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ER-associated Degradation and Cadmium Dependent Rescue of PCA1

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ER-associated Degradation and Cadmium Dependent Rescue of PCA1

By

Nathan James Smith

A DISSERTATION

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ER-associated Degradation and Cadmium Dependent Rescue of PCA1

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University of Nebraska, 2014

Adviser: Jaekwon Lee

Protein synthesis and proper folding is an essential process for all organisms. In eukaryotes proteins of the secretory pathway are synthesized and inserted into the lumen or membrane of the endoplasmic reticulum. Eukaryotic cells maintain a mechanism for removal of proteins unable to fold properly. This process is known as ER-associated degradation (ERAD). A poorly functioning ERAD can lead to a build-up of misfolded proteins which has been implicated in several degenerative diseases such as Alzheimer's, Amyotrophic lateral sclerosis, and Parkinson's. Thus, the study of how proteins are recognized, extracted from the ER, and degraded is essential for determining methods for maintaining protein solubility and stability, and prevention of toxic accumulation of protein aggregates.

Our lab has previously identified Pca1, a cadmium exporting P_{1B}-type ATPase in *Saccharomyces cerevisiae*. A genetic knockout screen led to the discovery that Pca1 expression is controlled post-translationally through the ERAD pathway. Specifically, the ERAD-Cytoplasm (ERAD-C, indicating the location of the misfolding) pathway utilizes the E3 ubiquitin ligase Doa10 to ubiquitinylate substrates.

We further tested the mechanism by which Pca1 an eight transmembrane domain containing protein was extracted from the ER membrane for degradation in the cytoplasm. Surprisingly, we determined that the proteasome itself is essential for this process.

Finally, we sought to determine the requirements of cadmium sensing and rescue from ERAD as well as the molecular factors involved in recognition of the degron of Pca1.

Biophysical characterization revealed cadmium specific binding. A random-mutagenesis screen identified residues required for degradation of Pca1. Bioinformatical study of the Pca1 degraon structure identified a hydrophobic patch that when broken with amino acid substitution stabilized the protein. It was also determined that interaction with a known recognition factor of ERAD, Ssa1, was much weaker in the presence of a hydrophilic substitution or cadmium supplementation.

Collectively, our results revealed a mechanism in which Pca1 is regulated post-translationally through the ubiquitin proteasome system. We were also able to apply our findings of Pca1 to another ERAD-C substrate. Pca1 is an excellent model for the study of the ERAD-C pathway as it is short-lived and rapidly stabilized by the supplementation of cadmium.

Dedication

To my wife, Danielle

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I would first like to thank my advisor, Dr. Jaekown Lee for instruction and guidance over the course of my Ph.D. training. I would also like to thank my advisory committee, Dr. Donald Becker, Dr. Jiri Adamec, Dr. Mark Wilson and Dr. Ken Nickerson for helpful discussion and guidance. I would also like to thank Lee lab members both former and current for helpful discussion and suggestions.

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ABBREVIATIONS

ABC, ATP binding cassette

Cd, cadmium

Cu, copper

Zn, Zinc

Ca, Calcium

Pi, inorganic phosphate

UPR, unfolded protein response

UPRE, unfolded protein response element

kD, kilodalton

MT, metallothionine

DTT, dithiothreitol

ICPMS, inductively coupled plasma mass spectrometry

GFP, green fluorescence protein

RFP, red fluorescence protein

GPD, glyceraldehyde-3-phosphate dehydrogenase

GSH, reduced glutathione

ER, endoplasmic reticulum

ERAD, endoplasmic reticulum degradation

HA, hemagglutinin

HRP, horse radish peroxidase

PBS, phosphate buffered saline

PGK, phosphoglycerate kinase

Hsp, heat shock protein

Chapter 1
Introduction

Forward.

Heavy metal toxicity is a major health concern as many diseases are related to its toxic accumulation. Many organisms have evolved highly regulated mechanisms to bind, sequester and extrude these metals. In this thesis I will concentrate on the Cd^{II} exporting $\text{P}_{1\text{b}}$ -Type ATPase in *S. cerevisiae*, Pca1. In particular I will focus on the mechanism of post translational regulation through ER-associated degradation. I will also investigate the elements of Pca1 which are essential for its ER-associated degradation and the constituents involved. The following is an introduction containing the current knowledge of Cd^{II} metabolism in eukaryotes, P-type ATPase ion transporters, and ER-function and ER-associated degradation.

1.1 Cadmium

This section is modified from the following chapter:

Cadmium Transport in Eukaryotes. Encyclopedia of Inorganic and Bioinorganic Chemistry. 1–12. 2013.
Smith, N., Wei, W. and Lee, J.

1.1.1 Properties

First identified as an element in 1817, Cd^{II} has garnered significant attention because of several cases of serious human diseases associated with Cd exposure and its high toxicity. Cd is a relatively abundant mineral normally present in the earth's crust. Cd^{II} has no physiological or biological role save in one organism, *Thalassiosira weissflogii* where Cd can replace Zn in carbonic anhydrase II under times of Zn deficiency [2]. The chemical similarities of Cd^{II} to several nutritional divalent metals such as Zn allow it to enter the cells and disrupt normal biological processes that rely on nutritional metals [3].

It is estimated that about 25,000 to 30,000 tons of Cd is released into the environment each year (1), approximately one-half is liberated from the weathering of rocks, and the other half is produced by industrial activities. Mining and smelting for copper (Cu) and zinc (Zn) produces Cd as a by-product. Human exposure via occupations and contaminated foods leads to serious damage to multiple organs, such as lung, kidney, bone, and testis depending on the route, dose, and duration of exposure. Gastrointestinal and respiratory symptoms were observed about 150 years ago among persons using Cd powder as a polishing agent [4]. Since then, diverse experimental studies on Cd toxicity using animal models and occasional cases of human intoxication were reported, which was culminated by the Itai-Itai disease in Japan in 1957. Consumption of rice harvested from paddy rice plants that were cultivated using Cd-contaminated water resulted in renal osteomalacia. More recent epidemiological studies have shown that Cd^{II} manifests adverse effects at low exposure levels on sensitive population groups, such as persons with diabetes [5]. The adverse effects of Cd on biological systems rely on diverse mechanisms that remain to be further elucidated. Fungi and plants can be exposed to high levels of Cd^{II} at the direct interface between organisms and growth environment.

1.1.2 Acquisition

Organisms are unlikely to have evolved mechanisms for active uptake of Cd^{II} as it does not have any functional role. Progress in mechanistic understanding of metal metabolism has shown that the pathways involved in acquisition of nutritional metals are the gateways for Cd^{II} because of broad substrate specificity and/or similar chemical characteristics of Cd^{II} with those metals, especially Zn^{II} and Ca^{II}. The Nramp family of transporters is widely distributed from bacteria to humans with some distinct tissue and organelle expression patterns [6]. In humans, Nramp2 (DMT1, DCT1) is responsible for the majority of non-heme iron uptake from the diet. DMT1 is located on the apical side of mature villi in enterocytes and the cell surface as well as

endosomal vesicles in peripheral organs and tissues suggesting tissue-specific distinct modes of action. DMT1 and other Nramp members transport not only Fe^{II} but also other nutritional and toxic metals such as Cd^{II}, Ni^{II}, Zn^{II}, Co^{II}, Mo^{II}, and Hg^{II} [7].

Smf1 and Smf2 are Nramp family metal transporters in yeast *S. cerevisiae* that display the same broad substrate specificity as other transporters in this family [8]. While these transporters are considered Mn^{II} importers, their expression levels are well correlated to Cd^{II} toxicity. This clearly supports the functional roles for Smf1 and Smf2 in Cd^{II} uptake. The third Nramp transporter in yeast, Smf3, localizes at the vacuolar membrane to mobilize vacuole-stored iron; however, its metal selectivity has not been defined.

Many different Zn transporters have been implicated in Cd^{II} uptake [6, 9]. In yeast *S. cerevisiae*, Zrt1 and Zrt2 are two major transporters responsible for high- and low-affinity Zn^{II} uptake, respectively. They are closely related (about 44% amino acid sequence identity) transporters that belong to the ZRT (zinc-regulated transporter) and IRT (iron-regulated transporter)-related Protein (ZIP) family of transporters. Cd^{II} transport and toxicity assays in the cells in which Zn transporters are deleted or over-expressed demonstrated the roles for these Zn^{II} transporters in Cd^{II} uptake. Zn^{II} as well as Cd^{II} and Co^{II} (but less effectively than Zn^{II}) trigger post-translational control of Zrt1 through ubiquitinylation followed by degradation in the vacuole [10].

The similar ionic radius between Cd^{II} and Ca^{II} (0.95 Å vs 1.00 Å) allows certain types of Ca^{II} channels/transporters to become the entry routes for Cd^{II} into cells [6]. In yeast *S. cerevisiae* Mid1, which functions as a stretch-activated Ca^{II}-permeable cation channel in response to pheromone, has been determined to have a Cd^{II} uptake capability [11]. Contribution of voltage-dependent Ca^{II} channels (VDCC) in Cd toxicity in mammals is supported by down regulation of the channels in Cd-resistant cell lines that were selected from Cd-sensitive cells. Cd^{II} was shown

to inhibit VDCC, which may be a mechanism for Cd^{II}-induced perturbation of Ca^{II} metabolism [12].

1.1.3 Mechanism of toxicity

High affinity for Cd to thiol groups is believed to be the primary determinant of its biological effects. Direct binding and indirect effects (e.g., oxidative modification) of Cd on Cys residues leads to impairment of various biochemical pathways such as redox homeostasis, DNA repair, signal transduction, and metabolism [3]. Chemical mimicry of nutritional divalent metals leads to competition of Cd^{II} for these ions' metabolic pathways and binding sites. For example Zn^{II} and Cd^{II} are located in the same column of the periodic table and exist as divalent cations in biological environments. *In vitro* assays have shown that Cd^{II} can functionally replace Zn^{II} in several enzymes; Cd^{II} competes with Zn^{II} for transcription factors Sp1 and p53, which control the expression of multiple genes that are critical for cell growth and death [13, 14]. Cd^{II} displays much higher affinity to thiol groups relative to Zn^{II} and other nutritional metal ions. Characterization of Cd^{II}-induced disorders has identified the molecules, pathways, and organs that are particularly sensitive to Cd toxicity [3].

1.1.4 Oxidative stress

Cd^{II} is a redox-inactive divalent metal; however, several lines of evidence indicate that Cd^{II} can exhibit its toxic effect through the production of reactive oxygen species (ROS) [15]. The presence of a thiol group on the cysteine residue of glutathione (GSH) and its high concentration (1~10 mM) in the cells allows for its role as a redox buffer. High levels of GSH in comparison to oxidized GSSG are vital for redox homeostasis. Cd^{II} disrupts the GSH/GSSG ratio by the formation of bis(glutathionato)cadmium (Cd-SG₂) complexes which leads to reduced GSH levels and impairment of GSH-dependent enzymes such as glutathione peroxidases, glutathione

S-transferases, and glutaredoxins. Moreover, likely through its binding to active site Cys residues, Cd^{II} inhibits glutathione reductase which reduces GSSG to replenish the GSH pool and thioredoxin that plays a role to reduction of oxidized cysteine residues. Consequently, inactivation of these critical anti-oxidant molecules and enzymes by Cd^{II} induces oxidative stress. ROS-induced damage of cellular macromolecules and perturbation of cell signaling are well-characterized causal factors of diverse disorders. This argument is also supported by high Cd^{II} sensitivity of cells that possess compromised anti-oxidant systems [3, 15].

1.1.5 Perturbation of the endoplasmic reticulum

Cd^{II} causes endoplasmic reticulum (ER) stress, however; the underlying mechanisms remain to be further defined. ER stress that is associated with mis-folding or assembly problems of secretory proteins activates ER stress response regulators to induce the unfolded protein response (UPR). It was shown that Cd^{II} activates UPR in yeast *S. cerevisiae*, and cells lacking Hac1 UPR regulator are hypersensitive to Cd^{II} but not to arsenite and mercury suggesting the ER as a sensitive target of Cd^{II} [16]. This ER stress and UPR in response to Cd^{II} is conserved in mammalian cells [17]. In addition to mRNA splicing of XBP1, the functional counterpart of yeast Hac1, two other known branches of mammalian UPR, PERK, and ATF6 are both activated by Cd^{II} stress [18]. The UPR plays a critical role for overcoming ER stress by inhibiting new protein synthesis and controlling expression of genes encoding molecular factors for protein refolding and degradation. However, ER stress and UPR also induce expression of apoptotic factors and activate signaling pathways involved in cell death. This might explain Cd^{II}-triggered tissue injury as a consequence of ER stress. Despite these observations supporting Cd^{II}-induced ER stress, the causing factor(s) mediating Cd^{II} effects, such as oxidative stress, selective Cd^{II} transport into the ER, and perturbation of Ca^{II} homeostasis are not well defined. It is intriguing despite Cd^{II}'s affinity for thiols, no obvious problem in disulfide bond formation in the ER was observed. Hence, oxidative stress might not be a primary factor of ER stress under Cd^{II} exposure conditions.

Disruption of the Ca^{II} homeostasis in the ER might change Ca^{II} -dependent processes to induce protein misfolding [19]. Alternately, either Ca^{II} release from the ER as a consequence of ER stress could mediate Cd^{II} toxicity or up regulation of Ca^{II} uptake pathways in response to ER stress might serve as a self-poisoning mechanism under Cd^{II} -induced ER stress. Disruption of Ca^{II} homeostasis in the ER leads to cell death by disruption of the mitochondria through depolarization, inhibition of ATP generation, and release of apoptosis inducing factor (AIF) and endonuclease G [19]. Cd-induced apoptosis is diminished by mitochondrial Ca^{II} uniporter inhibitors suggesting the roles for Ca^{II} in conveying Cd^{II} -triggered cell death. Hence, the mitochondria appears to be another major target organelle of Cd^{II} toxicity [20].

1.1.6 Detoxification

Once Cd^{II} enters cells, it interacts with various cellular molecules primarily due to its high affinity for thiol groups. To defend against these toxic effects, cells should have Cd detoxification mechanisms, such as chelation, conversion to less toxic forms, sequestration at subcellular compartments, or efflux. Heavy metal-binding peptides including metallothioneins (MTs) and glutathione (GSH) (γ -Glu-Cys-Gly) are the first defense players against Cd toxicity. Metallothioneins (MTs) are small (5~10 kD in most cases) Cys-rich (e.g., 20 Cys in mammalian MTs) peptides which play vital roles for detoxification of heavy metals through metal-thiolate cluster formation [21, 22]. They have been found throughout the animal and plant kingdoms, fungi, and some prokaryotes. The N- and C-terminal metal binding domains coordinate multiple metals (7 Cd^{II} ions in mammalian MTs, but 2-12 depending on the metal, MTs, and experimental conditions). Yeast *S. cerevisiae* genome carries two MTs, Cup1 (61 aa) or Crs5 (69 aa). Excess copper induces expression of Cup1 via transcription regulator Ace1 [23]. Although Cd^{II} and Zn^{II} do not induce Cup1 expression, Cup1 is able to bind to Cd^{II} as well and confers Cd^{II} resistance. Crs5-mediated copper resistance is evident only when Cup1 is not present indicating it may

function as a backup of Cup1 and/or possess distinct role(s). Consistently, basal expression of Crs5 is relatively high and induction by copper is moderate relative to Cup1 [24].

Glutathione (GSH) is an abundant (1~10 mM in most cell types) tripeptide composed of Glu, Cys, and Gly. It has been identified in eukaryotes, Gram-negative bacteria, and a few Gram-positive bacteria where it serves as a major redox buffer. Various cellular processes, including redox homeostasis, storage and transport of Cys, maintenance of structure and function of proteins, metabolism of xenobiotics and heavy metals, and enzyme reactions rely on GSH [21]. The thiol group (SH) of cysteine is primarily responsible for the biological activities of GSH through its redox activities and disulfide bond formation. GSH is essential for many organisms as indicated by lethality upon deletion of GSH synthesis enzymes.

GSH can form complexes with many heavy metal ions including Cd^{II}. It occurs non-enzymatically but an enzymatic reaction for this complex formation has been proposed as well. It was shown in *S. cerevisiae*, the bis(glutathionato)cadmium (Cd-SG₂) complex is a major species, which can be sequestered into the vacuole and may be exported out of the cells. GSH in the vacuole and extracellular milieu are recycled for GSH synthesis after degradation by γ -glutamyl-transpeptidase to Glu and Cys-Gly, which is further cleaved by dipeptidase. Cytoplasmic GSH, also cleaved by the Dug peptidase complex, is slowed down by Cd. It is interesting to note a report indicating that elevated GSH levels confers Cd resistance in mammalian cells by down regulation of ZIP8 Cd importer expression through the suppression of Sp1, a transcription factor for ZIP8 [25]. This illustrates a new mode of action of GSH in conferring Cd resistance.

Vacuolar sequestration of Cd that forms complexes with GSH via a transporter-mediated mechanism has been characterized in fungi. ATP-binding cassette (ABC) transporters (e.g., Ycf1 in *S. cerevisiae*) are known to be responsible for such transport activities. Despite conserved structural similarities in mammalian ABC (MRP) transporters (e.g., 12 ABCC transporters in

human genome) with those of yeast and plants, their roles in heavy metal metabolism remain to be ascertained [26, 27].

Of the two P_{1B}-Type ATPases present in the yeast genome, (Ccc2 and Pca1), Cu^I transport by Ccc2 to the secretory pathway has been confirmed in yeast *S. cerevisiae* while the function of Pca1 has been more obscure [28]. Recent studies clearly demonstrated that by mediating Cd extrusion Pca1 plays a major role in Cd defense in yeast *S. cerevisiae* [29, 30]. Intriguingly, Pca1 contains a G970R loss-of-function mutation in all examined yeast *S. cerevisiae* lab strains. Natural yeast strains express functional Pca1, which is a primary factor conferring higher Cd resistance in the strains relative to laboratory strains. Pca1 transcription is constitutive and steady state Pca1 protein levels are extremely low. However, Cd in the culture media rapidly induces Pca1 protein expression by inhibiting Pca1 turnover through a unique and interesting mechanism. This mode of Pca1 expression control appears to be important not only for rapid response to Cd toxicity but also for activation of Pca1 specifically by Cd^{II} but not by Zn^{II} or other nutritional divalent metals. Constitutive expression of Pca1 would lead to a loss of nutritional metals as a consequence of the broad metal specificity of Pca1 like other members in this family of transporters [29, 30]. Figure 1 represents the current understanding of Cd^{II} metabolism in *S. cerevisiae* and mammals.

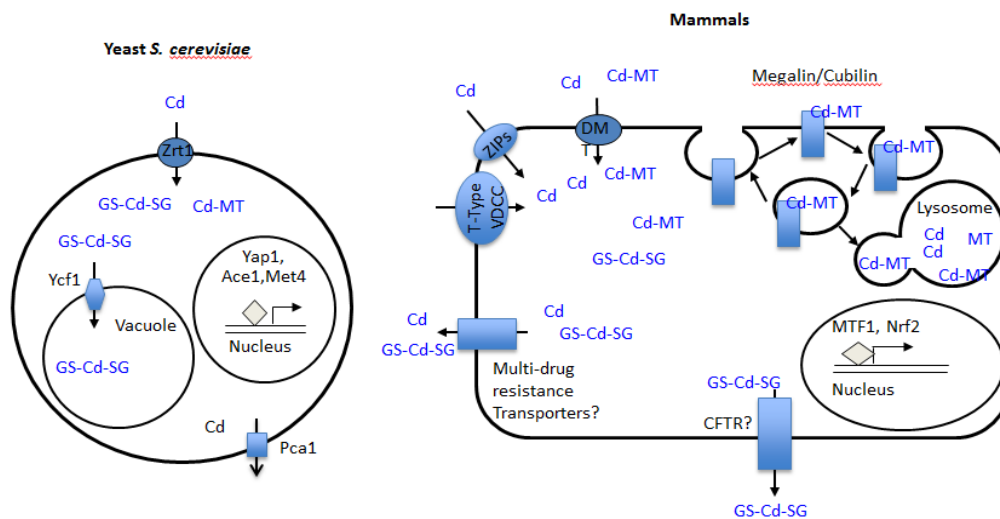


Figure 1. Cellular factors involved in cadmium uptake, chelation, sequestration, export, and transcription responses. Divalent metal ion transporters, calcium channels, cation channels/transporters, ATP-binding cassette transporters, and P_{1B}-type ATPases are responsible for cadmium translocation across the membrane. Glutathione (GSH) and metallothionein (MT) form complexes with cadmium, which chelates cadmium and provides substrates of some transporters. Cd-SG₂ indicates the bis(glutathionato)cadmium complex.

1.2 P-type ATPases

P-Type ATPases are a family of membrane proteins which utilize ATP hydrolysis to actively transport ions and lipids across membranes [31, 32]. This family is large and ubiquitously expressed. P-type ATPases are present in almost all organisms from bacteria to humans although a few parasitic bacteria appear to have no P-type ATPases [33]. They also have a wide range of function including establishing and maintaining electrochemical gradients, muscle contraction, cellular signaling, delivery of cofactors to specific cellular compartments, and removal of toxic heavy metals through extrusion from the cell [29, 31, 32, 34]. The P-type ATPase family is divided into five branches (I-V) where each branch can contain several subgroups. For example there are up to seven subfamilies for the P_{1b}-type ATPases [35]. Type I P-type ATPases are ion pumps which can be broken into two sub classes P_{1A}- and P_{1b}-Type. 1A is a relatively small group of bacterial ion pumps. Unlike most P-type ATPases which transport cations such as H^I, Na^I, K^I, Mg^{II}, and Ca²⁺, P_{1B}-Type is a much larger group ranging from bacteria to humans, and they are involved in the transport of transition metal ions such as Cu^I, Cu^{II}, Ag^I, Pb^{II}, Zn^{II}, and Cd^{II} [31]. Type II and III are involved in generating and maintaining membrane potential. IV and V are closely related to Type-I; however, IV is a class of transporter known as a “flipase” involved in lipid transport and have only been found in eukaryotes [31, 36]. Type-V is often referred to as the orphan transporters and no specific substrate has been identified for this class of transporters [32, 37].

The field of study regarding P_{1B}-Type ATPases has been well established due to the discovery that the mutations in two Cu transporting P_{1B}-type ATPases, ATP7a (MNK) and ATP7b (WND), are responsible for Menke’s and Wilson’s disease [38-40]. These proteins function in the secretion and excretion of Cu in humans. The yeast *S. cerevisiae* Ccc2 is a close homolog of these proteins and functions in Cu^I delivery to the secretory pathway [41, 42]. Pca1 is also a member of the P_{1B}-Type ATPase family and provides Cd^{II} resistance through excretion of

the toxic heavy metal [29]. Recently, the crystal structure of a homolog from *Legionella pneumophila* was solved opening new doors of study for determination of mutational changes, structure changes during the catalytic cycle, and domain function determination [43].

1.2.1 Mechanism of transport

The mechanism of ion transport for P-Type ATPases follows the Post-Albers cycle which was established using Na^+ and K^+ as the substrates being transported [44]. The metal ion binds to the E1-form of the ATPase at the cytoplasmic side of the transporter which triggers phosphorylation by ATP forming the E1-P state. This is then converted to the E2-P form which cannot phosphorylate ADP and subsequently leads to a reduced affinity for the metal and releasing of the metal to the other side of the membrane. The release of P_i allows for a reset of the protein to the E2 state so it can restart the cycle (Figure2) [31, 44].

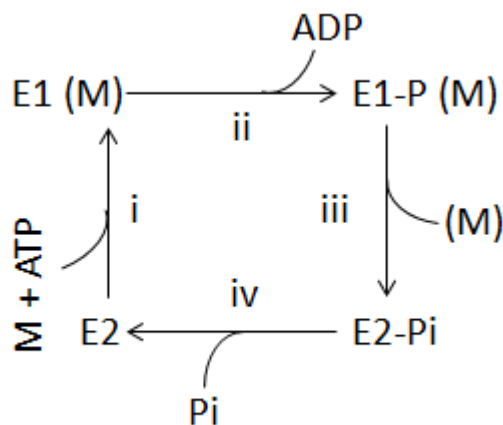


Figure 2. Mechanism of P-type ATPase metal transport. (i) Metal (M) and ATP bind to the E2 state of the protein followed by release of ADP to from the E1-P state (ii). The metal's affinity for the protein lessens and is released to form the E2-Pi state (iii). (iv) P_i is released to allow for the cycle to start over.

1.2.2 Structure

P-1B-type ATPases are comprised of three primary cytoplasmic domains essential for function, the N (nucleotide binding), P (phosphorylation), and A (actuator) domains [32]. P1B-type ATPases differ from other P-type ATPases because they contain eight transmembrane (TM) domains instead of the standard ten. They contain an extra two TM helices at the N-terminal end of the protein but have lost the final four TM helices present in other family members [31]. A conserved CPX/SPC motif present in the 6th TM helix serves as a putative metal transporting site although it is evident residues in the 7th and 8th TM domains are important for transport and ion selectivity as well [43, 45]. P1B-type ATPases also contain conserved N-terminal metal binding domains (MBD) in which the number of repeats can vary [46]. Characterization of these domains has been attempted for ATP7b and ATP7a as well as their yeast homolog CCC2 [47, 48]. The consensus GMTCXXCXXXIE where the CxxC forms the metal binding pocket was determined in ATP7a and ATP7b each of which have six of these N-terminal MBDs while Ccc2 contains two [48]. Cu^I chaperones ATOX1 in humans and Atx1 in yeast have been shown to deliver Cu^I to these MBDs [49, 50]. It has been hypothesized that these domains play a role in delivery of Cu^I to the transportation channel or a role in regulating the ATPase. It was found through truncation and chimera ATX1-CCC2MBD creation the MBDs appear to convey Cu to another Cu binding site in CCC2 indicating a functional role in activity [47].

Regulation of P-type ATPases is important for maintaining proper ion homeostasis within cells. This can occur at both the transcription and post-translational level. AtHMA4, a plant P_{1B}-type ATPase, is transcriptionally regulated in response to metal accumulation [51, 52]. ATP7a and ATP7b are regulated in response to Cu^I by their sub-cellular localization. Under times of high, Cu, ATP7a mobilizes from the golgi to the plasma membrane to export Cu and prevent toxicity [53, 54]. ATP7b functions under normal Cu levels as a transporter of Cu into the secretory pathway; however, under high Cu conditions it mobilizes to the membrane of liver cells

for excretion of Cu into the bile to prevent the toxic accumulation of Cu [54]. Pca1 is also regulated at the post translational level by its substrates levels. In the absence of Cd, Pca1 is rapidly turned over; however in the presence of Cd^{II}, Pca1 is rapidly stabilized [30]. Thus, Pca1 expression and stability is dependent on its substrate.

1.3 Protein synthesis and the secretory pathway

Protein members of the secretory pathway are synthesized by the ribosome and inserted into the endoplasmic reticulum (ER) lumen or membrane through the sec61 translocon [55-57]. This allows for maturation and folding, an essential step for the formation of functional proteins. The oxidative environment of the ER allows for di-sulfide bond formation through the actions of protein disulfide isomerases which can both form and break disulfide bonds [58]. Other modifications, such as N-linked glycosylation also take place in the lumen. Luminal chaperone-complexes, such as the Hsp70 Kar2 (Bip in mammalian cells) which includes the Hsp40/DnaJ-like proteins Jem1 and Scj1, are essential for holding and folding mis/unfolded proteins. These complexes also function in the degradation of the malformed peptides [59, 60]. Following successful maturation, proteins are then moved through the secretory pathway to reach their location of function. Transport of these cargo proteins through the secretory pathway requires vesicle formation. Budding and release from the ER, these vesicles are formed by the coating of a region of the ER membrane with Coat protein complex II (COPII). These COPII vesicles move to the golgi where they fuse with the golgi, and the COPII proteins are removed. Retro-grade transport is carried out in a similar fashion with COPI proteins forming the vesicle at the Golgi for returning escaped luminal proteins or proteins which escaped degradation to the ER [61].

1.3.1 ER quality control/UPR

In the event of failed protein folding/refolding at the ER, the malformed protein must be removed to prevent the toxic accumulation and aggregation of these proteins through activation

of the unfolded protein response (UPR) a conserved pathway from yeast to humans. In yeast, UPR, is triggered by the homodimerization of IRE1 an ER resident transmembrane protein kinase/endonuclease which splices the mRNA of Hac1 (Xbp1 in mammals) leading to its translation as a transcription factor [62, 63]. The mammalian pathway contains two additional branches in which Atf6 and PERK in addition to Ire1 function at the ER to activate a response [64]. In yeast Hac1 mobilizes to the nucleus and binds to DNA by recognizing the unfolded protein response element (UPRE) and up regulates several genes coding for proteins involved in the degradation of misfolded proteins as well as several chaperone-like proteins which maintain solubility and enhance secretion of functional proteins [65]. At the same time, transcription of nonessential secretory proteins is suppressed to limit the flux through the secretory pathway. Many of the targets are members of the ER-associated degradation (ERAD) system [66]. Through this system misfolded ERAD substrates are first recognized, ubiquitinated, and then degraded by the cytosolic proteasome. The malfunctioning of ERAD leads to the chronic accumulation of malformed proteins, and this continual induction of the unfolded protein response will result in cell death. The toxic accumulation and aggregation of misfolded proteins has been linked several degenerative disorders such as amyotrophic lateral sclerosis, Alzheimers, and Parkinson's disease [67-70].

1.3.2 ER-Associated degradation system of *Saccharomyces cerevisiae*

The ERAD system contains three major pathways in *Saccharomyces cerevisiae* commonly known as ERAD-L, ERAD-M, and ERAD-C [71]. These refer to the location of the misfolded region of the substrate: luminal, membrane, and cytosolic respectively. Proteins containing misfolded regions in the lumen and cytosol are ubiquitinated via the E3 ubiquitin ligase Hrd1. Membrane proteins containing a cytosolic misfolded region are ubiquitinated by the Doa10 E3 ligase. These three pathways are conserved in mammalian cells; however, mammalian cells contain several characterized E3 ubiquitin ligases which function in ERAD.

GP78, an orthologue of yeast Hrd1, hHrd1 an N-terminal homologue of Hrd1, and Teb4, a Doa10 homologue, have been identified in the ER membrane to carry out the ubiquitin ligase activity in mammalian ERAD. Cytoplasmic E3s such as CHIP, SCF^{FBX2}, and Parkin have also been identified in mammals as potential ERAD ubiquitin ligases [72-74]. Many additional factors are involved to deliver substrates to the ubiquitin ligase complex and following ubiquitinylation, extraction, and degradation at the proteasome (See table 1 for list of ERAD components and function and Figure 3 for a schematic of ERAD in *S. cerevisiae*).

1.3.3 Recognition of ERAD-L and ERAD-M substrates

The Hsp70, Kar2 (Bip in mammalian cells), has been implicated in the recognition of ERAD substrates containing a luminal misfolded region [75]. Kar2 is not alone in this process as several other proteins have also been connected to this process. Scj1 and Jem1 are two DnaJ/Hsp40 proteins which interact with Kar2 and help with recognition. Kar2 in conjunction with Scj1 and Jem1 recognize misfolded proteins by binding to exposed hydrophobic regions to maintain their solubility [60]. In the event of failure to re-fold, the protein is escorted to ERAD machinery in a Kar2 dependent manner. Other proteins involved in this process include the lectin yos9 which recognizes misfolded N-linked glycosylated proteins and helps with delivery of these to Hrd1 [76, 77]. Htm1/Mnl1, in conjunction with PDI1 again assists in the destruction of glycoproteins through modification of the N-linked glycan to Man7GlcNac2 (Mannose7, N-acetylGlucosamine2), which is a signal for destruction on the target substrate [78].

1.3.4 Recognition of ERAD-C substrates

ERAD substrates containing a cytoplasmic misfolded region appear to be recognized as degradation substrates through the action of resident cytoplasmic proteins. The cytoplasmic Hsp70 chaperones have long been considered as protein complexes which function to refold un/misfolded proteins by binding to exposed hydrophobic or oily patches [79]. Hsp70s contain

two functional domains: a nucleotide binding domain in which ATP binds and is hydrolyzed to ADP and a substrate binding domain [80, 81]. Hsp70s require a co-chaperone, Hsp40, for proper holdase and refolding function [82]. Hsp70 substrate binding is accomplished by binding to exposed hydrophobic regions on proteins. Release of the substrate requires nucleotide exchange factor activity to form an ATP-Hsp70-Hsp40 complex [79, 83]. Rebinding of a polypeptide stimulates hydrolysis of ATP. A nucleotide exchange factor allows for the switching of this complex from the ADP-ATP bound state. Yeast contains three types of nucleotide exchange factors Sse1/2, (Hsp110s), Fes1 (HspBP1) and Ssn1 (Bag-1, Bag protein homologues) [84, 85]. Recently however, it been determined that Hsp70s chaperones do not only function in the refolding of misfolded proteins but also in escorting misfolded proteins to degradation [84, 86, 87]. These protein complexes function in a similar manner as the luminal Hsp70 Kar2 as that they require both Hsp40 co-chaperone and nucleotide exchange factors.

Ssa1 and Ssa2 are two Hsp70s in yeast that have been implicated in the degradation of the ERAD-C substrate Ste6*, a mutated form of the alpha factor transporter Ste6. Their genetic mutation leading to a temperature dependent loss of function resulted in increased stability of Ste6*[87]. This role for Ssa1 and Ssa2 in degradation is not limited to ERAD. Ssa Hsp70s have also been identified as factors in cytoplasmic ubiquitin proteasome substrates specifically with Fes1 functioning as a nucleotide exchange factor. A proteasome substrate dihydrofolate reductase (DHFR) mutant requires Hsp70, ssa1 and Hsp40, ydj1 as well as a nucleotide exchange factor, Fes1, for its efficient degradation [84]. The mechanism by which proteins are selected for degradation over holding/refolding is still unclear. It does appear the nucleotide binding domain is very important for this activity. Ssa1 and Ssa2 were shown to have distinct activities regarding the prion propagation of [URE3] and the degradation through the vacuole of FBPase the gluconeogenic enzyme. Ssa1 and Ssa2 differ in only four residues. By mutating only a single residue G83 in the nucleotide binding domain of Ssa2 to the Ala contained in Ssa1, [URE3]

propagation was reduced in a similar manner to Ssa1 wild type expression indicating the importance of this domain in determining the function of stability versus degradation [88]. The interacting factors may also play an important role as the nucleotide exchange factor, Sse1, is indicated in the regulated degradation of ER substrates, whereas Fes1 has been linked to the degradation of cytoplasmic substrates [84, 85]. The Hsp40 bound to Ssa1 also appears to be important as Ydj1 is indicated in the degradation of Ste6* through ERAD, but the degradation of the short-lived GFP was shown to require Sis1 (HSP40), whereas Ydj1 expression led to its stabilization [89, 90]. It is important to note these mechanisms of chaperone assisted degradation are conserved in mammals. The NEF HSP110 has been shown to increase degradation of the pathogenic mutant form, cystic fibrosis transmembrane conductance regulator, CFTR Δ 508 [91]. Chaperone assisted degradation is also not limited to the ubiquitin proteasome pathway. Chaperone mediated autophagy is a developing field in which it has been determined the degradation of specific substrates through autophagy often times require the binding of a chaperone for direction to the autophagosome [92].

1.3.5 Ubiquitylation of misfolded proteins

After recognition, ERAD substrates are directed to ubiquitylation machinery where ubiquitin (ub) is covalently conjugated to the target substrate through the ϵ -amino group of lysine residues. This process requires three specific enzymes referred to as E1, E2, and E3. E1, or ubiquitin activating enzyme, generates a Ub thioester bond between itself and Ub in an ATP dependent manner. This Ub-E1 complex then transfers the Ub to the E2, ubiquitin conjugating enzyme, again through a thioester bond. Here with the help of an E3 ubiquitin ligase the Ub group is transferred to the substrate. This transfer is either direct from the E2 where the E3 acts as a facilitator as is the case with U-box and RING domain E3 active site containing proteins, or Ub is first transferred to the E3 from the E2 where it is then attached to the substrate as with HECT domain containing E3s [71]. Hrd1 and Doa10, the E3s of ERAD in *S. cerevisiae* are both

RING domain proteins facilitating Ub of substrates through the E2 conjugating enzymes Ubc7, Ubc6, and Ubc1 [93-95]. Ubc6 is an integral membrane protein of the ER but Ubc7 requires an adaptor protein, Cue1, to maintain ER association [96]. Doa10 utilizes both Ubc6 and Ubc7 whereas Hrd1 requires Ubc7 for function and at least in the case of CPY*, carboxy-peptidase yscY-S255R, a mutated form of the vacuolar carboxypeptidase Y. Ubc6 and 7 are conserved in higher organisms, UBC6e and UBCH7 in mammals. Ubc6e like its yeast counterpart is tethered to the ER membrane, whereas UBCH7 is recruited to the membrane by a Cue like domain present on the E3 ligase GP78 [60, 71].

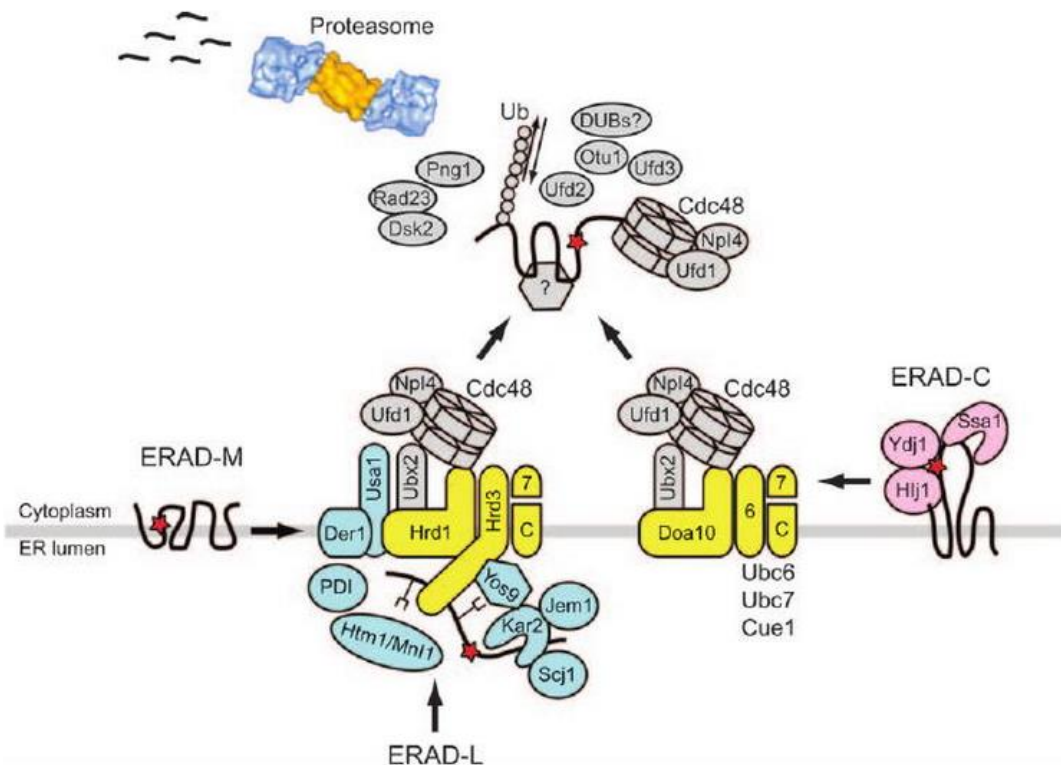


Figure 3. Working model of ERAD in yeast *S. cerevisiae*. ERAD-M and L substrates (red star indicates location of mutation/misfolding) are ubiquitinated through the hrd1 E3 ubiquitin ligase complex. Luminal factors involved in recognition and delivery of substrates to Hrd1 include, Yos9, Kar2-Jem1-Scj1 complex, Htm1/Mnl1, and PDI. Der1, Usa1 and Hrd3 are involved in recognition of substrates as well. Cue1, C, functions as a dock for the E2 ubiquitin conjugating enzyme Ubc7, 7, just as Ubx2 functions as the platform for Cdc48-npl4-ufd1 complex membrane association. The ERAD-C pathway substrates are recognized by cytoplasmic chaperones; Ssa1-Ydj1-Hlj1, and delivered to the Doa10 complex for ubiquitination. Ubc6, 6, is also involved in this pathway and functions as an E2 conjugating enzyme. Cytoplasmic components include poly-ubiquitination enzymes such as Ufd2 and deubiquitination enzymes, Otu1. The adaptor proteins Rad23, Dsk2, and Png1 have been found to help in degradation of ERAD substrates as well. The proteasome degrades the ubiquitinated substrates. This figure is adapted from [1].

1.3.6 Retro translocation, extraction and degradation of misfolded proteins

ERAD substrates are members of the secretory pathway and reside in the lumen or ER membrane and must eventually reach the proteasome for degradation. Several candidates have been proposed for the retrotranslocon, or channel which allows for movement of proteins from the ER to the cytosol, without a specific protein exclusively performing this role. Sec61 and derlin1 as well as the E3 ubiquitin ligases themselves have been proposed as possible retrotranslocation channels [97, 98]. There are several lines of evidence indicating Sec61 may

function not only as the translocon for insertion of secretory proteins into the ER following translation but also in the reverse for removal of proteins from the ER. Mutation of Sec61 results in the stalled ERAD of substrates, delaying the export of CPY* from the lumen. It was also found this does not work alone. Der1, Hrd1, and Kar2 were also indicated to work in conjunction with Sec61; however, a specific mechanism has not been determined [75, 99, 100]. This was also studied using the Deg1-Sec62 chimera, shows deg1 is a degradation signal that targets Mata2, a transcriptional repressor, for Doa10 dependent degradation. Here Sec61 mutation led to stabilization of the protein for post-translational modification, but mutation of the channel itself had no effect on the degradation of the chimera [101].

The idea that E3s may function as the retrotranslocon is an appealing hypothesis as both of the E3 ubiquitin ligases contain several TM domains which do not appear to be necessary for ubiquitin ligase function, (six for Hrd1 and fourteen for Doa10). *In vitro* study of the integral membrane ERAD-M substrate HMG2p (HMG CoA reductase yeast homolog) degradation revealed the necessity of Hrd1 for dislocation from the membrane. This dislocation from the membrane did not require Hrd1 transmembrane domain indicating it was not the translocon [102]. This study also ruled out Sec61 and Der1 as being involved in this process. This study emphasized the importance of the Cdc48 complex, homolog to the mammalian p97. Cdc48 is an AAA-ATPase and has been determined to work in dislocation of proteins from and through the ER membrane for ERAD. Cdc48 forms a complex with Npl4 and Ufd1 proteins which have been determined help bind poly-ubiquitylated proteins. These proteins may be involved in their recognition and are conserved in mammalian cells. Cdc48 is recruited to the ER membrane by Ubx2 allowing for close association with ERAD machinery and substrates [103]. It has also been shown to interact with ERAD components, Hrd1 and Der1 in yeast, and in mammalian cells, GP78 [104, 105]. The Cdc48-ufd1-Npl4 complex contains ubiquitin binding domains which function to bind to ubiquitylated substrates but are also able to bind non-ubiquitylated

substrates [106]. Once bound to the substrate, it can function in an ATP-dependent manner to extract proteins from the membrane. Mutation of Cdc48 prevents degradation of many ERAD substrates from all three pathways resulting in their inability to be removed from the ER lumen or membrane [71].

Aside from Ufd1 and Npl4, Cdc48 contains other interacting partners which have been implicated in the ERAD of some substrates. Ufd2, an E4 ubiquitin ligase, binds to the Cdc48 complex and functions to extend ubiquitin chains on degradation substrates [107]. Ufd2 binds to the C-terminal tail of Cdc48 and competes for binding with Ufd3, a protein involved in regulating ubiquitin and ubiquitin pools. Ufd2 is conserved in humans (UFD2a, E4B); however unlike the yeast Ufd2, E4B binds to the N-terminus of p97 and conveys the same function [107].

Ubiquitylation by an E3 ligase is not always sufficient for efficient degradation. E3s add ~3 ubiquitin groups to a given substrate further ubiquitylation is carried out by E4s which function as chain extension enzymes. The exact necessity for this is not known; however, it is certain that poly-ubiquitylation is important for efficient degradation of substrates and has been hypothesized to function as a ratcheting mechanism allowing ubiquitin binding proteins to maintain interaction with a substrate during degradation. There are several ubiquitin receptors on the 26S proteasome, specifically on the 19s regulatory particle, Rpn10, Rpn13, and ATPase5 [71]. E4 ligases also exist on the proteasome itself. Hul5 is a proteasome resident protein which has been implicated in the ERAD of a synthetic ERAD-L substrate through its function in ubiquitin chain extension [108, 109]. Recently another cdc48 resident protein (VMS1) has been identified to function in degradation of Ub-proteasome substrates. There is, however, conflicting data on whether it has a role in the degradation of ERAD substrates [110, 111].

Cdc48 associates directly with the 19s regulatory particle of the 26s proteasome which is mediated by Rad23 and Dsk2, ubiquitin-like domain and ubiquitin associated domain containing proteins. They bind poly-ubiquitylated substrates and direct them to the proteasome for

degradation. Rad23 has been shown to interact with both Ufd2 on Cdc48 and the proteasome, Rpn1, bridging the gap between the proteasome and Cdc48 and allowing for efficient degradation [71, 112]. Rad23 also interacts with Png1, a de-glycosylating enzyme, to degrade glycosylated ricin A chain [113]. A deglycosylation event may be necessary to allow the substrate to be fed into the proteasome for degradation and prevent any steric hindrance the glycosyl group may cause.

During the course of degradation and following poly-ub, de-ub occurs prior to proteolysis. This allows for the recycling of ubiquitin as well as the entrance of a substrate into the proteasomes catalytic core. In mammals the de-ubiquitinating enzyme for ERAD has been identified as ataxin-3, and its mutation slows the degradation of the ERAD substrate TCR α [114]. In addition mutation of ataxin-3 also induces the UPR further supporting its role in ERAD. De-ubiquitinylation is also a means of controlling expression of proteins in the cell. Usp25, a ubiquitin specific protease in mammalian cells, can extend the life of the ERAD substrate CD3 δ , which is ubiquitinated by Hrd1 for degradation. Usp25 removes the ubiquitin from CD3 δ thereby rescuing it from ERAD [115]. Despite the multitude of data regarding the extraction of proteins from and through the ER membrane during ERAD, retrotranslocation of substrates remains a hotly debated subject. There are several data supported hypotheses which exist about the fate of ERAD polytopic substrates following ubiquitinylation. First, the protein is fully extracted from the membrane through the action of the CDC48 complex and present in the cytoplasm as a full-length protein. The substrate is then escorted to the proteasome for degradation (Figure 4) [1, 87]. Second, the protein is cleaved by the proteasome prior to extraction. Then the small pieces are extracted and taken to the proteasome for degradation. This is a possibility as it has been displayed that the proteasome has endoproteolytic activity and is not simply used for degradation of the whole protein [116]. Finally the proteasome itself is involved in the extraction of the substrate for degradation. This is appealing as release of a hydrophobic

poly-topic protein into the hydrophilic environment of the cytoplasm could result in massive protein aggregation and would require many factors to maintain solubility. Supporting this, the 19s regulatory particle of the proteasome has been implicated in this process [117]. These hypotheses will be tested in Chapter two.

1.3.7 Proteasome structure and degradation

The 26s proteasome is a large multi-protein complex comprised of a catalytic core 20s and a 19s regulatory particle/cap. The 20s core can exist independently of the 19s and vice versa. The 20s core is comprised of twenty-eight subunits forming a cylinder containing four stacked rings of seven subunits each [118]. This ring structure is: $\alpha 1-7$, $\beta 1-7$, $\beta 1-7$, $\alpha 1-7$ in yeast where each subunit of each ring is different. Only the $\alpha 3$ subunit is non-essential as it is replaced with the $\alpha 4$ under certain circumstances [119]. The α rings provide a docking location for the 19s regulatory particle, whereas the β rings form the active sites. There are three different forms of active sites which differ in activity. There is a chymotrypsin-like site in the $\beta 5$ subunit as well as a trypsin-like domain contained in the $\beta 2$. The $\beta 1$ subunit houses a post-glutamyl peptide hydrolytic (PGPH) domain [120]. These differences allow for substrate specificity [121].

The 19s regulatory particle is comprised of seventeen subunits which function in recognition, unfolding, and feeding of substrates to the 20s core. There is also evidence that the 19s regulatory particle plays a pivotal role in extraction of proteins from/through the membrane [60, 122, 123]. This is accomplished through the activity of six AAA-ATPases, Rpt1-6. Other subunits of this structure are involved in binding ubiquitinated substrates and de-ubiquitinating substrates to allow for degradation through the 20s activity [124].

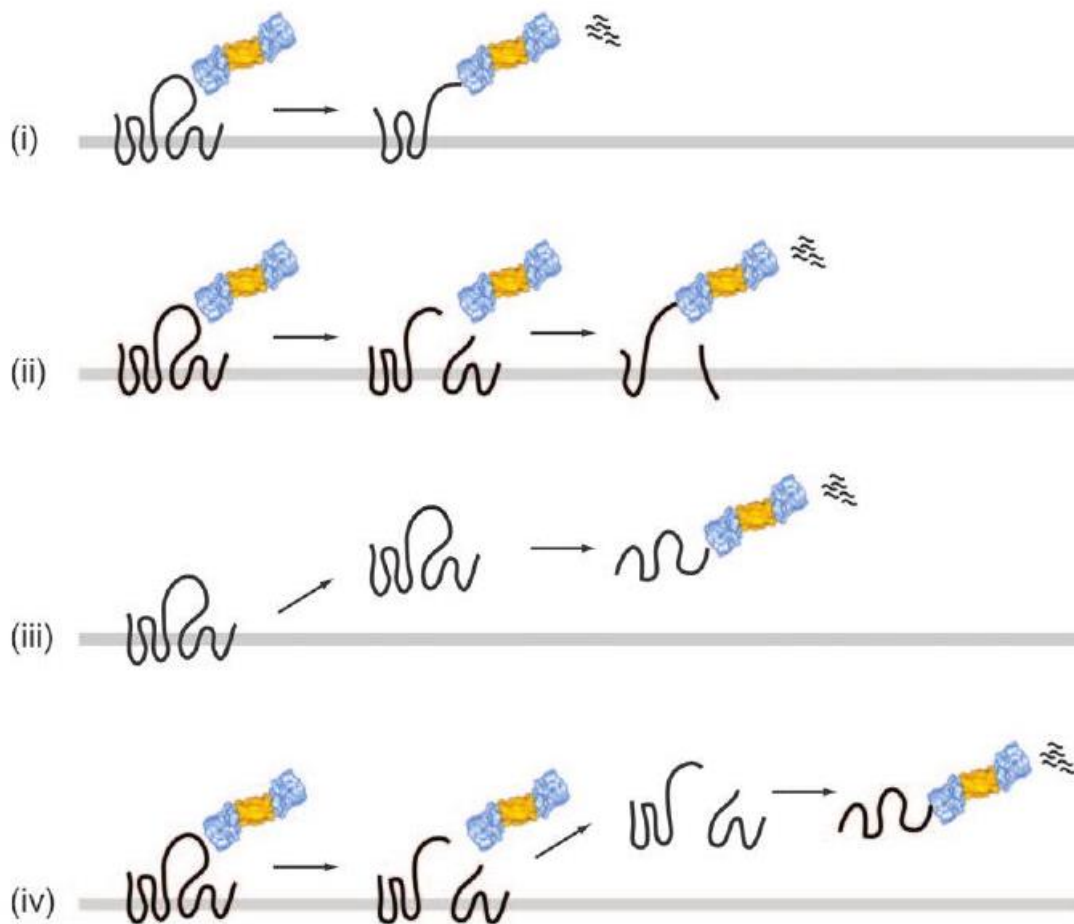


Figure 4. Potential mechanism for polytopic protein dislocation from the ER and the role of the proteasome. (i) Direct feeding of the polytopic protein into the proteasome without dislocation from the ER. (ii) The proteasome first cleaves cytoplasmic loops followed by direct feeding of the cleavage products into the proteasome for degradation. (iii) The protein substrate is first dislodged completely from the membrane followed by degradation in the cytoplasm by the proteasome. (iv) The cytoplasmic loops are first cleaved by the proteasome then the cleavage products are dislodged from the ER membrane and degraded in the cytoplasm. This figure is adapted from [1].

1.3.8 The specific ER-associated degradation of characterized substrates:

The ERAD of several substrates has been well characterized with the major factors involved being identified. This is especially true for the ERAD-M substrate 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase (HMG2 in yeast) and the ERAD-L substrate CPY*. Both are directed through Hrd1 for ubiquitinylation, although mammalian cells utilize gp78 and trc8 for degradation of HMG CoA reductase [125]. The mammalian HMG CoA-

reductase performs the rate-limiting step of cholesterol synthesis, and its degradation is regulated by sterol composition in the membrane. When there is an abundance of sterols integrated into the membrane, HMG CoA reductase is targeted to GP78 for ubiquitinylation and degradation. This occurs through interaction with insigs1 via its N-terminal domain [125]. The N-terminal transmembrane domain of HMG-CoA reductase functions as the required and sufficient domain for degradation [126]. This domain contains no catalytic activity, and when removed, the remaining catalytic domain remains functional [126, 127]. A sterol sensing domain within HMG CoA reductase recognizes the sterol composition of the membrane and leads to the binding of HMG CoA reductase to insigs-1 which interacts with GP78 allowing for GP78 dependent ubiquitinylation [125, 128]. Recently, it has been determined a similar mechanism, using either insigs-1 or insigs-2 functions to link HMG CoA reductase to the E3 Trc8 for ubiquitinylation and degradation [125]. This displays the role for multiple E3s in the degradation of a single substrate. Under times of low sterol levels, HMG CoA reductase is not degraded as it does not interact with insigs-1 or 2 and is able to perform its function in regulated cholesterol synthesis. The mechanism is very similar in yeast with HMG2 although the feedback is not through sterols, instead flux through the cholesterol synthesis pathway is key [129]. HMG2 recognizes farnesyl pyrophosphate (FPP) levels. FPP is an intermediate in the cholesterol biosynthetic pathway. Under conditions in which FPP levels are elevated, HMG2 is degraded rapidly, and conversely under times of limited FPP, HMG2 is more stable [130]. Recent studies have claimed that geranylgeranyl pyrophosphate (GGPP) is the more likely signal [127, 129]. When exposed to FPP or GGPP, HMG2 undergoes structural changes which leads to its recognition as a misfolded protein [129]. Although FPP and GGPP are not sterols, the conserved sterol sensing domain still stimulates the ERAD of HMG2 in a GGPP dependent manner [129].

CPY*, a mutated form of the vacuolar carboxy-peptidase Y containing a S255R, is turned over through the ERAD-L system. After translation and transportation into the ER lumen, it is

folded and glycosylated. With the 255R mutation, it is unable to be secreted to the vacuole and is instead recognized as an ERAD substrate and directed to Hrd1 for ubiquitinylation and degradation. CPY* has long been used as a model for the study of ERAD-L and determining the constituents involved. The study of CPY* has led to the discovery of a carbohydrate-retention mechanism for preventing the secretion of CPY* to the vacuole [60]. The glycosylation of CPY* is necessary for this function. The glycosylation modification is trimmed by glucosidases 1 and 2 followed by Mns1 [131]. This functions as a timer for folding. If folding is unsuccessful, CPY* is recognized by Htm1. As previously described, this binds $\text{Man}_8\text{GlcNAc}_2$ and leads to modification of this group to $\text{Man}_7\text{GlcNAc}_2$, which acts as a signal for degradation [78]. The previously discussed Hsp70 chaperone complex, Kar2 Jem1p and Scj1p, function in the regulated degradation of CPY* providing the escort services required for direction to the membrane resident machinery [75]. PDI is also required for efficient degradation of CPY* as removal of PDI enzymatic activity resulted in stabilization and loss of export of CPY* to the cytosol [132]. Other ERAD components identified in CPY* degradation include Der1 and Hrd3. These may play a role in recognition and retrotranslocation of CPY* for ubiquitinylation by the cytoplasmic Ring-domain of Hrd1. The hypothesized retrotranslocon for CPY* is sec61 where both hrd3 and Der1 may play a role in this [60]. The AAA-ATPase Cdc48 also plays an important role in the extraction and removal of CPY* from the ER. Study of the extraction of CPY* displayed Cdc48 itself is sufficient for extraction of CPY* from the membrane, and the components of the 26s proteasome, specifically the AAA-ATPases on the 19s proteasome, are not required for this process [95].

As for the determination of the factors involved in the degradation of ERAD-C substrates, Ste6* has been utilized. Ste6* contains a Q1249X which causes a premature stop codon and change in N-glycosylation leading to its degradation through the ERAD-C pathway via Doa10 dependent ubiquitinylation [133, 134]. Ste6* is recognized by cytoplasmic Hsp70

chaperone complexes directing it to the Ubc7 Doa10 complex for ubiquitinylation. Cdc48 activity as well as Ufd2 poly-ubiquitinylation is required for the efficient degradation of the protein [87].

A more unique substrate is the cytosolic/nuclear protein Mata2 whose degradation is dependent on the ERAD-C machinery, Doa10, ubc7, and ubc6 despite not being an ER-resident protein. Mata2 also contains a unique degron, (minimal region required for degradation) Deg1, a 67 amino acid stretch which can be transplanted onto other proteins leading to their regulated degradation through the ERAD system [94]. Mata2 is a transcriptional repressor involved in governing yeast, *Saccharomyces cerevisiae*, cell type. There are three cell types: two haploid forms referred to as, a and α , as well as a diploid a/ α formed by the mating of the two haploid cells [135]. These types are all controlled via the *MAT* locus, which codes for specific genes determining cell type such as Mata2. It was determined Mata2 was short lived in α cells but quite stable in the diploid a/ α cells [136]. Mata2 forms a heterodimer with Mata1 in diploid cells which is responsible for this stability. A 19aa region was determined to be the essential determinant of the Deg1 degron, aa14-32. This region forms an amphipathic helix necessary for the formation of a coiled-coil interaction between Mata2 and Mata1. In haploid cells, this interaction does not occur leading to rapid Mata2 turnover [135].

Pca1 is a cadmium-extruding P-type ATPase that plays a major role for cadmium detoxification in yeast *Saccharomyces cerevisiae*. PCA1 contains a cysteine rich N-terminal, cadmium responsive domain, which targets Pca1 for degradation in the absence of cadmium to the proteasome. Cadmium rapidly up regulates PCA1 by preventing its ubiquitinylation and subsequent degradation by the proteasome [30]. This makes Pca1 unique in that its expression is dependent on its substrate directly leading to its stabilization through a post-translational mechanism. Pca1 is not inherently misfolded, but it appears to be recognized as such. Removal of the N-terminal domain, aa1-392, results in complete stabilization of Pca1, and the specific

determinants of how this domain functions in Pca1 degradation will be discussed in later chapters [30]. As Pca1 is a P1b-type ATPase, multi-transmembrane domain containing protein member of the secretory pathway, degradation at the cytosolic proteasome requires a mechanism by which Pca1 is extracted from the membrane and degraded. The mechanism by which this occurs has not been established and will be discussed in the following chapters as well many of the players in Pca1 degradation (Figure 5).

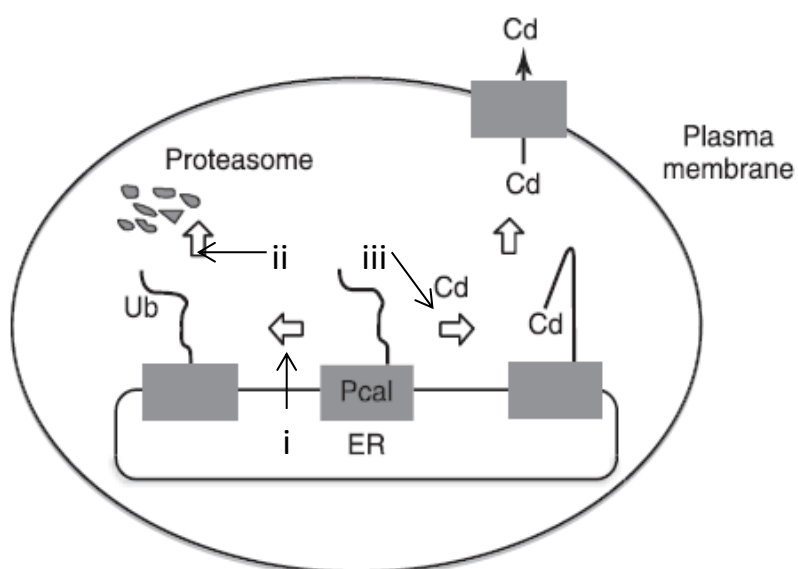


Figure 5. The following chapters will focus on the mechanism of Pca1 degradation (i). The mechanism of polytopic membrane protein extraction to the proteasome (ii). The mechanism of Cd^{II} sensing of Pca1 and the factors involved in recognition of Pca1 for degradation (iii).

Table 1

Component	Location	Yeast	Mammals
Recognition			
Hsp70 family	ER	BiP (also known as Kar2)	BIP (also known as GRP78)
	Cytoplasm	Ssa1	HSP70 and HSC70
Hsp40 family	ER	Scj1 and Jem1	ERDJ1–5 and p58IPK
	Cytoplasm	Ydj1 and Hlj1	HDJ1–2, HSJ1 and Cys string protein
Nucleotide-exchange factor	ER	Si11 (also known as Sls1) and Lhs1	SIL1, GRP170 and BAP
	Cytoplasm	Sn11, Fes1 and Sse1	BAG1–2, HSPBP1 and HSP110
Small Hsps	Cytoplasm	Hsp26 and Hsp42	α -Crystallin
Hsp90 family	ER	Unknown	GRP94
	Cytoplasm	Hsp82 and Hsc82	HSP90
Protein disulphide isomerase	ER	Pdi1 and Eps1	PDI, ERP29, ERP57, ERP72 and ERDJ5
α -Mannosidase-like	ER or ER membrane	Htm1 (also known as Mnl1)	EDEM1–3
Mannose-6-phosphate receptor-like	ER	Yos9	OS9 and XTP3-B
UBL domain containing	ER membrane	Usa1	HERP
Sec61 complex	ER membrane	Sec61 complex and Ssh1 complex	Sec61 complex
Derlins	ER membrane	Dfm1 and Der1	Derlin-1–3
Regulators?	ER membrane	Usa1	HERP
		Ubx2	unkown
		Unknown	VIMP, BAP31 and SVIP
E1 ubiquitin-activating enzyme	Cytoplasm	Uba1	UBE1
E2 ubiquitin-conjugating enzyme	ER membrane	Ubc6	UBC6e
	Membrane associated	Ubc7–Cue1 complex	UBCH7 (also known as UBC7)
	Cytoplasm	Not established	UBCH5
E3 ubiquitin ligase	ER membrane	Hrd1–Hrd3 complex	HRD1–SEL1L complex
		Doa10	TEB4 (also known as MARCH IV)
		Unknown	GP78 and RMA1 (also known as RNF5)
	Cytoplasm	Rsp5	NEDD4–2

		Unknown	Parkin, CHIP and SCFFBX2 or SCF2FBS2
E4 chain-extension enzyme	Cytoplasm	Ufd2	UFD2a
Cdc48 complex	Membrane associated	Cdc48-Ufd1-Npl4	p97-UFD1-NPL4
UBL and UBA domain containing	Cytoplasm	Rad23 and Dsk2	RAD23
Deglycosylating enzyme	membrane associated	Png1	Peptide N-glycanase
	or cytoplasm		
De-ubiquitylating enzyme	Cytoplasm	Unknown	Ataxin-3
Ubiquitin receptor	19S proteasomal cap	Rpn10	RPN10 (also known as S5a)
		Rpn13	RPN13
		Rpt5	RPT5 (also known as TBP1 or S6)

Table 1. This table contains a list of identified ERAD components and their function in yeast and with their mammalian counterparts. This table is adapted from [71] with minor modifications.

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Chapter 2:

Cadmium induces expression of its exporter through rescue from ER-associated degradation

Note: The results described in this chapter have been published all text is modified from the original version.

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2.1 Abstract

The environmental contaminant Cd is highly toxic and implicated in a number of diseases. In yeast *Saccharomyces cerevisiae* the P-type ATPase Pca1 has been identified to extrude Cd from cells and is a major component of the yeast Cd detoxification system. Pca1 expression is dictated by the presence of its N-terminal region. Pca1 is rapidly turned over in an N-terminal dependent manner; however, addition of Cd to growth media leads to a rapid stabilization of Pca1. In the following chapter, we will display that Pca1 degradation is mediated through the ER-associated degradation (ERAD) pathway which functions in maintaining homeostasis of the secretory pathway through degradation of misfolded proteins. Unexpectedly, this pathway is also involved in the Cd-dependent regulation of Pca1 and represents the role of this pathway in not only degradation of misfolded proteins but also expressional control of functional proteins. This mechanism allows yeast to rapidly respond to the presence of Cd by continual synthesis of Pca1. This mechanism may be true of other ERAD substrates in which Pca1 can be used as a model.

2.2 Introduction

Many metal ions such as copper, iron, and zinc play essential roles for the sustenance of life, acting as cofactors and structural elements for a wide variety of enzymes and proteins. However delicate control of metal homeostasis is required as too much of these essential metals are toxic. In addition there is a wide range of non-physiological metals which are purely detrimental to the cell. Organisms have refined excretion and detoxification methods for survival [2, 3]. Cd is a highly toxic, heavy metal pollutant which has been implicated in a wide variety of illnesses such as renal dysfunction, cancer, and reproduction problems [4]. *S. cerevisiae* contains Pca1, an eight transmembrane domain containing protein charged with excretion of Cd from the cell. Pca1 is a member of the P_{1b}-Type ATPase family which is highly conserved from mammals to bacteria [5, 6].

Previously our lab determined that Pca1 is a substrate of the ubiquitin proteasome system which is rapidly degraded in the absence of its substrate, Cd. This degradation is carried out via its N-terminal cytosolic domain (aa1-392) prior to secretion from the ER [7]. In the presence of Cd, Pca1 is rapidly stabilized in an N-terminal dependent manner. An independent degron (aa250-350) was determined to be sufficient for both degradation and Cd-dependent stabilization [7].

The normal mode of degradation of a secreted plasma membrane protein is via endocytosis following ubiquitinylation and subsequent lysosomal degradation. Given Pca1's Cd dependent stabilization and proteasome dependent degradation, Pca1 therefore represents a noteworthy manner in which expression and secretion of a functional protein is controlled.

To determine the components involved in Pca1 degradation, we developed a screen utilizing genetic knockouts to identify genes required for Pca1 turnover. We determined the ER-associated degradation system. Specifically, the Doa10 pathway was required for Pca1 expressional control. Member proteins of the secretory pathway are inserted into the ER following translation. Here they are properly folded and matured via post translational modification such as disulfide formation and glycosylation. In the event of mutation or stress, a protein may fail to fold properly, a large buildup of these malformed proteins leads to the induction of the unfolded protein response (UPR), which in turn up-regulates the expression of proteins charged with refolding or removing misfolded proteins [8]. The system involved in eliminating misfolded proteins is the ERAD pathway which alleviates the stress induced by the accumulation of abnormal proteins [9, 10]. Pca1 differs from these ERAD substrates as it is a naturally occurring un-mutated protein and apparent monomer which has its expression controlled through the ERAD system [9, 10]. As Cd is extremely toxic environmental contaminate and not always present, it is possible that yeast developed this system of control of Pca1 for a rapid response sensing and removal of Cd.

2.3 Experimental procedures

2.3.1 Selection of yeast mutants which stabilize expression of Pca1: Green fluorescent protein (GFP) was N-terminally fused to Pca1 and this construct was transformed into an individual deletion pool containing all non-essential deletions (4,848) in *S. cerevisiae* (Open Biosystems). Approximately 400,000 transformed colonies were collected and diluted to $A_{600}=1$ through re-suspension in sterilized water. Cells containing a high GFP-Pca1 fluorescence, indicative of slowed turnover of Pca1, were sorted by flow cytometry and plated on selective SC media. The strong GFP signal was confirmed by confocal microscopy. Specific gene deletions were identified using a PCR amplification primer set of unique 20-base “tag” sequences [11].

2.3.2 Half-life determination using cyclohexamide: Logarithmically growing cells were treated with 100 μ g/mL cyclohexamide to halt protein synthesis. Ice-cold kill buffer (PBS containing 15mM NaN_3 and 15mM NaF) was added to equal volume of cells (15mL) at the indicated time points. No cyclohexamide was added to time point 0. Protein extraction was performed on the cells in which extracts were used for SDS/PAGE and western blot analysis [7]. Membranes were probed using anti-HA primary antibodies and horseradish peroxidase (HRP) conjugated-anti-rabbit secondary antibodies. Chemiluminescence was used to detect Pca1. Phosphoglycerate kinase (PGK) was used as a loading control and probed for using anti-PGK primary antibodies and HRP-conjugated anti-mouse secondary antibodies. Quantification was performed using Total Lab TL 100 software; PGK was used for normalization.

2.3.3 Autoradiography and ^{64}Cu blotting: 3HA-tagged Pca1 aa250-350 and 3HA-tagged Pca1 aa250-350 Δ cys (all cysteine residues are mutated to alanine) were subjected to anti-HA immunoprecipitation from Δ doa10 cells using anti-HA conjugated beads (Pierce). Immunoprecipitates were used for SDS/PAGE and transferred onto a nitrocellulose membrane. Metal binding buffer [12] was used to equilibrate the membranes. The membranes were then

probed for 1hr with 10 μ Ci 64 Cu (~1 μ M CuCl₂) (Isotracer Technique) followed by extensive washing prior to autoradiography and protein staining (MemCode)(Pierce). 5-fold excess of non-radioactive competitor ions were used for 64 Cu competition assays.

2.3.4 Pca1 (aa250-350) limited Trypsin proteolysis: Δ do10 cells expressing 3HA-Pca1 aa250-350 or 3HA-tagged Pca1 aa250-350 Δ cys were cultured with or without 50 μ M CdCl₂ for 1hr. Cytosolic fractions were prepared and subjected to treatment with trypsin (Sigma) on ice for 10 minutes prior to addition of soybean trypsin inhibitor, 0.2 μ g/mL (Fluka BioChemika) for 15 minutes. Anti-HA immunoblotting was used to determine trypsin proteolysis patterns.

2.3.5 Yeast strains and growth conditions: The majority of the strains used in this study are from the *Saccharomyces* genome deletion project [11] purchased from Open Biosystems. Double knockouts were created by homologous recombination using PCR-based gene deletion pFA6a-His3MX6 as a template [13]. Gene specific primers were utilized to confirm deletion. Yeast cells were cultured in synthetic complete (SC) or YPD media as previously described [6].

2.3.6 Plasmid construction, expression, and transformation: A glyceraldehyde-3-phosphate dehydrogenase (GPD) gene promoter was used for expression of all constructs unless specified [14]. GFP and HA tags were inserted into a C- or N-terminal artificially generated site using flanking *Not1* restriction enzyme sites on the GFP or triple-HA. An unfolded protein response element UPRE reporter construct was generously provided by Peter Walter (University of California, School of Medicine, San Francisco). This construct contains four tandem repeats of a 22 nucleotide UPRE upstream of a disabled Cyc1 promoter which was fused to the LacZ gene [15]. An HA-tagged Ste6* expression plasmid was generously provided by Susan Michaelis (John Hopkins School of Medicine, Baltimore). The overlap extension method was utilized to carry out site directed mutagenesis [16]. The lithium acetate procedure was used for plasmid

transformation into yeast cells. All transformations were grown at 30°C on SC media for selection [17].

2.3.7 Protein extraction and Immunoblotting: Protein extracts were prepared using glass bead disruption in PBS containing protease inhibitor (complete Mini; Roche) 5mM EDTA and 1% Triton X-100. For SDS/PAGE and immunoblot, lysates were denatured for 15 minutes in SDS sample buffer containing 25mM DTT. Denatured protein was subjected to SDS/PAGE and transfer to nitrocellulose membrane for probing with specific antibodies.

2.3.8 Immunoprecipitation for Ubiquitin detection: Cell lysates were prepared by glass bead disruption in PBS containing protease inhibitors (Roche complete mini), 5mM N-ethylmaleimide, 1mM PMSF and 1% TritonX-100. HA tagged proteins were subjected to immunoprecipitation using HA-Tag IP/Co-IP kit (Pierce) according to manufacturer's specifications. Protein was eluted using 2X SDS sample buffer at 37°C for 15 minutes. 100mM DTT was added for denaturing of the protein for an additional 15 minutes at 37°C. Ubiquitin conjugation was detected using mouse monoclonal anti-mono-ubiquitin antibody (Covance).

2.3.9 Chemical crosslinking and microsome preparation: Preparation of microsomes was performed as previously described [18]. Cells were disrupted by glass bead vortexing for 10x30sec alternating on /off ice in 300µL lysis buffer (20mM HEPES, 50mM KOAc, 2mM EDTA, 100mM sorbitol, 1mM DTT, 1mM PMSF, and HALT protease inhibitors (Pierce)). 250µL of buffer88 (20mM HEPES, 50mM KOAc, 250mM sorbitol, and 5mM MgOAc) was then added. Lysate was then subjected centrifugation at 300g for 3 minutes to remove cell debris and glass beads. Supernatant was transferred to a clean microcentrifuge tube, and the beads were rinsed with an additional 250µL of buffer88 and collected. The supernatant was the subjected to centrifugation at 18,000rpm for 20 minutes at 4°C. The supernatant was removed, and the remaining microsomal pellet was re-suspended in 150µL of 0.2M Triethanolamine, pH 8.0.

100 μ g/mL of dimethyl 3,3'-dithiobispropionimidate (DTBP) a water soluble membrane permeable, thiol-reversible crosslinker was added to the microsomes and incubated for 1hr on ice. Cross-linked was quenched with 40 μ L of 1M Tris (pH7.5) on ice for an additional 20 minutes. Cell pellets were again attained by centrifugation at 18,000 RPM for 15 minutes at 4°C. Pellets were then washed in ice cold PBS and solubilized in 100 μ L of PBS containing 1% SDS at 37°C for 30 minutes. Triton X-100 was added for a final concentration of 1.5% and incubated on ice for an additional 30 minutes. Lysate was then subjected to anti-HA immunoprecipitation for isolation of HA tagged proteins using anti-HA antibody conjugated sepharose beads (Pierce) at 4°C overnight. Proteins were eluted using 2X SDS sample buffer and cross-linking was reversed with the addition of 100 μ M DTT at 37°C for 30 minutes. Elutates were then subjected to SDS/PAGE and immunoblot analysis.

2.3.10 LacZ Reporter assay: β -galactosidase expression/activity was determined as previously described [19]. 1 O.D._{600nm} of cells were re-suspended in Z buffer and permeabilized with 0.1% SDS and choloform. 200 μ L of 4mg/mL *ortho*-Nitrophenyl- β -galactoside (ONPG) in Z buffer was added to the permeabilized cell mixture. 500 μ L Na₂CO₃ was used to quench the reaction. B-Galactosidase levels are reported in miller units $[A_{420}/(T_{min})(V_{mL})(A_{600})]$

2.3.11 Oligomycin resistance assay: Wild-Type (WT) or Δ doa10 cells expressing either Yor1-GFP, Pca1 (aa1-392)-Yor1-GFP or empty vector were grown in selective SC media until mid-log phase. ~5 μ L of A₆₀₀ cells were spotted on solid YPEG media either containing 0 or 10 μ M CdCl₂. Cells were incubated at 30°C for 2 days prior to photograph.

2.3.12 In-vitro trypsin proteolysis of Pca1 degron: Pca1 aa250-350 was cloned into the PGEX-6p-1 (Amersham Pharmacia Biotech Inc.) vector for fusion to glutathione sulfur transferase (GST). The resulting GST-250-350Pca1 construct was expressed in *Escherichia coli* and protein lysate was prepared and subjected to incubation with glutathione sepharose for

immobilization of the GST fusion construct. The 250-350Pca1 region was liberated in metal binding buffer (100mM Tris-HCl pH 7.0, 50mM NaCl, 100mM sucrose 10% glycerol, 1mM DTT and 0.1% Triton X-100) with PreScission protease (Amersham). 1 μ g/mL of Trypsin (sigma) was added to the Pca1 250-350 peptides for 10 minutes on ice followed by addition of 0.2 μ g/mL of soybean trypsin inhibitor (Fluka BioChemika) for 15 minutes on ice. Coomassie blue staining was utilized to visualize proteolytic patterns.

2.4 Results

2.4.1 In the absence of cadmium, Pca1 expression is controlled via ER-associated degradation. To determine the components involved in Pca1 degradation, we performed a screen of genetic mutants which displayed defective Pca1 turnover. GFP-Pca1 was transformed into a collection of viable genetic knockouts with each cell lacking a single gene [11]. Cells were selected based on the emission of a high-GFP signal using a cell-flow cytometer. This indicated the turnover of Pca1 was disrupted in a particular cell. (Figure 1)

Surprisingly, cells lacking CUE1 were identified as containing high Pca1 expression (Figure 1B-D). CUE1 is an ER resident membrane protein which recruits the cytosolic E2 ubiquitin conjugating enzyme Ubc7 to the ER [20]. Cue1 is an important protein involved in the ERAD system, which led us to investigate other primary components of the ERAD pathway, specifically the E2 ubiquitin conjugating enzymes Ubc6 and Ubc7 as well as the E3 ubiquitin ligases Doa10, and Hrd1 [21-24]. ERAD substrates in yeast are degraded via Hrd1 or Doa10 depending on the location of misfolding. If the malformed region of the protein is in the lumen of the ER (ERAD-L) or membrane (ERAD-M) the protein is ubiquitinated by Hrd1, if the misfolded region is cytoplasmic (ERAD-C) the protein is ubiquitinated by doa10 [9, 10]. Pca1 was stabilized by the lack of UBC7 or DOA10 but not HRD1 (figure 2A). This is consistent with the location of the cytosolic degron (aa250-350). Cycloheximide chase corroborated the roles of Doa10 and Ubc7 in Pca1 degradation and established no role for Hrd1 (Figure 2B). Doa10 dependent ubiquitination was verified as it was dramatically reduced in Δ doa10 cells. Unfortunately, our screen was unable to pick-up any other mutants beside CUE1 indicating a lack of saturation in our library. A more thorough screening may reveal other factors involved in Pca1 turnover including Ubc7 and Doa10. Taken together these data demonstrate the role of the ERAD-C pathway in Pca1 degradation.

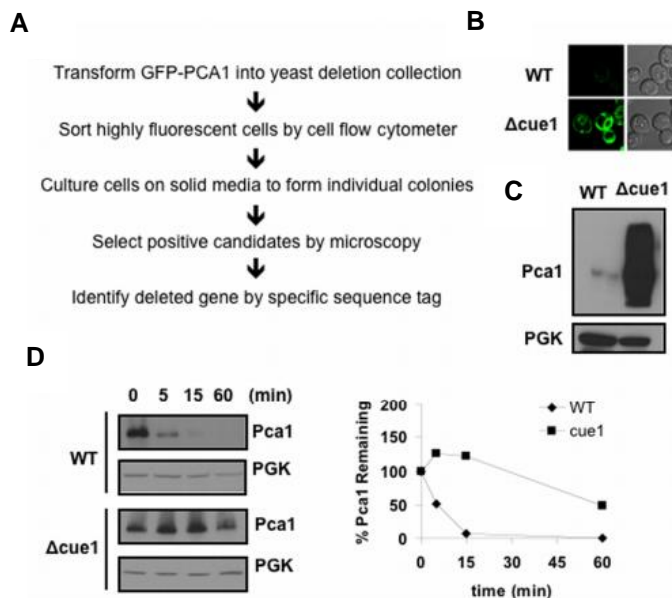


Figure 1 Genetic screen of factors involved in Pca1 degradation. (A) Schematic of genetic screen used to determine factors involved in Pca1 turnover. (B) Confocal microscopy of GFP-Pca1 in WT and Δ cue1 cells (identified in the screen) (C) Immunoblot of 3HA Pca1 expressed in WT or Δ cue1 cells, PGK used as a loading control. (D) Cyclohexamide (CHX) chase of 3HA Pca1 expressed in WT or Δ cue1 cells treated with CHX for the indicated time points the right panel displays quantification by pixel density at each time point.

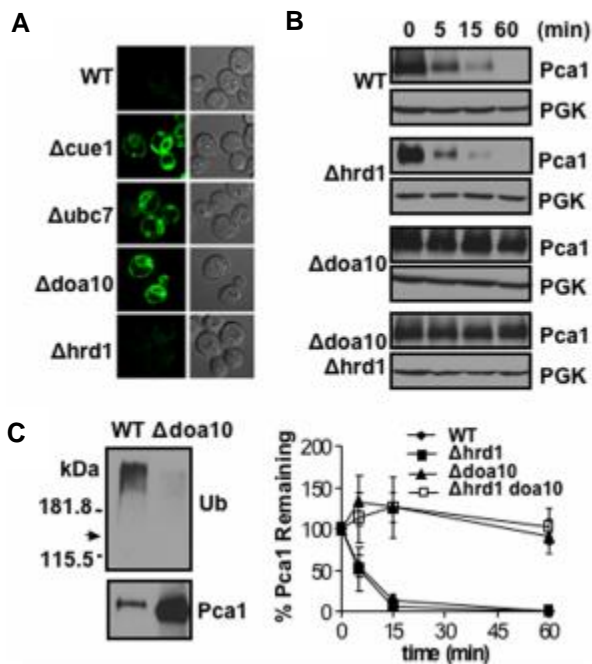


Figure 2 Pca1 is degraded through ERAD. (A) Confocal microscopy of GFP-Pca1 in WT, Δ cue1, Δ ubc7, Δ doa10, Δ hrd1 cells. (B) Cyclohexamide chase of 3HA-Pca1 in WT, Δ hrd1, Δ doa10, Δ hrd1 Δ doa10 cells. PGK is used as a loading control. The lower panel displays quantification based on pixel density normalizing to PGK. (C) Ubiquitylation determination of Pca1 in WT versus Δ doa10 cells. Immunoprecipitation of 3HA pca1 was followed by probing with anti-ubiquitin, and anti HA.

2.4.2 Pca1 Cd sensing and degradation occur prior to secretion from the ER. Since Pca1 is turned over through the ubiquitinylation activity of Doa10, an integral ER membrane protein, we believed Pca1 regulation through ERAD occurred prior to secretion from the ER. To test this line of reasoning, we utilized the Sec23-1 temperature sensitive mutant which is defective in COPII vesicle trafficking from the ER to the Golgi [25]. By preventing secretion of Pca1, we saw no change in the rate of Pca1 degradation indicating that this turnover does not require transport from the ER (Figure 3A). We next sought to determine the role for the Cdc48/Npl4/Ufd1 AAA-type ATPase, a known component in the degradation of other ERAD-C substrates such as Ste6*[26]. By using a temperature sensitive mutant of this complex, *Cdc48-3*, we determined that Cdc48/Npl4/Ufd1 complex plays a significant role in Pca1 degradation as Pca1 is more stable in the mutant strain vs its isogenic wild-type strain when incubated at restrictive temperature (Figure 3B). Because the degradation of Pca1 occurs at the ER, it would make sense that cadmium sensing would also occur at this location. Predictably presentation of Pca1 with Cd results in stabilization of Pca1 despite ER exit being blocked (Figure 3C).

2.4.3 Cd dependent stabilization is specific to Pca1.

To verify the role of Cd in stabilizing Pca1 is specific to Pca1, we examined the possibility that Cd may affect ER homeostasis and ERAD functionality. It is also possible that the ERAD system may become inundated by the accumulation of misfolded/damaged proteins through the toxic effects of Cd. We addressed this through examination of the unfolded protein response as this will be induced under misfolded protein accumulation [8, 27]. We expressed the UPR reporter construct (UPRE-LacZ) and monitored induction of the UPR [15]. Although Pca1 is an ERAD substrate, no appreciable increase in the expression of the reporter gene was identified when Pca1 was over expressed (Figure 4A). Cd levels required for rapid up-regulation of Pca1 expression also did not lead to any major response in UPR induction (Figure 4B). Further evidence indicating Cd dependent stabilization and rescue from ERAD is specific to Pca1

is displayed in figure 3C where Cd supplementation did not induce Ste6*_p stabilization. The cytoplasmic N-terminus of Pca1 is sufficient for targeting to ERAD.

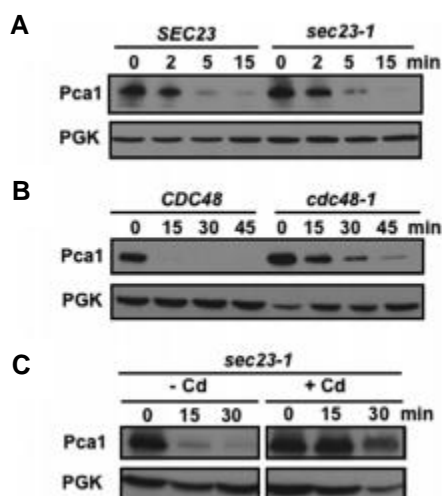


Figure 3 Pca1 cadmium sensing and regulation of Pca1 expression occurs at the ER. 3HA-Pca1 expressed in *Sec23* and the temperature sensitive *sec23-1* which blocks ER to Golgi transport when placed at restrictive temperature (37°C for 30 minutes) were subjected to cyclohexamide chase for the indicated times (A). (B) Cyclohexamide chase of 3HA-Pca1 was expressed in *CDC48* and the temperature sensitive mutant *cdc48-3* after being placed at restrictive temperature (37°C for 30 minutes) and anti-HA western blot analysis. PGK is used as a loading control. (C) Western blot analysis of 3HA Pca1 in *sec23-1* cells placed at restrictive temperature prior to culture with cyclohexamide and with or without 50μM CdCl₂.

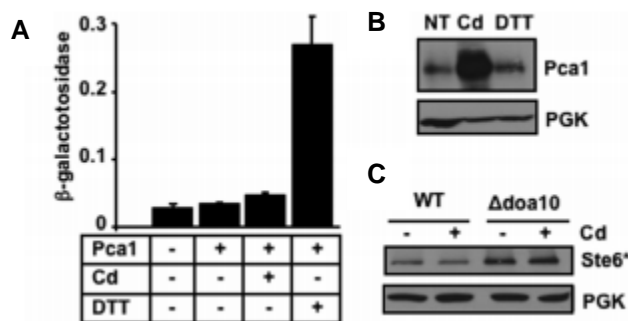


Figure 4 Specificity of cadmium dependent stabilization of Pca1. (A) a LacZ reporter assay of the unfolded protein response in WT cells expressing the UPRE-LacZ reporter construct co-currently with Pca1 (+), 50μM CdCl₂ 1hr [Cd (+)] and 2mM DTT 1hr [DTT (+)] where indicated. Western blot analysis of 3HA-Pca1 expression when cells are treated with 50μM CdCl₂ or 2mM DTT for 1hr. Western blot analysis of Ste6*-HA expressed in WT or Δ*doa10* cells with or without 50μM CdCl₂ 1hr (C). PGK is used as a loading control.

Abnormal proteins of the secretory pathway are often controlled via check points to ensure their quality, and this inhibits their secretion from the ER. For example, secretion is prevented when the ERAD pathway is disabled [26, 28]. We utilized the oligomycin exporting ATP-binding cassette (ABC) transporter, Yor1, to address this issue as it must be secreted to the plasma membrane for function. By fusing the N-terminal regulatory domain of Pca1 1-392 to Yor1, we could determine if Yor1 is able to escape the ER or if the degron of Pca1 prevented this [29]. If this is the case no oligomycin resistance would occur unless the cells were supplemented with Cd. We tested oligomycin resistance; in agreement with its rapid degradation, Pca1 (1-392) Yor1-GFP was unable to confer oligomycin resistance on media lacking Cd (Figure 5A middle). Supplementation of Cd resulted in resistance (Figure 5A right) as did genetic deletion of *doa10* with or without Cd supplementation. Collectively, these results display that Pca1 (1-392) Yor1-GFP is secreted when turnover is prevented despite the absence of Cd. This is consistent with our previous finding that deletion of the N-terminus (1-392) of Pca1 does not lead to a loss of function or secretion.

Next, we sought to determine if the degron of Pca1 is sufficient for degradation of a cytosolic substrate. By fusing the degron, Pca1 250-350, to GFP we determined that it is degraded through ERAD specifically Doa10 and in a Cd dependent manner (Figure 5B). Again indicating the specificity of Cd for the Pca1 degron, Cd supplementation had no effect on the cytoplasmic Doa10 substrate GFP-CL1 (Figure 5B) [30].

2.4.4 Pca1 Physically interacts with Doa10. We hypothesized that the degron of Pca1 is necessary for Doa10 interaction. Microsomes were prepared from cells containing Doa10-13myc, (Doa10-myc) and HA tagged Pca1 either with or without the N-terminus (HA-Pca1 Δ 392) [6, 18]. As a positive control for this interaction, HA-Ste6* was utilized [18]. Purified microsomes were treated with dimethyl dithiobispropionimidate (DTBP) a membrane permeable thio-reversible crosslinker. Immunoprecipitation was performed using anti-HA conjugated

Sepharose beads to pull down HA-tagged proteins. Doa10 was pulled down with HA-Pca1 and HA-Ste6* but not HA-Pca1 Δ 392 (Figure 6) indicating requirement of the N-terminal degron for Doa10-Pca1 interaction.

2.4.5 Metal binding and metal dependent conformational change of the Pca1 degron. As previously demonstrated, both Cd and Cu can prevent Pca1 degradation, thus we hypothesized that metal binding to this degron would result in hiding of the degradation signal [7]. To display metal binding of the degron, HA-Pca1 250-350 was immunoprecipitated using anti-HA Sepharose beads and mobilized to a nitrocellulose membrane. Autoradiography of the nitrocellulose membrane incubated with $^{64}\text{Cu}(\text{II})$ showed cysteine dependent binding to HA-Pca1 (250-350) as no binding was detected in the construct bearing a mutation of all seven cysteine residues to alanine (Figure 7A). Cu(II) and Cd(II) but not Zn(II) were able to compete with $^{64}\text{Cu}(\text{II})$ for binding to the HA-Pca1 (250-350) peptide as displayed by loss of signal in Figure 7B.

To identify conformational change in the degron upon the addition of Cd, we used a limited trypsin proteolysis assay. This allowed us to determine conformational change by observing a difference in trypsin degradation patterns. Cells were cultured with or without Cd and varying concentrations of trypsin (Figure 7C). We determined that there was some protection against trypsin proteolysis in cells treated with Cd, but there was no protection of the peptide lacking the seven cysteine residues [7].

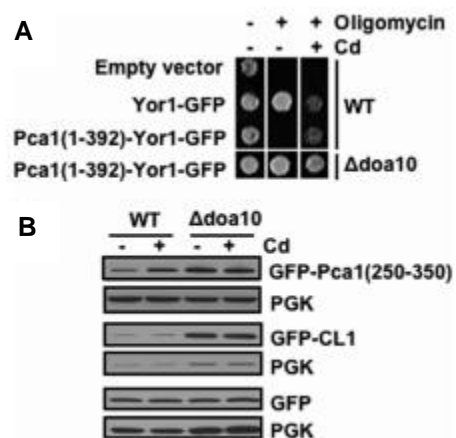


Figure 5 The N-terminal regulatory domain does not function as an ER retention signal. (A) Oligomycin resistance assay of WT or Δ doa10 cells expressing Yor1-GFP, or Pca1(1-392) Yor1-GFP. Cells were spotted on YPEG solid media plates with (+) or without (-) 2.5 μ g/mL oligomycin, or 10 μ M CdCl₂ (B) Western blot analysis of GFP-Pca1(250-350), GFP-CL1 or GFP expressed in WT or Δ doa10 cells cultured with (+) or without (-) 50 μ M CdCl₂ for 1hr. PGK is used as a loading control.

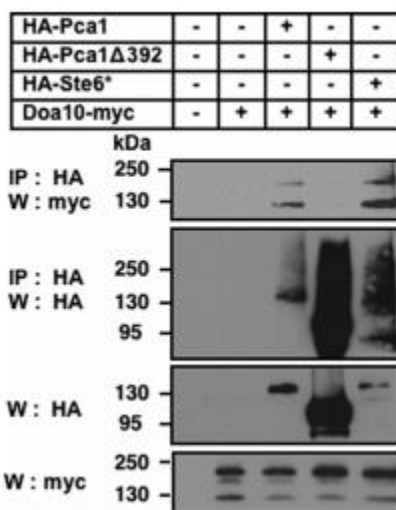


Figure 6 Pca1 N-terminal regulatory domain is essential for interaction with Doa10. Empty vector, 3HA-Pca1, 3HA- Δ 392Pca1 (lacking the first 392 amino acids) or Ste6*-HA were transformed into WT cells or WT cells containing a chromosomally integrated Doa10-13myc. These cells were subjected to microsomal isolation and chemical cross-linking followed by anti HA immunoprecipitation. Immunoprecipitates were subjected to reduction with DTT to break the cross-linking and SDS/PAGE for determination of interaction. Western blots were probed with anti-HA and Anti-myc antibodies.

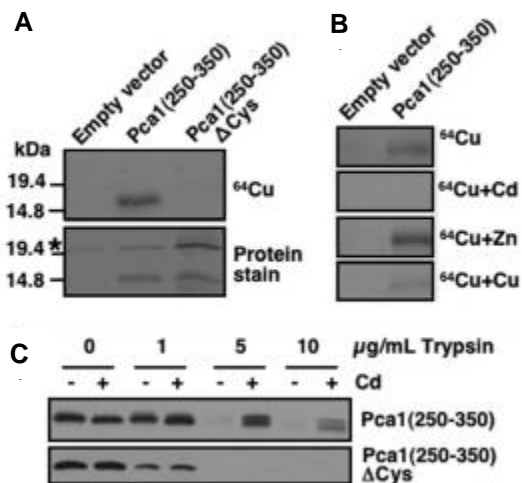


Figure 7 Conformational change and metal binding of the Pca1 degran. 3HA Pca1 250-350 with or without (Δ) cysteine residues were expressed in Δ doa10 cells. (A) HA-tagged Pca1 constructs were immunoprecipitated and subjected to ^{64}Cu blotting with $1\mu\text{M}$ CuCl_2 , μCi which was followed by autoradiography. The asterisk on the lower panel indicates a nonspecific band. (B) A 5-fold excess of non-radioactive metal ions were added and ^{64}Cu blotting was performed. (C) Anti-Ha immunoblot was performed on cell lysates prepared from cells treated with or without $50\mu\text{M}$ CdCl_2 for 1 hour and increasing concentrations of Trypsin (as indicated).

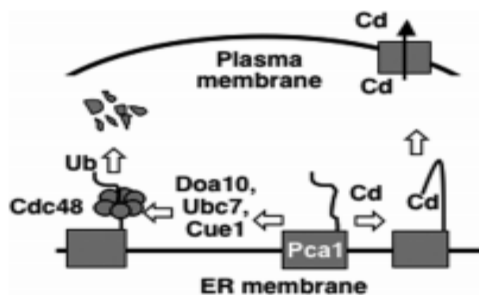


Figure 8 Model of Pca1 regulation through ERAD. Cadmium sensing occurs via the N-terminal cytosolic extension which lead to conformational changes rescuing Pca1 from degradation through the ERAD pathway.

2.5 Discussion

The data presented herein reinforces the model presented in Figure 8, in which Pca1 a plasma membrane protein is post translationally controlled via an unexpected mechanism at the endoplasmic reticulum. After synthesis, Pca1 is rapidly turned over by the ERAD-C ubiquitin proteasome system in the absence of Cd. This degradation is mediated by an N-terminal degron which senses Cd and induces a conformational change resulting in rescue from ERAD. This mechanism allows for rapid response to cadmium by the up-regulation of Pca1 protein levels.

As cadmium is a highly toxic environmental contaminant in which the levels fluctuate, it is valuable for the cell to continually synthesize a cadmium exporter. In the absence of Cd, there is no need for a high level of Pca1, so ERAD immediately following synthesis is able to maintain cellular homeostasis by keeping Pca1 levels low. Another possible advantage for maintenance of low Pca1 levels in the absence of Cd is the potential for non-specific metal export of essential metals. This has been identified in other P_{1B}-Type ATPases, thus the continual turnover of Pca1 would prevent the unwanted export of nutritional metals needed for cell growth and survival [31].

ERAD is in place to remove un- or mis-folded proteins preventing their accumulation which leads to toxicity and eventual cell death [9, 10]. About 30% of all proteins are rapidly turned over following synthesis by the proteasome [32]. It is commonly thought that these proteins are comprised of misfolded or partially synthesized ribosome products which come from translation errors. These small peptides can act as both viral and host peptides to be presented to MHC class I molecules [33]. Our results display another yet unexamined task for ERAD in which the careful manipulation of a plasma membrane protein is controlled via a substrate dependent degron.

Pca1 post translational regulation at the ER may serve as a largely uncharacterized mechanism by which the cell is able to control the expression of protein members of the secretory

pathway. Indeed ERAD component machinery have been shown to be involved in the regulated degradation of proteins. Apolipoprotein B is degraded at the ER in a co-translational manner, when lipid efflux from the liver is low [34, 35]. Sterol, in particular cholesterol, synthesis is regulated through the ERAD system in which the rate-limiting step is mediated by the ER-resident HMG-CoA reductase [36]. A sterol-sensing domain within HMG-CoA reductase dictates the ERAD of this protein. Additionally the yeast protein, MATa1 is degraded via Doa10 and the binding of MATa2 to Mata1 prevents its degradation. Pca1 differs from these examples in that its degradation is mediated through an N-terminal degron which is masked by the presence of its substrate [23, 37]. While there appears to be a mounting of evidence of expressional control of other ion channel members of the secretory pathway, the role of ERAD has not been studied in this process. For example, the ubiquitin proteasome system has been established in control of opioid receptors, aquaporin, ATP sensitive K⁺ channels, and acetylcholine receptor [38-41]. Opioid receptors are of special interest as up to 60% of newly synthesized delta opioid receptor proteins are degraded prior to secretion to the cell surface; however, the presence of the membrane permeable opioid ligand enhances opioid receptor maturation and secretion [42]. It is worthy to determine if this receptor functions in the manner we established for Pca1 in this study.

From this study, we were able to establish the interaction between Doa10 and Pca1. It is still unknown however; if any other molecular factors are necessary for recognition of Pca1 as a degradation substrate. Doa10 interaction may be downstream of initial recognition of Pca1, and other molecular factors involved in this have not been identified.

The mechanism by which Pca1's degron is recognized by degradation machinery is still unknown. The current prevailing hypothesis is that misfolded proteins expose normally buried hydrophobic residues to the cytosolic surface which is recognized and directed to degradation. Mata2 displays a degron, Deg1, comprised of an amphipathic helix which is hydrophobic in nature [37]. There is no clear sequence similarity between Pca1 degron and Deg1. Structural

studies of the Pca1 degron both with and without Cd would be ideal for identifying the structural components necessary for recognition by the ERAD machinery. The application of this study would be broad impacting as we can utilize the information, project the requirements for degradation of ERAD-C substrates, and further apply this to identify other substrates which contain similar degradation signals. While Pca1 250-350 degron does not have any significant sequence similarity with known proteins, we can identify potential metal binding sites of Pca1 family members in plants [31]. We were unable, however, to determine if these proteins are regulated in a similar manner. It will be worthy to study this further in the future.

Here, we have demonstrated that Pca1 is regulated in a degron and substrate dependent manner in a novel and intricate manner to dictate its expression immediately following transcription. These data display that small molecules substrates or metabolites may play an integral role in the regulation of protein secretion as is the case with Pca1.

2.6 Works Cited

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**Chapter 3:
ER-associated Degradation of Pca1, a Polytopic Protein,
via Interaction with the Proteasome at the Membrane**

Note: The results presented in this chapter are to be published with the following authors:

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3.1 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 cadmium exporter in the yeast *S. cerevisiae*. However, the mechanism by which ERAD substrates, including Pca1p and other polytopic proteins are targeted to the proteasome in the cytosol remains to be elucidated. Our study determined the roles for the molecular factors involved in dislodging Pca1p and two other ERAD substrates from the ER. In the cells where proteolytic activities of the 20S proteasome are inactivated, poly-ubiquitinated Pca1p is predominantly stabilized at the ER membrane suggesting that the proteasome is required for not only destruction but also extraction of Pca1p from the ER. Pca1p formed a complex with the proteasome at the membrane in a Doa10 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, the defect of Pca1p's poly-ubiquitination in *ufd2Δ* cells did not affect formation of a complex between Pca1p and the proteasome. Ste6^p, another integral membrane protein undergoing ERAD through the Doa10p-dependent pathway, 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 20S proteasome. Thus, identification of Pca1p as a new ERAD substrate and characterization of the process revealed that extraction and degradation of polytopic membrane proteins for ERAD is a coupled event in the 26S proteasome that is recruited to ubiquitinated substrates while they reside at ER membrane. This cellular strategy could be evolved to relieve the dilemma of solubilization of hydrophobic peptides in the cytosol during ERAD.

3.2 INTRODUCTION

Secretory proteins are inserted into the membrane or lumen of the endoplasmic reticulum (ER) for folding and maturation followed by subcellular trafficking [1-6]. A significant portion of proteins suffer failure in the processes which result in inactive conformation and aggregation [6]. Organisms have evolved the systems to deal with refolding and removal of terminally misfolded proteins. Disruption of folding or buildup of misfolded proteins in the ER induces unfolded-protein response (UPR) to enhance folding capacity and reduce new protein synthesis [8, 9]. Terminally misfolded proteins at the secretory pathway are targeted for the ubiquitin-proteasome dependent removal; this is known as ER-associated degradation (ERAD) [10-15]. Excess turnover or buildup of aggregated proteins is attributed to numerous diseases, such as cystic fibrosis, diabetes, amyotrophic lateral sclerosis, and Alzheimer's and Parkinson's disease.

Several individual substrates and molecular factors involved in ERAD have been characterized [16-19]. For instance 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 at the cytosolic region(s) are ubiquitinated by Doa10p. Some overlap between these two E3 enzymes was observed for a few substrates [20-23]. In congress with the E3 ubiquitin ligases, several other components, such as E2 ubiquitin conjugating enzymes, E4 ubiquitin extension enzymes, and molecular factors (e.g., chaperones) work for recognition and direction of substrates to ubiquitin ligases and the proteasome [11, 19, 22]. As the proteasome is in the cytosol, ERAD substrates should be mobilized from the ER lumen or dislodged from the membrane to be destructed [1]. The Cdc48p AAA-ATPase (p97 in mammals) is thought to provide a primary driving force in the process [16, 20, 24]. Translocation of luminal ERAD substrates to the cytosol could occur through translocon (translocation channel) formed with several proteins, such as Sec61p [5, 25-32], and E3 ubiquitin ligases possessing multi-transmembrane domains (e.g., Hrd1p) [33-35]. E4 ubiquitin chain

extension enzymes (e.g., Ufd2p, Hul5p) facilitate ERAD through poly-ubiquitinylation [16, 36, 37].

Despite significant progress in the identification and characterization of molecular factors involved in ERAD, the mechanisms how proteins in the ER are targeted to the cytoplasmic proteasome remained to be elucidated [11, 16, 36]. Integral membrane proteins might be dislodged from the membrane and escorted to the proteasome for destruction [16]. However, this requires the cells to extract proteins containing hydrophobic regions and maintain their solubility in the cytosol. Thus, direct loading into the proteasome during the extraction from the membrane as a full-length protein or fragmented pieces could be a mechanism resolving the problem [36].

Our previous study showed that expression of Pca1p cadmium exporting P-type ATPase in the yeast *S. cerevisiae* is dependent upon the ERAD pathway [38]. In the absence of cadmium Pca1p is rapidly turned over through Doa10p-mediated ubiquitinylation and the proteasome; however, when cadmium is present the protein is rescued from ERAD and secreted to the plasma membrane where it functions as a cadmium exporter [38, 39]. A degron at the N-terminal cytosolic domain is responsible for ERAD of Pca1p and also senses cadmium to rescue Pca1p from ERAD. Given its rapid turnover, degron rather than misfolding dependent ERAD, and control of the 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 ERAD substrates from the ER including Pca1p, another membrane protein Ste6*p [40] and the luminal protein Cpy1*p [19]. Subcellular location, physical interaction of the substrates with the proteasome, and their turnover rates were determined. Our data suggests that distinct from Cpy*p, extraction and degradation of Pca1p and Ste6*p are coordinated by their interaction with the

proteasome to the ER membrane, which is likely significant for avoiding the release of membrane proteins to the cytosol in the process of ERAD.

3.3 Experimental procedures

3.3.1 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::KanMX6* (*hul5Δ*), *ufd2::KanMX6* (*ufd2Δ*), *pdr5::KanMX6* (*rpn5Δ*), and *doa10::KanMX6* (*doa10Δ*) were purchased from OpenBiosystems. A *pdr5::KanMX6* (*rpn5Δ*) strain background was used for experiments in which new protein synthesis was blocked by cycloheximide (CHX) co-culture. Strains of *doa10::His3* or *ufd2::His3* in a *rpn5Δ* strain (*doa10Δrpn5Δ*, and *ufd2Δrpn5Δ*, respectively) were generated by homologous recombination of a deletion cassette as previously described [41]. Yeast cells were cultured in synthetic complete (SC) medium (2 % dextrose, 0.2 % amino acid mixture, and 0.67 % yeast nitrogen base) lacking specific amino acid(s) if plasmid selection is necessary. Cells were cultured at 30 °C unless specified. To inactivate Cdc48p, a strain expressing a temperature-sensitive *CDC48* allele (*cdc48-3*) was shifted to restrictive temperature 37 °C for 30 min [42]. For cycloheximide (CHX) chase, cells at the mid-log phase were co-cultured with CHX (Sigma, 100ug/mL) for a period as indicated in each experiment.

3.3.2 Plasmid Construction - A single copy yeast vector p416-GPD [43] was used for glyceraldehyde-3-phosphate dehydrogenase gene promoter-mediated constitutive expression of *PCAI*, N-terminal truncated *PCAI*, and *CPY1** [39]. Hemagglutinin (HA) epitope tagging and green fluorescent protein (GFP) fusion at the N-terminus of Pca1p were conducted as previously described [39]. 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 [19]. Site-directed mutagenesis was accomplished by a primer overlap extension method [44]. Two c-myc epitopes were inserted in the C-terminus of *CPY1** for Western blotting analysis using anti-myc antibodies. Common molecular biology techniques, including plasmid amplification using *Escherichia coli* and purification, followed

previously established methods [92]. Plasmid transformation into yeast was performed using the lithium acetate method [45].

3.3.3 Fluorescence Microscopy - Cells cultured in SC media were mixed with phosphate-buffered saline (PBS) containing NaN_3 (15 mM) and NaF (15 mM) to limit cellular activities. Cells were collected by centrifugation and subjected to confocal microscopy as described [39].

3.3.4 Fractionation of Cell Lysates – Yeast cells were broken by vortexing (1 min X 5 times) with glass beads in the PBS lysis buffer containing 1mM PMSF 5mM EDTA HALT protease inhibitor cocktail and subjected to centrifugation (4 °C, 1,000 x g, 10 min) to remove large cell debris and unbroken cells. The supernatant was collected for fractionation of soluble proteins and the pellet containing integral membrane proteins by centrifugation (4 °C, 100,000 x g, 1 hr). Flotation sucrose gradient fractionation was performed as previously described [46]. Briefly, yeast cells were broken using glass beads in the lysis buffer (50 mM HEPES, 150 mM NaCl, 5mM EDTA, 1 mM DTT, 1 mM PMSF, HALT protease inhibitors). Sucrose gradient (0.25 M, 1.5 M, ~1.7 M [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.

3.3.5 In Vivo Crosslinking and Immunoprecipitation - Cells expressing Pca1p tagged with HA epitope were co-cultured with a 20S proteasome inhibitor MG132 (20 μM) for 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 $\mu\text{g}/\mu\text{l}$ 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, protease inhibitor cocktail (Halt, thermo scientific), and 1 mM phenylmethanesulfonyl fluoride (PMSF) (Sigma) to quench crosslinking reaction. Cells were then broken by glass bead disruption (10 X 1 min in ice). Lysates were collected and fractionated by centrifugation at 100000g for 30 minutes. The resulting pellet was washed two times in ice-cold PBS and re-suspended in PBS containing

Triton X-100 (1 %) 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.

3.3.6 Immunoblotting - Yeast cells broken by vortexing (5 X 1 min) with glass beads in the lysis buffer (PBS containing 1 mM PMSF, 50 mM Halt protease inhibitor cocktail, 5 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100). Soluble fraction was obtained by centrifugation (15 minutes at 21000g). 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 samples were subjected to polyacrylamide gel electrophoresis (PAGE). 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), c-myc epitope (ABM, G019), or ubiquitin (Covance, MMS-257P). 3-phosphoglycerate kinase (PGK) was detected using anti-PGK antibodies (Invitrogen, 459250) to determine equal loading. Horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology Inc.) 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.

3.4 RESULTS

3.4.1 Pca1p remains in the membrane when the proteasome is inhibited – Pca1p, a cadmium-exporting P_{1B}-type ATPase in yeast, is a polytopic protein (Fig. 1A) that undergoes ERAD in the absence of cadmium [38]. Doa10p, an E3 ligase, the proteasome, and a degron identified within amino acids 250 and 350 at the N-terminal cytosolic domain (Fig. 1A) are required for Pca1p turnover [39]. Given this uniqueness and eight predicted transmembrane helices of Pca1p, we sought to determine if ERAD of Pca1p relies on known molecular factors of ERAD and how this polytopic protein is degraded by the proteasome working in the cytosol. 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). All 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 [47] co-culture or Cdc48 AAA-ATPase is inactivated at a restrictive temperature (Fig. 1B). Cdc48p is a known critical player in dislodging ERAD substrates from the ER [48]. A strain possessing temperature-sensitive *CDC48* allele (*cdc48-3*) was used to inactivate this essential gene [42] at a restrictive temperature 37°C. Cells were pre-cultured with cycloheximide to inhibit new Pca1p synthesis. Given that the half-life of Pca1p is less than 5 min [39], the majority of remaining Pca1p should be ubiquitinated species. 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 [39]; however, Pca1p is highly expressed when the proteasome or Cdc48p is inactivated (Fig. 1B). Co-localization of Pca1-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 the function of proteolytic activity of the proteasome and inactivation of Cdc48 AAA-type ATPase.

We next addressed the possibility of denatured or fragmented Pca1p might be accumulated in the cytosol. Pca1p fused with 3HA and 2Myc (Fig. 1A) was expressed in WT, *cdc48-3*, and *doa10Δ* cells. Western blotting analyses of total cell extract using anti-HA or Myc antibodies displayed signals at the locations corresponding to full-length Pca1p (* in Fig. 1C). The signals of higher molecular weight than full-length Pca1p particularly in WT cells co-cultured with a proteasome inhibitor MG132 likely reflect ubiquitinated Pca1p species. All Pca1p fragments below full-length Pca1p were also in cells lacking Doa10p (*doa10Δ*), the major E3 ligase of Pca1p. Considering the fact that Pca1 ubiquitinylation is hardly detectable in *doa10Δ* cells [38], those fragments are irrelevant to ERAD. Unfortunately 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 (lane 1 Figure 1C middle panel)

To determine distribution of Pca1p in membrane and cytosol, total cell lysates were fractionated by ultra-centrifugation (100,000 x g for 30 min) to supernatant (S) and pellet (P) containing soluble and integral membrane proteins, 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-ubiquitin antibodies (Fig. 1E) further confirmed that most of ubiquitinated Pca1p species are in the “P” fraction. These results suggest that Pca1p in the cells deficient of proteolytic activities of the proteasome or Cdc48p function resides in the membrane.

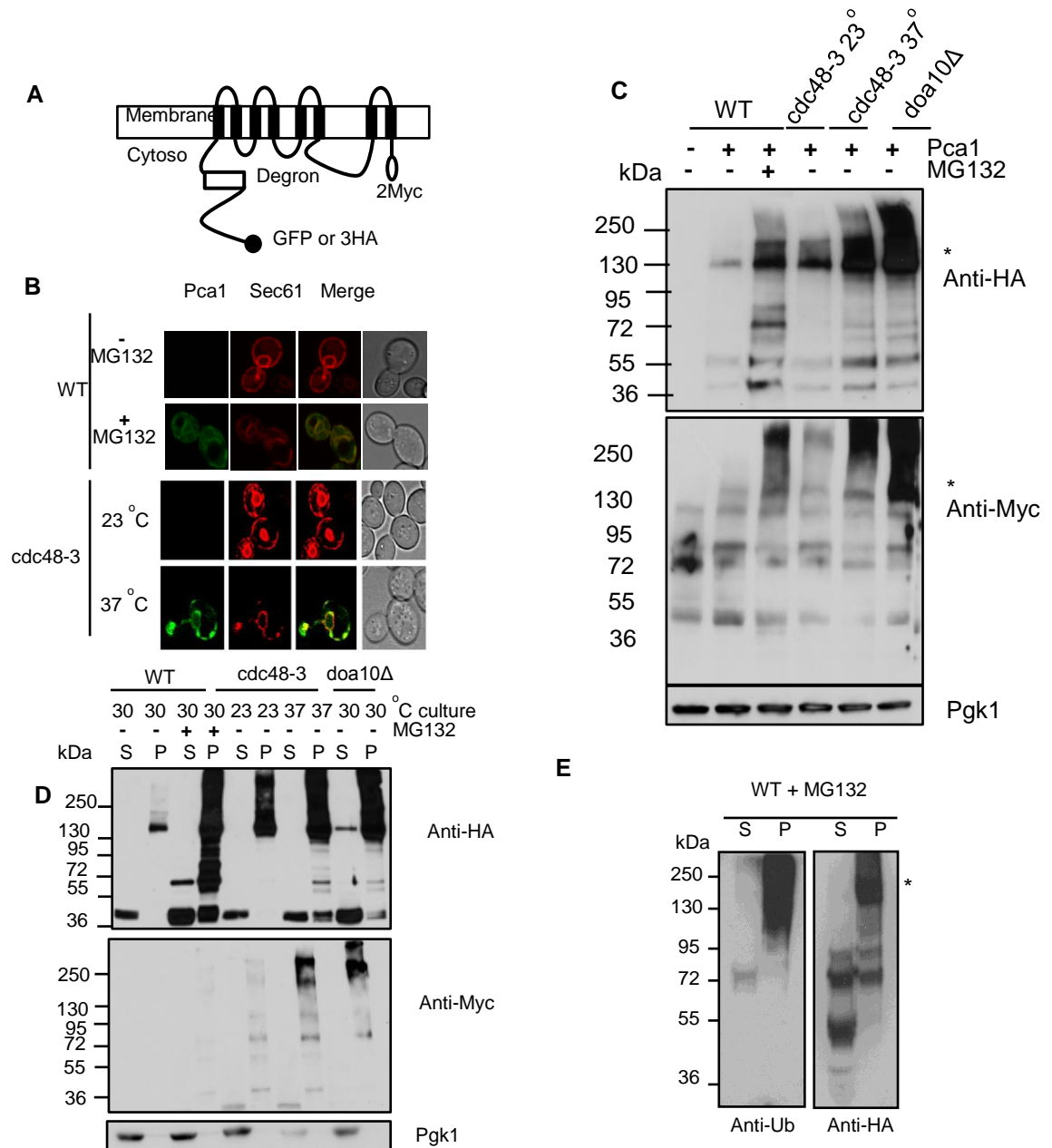


Figure 1 Localization of Pca1p at the membrane of the cells in which the proteasome or Cdc48 AAA-type ATPase is inactivated. (A) A schematic depiction of Pca1p. The black squares indicate eight transmembrane helices. Green fluorescent protein (GFP) or triple HA epitope was fused at the N-terminus (filled circle). Myc epitope is fused at the C-terminus (empty circle). 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 Sec61p, an ER-resident protein, were co-transformed into WT control cells and a strain containing a temperature-sensitive allele of CDC48 (*cdc48-3*). Mid-log phase cells were co-cultured at the indicated temperature with and without MG132 for 2hr. Cyclohexamide 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 Pca1-GFP was visualized by confocal fluorescent microscopy. (C) Detection of Pca1p and its fragments by immuno-blotting. Pca1p fused with 3HA and Myc epitope was expressed in a control strain lacking PDR5 (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 blotting using anti-HA and -Myc antibodies. The blots were also probed with anti-Pgk1p antibodies to determine equal loading. (D) Fractionation of Pca1p to determine subcellular distribution of Pca1p. Total lysates (T) 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 T, S and P fractions were solubilized (1% Triton X-100) and subjected to Western blot analysis using Anti-HA and -Myc antibodies. (E) Detection of ubiquitinated 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. Pca1p protein and its ubiquitinylation status were determined by Western blotting using anti-HA and -ubiquitin (Ub) antibodies, respectively. The asterisk indicates the location where full-length non-ubiquitinated Pca1p migrates.

3.4.2 Pca1p in the pellet fraction exists as membrane-integrated species - The results presented in Fig. 1 suggest that Pca1p is not dislodged from the membrane without the activities of the proteasome and Cdc48p. However, Pca1p could be extracted out of the membrane but associated with the membrane or form cytosolic aggregates or large 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) to release peripheral membrane proteins to soluble (S) fraction [49]. Under this condition all Pca1p species, including Pca1p fragments that migrate faster than full-length Pca1p, remain in the pellet fractions of WT cells co-cultured with MG132, Cdc48p-inactive cells, and *doa10Δ* cells (Fig. 2A). However, Triton X-100 (1%) released Pca1p to the “S” fraction near completely (Fig. 2A), suggesting that Pca1p does not form a large complex or aggregate that can be pelleted by centrifugation (100,000 x g).

To confirm further Pca1p's membrane localization, cell lysates were subjected to a sucrose density gradient fractionation [46] (Fig. 2B). If Pca1p is in membrane vesicles, it will float to the low-density fractions. Cell lysate was loaded at the layer of 1.7 M sucrose. Centrifugation followed by Western blotting analysis of collected fractions displays that the majority of full-length Pca1p is floated to the low-density fractions similar to that of Pca1p(Δ 392) lacking the degon, which is distinct from Pgk1p, a soluble protein (Fig. 2B). Treatment of the cell lysate with Triton X-100 (1%) followed by the same experiment resulted in migration of Pca1p at the fractions detecting Pgk1p (Fig. 2B). Most of Pca1p species, excluding a few small fragments, were detected at the membrane fractions (Fig. 1C, upper panel).

Pca1p with different degrees of ubiquitinylation could be spread widely on SDS-PAGE as Pca1p species of diverse molecular weight, which results in no detection by Western blot. Samples obtained by sucrose density gradient fractionation were subjected to treatment of a de-ubiquitinylation enzyme (Usp2) followed by Western blotting analysis of Pca1 using anti-HA antibodies (Fig. 1C, lower panel). No Pca1p was identified in the fractions containing soluble

proteins. Next to ascertain ubiquitylation status of Pca1p in the fractions containing membrane vesicles (including 4, 5, and 6) and soluble proteins (including 10, 11, and 12), Pca1p was immunoprecipitated using anti-HA antibodies and then probed with antibodies against ubiquitin and HA epitopes (Fig. 2D). Most of ubiquitylated Pca1p species were detected at the fractions containing floated membrane vesicles. The soluble protein fractions contain only a few ubiquitin-conjugated Pca1p fragments likely corresponding to cleaved N-terminal cytosolic portion of Pca1p. Collectively, these results suggest that Cdc48p alone cannot dislodge Pca1p from the membrane for ERAD if Mg132 inhibits the proteolytic activities of the proteasome.

3.4.3 Pca1p interacts with the proteasome at the ER membrane in an E3 ligase and its

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 2Myc epitope-tagged wild-type control Pca1p and Pca1p lacking N-terminal 392 amino acids which contains a degron (Pca1(degronΔ)) were co-cultured with an inactivator of the 20S proteasome, MG132, and then a membrane permeable thiol-reversible cross-linker, dimethyl 3,3'-dithiobispropionimidate (DTBP). Membrane fractions were isolated as described in Fig. 1D, washed, and solubilized (1% Triton X-100). Samples were then subjected to anti-myc immunoprecipitation. After elution, cross-links were broken and protein interaction was visualized by Western blot. Pca1p was found to form a complex with Rpn5p, a 19s proteasomal subunit [50], and 20S subunits at the membrane (Figs. 3A and 3B). The complex formation between Pca1p and Rpn5p was dependent on *DOA10* encoding an E3 ligase for Pca1p and a degron in Pca1p (Fig. 3A). These results indicate that when Pca1p is ubiquitylated by Doa10p, it recruits the proteasome while it resides at the membrane.

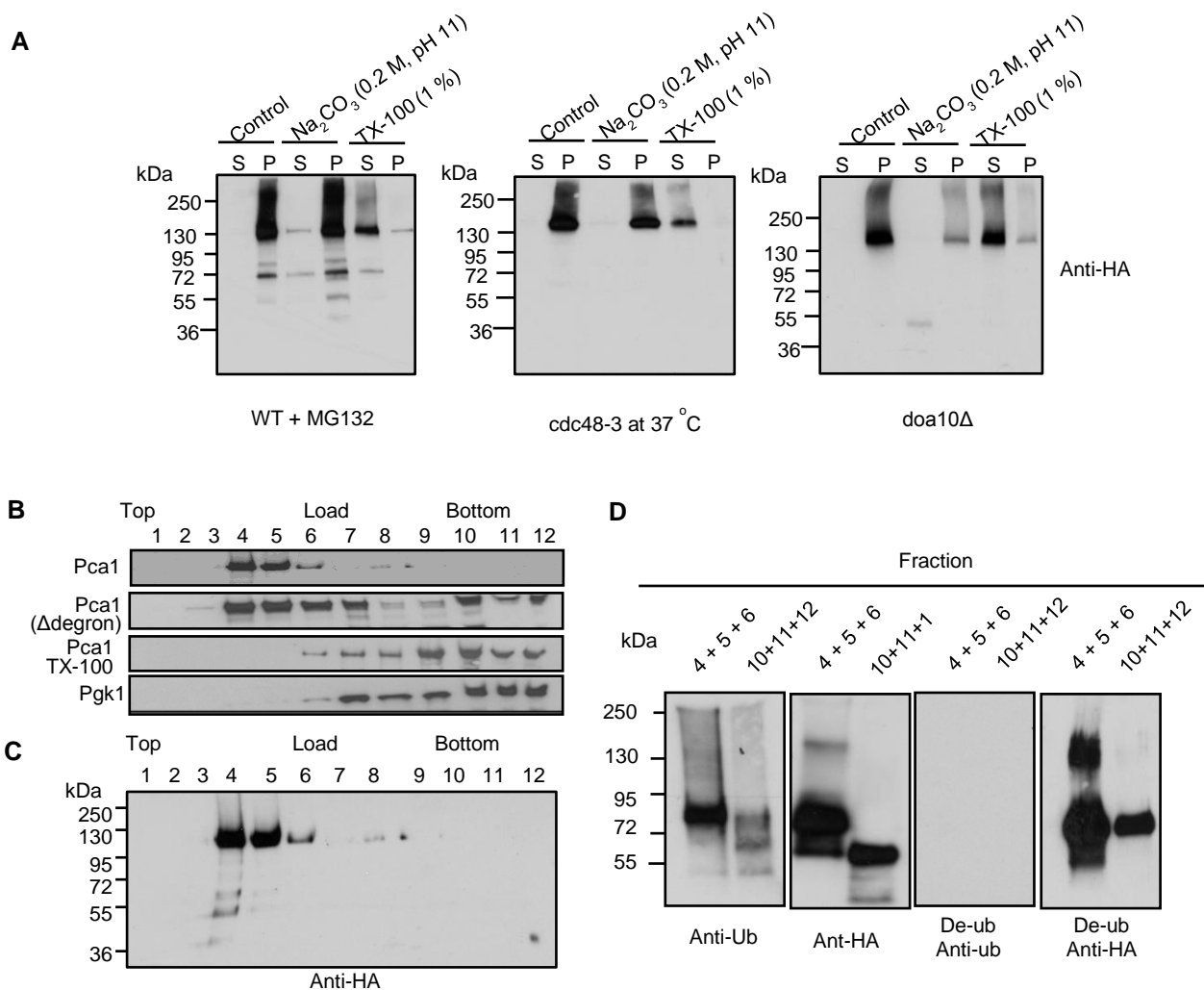


Figure 2 Detection of fragmented or ubiquitinated Pca1p by subcellular fractionation. (A) Pca1p and its degradation intermediates are embedded in the membrane. Pca1p fused with triple HA and Flag epitopes at the N- and C-termini, respectively, was expressed in a control strain lacking PDR5 (WT), a strain expressing *cdc48-3* temperature sensitive allele, and a *doa10Δ* strain. WT and *cdc48-3* cells were pre-cultured with MG132 (20 μ M, 2 hrs) and at 37 oC (30 min), respectively. Total cell lysates were prepared by glass bead disruption followed by centrifugation (300 x g for 10 min) to remove unbroken cells. Pellet fractions obtained by centrifugation (100,000 x g for 30 min) of total cell lysate were re-suspended in phosphate-buffered saline (PBS) with and without Na_2CO_3 or Triton X-100. The suspensions were incubated for 30 min on ice and then centrifuged at 100,000 x g for 30 min. The supernatant (S) was precipitated in trichloroacetic acid (10 %) and washed two times with acetone. The pellet (P) was solubilized with PBS containing detergent (1% Triton X-100). Samples were denatured in SDS sample buffer (37 $^\circ\text{C}$, 15 min) and analyzed by Western blot using anti-HA antibodies. **(B)** Fractionation of Pca1p by flotation sucrose gradient. WT cells expressing 3HA and Myc tagged Pca1p or Pca1(degron Δ)p lacking first 392 amino acid containing a degron were co-cultured with MG132 for 2 hrs. Cyclohexamide 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 for 10 min) to remove un-broken cells. Samples were loaded at the layer of 1.7M 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 Pca1(degron Δ)p using anti-HA antibodies. Presented data reflects full-length Pca1p and Pca1(degron Δ)p. Pgk1p was detected as a marker of soluble proteins. **(C)** Distribution of full-length, ubiquitinated, and fragmented Pca1p species determined by flotation sucrose gradient. Fractions obtained as described in (B) were subjected to Western blot analysis of Pca1p with and without treatment of deubiquitylation enzyme after fractionation as described previously [7]. **(D)** Detection of ubiquitinated Pca1p from fractions containing membrane and cytosolic proteins. Total cell lysates were fractionated as described in (B) and solubilized by detergent (1% Triton X-100). Using anti-HA antibodies, Pca1p was immuno-precipitated from pooled fractions 4, 5 and 6 (4+5+6) and 10, 11 and 12 (10+11+12) containing membrane and cytosolic proteins, respectively. The samples were subjected to Western blotting using anti-ubiquitin (Ub) and -HA antibodies.

A remaining question is if Pca1p is a unique case of interaction with the proteasome at the membrane in the process of ERAD or other poly-topic membrane proteins undergoing ERAD manifest a similar pattern observed for Pca1p. Ste6**p* is a mutated form of the alpha factor transporter Ste6p in yeast [40], which causes a premature stop codon and a change in N-glycosylation. Ste6**p* is also targeted for degradation via the Doa10p, Cdc48p, and proteasome dependent pathway [16, 40, 51]. Subcellular fractionation showed that Ste6**p* remained in the membrane fraction under proteasome inhibition conditions (data not shown). Flotation sucrose gradient fractionation also showed that Ste6**p* remains in the membrane when cells are co-cultured with a proteasome inhibitor (data not shown). The same experiment described in Fig. 3A showed that Ste6**p* pulled down a proteasome subunit Rpn5p in a Doa10p-dependent manner (Fig. 3C). Therefore, Ste6**p* also attracts the proteasome to the ER membrane when it is ubiquitinated by Doa10p.

3.4.4 A need of Cdc48p in the complex formation between Pca1p and the proteasome –

Cdc48p is a critical molecular factor for ERAD of Pca1p and many other proteins [16, 38, 42]. Nevertheless, its role(s) for ERAD of integral membrane proteins remain to be determined. In particular, given the requirement of both the proteasome and Cdc48p for extraction and degradation of Pca1p (Figs. 1 and 2A), the role of Cdc48 in the ERAD of Pca1p in conjunction with the proteasome is elusive. ATPase activities of Cdc48p are believed to contribute to the retro-translocation of ubiquitinated luminal proteins for ERAD [42], and Cdc48p also escorts them to the proteasome as demonstrated by a physical interaction between Cdc48p and the proteasome [42]. To test a hypothesis that Cdc48p might recognize ubiquitinated Pca1p to recruit the proteasome, we ascertained if the proteasome forms a complex with Pca1p in the cells expressing inactive Cdc48p. Indeed, no significant physical interaction between Pca1p and Rpn5p subunit of the proteasome was detected when a strain expressing a temperature sensitive *cdc48-3* allele was cultured at a restrictive temperature (Fig. 4A).

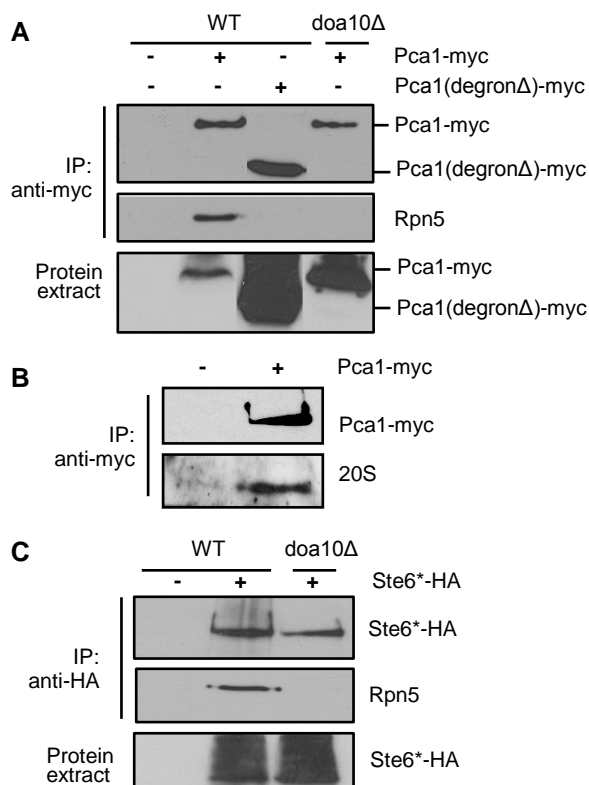


Figure 3. Pca1p forms a complex with the proteasome at the membrane in a Doa10p dependent manner. Pca1p, Pca1(degronΔ)p deleting first 392 amino acids, and Ste6*p were expressed in WT and DOA10 E3 ligase knockout (doa10Δ) strains. Two Myc and three HA epitope were tagged at the C-terminus of Pca1p and Ste6*, respectively. Cells were co-cultured with cell permeable reversible cross-linker (DTBP, 100 ug/ml) and MG132 (20 uM), an inhibitor of proteasomal proteolytic activities, for 2 hrs. Protein extracts were obtained from the cells by glass bead disruption and Triton X-100 (1%) solubilization. The samples were subjected to immune-precipitation using anti-Myc. Co-immunoprecipitation of Pca1p and Ste6*p with Rpn5p, a subunit of 19S regulatory particle (A and C), and 20S catalytic core particle (B) was detected by Western blotting.

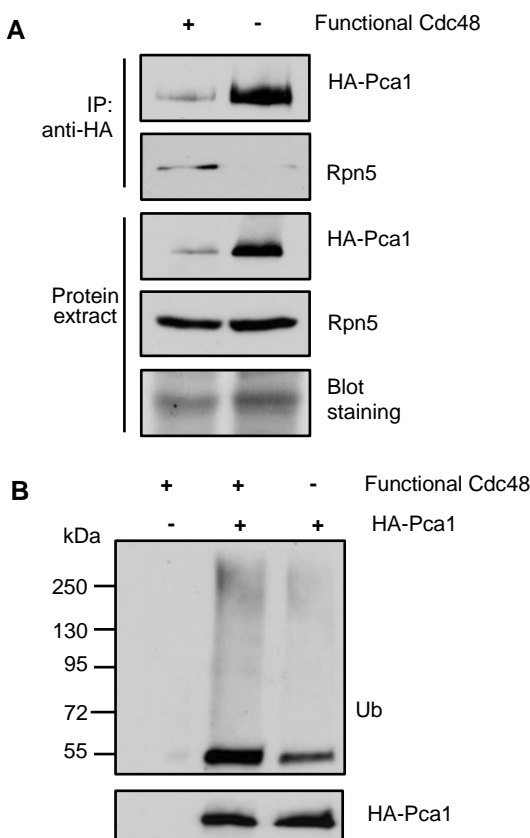


Figure 4 Cdc48p plays a role in Pca1p's complex formation with the proteasome and poly-ubiquitylation. (A) A physical association between Pca1p and Rpn5p, a subunit of 19S regulatory particle, determined by co-immunoprecipitation. Pca1p tagged with triple HA epitope at the N-terminus (HA-Pca1) was expressed in a strain carrying a temperature-sensitive CDC48 allele (*cdc48-3*). The cells at mid-log phase were cultured at a permissive temperature (23 °C, + Functional Cdc48) or shifted to a restrictive temperature (37 °C, - Functional Cdc48) for 30 min with a cell permeable reversible cross-linker (DTBP, 100 ug/ml) and MG132 (20 uM), an inhibitor of proteolytic activities of the proteasome, for 2 hrs. Protein extracts obtained by glass bead disruption isolation of the pellet fraction as previously described (see methods and materials) and Triton X-100 (1%) solubilization used for immunoprecipitation using anti-HA antibodies. Co-immunoprecipitation of Rpn5p, a subunit of 19S regulatory particle was detected by Western blot. (B) Poly-ubiquitylation of Pca1p as a functional of Cdc48p activities. To determine ubiquitylation status of Pca1p, the samples obtained by immuno-precipitation as described above were subjected to Western blotting using anti-ubiquitin antibodies.

Cdc48 also interacts with other molecular factors involved in ERAD such as E4 ubiquitin chain extension enzyme Ufd2p [52]. To gain a better understanding of the defect in Pca1p's ERAD and complex formation with the proteasome, we determined ubiquitinylation status of Pca1p in the Cdc48 inactive cells. Immunoprecipitation of Pca1p using anti-HA antibodies followed by West blot analysis of Pca1p using anti-ubiquitin antibodies showed a slight ubiquitinylation deficiency in the cells expressing non-functional Cdc48p (Fig. 4B). This could reflect that defect in Cdc48p leads to a problem in recruiting E4 ligase such as Ufd2p and/or presenting mono-ubiquitinated Pca1p to Ufd2p.

3.4.5 Ufd2p, an E4 Ubiquitin Extension Enzyme for Pca1p, did not affect complex

formation between Pca1p and the proteasome - Deficiency of ubiquitin chain extension

observed in Cdc48p-defective cells (Fig. 4B) could cause the lack of a complex formation

between Pca1p and the proteasome (Fig. 4A) and ultimately, ERAD deficiency of Pca1p (Fig.

1B). Following ubiquitinylation 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

[16, 36, 52, 53]. 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 [37], and Hul5p is a component of the proteasome [36, 54, 55]. We ascertained the

roles for poly-ubiquitinylation in ERAD and the interaction of Pca1p with the proteasome in

ufd2Δ and *hul5Δ* strains. Cycloheximide chase of Pca1p revealed no significant difference in

half-life ($T_{1/2}$) of Pca1p expressed in WT and *hul5Δ* cells; however, *ufd2Δ* leads to significant

extension of the $T_{1/2}$ of Pca1p (~30 min) (Figs. 5A and 5B). This result suggests that Ufd2p is

required for efficient turnover of Pca1p. Immuno-precipitation of Pca1p followed by

immunoblotting using anti-ubiquitin antibodies supported that Ufd2p is a major E4 enzyme of

Pca1p (Fig. 5C). In the *ufd2Δ* cells, Pca1p was detected at the membrane fractionation but not at

the soluble fraction (Fig. 5D) suggesting that in the *ufd2Δ* cells the majority of Pca1p remains in the membrane.

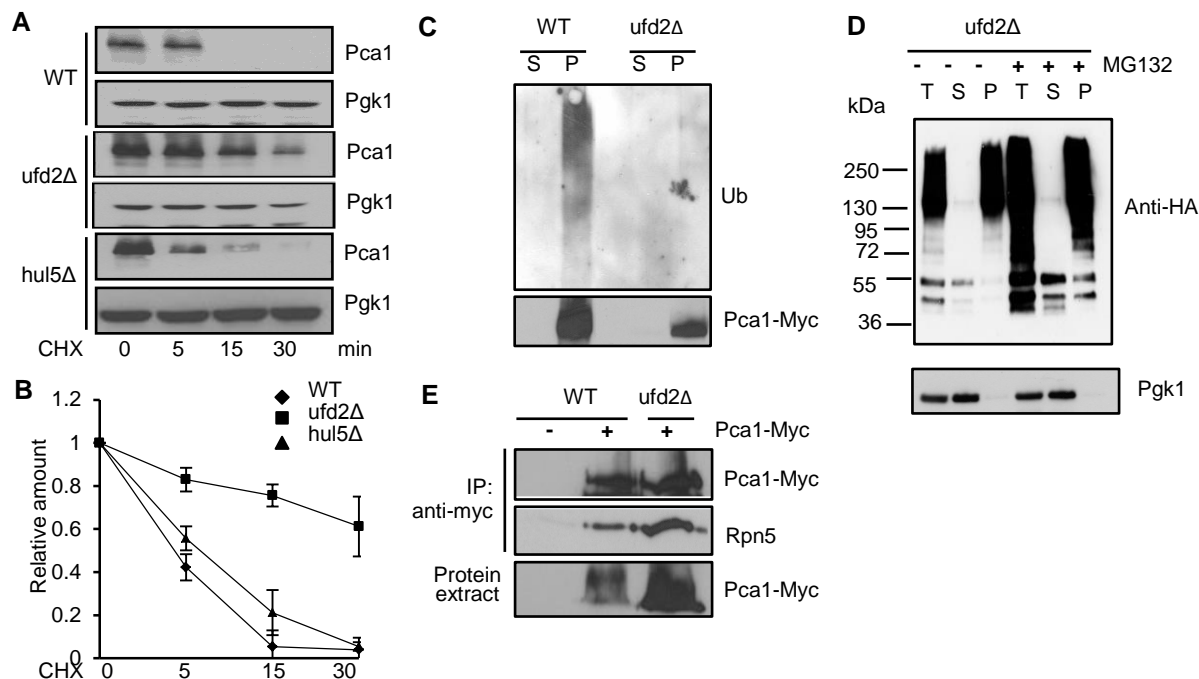


Figure 5 Formation of a complex between Pca1p and the proteasome is independent of poly-ubiquitylation status of Pca1p. (A) Significant reduction of turnover rate of Pca1p in Ufd2 gene knockout (*ufd2Δ*) cells. Pca1p tagged with two Myc epitope at the C-terminus (Pca1-Myc) was expressed in WT, *ufd2Δ*, and *hul5Δ* strains. Cycloheximide (CHX) chase and Western blotting determined Pca1p levels. Protein extracts were prepared by glass bead disruption and Triton-X 100 (1%) solubilization. Pgk1p was probed as a loading control. (B) Pca1p levels presented in (A) were quantitated from four repeats. (C) Defect in poly-ubiquitylation of Pca1p in a *ufd2Δ* strain. An expression construct of Pca1p tagged triple HA and one Flag epitope at the N and C-terminus, respectively, was transformed in a *ufd2Δ* strain. Total cell lysates (T) were obtained from *ufd2Δ* cells with and without co-culture with MG132 for 30 min by glass bead disruption and then removing un-disrupted cells and large particles by centrifugation (300 x g for 5 min). The detergent-solubilized S and P fractions were subjected to immune-precipitation using anti-HA antibodies followed by West blotting using anti-ubiquitin (Ub) and -HA antibodies. (D) Total cell lysates (T) described in (C) 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%), Western blot using Anti-HA, -Flag, and -Pgk1p antibodies determined distribution of Pca1p species in the S and P fractions. (E) Formation of a complex between Pca1p and Rpn5p, a subunit of the proteasome. After co-culturing the cells with a permeable reversible cross-linker (DTBP, 100 ug/ml) and MG132, an inhibitor of proteasomal proteolytic activities, protein extracts were obtained from the cells by glass bead disruption and detergent (1% Triton X-100) solubilization. The samples were subjected to immunoprecipitation using anti-Myc antibodies. Rpn5p in the samples was detected by Western blotting using anti-Rpn5p antibodies.

As Ufd2p is required for efficient degradation of Pca1p, we next sought to determine if polyubiquitinylation by Ufd2p is necessary for the interaction of Pca1p with the proteasome. In vivo cross-linking and co-immunoprecipitation 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 presented in Fig. 5C is not required for formation of a complex between Pca1p and the proteasome.

3.4.6 Retro-translocation of a soluble ERAD substrate without functional proteasome –

Maturation and trafficking of vacuolar carboxypeptidase Y (Cpy1p) in yeast is dependent on ER [56]. Cpy1p containing a G255R mutation (Cpy1*p) is degraded through the ERAD pathway [19, 56-59]. We have employed Cpy1*p as an example of an ER luminal protein to elaborate the hypothesis that soluble ERAD substrates can be retro-translocated prior to being targeted to the proteasome.

When WT yeast cells were co-cultured with a proteasome inhibitor MG132, Cpy1*p was detected as full-length and poly-ubiquitinated forms in the soluble fraction (Fig. 6A, 3rd and 4th panels). Rpn5p, a subunit of 19S proteasome, was co-immunoprecipitated with Cpy1*p in soluble protein fraction but not pellet fraction (Fig. 6A, 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. 6B); thus, detection of Cpy1*p in soluble fraction followed by poly-ubiquitinylation (Fig. 6A) is a consequence of ERAD progress of Cpy1*p rather than it leaking from the ER during sample preparation. Collectively, these results suggest that Cpy1*p ubiquitinated by Hrd1p is retro-translocated to the cytosol without proteolytic activities of the proteasome. This is distinct from Pca1p.

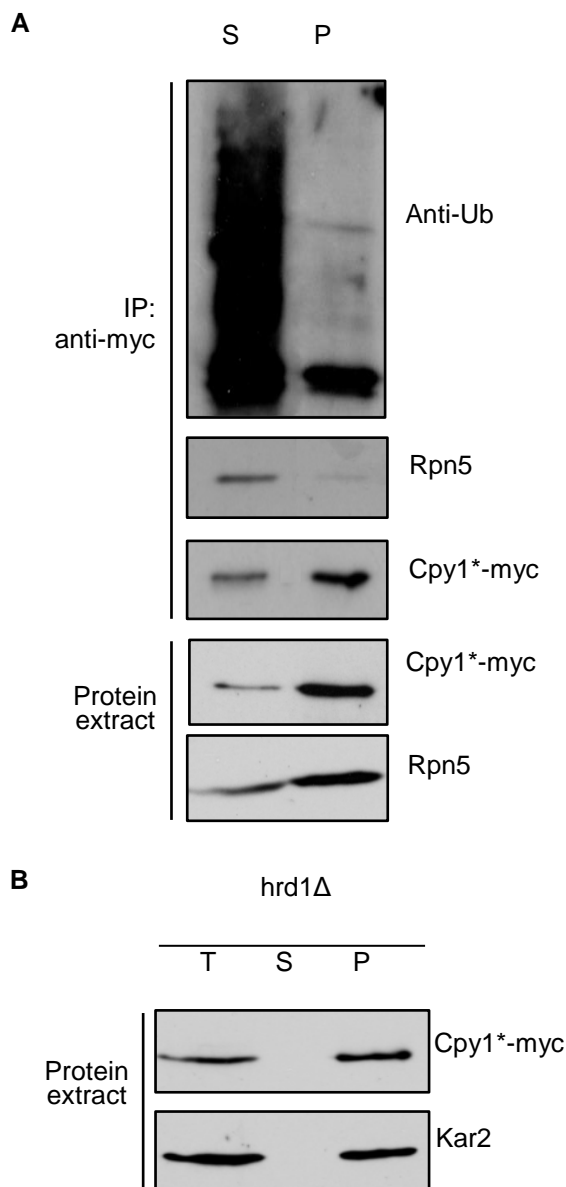


Figure 6 Distribution Cpy1*^p in the cells where the proteolytic activities of the proteasome are inhibited. Cpy1*^p tagged with one Myc epitope at the C-terminus was expressed in WT control and *hrd1Δ* cells. Cells were co-cultured with MG132 (20 μM, 2 hrs), an inhibitor of proteasomal proteolytic activities, and a cell permeable reversible cross-linker (DTBP, 100 μg/ml, 30 min). The reaction was stopped with Tris-HCl (50 mM, pH 7.4). Total cell lysates (T) were prepared by glass bead disruption, and then un-broken cells were removed by centrifugation (300 x g for 5 min). Samples were subjected to separation of soluble (S) and pellet (P) fractions by centrifugation (100,000 x g for 30 min). Protein extracts were obtained by solubilization the samples with detergent (1% Triton X-100). To determine the physical interaction between Cpy1*^p and Rpn5p, the samples were subjected to immunoprecipitation using anti-Myc antibodies followed by Western blotting using anti-Rpn5 and -Myc antibodies (**A** 2nd and 3rd panels). Ubiquitylation status was determined by probing the blots with anti-ubiquitin (Ub) antibodies (**A**, Top panel). Cpy1*^p and were detected by Western blot using anti-Myc antibodies (**A-B**). The blots were also probed with antibodies against Kar2p, an ER-resident protein (**B** and **D**).

3.5 DISCUSSION

The mechanisms underlying retro-translocation of substrates of ERAD from the ER to the cytosol for destruction remain unclear. This process for membrane proteins is additionally complex because they should be dislodged from the lipid bilayer to a hydrophilic environment. Our data indicates that polytopic ERAD substrates undergo proteasomal degradation at the ER membrane via recruiting the proteasome rather than being extracted to the cytosol prior to being targeted to the proteasome, which is distinct from the process of Cpy1*^p, a soluble substrate in the lumen of the ER. Several lines of evidence suggest that membrane proteins undergoing ERAD are fed into the proteasome while they reside in the membrane in an E3 ligase and Cdc48p but not poly-ubiquitinylation dependent manner. Destruction of the substrates at the ER membrane would be advantageous for cells by minimizing the burdens of extraction and solubilization of membrane proteins in the cytosol. These results shed some new light on this vital but under characterized cellular process.

Our study confirmed a significant role for Cdc48p in ERAD of Pca1p; however, the mode of action of Cdc48p appears to be substrate specific. Three distinct but overlapping ERAD pathways L, M, and C recognize misfolded lesions in luminal proteins, transmembrane helix(s), and cytosolic domain(s) of integral membrane proteins, respectively [20]. Given the catalytic domain of E3 ligases involved in ERAD is on the cytosolic face of the ER membrane, luminal ERAD substrates should be trans-located first at least partially to the cytosolic face of the ER membrane [60]. It has been proposed that Sec61p, Hrd1p, Der1p, and/or Usa1p form a complex for substrate translocation across the membrane [33, 61, 62]. Cdc48p (p97/VCP in higher eukaryotes), a hexameric complex of AAA-type ATPase, plays diverse roles in ERAD [63, 64] although the mechanistic details remain to be defined and there is experimental evidence arguing a rather non-essential role [65]. Cdc48p and its cofactors, Ufd1 and Npl4, are recruited to the ER via Ubx2p to disassemble ubiquitin-conjugated substrates from the ubiquitinylation machinery in the membrane and unfold for the delivery to the proteasome. By interacting with E4 ligase (e.g.,

Ufd2p) and other proteolytic factors (e.g., Rad23p), Cdc48p appears to serve as a scaffold as well. Thus, Cdc48p is a critical player in dislodging ubiquitinated substrates from the ER [63, 64]. Our data indicates that Cdc48p is a significant player in ERAD of Pca1p, which is consistent to previous reports. Nevertheless, it is interesting to note an impaired complex formation between the proteasome and Pca1p if Cdc48p is inactivated. Cdc48p might be involved in initial recruitment of the proteasome by unfolding polytopic proteins and presenting them to the proteasome. This argument is in line with a previous report displaying Cdc48's cooperation with the proteasome for turnover of Insig-1, an ERAD-M substrate [66]. However, a recent report indicated that functional defect of Cdc48p does not affect association of the proteasome with the Hrd1p E3 ligase complex [67]. Given differences in ERAD pathways, substrates, and experimental systems (e.g., *in vitro* vs *in vivo* experiments, natural vs artificial substrates, and soluble vs membrane substrates) in the previous reports, a model encompassing all studies about functions and mechanisms of action of Cdc48p cannot be established yet. Nevertheless, our *in vivo* chemical cross-linking of the Pca1p complex clearly indicates that Cdc48p plays a significant role for recruiting the proteasome to Pca1p in the ERAD process.

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. Several models addressing this issue have been proposed [16, 68]. Integral membrane proteins could be first dislodged from the membrane to the cytosol and then delivered to the proteasome. Cdc48p and/or unknown translocation machinery in the membrane could be involved in the process. Alternatively, the proteasome could be recruited to ubiquitinated substrates while they are at the membrane. AAA-type ATPases in the 19S regulatory particle of proteasome could be a driving force of substrates extraction from the membrane followed by direct feeding to the 20S. Given the endo-proteolytic activities of the proteasome [69], fragmentation of substrates *via* proteolysis of cytosolic loop might facilitate extraction of transmembrane helices. Intra-membrane proteases, such as rhomboid-family

proteins, could also play a role in the substrate fragmentation process [70-72]. It is intriguing to note previous studies reporting somewhat inconsistent data regarding this process. Several studies detected full-length and fragments of integral membrane proteins (e.g., Ste6*_p, CFTR, and MHCI) in the cytosol when the proteins undergo ERAD [16, 73-75]. An *in vitro* reconstitution assay system found that prior to degradation, Ste6*, a well-characterized ERAD-C substrate, is dislodged from the membrane [16]; however, it is worth pointing out that only a small fraction of Ste6*_p was released from the membrane under the experimental conditions. Inactivation of 20S proteasome in an *in vitro* ERAD assay of CFTR displayed relatively minor effects on membrane extraction and release of its degradation intermediates [73]. Distinct from these *in vitro* experiments, our study conducted in cells showed no detectable cytoplasmic full-length Pca1p or its fragments associated with ubiquitinylation when 20S activities of the proteasome are inhibited. Rather, Pca1 remained in membrane fraction despite functional Cdc48p and active 19S proteasome. Therefore, only minimal amount of Pca1p and Ste6*_p might be extracted to the cytosol, and the 20S proteasome could play a vital role for removing polytopic proteins from the membrane. These results suggest that cooperation between Cdc48p and the 26S proteasome is required for dislodging polytopic proteins from the membrane. Pca1p extraction and proteolysis by the 20S therefore could be coupled; thus, our results reflect that in the absence of 20S peptidase activities, a complex containing Pca1p and the 19S and 20S proteasomes stalls at the ER membrane.

Detection of the complex containing Pca1p and subunits of 19S and 20S proteasome at the membrane in a ubiquitin ligase dependent manner suggests that Pca1p ubiquitinylated by an E3 enzyme attracts the proteasome while it resides at the ER membrane. Consistently, no full-length Pca1p was detected in the cytosol of cells which is distinct from accumulation of poly-ubiquitinylated Cpy*_p, a luminal protein undergoing ERAD, in the cytosol as a complex with the proteasome. The proteasomes are localized mainly in the nucleus and cytosol as free or nuclear envelope and ER network-attached forms [76-78]. The clustering of the proteasomes on the ER

membrane is particularly eminent in the yeast *S. cerevisiae* (>80% total proteasome) relative to mammals (<20%) [77]. Sts1p (Cut8p in fission yeast) is a critical molecular factor involved in enriching the proteasome at the nuclear envelope [79, 80], while the counterpart at the ER membrane remains to be identified. The proteasome might be associated with the ER via physical interaction with ER membrane protein(s), which could be reminiscent of Ubc6p/7p and Cdc48p that are recruited to the ER membrane by Cue1 and Ubx2p, respectively [81-83]. Physical proximity of the proteasome to the ER could be in favor of efficient recognition of substrates for ERAD. Moreover, as we showed for Pca1p and Ste6*_p, 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 composition and dynamics 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 [78]. The assembly, subunit composition, modifications of amino acid residues, interaction with regulators(s) if any, and subcellular distribution are changed in response to cellular cues and stresses [76]. It would be interesting to examine if ER stresses would lead to recruitment of the proteasomes to the ER membrane. This could be better accessed in mammals displaying relatively minor steady state localization of the proteasome to the ER [84, 85]

The significance of polyubiquitination of membrane proteins in ERAD remains as an intriguing question. The regulatory particles of the proteasome contain several functional components, including AAA-Type ATPase, E4 ubiquitinating ligase(s), and de-ubiquitinylation enzyme(s). These enzymes are significant players for recognition of ubiquitin-tagged substrates, unfolding, poly-ubiquitinylation, translocation into the catalytic sites, and deubiquitinylation for rescuing ubiquitin from substrates [86]. Hul5p, an E4 enzyme associated in the regulatory particle of yeast, is involved in proteolysis of proteins by the proteasome [55]. It was shown that Hul5p is involved in ERAD of chimeric derivatives (e.g., CTL* and Sec61-2L) of well-characterized substrates [36] and proteasome-dependent degradation of cytosolic proteins that are damaged

under heat stress [54]. However, knockout of UFD2 gene encoding another E4 ligase did not affect the heat-induced ubiquitylation [54], indicating target specificity in E4 enzyme activation and/or requirement. 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. Significant remaining turnover of Pca1p in a *ufd2Δ* strain ($t_{1/2} = \sim 0.5$ hr) relative to that in a strain lacking DOA10, encoding E3 ligase for Pca1p ($t_{1/2} = \sim 2$ hr) [38] suggests that poly-ubiquitylation may not be essential for ERAD but rather enhance its efficiency. Alternatively, given three other E4 ligases in addition to HUL5 and UFD2 in the genome of the yeast *S. cerevisiae* [87], not only Ufd2p but also other E4 ligase(s) might target Pca1p for ERAD. However, we disproved this by showing near absence of poly-ubiquitylated Pca1p in *ufd2Δ* cells. The molecular basis of substrate specificity of E4 ubiquitin ligase and the significance(s) of poly-ubiquitylation in ERAD remain to be defined. It is interesting to note that although Pca1p turnover is slow in *ufd2Δ* cells and there is drastic reduction of poly-ubiquitylation of Pca1p in the cells, Ufd2p deficiency does not result in any significant effect on the formation of a complex between Pca1p and the proteasome at the membrane. These results indicate that polyubiquitylation of substrates might not be a determinant of initial interaction with the proteasome but rather promotes efficient processing of substrates by the proteasomes.

The underlying mechanism of Hrd1p E3 ligase dependent ERAD-L and M pathways are better characterized relative to those of ERAD-C pathway dealing with Pca1p and Ste6*p. Nevertheless, it appears that there is no convincing model accommodating the results of previous studies. It has been proposed that Hrd1p's transmembrane helices play a critical role in retro-translocation of Cpy1*p, a luminal ERAD substrate [33, 62]; however, the roles for Sec61p as a retro-translocation channel were reported as well [88, 89]. While Hrd1p is required for ubiquitylation of ERAD-M substrates [90], *in vitro* retro-translocation study of Hmg2p showed that it occurs in the absence of Hrd1p transmembrane helices [91].

Identification of Pca1p as a target for ERAD-mediated expression control [38, 39] and results presented herein provided an opportunity for a better understanding of ERAD of polytopic proteins. Further studies on proteasome-dependent turnover of membrane proteins in the ER and other organelles of yeast and higher eukaryotes would determine if the recruitment of the proteasome to ubiquitinated substrates while those are embedded in the membrane is a conserved mechanism.

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Chapter 4:
Cadmium Sensing and Amino Acid Composition of a Cis-acting Degron
And Determination of Its Role for ER-associated Degradation of Pca1
Cadmium Exporter

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4.1 ABSTRACT

The active control of protein turnover is a critical cellular process regulating biochemical pathways and destructing terminally misfolded or damaged proteins. Pca1p, a cadmium exporter in yeast, is rapidly degraded by the ER-associated degradation (ERAD) system in the absence of cadmium via a cis-acting degron rather than folding defect. Cadmium induces Pca1p expression in a manner dependent on the degron, suggesting cadmium-mediated masking of the signal that recruits molecular factors involved in ERAD of Pca1p. However, the characteristics and mechanisms of action of the degron in Pca1p and most of those in other proteins remain to be determined. Our data presented herein indicates the Pca1's degron senses cadmium via specific cysteine residues to induce conformational change. Random mutation of the degron followed by selection of those losing its degron functionality revealed that distribution of amino acid residues and secondary structure comprise the signal for recruiting molecular factors for degradation. In particular, hydrophobic amino acids and cadmium binding of the degron are critical for determining its interaction with the an Hsp70 chaperone, Ssa1p. These results illustrate a new mode of regulation of a degron. This might be useful for a better understanding of other degrons and regulated turnover of proteins.

4.2 INTRODUCTION

Protein degradation plays a significant role for the expression control of proteins in response to cellular cues followed by regulation of a number of biochemical pathways [1-4]. The system also destructs terminally misfolded and damaged proteins to prevent their accumulation and aggregation [5-8]. In line with these vital roles, defects in proteostasis in association with genetic and epigenetic problems, cellular stress, and/or reduced cellular capacity are attributed to multiple diseases such as neurodegenerative diseases, cystic fibrosis, diabetes, and cancer [12-14].

Regulated turnover of proteins should be highly target specific and dynamic to be able to respond to cellular needs. Molecular factors recognize the degradation signal or 'degron' in targets to initiate the process. The conditional exposure of degrons and/or activities of molecular factors involved in the process could determine the fate of targets [1, 15, 16]. Degrons can be masked or exposed through diverse mechanisms such as post-translational modifications (e.g., phosphorylation, hydroxylation, glycosylation), protein-protein interactions, and binding of specific metabolites [18-22]. Despite this conceptual frame and many examples undergoing regulated turnover, only a few degrons have been identified and characterized. For instance, I κ B phosphorylation or Cys modification of Keap1 leads to their dissociation from NF- κ B and Nrf2, respectively, to stabilize these transcription regulators [21]. Hydroxylation of specific proline residues of HIF-1 recruits a ubiquitin ligase complex [23, 24]. Given defining degron as a regions of proteins that can be transferred to other proteins with the maintenance of their characteristics [1, 25], a degron in Met α 2p transcription repressor in yeast has been relatively well characterized. Dimerization of Met α 2p with Met α 1p hides the degron [26], indicating an exposure control of a degron through protein multimerization. The degron forms an amphipathic helix, and hydrophobic face of the helix appears to be a primary signal for recruiting degradation machinery [26]. Similar characteristics were also found in a degron of mammalian GSK1 [27]. These characterizations of degrons reveal diverse and sometimes similar mechanisms in regulated

turnover of proteins. Nevertheless, the biophysical characteristics and mechanisms of regulation of degrons remain unknown in most cases.

The cells also degrade unassembled, misfolded, or damaged proteins to prevent toxicity of these abnormal proteins. Translational error, protein denaturation and damage by stresses such as heat, metals, and defects in maturation, including multimerization, glycosylation, or cofactor binding, all result in production of proteins that are non-functional and prone to aggregation [28, 29]. For instance, the ER-associated degradation (ERAD) system controls the quality of proteins that are first targeted to the lumen or membrane of the ER for distribution to different cellular compartments and membranes [30-34]. Defect in protein maturation, such as folding, multimer assembly, and glycosylation, results in destruction primarily by the ubiquitin/proteasome system in the cytosol. Several model substrates possessing mutations, such as CFTR, Cpy1**p*, and Ste6**p*, as well as natural proteins, such as ApoB, and Hmg2*p*, have been extensively studied [28, 35-38]. While many different molecular factors involved in the process have been identified [39], the mechanistic details remain to be investigated. For instance, it is still unclear how the degradation machinery recognizes terminally misfolded proteins relative to those undergoing folding, refolding, or maturation.

Ubiquitin is often utilized as a "tag" for degradation of proteins [4, 15, 39, 40]. The proteasome is a major destination for degradation of those proteins [41, 42]. The consecutive action of E1 activation enzyme, E2 conjugating enzyme, and E3 ligase of ubiquitin attach ubiquitin to mainly Lys residue(s) of targets [39]. Multiple E1, E2, and E3 enzymes in organisms displayed target specificity as well as redundancy [28, 39]. E4 enzymes extend the ubiquitin length by adding additional ubiquitin to existing ubiquitin moiety to promote degradation presumably via efficient delivery to and/or recognition by the proteasome [39]. There is also mounting evidence for the role of the Hsp70*p* chaperones in the degradation of proteins including several ERAD substrates [28, 43-46], which can resolve at least partially how the substrates of degradation pathways are

recognized initially. The cells express diverse molecular chaperones, including Hsp110, Hsp90, Hsp70, Hsp40, and smaller chaperones [35, 47, 48]. It has been believed that they are essential molecular factors for prevention of protein aggregation during synthesis, folding, refolding after denaturation, and delivery of substrates of ubiquitylation to E3 ligase [49]. For instance, Hsp70 chaperones are often considered to be involved in refolding of misfolded proteins in an attempt to maintain solubility and stability of the protein [49]. These proteins often work in congress with a co-chaperone (Hsp40), and their activity is dependent on nucleotide exchange (ATP and ADP) mediated by nucleotide exchange factors (NEFs) [49, 50].

We have documented that Pca1p in the yeast *S. cerevisiae* is a cadmium exporting P-type ATPase contains a degron at the cytoplasmic N-terminus [18, 51]. The degron residing between 250th and 350th amino acids is responsible for rapid turnover of Pca1p and another protein fused with the sequence via the ER-associated degradation (ERAD) pathway [18, 51]. Cadmium in the culture media rapidly upregulates Pca1p via the degron dependent mechanism [51]. Given high affinity of cadmium to the thiol and seven Cys residues within the degron, cadmium could directly bind to the degron as a mechanism of cadmium sensing [18] followed by masking the degron. This ERAD-mediated expression control of cell surface protein via a degron rather than misfolding or assembly defect as well as the masking of the degron by cadmium illustrates a unprecedented mechanism of protein turnover.

This study employed Pca1p, as a new and unique ERAD substrate to gain better insights into the characteristics of degrons and the mechanism by which cadmium controls accessibility of the degron. Our results presented herein suggest that direct cadmium binding via Cys residues in the degron in Pca1p alter the secondary structure and exposure of hydrophobic residues of the degron to avoid attraction of molecular chaperons and ubiquitylation enzymes.

4.3 Experimental procedures

4.3.1 Yeast strains and growth conditions - BY4741 haploid *S. cerevisiae* strain (*MATa his3_1, leu2_0, met15_0, ura3_0*) and null mutants including *pdr5::KanMX6*, *doa10::KanMX6*, *ydj1::KanMX6*, *Sse1::KanMX6*, *Sse2::KanMX6*, *Snl1::KanMX6*, *Fes1::KanMX6* were obtained from the Open Biosystems. All yeast cells were cultured in synthetic complete (SC) medium which contains 2% dextrose, 0.2% amino acid mixture, and 0.67% yeast nitrogen base and lacks the specific amino acid(s) necessary for plasmid selection. Cells were cultured at 30°C unless specified. The cells were co-cultured with a proteasome inhibitor (MG132, 20 uM for 2 hrs) as indicated in experiments.

4.3.2 Plasmid construction – A single copy yeast plasmid, p416-GPD [52], was used for GPD1 gene promoter-mediated constitutive expression of PCA1 and its mutant alleles, including L296S, C298A/C300A, C311A/C312A, S291C/C298S/L306C/C311S, I299N, I299T, F318L, I299L, L296S, Y203S, and N301L. Triple hemagglutinin (3HA) and double c-myc (2Myc) epitope were inserted after start codon and before stop codon of PCA1, respectively, for detection of Pca1p expression. The fusion of these epitopes did not alter function of Pca1p [51]. PCA1 mutant alleles were created with PCR-based site-directed mutagenesis using an over-lapping primer method [53]. The coding sequence of SSA1 was PCR amplified using a gene-specific primer set and inserted into the BamHI/XhoI restriction sites of p415-GPD plasmid [53] for expression in yeast. HA epitope was fused at the C-terminus. To purify glutathione sulfur transferase (GST)-fused Pca1p fragment encompassing amino acids 250 to 350 region (GST-Pca1(250-350)) by *E. coli* expression, the nucleotide sequence obtained by PCR using a gene-specific primer set flanked with BamHI restriction site and a stop codon followed by XhoI at the 5' and 3', respectively, was inserted into the pGEX-6P-1 vector.

YOR1 expression construct in p415-GPD vector was prepared by PCR amplification of the coding sequence of YOR1 gene in BY4741 strain using a gene specific primer set followed by ligation of it into XbaI and XhoI restriction enzyme sites of the vector. NotI site was artificially

inserted right after the start codon. 3HA epitope-fused PCA1(1-390) was inserted into the *NotI* site. Site-directed mutagenesis was conducted by the primer overlap extension method [54] to create HindIII and PstI sites flanking the PCA1(250-350) region without altering amino acids.

Plasmid amplification and purification using *Escherichia coli* was followed previously established molecular biology methods [55, 56] [110]. Yeast plasmid transformation was performed using a lithium acetate method [57].

4.3.3 Purification of Pca1(250-350)p peptide - BL21 *E. coli* cells expressing pGEX-6P-1 vector containing GST-Pca1(250-350)p or GST were grown to mid log phase and induced with 50uM IPTG over night at 18°C. Cells were collected and lysed by sonication in the lysis buffer (50mM Tris-HCl pH 7.2, 100mM sucrose, 10% glycerol, 500mM NaCl, 1mM *tris*(2-carboxyethyl)phosphine (TCEP), 1mM Pepstatin A and 1mM PMSF). Lysates were then incubated with Triton X-100 (1%) at 4°C for 30 min and then centrifuged at 11,000g to remove all debris. The resulting lysate was added to a glutathione agarose column (Pierce) and incubated overnight with gentle rocking at 4°C. The column was washed with the wash buffer (20mM Tris-HCl pH7.2, 100mM sucrose 10% glycerol, 150mM NaCl, 1mM TCEP, and 15mM octyl-beta-glucoside (BOG)). Then treated with 100 units Prescission protease (GE Life Sciences) in the wash buffer. This protein was then used for biophysical characterization of Pca1(250-350)p [56].

4.3.4 Metal-induced tyrosine quenching of Pca1(250-350) determined by spectroscopy.-

Binding of Cd²⁺ to Pca1250-350 [in 50 mM Tris-HCl (pH 7.4) in the presence of 1 mM TCEP] through addition of increasing concentrations of CdCl₂ (0.625 μM after mixing) was monitored by the quenching of Pca1250-350 tyrosine fluorescence. Excited at 280 nm where the emission was collected and filtered ensuring only emissions past 280 nm were collected [58].

4.3.5 Isothermal titration calorimetry (ITC) - Purified Pca1(250-350)p (10uM X amount) was placed into the reaction chamber of MicroCal VP-ITC microcalorimeter and 5uM CdCl₂ was added. The reaction was monitored for eighteen injections with at total runtime of 90 min and the resulting data was plotted using MicroCal Origin. This was repeated with CdCl₂ against the

sample buffer to exclude CdCl₂ binding to the components in the reaction buffer specifically TCEP.

4.3.6 Inductively coupled plasma mass spectrometry (ICPMS) - Cells were co-cultured with CdCl₂ (1μM for 0 - 60 min) at the mid log phase (OD₆₀₀~0.8). At each time point cells were collected and washed once in the worm media containing 10mM EDTA to remove CdCl₂ sticking to the cell surface. Cells were then thoroughly washed in ice-cold H₂O containing 10mM EDTA. Cells (OD₆₀₀=5) were collected and digested in 600μL of 10% nitric acid 70°C for 2hrs. Metal levels in the samples were measured by ICPMS as described previously [59]. Cadmium concentration in the cell was calculated using the average cell number 3×10^7 per each OD₆₀₀=1 and the average cell volume 42×10^{-15} L of cells at exponential growth phase [60].

4.3.7 Generation of a PCA1(250-350) library containing random mutations – PCA1(250-350) was PCR amplified under an error-prone condition [61] using a primer set flanked with HindIII and PstI sites at the 5' and 3' ends, respectively. This generated a library of PCA1(250-350) fragments containing a single amino acid change randomly per three fragments. PCR products were replaced the corresponding fragment of a plasmid containing a N-terminal in-frame fusion of PCA1(1-390) with YOR1 (described above 'Plasmids' section). After transformation approximately 6,000 E. coli colonies were obtained, which is corresponding to the possibility that the library covers 20X possibilities of each amino acid mutation. The resulting colonies were pooled together and subjected to plasmid extraction.

4.3.8 Selection of PCA1(1-390)-YOR1 plasmids containing mutation(s) in Pca1(250-350) which stabilize Yor1p to confer oligomycin resistance - PCA1(1-390)-YOR1 plasmids containing random mutation(s) in Pca1(250-350) were transformed to yor1Δ yeast strain. Replicative plating of the colonies (~30,000) to plates containing oligomycin (1μg/mL) selected yeast cells conferring Yor1p-dependent oligomycin resistance. Cells able to grow on oligomycin plates were considered positive and selected for culturing for plasmid extraction. The plasmid was extracted from the yeast cells as described previously [62] and amplified using E. coli.

Sequencing of the plasmids identified the residues that were mutated within the PCA1(250-350) of the plasmids.

4.3.9 Cell growth assay on solid media containing oligomycin and/or cadmium – Cell growth assays were completed as previously described [18]. Briefly wild-type strains expressing Pca1 (1-392)-Yor1, Yor1 or mutant Pca1 (1-392)-Yor1 were cultured in SC selective media to mid-log phase. Cells (~5 μ L, A₆₀₀ = 0.1) were spotted on solid YPEG media[18], prepared without the addition of cadmium and with or without the addition of oligomycin (1 μ g/mL). Plates were incubated at 30 °C for 2 days prior to photography.

4.3.10 Immunoblotting - Cells were broken using glass bead disruption in lysis buffer (PBS containing 0.1mM PMSF (phenylmethanesulfonylfluoride, Sigma), protease inhibitor cocktail (Complete mini, Roche), 0.1mM EDTA and 1% Triton X-100). Protein concentration of samples was measured using a BCA kit (Pierce) following manufacturer's instructions. Samples were denatured using the sample buffer containing dithiothreitol (25 mM) and subjected to denaturation at 37 °C for 15 minutes. SDS-PAGE was transferred to nitrocellulose membrane and proteins were detected using rabbit anti-HA monoclonal antibodies (Rockland, 600-401-384), mouse anti-myc antibodies (ABM, G019), and mouse anti-3-phosphoglycerate kinase antibodies (P_{gk1}) (Invitrogen, 459250). Horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology Inc., SC-2005) and goat anti-mouse IgG (Santa Cruz Biotechnology Inc., SC-2004) were secondary antibodies. West Pico Chemilluminescence (Thermo Scientific) was used for illumination of antibody bound proteins.

4.3.11 Co-immunoprecipitation to determine physical interaction between Pca1p and Ssa1p - 3HA-Pca1-2Myc with and without L296S substitution and empty vector (p416-GPD) were co-expressed with Ssa1-HA in Δ pdr5 cells. Cells at the mid log phase (OD₆₀₀= ~0.8) were treated with MG132 (20 μ M) for 2hr followed by collection and washing in ice cold PBS and then re-suspended in PBS containing MG132 (20 μ M) and 100 μ g/ μ L of the membrane permeable thiol-reversible crosslinker dimethyl 3,3'-dithiobispropionimidate (DTBP) (Thermo Scientific)

reversible crosslinker dimethyl 3,3'-dithiobispropionimidate (DTBP) (Thermo Scientific) (producer), a cell-permeable cross-linker, or 30min at room temperature with gentle rocking. Cells were then placed on ice and washed in the lysis buffer (50mM Tris-HCl pH7.4, 0.1mM EDTA, 0.1mM PMSF, protease inhibitor cocktail (Complete Mini, Roche) to quench the reaction. Cells lysates were obtained via glass bead disruption in the lysis buffer. Samples were spun down at 300g for 3min to remove debris and unbroken cells. The lysates were then centrifuged at 100,000g for 30min to create a pellet fraction. Pellets were treated with Triton X-100 (1%) for 30min at 4°C with gentle rocking for membrane solubilization. Protein concentration was measured using a BCA kit (Pierce). Cell lysates (1 mg) were incubated with anti-myc beads using a Profound™ c-Myc Tag IP/Co-IP kit (Thermo Scientific) according to the manufacturer's specifications. Eluted proteins were treated with DTT (150 mM) to break the cross links and incubated at 37°C for 30 min. the resulting solution was run on SDS-PAGE followed by immunoblotting to detect interaction between Pca1p and Ssa1p.

4.3.12 Alignment of Pca1(271-320) with the corresponding sequence of Pca1-like proteins in

other fungi – Pca1 (271-320) sequence was obtained from the yeast genome database, yeastgenome.org and NCBI BLAST was used for identification of closely related sequences [63]. After obtaining related sequences from other fungi (*Aspergillus fumigatus*, A.f., *Gibberella zeae*, G.z. *Penicillium janthinellum*, P.j. *Aspergillus clavatus*, A.c. *Botryotinia fuckeliana*, B.f. and *Sclerotinia sclerotiorum* S.s) these sequences were aligned using ClustalW2

<http://www.ebi.ac.uk/Tools/msa/clustalw2/> [11].

4.3.14 Prediction of secondary structure of Pca1(250-350) [and Pca1(271-320)] – Pca1 230-350 sequence was submitted I Tasser, <http://zhanglab.ccmb.med.umich.edu/I-TASSER/> for 3D structure prediction. The Highest scoring match was selected [64].

Helical wheel projections were completed by submitting Pca1 271-306 to <http://rzlab.ucr.edu/scripts/wheel/wheel.cgi>. Secondary structure prediction was also carried out

through submission of Pca1 271-320 and Pca1 271-320(I299N) to I-Tasser. Hydrophobic cluster analysis was performed using Pca1 250-350, and Pca1 250-350 containing either the I299N or L296S mutations [17] using the HCA portal through Expasy, <http://mobylye.rpbs.univ-paris-diderot.fr/cgi-bin/portal.py?form=HCA#forms::HCA>.

4.4 RESULTS

4.4.1 Determination of cadmium sensing by Pca1(250-350) peptide – Several lines of evidence [18, 51] indicate that Pca1p in the yeast *S. cerevisiae* undergoes a rapid turnover by the ER-associated degradation (ERAD) system in a cadmium and degron-dependent mechanism. To gain a better understanding of how the ERAD machinery recognizes Pca1p in the absence of cadmium and how cadmium masks the signal, we thought to further characterize the degron in Pca1p. Glutathione S-transferase (GST) fused Pca1(250-350) peptide encompassing from 250th to 350th amino acids was expressed in *E. coli* and subsequently purified using glutathione (GSH) agarose followed by cleavage of the GST (Fig. 1A). The purified peptide was subjected to UV fluorescence spectroscopy. Although this region does not contain any Trp residues, it does contain six Tyr residues and we were able to follow the change in emission spectra with the introduction of cadmium (Fig. 1B). Cadmium and copper both affected the emission spectra of the peptide but not zinc; this is consistent with *in vivo* data displaying that cadmium and copper but not zinc inducing stabilization of Pca1p in a manner dependent on the degron [9, 18, 51]. Isothermal titration calorimetry (ITC) was utilized to determine the binding affinity of cadmium with Pca1(250-350). As expected the peptide was able to bind cadmium (Fig. 1C). The disassociation constant of $K_d = \sim 6 \mu\text{M}$ was determined for this peptide when presented with CdCl_2 by $1/K$ (Fig. 1C lower panel). The observed stoichiometric ratio of Cd^{2+} binding was one Cd^{2+} ion to one peptide.

Given Pca1p's rescue from the ERAD when cells are co-cultured with cadmium less than $1 \mu\text{M}$ concentration, the K_d value was higher than anticipated. We addressed a possibility of intracellular cadmium accumulation over time to induce Pca1p stabilization. Indeed, cadmium concentration of the cells co-cultured with $1 \mu\text{M}$ CdCl_2 reached $\sim 6 \mu\text{M}$ and $\sim 15 \mu\text{M}$ after 5 min and 30 min, respectively (Fig. 2A). The cadmium accumulation was correlated well with Pca1p stabilization (Fig. 2B). While it is necessary to note the differences between *in vivo* and *in vitro* environments affecting available cadmium to Pca1(250-350), this data indicates that the $\sim 6 \mu\text{M}$ K_d for cadmium binding to the Pca1(250-350) is relevant.

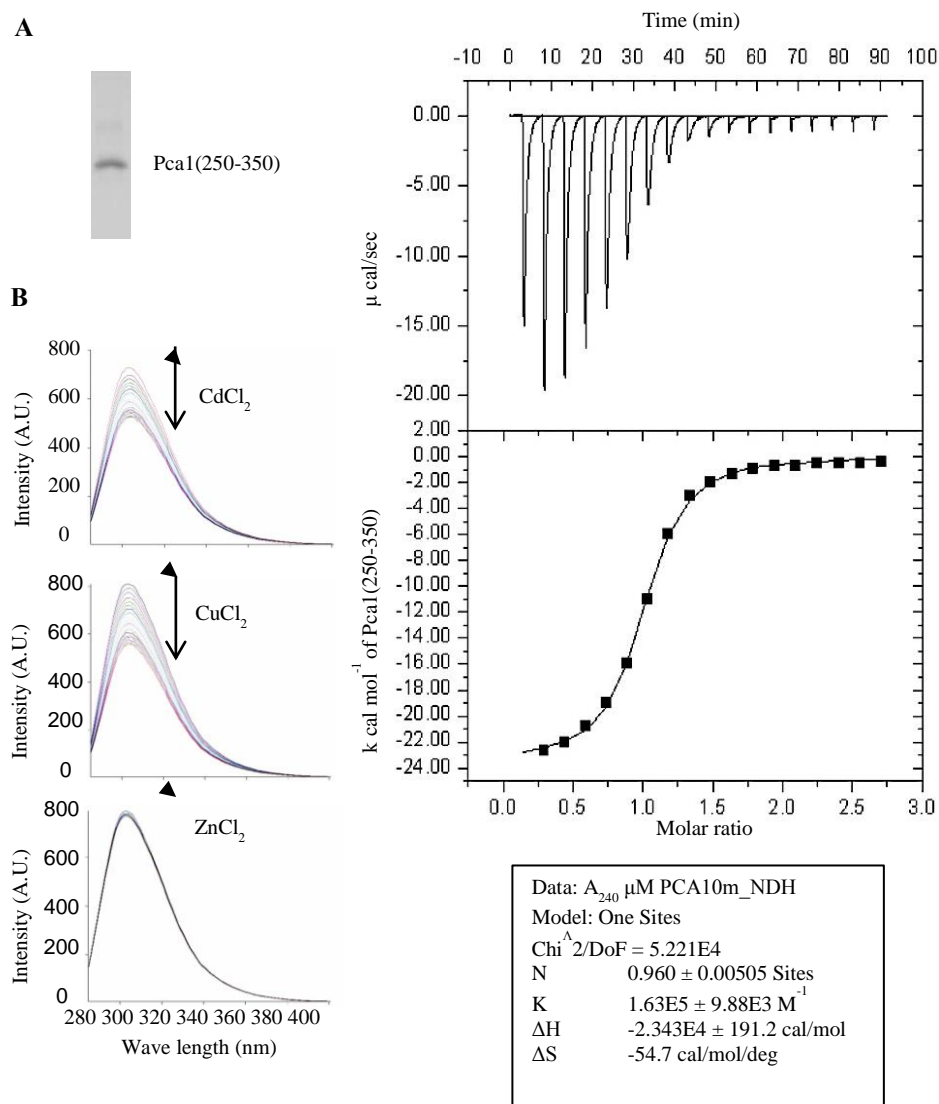


Figure 1. Cadmium binding to Pca1(250-350). *A*, Purification of Pca1(250-350) peptide containing a cadmium-responsive degen. *B*, Tyrosine quenching with the addition of metals using excitation emission spectroscopy. 2μM Pca1 + CdCl₂, CuCl₂, and ZnCl₂ at the concentrations from 0 to 6.25 μM in a cumulative manner and emission intensity were monitored. (How is data converted to arbitrary unit?) *C*, Binding of cadmium to Pca1(250-350) determined by *Isothermal titration calorimetry* (ITC). 5μM Pca1 (250-350) was titrated with 5μM CdCl₂ over 90 minutes.

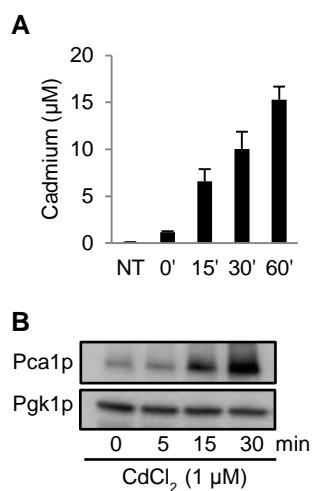


Figure 2. Accumulation of cadmium in the yeast *S. cerevisiae* and Pca1p up-regulation. **A**, BY4741 yeast cells in which PCA1 is non-functional because of natural mutation [9] were co-cultured with CdCl₂ (1µM in the media) at the mid log phase and collected at the indicated time points. Total cell-associated cadmium was measured by inductively coupled plasma mass spectrometry. Cellular cadmium concentration was calculated based on cell numbers of the samples and volume of each cell. The data indicates average and standard deviation of nine experiments. **B**, Determination of Pca1p stabilization as an indicator of cellular cadmium accumulation. BY4741 cells expressing functional Pca1p fused with N-terminal triple HA epitope (3HA) by the constitutive GPD1 gene promoter were cultured as described above **A**. Cell lysates were prepared. Western blotting analysis using anti-HA antibodies visualized cadmium-induced expression of Pca1p. Pgk1p was detected with specific antibodies to determine equal loading. A representative figure of two repeats is presented.

4.4.2 Roles for cysteine residues of Pca1(250-350) in cadmium sensing - Cys residues are openly targeted by cadmium because of its high affinity for thiols [65]. Pca1(250-350) contains seven Cys residues including those in the CXCX₁₀CC motif (X= any amino acid) (Fig. 3A). To determine if these residues are involved in the cadmium sensing, the Cys residues were substituted with Ala (Fig. 3B). Cells expressing PCA1 alleles possessing the mutations were cultured with and without cadmium (1 uM, 30 min), and steady state Pca1p levels was measured by Western blot. As expected WT Pca1p showed dramatic up-regulation of Pca1p when cells were treated with cadmium. Both the CxC and CC motif mutations revealed that Pca1p responds to cadmium albeit less effectively (less than 50% relative to control Pca1p) indicating that substitution of CxC or CC to Ala is unable to completely abolish cadmium sensing. Given the remaining sensing, the other five Cys residues could sense cadmium in the absence of two Cys residues at the CXC or CC. However, when all seven Cys residues were substituted to Ala, there was a complete loss of cadmium-induced Pca1p stabilization. It is also intriguing to note the elevated steady state expression levels of Pca1p possessing Ala substitution of CC or all seven Cys. This could suggest inefficient recognition of Pca1p by the ERAD machinery. Nevertheless, the expression of Pca1p possessing Ala substitution of all Cys residues in Pca1(250-350) is less than 10% levels of Pca1p in the cells co-cultured with cadmium (Fig. 3B) suggesting that the Ala substitutions do not perturb the major signal attracting the ERAD machinery.

In an effort to determine if the Cys residues work in congress to coordinate cadmium or if it was simply due to nonspecific thiol affinity of cadmium, Cys298 in the CxC and C311 in the CC were switched with S291 and S306, respectively. This broke the CXC and CC motifs but maintained the same seven Cys residues in Pca1(250-350). The complete loss of cadmium sensing of Pca1(S291C,C298S,S306C,C311S) (Fig. 3C), which was reflected by the lack of cadmium-induced stabilization, indicates the existence of a specific binding site for cadmium within Pca1(250-350). Pca1(S291C,C298S,S306C,C311S) still displayed a Doa10p-dependent turnover as its expression is high in a *doa10*Δ strain lacking E3 ligase which is responsible for

Pca1p turnover. Collectively, these results suggest that the distribution of Cys residues is critical for cadmium sensing likely via cadmium coordination.

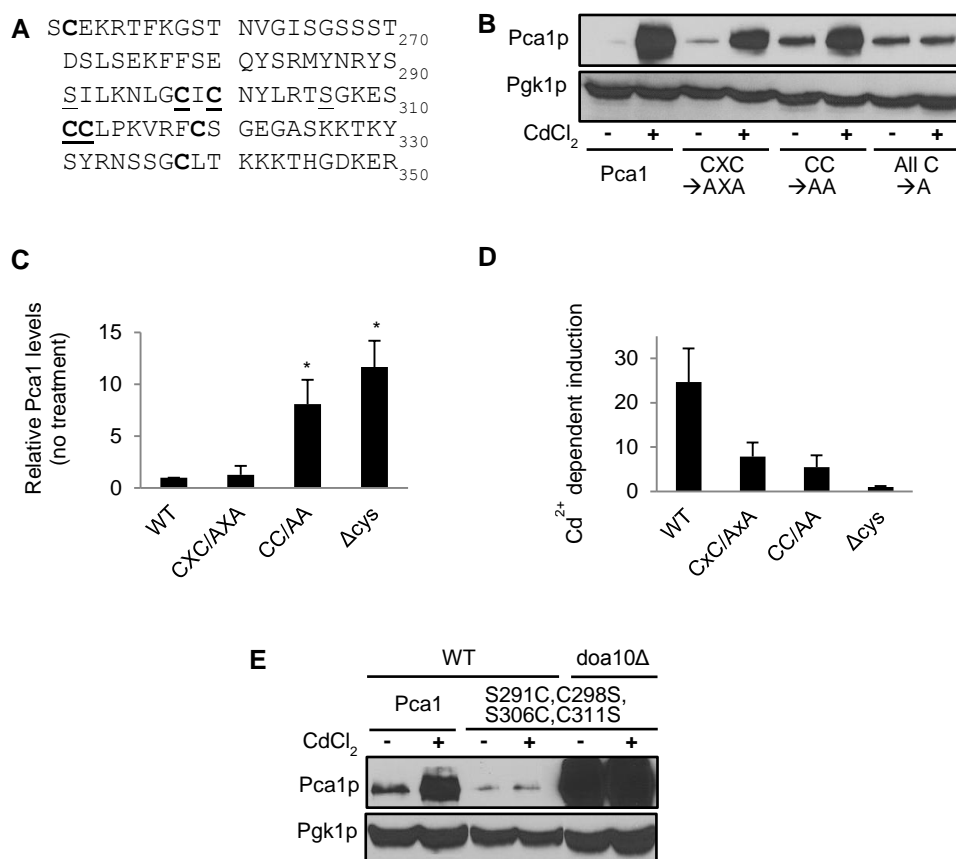


Figure 3. Roles for cysteine residues of Pca1(250-350) in cadmium-induced Pca1p stabilization. **A**, Amino acid sequence of Pca1(250-350) with cysteine (C) residues in bold. CXC and CC are underlined. Serine (S) residues that exchange their positions with the first C residues of the CXC and CC are underlined. **B**, The GPD1 gene promoter-mediated expression constructs of functional Pca1p with and without site directed mutation of Cys residues in Pca1(250-350) were transformed to BY4741 yeast strain where the chromosomal PCA1 is deleted. All these PCA1 were fused with N-terminal triple HA epitope (3HA) to detect Pca1p by Western blotting. Cells co-cultured with CdCl₂ (1 μM for 30 min) at the mid log phase were subjected to total cell lysate preparation by glass bead disruption in the buffer containing Triton X-100 (1%). Western blotting with anti-HA antibodies determined expression levels of Pca1p. Pgk1p levels were used to determine equal loading. A representative figure of four repeats is presented. **C**, The graph presents average and standard deviation of four experiments. Data reflects the steady state expression levels Pca1p possessing site-directed mutation(s) relative to those of wild-type control Pca1p. **D**, A graph representing the relative induction of Pca1 by supplementation of 1μM CdCl₂ vs no supplementation for each construct. **E**, Expression levels of Pca1p possessing S291C, C298S, S306C and C311S were determined in WT control and *doa10Δ* cells as described in **B**. A representative data of two experiments is presented.

4.4.3 Identification of residues in Pca1(250-350) that are involved in its functionality as a

degron - While our data presented in Figs. 1, 2 and 3 and published previously [51] indicates that a degron in Pca1(250-350) is masked by cadmium sensing by Cys residues, these Cys residues are not essential for recognition of Pca1p by the ERAD system. We next sought to identify amino acid residues composing the degron in Pca1(250-350). This is a significant question because the identity and characteristics of most of degrons are poorly defined despite regulated turnover of numerous proteins.

To be defined as a degron, the in frame fusion of Pca1(1-392) containing Pca1(250-350) with other proteins, such as CaCRP1p and Yor1p [18, 51], results in regulation of their expression in degron, ERAD, and cadmium-dependent manners. Yor1p is a multidrug resistance protein that effluxes oligomycin, a mitochondrial toxin [66], to confer cell growth. Given the rapid turnover of Pca1(1-392)-Yor1p, expression of it does not confer oligomycin resistance in yeast cells (Fig. 4A); however, cadmium co-culture stabilizes Pca1(1-392)-Yor1p to allow cell growth on the media containing lethal concentration of oligomycin (Fig. 4A). If the amino acid residue(s) within Pca1(250-350) necessary for its degron characteristics are substituted by different amino acid(s), Pca1(1-392)-Yor1p would be stabilized without cadmium co-culture followed by oligomycin resistance. We employed this experimental system to identify residues in Pca1(250-350) that are required for recognition by the ERAD machinery. PCA1(250-350) fragments possessing random mutation(s) were generated by an error-prone PCR method [61]. The pool of PCA1(250-350) fragments flanked with restriction enzyme sites was ligated with the corresponding enzyme sites artificially created at the same location in Pca1(1-392)-Yor1p. This generated a Pca1(1-392)-Yor1p expression library containing random mutation(s) at the region of Pca1(250-350). This library was transformed in yeast cells, and then the cells were selected on solid media containing oligomycin but not cadmium at the concentration displaying no growth of cells that express wild-type Pca1(1-392)-Yor1p (Fig. 4B). Pca1(1-392)-Yor1p expression plasmids were retrieved from

growing colonies. Sequencing of Pca1(250-350) region of the plasmids identified mutations. Retransformation of each plasmid to yeast cells followed by oligomycin resistance confirmed functionality of Pca1(1-392)-Yor1p (Fig. 4B). Pca1(1-392)-Yor1p possessing mutation(s) conferred growth of cells at different degrees suggesting each mutation resulted in different effects. When mobilized PCA1(1-392) possessing mutations (e.g., I299N, I299T, and L296S) that confer the strongest growth of cells on oligomycin plates back to the full-length PCA1, these mutations result in cadmium independent stabilization of the protein (Figs. 4C and 4D). To determine specificity of the identified mutation, we also introduced unidentified mutations, including I299L, Y302S, and N301L. These PCA1 alleles did not lead to Pca1p stabilization but resulted in slight less steady state Pca1p levels. This effect is more evident for PCA1(N301L) (Fig. 4D). Collectively, these results suggest that amino acid composition is a critical determinant of the degron.

While mutations were introduced randomly throughout PCA1(250-350), all of mutations except a few that were identified in combination with other mutation(s) were within PCA1(271-320) (Fig. 4B). This suggests that this region might contain major signal(s) to be a degron. Database searches for proteins containing homologous sequences of Pca1(271-320) identified only Pca1-like proteins in other fungi (Fig. 5). The corresponding sequence of Pca1(271-320) in the identified proteins displayed several conserved amino acids (Fig. 5), including CXC and CC that are important for cadmium sensing, and L299, L296, and E309 that are critical for the functionality of the degron (Fig. 4B). This indicates that Pca1p in other fungi might be regulated by cadmium in a similar manner in *S. cerevisiae* but the primary amino acid sequence of Pca1(250-350) is not sufficient to identify other degrons.

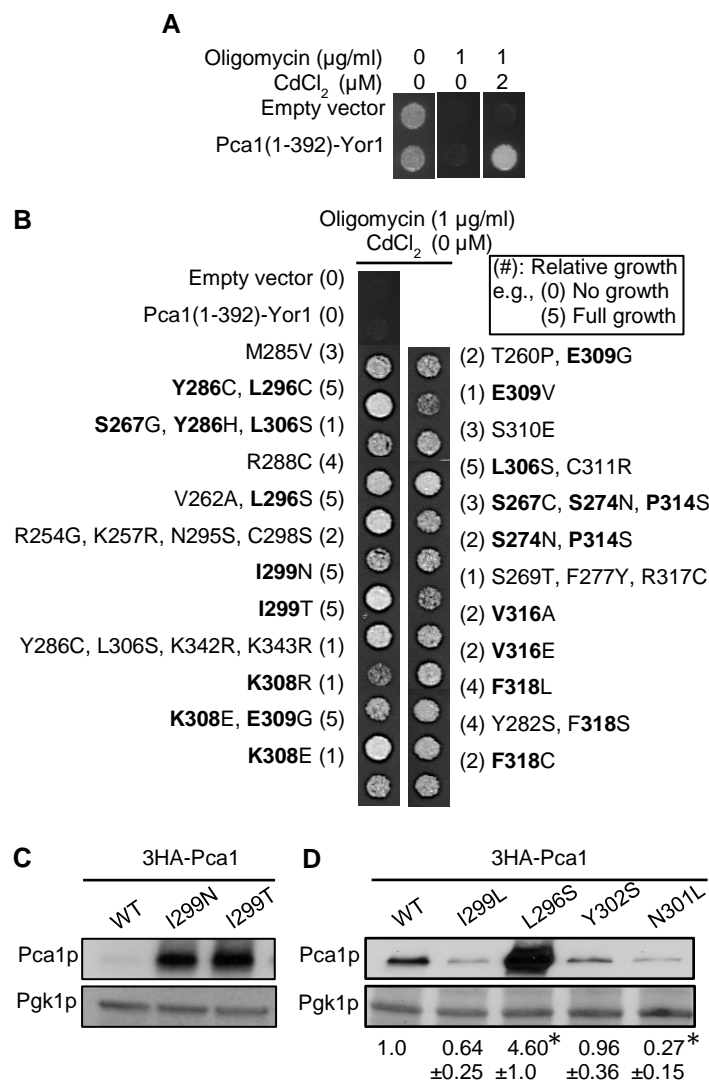


Figure 4. Identification of residues required for the functional role of Pca1(250-350) as a degron. **A**, A GPD1 gene promoter-mediated expression construct of Yor1p fused with Pca1(1-390) at the N-terminus (Pca1(1-390)-Yor1) was transformed into BY4741 yeast cells. Triple HA epitopes (3HA) were fused right after start codon. Growth of the cells expressing empty vector and Pca1(1-390)-Yor1 was examined on solid synthetic complete (SC) media lacking leucine (SC-leu) to select plasmid with and without supplementation of oligomycin and/or CdCl_2 at the indicated concentrations. Cells (~5 μl) at the mid log phase in liquid SC-leu media were spotted on the plates and cell growth was photographed in 2 days. **B**, Pca1(250-350) fragments containing random mutation(s) generated by error-prone PCR and flanked with HindIII and PstI sites were replaced the corresponding fragment in Pca1(1-392)-Yor1 by restriction enzyme digestion followed by ligation of the fragments. The plasmid library was transformed into yeast cells to select cells that can grow on solid media containing lethal concentration of oligomycin. Plasmids were retrieved from growing cells and subjected to sequencing. Transformation of the obtained plasmids into yeast cells and examination of oligomycin resistance confirmed specificity of the function conferred by Pca1(1-392)-Yor1 possessing mutation(s). Growth of BY4741 yeast cells expressing empty vector, wild-type control Pca1(1-392)-Yor1, or selected plasmids containing amino acid substitution(s) within the Pca1(250-350) were examined on solid SC-leu media containing oligomycin (1 $\mu\text{g/ml}$). Cells (~5 μl) at the mid log phase in the liquid SC-leu were spotted on the solid SC-leu media and cell growth was photographed in 3 days. The relative oligomycin resistance of cells were rated using cells expressing Pca1(1-392)-Yor1 containing Pca1(I299N) substitution as the highest “5” (full growth) and wild-type control Pca1(1-392)-Yor1 as the lowest “0” (no growth). Several plasmids contain more than one mutation. The quantitation reflects a confirmation by at least two experiments. **C and D**, Western blot using anti-HA antibodies determined steady state expression levels of Pca1p with and without indicated site-directed mutations. Triple HA epitope (3HA) were fused at the N-terminus (3HA-Pca1p). The GPD1 gene promoter-mediated expression constructs of 3HA-Pca1p were transformed into BY4741 cells lacking chromosomal PCA1. Cell lysates were prepared using a buffer containing Triton X-100 (1%) by glass bead disruption of cells that were collected at the mid log phase. A representative figure of four experiments is presented. **D**, Three other mutations (I299L, Y302S, N301L) that were not identified by the selection presented in **B** determined specificity of the effects of amino acid substitution on Pca1p stability. The results are quantitated and presents as folds change in expression levels of Pca1p possessing indicated mutation relative to that of control wild-type Pca1p. The asterisks indicate $p < 0.01$.

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S.c.      271    DSLSEK-FFSEQYSRMYNRYSSILKNLGCICNYLRTLKGKESCCLPKVRFCS 320
A.f.      122    GATDHK-ACSQHLSALDRYGATLQALGICRALIALGQETCCEIRERRSL 171
G.z.      23     GATDGK-PCNKHIVSALDRYGATLQALGCLCRTLISMGQESCCETRDRPA- 71
P.j.      1     ----HK-ACSQHLSALDRYGATLQALGICRALIALGQETCCEIRERQTL 46
A.c.      122    GATDRK-ACSQHNSALDRYGATLQALGICRALIALGQETCCEIRGRQAL 171
B.f.      71     LPSDEE-PCQSHLSKAKAAYSEILNNFGCICKVLLSLNLESCCALESQTVR 120
S.s.      130    -TKNKKGPCQSHMQSAKSRFQPTLDALEICRITLFAFNLESCCSKIDNTSC 179
          :   ..:           :   * . : *:* . * :.. *:*

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Figure 5. Alignment of Pca1(271-320) sequence with the corresponding sequence of Pca1-like proteins in other fungi. Blast search of the NCBI database for proteins that possess amino acid sequence similarities with Pca1p was conducted. Other fungi, including *A. fumigatus* (A.f), *G. zeae* (G.z), *P. janthinellum* (P.j), *A. clavatus* (A.c), and *B. fuckeliana* (B.f), possess Pca1-like proteins that are corresponding to Gene bank numbers 3510005, 156536662, 4708560, 2791550, and 5432339, respectively. The amino acid sequence of Pca1(271-320) was aligned with the corresponding sequence of those in the identified proteins ClustalW2, <http://www.ebi.ac.uk/Tools/msa/clustalw2/> [11]. Asterisk indicates all sequences aligned contain the same residue at that location. A double dot (:) indicates that most of the amino acids at the indicated location are very similar. A single dot (.) represents a somewhat similar conservation of amino acid type.

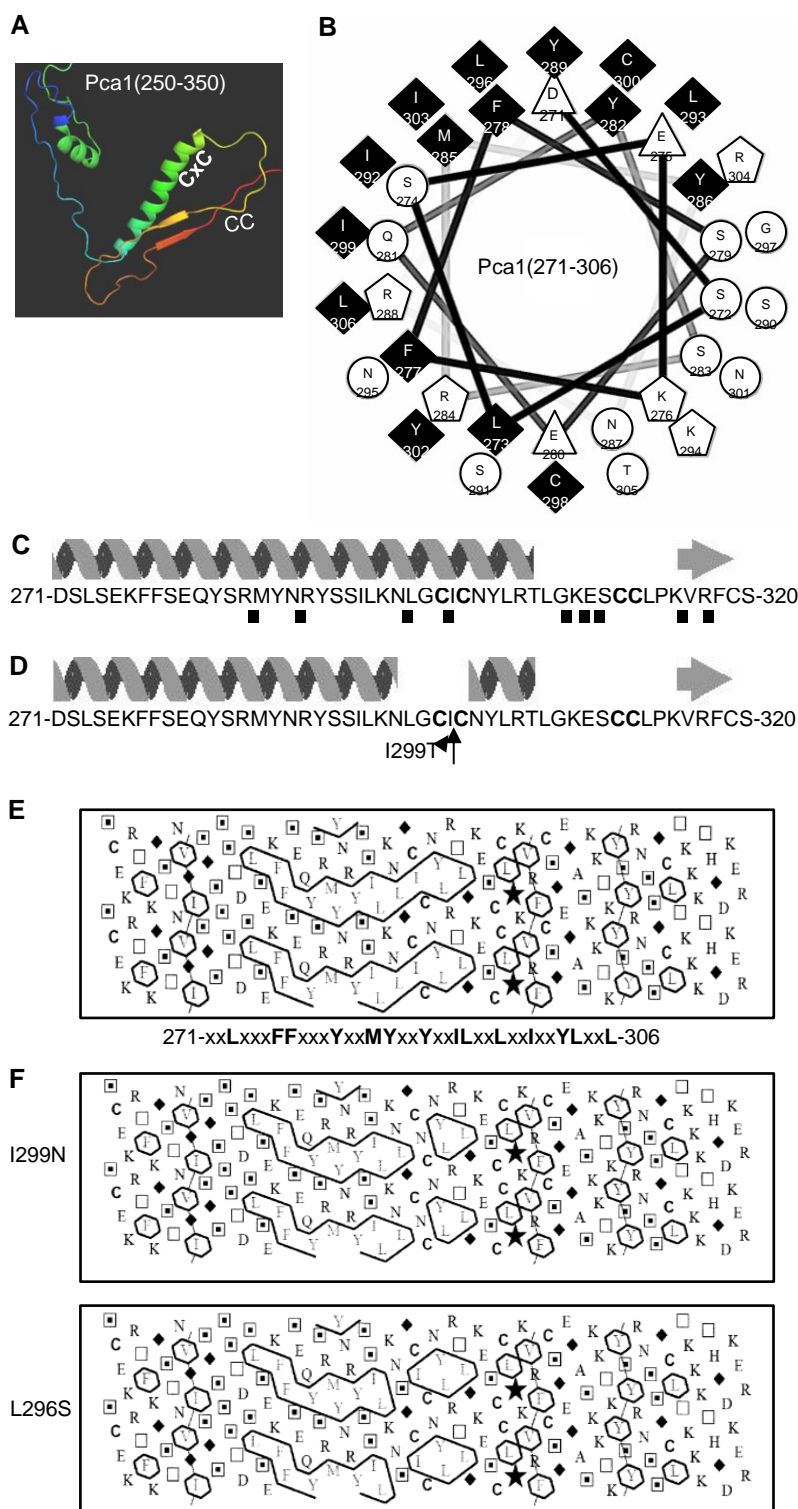


Figure 6. Prediction of secondary structure of Pca1(250-350) and distribution of amino acid substitution affecting Pca1p expression. **A**, Predicted structure of Pca1(250-350) obtained by I-TASER (Zhang Y, 2008). **B**, Distribution of amino acids within the Pca1(271-306) helix predicted by <http://rzlab.ucr.edu/scripts/wheel/wheel.cgi?sequence=ABCDEFGHIJKLMNQP&submit=Submit> [10]. Diamonds, circles, triangles, and pentagons indicate hydrophobic, hydrophilic, potentially negative charged, and potentially positively charged residues, respectively. **C**, Predicted secondary structure of Pca1(271-320) containing all confirmed mutations that affects YoriP-mediated oligomycin resistance when fused with YoriP (Fig. 4C). **D**, Perturbation of the secondary structure of Pca1(271-320) by I299T substitution. **E**, Formation of a hydropholic patch by amino acids within Pca1(271-306) obtained by hydrophobic cluster analysis <http://mobylye.rpbs.univ-paris-diderot.fr/cgi-bin/portal.py?form=HCA#forms::HCA> [17]. Amino acids that are involved in the formation of the hydropholic patch are indicated by the outline. The amino acids are present via their one letter identification except for glycine residues which are represented by a black diamond, serine, which are black squares with a dot in the middle, and threonine which are represented by black open squares. **F**, Disruption of a hydropholic patch within Pca1(271-320) by I299N or L296S substitution.

4.4.4 Characteristics of the degron in Pca1(250-350) – Given no sequence similarity of Pca1(250-350) with any known protein, we predicted its secondary structure of it using the I-TASSER program [64]. A long helix encompassing Pca1(271-306) followed by a unstructured loop and two short beta sheets was observed (Fig. 6A). The CXC and CC motifs are localized near the end of the helix and in the middle of loop, respectively. Another Cys residue is at the 1st beta sheet. The helix was amphipathic (Fig. 6B). It was intriguing to note that all single mutations negating the degron in Pca1(250-350) were all distributed within Pca1(271-320) corresponding to the helix, loop, and first beta sheet (Fig. 6C). Mutations within the helix reflect the reduction of hydrophobicity (Figs. 4C, 4D, and 4E, and Fig. 6C). The hydrophobic residues in the helix form a patch (Fig. 6E), which is consistent to amphipathic nature of the helix (Fig. 6B). Three mutations, including I299T, I299N, L296S, that lead to a dramatic increase in Pca1 stabilization (Fig. 4C and 4D) perturbed the cluster to alter the helix structure (Figs. 6D and 6F). Collectively, these data suggest that the pattern of amino acid distribution is a critical determinant of the degron and cadmium sensing by the Cys residues at the region perturb the characteristic to negate its functionality as a degron.

4.4.5 Interaction between Pca1(250-350) and Ssa1, a Hsp70, in cadmium and amino acid composition dependent manner – In addition to protein maturation and refolding of denatured proteins, previous research implied critical roles for molecular chaperones in protein turnover [28, 43, 67, 68]. For instance, ERAD substrates are recognized by molecular chaperones, especially Hsp70s, to be targeted to ubiquitinylation machinery [28, 69]. Surface exposure of hydrophobic residues in unfolded, misfolded or denatured proteins is considered as a primary determinant of recruiting molecular chaperones [49]. The hydrophobic patch in the helix of Pca1(250-350) (Fig. 6B and 6C) which is predicted to localized in the cytosol [9] might attract molecular chaperones, and cadmium sensing could induce conformational change to hide the signal. We elaborated this hypothesis by determining a physical interaction between Ssa1p, a Hsp70, and Pca1p in the cells expressing WT control PCA1 or PCA1 possessing L296S

substitution which leads to stabilization of Pca1p without cadmium co-culture. The cells co-expressing double Pca1p tagged with two c-myc epitopes (Pca1-2myc) and HA epitope tagged Ssa1p (Ssa1-HA) using the constitutive GPD1 gene promoter were cultured with and without cadmium in the media (1 μ M, 1hr). To prevent rapid turnover of Pca1p, MG132, a proteasome inhibitor was also added to the media (20 μ M 30min prior to collection). After treating cells with a cell-permeable cross linker (DTBP, 100 μ g/ml for 30 min), membrane fractions were obtained by glass bead disruption followed by Triton X-100 solubilization. Pca1p-2Myc was immunoprecipitated using anti-myc antibodies and probed with anti-myc antibodies and anti-HA antibodies to detect Pca1p and Ssa1p, respectively (Fig. 7A). While similar levels of Pca1p was immunoprecipitated, Ssa1p association with Pca1p was less than 15% ($13.4 \pm 1.5\%$, n=2) of control cells if the cells were co-cultured with cadmium (Fig. 7A). This result clearly suggests that cadmium sensing by Pca1p interferes with recruitment of Ssa1 protein presumably by masking the hydrophobic patch within Pca1(271-320). To address this, we compared complex formation of Ssa1p with control Pca1p and Pca1p(L296S) which is stable and perturbs the hydrophobic patch in the Pca1(271-320) region (Fig. 6F). Indeed, the pull-down efficiency of Ssa1p by Pca1(L296S)p was less than 20% ($15.7 \pm 5\%$, n=2) relative to that of control Pca1p. These results indicate that cadmium and the characteristics of amino acids within the degron, such as hydrophobicity, affect Pca1p's interaction with Ssa1p, a known molecular factor of ERAD.

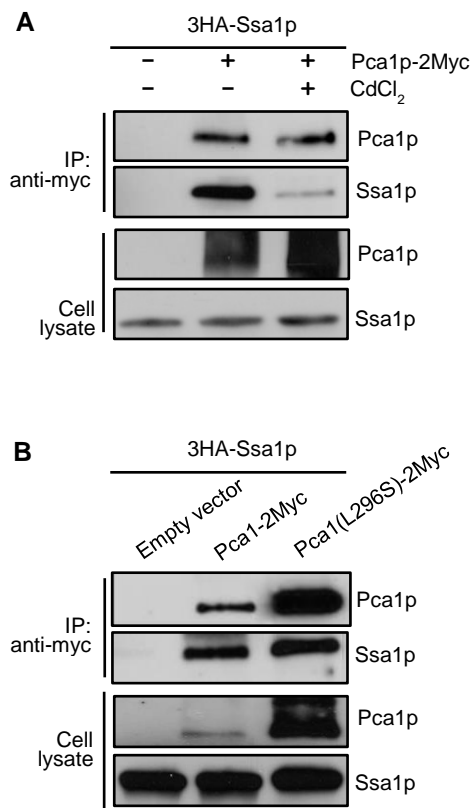


Figure 7. Physical interaction between Pca1(250-350)p and a Hsp70p in a cadmium and amino acid composition-dependent manner. **A**, BY4741 yeast cells expressing Pca1p tagged with two c-myc epitope (Pca1-2Myc) and Ssa1p tagged with triple HA epitope (3HA-Ssa1) by the constitutive GPD1 gene promoter were co-cultured with and without CdCl₂ (1 μM) for 1hr at the mid log phase and treated with the membrane permeable cross linker DTBP (100ug/mL). Cell lysates were prepared by glass bead disruption with isolation of the pellet fraction followed by Triton X-100 (1%) solubilization. Samples were subjected to Western blot using anti-HA and -myc antibodies. Pgk1p was detected using specific antibodies to determine equal loading. Immunoprecipitation of Pca1-2Myc in the lysates was carried out using anti-myc conjugated beads (Pierce). Pca1-2Myc and 3HA-Ssa1 in the eluted samples were detected by Western blot using anti-myc and -HA antibodies. **B**, The experiments described in **A** were conducted in cells expressing an empty vector, Pca1-2Myc, and Pca1(L296S)-2Myc along with 3HA-Ssa1. L296S indicates the substitution of Leu at 296th amino acid in Pca1p to Ser. Cells were collected at the mid log phase without cadmium co-culture.

4.5 DISCUSSION

Every single protein in cells theoretically contain embedded signal(s) determining its life span and potential to be a target of quality control in association with diverse reasons ranging from genetic mutation to translational error to cellular stress. Nevertheless, it remains to be determined if there are common and/or client-specific characteristic(s) in the signals. With the continuous efforts for identification of such signals from different targets, one could come up with algorithms by which the signal(s) in each protein could be predicted and provide methods for controlling the signals via physiological or pharmacological approaches. This is an important issue because various diseases are implicated with imbalance in protein expression and turnover [12, 70-72].

Despite many examples of regulated turnover of proteins, the identity and mode of control of degradation signals have been defined for only few of them. Our study illustrates the characteristics of a degron in Pca1p, which leads to a better understanding of the mechanism underlying regulated turnover of Pca1p cadmium exporter and other proteins. While the amphipathic helix characteristics of a portion of Pca1p's degron are similar to those of Mat α 2p in yeast [26] and GSK1 in mammals [27], it contains unique amino acid residues that appear to be involved in inactivation of the degron in response to cadmium sensing. The results presented in this manuscript reflect one of best-characterized degrons.

Cadmium inactivates the degron in Pca1p [18, 51], which explains the mechanism underlying rapid up-regulation of Pca1p expression at the cell surface when cadmium accumulation is high. Our *in vitro* studies conducted using purified Pca1(250-350) clearly displayed cadmium and copper but not zinc sensing by the peptide. This result is consistent to that of *in vivo* determination of metal-induced stabilization of Pca1p determined in cells [9, 51]. It is worth noting that copper-induced stabilization was evident in Δ ace1 cells defective in metallothionein (MT) induction but not wild type (WT) cells [9], which indicates that copper sensitivity to Pca1(250-350) is low relative to Ace1p and MT. Despite cadmium binding to MT distinct from

mammalian cells in which MT expression is regulated by cadmium and several other metal ions, the activation Ace1p in yeast *S. cerevisiae* that control MT expression, copper-specific [73]. Consistently, cadmium-induced stabilization of Pca1p was observed in WT cells [9]. The selectivity of Pca1(250-350) to cadmium versus zinc is intriguing because of the similar characteristics between the elements and documented examples of sharing binding sites and transporters [65, 74-76].

Given the critical role of Pca1(250-350) as a sensor of cadmium to stabilize Pca1p, the relatively high $K_d \sim 6 \mu\text{M}$ of Pca1(250-350) for cadmium was an unanticipated outcome. It was shown that CueR transcription activator of copper efflux genes in *E. coli* manifests zeptomolar sensitivity to free Cu^+ [77]. It was surprising to note that cells co-cultured with $1 \mu\text{M CdCl}_2$ accumulate $\sim 6 \mu\text{M}$ cadmium within 5 min, which was correlated with Pca1p stabilization. Therefore, our *in vitro* results could reflect cadmium sensing by Pca1(250-350) *in vivo*. Another possibility is that cadmium sensing by Pca1(250-350) is promoted by specific factor(s). Glutathione (GSH) is an abundant (low mM concentration) tri-peptide that is vital for cell growth and cadmium detoxification through chelation and conveying cadmium complexes to YCF1 cadmium transporter in the vacuole [111]. Our study however indicated that Pca1(250-350)'s cadmium binding *in vitro* is not affected by reduced and oxidized GSH and cysteine in the reaction buffer (data not shown). This suggests that cadmium binding affinity is higher for Pca1(250-350) relative to that of GSH and free cysteine. Assessment of cadmium-induced Pca1p stabilization in the absence of GSH *in vivo* was not lead to clear answer (data not shown), which seems to be primarily attributed to essential roles of GSH in yeast cell growth [78]. Studies on characterization of molecular factors for copper metabolism revealed the roles for metallochaperones for subcellular distribution of copper [79]. It is intriguing that Atx1p metallochaperone can bind both cadmium and copper [80]; thus, Atx1p may be involved in subcellular trafficking of cadmium for Pca1p, which could increase the efficiency of cadmium sensing. However, our previous data showed that Pca1p-mediated cadmium resistance is not

affected in the absence of known copper chaperones [59]. These results do not ignore the possibility of unidentified cadmium-specific metallochaperone(s) that are involved in cadmium delivery to the degron in Pca1(250-350).

The half-life of proteins is determined by various mechanisms such as intrinsic signatures (e.g., N-terminal amino acid, PEST sequences), cellular regulation (e.g., post-translational modification of targets recruiting degradation machinery), folding failure, and damage [1, 81-83]. Cadmium-induced masking of the degron is an interesting example of regulation of protein turnover. Small molecules such as inorganic elements, xenobiotics, substrates, ligands, and metabolites could be widely involved in cellular control of protein stability. This process is analogous to allosteric and feedback regulation of protein activities, but only a few examples have been reported. It was shown that cell permeable agonists and antagonists elevated expression of wild-type and mutated δ opioid receptors that are degraded by ERAD [84]. Binding of estrogen to the estrogen receptor α (ER α) controls the pathways of its turnover [85]. Turnover of apolipoprotein B (ApoB) and Hmg2p in response to limitation of lipid molecules and surplus of intermediates of sterol biosynthesis, respectively, are other examples of ligand-dependent protein turnover [22, 86, 87]. Further studies on the mechanisms of the processes and identification of additional targets undergoing similar mode of regulation could confirm if it is a prevalent way of controlling protein turnover.

The core sequence of the degron Pca1(271-320) contains 5 Cys residues and most of the identified residues that affect functionality of the degron. While no metal-sensing degron has been characterized previously, Cys is a common residue of metal binding sites in proteins [88]. It is worth noting that several other P_{1B}-type ATPase family members in mammals and plants to which Pca1p belongs, possess under-characterized metal-binding sites at the N- or C-terminus [89, 90]. Several of them are required for the function of the transporters [90], but their roles for regulation of activities, expression, and/or subcellular trafficking remain to be defined. Metal sensing transcription regulators such as Mac1p and Ace1p in yeast and MTF-1 in higher

eukaryotes, including mammals, also rely on Cys residues that likely bind with metal ions as a sensing mechanism [91]. Other common amino acid residues involved in metal sensing are histidine and methionine. For instance, a histidine-rich cluster mediates the ubiquitylation and degradation of a human zinc importer to protect against zinc cytotoxicity [92]. The degron in Pca1p contains one Met and His residues and manifests a specificity in sensing cadmium and copper but not zinc and iron, which illustrates a metal specificity ([9]; Fig. 1B).

Only Pca1-like proteins in other fungi contain a similar amino acid sequence to Pca1(271-320), which indicates that the primary sequence of the degron in Pca1p does not provide useful information for identifying degrons in other unrelated proteins. Our several attempts for obtaining secondary and tertiary structures of Pca1(250-350) containing the degron with and without cadmium sensing have not been successful which is primarily attributed to solubilization problem of purified peptide at concentrations required for in solution experiments and crystallization. Nevertheless, identification of amino acid residues within Pca1(271-350) that are responsible for its function as a degron revealed a critical role of the residues forming a hydrophobic patch on a predicted amphipathic helix of Pca1(271-306). The exposure of hydrophobic residues at the surface of proteins resulting from misfolding and denaturation has been considered as a signal for recruiting molecular factors involved in refolding and destruction [26, 93]. Given Pca1p stabilization by site-directed substitution of several other amino acids in the predicted loop and beta-sheet that follows, the amphipathic helix suggests that those mutations could mimic cadmium sensing to induce conformational changes of the helix to perturb the degradation signal and/or be part of the degradation signal. The degradation signals of two known ERAD substrates, Met α 2 of yeast and mammalian Sgk1, have been characterized as amphipathic helices [26, 27]. Intermolecular interaction masks the signal in Met α 2 [26]; however, it is uncertain whether the degron in Sgk1 is regulated by cellular cues as a mechanism of expression control. A non-biased selection of peptides that can serve as signals for ERAD in yeast *S. cerevisiae* when it is fused with another protein identified 16 to 50 amino acid peptides that are highly hydrophobic [94].

Determination of the contribution of each amino acid in one of the peptide by site-directed mutagenesis confirmed that both a patch of bulky hydrophobic residues and positive charged residues were found to be essential, which was distinct from the degrons in *Meta2* and *Sgk1* [95]. These previous reports are consistent to our results indicating the roles for hydrophobic residues and two positively charged residues, including R288 and K309, in ERAD of *Pca1p* (Fig. 6C). Our study also identified a negative charged residue E309 as an important component of the degron (Fig. 6C), which is unique for *Pca1p*.

Nevertheless, all these characterized signals targeting proteins for ERAD do not display any sequence homology with other known proteins except their orthologues in other organisms. With these limited examples of characterized degrons for ERAD, it is evident that the primary amino acid sequence is not sufficient to predict degrons in proteins undergoing regulated turnover. Integration of secondary and tertiary structures of particular protein and its interactions with other proteins and small molecules might achieve the goal.

Regulated turnover and ERAD are largely dependent on ubiquitinylation followed by degradation at the proteasome [39]. E3 ubiquitin ligases are major molecular factors for making substrates expose degradation signals [96]. While substrate-specific mediators for recruiting protein degradation apparatus have been identified [97], several lines of evidence indicate that molecular chaperones rather than E3 enzymes are primarily responsible for recognition of the substrates [47, 98-100]. Many of molecular chaperones were initially characterized as heat-stress induced proteins (HSP) that play critical roles for protein folding [49]. Thus, the dual roles for HSP, folding and degradation, are intriguing, and it is still unclear exactly what determines the fate of clients of HSP. Our data displayed that *Ssa1p*, an Hsp70, physically interacts with *Pca1p* in a cadmium and degron functionality dependent manner, and the reduced interaction assessed by less efficient immune-precipitation of *Ssa1p* with *Pca1p* is correlated with stabilization of *Pca1p*. Given surface exposure of hydrophobic residues as a signal for recruiting molecular chaperones, the result supports the notion that the patch of hydrophobic amino acids at the helix

Pca1(271-306) is recognized by Ssa1p. Identification and characterization of other molecular factors involved in presenting Pca1p for ubiquitinylation such as Pca1p specific mediator(s), Ssa1p co-chaperones, nucleotide exchange factors of Ssa1p, and/or other HSP family members warrants further studies.

Our results indicate that cadmium serves as a natural chemical chaperone masking the degron in Pca1p for elevating its cell surface expression. Given the implications of rapid and inefficient turnover of proteins in various human diseases, therapeutic control of protein turnover is an important research topic. Identification of small molecules that can improve maturation and secretion of CFTR Δ F508, which is associated with the most common lethal genetic disease in Caucasians, has been actively explored [101]. For instance, co-culture of cells expressing CFTR Δ F508 with glycerol, known to stabilize proteins in their native conformation, restored its chloride transport function [102, 103]. The effects of glycerol were also observed for a mutated hERG potassium channel [104] and yeast Hmg2p [105]. The therapeutic potential of chemical chaperones in neurodegenerative diseases manifesting accumulation of misfolded proteins, such as Alzheimer, Parkinson, Huntington, and prion diseases has been proposed as well [106]. It is intriguing to note that inorganic elements are contributing factors of these disorders; this could be through direct binding, metal-catalyzed generation of reactive oxygen followed by protein damage, and/or stabilization of specific conformation of peptide implicated in each disease [107-109]. Nevertheless, our studies on cadmium and copper as a folding factor of a degron in Pca1p to lead to beneficial outcomes provides a new conceptual frame for further determination of chemical chaperone roles of other element, especially abundant ions, such as potassium, sodium, magnesium, and chloride.

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5. Summary and Conclusions

5.1 Final discussion

The results discussed in this thesis demonstrate a mechanism by which Pca1, an ERAD-C substrate, is regulated via a specific sequence known as a degron. The degradation of Pca1 is predicated on the presence or absence of cadmium. Under conditions in which cadmium is not present, Pca1 is degraded through the ERAD pathway in which Doa10 is the E3 ubiquitin ligase and essential for this process [1]. These results display the first example of ERAD in which a fully functional secretory protein is regulated by its substrate for rapid expression.

It will be beneficial to determine if the mechanism by which Pca1 is degraded/regulated can be applied to other substrates of the secretory pathway. Expressional control of many proteins that are not required to be constitutively expressed may be regulated in such a manner. The advantage of this mechanism for Pca1 to the cell is due to the toxicity of cadmium. The continual synthesis and degradation of the protein allows for a rapid up-regulation of expression of Pca1 in the event of exposure to cadmium. However, the cell may not want to continually express Pca1 as many transporters lack high levels of fidelity and export of essential elements could be detrimental to the cells survival.

We also determined that the proteasome is required for extraction of both Pca1 and another ERAD-C substrate Ste6* from the ER membrane [2]. This result displayed a mechanism in which cells are able to degrade polytopic proteins and prevent release of these proteins into the cytosol where maintenance of solubility would be a taxing factor. Cdc48 was also determined to be important for substrate interaction with the proteasome, specifically in the case for Pca1. The exact function of Cdc48 in removing proteins from the membrane has yet to be determined but we can speculate that it may be important for recruiting the proteasome to the ubiquitylated substrate. This will be worthy to test to determine the exact mechanism by which Cdc48 is required for substrate-proteasome interaction as Cdc48 is implicated in the turnover of many different substrates [3, 4]. We further displayed the requirement of Ufd2, a Cdc48 resident E4

ubiquitin chain extension enzyme, for efficient degradation of Pca1 [5]. We also determined that although Ufd2 functions in poly-ubiquitinylation of Pca1, this process is not essential for Pca1 interaction with the proteasome. It is interesting to note that the poly-ubiquitinylation enzyme not Hul5 was implicated in Pca1 degradation despite its convenient location on the 26S proteasome [6]. This indicates that there is substrate specificity for poly-ubiquitinylation, and it would be worthwhile to define the determinants for this process.

Finally, we sought to determine the structural and environmental elements which led to the recognition of Pca1 for degradation and what changes occurred by the addition of cadmium to allow for rescue from said degradation. Utilizing the degron of Pca1 (250-350), we determined specificity and K_D for cadmium binding. Unfortunately, we were unable to go beyond this point with biophysical characterization due to the propensity of this peptide to aggregate. We also determined that two cysteine motifs were required for the sensing of cadmium and rescue of Pca1 from ERAD. We further established a random mutagenesis screen which led to the discovery of single mutations within the degron that rescued Pca1 from ERAD. These mutations seemed to be clustered in the Pca1 (271-320) region. Modeling of this region revealed a hydrophobic patch as well as an amphipathic helix. Site directed mutagenesis to disrupt this hydrophobic patch and amphipathic helix stabilized Pca1 indicating that the mechanism of sensing may be through the exposure of hydrophobic residues. We hypothesized that cadmium presentation to the degron may also disrupt/mask this hydrophobic exposure and lead to stabilization of the protein. Indeed, we saw a dramatic decrease in interaction with both site directed mutation and cadmium supplementation with the Hsp70 Ssa1, a known component of the ERAD-C system involved in recognition of substrates as well as maintaining an established role for refolding of misfolded proteins [2, 7]. We also sought to determine if Pca1 degron was conserved. We found that it is only conserved in other fungi; however, the discovery of an amphipathic helix within Pca1 (271-320) could allow for the establishment of a mechanism for other ERAD-C substrates. A cytosolic Doa10 substrate Mat α 2 has been shown to contain an amphipathic helix [8]. The development of

mechanism by which known substrates are recognized and degraded. Further understanding of protein degradation as demonstrated in this thesis can also lead to therapeutic approaches for increased secretion of degradation of desired proteins. As mentioned above, increased secretion of CFTR Δ F508 is of particular interest in the battle against cystic fibrosis [9].

A further interesting note is the role of chaperones. We displayed the interaction of Ssa1 with Pca1 in a cadmium and hydrophobic patch dependent manner. Chaperones are commonly considered to be involved in refolding/rescue of proteins from degradation [7]. There is also a plethora of evidence suggesting a role in protein degradation [2, 10, 11]. The determinants of what signals refolding as opposed to degradation for these chaperones is poorly understood. Pca1 will be an important tool for defining this mechanism as it is a short-lived protein that can be easily rescued from degradation and its interaction with Ssa1 is dependent on the nature of its degron.

5.2 Works Cited

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