Cell

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

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http://dx.doi.org/10.1016/j.cell.2014.07.050

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, in-

side 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.

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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, crosslinking 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
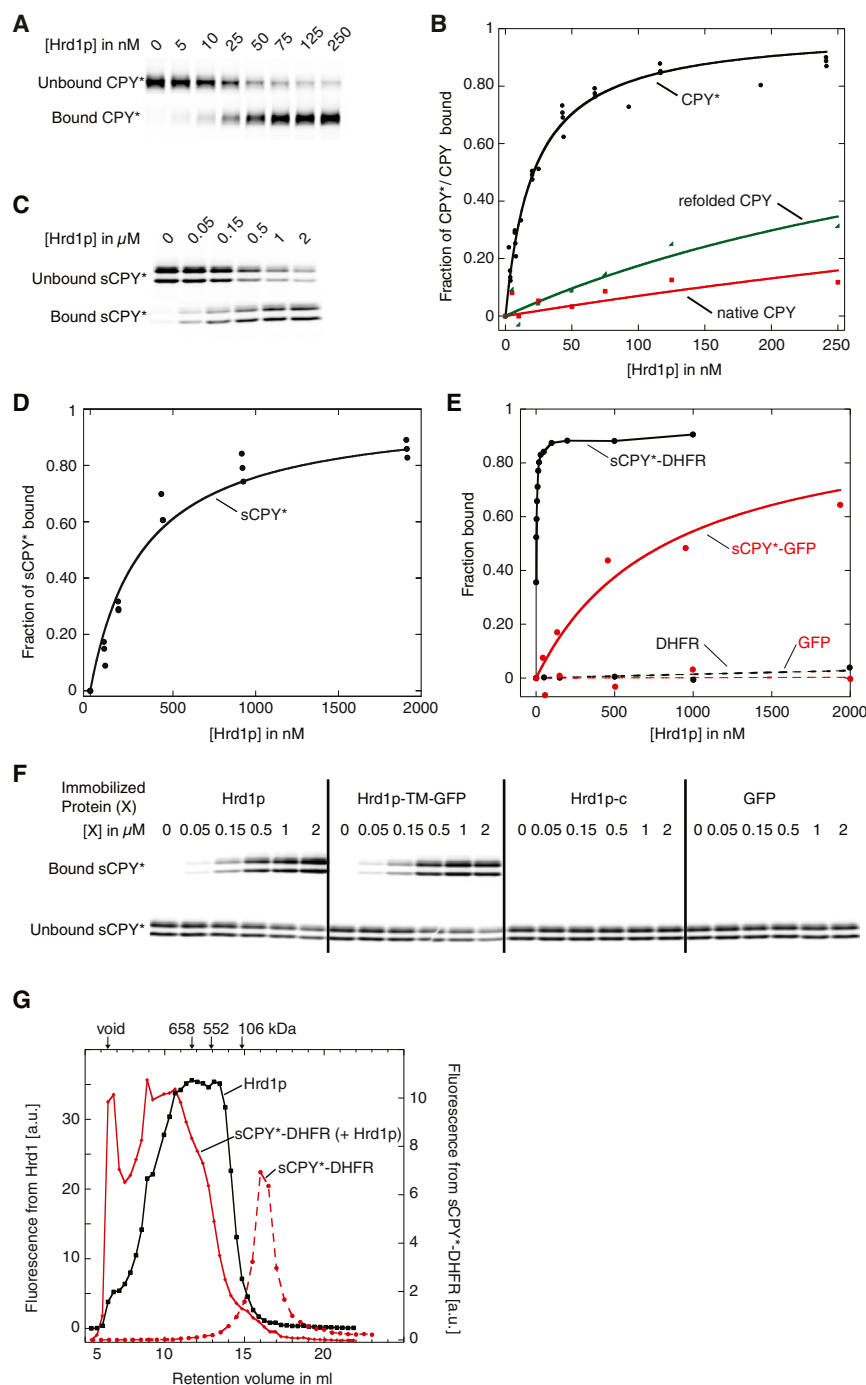
Figure 1. Substrate Interaction with Hrd1p

(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 Dy-Light680. 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.
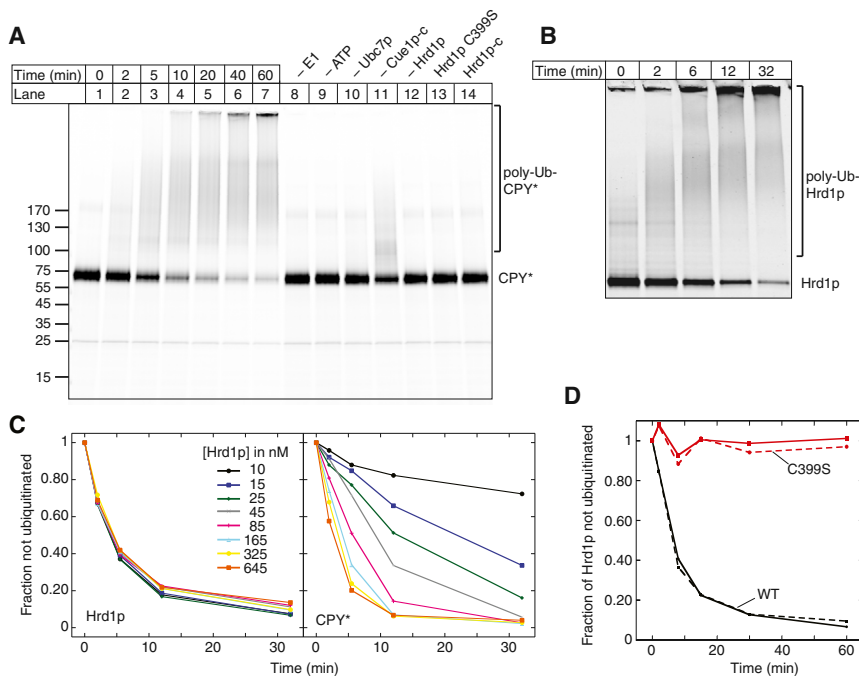
See also Figure S1.

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.

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),

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
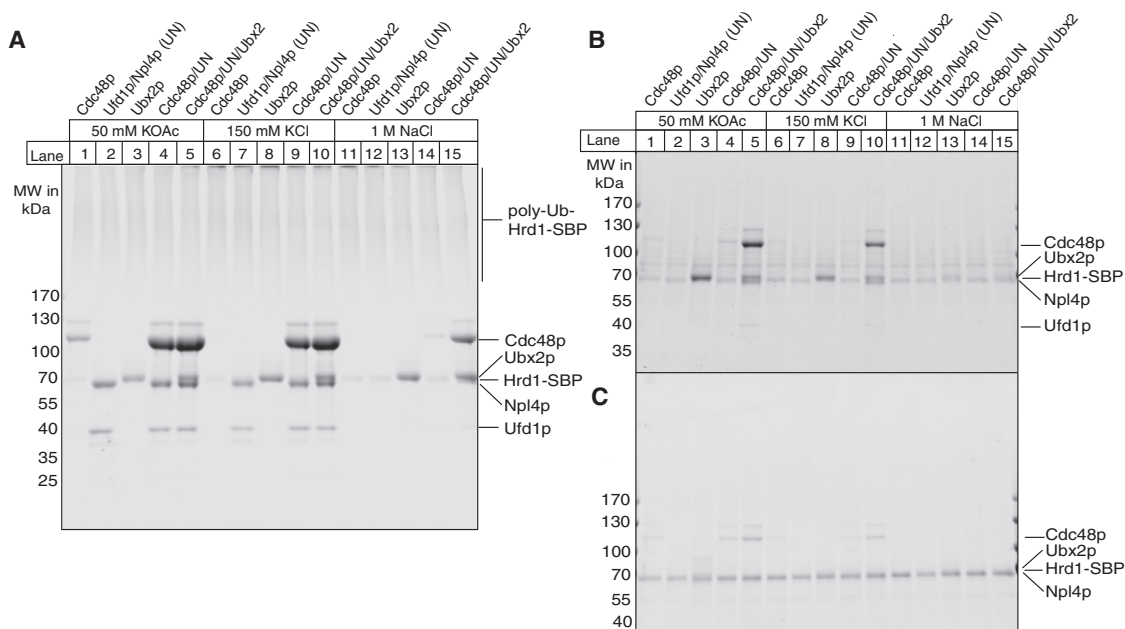
Figure 3. Recruitment of Cdc48p to Ubiquitinated Hrd1p

(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).
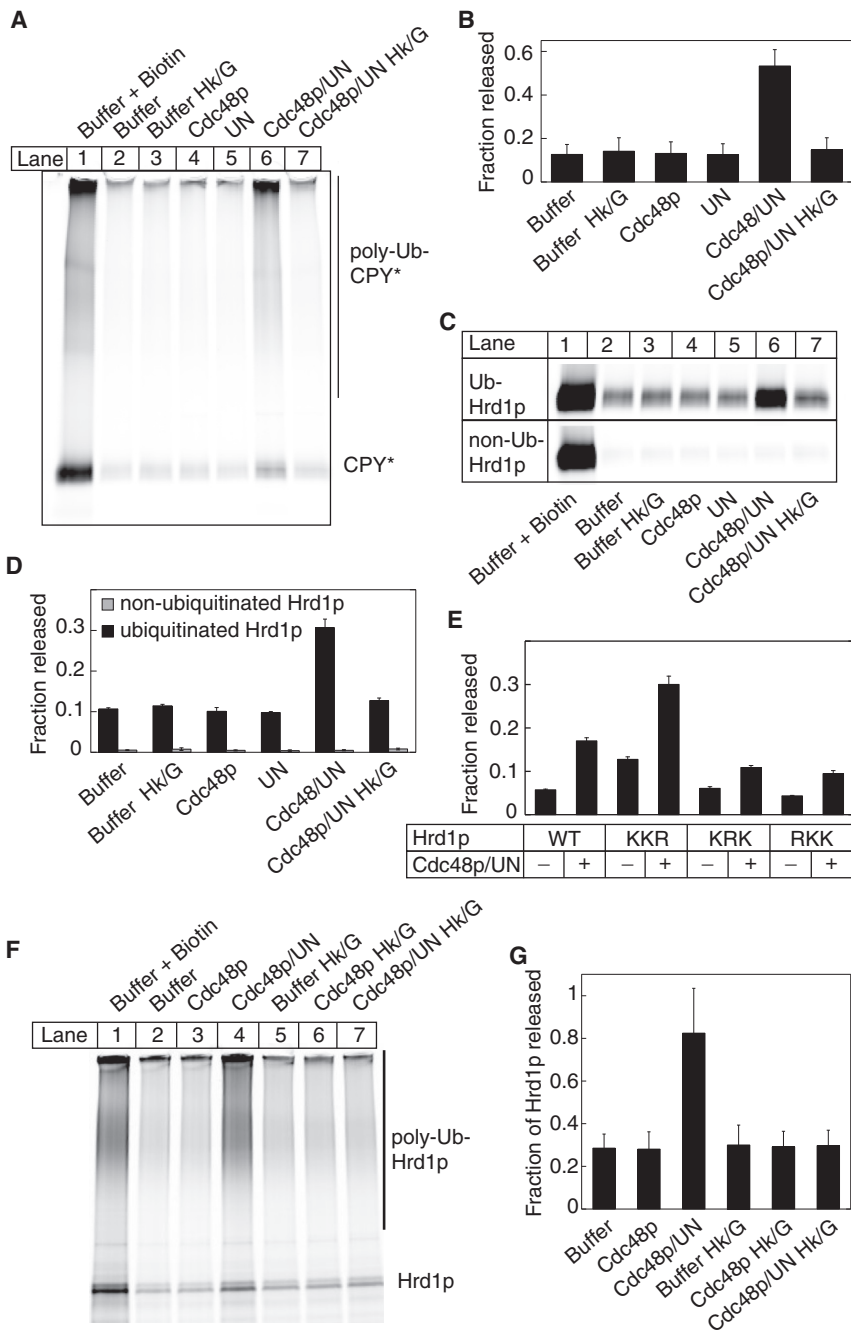
Cdc48p-Dependent Substrate Release from Hrd1p

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.

A

B

C

D

E

F

G

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).
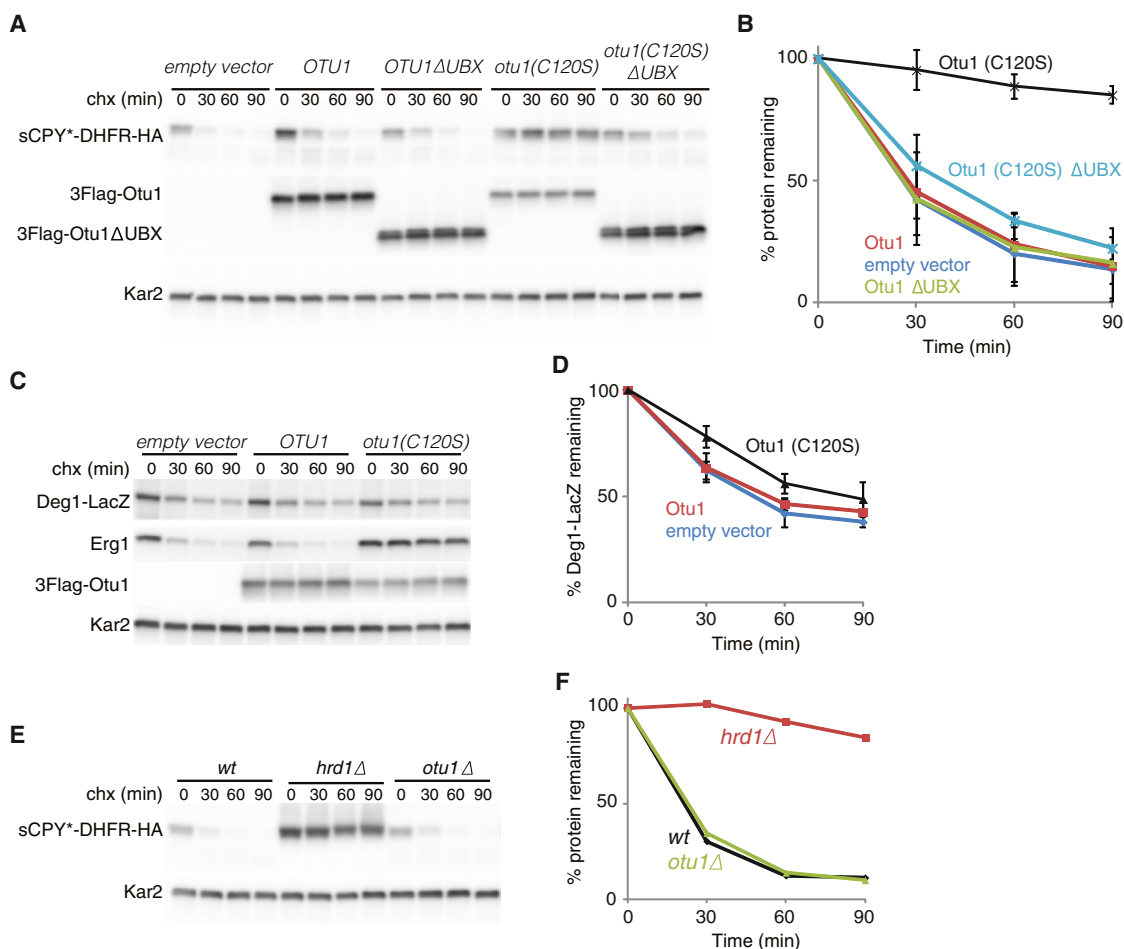
See also Figure S4.

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,

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

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
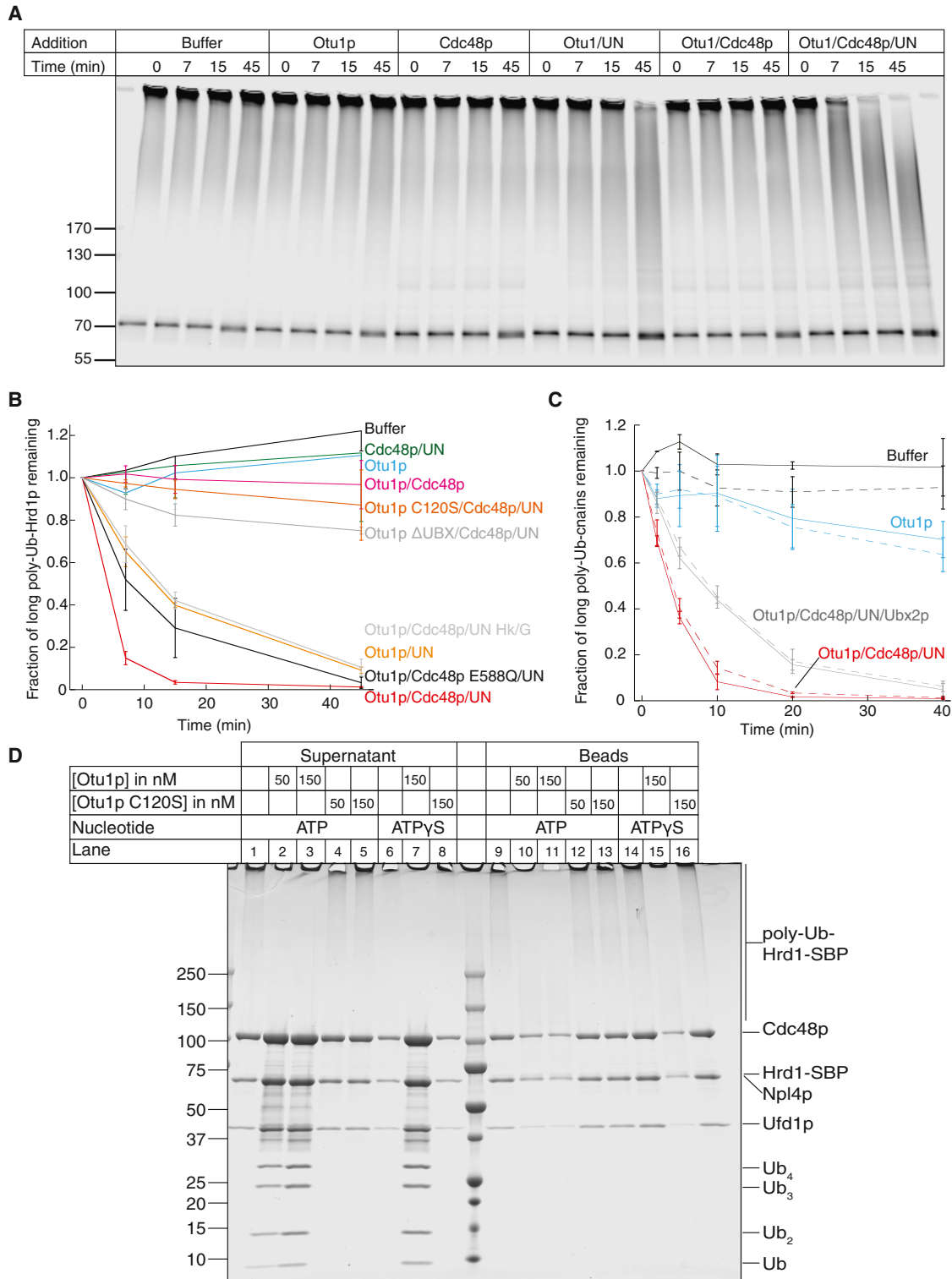
Figure 6. Cdc48p-Dependent In Vitro Deubiquitination by Otu1p

(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. Flota-

tion 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 polyubiquitinated 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.
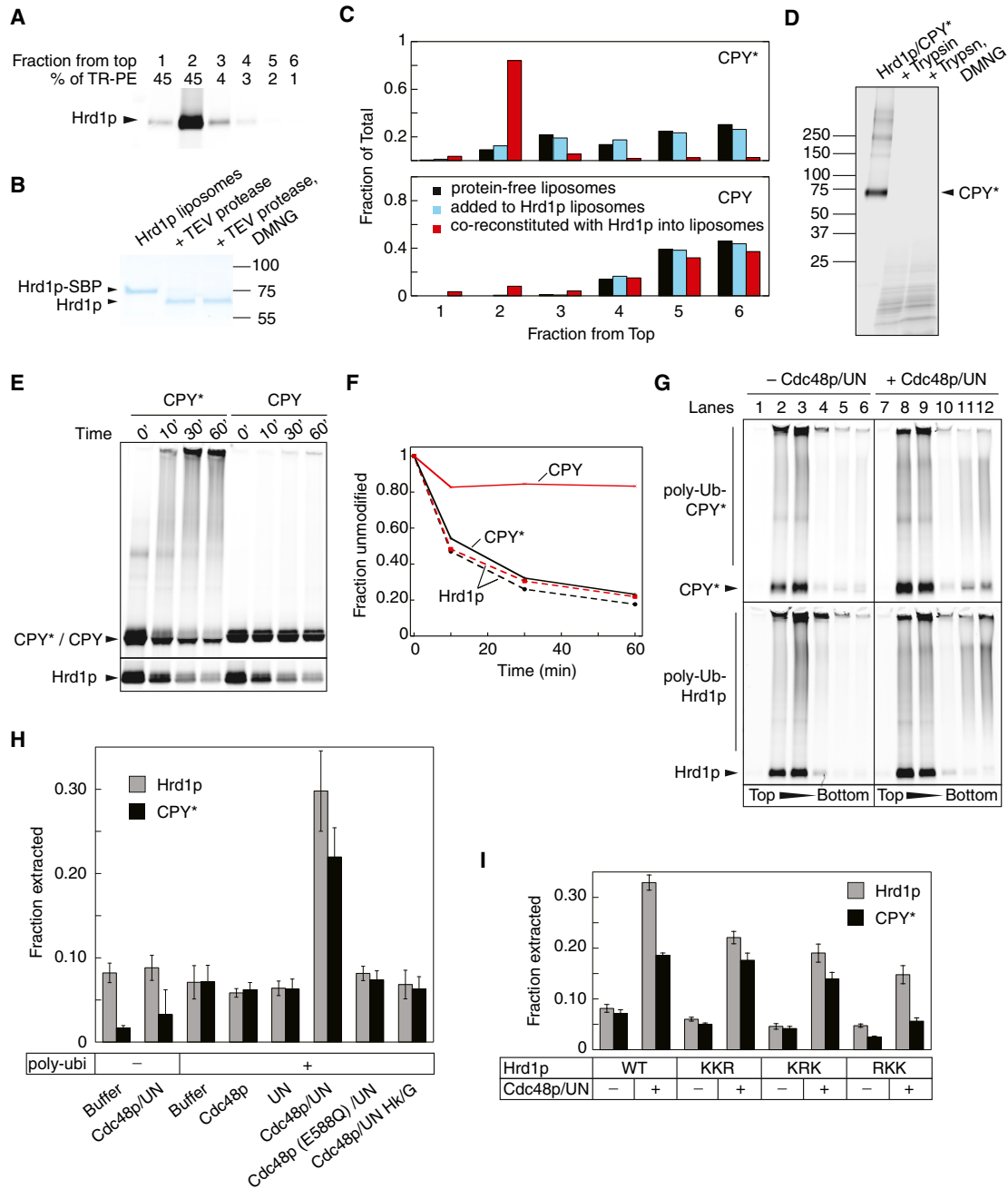
Figure 7. Membrane Extraction of Polyubiquitinated Proteins by Cdc48p

(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.

(legend continued on next page)

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 poly-ubiquitination, 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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