Endoplasmic Reticulum-associated Degradation of Pca1p, a Polytopic Protein, via Interaction with the Proteasome at the Membrane*

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Endoplasmic reticulum-associated degradation (ERAD) plays a critical role in the destruction of terminally misfolded proteins at the secretory pathway. The system also regulates expression levels of several proteins such as Pca1p, a cadmium exporter in yeast. To gain better insight into the mechanisms underlying ERAD of Pca1p and other polytopic proteins by the proteasome in the cytosol, our study determined the roles for the molecular factors of ERAD in dislodging Pca1p from the endoplasmic reticulum (ER). Inactivation of the 20S proteasome leads to accumulation of ubiquitinated Pca1p in the ER membrane, suggesting a role for the proteasome in extraction of Pca1p from the ER. Pca1p formed a complex with the proteasome at the membrane in a Doa10p E3 ligase-dependent manner. Cdc48p is required for recruiting the proteasome to Pca1p. Although the Ufd2p E4 ubiquitin chain extension enzyme is involved in efficient degradation of Pca1p, Ufd2p-deficient cells did not affect the formation of a complex between Pca1p and the proteasome. Two other polytopic membrane proteins undergoing ERAD, Ste6*p and Hmg2p, also displayed the same outcomes observed for Pca1p. However, poly-ubiquitinated Cpy1*p, a luminal ERAD substrate, was detected in the cytosol independent of proteasomal activities of the proteasome. These results indicate that extraction and degradation of polytopic membrane proteins at the ER is a coupled event. This mechanism would relieve the cost of exposed hydrophobic domains in the cytosol during ERAD.

Buildup of misfolded proteins in the endoplasmic reticulum (ER) induces the unfolded protein response to enhance folding capacity and reduce new protein synthesis (1–4). Terminally misfolded proteins at the secretory pathway are targeted to the ubiquitin-proteasome-dependent removal system, which is known as ER-associated degradation (ERAD) (3, 5–9). Accumulation of misfolded proteins or excess turnover is attributed to multiple diseases, such as cystic fibrosis, diabetes, and amyotrophic lateral sclerosis as well as Alzheimer and Parkinson diseases (3, 6, 7).

Several molecular factors involved in ERAD have been characterized (10–13). In the yeast Saccharomyces cerevisiae, misfolded ER luminal proteins and proteins carrying misfolding(s) at the transmembrane region(s) are ubiquitinated by the E3 ubiquitin ligase Hrd1p, whereas proteins carrying misfolding(s) at the cytosolic region(s) are ubiquitinated by Doa10p. Although these E3 enzymes display substrate specificity, some overlap has also been observed (14–17). In congress with the E3 ubiquitin ligases, several other components, such as E2 ubiquitin-conjugating enzymes, E4 ubiquitin extension enzymes, and molecular chaperones, work for recognition and direction of substrates to ubiquitin ligases and the proteasome (3, 13, 16). Because the proteasome is in the cytosol, ERAD substrates must be mobilized from the ER lumen or dislodged from the membrane to be destroyed (18). The Cdc48p AAA-ATPase (p97 in mammals) is thought to provide a primary driving force in the process (10, 14, 19). It has been proposed that translocation of luminal ERAD substrates to the cytosol may occur through a translocon (translocation channel) formed with several proteins, such as Sec61p (20–28), and E3 ubiquitin ligases possessing multitransmembrane domains (e.g. Hrd1p) (29–31). E4 ubiquitin chain extension enzymes (e.g. Ufd2p and Hul5p) facilitate ERAD through poly-ubiquitination (10, 32, 33).

Despite significant progress in the identification and characterization of molecular factors involved in ERAD, the mechanisms of how proteins in the ER are targeted to the cytoplasmic proteasome remained to be elucidated (3, 10, 32). Integral membrane proteins might be dislodged from the membrane and escorted to the proteasome for destruction (10), which requires the cells to extract proteins containing hydrophobic regions and maintain their solubility in the cytosol. Thus, direct loading of full-length substrates or fragmented pieces into the proteasome during extraction from the membrane could be a mechanism resolving the problem (32).

Our previous study showed that expression of the Pca1p cadmium efflux transporter in the yeast S. cerevisiae is dependent upon the ERAD pathway (34). In the absence of cadmium, Pca1p is rapidly turned over through a Doa10p- and proteasome-dependent mechanism. However, when cadmium is present, Pca1p escapes from ERAD and is secreted to the plasma membrane to export cadmium (34, 35). A degron at the N-terminal

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‡The abbreviations used are: ER, endoplasmic reticulum; ERAD, endoplasmic reticulum-associated degradation; IP, immunoprecipitation; DTBP, dimethyl 3,3’-ditiobispropionimidate; 3HA, triple HA; 2Myc, two c-myc; Ub, ubiquitin; S, supernatant; N, nonsoluble fraction; P, pellet.
cytosolic domain is responsible for the ERAD of Pca1p and also senses cadmium to rescue Pca1p from ERAD. Because of its rapid turnover ($t_{1/2} < 5$ min) (34), a degron- rather than misfolding-dependent ERAD, and control of this process by cadmium, Pca1p is a unique example among ERAD substrates. Moreover, the well-established experimental systems in yeast and conserved mechanisms for ERAD between yeast and mammals allow Pca1p to be a useful model substrate for gaining a better understanding of the mechanisms underlying ERAD of polytopic membrane proteins.

Here we characterized the roles for the molecular factors involved in dislodging Pca1p from the ER for ERAD. Subcellular location, physical interaction with the proteasome, and turnover rates of Pca1p were determined in yeast cells using genetic and biochemical approaches. Our data indicate that extraction and degradation of Pca1p and two other polytopic membrane proteins, Ste6*p and Hmg2p, are coordinated by complex formation with the proteasome while they reside at the ER membrane. The mechanism is likely significant for avoiding the release of membrane proteins to the cytosol.

**Experimental Procedures**

**Yeast Strains and Growth Conditions**—A BY4741 haploid *S. cerevisiae* strain (MATa his3Δ1, leu2Δ0, met15Δ0, and ura3Δ0) and null mutants of particular gene(s), including *hul5::KanMX6 (hul5Δ), ufd2::KanMX6 (ufd2Δ), rpn5::KanMX6 (rpn5Δ), and doa10::KanMX6 (doa10Δ),* were obtained from OpenBiosystems. A *pdr5::KanMX6 (pdr5Δ)* strain background was used for experiments in which cells were co-cultured with cycloheximide, MG132, or bortezomib. The strain background was used for experiments in which cells were co-cultured with cycloheximide, MG132, or bortezomib. Soluble and membrane-associated proteins were separated by centrifugation (4 °C, 100,000 g, 1 h).

To differentiate membrane association versus membrane integration of Pca1p, membrane fractions of *pdr5Δ, cdc48–3* (cultured at 37 °C), and *doa10Δ* cells expressing 3HA epitope-tagged Pca1p were resolubilized in PBS with and without Na$_2$CO$_3$ or Triton X-100. After incubation at 4 °C for 30 min, samples were fractionated by centrifugation (4 °C, 100,000 g, 30 min). Supernatant (S) was precipitated using TCA and denatured in SDS sample buffer. The unsoluble pellet (N) was denatured in SDS sample buffer. Both S and N samples were subjected to Western blotting analysis.

**In Vitro Deubiquitination**—The flotration sucrose gradient fractionation was performed as described previously (45). Yeast cells were broken using glass beads in lysis buffer (50 mM HEPES, 150 mM NaCl, 5 mM EDTA, 1 mM DTT, 1 mM PMSF, 50 mM protease inhibitor mixture). Sucrose gradient (0.25M, 1.5M, 1.7M (mixture of 200 μl of cell lysate and 600 μl of 2.3 M sucrose), and 2.3 M in descending order) was spun at 4 °C at 100,000 × g for 5 h. Twelve fractions (300 μl each) were then taken from the top.

**Flow Cytometry**—Cells were co-cultured with 20S proteasome inhibitor, MG132 (20 μM, 2 h). Cells were collected by washing twice in ice-cold PBS and resuspended in PBS containing MG132 and a membrane-permeable thiol-reversible cross-linker, dimethyl 3,3’-dithiobispropionimidate (DTBP) (100 μg/ml final concentration), for 30 min at room temperature with gentle rocking. Cells were then
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We first determined the subcellular distribution of GFP-Pca1p when proteolytic activities of the proteasome are inhibited by MG132 co-culture (39) or when Cdc48p AAA-ATPase is inactivated (Fig. 1B). Cdc48p is a known critical player in dislodging ERAD substrates from the ER (47). A strain possessing a temperature-sensitive CDC48 allele (cdc48–3) was used to inactivate this essential gene (37) at a restrictive temperature, 37 °C. Cells were precultured with cycloheximide to inhibit new Pca1p synthesis. Pca1p fused with GFP at the N terminus (GFP-Pca1p) is not detectable in WT control cells because of rapid turnover, as demonstrated previously (35). However, Pca1p is highly expressed when the proteasome or Cdc48p is inactivated (Fig. 1B). Co-localization of Pca1p-GFP with Sec63p, an ER-resident protein, indicates that Pca1p remains in the ER membrane under the experimental conditions. These results suggest that ubiquitinated Pca1p cannot be extracted from the ER membrane without proteolytic activities of the proteasome or upon inactivation of Cdc48p AAA-type ATPase.

We next ascertained the cellular levels of Pca1p and its fragments when the proteasome or Cdc48p is inactivated. Pca1p fused with 3HA and 2Myc (Fig. 1A) was expressed in WT, cdc48–3, and doa10Δ cells. Western blotting analyses of cell lysates using anti-HA or c-myc antibodies displayed signals at the locations corresponding to full-length Pca1p (Fig. 1C, arrowheads). Given that the half-life of Pca1p is less than 5 min (35), the majority of remaining Pca1p in WT cells co-cultured with cycloheximide should be ubiquitinated species. Pca1p fragments below full-length Pca1p, including ∼60-, 70-, 75-, and 90-kDa bands, were detected in WT, cdc48–3, and doa10Δ cells (Fig. 1C, top panel). We previously reported that Pca1p ubiquitination is absent in doa10Δ cells (34, 35). Therefore, fragmentation of Pca1p, observed in doa10Δ cells, is not likely to be a ubiquitination-dependent process. When probing with anti-myc antibodies, only full length was detectable, and all lower molecular weight fragments were nonspecific, as seen by the empty vector control (Fig. 1C, center panel, first lane). To determine the distribution of Pca1p, total cell lysates were fractionated by ultracentrifugation (100,000 × g for 30 min) to supernatant (S) and pellet (P) containing soluble proteins and membranes, respectively (Fig. 1D). Western blotting analysis detected full-length and higher molecular weight Pca1p species at the P fraction but not the S fraction (Fig. 1D). Immunoprecipitation of Pca1p from the S and P fractions with anti-HA antibodies followed by Western blotting using anti-HA and anti-ubiquitin (Ub) antibodies (Fig. 1E) further suggested that most ubiquitinated Pca1p species, except a portion of the ∼75-kDa fragment, reside in the membrane.

To rule out any off-target effect of MG132 on proteasome inhibition, we also conducted experiments using another proteasome inhibitor, bortezomib (40), and a strain expressing a temperature-sensitive allele of CIM3 (cim3–1) encoding a proteasome subunit (38). Fluorescent confocal microscopy of cells co-cultured with bortezomib displayed virtually identical results to those obtained using MG132. However, cells expressing CIM3–1 manifested Pca1p stabilization at the ER at both permissive (23 °C) and restrictive (37 °C) temperature (data not shown), which was confirmed by Western blotting analysis (Fig. 1F, fourth versus fifth lane). This indicates that Cim3–1p is not
fully functional at both 23 °C and 30 °C. Fractionation of soluble and membrane proteins followed by Western blotting of Pca1p also displayed similar results in bortezomib and MG132 co-cultures (Fig. 1G). Collectively, the data presented in Fig. 1 suggests that most of Pca1p and its fragments, except for an ~60-kDa fragment, are not dislodged from the membrane without the activities of the proteasome and Cdc48p.

**Pca1p in the Pellet Fraction Exists as Membrane-integrated Species**—We next considered the possibilities that Pca1p could be extracted out of the lipid bilayer but attached to the mem-

**FIGURE 1. Localization of Pca1p at the membrane of the cells in which the proteasome or Cdc48p is inactivated.** **A**, schematic of Pca1p. The black squares indicate eight transmembrane helices. GFP or 3HA is fused at the N terminus (filled oval), 2Myc is fused at the C terminus (empty oval). The empty square indicates the amino acid 250–350 region containing a degron. **B**, subcellular distribution of Pca1p determined by fluorescent microscopy. Expression plasmids of GFP-fused Pca1p and red fluorescent protein-fused Sec63p, an ER-resident protein, were co-expressed in a control strain lacking PDR5 (WT) and a strain containing a temperature-sensitive allele of CDC48 (cdc48−3). Mid-log phase cells were cultured with and without MG132 for 2 h. Cycloheximide was added to the medium 1 h before collecting cells. The cdc48−3 cells cultured at 23 °C were shifted to 37 °C for 30 min. Subcellular distribution of Pca1p-GFP was visualized by confocal fluorescent microscopy. **C**, detection of Pca1p and its fragments by immunoblotting. Pca1p fused with 3HA and 2Myc was expressed in a WT, a strain expressing the cdc48−3 allele, and a doa10 strain. WT and cdc48−3 cells were precultured with MG132 for 2 h and at 37 °C for 30 min, respectively. Total cell extracts prepared by glass bead disruption and Triton X-100 (1%) solubilization were subjected to Western blotting using anti-HA and anti-myc antibodies. The blots were also probed with anti-Pgk1 antibodies to determine equal loading. **D**, determination of the subcellular distribution of Pca1p. Lysates of the cells that were cultured as described in C were prepared by glass bead disruption, followed by removing unbroken cells (centrifugation at 300 × g for 10 min). The samples were separated to soluble and pellet fractions by centrifugation (100,000 × g for 30 min). The S and P fractions were solubilized (1% Triton X-100) and subjected to Western blotting analysis using anti-HA antibodies. **E**, detection of the majority of ubiquitinated Pca1p at the P fraction. Pca1p in the S and P fractions obtained from WT cells as described above was immunoprecipitated using anti-HA antibodies. The expression levels and ubiquitination status of Pca1p were determined by Western blotting using anti-HA and anti-ub antibodies, respectively. **F and G**, the experiments presented in C and D were conducted using bortezomib, a proteasome inhibitor, and a strain carrying a temperature-sensitive cim3−1 allele. The arrowheads (C–G), asterisks (C, F, and G), and arrows (C and F) indicate the location of full-length non-ubiquitinated Pca1p, nonspecific bands detected by anti-myc (C) or anti-HA (F) antibodies, and a Pca1p fragment dominant in proteasome-inactive WT cells relative to other cells, respectively. Each experiment was conducted three or more times using different clones, and representative data are presented.
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FIGURE 2. Detection of fragmented or ubiquitinated Pca1p by subcellular fractionation. A, the majority of Pca1p and its fragments are embedded in the membrane. Pca1p fused with triple HA at the N terminus was expressed in WT cells expressing 3HA-tagged Pca1p or Pca1p(degronΔ) using anti-HA antibodies. Pca1p was detected as a marker of membrane solubilization of cell lysate with Triton X-100 (1%, v/v). Immunoprecipitation of Pca1p. The formation of a complex containing one or more transmembrane helices (Fig. 1A) is expected to pull down Pca1p and its membrane-bound fragments into the Triton X-100-soluble fraction. Therefore, Triton X-100-soluble fractions were isolated as described in Fig. 1A, and these fractions were subjected to Western blotting analysis of collected fractions (Fig. 2B, first panel). Anti-HA antibodies detected primarily a non-ubiquitinated Pca1p fragment, are not dislodged from the membrane. It was further determined that poly-ubiquitinated full-length Pca1p species were not present in the soluble fractions because deubiquitination by the purified catalytic subunit of the deubiquitinating enzyme, USP2, did not lead to an enrichment of full-length Pca1p (Fig. 2C, second panel). Anti-HA antibodies detected primarily a fragment of ~60 kDa. Given the predicted N-terminal cytosolic domain (61 + ~3 kDa corresponding to tagged 3HA epitopes), the ubiquitinated Pca1p fragment detected in the soluble fraction could be a cleaved N-terminal cytosolic domain. However, a portion of the fragment was also detected in the membrane fraction as a peptide integrated into the membrane (Figs. 1, D, and G, and 2, A and B). Therefore, it is likely that the fragment contains one or more transmembrane helices (Fig. 1A) and/or domain(s) incorporated into the lipid bilayer. Collectively, these results indicate that, in the absence of proteasomal activities, Pca1p and most of its fragments, except for the ~60-kDa fragment, are not dislodged from the membrane.

Pca1p interacts with the proteasome at the ER Membrane in an E3 Ligase- and Degron-dependent Manner—Given the potential role for the proteasome in extraction of Pca1p from the ER, we next determined whether the proteasome forms a complex with Pca1p at the ER membrane. WT and doa10Δ cells expressing C-terminal two c-myc epitope-tagged wild-type control Pca1p and Pca1p lacking the N-terminal 392 amino acids that contain a degron (Pca1p(degronΔ)) were co-cultured with MG132 to inactivate the 20S proteasome. Membrane fractions were isolated as described in Fig. 1D, washed, and solubilized (Triton X-100, 1% (v/v)). Samples were then subjected to immunoprecipitation of Pca1p. The formation of a complex

branied or form large cytosolic complexes that could be pulled down to the pellet fraction. To address these concerns, the membrane fractions were incubated with Na2CO3 (0.2 M, pH 11) (48) to release peripheral membrane proteins to the supernatant (S, Fig. 2A). However, all Pca1p species, including Pca1p fragments that migrate faster than full-length Pca1p, remained in the non-soluble pellet (N) fractions of WT cells co-cultured with MG132 (Fig. 2A, left panel). Na2CO3 dissociates only a small portion (<5%) of full-length and fragmented Pca1p from the membrane (Fig. 2A, left panel, third lane). Only a small portion of Pca1p detected in the membrane fraction was disso-
between Pca1p and Rpn5, a proteasome subunit (49), was visualized by Western blotting. However, no detectable association was observed without cross-linker co-culture (Fig. 3A, lane 3). This could reflect a transient binding between Pca1p and the proteasome in the process of degradation. To address this concern, we conducted the experiment using a membrane-permeable thiol-reversible cross-linker, DTBP. After breaking the cross-links by denaturing samples in dithiothreitol-containing SDS sample buffer, the physical interactions of the proteasome subunits (39) with Pca1p were visualized by Western blotting.

Indeed, Pca1p was found to form a complex with Rpn5p, a 19S proteasomal subunit (Fig. 3, A, fourth lane, and C, second lane), and the α and β subunits of 20S the proteasome (Fig. 3D) at the membrane. Although the immunoprecipitation efficiency of Pca1p was over 90% (Fig. 3B, top panel, second versus fourth lane), most Rpn5p (>81%) still remained in the flow-through (Fig. 3B, center panel, second versus fourth lane). This suggests that Rpn5p is associated not only with Pca1p but also with other membrane proteins.

The complex formation between Pca1p and Rpn5p was dependent on Doa10p and the degron in Pca1p (Fig. 3C). Although the expression levels of Pca1p in doa10Δ cells and Pca1p lacking the degron (Pca1p(degronΔ)) were strikingly higher than Pca1p levels in wild-type control cells (Fig. 3C, third lane) (34, 35), immunoprecipitation of Pca1p and Pca1p(degronΔ) in doa10Δ and WT cells, respectively, did not pull down Rpn5p at detectable levels (Fig. 3C, second panel). These results confirm the specificity in complex formation between Pca1p and Rpn5p and indicate that, when Pca1p is ubiquitinated by Doa10p, the proteasome is recruited while Pca1p resides at the membrane.

Cdc48p Is Required for Complex Formation between Pca1p and the Proteasome—Cdc48p is a critical molecular factor for ERAD of Pca1p (Figs. 1, 2A, and 4A) and other proteins (10, 34, 37). The ATPase activities of Cdc48p are believed to contribute to the retrotranslocation of ubiquitinated luminal proteins for ERAD (37), and Cdc48p may escort them to the proteasome, as demonstrated by a physical interaction between Cdc48p and the proteasome (37). To determine the role of Cdc48p in the ERAD of Pca1p in conjunction with the interaction between Pca1p and the proteasome, we ascertained whether the proteasome forms a complex with Pca1p in cells expressing inactive Cdc48p. The levels of ubiquitinated proteins in cells expressing non-functional Cdc48p (37 °C culture) are comparable (1.26 ± 0.62-fold, n = 3, p = 0.53) with those of control (23 °C culture) cells (Fig. 4A, first panel). Despite higher Pca1p expression in Cdc48p-defective cells (Fig. 4A, second panel), immunoprecipitation of Pca1p followed by Rpn5p detection revealed that formation of a complex between Pca1p and the proteasome is dramatically reduced in cells expressing non-functional Cdc48p (Fig. 4B). This result suggests that Cdc48p recognizes ubiquitinated Pca1p for recruitment to the proteasome.

It was known that Cdc48p also interacts with other molecular factors involved in ERAD, such as the E4 ubiquitin chain extension enzyme (50). The observed defect of Pca1p ERAD (Fig. 1B) and the lack of Pca1p interaction with the proteasome...
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**FIGURE 4.** Cdc48p plays a role in Pca1p complex formation with the proteasome but not its poly-ubiquitination. A, no significant change of protein poly-ubiquitination in cells expressing non-functional Cdc48p. Pca1p tagged with the triple HA epitope was expressed in a strain carrying temperature-sensitive cdc48Δ3. Cells at mid-log phase were cultured at a permissive temperature (23 °C) or shifted to a restrictive temperature (37 °C) for 30 min with a cell-permeable and reversible cross-linker (DTBP, 100 μg/ml) and MG132 (20 μM), a proteasome inhibitor, for 2 h. Membrane fractions were solubilized with Triton X-100 (1%). The expression levels of Pca1p and Rpn5p and the overall ubiquitination status were determined by Western blotting. B, Cdc48p-dependent physical association between Pca1p and Rpn5p, a subunit of the 19S regulatory particle, was determined by co-immunoprecipitation. The Triton X-100 (1%)-solubilized membrane fraction was used for immunoprecipitation of Pca1p using anti-HA antibodies. Co-immunoprecipitation of Rpn5p, a subunit of the 19S regulatory particle, was detected by Western blotting. C, poly-ubiquitination of Pca1p as a function of Cdc48p activities. To determine the ubiquitination status of Pca1p, the samples obtained as described in B were subjected to Western blotting using anti-ubiquitin antibodies. To compare the relative ubiquitination of Pca1p, a 5-fold amount of immunoprecipitated Pca1p from cells cultured at 23 °C relative to those at 37 °C was loaded. Data were confirmed by triplicate experiments.

(Fig. 4B) in cells expressing non-functional Cdc48p may be attributed to a deficiency in recruiting E4 ligase(s) and/or presenting monoubiquitinated Pca1p for poly-ubiquitination. To address this, we determined the ubiquitination status of Pca1p in Cdc48p-inactive cells. Immunoprecipitation of Pca1p using anti-HA antibodies followed by Western blotting analysis of Pca1p using anti-ubiquitin antibodies determined poly-ubiquitination of Pca1p in cells expressing non-functional Cdc48p (Fig. 4C). Given that Pca1p levels in cells expressing non-functional Cdc48p are 5-fold higher relative to those in control cells (Fig. 4, A and B), for the experiment presented in Fig. 4C, we loaded a 5-fold higher immunoprecipitated sample obtained from cells cultured at 23 °C. Our data showed that cells expressing non-functional Cdc48p accumulate poly-ubiquitinated Pca1p comparable with control cells (Fig. 4C). Therefore, the higher Pca1p expression (Fig. 4A, second panel) and the reduced association of Pca1p with the proteasome (Fig. 4B) in Cdc48p-inactive cells are not likely attributable to a defect in poly-ubiquitination of Pca1p.

**FIGURE 5.** Formation of a complex between Pca1p and the proteasome is independent of poly-ubiquitination of Pca1p. A, significant reduction of turnover rate of Pca1p in ufd2Δ gene knockout (ufd2ΔΔ) cells. Pca1p tagged with the two c-myc epitope at the C terminus was expressed in WT, ufd2ΔΔ, and hul5ΔΔ strains. Cycloheximide (CHX) chase and Western blotting determined Pca1p levels. Protein extracts were prepared by glass bead disruption and detergent (1% Triton X-100) solubilization. Pgk1p was probed to determine equal loading. B, the Pca1p levels presented in A were quantitated (n = 4). Pca1p levels relative to those at time 0 are presented. *, p < 0.01. C, defect in poly-ubiquitination of Pca1p in an ufd2ΔΔ strain. The Pca1p-tagged c-myc epitope was expressed in an ufd2ΔΔ strain. Total cell lysates were obtained with and without co-culture with MG132 (20 μM, 2 h). Samples were subjected to separation to soluble and pellet fractions by centrifugation (100,000 × g for 30 min). After solubilization with Triton X-100 (1%), the S and P fractions were subjected to immunoprecipitation using anti-myc antibodies, followed by Western blotting using anti-UB and anti-myc antibodies to detect Pca1p. D, distribution of Pca1p species in the S and P fractions in ufd2ΔΔ cells. Total cell lysates (T) and the soluble and pellet fractions described in C were subjected to Western blotting using anti-myc and -Pgk1p antibodies. E, formation of a complex between Pca1p and Rpn5p, a subunit of the 19S proteasome. After co-culturing the cells with a permeable and reversible cross-linker (DTBP, 100 μg/ml) and MG132, an inhibitor of proteasomal proteolytic activities, cell lysates were obtained by glass bead disruption and detergent solubilization. The samples were subjected to immunoprecipitation using anti-myc antibodies. Rpn5p in the samples was detected by Western blotting using anti-Rpn5p antibodies. The arrowheads (C and D) indicate the location where full-length Pca1p migrates. The experiment (B–D) was conducted twice using two independent clones, and representative data are presented.

Ufd2p, an E4 Ubiquitin Extension Enzyme for Pca1p, and Poly-ubiquitination of Pca1p Did Not Affect Complex Formation between Pca1p and the Proteasome—Following ubiquitination by E3 ligase(s), E4 ubiquitin chain extension enzymes such as Ufd2p and Hul5p in yeast have been implicated in the degradation of several ERAD substrates (10, 32, 50, 51). Ufd2p interacts with Cdc48p and Rad23p in the process of substrate delivery to the proteasome (28). It is intriguing that Ufd2p regulates turnover of only a subset of Doa10p substrates (33), and Hul5p has been found to be associated with the 19S proteasome (32, 52, 53). We determined the t1/2, poly-ubiquitination of Pca1p, and formation of a complex between Pca1p and the proteasome in ufd2ΔΔ and hul5ΔΔ strains. Cycloheximide chase of Pca1p revealed no significant difference in Pca1p degradation in WT and hul5ΔΔ cells; however, UFD2 deletion dramatically extended the t1/2 of Pca1p (Fig. 5, A and B). This result suggests that Ufd2p is required for efficient turnover of Pca1p. Immunoprecipitation of Pca1p followed by immunoblotting using anti-ubiquitin antibodies supported that Ufd2p is the major E4
enzyme of Pca1p (Fig. 5C). In ufd2Δ cells, most Pca1p species were detected at the membrane fraction but not at the soluble fraction (Fig. 5D), indicating that, in ufd2Δ cells, the majority of Pca1p remains in the membrane.

Because Ufd2p is required for efficient degradation of Pca1p (Fig. 5B), we next sought to determine whether poly-ubiquitination by Ufd2p is necessary for the interaction of Pca1p with the proteasome. In vivo cross-linking and co-immunoprecipitation followed by Western blotting analysis showed that Pca1p is still able to interact with the proteasome in the absence of Ufd2p (Fig. 5E). This indicates that Ufd2p-dependent ubiquitin chain extension (Fig. 5C) is not critical for the formation of a complex between Pca1p and the proteasome. This result also further confirmed that the reduced interaction of Pca1p with the proteasome in cells expressing nonfunctional Cdc48p (Fig. 4, B and C) is not relevant to the poly-ubiquitination status of Pca1p.

Proteasome-dependent Extraction of ERAD Substrates Is Specific for Polytopic Proteins—We next determined whether other polytopic membrane proteins undergoing ERAD manifest an interaction with the proteasome at the membrane as observed for Pca1p. Ste6p is a mutated form of the a factor transporter Ste6p in yeast (54), which causes a premature stop codon and a change in N-glycosylation. Ste6p is also targeted for degradation via Doa10p, Cdc48p, and a proteasome-dependent pathway (10, 54, 55). Subcellular fractionation and flotation sucrose gradient fractionation showed that Ste6p remained in the membrane fraction under proteasome inhibition conditions (data not shown), as observed for Pca1p. The same experiment described in Fig. 3A showed that Ste6p pulled down a proteasome subunit, Rpn5p, in a Doa10p-dependent manner (Fig. 6A). Therefore, Ste6p also interacts with the proteasome at the ER membrane when it is ubiquitinated by Doa10p.

The expression of Hmg2p, an ER-resident membrane enzyme required for cholesterol synthesis, is controlled through the ERAD-M pathway. When the proteasome is inactivated by MG132 co-culture, Hmg2p also forms a complex with the proteasome at the membrane and not in the cytosol (Fig. 6B). Therefore, as observed for Pca1p and Ste6p, inhibition of the proteolytic function of the proteasome prevents Hmg2p release from the ER membrane.

Vaccular carboxypeptidase Y (Cpy1p) containing a G255R mutation (Cpy1* p) is degraded through the ERAD-C pathway (13.56–59). We have employed Cpy1p as an example of an ER luminal protein to elaborate the hypothesis that soluble ERAD substrates may be retrotranslocated prior to being targeted to the proteasome. When WT yeast cells expressing c-myc epitope-tagged Cpy1p were co-cultured with MG132, a proteasome inhibitor, Cpy1p was detected as full-length and poly-ubiquitinated forms in the soluble fraction (Fig. 6C). Rpn5p, a subunit of the 19S proteasome, was co-immunoprecipitated with Cpy1p in the soluble fraction (Fig. 6C, second panel), indicating that Cpy1p forms a complex with the proteasome in the cytosol. However, Cpy1p in cells lacking Hrd1p E3 ligase targeting Cpy1p for ERAD remains at the pellet fraction along with Kar2p, an ER-luminal protein (Fig. 6D). Therefore, detection of Cpy1p in the soluble fraction (Fig. 6C) is a consequence of ERAD progress of Cpy1p rather than its leaking from the ER during sample preparation followed by interaction with the proteasome. These results suggest that Cpy1p ubiquitinated by Hrd1p is retrotranslocated to the cytosol without proteolytic activities of the proteasome. This is distinct from other examined polytopic proteins (Pca1p, Ste6p, and Hmg2p).

Discussion

ERAD involves retrotranslocation of ER luminal proteins and dislodging of membrane proteins. Our data suggest that polytopic ERAD substrates undergo proteasomal degradation at the ER membrane via recruiting the proteasome rather than being extracted to the cytosol prior to delivery to the proteasome. This is distinct from ERAD of Cpy1p, a soluble protein in the lumen of the ER. The coupling of dislodging and destruction of membrane proteins would be advantageous for cells by minimizing the energy cost associated with preventing detrimental effects of hydrophobic proteins in the cytosol. These results shed new light on this vital but undercharacterized process.
Cdc48p (p97/VCP in higher eukaryotes), a hexameric AAA-type ATPase, plays diverse roles in ERAD (60, 61), although the mechanistic details remain to be defined. Despite experimental evidence arguing for a non-essential role of Cdc48p in the ERAD process (62), our study confirmed that Cdc48p is a significant player for ERAD of Pca1p by promoting its binding to the proteasome. In cells expressing inactive Cdc48p, the complex formation between the proteasome and Pca1p and Pca1p degradation is impaired. This result is consistent with a previous report displaying cooperation between Cdc48p and the proteasome in turnover of Insig-1p, an ERAD-M substrate (63).

Several lines of evidence support that Cdc48p and its cofactors Ufd2p and Npl4p are recruited to the ER via Ubx2p to disassemble ubiquitin-conjugated substrates from the membrane and for the delivery of those to the proteasome (60, 61). Therefore, it is likely that Cdc48p facilitates the interaction between ubiquitinated Pca1p and the proteasome.

Distinct from the ERAD-L pathway dealing with soluble substrates, a concern in ERAD of integral membrane proteins (ERAD-M and -C pathways) would be the extraction of proteins from the lipid bilayer and solubilization at the cytosol (10, 32). The results reported previously were not consistent with each other. An in vitro reconstitution assay found that, prior to degradation, Ste6p, a well characterized ERAD-C substrate, is dislodged from the membrane (10). However, it is worth pointing out that only a small fraction of Ste6p was released from the membrane under the experimental conditions. Secondly, an in vitro ERAD assay of cystic fibrosis transmembrane conductance regulator (CFTR) displayed relatively minor effects of proteasome inactivation on extraction and release of its degradation intermediates from the ER membrane (65).

Our study was conducted in cells, and most ubiquitinated Pca1p and its fragments remained in the membrane and formed a complex with the proteasome. This finding was confirmed with two other polytopic proteins. Therefore, our data support the hypothesis that dislodging of membrane proteins does not occur prior to their delivery to the proteasome.

Detection of the complex containing Pca1p and subunits of the 19S and 20S proteasomes from the membrane fractions is dependent on Doa10p, the major E3 ubiquitin ligase of Pca1p. This suggests that ubiquitinated Pca1p attracts the proteasome while residing in the ER membrane. The proteasomes are localized mainly in the nucleus and cytosol as free or nuclear envelope- and ER network-attached forms (66–69). The clustering of the proteasomes on the ER membrane is particularly high in the yeast S. cerevisiae (80% total proteasome) relative to mammals (<20%) (67). The proteasome might be associated with the ER via physical interaction with ER membrane protein(s), which is reminiscent of Cue1 and Ubx2p recruiting Ubc6p/7p and Cdc48p to the ER membrane, respectively (71–73). This may allow for rapid recognition of ERAD substrates by the proteasome. Sts1p is critical for enriching the proteasome at the nuclear envelope (70).

However, the counterpart at the ER membrane remains to be identified. Alternatively, the enrichment of the proteasome at the ER membrane might reflect the complexes between the proteasome and proteins undergoing ERAD. High demand of ERAD might control the distribution of the proteasomes for an efficient completion of the task. The 26S, 20S, and 19S proteasomal particles are known to exist in a dynamic equilibrium (68). The assembly, subunit composition, posttranslational modifications of amino acid residues, and interaction of the proteasome with regulators(s), if any, could be changed in response to cellular cues and stresses (66). It would be interesting to examine whether ER stresses would lead to an enrichment of the proteasomes to the ER membrane. This could be better accessed in mammals displaying relatively minor steady-state ER localization of the proteasome (74, 75).

The significance of poly-ubiquitination of membrane protein degradation is impaired. This result is consistent with a previous report displaying cooperation between Cdc48p and the proteasome. Sts1p is critical for enriching the proteasome at the ER membrane. The recruitment of the proteasome to ubiquitinated proteins while they reside in the membrane could be a conserved mechanism in ERAD of higher eukaryotes. It would be also interesting to define whether the proteasome-dependent destruction of membrane proteins in other organelles occurs in a similar manner.

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References

1. Ellgaard, L., Molinari M., and Helenius, A. (1999) Setting the standards: quality control in the secretory pathway. Science 286, 1882–1888
2. 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
3. Vembar, S. S., and Brodsky, J. L. (2008) One step at a time: endoplasmic reticulum-associated degradation. Nat. Rev. Mol. Cell Biol. 9, 944–957
4. Casagrande, R., Stern P., Diehn, M., Shamu, C., Osario, M., Zúñiga, M., Brown, P. O., and Ploegh, H. (2000) Degradation of proteins from the ER of S. cerevisiae requires an intact unfolded protein response pathway. Mol. Cell 5, 729–735
5. Brodsky, J. L., and Skach, W. R. (2011) Protein folding and quality control in the endoplasmic reticulum: recent lessons from yeast and mammalian cell systems. Curr. Opin. Cell Biol. 23, 464–475
6. Smith, M. H., Ploegh, H. L., and Weissman, J. S. (2011) Road to ruin: translation and degradation of misfolded proteins. J. Cell Biol. 193, 729–735
7. Mehnert, M., Sommer, T., and Jarosch, E. (2010) ERAD ubiquitin ligases: multifunctional tools for protein quality control and waste disposal in the endoplasmic reticulum. BioEssays 32, 905–913
8. Lippincott-Schwartz, J., Bonifacino, J. S., Yuan, L. C., and Klausner, R. D. (1988) Degradation from the endoplasmic reticulum: disposing of newly synthesized proteins. Cell 54, 209–220
9. Heinemeyer W., Klein schmidt, J. A., Saidowski, J., Escher, C., and Wolf, D. H. (1991) Proteinase yscE, the yeast proteasome/multicatalytic-multi-functional protease: mutants unravel its function in stress induced proteolysis and uncover its necessity for cell survival. EMBO J. 10, 555–562
10. Nakatsu kasa, K., Huyer, G., Michaelis, S., and Brodsky, J. L. (2008) Dissecting the ER-associated degradation of a misfolded polytopic membrane protein. Cell 132, 101–112
11. Sommer, T., and Jentsch, S. (1993) A protein translocation defect linked to ubiquitin conjugation at the endoplasmic reticulum. Nature 365, 176–179
12. Werner, E. D., Brodsky, J. L., and McCracken, A. A. (1996) Proteasome-dependent endoplasmic reticulum-associated protein degradation: an unconventional route to a familiar fate. Proc. Natl. Acad. Sci. U.S.A. 93, 13797–13801
13. Hiller, M. M., Finger, A., Schweiger, M., and Wolf, D. H. (1996) ER degradation of a misfolded luminal protein by the cytosolic ubiquitin–proteasome pathway. Science 273, 1725–1728
14. Carvalho, P., Gorder, V., and Rapoport, T. A. (2006) Distinct ubiquitin-ligase complexes define convergent pathways for the degradation of ER proteins. Cell 126, 361–373
15. Kret, S. G., Wang, L., and Hochstrasser, M. (2006) Membrane topology of the yeast endoplasmic reticulum–localized ubiquitin ligase Dua10 and comparison with its human ortholog TEB4 (MARCH-VI). J. Biol. Chem. 281, 4646–4653
16. Kato, J., Gilstring, C. F., and Ljungdahl, P. O. (2007) Membrane chaperone Sec61 in folding amino acid permeases preventing precocious dislocation of a misfolded secretory protein from the endoplasmic reticulum to the proteasome for destruction. Nature 384, 832–838
17. Ye, Y., Shibata, Y., Yun, C., Ron, D., and Rapoport, T. A. (2004) A membrane protein complex mediates retro-translocation from the ER lumen into the cytosol. Nature 429, 841–847
18. Devon, R. J., Sanders, S. L., Feldheim, D. A., and Schekman, R. (1991) Assembly of yeast Sec proteins involved in translocation into the endoplasmic reticulum into a membrane-bound multisubunit complex. Nature 349, 806–808
19. Schulze, A., Standera, S., Buergel, E., Kikker, M., van Voorden, S., Wiertz, E., Koning, F., Kloe tzel, P. M., and Seeger, M. (2005) The ubiquitin-domain protein HERP forms a complex with components of the endoplasmic reticulum associated degradation pathway. J. Mol. Biol. 354, 1021–1027
20. Pilone, M., Schekman, R., and Römisch, K. (1997) Sec61p mediates export of a misfolded secretory protein from the endoplasmic reticulum to the cytosol for degradation. EMBO J. 16, 4540–4548
21. M. Scott, D. C., and Schekman, R. (2008) Role of Sec61p in the ER-associated degradation of short-lived transmembrane proteins. J. Cell Biol. 181, 1095–1105
22. Pilone, R. K., and Wolf, D. H. (1999) Retrograde protein translocation: ERADication of secretory proteins in health and disease. Trends Biochem. Sci. 24, 266–270
23. 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
24. Stein, A., Ruggiano, A., Carvalho, P., and Rapoport, T. A. (2014) Key steps in ERAD of luminal ER proteins reconstituted with purified components. Cell 158, 1375–1388
25. Pilone, R. K., Bordallo, D., Deak, P., M., Taxis, C., Hirt, R., and Wolf, D. H. (1999) Genetic interactions of Hrd3p and Der3p with Sec61p suggest a retro-translocation complex mediating protein transport for ER degradation. J. Cell Sci. 112, 4123–4134
26. Kohlmann, S., Schaifer, A., and Wolf, D. H. (2008) Ubiquitin ligase Hul5 is required for fragment-specific substrate degradation in endoplasmic reticulum-associated degradation. J. Biol. Chem. 283, 16374–16383
27. Liu, C., van Dyk, D., Xu, P., Choe, Y., Pan, H., Peng, J., Andrews, B., and Rao, H. (2010) Ubiquitin chain elongation enzyme UBA2 regulates a subset of Doa10 substrates. J. Biol. Chem. 285, 10265–10272
28. Adle, D. J., Wei, W., Smith, N., Bies, I. J., and Lee, J. (2009) Cadmium-mediated rescue from ER-associated degradation induces expression of its exporter. Proc. Natl. Acad. Sci. U.S.A. 106, 10189–10194
29. Adle, D. J., and Lee, J. (2008) Expressional control of a cadmium-transporting P1B-type ATPase by a metal sensing degradation signal. J. Biol. Chem. 283, 31460–31468
30. Longtine, M. S., McKenzie, A., 3rd, Demarini, D. J., Shah, N. G., Wach, A., Brachat, A., Philippsen, P., and Pringle, J. R. (1998) Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. Yeast 14, 953–961
31. Ye, Y., Meyer, H. H., and Rapoport, T. A. (2001) The AAA ATPase Cdc48/p97 and its partner transport proteins from the ER into the cytosol. Nature 414, 652–656
32. Ghislain, M., Udvardy, A., Mann C. (1993) S. cerevisiae 26S protease mutants arrest cell division in G2/metaphase. Nature 366, 358–362
33. Bogoy, M., McMaster, J. S., Gaczynska, M., Tortorella, D., Goldberg, A. L., and Ploegh, H. (1997) Covalent modification of the activitee threnine of proteasomal $p$ subunits and the Escherichia coli homolog HslV by a new class of inhibitors. Proc. Natl. Acad. Sci. U.S.A. 94, 6629–6634
34. Fleming, J. A., Lightcap, E. S., Sadis, S., Thoroddsen, V., Bulawa, C. E., Blackman, R. K. (2002) Complementary whole-genome technologies reveal the cellular response to proteasome inhibition by PS-341. Proc. Natl. Acad. Sci. U.S.A. 99, 1461–1466
35. Mumberg, D., Muller, R., and Funk, M. (1995) Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. Gene 156, 119–122
36. Overton, M. C., and Blumer, K. J. (2002) The extracellular N-terminal domain and transmembrane domains I and 2 mediate oligomerization of proteasome recruitment to the endoplasmic reticulum.
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A yeast G protein-coupled receptor. J. Biol. Chem. 277, 41463–41472

43. Hampton, R. Y., and Bhakta, H. (1997) Ubiquitin-mediated regulation of 3-hydroxy-3-methylglutaryl-CoA reductase. Proc. Natl. Acad. Sci. U.S.A. 94, 12944–12948

44. Gretz, R. D., Schiestl, R. H., Willems, A. R., and Woods, R. A. (1995) Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. Yeast 11, 355–360

45. Metzger, M. B., Maurer, M. J., Dancy, B. M., and Michaelis, S. (2008) Degradation of a cytosolic protein requires endoplasmic reticulum-associated degradation machinery. J. Biol. Chem. 283, 32302–32316

46. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1987) Curr. Protoc. Mol. Biol., Greene Publishing Associates/Wiley Interscience, New York

47. Ye, Y., Meyer H. H., and Rapoport, T. A. (2003) Role of the p97-Ufd1-Npl4 complex in retrotranslocation from the ER to the cytosol: dual recognition of nonubiquitinated polypeptide segments and polyubiquitin chains. J. Cell Biol. 162, 71–84

48. Tandy, S., Williams, M., Leggett, A., Lopez-Jimenez, M., Dedes, M., Ramesh, B., Srai, S. K., and Sharp, P. (2000) Nramp2 expression is associated with pH-dependent iron uptake across the apical membrane of human intestinal Caco-2 cells. J. Biol. Chem. 275, 1023–1029

49. Finley, D., Tanaka, K., Mann, C., Feldmann, H., Hochstrasser, M., Vierstra, R., Johnston, S., Hampton, R., Haber, J., McCusker, J., Silver, P., Frontali, L., Thorsness, P., Varshavsky, A., Byers, B., et al. (1998) Unified nomenclature for subunits of the Saccharomyces cerevisiae proteasome regulatory particle. Trends Biochem. Sci. 23, 244–245

50. Koegl, M., Hoppe, T., Schlenker, S., Ulrich, H. D., Mayer, T. U., and Jenatsch, S. (1999) A novel ubiquitination factor, E4, is involved in multiquitin chain assembly. Cell 96, 635–644

51. Aviram, S., and Kormitzer, D. (2010) The ubiquitin ligase Hul5 promotes proteasomal processivity. Mol. Cell. Biol. 30, 985–994

52. Fang, N. N., Ng, A. H., Measday, V., and Mayor, T. (2011) Hul5 HECT ubiquitin ligase plays a major role in the ubiquitylation and turnover of cytosolic misfolded proteins. Nat. Cell Biol. 13, 1344–1352

53. Cossar, B., Hanna, J., Kirkpatrick, D. S., Zhang, D. P., Tone, Y., Hathaway, N. A., Buecker, C., Leggett, D. S., Schmidt, M., King, R. W., Giygi, S. P., and Finley, D. (2006) Ubiquitin chains are remodeled at the proteasome by opposing ubiquitin ligase and deubiquitinating activities. Cell 127, 1401–1413

54. Huyer, G., Piluik, W. F., Fansiler, 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

55. Huyer, G., Longsworth, G. L., Mason, D. L., Mallampalli, M. P., McCaffery, J. M., Wright, R. L., and Michaelis, S. (2004) A striking quality control subcompartment in Saccharomyces cerevisiae: the endoplasmic reticulum-associated compartment. Mol. Biol. Cell. 15, 908–921

56. Wolf, D. H., and Schäfer, A. (2005) CPY* and the power of yeast genetics in the elucidation of quality control and associated protein degradation of the endoplasmic reticulum. Curr. Top. Microbiol. Immunol. 300, 41–56

57. 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

58. Kostova, Z., and Wolf, D. H. (2005) Importance of carbohydrate positioning in the recognition of mutated CPY for ER-associated degradation. J. Cell Sci. 118, 1485–1492

59. Spear, E. D., and Ng, D. T. (2005) Single context-specific glycans can target misfolded glycoproteins for ER-associated degradation. J. Cell Biol. 169, 73–82

60. Meyer, H., Bug, M., and Bremer, S. (2012) Emerging functions of the VCP/p97 AAA-ATPase in the ubiquitin system. Nat. Cell Biol. 14, 117–123

61. Wolf, D. H., and Stolz, A. (2012) The Cdc48 machine in endoplasmic reticulum associated protein degradation. Biochim. Biophys. Acta. 1823, 117–124

62. Carlson, E. J., Pitonzo, D., and Skach, W. R. (2006) p97 functions as an auxiliary factor to facilitate TM domain extraction during CFTR ER-associated degradation. EMBO J. 25, 4557–4566

63. Ikeda, Y., Demartino, G. N., Brown, M. S., Lee, J. N., Goldberg, J. L., and Ye, J. (2009) Regulated endoplasmic reticulum-associated degradation of a polytopic protein: p97 recruits proteasomes to Insig-1 before extraction from membranes. J. Biol. Chem. 284, 34899–34900

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

65. Oberdorf, J., Carlson, E. J., and Skach, W. R. (2006) Uncoupling proteasome peptide and ATPase activities results in cytosolic release of an ER polytopic protein. J. Cell Sci. 119, 303–313

66. Rivett, A. J. (1998) Intracellular distribution of proteasomes. Curr. Opin. Immunol. 10, 110–114

67. Enenkel, C., Lehmann, A., and Kloetzel, P. M. (1998) Subcellular distribution of proteasomes implicates a major location of protein degradation in the nuclear envelope-ER network in yeast. EMBO J. 17, 6144–6154

68. Peters, J. M., Franke, W. W., and Kleinschmidt, J. A. (1994) Distinct 19S and 20S subcomplexes of the 26S proteasome and their distribution in the nucleus and the cytoplasm. J. Biol. Chem. 269, 7709–7718

69. Takeda, K., and Yanagida, M. (2005) Regulation of nuclear proteasome by Rhp6/Ubc2 through ubiquitination and destruction of the sensor and anchor Cut8. Cell 122, 393–405

70. Chen, L., Romero, L., Chuang, S. M., Tournier, V., Joshi, K. K., Lee, J. A., Kovalvi, G., and Madura, K. (2011) Sts1 plays a key role in targeting proteasomes to the nucleus. J. Biol. Chem. 286, 3104–3118

71. Biederer, T., Volkwein, C., and Sommer, T. (1997) Role of Cuel1p in ubiquitination and degradation at the ER surface. Science 278, 1806–1809

72. Schubert, 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

73. 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

74. Rivett, A. J., Palmer, A. J., and Knecht, E. (1992) Electron microscopic localization of the multicatalytic protease complex in rat liver and in cultured cells. J. Histochem. Cytochem. 40, 1165–1172

75. Palmer, A., Rivett, A. J., Thomson, S., Hendil, K. B., Butcher, G. W., Fuertes, G., and Knecht, E. (1996) Subpopulations of proteasomes in rat liver nuclei, microsomes and cytosol. Biochem. J. 316, 401–407

76. Fang, N. N., and Mayor, T. (2012) Hul5 ubiquitin ligase: good riddance to bad proteins. Proc. Natl. Acad. Sci. U.S.A. 109, 240–244