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 of Pca1p, a Polytopic Protein, via Interaction with the Proteasome at the Membrane

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ABSTRACT

Endoplasmic reticulum-associated degradation (ERAD) plays a critical role for 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 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. While Ufd2p E4 ubiquitin chain extension enzyme is involved in efficient degradation of Pca1p, Ufd2p-deficient cells did not affect 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 proteolytic 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 (UPR) 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’s and Parkinson’s 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 ubiquitinylated by Doa10p. While these E3 enzymes display substrate specificity, some overlap also has 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). As 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 multi-transmembrane domains (e.g., Hrd1p) (29-31). E4 ubiquitin chain extension enzymes (e.g., Ufd2p, Hul5p) facilitate ERAD through poly-ubiquitinylation (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 the extraction from the membrane could be a mechanism resolving the problem (32).

Our previous study showed that expression of 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 cytosolic domain is responsible for the ERAD of Pca1p and also senses cadmium to rescue Pca1p from ERAD. Because of its rapid turnover ($T_{1/2} = \leq 5$ 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 indicates 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 ($MATa\_his3_1, \_leu2_0, \_met15_0, \_ura3_0$) and null mutants of particular gene(s) including $hul5::\text{KanMX6}$ ($hul5\Delta$), $ufd2::\text{KanMX6}$ ($ufd2\Delta$), $rpm5::\text{KanMX6}$ ($rpm5\Delta$), and $doa10::\text{KanMX6}$ ($doa10\Delta$) were obtained from OpenBiosystems. A $pdr5::\text{KanMX6}$ ($pdr5\Delta$) strain background was used for experiments in which cells were co-cultured with cycloheximide, MG132, or bortezomib. Strains of $doa10::\text{His3}$ or $ufd2::\text{His3}$ in $pdr5\Delta$ background were generated by homologous recombination of a deletion cassette as previously described (36).

Yeast cells were cultured in synthetic complete (SC) medium [2 % (v/v) dextrose, 0.2 % (v/v) amino acid mixture, and 0.67 % (v/v) yeast nitrogen base] lacking specific amino acid(s) if plasmid selection is necessary. Cells were cultured at 30 °C unless specified.

To inactivate Cdc48p and Cim3p, strains expressing a temperature-sensitive $CDC48$ ($cdc48\Delta$) or $CIM3$ ($cim3\Delta$) allele were cultured at permissive temperature (23 °C) and then shifted to restrictive temperature (37 °C) for 30 min (37, 38). To inhibit translation, cells at the mid-log phase were co-cultured with cycloheximide (CHX) (Sigma, 100 µg/mL) for a period as indicated in each experiment. To inhibit the proteasome, cells were co-cultured with MG132 (Cayman Chemical, 20 µM) (39) or bortezomib (Selleck Chemicals, 20 µM) (40) for 2 hrs.

**Plasmid Construction** - A single copy yeast vector p416-GPD (41) was used for glyceraldehyde-3-phosphate dehydrogenase gene promoter-mediated constitutive expression of $PCA1$, N-terminal truncated $PCA1$ (35), $CPY1*$, $STE6*$ and $HMG2$. Plasmids for hemagglutinin
(HA) epitope tagging and green fluorescent protein (GFP) fusion at the N-terminus of Pca1p were conducted as previously described (35). C-terminal c-myc epitope tagging of Pca1p was achieved by PCR cloning using a primer containing c-myc sequence prior to the stop codon. Cpy1*p possesses the G255R substitution to be targeted to ERAD (13). Site-directed mutagenesis was accomplished by a primer overlap extension method (42). Two c-myc epitopes were inserted in the C-terminus of CPY1* for Western blotting analysis using anti-myc antibodies. HMG2 containing one c-myc epitope (43) was subcloned in p416-GPD vector. Common molecular biology techniques, including plasmid amplification using Escherichia coli containing one c-myc epitope (43) was subcloned in p416-GPD vector. Common molecular biology techniques, including plasmid amplification using Escherichia coli and purification, followed previously established methods (77). Plasmid transformation into yeast was performed using the lithium acetate method (44).

Fluorescence Microscopy - Cells cultured in SC media were mixed with phosphate-buffered saline (PBS) containing NaCl (15 mM) and NaF (15 mM) to inactivate cells. Cells were collected and fractionated by centrifugation and subjected to confocal microscopy (Olympus FV500) as described (35).

Fractionation of Cell Lysates - Yeast cells were broken by vortexing (1 min, 5 times) with glass beads in the PBS lysis buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM ethylenediaminetetraacetic acid (EDTA), and 50 mM protease inhibitor cocktail (HaltTM, Thermo Scientific). Centrifugation (4 °C, 1,000 x g, 10 min) removed large cell debris and unbroken cells. Soluble and membrane-associated proteins were separated by centrifugation (4 °C, 100,000 x g, 1 hr).

To differentiate membrane association vs membrane integration of Pca1p, membrane fractions of prd5Δ, cdc48-3 (cultured at 37 °C), and doa10Δ cells expressing 3HA epitope-tagged Pca1p were re-solubilized in phosphate-buffered saline (PBS) with and without Na2CO3 or Triton X-100. After incubation at 4 °C for 30 min, samples were fractionated by centrifugation (4 °C, 100,000 x g, 30 min). Supernatant (S) was precipitated using Trichloroacetic acid (TCA) and denatured in SDS sample buffer. The un-soluble pellet (N) was denatured in SDS sample buffer. Both S and N samples were subjected to Western blot analysis.

Flotation sucrose gradient fractionation was performed as previously described (45). Yeast cells were broken using glass beads in the lysis buffer (50 mM HEPES, 150 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol (DTT), 1 mM PMSF, 50 mM protease inhibitor cocktail). Sucrose gradient [0.25 M, 1.5 M, ~1.7 M (mixture of 200 µl cell lysate and 600 µl 2.3 M sucrose), and 2.3 M in descending order] was spun at 4 °C at 100,000 x g for 5 hrs. Twelve fractions (300 µL each) were then taken from the top.

In vitro De-ubiquitylation - The flotation sucrose gradient fractions (10-12) were subjected to buffer exchange using Slide-A-Lyzer dialysis cassette (Thermo Scientific) in 500 mL of de-ubiquitylation buffer (50 mM HEPES, 150 mM NaCl, 5 mM EDTA, 1 mM DTT, 1 mM PMSF, protease inhibitor cocktail without sucrose), followed by incubation with 5 ng of USP2 catalytic domain (BML-UW9850-0100 Enzo-life sciences) rocking at 37 °C for 1 hr. Samples were then subjected to a second round of buffer exchange in the same buffer without DTT. Immunoprecipitation was conducted using a kit (Profound™ c-Myc Tag IP/Co-IP application set, Thermo Scientific) according to the manufacturer’s specifications.

In Vivo Cross-linking and Immunoprecipitation - Cells expressing Pca1p tagged with HA epitope were co-cultured with a 20S proteasome inhibitor MG132 (20 µM, 2 hrs). Cells were collected by washing twice in ice-cold PBS and re-suspended 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 washed in the lysis buffer [50 mM Tris-HCl (pH 7.4), 5 mM EDTA, 50 mM protease inhibitor cocktail, and 1 mM PMSF] to quench crosslinking reaction. Cells were then broken by glass bead disruption (10 x 1 min on ice). Cells were then washed twice in ice-cold PBS and re-suspended in PBS containing Triton X-100 (1 %, v/v) and protease inhibitors. Immunoprecipitation was performed using a kit (Profound™ c-Myc Tag IP/Co-IP application set, Thermo Scientific) according to the manufacturer’s specifications.
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DTT in the SDS sample buffer cleaved the crosslinks.

Immunoblotting - Yeast cells were broken by vortexing (5 x 1 min) with glass beads in the lysis buffer (PBS containing 1 mM PMSF, 50 mM protease inhibitor cocktail, 5 mM EDTA, 1% Triton X-100). Soluble fraction was obtained by centrifugation (21,000 x g, 15 min). Protein concentrations were measured using a kit (BCA, Pierce) according to the manufacturer’s specifications. Samples were denatured in a reducing SDS sample buffer containing dithiothreitol (DTT, 25 mM) for 15 min at 37 °C and then subjected to polyacrylamide gel electrophoresis (PAGE). This mild denaturation condition avoided aggregation of Pca1p which contains eight predicted transmembrane helices. Gels were transferred to nitrocellulose membrane and hybridized with primary antibodies against HA epitope (Rockland, 600-401-384), Rpn5p subunit of the 19S proteasome (Abnova, PAB 15594), α and β subunits of 20S proteasome (Abcom, ab22673), c-myc epitope (ABM, G019), or ubiquitin (Covance, MMS-257P). 3-phosphoglycerate kinase (Pgk1p) was detected using anti-Pgk1p antibodies (Invitrogen, 459250) to determine equal loading. Horseradish peroxidase-conjugated goat anti-rabbit IgG or sheep anti-mouse IgG (Santa Cruz Biotechnology Inc) was used as secondary antibodies. Western Pico Chemiluminescence (Pierce) was used to detect antibody bound proteins.

Statistical Analysis - Experiment were conducted using minimum two independent clones, and representative data was presented. Results were presented as means ± SD. Student’s t-test determined the statistical differences. P < 0.05 was considered to be significant.

Results

Pca1p remains in the membrane when the proteasome is inactivated – Pca1p, a cadmium-exporting P13-type ATPase in yeast, is a polytopic protein (Fig. 1A) that undergoes ERAD in the absence of cadmium (34). A degron identified within amino acids 250 and 350 at the N-terminal cytosolic domain (1-557 amino acids, ~61 kDa) (Fig. 1A), Doa10p, an E3 ligase, and the proteasome are required for Pca1p turnover (35). Given cadmium-induced rescue of Pca1p from ERAD (34, 35) and its eight predicted transmembrane helices, we sought to determine if ERAD of Pca1p relies on known molecular factors for ERAD of misfolded proteins and how this polytopic protein is degraded by the proteasome. To determine subcellular localization and turnover rates of Pca1p, we fused green fluorescent protein (GFP) or epitope, such as triple hemagglutinin (3HA) and two c-myc (2Myc) at the N- or C-terminus (Fig. 1A). These PCA1 alleles are fully functional and display cadmium-dependent ERAD (data not shown).

We first determined subcellular distribution of GFP-Pca1p when proteolytic activities of the proteasome are inhibited by MG132 co-culture (46) or Cdc48p AAA-ATPase is inactivated (Fig. 1B). Cdc48p is a known critical player in dislodging ERAD substrates from the ER (47). A strain possessing temperature-sensitive CDC48 allele (cdc48-3) was used to inactive this essential gene (37) at a restrictive temperature, 37 °C. Cells were pre-cultured with cycloheximide to inhibit new Pca1p synthesis. Pca1p fused with GFP at the N-terminus (GFP-Pca1p) is not detectable in wild type (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 ubiquitinylated 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 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 (the triangle in Fig. 1C). Given that the half-life of Pca1p is less than 5 min (35), the majority of remaining Pca1p in wild-type (WT) cells co-cultured with cycloheximide should be ubiquitinylated species. Pca1p fragments below full-length Pca1p, including approximately 60, 70, 75, and 90 kDa bands, were detected in WT, cdc48-3, and doa10Δ cells (Fig. 1C, upper panel). We previously reported that Pca1p ubiquitinylation
is absent in doa10Δ cells (34, 35). Therefore, fragmentation of Pca1p, observed in doa10Δ cells, is unlikely a ubiquitinylation-dependent process. When probing with anti-myc antibodies, only full length was detectable and all lower molecular weight fragments were non-specific as seen by the empty vector control (Fig. 1C, middle panel, lane 1). To determine distribution of Pca1p, total cell lysates were fractionated by ultra-centrifugation (100,000 x g for 30 min) to supernatant (S) and pellet (P) containing soluble proteins and membranes, respectively (Fig. 1D). Western blot analysis detected full-length and higher molecular weight Pca1p species at the “P” fraction but not “S” fraction (Fig. 1D). Immuno-precipitation of Pca1p from the “S” and “P” fractions with anti-HA antibodies followed by Western blotting using anti-HA and -ubiquitin (Ub) antibodies (Fig. 1E) further suggested that most ubiquitylated Pca1p species, except a potion of the ~75 kDa fragment, reside in the membrane.

To rule out any off-target effect of MG132 on proteasome inhibition, we also conducted the 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 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 blot analysis (Fig. 1F, 4th lane vs 5th lane). This indicates that Cim3-1p is not fully functional at both 23 and 30 °C. Fractionation of soluble and membrane proteins followed by Western blotting of Pca1p also displayed similar results between bortezomib and MG132 co-cultures (Fig. 1G).

Collectively, the data presented in Fig. 1 suggests that most Pca1p and its fragments, except for a ~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 membrane 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 supernatant (S) (Fig. 2A). However, all Pca1p species, including Pca1p fragments that migrate faster than full-length Pca1p, remain in the non-soluble pellet (N) factions of WT cells co-cultured with MG132 (Fig. 2A, left panel). Na₂CO₃ dissociates only a small potion (< 5%) of full-length and fragmented Pca1p from the membrane (Fig. 2A, left panel, 3rd lane). Only a small portion of Pca1p detected in the membrane fraction was dissociated by Na₂CO₃ in Cdc48p inactive cells and in doa10Δ cells (Fig. 2A, middle and right panels), indicating membrane integration of Pca1p species. Triton X-100 (1 %, v/v) released Pca1p to the “S” fraction near completely (Fig. 2A), suggesting that most Pca1p and its fragments are integrated in the membrane.

To confirm Pca1p’s localization in the membrane rather than aggregated complexes, cell lysates were subjected to a sucrose density gradient fractionation (45) (Fig. 2B). If Pca1p is in membrane vesicles, it will “float” to the low-density fractions. WT cells expressing 3HA-tagged Pca1p or Pca1p(degronΔ) lacking first 392 amino acids containing the degron were co-cultured with MG132 for 2 hrs. Glass bead-disrupted cells were loaded at the layer of 1.7 M sucrose. Centrifugation followed by Western blotting analysis of collected fractions displayed that the majority of full-length Pca1p and Pca1p(Δ392) are within the membrane-containing low density fractions, unlike Pgk1p, a soluble protein (Fig. 2B, 4th panel). Membrane solubilization of cell lysate with Triton X-100 (1 %, v/v) resulted in co-fractionation of Pca1p with Pgk1p (Fig. 2B, 3rd panel).

Pca1p species with different degrees of ubiquitinylation may exist in the cytosol below detectable limit by Western blot. To address this concern, we assessed the ubiquitinylation status of Pca1p in the fractions 10, 11 and 12 containing soluble proteins. Pca1p was immuno-precipitated using anti-HA antibodies and then probed with antibodies against ubiquitin and HA epitopes (Fig. 2C). The soluble protein fractions contain three major ubiquitin-conjugated Pca1p fragments (~60, ~75, and ~90 kDa) (Fig. 2C, 1st panel). It was further determined that poly-ubiquitylated full-length Pca1p species were not present in the
soluble fractions since deubiquitinylation by the purified catalytic subunit of the de-ubiquitylating enzyme, USP2, did not lead to an enrichment of full-length Pca1p (Fig. 2C, 2nd panel). Anti-HA antibodies detected primarily a fragment of ~60 kDa. Given the predicted N-terminal cytosolic domain (61 kDa + ~3 kDa corresponding to tagged 3HA epitopes), the ubiquitylated Pca1p fragment detected in the soluble fraction could be a cleaved N-terminal cytosolic domain. However, a portion of the fragment is also detected in the membrane fraction as a peptide integrated into membrane (Figs. 1D, 1G, 2A, and 2B). Therefore, it is likely that the fragment contains one or more transmembrane helixes (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 a degron dependent manner – Given the potential role for the proteasome in extraction of Pca1p from the ER, we next determined if 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 N-terminal 392 amino acids which contains 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 immuno-precipitation of Pca1p. The formation of a complex between Pca1p and Rpn5, a proteasome subunit (49), was visualized by Western blot. 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, dimethyl 3,3′-dithiobispropionimidate (DTBP). After breaking cross-links by denaturing samples in dithiothreitol-containing SDS sample buffer, physical interactions of proteasome subunits (46) with Pca1p were visualized by Western blotting. Indeed, Pca1p was found to form a complex with Rpn5p, a 19S proteasomal subunit (Fig. 3A, lane 4; Fig. 3C, lane 2), and the α and β subunits of 20S proteasome (Fig. 3D) at the membrane. While immuno-precipitation efficiency of Pca1p was over 90 % (Fig. 3B, 1st panel, lane 2 vs 4), most Rpn5p (~81%) still remained in the flow-through (Fig. 3B, 2nd panel, lane 2 vs 4). This suggests that Rpn5p is associated with not only Pca1p but also other membrane proteins.

The complex formation between Pca1p and Rpn5p was dependent on Doa10p and the degron in Pca1p (Fig. 3C). Although 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, lane 3) (34, 35), immuno-precipitation of Pca1p and Pca1p(degronΔ) in doa10Δ and WT cells, respectively, did not pull down Rpn5p at detectable levels (Fig. 3C, middle panel). These results confirm the specificity in the complex formation between Pca1p and Rpn5p and indicate that when Pca1p is ubiquitylated by Doa10p, the proteasome is recruited while Pca1p resides at the membrane.

Cdc48p is required for the complex formation between Pca1p and the proteasome – Cdc48p is a critical molecular factor for ERAD of Pca1p (Figs. 1, 2A, 4A) and other proteins (10, 34, 37). ATPase activities of Cdc48p are believed to contribute to the retro-translocation of ubiquitylated 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 if the proteasome forms a complex with Pca1p in the cells expressing inactive Cdc48p. The levels of ubiquitylated proteins in the cells expressing non-functional Cdc48p (37 °C culture) are comparable (1.26 ± 0.62 fold, n=3, p=0.53) to those of control (23 °C culture) cells (Fig. 4A, upper panel). Despite higher Pca1p expression in Cdc48p-defective cells (Fig. 4A, 2nd panel), immuno-precipitation 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 ubiquitylated Pca1p for recruitment to the proteasome.
It was known that Cdc48p also interacts with other molecular factors involved in ERAD such as E4 ubiquitin chain extension enzyme (50). The observed defect of Pca1p ERAD (Fig. 1B) and the lack of Pca1p interaction with the proteasome (Fig. 4B) in the cells expressing non-functional Cdc48p may be attributed to a deficiency in recruiting E4 ligase(s) and/or presenting mono-ubiquitinylated Pca1p for polyubiquitination. To address this, we determined ubiquitinylation status of Pca1p in Cdc48p inactive cells. Immunoprecipitation of Pca1p using anti-HA antibodies followed by Western blot analysis of Pca1p using anti-Cdc48p antibodies determined ubiquitinylation status of Pca1p in Cdc48p inactive cells. Immunoprecipitation of Pca1p for polyubiquitination. To address this, we determined ubiquitinylation status of Pca1p in Cdc48p inactive cells. Immunoprecipitation of Pca1p using anti-HA antibodies followed by Western blot analysis of Pca1p using anti-Cdc48p antibodies determined ubiquitinylation status of Pca1p in Cdc48p inactive cells. Immunoprecipitation of Pca1p for polyubiquitination. To address this, we determined ubiquitinylation status of Pca1p in Cdc48p inactive cells. Immunoprecipitation of Pca1p using anti-HA antibodies followed by Western blot analysis of Pca1p using anti-Cdc48p antibodies determined ubiquitinylation status of Pca1p in Cdc48p inactive cells. Immunoprecipitation of Pca1p for polyubiquitination. To address this, we determined ubiquitinylation status of Pca1p in Cdc48p inactive cells.

As Ufd2p is required for efficient degradation of Pca1p (Fig. 5B), we next sought to determine if poly-ubiquitinylation by Ufd2p is necessary for the interaction of Pca1p with the proteasome. In vivo cross-linking and co-immunoprecipitation followed by Western blot 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 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 non-functional Cdc48p (Figs. 4B and 4C) is not relevant to poly-ubiquitinylation status of Pca1p.

Proteasome dependent extraction of ERAD substrates is specific for polytopic proteins – We next determined if other poly-topic membrane proteins undergoing ERAD manifest an interaction with the proteasome at the membrane as observed for Pca1p. Ste6*p is a mutated form of the alpha factor transporter Ste6p in yeast (54), which causes a premature stop codon and a change in N-glycosylation. Ste6*p is also targeted for degradation via Doa10p, Cdc48p, and proteasome dependent pathway (10, 54, 55). Subcellular fractionation and flotation sucrose gradient fractionation showed that Ste6*p 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 Ste6*p pulled down a proteasome subunit Rpn5p in a Doa10p-dependent manner (Fig. 6A). Therefore, Ste6*p also interacts with the proteasome at the ER membrane when it is ubiquitinylated 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 Ste6*p, inhibition of proteolytic function of the proteasome prevents Hmg2p release from the ER membrane.

Vacuolar carboxypeptidase Y (Cpy1*p) containing a G255R mutation (Cpy1*p) is degraded through the ERAD-C pathway (13, 56-59). We have employed Cpy1*p as an example of an ER luminal protein to elaborate the hypothesis that soluble ERAD substrates may be retro-translocated prior to being targeted to the proteasome. When WT yeast cells expressing c-myc epitope tagged Cpy1*p were co-cultured with MG132, a proteasome inhibitor, Cpy1*p was detected as full-length and poly-ubiquitinylated forms in the soluble (S) fraction (Fig. 6C). Rpn5p, a subunit of 19S proteasome, was co-immunoprecipitated with Cpy1*p in soluble fraction (Fig. 6C, 2nd panel) indicating that Cpy1*p forms a complex with the proteasome in the cytosol. However, Cpy1*p in the cells lacking Hrd1p E3 ligase targeting Cpy1*p for ERAD remains at the pellet fraction along with Kar2p, an ER luminal protein (Fig. 6D); therefore, detection of Cpy1*p in soluble fraction (Fig. 6C) is a consequence of ERAD progress of Cpy1*p rather than its leaking from the ER during sample preparation followed by interaction with the proteasome. These results suggest that Cpy1*p ubiquitinylated by Hrd1p is retro-translocated to the cytosol without proteolytic activities of the proteasome. This is distinct from other examined polytopic proteins, Pca1p, Ste6*p, and Hmg2p.

Discussion

ERAD involves retro-translocation 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 Cpy1*p, 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 dealing hydrophobic proteins in the cytosol. These results shed new light on this vital but under characterized 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 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 to a previous report displaying a 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, Ufd1p 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 ubiquitinylated 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, 64). While several potential mechanisms have been proposed (3, 10, 32), the results reported previously were not consistent with each other. An in vitro reconstitution assay found that prior to degradation, Ste6*p, a well-characterized ERAD-C substrate, is dislodged from the membrane (10); however, it is worth pointing out that only a small fraction of Ste6*p was released from the membrane under the experimental conditions. Secondly, an in vitro ERAD assay of 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 ubiquitinylated 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 supports 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 19S and 20S proteasome from the membrane fractions is dependent on Doa10p, the major E3 ubiquitin ligase of Pca1p. This suggests that ubiquitinylated 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 (66-68). 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, post-translational 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 if 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 polyubiquitylation of membrane proteins in ERAD remains an intriguing question. The regulatory particles of the proteasome contain several functional components, including AAA-Type ATPase, E4 ubiquitin ligase(s), and de-ubiquitylation enzyme(s). Hul5p, an E4 enzyme associated with the regulatory particle of yeast, is involved in ERAD of reporter substrates (e.g., CTL* and Sec61-2L) (32) and proteasome-dependent degradation of cytosolic proteins that are damaged under heat stress (52). However, knockout of *UFD2* gene encoding another E4 ligase did not affect the heat-induced ubiquitylation (52). Our study on the turnover rate of *Pca1p* in *hul5Δ* and *ufd2Δ* strains showed that the *Ufd2p* but not *Hul5p* is involved in ERAD of *Pca1p*, confirming target specificity of E4 ligases. Nevertheless, it’s worth noting that the relative stability of *Pca1p* in *ufd2Δ* strain was still significantly lower than that of the *doa10Δ* strain (*T1/2 = 30 min vs > 2 hrs*), which suggests that poly-ubiquitylation is not essential for ERAD but enhances the efficiency. One might argue that, given three other E4 ligases in addition to *Hul5p* and *Ufd2p* in the genome of the yeast *S. cerevisiae* (76), there may be some redundancy. However, we disproved this by showing near absence of poly-ubiquitylated *Pca1p* in *ufd2Δ* cells. It is also interesting to note that, although *Pca1p* turnover is slow in *ufd2Δ* cells and a drastic reduction of poly-ubiquitylation of *Pca1p* in the cells, *Ufd2p* deficiency does not affect the formation of a complex between *Pca1p* and the proteasome. These results indicate that poly-ubiquitylation of *Pca1p* is not a critical determinant of initial interaction with the proteasome but rather promotes efficient processing of substrates by the proteasome.

This study reflects a comprehensive in vivo study determining how polytopic proteins are targeted to the ERAD pathway in yeast. The recruitment of the proteasome to ubiquitylated 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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**Conflict of Interest** - The authors declare that they have no conflicts of interest with the contents of this article.

**Author Contributions** – NS, DJA and JL designed the research. NS, DJA, MZ, XQ and HK performed
the experiments. NS, DJA and JL analyzed the data. NS, DJA and JL wrote the manuscript. All authors reviewed the results and approved the final version of the manuscript.

References


Proteasome Recruitment to the Endoplasmic Reticulum

FIGURE 1. Localization of Pca1p at the membrane of the cells in which the proteasome or Cdc48p is inactivated. A. A schematic depiction of Pca1p. The black squares indicate eight trans-membrane helixes. Green fluorescent protein (GFP) or triple HA epitope (3HA) is fused to the N-terminus (filled oval). Two c-myc epitope (2Myc) is fused at the C-terminus (empty oval). The unfilled 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 (RFP)-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 hrs. Cycloheximide was added to the media 1 hr 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 immuno- blotting. Pca1p fused with 3HA and 2Myc was expressed in a WT, a strain expressing cdc48-3 allele, and a doa10Δ strain. WT and cdc48-3 cells were pre-cultured with MG132 for 2 hrs 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 blot using anti-HA and -myc antibodies. The blots were also probed with anti-Pgk1p antibodies to determine equal loading. D. Determination of 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 (300 x g for 10 min centrifugation). The samples were separated to soluble (S) and pellet (P) fractions by centrifugation (100,000 x g for 30 min). The S and P fractions were solubilized (1 % Triton X-100) and subjected to Western blot analysis using Anti-HA antibodies. E. Detection of majority of ubiquitylated Pca1p at the P fraction. Pca1p in the S and P fractions obtained from WT cells as described above was immuno-precipitated using anti-HA antibodies. Expression levels and ubiquitylation status of Pca1p were determined by Western blot using anti-HA and -ubiquitin (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 triangle (C - G), asterisks (C, F, G), and arrows (C, F) indicate the location of full-length non- ubiquitylated Pca1p, non-specific 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 is presented.

FIGURE 2. Detection of fragmented or ubiquitylated Pca1p by subcellular fractionation. A. Majority of Pca1p and its fragments are embedded in the membrane. Pca1p fused with triple HA at the N-terminus was expressed in a control strain lacking PDR5 (WT), a strain expressing a cdc48-3 temperature sensitive allele, and a doa10Δ strain. WT and cdc48-3 cells were cultured with MG132 (20 µM, 2 hrs) and at 37°C (30 min), respectively. Membrane fractions obtained by centrifugation (100,000 x g, 30 min) of total cell lysate were re-suspended in phosphate-buffered saline (PBS) with and without Na2CO3 (0.2 M, pH 11) or Triton X-100 (1 %). The samples were incubated for 30 min on ice and then centrifuged at 100,000 × g for 30 min. The supernatant (S) was precipitated in trichloroacetic acid (10 %) and washed twice with acetone. The nonsoluble pellet (N) was re-suspended in PBS containing detergent (1 % Triton X-100). Samples were denatured in SDS sample buffer (37 °C, 15 min) and analyzed by Western blot using anti-HA antibodies. B. Fractionation of Pca1p by flotation sucrose gradient. WT cells expressing 3HA-tagged Pca1p or Pca1p(degronΔ) lacking first 392 amino acids containing a degron were co-cultured with MG132 for 2 hrs. Cycloheximide was added to the media 1 hr before collecting cells. Total cell lysates were prepared by glass bead disruption followed by centrifugation (300 x g, 10 min) to remove unbroken cells. Samples were loaded at the layer of 1.7 M sucrose density at a 0.25 to 2.3M gradient from the top to bottom. After centrifugation (100,000 x g, 5 hrs), the collected fractions were subjected to Western blotting. An aliquot was incubated with Triton X-100 (1 %) on ice for 30 min before
the fractionation to extract Pca1p out of the membrane. Obtained fractions were subjected to Western blot analyses of Pca1p and Pca1p(degronΔ) using anti-HA antibodies. Pgk1p was detected as a marker of fractions containing soluble proteins. C, Distribution of full-length, ubiquitylated, and fragmented Pca1p species. Fractions obtained as described in B were subjected to Western blot analysis of Pca1p with and without treatment of deubiquitylation enzyme. Fractions 10, 11 and 12 containing cytosolic proteins were pooled, deubiquitylated (De-Ub), and then solubilized by detergent (1 % Triton X-100). Pca1p was immuno-precipitated using anti-HA antibodies. The samples were subjected to Western blotting using anti-ubiquitin (Ub) and -HA antibodies. The triangle indicates the location where full-length non-ubiquitylated Pca1p migrates. Results were confirmed by three or more repeats, and representative data is presented.

FIGURE 3. Pca1p forms a complex with the proteasome at the membrane in a Doa10p dependent manner. Pca1p, Pca1p(degronΔ) deleting first 392 amino acids, and control empty vector were expressed in WT and doa10Δ strains. Two e-myc epitopes were tagged at the C-terminus of Pca1p. Cells were cultured with and without a cell permeable and reversible cross-linker (DTBP, 100 µg/ml) and MG132 (20 µM) for 2 hrs. Cell lysates were obtained by Triton X-100 (1 %) solubilization of membrane fraction. The samples were subjected to immunoprecipitation using anti-myc antibodies. Co-immunoprecipitation of Pca1p with Rpn5p, a subunit of 19S regulatory particle (A, C), and α and β subunits of 20S catalytic core (D) was determined by Western blotting. B, Pca1p and Rpn5p levels in the input (cell lysate) and flow-through of Pca1p immune-precipitation (IP flow-through) were compared by Western blotting. Each experiment was conducted twice using two independent clones, and representative data is presented.

FIGURE 4. Cdc48p plays a role in Pca1p’s complex formation with the proteasome but not its poly-ubiquitylation. A. No significant change of protein poly-ubiquitylation in cells expressing non-functional Ccd48p. Pca1p tagged with triple HA epitope was expressed in a strain carrying a temperature-sensitive cdc48-3. The 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 hrs. Membrane fractions were solubilized with Triton X-100 (1 %). Expression levels of Pca1p and Rpn5p and the overall ubiquitylation status were determined by Western blot. B, Cdc48p-dependent physical association between Pca1p and Rpn5p, a subunit of 19S regulatory particle, was determined by co-immunoprecipitation. Triton X-100 (1 %) solubilized membrane fraction was used for immune-precipitation of Pca1p using anti-HA antibodies. Co-immunoprecipitation of Rpn5p, a subunit of 19S regulatory particle was detected by Western blot. C, Poly-ubiquitylation of Pca1p as a functional of Cdc48p activities. To determine ubiquitylation status of Pca1p, the samples obtained as described in B were subjected to Western blot using anti-ubiquitin antibodies. To compare relative ubiquitylation of Pca1p, five-fold amount of immune-precipitated Pca1p from cells cultured at 23 °C relative to those at 37 °C was loaded. Each data was confirmed by triplicate experiments.

FIGURE 5. Formation of a complex between Pca1p and the proteasome is independent of poly-ubiquitylation of Pca1p. A, Significant reduction of turnover rate of Pca1p in UFD2 gene knockout (ufd2Δ) cells. Pca1p tagged with two e-myc epitope at the C-terminus was expressed in WT, ufd2Δ, and hul5Δ strains. Cycloheximide (CHX) chase and Western blot 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, Pca1p levels presented in A were quantitated (n=4). Pca1p levels relative to those at time 0 are presented. Asterisk indicates p<0.01. C, Defect in poly-ubiquitylation of Pca1p in a ufd2Δ strain. Pca1p tagged e-myc epitope was expressed in a ufd2Δ strain. Total cell lysates (T) were obtained with and without co-culture with MG132 (20 µM, 2 hrs). Samples were subjected to separation to soluble (S) and pellet (P) fractions by centrifugation (100,000 x g for 30 min). After solubilization with Triton-X 100 (1 %), S and P fractions were subjected to immune-precipitation using
anti-myc antibodies followed by Western blotting using anti-ubiquitin (Ub) and -myc antibodies to detect Pca1p. D, Distribution of Pca1p species in the S and P fractions in ufd2Δ cells. Total cell lysates (T) and soluble (S) and pellet (P) fractions described in C were subjected to Western blot 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 triangle (C and D) indicates the location where full-length Pca1p migrates. Experiment (B - D) was conducted twice using two independent clones, and representative data is presented.

FIGURE 6. Ste6*p, Hmg2p, and Cpy1*p form a complex with the proteasome at the membrane. Ste6*p, Hmg2p, or Cpy1*p tagged with either HA or c-myc epitope was expressed in WT control (A-C) doa10Δ (A) and hrd1Δ (D) cells. Cells were co-cultured with MG132 (20 μM, 2 hrs), an inhibitor of proteasomal proteolytic activities, and a cell permeable reversible cross-linker (DTBP, 100 μg/ml, 30 min). Total cell lysates (T) were prepared by glass bead disruption. Samples were subjected to separation of soluble (S) and membrane (P) fractions by centrifugation (100,000 x g, 30 min). Protein extracts were obtained by solubilizing samples with detergent (1% Triton X-100). A, The membrane (P) fractions were used to determine an interaction of Ste6*p with the proteasome. HA-Ste6*p were subjected to anti-HA immunoprecipitation followed by Western using anti-HA and anti-Rpn5 antibodies. B and C, Lysates of WT cells expressing Hmg2p-myc or Cpy1*p-myc were subjected to fractionation (S and P) followed by detergent solubilization. Anti-myc immunoprecipitation and Western blotting using anti-Rpn5p and -myc antibodies determined their physical interaction. Ubiquitinylation status was determined by probing the blots with anti-ubiquitin (Ub) antibodies (C, middle panel). D, Cpy1*p expressed in total cell lysate (T) and S and P fractions of hrd1Δ cells was detected by Western blot using anti-myc antibodies. The blots were also probed with antibodies against Kar2p and Pgk1p, an ER-resident protein and cytosolic protein, respectively. Representative figures of two or more independent experiments are presented.
Fig. 1

A. Membrane
   Cytosol
   Degron
   GFP or 3HA

B. MG132
   23 °C
   WT
   -
   +
   MG132
   37 °C
   Pca1
   Sec63
   Merge
   Cells

C.WT  +  MG132
   kDa
   250
   130
   95
   72
   55
   HA
   Pgk1
   myc
   *
   Pca1
   MG132

D. Bortezomib
   - + + + + - - - -
   HA
   180
   130
   95
   72
   55
   *
   Pgk1
   Bortezomib

E. WT
   +
   -
   +
   MG132
   kDa
   250
   130
   95
   72
   55
   HA
   Ub
   Pca1
   **
   *
   Pgk1
Fig. 2

A

WT + MG132
Na$_2$CO$_3$  
TX-100

B

WT + MG132

C

Fractions

Fractions

Fractions

Pca1
Pca1 (degronΔ)
TX-100
Pgk1

Fractions

De-Ub
Ub
HA

kDa

kDa

kDa

kDa

Top

Load

Bottom

123456789101112

130 130 250 250

130 250

95 95 72 72

55 55 36 36

- + + - - - - + +

- + + - - - - + +

- - + + - - - - + +

- - + + - - - - + +

- - + + - - - - + +

- - + + - - - - + +

- - + + - - - - + +

- - + + - - - - + +
Fig. 3

A

IP:
anti-myc

Pca1-myc

Cross linker

Pca1
Rpn5

Cell lysate

Membrane staining

B

Cell lysate

IP flow-through

Pca1-myc
Pca1
Rpn5

Membrane staining

C

WT
doa10Δ

Pca1-myc
Pca1(degronΔ)-myc

Pca1
Pca1(degronΔ)
Rpn5

Cell lysate

Membrane staining

D

Pca1-myc

Pca1
20S

Cell lysate

IP flow-through

Pca1

20S
Fig. 4