitinated Hrd1p was tested by first immobilizing Hrd1p-SBP on beads at room temperature for 1 hr and then adding the ubiquitination machinery.

Reconstitution into Proteoliposomes and Density Gradients

For reconstitution of Hrd1p, protein-free liposomes (final lipid concentration 10 mM, containing 0.5 mol% Texas Red-labeled phosphatidyl ethanolamine) were mixed with DMNG (15 mM) and Hrd1p (2 μM) (and optionally with substrate [0.5 μM]) and incubated for 1 hr at room temperature. This mixture was applied to detergent-removal spin columns (Pierce).

In Vivo ERAD-Substrate Degradation Experiments

Cycloheximide shutoff experiments were performed essentially as described previously (Carvalho et al., 2010).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2014.07.050.

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REFERENCES

Bagola, K., Mehnert, M., Jarosch, E., and Sommer, T. (2011). Protein dislocation from the ER. Biochim. Biophys. Acta *1808*, 925–936.

Bays, N.W., Gardner, R.G., Seelig, L.P., Joazeiro, C.A., and Hampton, R.Y. (2001a). Hrd1p/Der3p is a membrane-anchored ubiquitin ligase required for ER-associated degradation. Nat. Cell Biol. *3*, 24–29.

Bays, N.W., Wilhovsky, S.K., Goradia, A., Hodgkiss-Harlow, K., and Hampton, R.Y. (2001b). HRD4/NPL4 is required for the proteasomal processing of ubiquitinated ER proteins. Mol. Biol. Cell *12*, 4114–4128.

Bernardi, K.M., Williams, J.M., Inoue, T., Schultz, A., and Tsai, B. (2013). A deubiquitinase negatively regulates retro-translocation of nonubiquitinated substrates. Mol. Biol. Cell *24*, 3545–3556.

Biederer, T., Volkwein, C., and Sommer, T. (1997). Role of Cue1p in ubiquitination and degradation at the ER surface. Science *278*, 1806–1809.

Bordallo, J., and Wolf, D.H. (1999). A RING-H2 finger motif is essential for the function of Der3/Hrd1 in endoplasmic reticulum associated protein degradation in the yeast Saccharomyces cerevisiae. FEBS Lett. *448*, 244–248.

Bordallo, J., Plemper, R.K., Finger, A., and Wolf, D.H. (1998). Der3p/Hrd1p is required for endoplasmic reticulum-associated degradation of misfolded lumenal and integral membrane proteins. Mol. Biol. Cell *9*, 209–222.

Brodsky, J.L. (2012). Cleaning up: ER-associated degradation to the rescue. Cell *151*, 1163–1167.

Carvalho, P., Goder, V., and Rapoport, T.A. (2006). Distinct ubiquitin-ligase complexes define convergent pathways for the degradation of ER proteins. Cell *126*, 361–373.

Carvalho, P., Stanley, A.M., and Rapoport, T.A. (2010). Retrotranslocation of a misfolded luminal ER protein by the ubiquitin-ligase Hrd1p. Cell *143*, 579–591.

Denic, V., Quan, E.M., and Weissman, J.S. (2006). A luminal surveillance complex that selects misfolded glycoproteins for ER-associated degradation. Cell *126*, 349–359.

Ernst, R., Mueller, B., Ploegh, H.L., and Schlieker, C. (2009). The otubain YOD1 is a deubiquitinating enzyme that associates with p97 to facilitate protein dislocation from the ER. Mol. Cell *36*, 28–38.

Finger, A., Knop, M., and Wolf, D.H. (1993). Analysis of two mutated vacuolar proteins reveals a degradation pathway in the endoplasmic reticulum or a related compartment of yeast. Eur. J. Biochem. *218*, 565–574.

Flierman, D., Ye, Y., Dai, M., Chau, V., and Rapoport, T.A. (2003). Polyubiquitin serves as a recognition signal, rather than a ratcheting molecule, during retrotranslocation of proteins across the endoplasmic reticulum membrane. J. Biol. Chem. *278*, 34774–34782.

Gardner, R.G., Swarbrick, G.M., Bays, N.W., Cronin, S.R., Wilhovsky, S., Seelig, L., Kim, C., and Hampton, R.Y. (2000). Endoplasmic reticulum degradation requires lumen to cytosol signaling. Transmembrane control of Hrd1p by Hrd3p. J. Cell Biol. *151*, 69–82.

Garza, R.M., Sato, B.K., and Hampton, R.Y. (2009). In vitro analysis of Hrd1p-mediated retrotranslocation of its multispanning membrane substrate 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase. J. Biol. Chem. *284*, 14710–14722.

Gauss, R., Jarosch, E., Sommer, T., and Hirsch, C. (2006). A complex of Yos9p and the HRD ligase integrates endoplasmic reticulum quality control into the degradation machinery. Nat. Cell Biol. *8*, 849–854.

Guerriero, C.J., and Brodsky, J.L. (2012). The delicate balance between secreted protein folding and endoplasmic reticulum-associated degradation in human physiology. Physiol. Rev. *92*, 537–576.

Hänzelmann, P., Buchberger, A., and Schindelin, H. (2011). Hierarchical binding of cofactors to the AAA ATPase p97. Structure *19*, 833–843.

Hassink, G.C., Barel, M.T., Van Voorden, S.B., Kikkert, M., and Wiertz, E.J. (2006). Ubiquitination of MHC class I heavy chains is essential for dislocation by human cytomegalovirus-encoded US2 but not US11. J. Biol. Chem. *281*, 30063–30071.

Huyer, G., Piluek, W.F., Fansler, Z., Kreft, S.G., Hochstrasser, M., Brodsky, J.L., and Michaelis, S. (2004). Distinct machinery is required in Saccharomyces cerevisiae for the endoplasmic reticulum-associated degradation of a multispanning membrane protein and a soluble luminal protein. J. Biol. Chem. *279*, 38369–38378.

Jarosch, E., Taxis, C., Volkwein, C., Bordallo, J., Finley, D., Wolf, D.H., and Sommer, T. (2002). Protein dislocation from the ER requires polyubiquitination and the AAA-ATPase Cdc48. Nat. Cell Biol. *4*, 134–139.

Liu, Y., and Ye, Y. (2012). Roles of p97-associated deubiquitinases in protein quality control at the endoplasmic reticulum. Curr. Protein Pept. Sci. *13*, 436–446.

Mehnert, M., Sommer, T., and Jarosch, E. (2014). Der1 promotes movement of misfolded proteins through the endoplasmic reticulum membrane. Nat. Cell Biol. *16*, 77–86.

Meyer, H.H., Wang, Y., and Warren, G. (2002). Direct binding of ubiquitin conjugates by the mammalian p97 adaptor complexes, p47 and Ufd1-Npl4. EMBO J. *21*, 5645–5652.

Neuber, O., Jarosch, E., Volkwein, C., Walter, J., and Sommer, T. (2005). Ubx2 links the Cdc48 complex to ER-associated protein degradation. Nat. Cell Biol. *7*, 993–998.

Park, E., and Rapoport, T.A. (2012). Mechanisms of Sec61/SecY-mediated protein translocation across membranes. Annu. Rev. Biophys. *41*, 21–40.

Plemper, R.K., Bordallo, J., Deak, P.M., Taxis, C., Hitt, R., and Wolf, D.H. (1999). Genetic interactions of Hrd3p and Der3p/Hrd1p with Sec61p suggest a retro-translocation complex mediating protein transport for ER degradation. J. Cell Sci. *112*, 4123–4134.

Rabinovich, E., Kerem, A., Fröhlich, K.U., Diamant, N., and Bar-Nun, S. (2002). AAA-ATPase p97/Cdc48p, a cytosolic chaperone required for endoplasmic reticulum-associated protein degradation. Mol. Cell. Biol. *22*, 626–634.

Ravid, T., Kreft, S.G., and Hochstrasser, M. (2006). Membrane and soluble substrates of the Doa10 ubiquitin ligase are degraded by distinct pathways. EMBO J. *25*, 533–543.

Rumpf, S., and Jentsch, S. (2006). Functional division of substrate processing cofactors of the ubiquitin-selective Cdc48 chaperone. Mol. Cell *21*, 261–269.

Sato, B.K., Schulz, D., Do, P.H., and Hampton, R.Y. (2009). Misfolded membrane proteins are specifically recognized by the transmembrane domain of the Hrd1p ubiquitin ligase. Mol. Cell *34*, 212–222.

Schuberth, C., and Buchberger, A. (2005). Membrane-bound Ubx2 recruits Cdc48 to ubiquitin ligases and their substrates to ensure efficient ER-associated protein degradation. Nat. Cell Biol. *7*, 999–1006.

Shcherbik, N., and Haines, D.S. (2007). Cdc48p(Npl4p/Ufd1p) binds and segregates membrane-anchored/tethered complexes via a polyubiquitin signal present on the anchors. Mol. Cell *25*, 385–397.

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.

Vashist, S., and Ng, D.T. (2004). Misfolded proteins are sorted by a sequential checkpoint mechanism of ER quality control. J. Cell Biol. *165*, 41–52.

Wang, X., Yu, Y.Y., Myers, N., and Hansen, T.H. (2013). Decoupling the role of ubiquitination for the dislocation versus degradation of major histocompatibility complex (MHC) class I proteins during endoplasmic reticulum-associated degradation (ERAD). J. Biol. Chem. *288*, 23295–23306.

Xie, W., and Ng, D.T. (2010). ERAD substrate recognition in budding yeast. Semin. Cell Dev. Biol. *21*, 533–539.

Ye, Y., Meyer, H.H., and Rapoport, T.A. (2001). The AAA ATPase Cdc48/p97 and its partners transport proteins from the ER into the cytosol. Nature *414*, 652–656.

Yu, H., and Kopito, R.R. (1999). The role of multiubiquitination in dislocation and degradation of the alpha subunit of the T cell antigen receptor. J. Biol. Chem. *274*, 36852–36858.

EXTENDED EXPERIMENTAL PROCEDURES

Strains Used for Protein Expression

For the expression of proteins in *E. coli*, strain BL21-CodonPlus (DE3)-RIPL (Stratagene) was used. For the expression of Ubx2p and Uba1p in *S. cerevisiae*, strain InvSc1 strain (Invitrogen) was used. All Hrd1p variants were expressed in a ubc7 knock-out strain derived from BY4742 (OpenBiosystems) (*MATα ura3Δ0 his3Δ1 leu2Δ0 lys2Δ0 ubc7::KANR*). For the expression of ERAD-substrates, a strain lacking hrd3 and alg3, derived from BY4741 (OpenBiosystems), was used (*MATa ura3Δ0 his3Δ1 leu2Δ0 lys2Δ0 hrd3::KANR alg3::HIS3)*.

Constructs

Expression of Proteins in *S. cerevisae*

All plasmids designed for the overexpression of Hrd1p were derived from 2μ plasmids of the pRS42X series with a Gal1 promoter (Mumberg et al., 1994). For expression of Hrd1p and the Hrd1p(C399S) mutant, HRD1 was C-terminally tagged with a tobacco etch virus (TEV) protease cleavage site followed by a streptavidin binding peptide (SBP) (Keefe et al., 2001). A Lys residue in the SBP-tag was mutated to Arg to prevent ubiquitination of the SBP-tag. This results in a C-terminal tag with the sequence SG-ENLYFQS-GGG-MDERTTGWRGGHVV-EGLAGELEQLRARLEHHPQGQREP. For sortase mediated labeling, a sequence coding for LPETGG was fused to the C terminus of the SBP-tag.

For the expression of a lysine-free Hrd1p variant a construct was synthesized in which all 27 Lys residues were mutated to Arg (Genscript). Attempts to express and purify this mutant failed. We used this construct to generate mutants by Gibson assembly (NEB) in which Lys residues in three different regions of the protein were mutated to Arg. The regions comprised residues 1-301, 302-407, and 407-551.

Hrd1TM-GFP contains residues 1-205 of Hrd1p, followed by a short linker (Ser-Gly-Gly-Ser-Gly), Superfolder GFP (Pédelacq et al., 2006), another short linker (Ser-Gly-Gly-Ser-Gly), and the same SBP tag as described for full-length Hrd1.

To generate a labeled, un-tagged Hrd1p variant (Figures 4E and 4F), we first removed all Cys residues outside the RING domain (C24V, C168A, C208A). This mutant was purified and behaved indistinguishably from the wild-type protein in substrate binding and ubiquitination activity. It did not react with a maleimide-coupled dye, indicating that the cysteines in the RING finger domain are not reactive. Then, a Cys residue was introduced at the C terminus of Hrd1p just before the TEV cleavage site. This results in the following C-terminal extension to Hrd1p after removal of the SBP tag with TEV protease: GCGENLYFQ. The protein was then labeled at the introduced Cys with a maleimide-coupled dye.

For overexpression of proteins that require the presence of a signal sequence for targeting to the ER lumen (CPY, CPY*, sCPY*, and sCPY*-DHFR), a sequence encoding the signal sequence of prepro-α-factor, followed by a His_{14}-tag and a 3C-protease cleavage site was ligated into the pRS425-PGal1 vector using SpeI/SmaI sites. This results in the N-terminal sequence MRFPSIFTAVLFAAS SALA-SKHHHHSGHHHTGHHHHSGSHHHGSG-LEVLFQ-GP. The SmaI site was then used to insert sequences encoding for CPY* (Ng et al., 2000), sCPY* (Carvalho et al., 2010), or sCPY*-DHFR-3HA (Carvalho et al., 2010). We also introduced a Cys residue at the C terminus of sCPY* that was used for maleimide-labeling. In the case of CPY and CPY*, an SBP-tag followed by the sequence HDEL for ER retention was attached to the C terminus, separated by a TEV-cleavage site, resulting in the C-terminal sequence GGG-EN LYFQS-GGG-MDEKTTGWRGGHVVEGLAGELEQLRARLEHHPQGQ-REPHDEL.

For overexpression of Ubx2p and Uba1p with an N-terminal His-tag, a sequence was inserted into pRS426PGal1 coding for a His_{14}-tag and a TEV-protease cleavage site, resulting the N-terminal sequence MSKHHHHSGHHHTGHHHH-SGSHHHSG-EN LYFQS-G. An SfoI restriction site following the sequence was used for cloning of the inserts.

Expression of Proteins in *Escherichia coli*

The K27Sumo vector was used for the expression of GFP-SBP, the cytosolic fragment of Hrd1p (residues K325-I551), Cdc48p, Ufd1p, a C-terminal fragment of Ubx2p, and Otu1p and its variants. This vector (kindly provided by Dr. Dirk Görlich, Max-Planck-Institut für biophysikalische Chemie, Göttingen, Germany) encodes an N-terminal His_{14}-SUMO-tag. DNA fragments coding for the proteins were ligated into this vector using the SfoI/NotI restriction sites. Expression from this vector is under the control of a T5 promoter.

To generate an expression construct for untagged Npl4p, the coding sequence was ligated into the pET21b vector (Novagen) using NdeI/XhoI restriction sites. The stop codon TAA was introduced before the XhoI site.

To generate an expression construct for Ubc7p with an N-terminal His_6-tag, the coding sequence was ligated into pET28b (Novagen) using NdeI/XhoI restriction sites. The stop codon TAA was introduced before the XhoI site.

To generate an expression construct for Cue1p with a C-terminal His_6-tag, the coding sequence was ligated into pET21b (Novagen) using NdeI/XhoI restriction sites. For the cytoplasmic fragment of Cue1p, a fragment coding for amino acids 24-203 was used.

To generate constructs for the expression of C-terminally His_6-tagged DHFR and GFP, the coding sequences were ligated into pET21b using NdeI/XhoI restriction sites. The superfolder GFP variant was used (Pédelacq et al., 2006), and for DHFR, a Cys-free variant (Cys85Ala, Cys152Ser) (Iwakura et al., 1995). For labeling with a maleimide dye, a single Cys residue was attached to both GFP and DHFR resulting in the C-terminal tag: CLEHHHHHH. A fusion of sCPY* and GFP, separated by a Ser-Gly linker, was generated in a similar way.

To generate an expression construct for Otu1p ΔUBX, a fragment coding for amino acids 80-301 was used.

For the expression of N-terminally labeled ubiquitin, the sequence for yeast ubiquitin was inserted into the K27SUMO vector with a single cysteine residue as a spacer.

Protein Expression and Purification

Proteins Expressed in *S. cerevisiae*

For expression of proteins in yeast, strains were transformed with the corresponding plasmids. A starter culture was inoculated and grown for 24 hr at 30°C in synthetic complete medium with 2% (w/v) glucose and amino acid drop-out supplements. This culture was then diluted 1:40 into fresh medium and grown for additional 24 hr. Expression was then induced by adding 1/3 of the culture volume of 4x YEP broth containing 8% (w/v) galactose. The culture was grown for an additional 12 – 16h at 25°C for Hrd1p and all its variants, and at 30°C for all other proteins. Cells were then pelleted at 3000 x g, washed once in H_2O and stored at −80°.

Cell lysates were prepared as follows. Approximately 100 g of yeast cells were resuspended in 150 ml of buffer A (50 mM Tris/HCl (pH 8.0), 300 mM KCl, 40 mM imidazole) supplemented with 1 mM PMSF, 1.5 μM pepstatin A. Glass beads (0.5 mm diameter, BioSpec Products) were added equivalent to about 1/3 to 1/2 of the volume of the cell suspension. Cells were then broken in a BioSpec BeadBeater for 15-20 min with 30 s/60 s on/off cycles in a water/ice bath. After removal of the glass beads, the lysate was centrifuged twice in 50 ml conical tubes at 2000 x g for 10 min. The supernatant was then subjected to ultracentrifugation in a Ti45 rotor at 42,000 x g for 45 min. To prepare a crude membrane fraction, the resulting pellet was washed twice by resuspension and centrifugation. The crude membrane fraction was resuspended in a minimal amount of buffer A, flash-frozen in liquid N_2 and stored at −80°C.

Hrd1p and its variants were purified as follows. The crude membrane fraction was resuspended in approximately 1 ml of buffer S (50 mM HEPES, 300 mM KCl, 5 mM magnesium acetate, 0.5 mM Tris(2-carboxyethyl)phosphine (TCEP), 1% (w/v) decyl maltose neopentyl glycol (DMNG)) per 1 g of original cell pellet and incubated for 30 min at 4°C. After pelleting insoluble material by ultracentrifugation (Ti45, 30 min, 42k rpm), 3 ml of High Capacity Streptavidin Agarose resin (Pierce) were added per 100 ml of solubilized membranes and incubated for 3h on a rolling incubator. Beads were then washed with 20 column volumes (CV) of buffer H (20 mM HEPES, 300 mM KCl, 5 mM magnesium acetate, 120 μM DMNG, 0.5 mM TCEP), followed by 20 CV of buffer H supplemented with 0.25 mM ATP at room temperature, followed by 100 CV of buffer H. The protein was then eluted with buffer H supplemented with 2 mM biotin. The protein was finally purified by size exclusion chromatography on a Superose 6 16/60 column equilibrated with buffer H using a flow rate of 0.5 ml/min.

To purify Ubx2p, the crude membrane fraction was resuspended in approximately 1 ml of buffer A supplemented with 1% DMNG (w/v) per 1 g of original cell pellet and incubated for 30 min at 4°. After pelleting insoluble material by ultracentrifugation (Ti45, 30 min, 42k rpm), the solubilized material was loaded onto a 5 ml HisTrap FF column (GE healthcare) using the sample pump of an Äkta purifier (GE healthcare). The column was equilibrated with buffer A supplemented with 120μM DMNG. After loading, the column was washed with 10 CV of buffer A supplemented with 120μM DMNG, followed by a linear increase in imidazole concentration to 100 mM over 10 CV. Raising the imidazole concentration to 500 mM eluted the protein. Immediately after elution, EDTA and DTT were added to final concentrations of 1 mM each. To remove the N-terminal His-tag, TEV protease was added (molar ratio Ubx2p/TEV protease was 100:1). Cleaved-off tags and TEV protease were removed by gel filtration on a Superose 6 16/60 column equilibrated with buffer H, using a flow rate of 0.5 ml/min.

To purify Uba1p, yeast cells expressing His_{14}-Uba1p were subjected to glass-bead lysis followed by a 2000xg centrifugation to remove unbroken cells and debris, and a 100,000xg ultracentrifugation step. The supernatant was then loaded onto a 5 ml HisTrap FF column (GE healthcare) equilibrated with buffer A, using the sample pump of an Äkta purifier (GE healthcare). The column was washed with 10 CV of buffer A, followed by a linear increase in imidazole concentration to 100 mM over 10 CV. Raising the imidazole concentration to 500 mM eluted the protein. Immediately after elution, EDTA and DTT were added to final concentrations of 1 mM each. To remove the N-terminal His-tag, TEV protease was added (molar ratio Uba1p/TEV protease 100:1). The protein was dialyzed against 50 mM Tris/HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 1 mM DTT for 16 hr using a 30 kDa molecular weight cut-off dialysis membrane, and loaded onto a MonoQ 10/100 GL column (GE healthcare) equilibrated with 50 mM Tris/HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 1 mD DTT. Linearly increasing the NaCl concentration to 500 mM over 10 CV eluted the protein at a conductivity of approximately 25 mS/cm. Peak fractions were pooled and subjected to size-exclusion chromatography on a Superdex 200 16/60 column equilibrated with 20 mM HEPES/KOH (pH 7.4), 150 mM KCl, 300 mM sorbitol, 1 mM DTT.

The ERAD model substrate His_{14}-CPY*-SBP-HDEL was purified as follows. A crude membrane fraction was resuspended in buffer A supplemented with 6M urea (buffer AU) and incubated at RT for 30 min. Insoluble material was removed by ultracentrifugation (Ti45, 30 min, 42k rpm). The supernatant was loaded onto a 5 ml HisTrap FF column (GE healthcare) equilibrated with buffer AU. The column was washed with 10 CV of buffer AU, followed by 10 CV of buffer A to remove urea, followed by a linear increase in imidazole concentration to 100 mM over 10 CV. Further increasing the imidazole concentration to 500 mM eluted the protein. Immediately after elution, EDTA and DTT were added to final concentrations of 1 mM each. The protein was then purified on High Capacity Streptavidin Agarose Resin. To remove both the His_{14}- and SBP-tags, 3C protease and TEV protease were added (molar ratio CPY*/protease approximately 20:1) and incubated for 16h at 4°C. Imidazole was added to a final concentration of 30 mM, and the proteases were removed by applying the solution to a 1ml HisTrap HP column (GE healthcare). Finally the protein was run over a PD10 desalting column to adjust the buffer to 20 mM HEPES/NaOH (pH 7.5), 500 mM NaCl, 0.5 mM DTT.

Wild-type CPY was purified by two different methods. In both cases, the starting material was a membrane fraction from yeast cells expressing His_{14}-CPY-SBP-HDEL. To purify CPY under native conditions, the membranes were solubilized with buffer A supplemented with 1% n-decyl-β-D-maltopyranoside (w/v, DM)). After removal of insoluble material by ultracentrifugation, the extract was loaded onto a 5 ml HisTrap FF column (GE healthcare) equilibrated with buffer A supplemented with 0.1% DM (w/v). The column was then washed with 10 CV of buffer A, followed by a linear increase in imidazole concentration to 100 mM over 10 CV. Further increasing the imidazole concentration to 500 mM eluted the protein. The protein solution was diluted 1:5 with 20 mM Tris/HCl (pH 8.0), 5 mM β-mercaptoethanol, 1 mM EDTA. TEV and 3C protease were added (molar ratio CPY*/protease approximately 20:1), and incubated for 16h at 4°C. The protein was then loaded onto a MonoQ 10/100 GL column (GE healthcare) equilibrated with 50 mM Tris/HCl (pH 8.0), 100 mM NaCl, 5 mM β-mercaptoethanol. Linearly increasing the NaCl concentration to 500 mM over 10 CV eluted the protein at a conductivity of approximately 25 mS/cm. Peak fractions were pooled and subjected to gel filtration on a Superdex 200 16/60 column equilibrated with 20 mM HEPES/KOH (pH 7.4), 500 mM NaCl, 5 mM β-mercaptoethanol.

To purify CPY under denaturing conditions, membranes were resuspended in buffer AU. Insoluble material was removed by ultracentrifugation (Ti45, 30 min, 42k rpm). The supernatant was loaded onto a 5 ml HisTrap FF column (GE healthcare) equilibrated with buffer AU. The column was washed with 10 CV of buffer AU, followed by 10 CV of buffer A to remove urea. All following steps were identical to the ones employed for the purification under native conditions.

sCPY* and sCPY*-DHFR were purified as follows. These proteins precipitated in the absence of urea, so the purification was performed in the presence of 6M urea, unless indicated otherwise. Both proteins were solubilized from a crude membrane fraction in buffer AU and purified on a 5 ml HisTrap FF column (GE healthcare), as described above. After elution, the proteins were dialyzed against 20 mM HEPES, 500 mM NaCl, 0.5 mM DTT, 1M urea for 4 hr. 3C protease was added (molar ratio approximately 1:1) and the mixture incubated for 16h at 4°C. The protease was removed by applying the solution (supplemented with imidazole and urea to final concentrations of 30 mM and 6M, respectively) to a 1ml HisTrap HP column (GE healthcare). Finally the proteins were run on a Superdex200 10/300 Gl equilibrated with 20 mM HEPES/NaOH (pH 7.5), 500 mM NaCl, 0.5mM DTT, 6M urea.

Proteins Expressed in *E. coli*

Unless indicated otherwise, the initial steps in the purification of all His-tagged proteins expressed in *E. coli* were as follows. Cells were grown in Terrific Broth, and expression was induced with 0.5 mM IPTG followed by incubation at 18°C for 16h. Cells were then pelleted at 4,000 x g, resuspended in buffer N (50 mM Tris/HCl (pH 8.0), 500 mM NaCl, 40 mM imidazole), supplemented with 1 mM PMSF and 1.5 μM pepstatin A, and lysed in a microfluidizer.

After ultracentrifugation (Ti45, 40,000 rpm, 30 min), the clarified lysate was incubated with 1 ml Ni-NTA / 1L culture for 2h at 4°C on a rolling incubator. Beads were then washed in a BioRad Econo column with 4 × 50 ml buffer N and eluted with buffer N containing 500 mM imidazole.

Purification of Hrd1p (residues 325-551; Hrd1p-c) was performed as follows. Cells were grown at 30°C for 2h after addition of IPTG. After elution from the Ni-NTA resin, TCEP was added to a final concentration of 1 mM and SUMO protease was added at a molar ration of approximately 1:500. The protein was then dialysed against 50 mM Tris/HCl (pH 8.0), 100 mM NaCl, 0.5 mM TCEP for 16 hr using a 10 kDa molecular weight cut-off dialysis membrane, and loaded onto a MonoQ 10/100 GL column (GE healthcare) equilibrated with 50 mM Tris/HCl (pH 8.0), 100 mM NaCl. Linearly increasing the NaCl concentration to 500 mM over 10 CV eluted the protein at a conductivity of approximately 35 mS/cm. Peak fractions were pooled and subjected to size-exclusion chromatography on a Superdex 200 16/60 column equilibrated with 20 mM HEPES/KOH (pH 7.4), 200 mM NaCl, 0.5mM TCEP. The same protocol was used for the purification of a C-terminally SBP tagged version of Hrd1p-c, and for Ubx2p-c and Otu1p and its variants (also expressed at 30°C for 2h after addition of IPTG).

Cdc48p was purified as follows. Cells expressing His_{14}-SUMO-Cdc48p were grown at 30°C for 2h after addition of IPTG and then lysed by incubation with lysozyme (1 mg/ml) and DNaseI (0.05 mg/ml) for 30 min at 22°C. 5 mM magnesium acetate and 100 μM ATP were present throughout the entire purification. After elution from the Ni-NTA resin, SUMO-protease was added at a molar ratio of 1:500 and incubated for 30 min on ice. To further enrich for hexameric Cdc48p, the solution was loaded on a Superose 6 16/60 column equilibrated to 20 mM HEPES, 300 mM sorbitol, 150 mM NaCl, 5 mM magnesium acetate, 0.5 mM TCEP, 0.1 mM ATP) with a flow rate of 0.5 ml/min. Peak fractions were pooled and concentrated to about 4 mg/ml and flash-frozen in liquid nitrogen.

A complex of Ufd1p and Npl4p was purified as follows. His_{14}-SUMO-Ufd1p and untagged Npl4p were expressed separately, and the bacterial pellets were then mixed at a 2:1 ratio (Ufd1p: Npl4p). The protein complex was purified on Ni-NTA resin according to the standard protocol. After elution from the Ni-NTA resin, TCEP was added to 0.5 mM, and the His_{14}-SUMO-tag removed by SUMO-protease. The solution was dialyzed for 2h against 50 mM Tris/HCl (pH 8.0), 100 mM NaCl, 0.5 mM TCEP and then loaded onto a MonoQ 10/100 GL column (GE healthcare) equilibrated with 50 mM Tris/HCl (pH 8.0), 100 mM NaCl. Linearly increasing the NaCl concentration to 500 mM over 10 CV eluted the protein at a conductivity of approximately 30 mS/cm. Peak fractions were pooled and subjected to gel filtration on a Superdex 200 16/60 column equilibrated with 20 mM HEPES/KOH (pH 7.4), 200 mM NaCl, 5 mM magnesium acetate, 0.5 mM TCEP.

A C-terminally His_6-tagged cytoplasmic fragment of Cue1p (Cue1p-c-His_6), containing residues 24-203 of Cue1p, was purified according to the standard protocol described above, but with only 20 mM imidazole in the lysis and wash buffers, and 400 mM imidazole in the elution buffer. After elution from the Ni-NTA resin, the protein was dialyzed against 20 mM Tris/HCl (pH 8.0), 50 mM NaCl, 1 mM EDTA, 1 mM DTT and loaded onto a MonoQ 10/100 GL column (GE healthcare) equilibrated with 50 mM Tris/HCl (pH 8.0),

50 mM NaCl, 1 mM DTT. Linearly increasing the NaCl concentration to 500 mM over 10 CV eluted the protein at a conductivity of approximately 15 mS/cm. Peak fractions were pooled and flash frozen in liquid nitrogen.

Ubc7p was purified as follows. N-terminally His_6-tagged Ubc7p was purified from inclusion bodies. A 10,000 x g pellet following cell lysis was resuspended in 50 mM Tris/HCl (pH 8.0), 500 mM NaCl, 6 M urea, 10 mM imidazole and incubated for 30 min at RT. After ultracentrifugation, soluble Ubc7p was purified by Ni-NTA chromatography as described above, except that wash and elution buffers contained 20 mM and 400 mM imidazole, respectively, and 6 M urea. After elution from the Ni-NTA resin, the protein was dialyzed against 20 mM Tris/HCl (pH 8.0), 500 mM NaCl, 1 M urea, 1 m mM EDTA, 1 mM DTT. To remove the N-terminal His_6-tag, 500 μl of a 5 mg/ml thrombin solution (bovine thrombin, lyophilized powder from MPbio: dissolved in 50% (w/v) glycerol)) was added (initial culture volume of 4L), and incubated for 8 hr at RT. The protein was then dialyzed against 20 mM Tris/HCl (pH 8.0), 50 mM NaCl, 1 mM EDTA, 1 mM DTT and loaded onto a MonoQ 10/100 GL column (GE healthcare) equilibrated with 50 mM Tris/HCl (pH 8.0), 50 mM NaCl, 1 mM DTT. Linearly increasing the NaCl concentration to 250 mM over 10 CV eluted the protein at a conductivity of approximately 15 mS/cm. Peak fractions were pooled and additionally purified by gel filtration on a Superdex 75 16/60 column (GE healthcare) equilibrated with 20 mM HEPES/NaOH (pH 7.4), 200 mM NaCl, 1 mM DTT.

sCPY*-GFP was purified as follows. C-terminally His_6-tagged sCPY*-GFP was purified from inclusion bodies. A 10,000 x g pellet following lysis was resuspended in 50 mM Tris/HCl (pH 8.0), 500 mM NaCl, 4 M urea, 10 mM imidazole and incubated for 30 min at room temperature. After ultracentrifugation, solubilized sCPY*-GFP was purified by Ni-NTA chromatography as described above, except that wash and elution buffers contained 20 mM and 400 mM imidazole, respectively, and 2 M urea. After elution from Ni-NTA, the protein was dialyzed against 20 mM Tris/HCl (pH 8.0), 100 mM NaCl, 2 M urea, 1 m mM EDTA, 1 mM DTT. The protein was then loaded onto a MonoQ 10/100 GL column (GE healthcare) equilibrated with 100 mM Tris/HCl (pH 8.0), 100 mM NaCl, 1 mM DTT, 2 M urea and eluted with a linear gradient to 500 mM NaCl. Peak fractions were pooled and additionally purified by gel filtration on a Superdex 200 column (GE healthcare) equilibrated with 20 mM HEPES/NaOH (pH 7.4), 200 mM NaCl, 1 mM DTT, 2 M urea.

GFP and DHFR were both purified as C-terminally His_6-tagged proteins by Ni-affinity chromatography, followed by anion-exchange chromatography and gel filtration using a Superdex 200 column. Different from the general protocol, cells were grown at 30°C for 2h after addition of IPTG.

Protein Labeling with Fluorescent Dyes

Cys-specific labeling with maleimide dyes was performed at RT with a 3-fold excess of dye for 1h. Free dye was then removed by gel filtration using Sephadex G50 superfine (GE healthcare). The labeling efficiency ranged from 60%–90%.

For labeling with amine-reactive N-hydroxy-succinimidyl (NHS) esters, equimolar amounts of dye and protein were incubated for 30 min at RT. Addition of 100 mM Tris/HCl (pH 8.0) stopped the reaction. Free dye was removed by gel filtration using Sephadex G50 superfine (GE healthcare).

Hrd1p-SBP containing the C-terminal sequence LPETGG was labeled with the sortase technique (Popp et al., 2009). A peptide with the sequence Gly-Gly-Gly-Cys was purchased from Genscript and labeled with DyLight680 maleimide. The labeled peptide was subsequently purified on a C18 reverse-phase column. A pentamutant P94R/D160N/D165A/K190E/K196T of SrtA from *S. aureus* was purified from *E. coli* (Chen et al., 2011). For the labeling reaction, 20 μM Hrd1p was incubated with 10 μM SrtA, and 100 μM labeled peptide in buffer H supplemented with 5 mM calcium chloride for 24 hr at 4°C. Hrd1p was then purified by gel filtration on a Superose 6 column. The labeling efficiency was about 50%.

Binding Assays

All binding assays were performed at room temperature in buffer U (20 mM HEPES/KOH (pH 7.4), 150 mM potassium chloride, 5 mM magnesium acetate, 0.2 mM TCEP, 0.12 mM DMNG) supplemented with 0.2 mg/ml BSA. Fixed concentrations of substrate were mixed with increasing concentrations of Hrd1p. 20 μl of binding reaction were then incubated with 20 μl washed magnetic streptavidin beads (Pierce) for 1h. The beads were briefly washed three times and bound protein eluted with buffer U supplemented with 2 mM biotin. Supernatants and eluted protein were analyzed by SDS-PAGE and scanning of the gel with an Odyssey scanner (Li-Cor). Binding curves were generated by quantification of the unbound protein. Apparent affinities (K_D) were estimated by fitting the data (fraction of bound substrate (r) as a function of total Hrd1p concentration ([Hrd1p]) to the following equation:

$$r = [Hrd1p]/(K_D + [Hrd1p]).$$

This analysis assumes a 1:1 stoichiometry of substrate:Hrd1p, which is likely incorrect (see Figure 1G). The exact stoichiometry of the interaction is unknown, so the determined constants only serve to compare the apparent affinities of different substrates.

Ubiquitination Assay

Ubiquitination assays were performed at 30°C in buffer U supplemented with 0.2 mg/ml BSA. Unless indicated otherwise, the concentrations of the individual components were: 0.1 μM Uba1p, 1 μM Ubc7p, 1 μM Cue1p-c, 0.1 μM Hrd1p (labeled with DyLight680), 0.1 μM substrate (labeled with DyLight800), 100 μM ubiquitin (from yeast, purchased from R&D Systems), 2.5 mM ATP. The ubiquitin variants K48R, and methylated ubiquitin were both derived from human ubiquitin (R&D Systems). For the ubiquitination reaction shown in Figure S2C, the concentration of the individual components were higher: 0.4 μM Uba1p, 4 μM Ubc7p and Cue1p-c,

2.5 μM Hrd1p / Hrd1p-c. For time-course experiments, aliquots were taken at the indicated times, 3 x sample buffer was added (12% SDS (w/v), 30% glycerol (w/v), 0.05% Coomassie blue G-250 (Serva), 150 mM Tris/HCl (pH 7.0)), followed by heating to 70°C. The samples were analyzed by SDS-PAGE and scanning of the gel with an Odyssey scanner (Li-Cor). For quantification, the intensity of the band of the unmodified protein was measured using the ImageJ software.

Cdc48p Binding Assay

For the experiments presented in Figures 3 and S3C, Hrd1p-SBP was immobilized on magnetic streptavidin beads and then incubated with ubiquitination machinery at standard concentrations (but [Hrd1p] = 1 μM) for 1h at room temperature. In separate reactions, ubiquitin was either replaced by methylated ubiquitin (for Figure 3B) or buffer (for Figure 3C). The beads were washed and incubated with 2 μM solutions of the indicated components in buffer U supplemented with 250 μM ATPγS for 15 min. The beads were briefly washed three times with buffer U supplemented with 100 μM ATPγS, and bound protein eluted with buffer U supplemented with 2 mM biotin. Eluted fractions were analyzed by SDS-PAGE. The gel was stained with IRDye Blue Protein Stain (Li-Cor) and scanned with an Odyssey scanner (Li-Cor).

Assays for Substrate Release and Dissociation of Hrd1p Oligomers

For the experiment presented in Figures 4A and 4B, Hrd1p (0.5 μM) was first immobilized on magnetic streptavidin resin in the presence of fluorescently labeled CPY* (0.1 μM), and then incubated with ubiquitination machinery at standard concentrations for 1h at 30°C. After removal of the ubiquitination machinery, the beads were incubated for 30 min at 30°C with the indicated components (100 nM each) in buffer U supplemented with 2 mM ATP. To determine the total amount of bound material, beads were incubated with buffer U supplemented with 2 mM biotin. Samples were subjected to SDS-PAGE and scanning of the gel with an Odyssey scanner. For quantification of the release of ubiquitinated protein, the intensity of the region indicated in the figure as "poly-Ub-CPY*" was measured using the ImageJ software.

In the experiment shown in Figures 4C, 4D, and 4E, release of nonubiquitinated CPY* from poly-ubiquitinated or nonubiquitinated Hrd1p was measured. To this end, bead-immobilized Hrd1p (0.5 μM) was incubated with ubiquitination machinery or buffer. The beads were then washed and incubated for 30 min at RT with 100 nM CPY*. After an additional wash step, beads were then incubated with the indicated factors in buffer U supplemented with 2 mM ATP or, as a control for full release, with buffer U supplemented with 2 mM biotin. In control experiments, ATP was depleted by prior incubation with hexokinase and glucose (Hk/G) for 10 min on ice). The supernatants were analyzed by SDS-PAGE and scanning of the gel with an Odyssey scanner. The amounts of nonubiquitinated CPY* were quantified with the ImageJ software.

25 x stocks of Hk/G were prepared according to this protocol: 267 μl Hexokinase (1500 U/ml in ammonium sulfate, purchased from Roche) were mixed with 400 μl 1 M glucose in a total volume of 1 ml. The mixture was aliquoted and stored at −80°C.

For the experiment presented in Figures S4A and S4B, Hrd1p (0.5 μM) was first immobilized on magnetic streptavidin resin in the presence of fluorescently labeled sCPY* (0.1 μM), and then incubated with ubiquitination machinery at standard concentrations for 1h at room temperature. After removal of the ubiquitination machinery, the beads were incubated for 15 min at room temperature with the indicated components (100 nM each) in buffer U supplemented with 0.5 mM ATPγS. After an additional wash step with buffer U supplemented with 0.1 mM ATPγS, the beads were incubated with either 1 mM ATP or ATPγS for 10 min at RT (unbound fraction in figure). In a final step, the material still bound was released by incubation with buffer U supplemented with 2 mM biotin (bound fraction). Samples were subjected to SDS-PAGE and scanning of the gel with an Odyssey scanner. For quantification of the release of ubiquitinated protein, the intensity of the region indicated in the figure as "poly-Ub-sCPY*" was measured using the ImageJ software. Total ubiquitinated sCPY* was determined as the sum of the intensities measured in the bound and unbound fractions.

For the experiments shown in Figures S4C and S4D, bead-immobilized ubiquitinated or nonubiquitinated Hrd1p (0.5 μM) was incubated with 0.1 μM fluorescently labeled sCPY* or sCPY*-DHFR in buffer U supplemented with 0.2 mg/ml BSA. The beads were then washed and incubated for 30 min at RT with the indicated components. Bound material was then eluted with buffer U supplemented with 2 mM biotin. Supernatants and eluted material were analyzed by SDS- PAGE and scanning of the gel with an Odyssey scanner. The fraction released was determined by dividing the intensities in the unbound fraction by the sum of the intensities in unbound and bound fractions.

For the experiments in Figures 4F and 4G, 400 nM of Hrd1-SBP were mixed with 100 nM of fluorescently labeled untagged Hrd1p and immobilized on magnetic streptavidin beads. The beads were incubated with ubiquitination machinery for 45 min at RT. After washing, the beads were incubated with the indicated components (100 nM in buffer U with 1 mM ATP) or with buffer U supplemented with 2 mM biotin. The supernatants were analyzed by SDS-PAGE and scanning of the gel with an Odyssey scanner. For quantification, the intensity of the region indicated in the figure as "poly-Ub-Hrd1p" was measured using the ImageJ software and compared to the amount released by incubation with biotin.

DUB Activity Assay

For the experiment in Figures 6A and 6B, beads with immobilized fluorescently labeled Hrd1p were incubated with ubiquitination machinery, washed, and bound protein eluted with buffer U supplemented with biotin. Eluted Hrd1p was mixed on ice with the indicated components, resulting in a final concentration of approximately 150 nM Hrd1p and 100 nM of the other components, except for Otu1p, which was present at 5 nM. The mixtures were then incubated at 30°C. At the indicated times, aliquots were taken and mixed

with SDS sample buffer, immediately followed by heating of the samples to 90°C. The samples were analyzed by SDS-PAGE and scanning of the gel with an Odyssey scanner.

For the experiment in Figure 6C, complexes of Hrd1p and CPY* were immobilized on beads, instead of only Hrd1p.

For the experiment in Figure 6D, immobilized Hrd1p (1μM) was ubiquitinated and then incubated with 2μM complex of Cdc48p, Ufd1p, and Npl4p in the presence of 2 μM ATPγS. After removal of unbound Cdc48p complex, the beads were incubated for 1 hr at 30°C with the indicated components. Supernatants and beads were analyzed by SDS-PAGE and Coomassie-Blue staining.

For the experiment in Figures S6A and S6B, 0.5 μM ubiquitin, N-terminally labeled with a fluorescent dye, was present during the ubiquitination reaction.

Reconstitution into Proteoliposomes

The following synthetic lipids from Avanti Polar Lipids were used: 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (DOPS). Texas Red labeled 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (TR-PE) was purchased from Invitrogen.

For the preparation of protein-free liposomes, chloroform solutions of POPC, DOPE, DOPS, and TR-PE were mixed at a molar ratio of 69.5:20:10:0.5, respectively, and chloroform was removed in a SpeedVac. The resulting lipid film was resuspended in 20 mM HEPES/KOH (pH 7.4), 150 mM potassium chloride, 5 mM magnesium acetate, TCEP, and after brief sonication, extruded through 200 nm pore-size filters. The final total lipid concentration was 20 mM.

For the preparation of Hrd1p-containing proteoliposomes, protein-free liposomes (10 mM final lipid concentration) were mixed with DMNG (15 mM), Hrd1p (2 μM) and, optionally, substrate (0.5 μM), and incubated for 1h at room temperature. The detergent was then removed with detergent removal spin columns (Pierce) at RT. For the preparation of 150 μl proteoliposome solution, the mixture of lipids, detergent and protein was successively applied to four detergent removal spin columns with 500 μl resin each. Each time the mixture was incubated for 30 min with the resin before spin-elution.

For binding experiments, 25 μl of liposomes were mixed with fluorescently labeled substrate at a final concentration of 25 nM in a total volume of 50 μl, and incubated at RT. This mixture was then subjected to flotation in a Nycodenz step-gradient. To this end, 50 μl of a solution containing liposomes was mixed with the same volume of an 80% (w/v) Nycodenz solution, and overlaid with 50 μl of 30% Nycodenz, 50 μl of 15% Nycodenz, and 50 μl of buffer (20 mM HEPES/KOH (pH 7.4), 150 mM potassium chloride, 5 mM magnesium acetate, 0.2 mM TCEP). Gradients were subjected to ultracentrifugation in a TLS-55 rotor for 1h at 48,000 x g. The gradient was separated into six fractions of equal volume starting from the top. Fractions were pipetted into a 384-well plate and the plate scanned in an Odyssey scanner (Li-Cor), or analyzed by SDS-PAGE and scanning of the gel.

For ubiquitination reactions, proteoliposomes were diluted 1:10 into the other components, resulting in final concentrations of Hrd1p and substrate of ~200 nM and ~50 nM, respectively. Since ubiquitination was less efficient than in detergent solution, the concentrations of Uba1p, Ubc7p, and Cue1p-c were doubled in these experiments.

To estimate the fraction of Hrd1p oriented in reconstituted liposomes with the cytoplasmic domain facing outside, liposomes were incubated with TEV protease (equimolar to Hrd1p) for 1h at RT in the absence or presence of 1% DMNG.

For the release reaction presented in Figures 7F and 7G, the indicated components were added to liposomes after a 1h ubiquitination reaction and incubated for an additional 30 min at 30°C. The mixture was then analyzed by flotation in a Nycodenz gradient as described.

SUPPLEMENTAL REFERENCES

Chen, I., Dorr, B.M., and Liu, D.R. (2011). A general strategy for the evolution of bond-forming enzymes using yeast display. Proc. Natl. Acad. Sci. USA *108*, 11399–11404.

Iwakura, M., Jones, B.E., Luo, J., and Matthews, C.R. (1995). A strategy for testing the suitability of cysteine replacements in dihydrofolate reductase from Escherichia coli. J. Biochem. *117*, 480–488.

Keefe, A.D., Wilson, D.S., Seelig, B., and Szostak, J.W. (2001). One-step purification of recombinant proteins using a nanomolar-affinity streptavidin-binding peptide, the SBP-Tag. Protein Expr. Purif. *23*, 440–446.

Mumberg, D., Müller, R., and Funk, M. (1994). Regulatable promoters of Saccharomyces cerevisiae: comparison of transcriptional activity and their use for heterologous expression. Nucleic Acids Res. *22*, 5767–5768.

Ng, D.T., Spear, E.D., and Walter, P. (2000). The unfolded protein response regulates multiple aspects of secretory and membrane protein biogenesis and endoplasmic reticulum quality control. J. Cell Biol. *150*, 77–88.

Pédelacq, J.D., Cabantous, S., Tran, T., Terwilliger, T.C., and Waldo, G.S. (2006). Engineering and characterization of a superfolder green fluorescent protein. Nat. Biotechnol. *24*, 79–88.

Popp, M.W., Antos, J.M., and Ploegh, H.L. (2009). Site-specific protein labeling via sortase-mediated transpeptidation. Curr. Protoc. Protein Sci. *Chapter 15*, Unit 15.3.

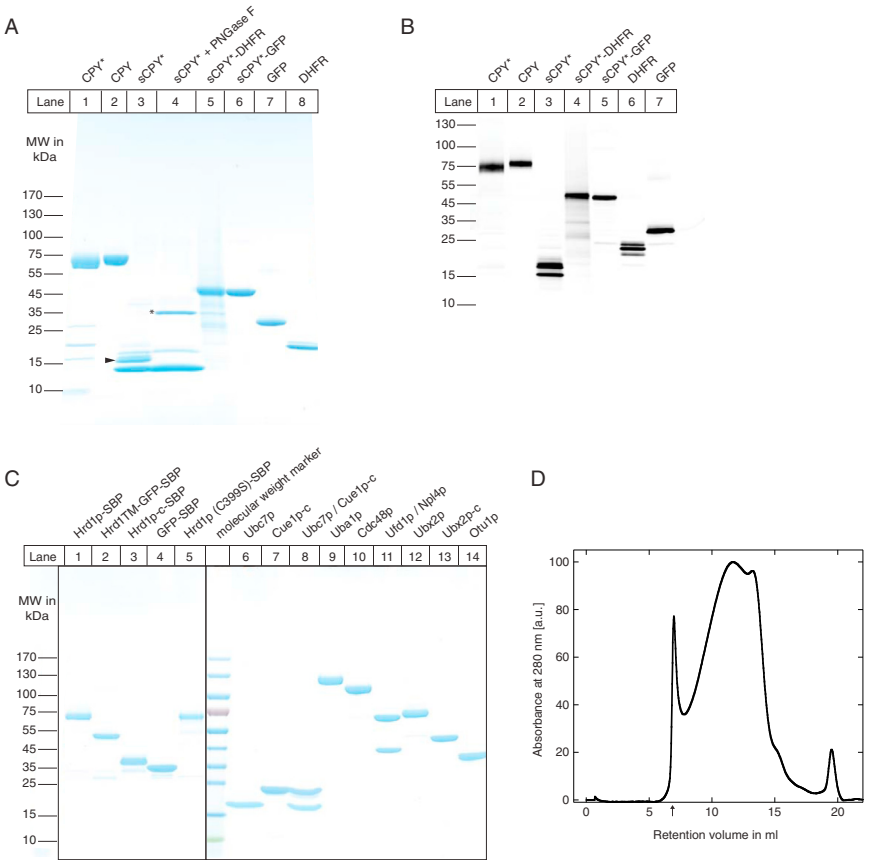

Figure S1. Characterization of the Purified ERAD Components and Substrates, Related to Figure 1

(A) The indicated proteins were tested as ERAD substrates. The purified proteins were subjected to SDS-PAGE, followed by staining with Coomassie blue. The upper band seen with sCPY* is glycosylated (arrowhead), as shown by its down-shift after treatment with peptide N-glycosidase F (PNGase F). The asterisk indicates the position of PNGase F.

(B) The proteins in (A) were labeled with DyLight800 and subjected to SDS-PAGE, followed by fluorescence scanning of the gel.

(C) The indicated purified ERAD components were subjected to SDS-PAGE, followed by staining with Coomassie blue.

(D) Purified wild-type Hrd1p was subjected to gel filtration on a Superose 6 column and the absorbance at 280 nm was monitored. The arrow indicates the void volume.

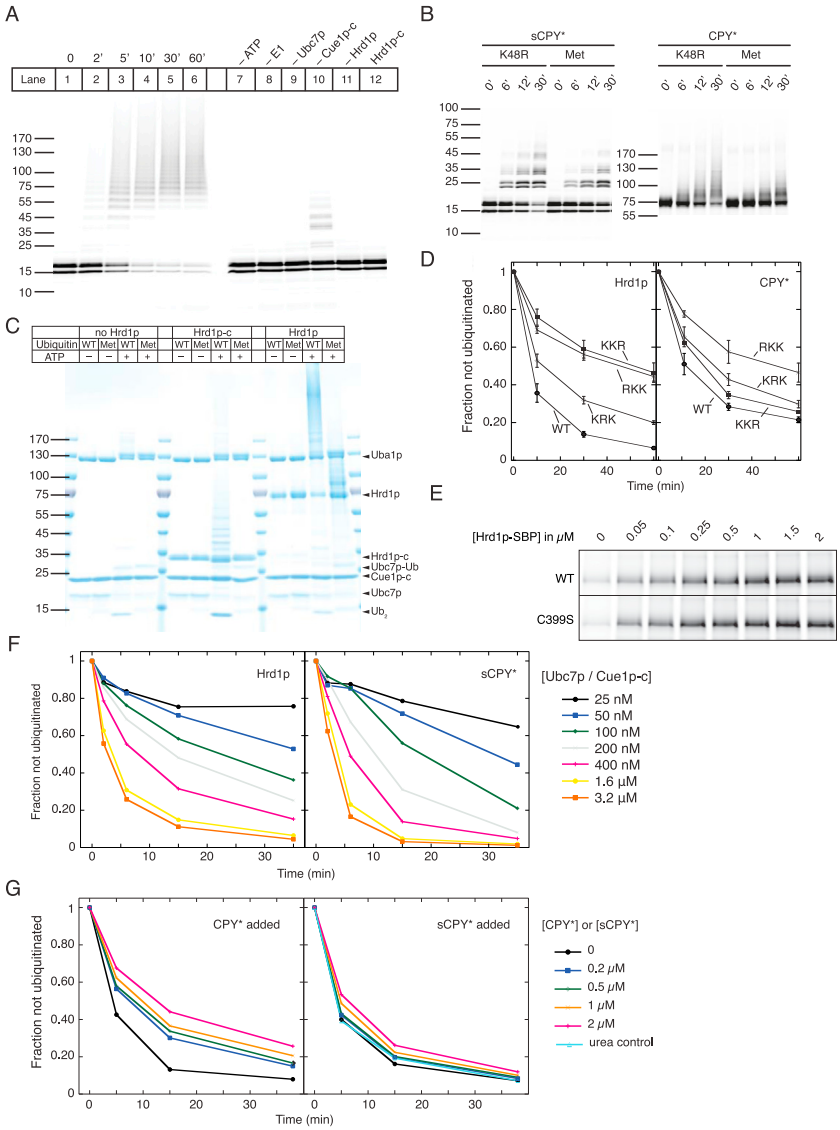

Figure S2. Ubiquitination of Substrate and Hrd1p, Related to Figure 2

(A) Lanes 1-6 show a time-course of sCPY* (100 nM) poly-ubiquitination with all components present. Lanes 7-11 show reactions after 60 min with the indicated components omitted. In lane 12, wild-type Hrd1p (100 nM) was replaced with 1 μM of the cytoplasmic domain of Hrd1p (Hrd1p-c).

(B) The ubiquitination of CPY* or sCPY* (both at 100 nM) was followed over time with ubiquitin replaced with either a K48R ubiquitin mutant or with ubiquitin in which all lysines were methylated (Met).

(C) Ubiquitination reactions were performed in the absence of Hrd1p, with a cytoplasmic fragment of Hrd1p (Hrd1p-c), or with full-length Hrd1p. The reactions were performed with either wild-type (WT) ubiquitin or methylated ubiquitin (Met) in the presence or absence of ATP. After 30 min incubation, the samples were analyzed by SDS-PAGE and staining with Coomassie blue. Note that in the absence of Hrd1p, di-ubiquitin and an Ubc7p-ubiquitin adduct were formed. Full-length Hrd1p, but not Hrd1p-c, catalyzes efficient auto-ubiquitination, although Hrd1p-c generates ubiquitin chains.

(D) The time-course of auto- and substrate-ubiquitination was determined in parallel for Hrd1p mutants that carry Lys to Arg mutations in three different regions (Hrd1p at 100 nM). Shown are the means and standard deviations of three experiments.

(E) The indicated amounts of wild-type SBP-tagged Hrd1p or of the C399S mutant were immobilized and incubated with 100 nM fluorescently labeled untagged wild-type Hrd1p. The bound fractions were analyzed by SDS-PAGE and fluorescence scanning of the gel. All samples were analyzed in the same gel and the images scaled identically.

(F) The poly-ubiquitination of fluorescently labeled sCPY* (100 nM) and Hrd1p (100 nM) was followed in parallel over time. The concentrations of Ubc7p and Cue1p-C were varied, as indicated, keeping the 1:1 molar stoichiometry between the two components constant. The disappearance of unmodified sCPY* or Hrd1p was quantitated.

(G) The auto-ubiquitination of fluorescently labeled Hrd1p (50 nM) was followed over time in the presence of different concentrations of CPY* or sCPY*.

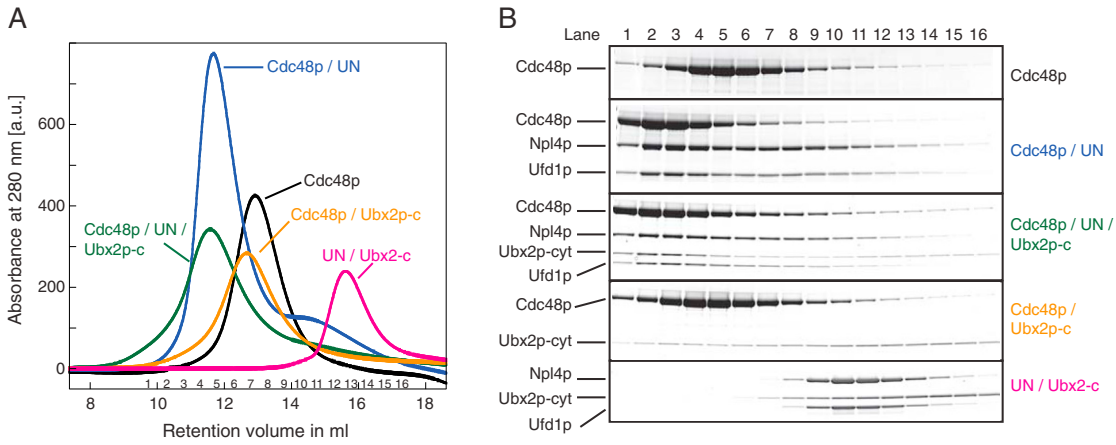

Figure S3. Recruitment of Cdc48p to Ubiquitinated Hrd1p, Related to Figure 3

(A) Cdc48p (12 μM), Ufd1p/Npl4p (U/N) complex (15 μM), and the UBX domain of Ubx2 (Ubx2p-c, 15 μM) were mixed, as indicated, in the presence of 1 mM ATPγS at 4°C, and subjected to gel filtration on a Superose 6 column equilibrated with 20 mM HEPES/KOH (pH 7.4), 150 mM potassium chloride, 5 mM magnesium acetate, 0.2 mM TCEP, 100 μM ATPγS. The absorbance at 280 nm was monitored.

(B) Fractions of the gel filtration experiments in (A) were analyzed by SDS-PAGE and Coomassie staining.

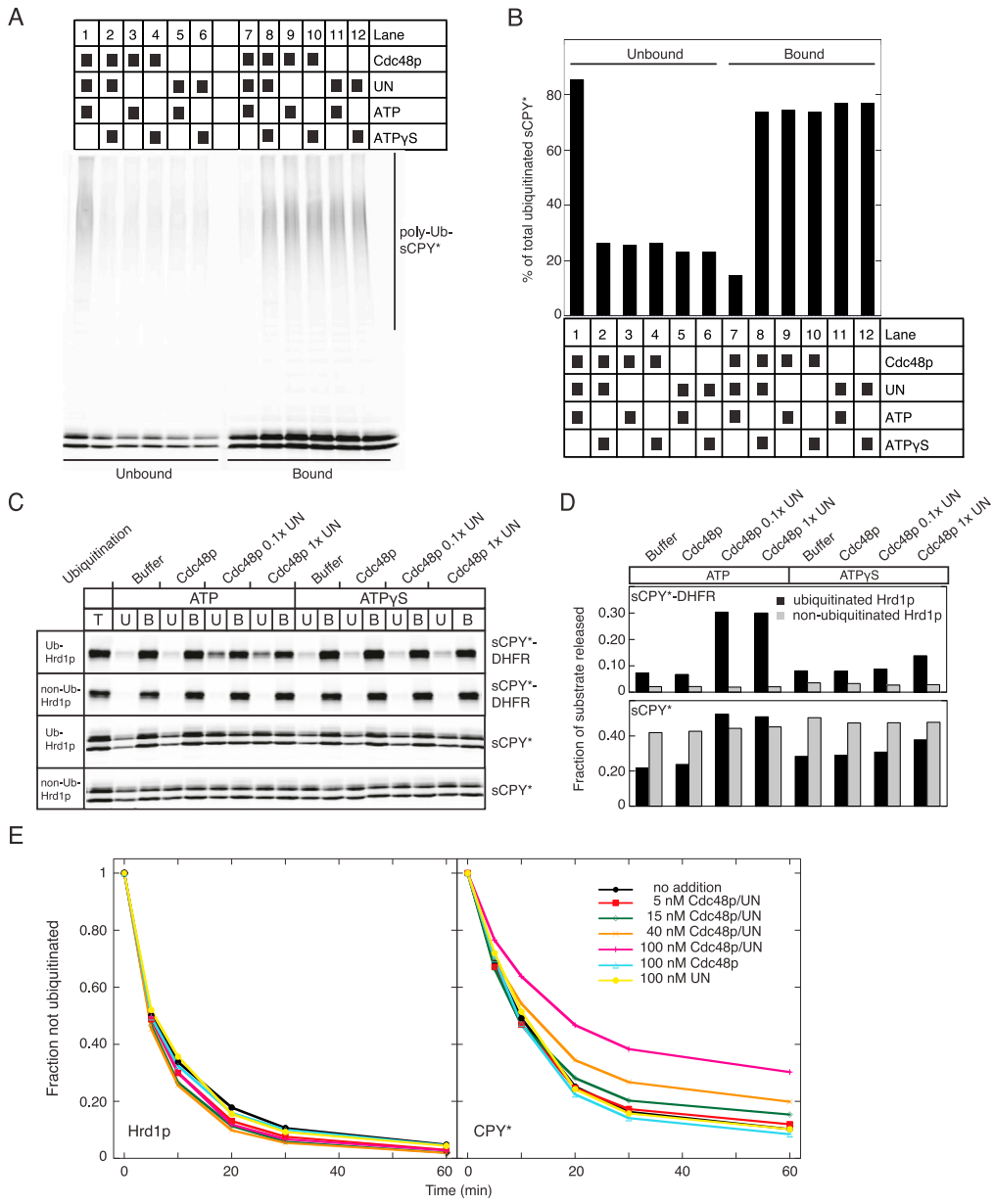

Figure S4. Release of Substrate from Hrd1p by Cdc48p, Related to Figure 4

(A) Bead-immobilized Hrd1p was incubated with the ubiquitination machinery in the presence of fluorescently labeled sCPY*. After washing, the beads were incubated with Cdc48p in the absence or presence of the Ufd1p/Npl4p (UN) complex, or with UN alone. These reactions were performed in the presence of ATPγS to allow Cdc48p binding, but not Cdc48p activity. The beads were then washed and incubated with ATP or ATPγS, and the bound and unbound fractions analyzed by SDS-PAGE and fluorescence scanning of the gel.

(B) Quantification of bound and unbound ubiquitinated sCPY* in the experiment shown in (A).

(C) SBP-tagged Hrd1p was bound to streptavidin beads and incubated with the ubiquitination machinery to generate ubiquitinated Hrd1p (Ub-Hrd1p). The ubiquitination machinery was removed and the beads incubated with fluorescently labeled sCPY*-DHFR or sCPY*. After washing, the beads were incubated in ATP or ATPγS with 100 nM Cdc48p in the absence or presence of UN (10 or 100 nM; 0.1x or 1x, respectively). Controls were performed with non-ubiquitinated Hrd1p (non-Ub-Hrd1p). The bound (B) and unbound (U) fractions were analyzed by SDS-PAGE and fluorescence scanning of the gels. The total releasable material (T) was determined after addition of biotin to the beads.

(D) Quantification of the experiments in (C) (released from Ub-Hrd1p, black columns; released from non-Ub-Hrd1p, gray columns).

(E) The poly-ubiquitination of fluorescently labeled CPY* (100 nM) and Hrd1p (100 nM) was followed in parallel over time in the presence of the indicated amounts of Cdc48p/UN complex, keeping the 1:1 molar stoichiometry between the two components constant. The disappearance of unmodified CPY* or Hrd1p was quantitated.

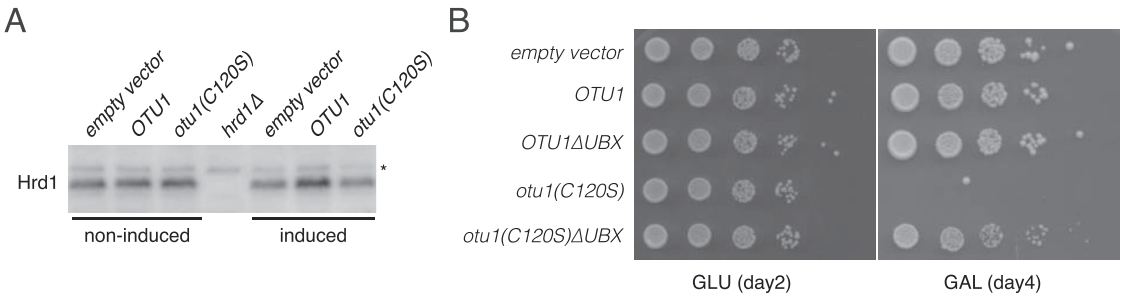

Figure S5. Otu1p Function In Vivo, Related to Figure 5

(A) The level of Hrd1p was analyzed in cells in which the expression of Otu1p or Otu1(C120S) was induced or not induced. A control was performed with cells lacking Hrd1p (hrd1Δ). The asterisk indicates an unrelated protein reacting with Hrd1p antibodies.

(B) Yeast cells transformed with plasmids containing the indicated genes under the Gal1 promoter were spotted in serial dilutions on glucose- or galactose-containing plates and grown for two or four days, respectively.

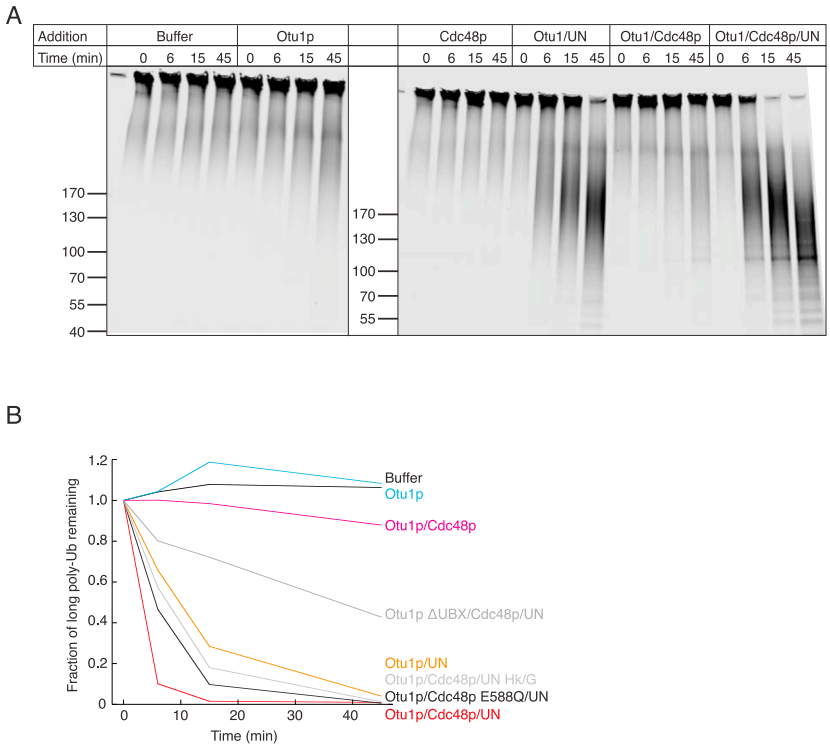

Figure S6. Cdc48p-Dependent In Vitro Deubiquitination by Otu1p, Related to Figure 6

(A) Bead-immobilized Hrd1p-SBP was incubated with a mixture of unlabeled ubiquitin and fluorescently labeled ubiquitin in the presence of the other components of the ubiquitination machinery. After washing, Hrd1p was eluted from the beads with biotin and incubated with the indicated components (Ufd1/Npl4p; UN) for different time periods in the presence of ATP. Hrd1p was in a 30-fold excess over Otu1p, whereas all other components were about equimolar to Hrd1p. The samples were analyzed by SDS-PAGE and fluorescence-scanning.

(B) Quantification of experiments performed as in (A). The disappearance of the longest ubiquitin chains was quantified under different conditions. ATP was depleted with hexokinase/glucose (Hk/G). Where indicated, an ATPase-defective Cdc48p mutant (Cdc48p E588Q) or an Otu1p mutant lacking the UBX domain were used.

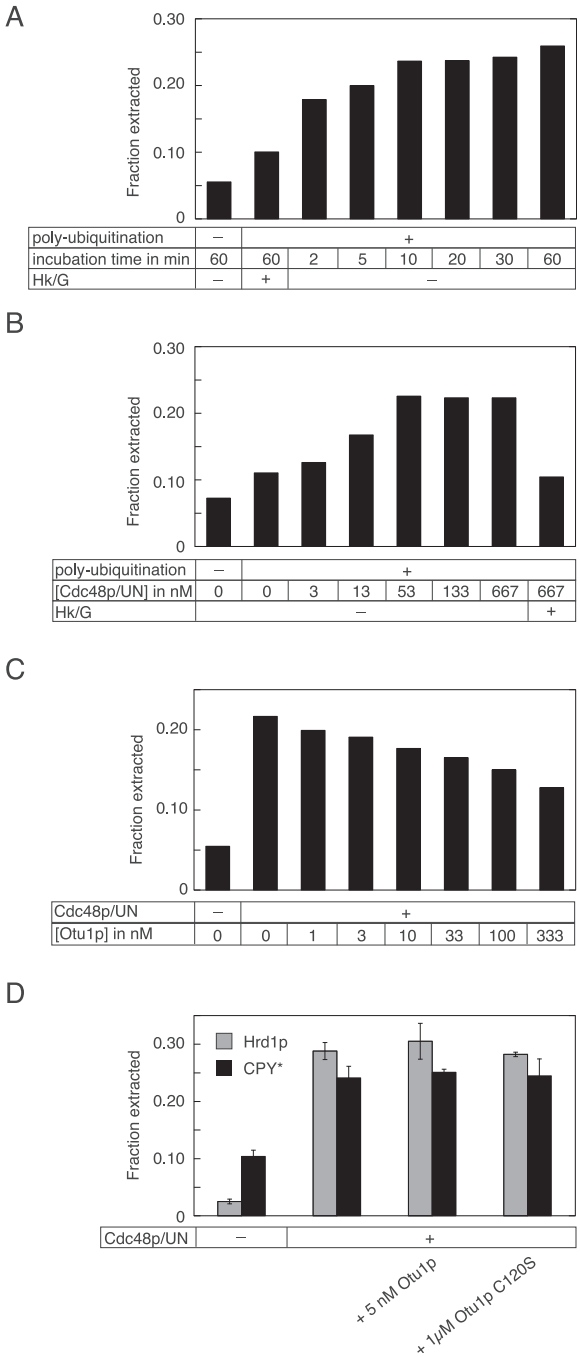

Figure S7. Membrane Extraction of Polyubiquitinated Proteins by Cdc48p, Related to Figure 7

(A) Hrd1p and fluorescently labeled CPY* were co-reconstituted into proteoliposomes. The vesicles were incubated with the ubiquitination machinery, followed by incubation with 100 nM Cdc48p complex for different time periods. The vesicles were floated in a Nycodenz gradient and fractions analyzed for total fluorescence. Shown is the fraction of CPY* extracted from the proteoliposomes. Where indicated, the ubiquitination reaction was omitted or hexokinase/glucose (Hk/G) was added before addition of Cdc48p complex.

(B) As in (A), but the incubation was performed for 30 min with different concentrations of the Cdc48p complex.

(C) As in (A), but the incubation was performed in the presence of different concentrations of Otu1p.

(D) Hrd1p and CPY* labeled with different fluorescent dyes were co-reconstituted into proteoliposomes and incubated with the ubiquitination machinery, followed by incubation with 100 nM Cdc48p complex. Where indicated, either 5 nM wild-type Otu1p or 1 μM enzymatically inactive Otu1p C120S were present during the last incubation. Shown is the fraction of Hrd1p or CPY* extracted from the proteoliposomes (means and standard deviations of three experiments).