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Identification and Characterization of a Cadmium-Transporting P-Type ATPase in Yeast *Saccharomyces cerevisiae*

David J. Adle
University of Nebraska - Lincoln, david.adle@huskers.unl.edu

IDENTIFICATION AND CHARACTERIZATION OF A CADMIUM-TRANSPORTING P-TYPE ATPASE IN YEAST *SACCHAROMYCES CEREVISIAE*

By

David James Adle

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IDENTIFICATION AND CHARACTERIZATION OF A CADMIUM-TRANSPORTING P-TYPE ATPASE IN YEAST *SACCHAROMYCES CEREVISIAE*

David James Adle, Ph.D.

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Adviser: Jaekwon Lee

Detoxification and homeostatic acquisition of metal ions are vital for all living organisms. Non-physiological heavy metals are toxic at low concentrations and represent major environmental hazards to human health. In particular, cadmium is a toxic environmental pollutant linked to a number of ailments including cancer, kidney and bone disease and reproductive disorders. The biological effects of cadmium toxicity which lead to human disease and the cellular mechanisms for cadmium defense are ill defined. Thus, the study of heavy metal detoxification systems represents an important research avenue to help combat cadmium related disorders.

The goal of this research project was to identify novel factors involved in metal ion defense in the model eukaryote, *Saccharomyces cerevisiae.* A genetic screen led to the discovery of Pca1, a P-type ATPase which functions as a cadmium-specific efflux pump. Unexpectedly, the *PCA1* allele in common laboratory yeast strains possesses an inactivating mis-sense mutation which has complicated previous attempts to characterize its function.

A unique feature of Pca1 is a cysteine-rich cytosolic amino terminal extension that can participate in metal coordination and regulate expression levels. Under normal

conditions, Pca1 is an unstable protein that is ubiquitinated and rapidly degraded by th proteasome. However, in the presence of cadmium, ubiquitination is inhibited resulting i rapid up-regulation and trafficking of Pca1 to the cell surface for cadmium efflux. An autonomous degradation signal within the cysteine-rich domain is necessary and sufficient for metal responsive regulation.

Finally, a second genetic screen was devised to uncover factors involved in Pca1 degradation. Unexpectedly, components of the ER-associated degradation (ERAD) pathway were found to be required for Pca1 turnover in the absence of cadmium. Conformational changes associated with cadmium binding to the metal sensing degradation signal lead to the escape of Pca1 from ERAD.

In summary, our data have revealed a novel metal detoxification system in a eukaryotic organism mediated by a P-type ATPase that is unique in structure and substrate specificity. Furthermore, we have described an unprecedented mode of ligand regulated degradation of a cell surface transporter at the ER and have provided the mechanistic basis of this regulation.

DEDICATION

To my wife Angela and son Caleb

ACKNOWLEDGMENTS

First of all I thank my academic adviser, Dr. Jaekwon Lee for his continuous support and guidance over the last 5 years. I also appreciate the helpful suggestions by my supervisory committee members, Dr. Audrey Atkin, Dr. Donald Becker, Dr. Vadim Gladyshev and Dr. Melanie Simpson. I would like to thank all of the Lee lab members for all of their hard work and helpful discussions. Finally, I would like to thank my wife, Angela, for her support and understanding over these years and my parents for instilling in me the work ethic to achieve this accomplishment.

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ABBREVIATIONS

ABC, ATP binding cassettte

Cd, cadmium

Cu, copper

DTT, dithiothreitol

GFP, green fluorescence protein

GPD, glyceraldehyde-3-phosphate dehydrogenase

GSH, reduced glutathione

ER, endoplasmic reticulum

ERAD, endoplasmic reticulum degradation

HA, hemagglutinin

HRP, horse radish peroxidase

ICPMS, inductively coupled plasma mass spectrometry

PBS, phosphate buffered saline

PGK, phosphoglycerate kinase

ORF, open reading frame

UPR, unfolded protein response

UPRE, unfolded protein response element

kD, kilodalton

MT, metallothionine

Chapter 1

Introduction

1.1. Cadmium, a Toxic Heavy Metal

1.1.1. Physical Properties. Cadmium is a non-physiological heavy metal that is one of the most serious environmental pollutants. The only known example of a biological function for cadmium has been described for the marine diatom *Thalassiosira weissflogii* in which cadmium can replace the zinc cofactor in carbonic anhydrase II when cells are growing under extreme zinc deficiency (1). Zinc and cadmium belong to the same column on the periodic table (2B) with filled 3d and 4d orbitals and consequently they share many physical properties. Cadmium (II) is the only accessible reduction state following the loss of two 5s electrons under physiological conditions. Thus cadmium is not a redox active metal and does not initiate Fenton reactions directly. Cadmium is classified as a soft Lewis acid and has a very high affinity for easily oxidized ligands, such as oxygen and sulfur. Cadmium is then expected to displace zinc from protein ligands containing a sulfur dominated coordination sphere. Cadmium occurrence is mainly associated with other heavy metals including zinc, and to a lesser extent copper and lead thus cadmium is an inescapable by-product of metallurgy. The chemical properties of cadmium have been implemented into a wide range of technical applications that include nickel-cadmium batteries, pigments, and various metal alloys just to name a few.

1.1.2. Human Exposure to Cadmium. It is estimated that up to 30,000 tons of cadmium are released into the environment each year where one-half is liberated by weathering rocks and the other half is produced by industrial activities (2). The manufacturing and disposal of cadmium-containing compounds has led to a significant

increase of cadmium exposure to humans. Approximately 512,000 employees in the United States work in environments which potentially expose them to cadmium (2). Air pollution can also arise from non-human processes such as wind blown cadmium powder suspensions at the surface of the earth or dispersion of volcanic gases. Cadmium released into the environment ultimately enters the food chain and water supply. Agricultural activities contribute to the incorporation of cadmium from soil and minerals into crops which are used for human consumption. The cadmium content in soil determines metal accumulation in plants which is influenced by natural geological features or industrial pollution. Cadmium concentrations in plants typically range from 0.5 - 5μM but can reach more than 100μM in hyper-metal-accumulating plants (3). Use of fertilizers greatly increases soil cadmium concentrations and acidification enhances solubility and uptake into plants (3). The greatest risk for cadmium exposure occurs through cigarette smoke. One cigarette contains about 1-2μg of cadmium of which 10% is inhaled into the lungs while smoking. While only a small percentage of cadmium is absorbed in the digestive tract, up to 50% of inhaled cadmium enters the blood stream (4). Under conditions of iron deficiency such as in a low iron diet and pregnancy, cadmium uptake is increased due to up-regulation of iron transporters which exhibit broad substrate specificity (4).

1.1.3. Toxic Cellular Effects and Pathologies of Cadmium Exposure. The toxic effects of cadmium on cellular functions are multifarious. The deleterious effects of cadmium are believed to stem from its ability to replace essential metal cofactors in metalloenzymes and binding to thiol-containing proteins involved in oxidative stress defense (e.g., glutathione, thioredoxin and thioredoxin reductase). Consequences include

changes in gene expression, cell cycle progression, proliferation, differentiation, signal transduction, inhibition of DNA replication and repair, induced apoptosis, endocrine disruption and alterations of metal and redox homeostasis (5).

The human clinical symptoms of cadmium poisoning are correlated to the organ distribution of the metal. Emphysema and chronic rhinitis have been linked to cadmium inhalation. Cadmium is classified as a class I pulmonary carcinogen because of the high incidence of lung cancer of individuals who were occupationally exposed to cadmium (4). Cadmium is likely responsible for the onset of other cancers but the scarcity of data has not extended the range of carcinogenicity beyond lung carcinoma. One of the main target organs of cadmium toxicity is the kidney. Renal tubular damage is an adverse effect resulting from chronic cadmium exposure (4,6,7). Renal damage results in inadequate activation of vitamin D which leads to decreased calcium absorption and impaired bone mineralization. Bone disease associated with prolonged cadmium exposure was first reported in a cadmium contaminated river basin in Japan where several cases of Itai-Itai disease were recognized (4).

Cadmium has also been associated with reproductive and developmental defects. Cadmium exposure during pregnancy is correlated with an increased incidence of low birth weight and spontaneous abortion (4). Cadmium affects steroid synthesis pathways and secretion of progesterone, a hormone that plays a critical role in maintaining pregnancy (8). In mammals the estrogenic effects of cadmium induce uterine hyperplasia, mammary gland development, early onset of puberty, male infertility and breast cancer (9,10). Alarmingly, the doses of cadmium which exhibited estrogenic effects were similar

to human dietary exposure. Moreover, cadmium could exhibit synergistic estrogenic effects in combination with dietary phytoestrogens (9).

1.1.4. Cadmium Absorption. Cadmium is a non-essential metal and is unlikely to enter cells through a transport mechanism specific for cadmium. Cadmium particles breathed in through the nose move through the primary olfactory neurons to their final destination in the olfactory bulb. In the lungs, cadmium can pass through alveolar cells directly into the bloodstream despite an efficient barrier in the lung epithelium for other toxic molecules and heavy metals (11). The mechanism by which cadmium reaches circulation is not well characterized but likely involves uptake through channels or transporters dedicated for essential ions or other biomolecules (Figure 1.1). Ingested cadmium enters the digestive tract where the acidic environment favors absorption at the apical membrane of enterocytes. The proton-metal Iron(II) cotransporter, DMT1 (DCT1, Nramp2), appears to be involved in this process (11). Zinc transporters have also been implicated in cadmium uptake. The putative mouse zinc transporter ZIP8 has been attributed with cadmium uptake in vascular epithelial cells of testis (12). Moreover, deletion or overexpression of Zrt1, a plasma membrane zinc transporter in yeast *Saccharomyces cerevisiae*, affects cadmium uptake (13). There is also evidence that cadmium may permeate cells through calcium channels (14-16). Cadmium uptake may also occur by other mechanisms which may include endocytosis of Cadmium-bound molecules, organic cation transporters (OCT) or transporters of other metal ions such as manganese (17) .

1.1.5. Subcellular Distribution and Sequestration. Once inside the cell, cadmium is sequestered by metallothionine (MT), a cysteine-rich peptide, as well as glutathione (GSH) (Figure 1.1). In eukaryotes this is thought to be the first line of defense for neutralizing cadmium and other toxic heavy metals (18-22). In *S. cerevisiae*, GSH-conjugated cadmium is compartmentalized into the vacuole by the ATP-binding cassette (ABC) transporter, Ycf1 (23). In plants and fission yeast *Schizosaccharomyces pombe,* cadmium is sequestered by phytochelatin, a GSH polymer (20). It is possible that other unidentified cadmium-binding proteins or molecules may exist as has been demonstrated for other heavy metals. For example, cytosolic metallochaperones deliver copper, a vital yet toxic trace element, as a cofactor to copper-requiring proteins through direct protein-protein interactions. This strategy ensures that there is virtually no unbound copper in the cytoplasm where it can catalyze unwanted Fenton and Haber-Wiess reactions (24). Recently, an iron-binding protein was discovered that ferries iron to its storage protein, ferritin (25). In *E. coli*, arsenic, a toxic non-physiological metalloid, is delivered to an efflux pump by a cytoplasmic protein (26). Thus, intracellular trafficking of cadmium may be a carrier mediated process.

1.1.6. Cadmium Extrusion. In prokaryotes, a plethora of metal efflux pumps, including P-type ATPases, ATP binding cassette (ABC) transporters, resistance-nodulation-cell division transporters (RND) and cation diffusion facilitators (CDF), have all been described (27-29). In Gram-Positive bacteria cadmium extrusion is mediated through the P_{1B}-type ATPase, CadA (30). Aside from known bacterial metal efflux systems, the mechanisms of cadmium efflux in eukaryotes remain largely unknown.

Recently, the yeast ABC transporter, Yor1, has been proposed to efflux GSH-conjugated cadmium out of the cell (31). In plants, several P-type ATPases which resemble the bacterial divalent (zinc(II)/cobalt(II)/cadmium(II)/lead(II)) cation transporters and an ABC transporter have been implicated in cellular cadmium efflux (32-34). In humans, the excretion rate of cadmium is relatively low (35). The long residence time in the body likely reflects the high affinity of cadmium for sulfhydryl groups of cellular ligands and the lack of an efficient excretion system. Mammalian studies have suggested that multidrug resistance proteins (MRP's) could transport GSH or cysteine conjugated cadmium out of hepatocytes and renal epithelial cells (36,37). Further characterization of cadmium extrusion systems will ultimately enhance our ability to combat cadmium related disorders.

1.1.7. Protective Cellular Responses to Cadmium. Cells regulate a number of biochemical pathways to protect themselves from cadmium toxicity. In mammals, both GSH and MT gene transcription are induced by the MTF-1 transcription factor in response to cadmium and oxidative stress (22,38). In *S. cerevisiae,* copper sensing by the Ace1 transcription factor induces MT production (39), however cadmium induction of MT is strain specific and the transcription factor responsible is unknown (40). Cadmium up-regulates GSH and other antioxidant enzymes (superoxide dismutase, thioredoxin and thioredoxin reductase) by thiol dependent activation of the Yap1 stress response regulator (41). Remarkably, sulfur metabolism in both yeast and mammals can be reorganized in response to cadmium resulting in a significant increase in GSH production. This restructuring of transcriptional circuitry is accomplished by cadmium induced

inactivation of *S. cerevisiae* Met4 and mammalian Nrf2 or the ubiquitination and proteasomal degradation of *S. pombe,* Zip1 transcription factor (42). Cadmium also modifies expression levels of other stress responsive proteins such as heat shock proteins (HSPs). HSPs play an important role in protein folding and in the degradation of damaged or mis-folded proteins. Induction of HSPs is most likely a response to cadmium-mediated denaturation and/or oxidative damage of proteins. Cells can also protect themselves by curbing cadmium uptake. In *S. cerevisiae*, zinc, cobalt or cadmium can trigger the degradation of the ZRT1 zinc transporter preventing excessive metal uptake (43). The evolution of these diverse cellular responses underscores the significance of cellular defense against cadmium toxicity.

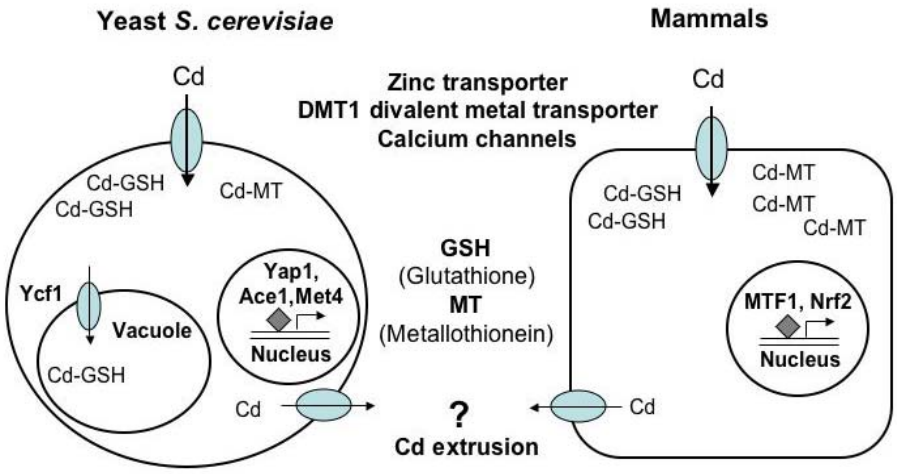

Figure 1.1. A model of Cd metabolism in yeast *S. cerevisiae* and mammals. Divalent nutritional metal transporters mediate Cd uptake across the plasma membrane. Intracellular glutathione (GSH) and metallothionine (MT) play critical roles in Cd chelation. In yeast, Ycf1 sequesters GSH-conjugated Cd into the vacuole. Cd induces detoxification pathways through several mechanisms, including enhanced gene expression by Yap1, Ace1, Met4 in yeast and MTF1 and Mrf2 in mammals. Cadmium extrusion factors are largely unknown.

1.2. P_{1B}-Type ATPases

1.2.1. Function and Classification. P-type ATPases are an important family of active membrane transporters that play fundamental roles in cell function by maintaining ion gradients. They are ubiquitous and are involved in transport processes in virtually all living organisms. P-type pumps are required for nutrient uptake, muscle contraction, neurotransmission, signaling and excretion of toxic heavy metals (29,32,33,44-48). P-type ATPases are categorized into 5 main groups (Types I-V) based on sequence homology. Within these branches several subtypes or classes can be distinguished based on substrate specificity. For instance, the P_{1B} class transports "soft" transition metals copper(I), silver(I) copper(II), zinc(II), lead(II), mercury(II), cobalt(II) and cadmium(II)). P_{1B} ATPases are found in almost all organisms ranging from bacteria to humans. Based on substrate specificities, they are further divided into monovalent and divalent translocating ATPases (49). P_{1B} ATPases are vital for metal detoxification in bacteria (29). In eukaryotes copper translocating ATPases are linked to two human genetic diseases, Wilson and Menkes disease. Consequently, because of their medical relevance, these copper pumps have received much attention regarding their mechanism of action and regulation. Cloning of the genes that are defective in Wilson and Menkes disease revealed that two P_{1B}-type ATPases, ATP7a (MNK) and ATP7b (WND) are essential for copper acquisition and excretion in humans. Yeast *S. cerevisiae* also possesses a homologue of WND and MNK (50). The pathogenic yeast, *C. albicans,* expresses an additional copper efflux ATPase (CaCRP) at the plasma membrane which leads to high copper tolerance (51). Plants contain several P_{1B}-type ATPases (eight in *A. thaliana*) that

appear to be involved in subcelluar distribution and tissue compartmentalization of metal ions (32,33).

1.2.2. Structure and Mechanism of Action. Crystal structures have been solved for Type III ATPases (Ca^{2+}-ATPase and Na^{+},K^{+}-ATPase) and a Type II ATPase (H^{+}-ATPase). However, little is known about the structure and mechanism of action of metal transporting P_{IB}-type ATPases which are structurally distinct from Type II and III P-type ATPases. P_{1B} ATPases are composed of three principle domains, an ATP-binding domain (N domain), a phosphorylation domain (P domain), and an actuator domain (A domain) (44) (Figure 1.2.). Unlike other P-type ATPase family members, P_{1B}-type ATPases possess 8 transmembrane domains, a CPX or SPC motif at the 6th transmembrane helix, and metal binding domains (MBDs) at the N and/or C terminus. Structures of these domains have been solved separately. However, a complete structure of a full length P_{1B}-type ATPase is yet to be solved.

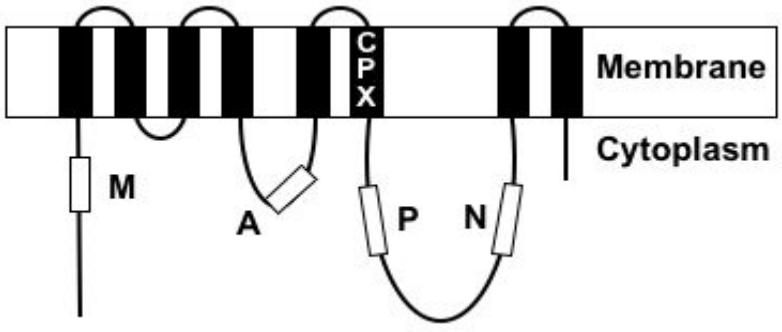

Figure 1.2. Schematic depiction of P_{1B}-type ATPase Conserved Features. Eight transmembrane helixes, metal binding domains (M) containing GMXCXXC motif, CPX motif within 6th transmembrane helix, an actuator domain (A), a phosphorylation domain (P), and a nucleotide binding domain (N) are indicated.

The postulated mechanism of ion translocation of P_{1B}-type ATPases is based on the Post-Albers cycle for the Na^+/K^+ ATPase (45) (Figure 1.3.). Cytosolic ATP and metal ions bind to the E2 state, which converts the enzyme to the E1 form. Conformational changes resulting from ATP phosphorylation and dephosphorylation drive metal ion translocation across the membrane. After releasing Pi, the enzyme is converted to the E2 form to initiate the next cycle. It is not known yet how many metal ions are translocated per cycle and whether there is counter translocation of ion(s) from the other side of the membrane

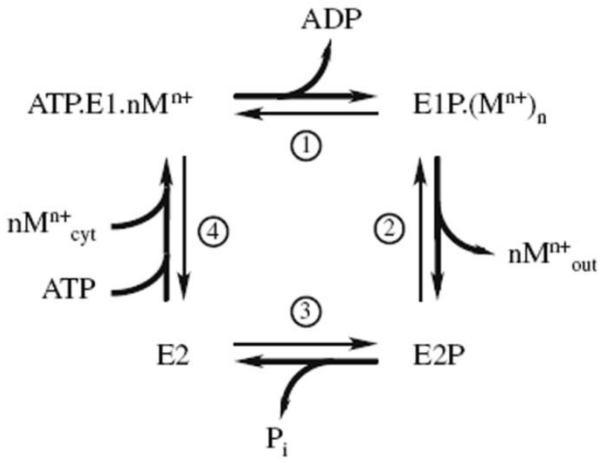

Figure 1.3. P1B-Type ATPase catalytic cycle. E1, E2, E1P and E2P represent the conformations based on the Post-Albers model for the Na^+-K^+ ATPase. M^{n+} represents a metal transported by the enzymes. "n" indicates the uncertainty on the specific stoichiometry of transport. M^{n+}cyt and M^{n+}out represent the cytoplasmic or extracellular/luminal localization of the transported metals. Figure adapted from (49)

The CPX/SPC motif at the 6th transmembrane helix is thought to coordinate metal ions during translocation. Sequence alignments and site-directed mutagenesis have predicted other potential metal binding residues located in the 7th and 8th trans-membrane domains as well (49,52). However, metal coordination during transport and the mechanisms of P_{1B}-type ATPases remain to be determined. Moreover, the molecular determinant(s) that define substrate specificity are not known.

Most P_{1B}-type ATPases possess N and/or C-terminal MBDs that are believed to play roles in catalysis and/or regulation (52). The human Cu^+ transporting ATPases, WND and MNK, possess 6 MBDs (~70 amino acids, βαββαβ fold) at the N-terminus (53,54). This domain is conserved in many P_{1B}-type ATPases, and the number of repeats can vary. Several atypical MBDs have also been identified (55,56). The conserved MBD contains a GMXCXXC motif in which two cysteine residues coordinate a single Cu^+ ion (53,54) . The Atx1 metallochaperone delivers copper(I) to these domains through direct docking to the 2nd and 4th domains of WND, suggesting distinct functions of these domains (57,58). Despite structural and metal binding properties have been well characterized, their roles in ATPase function are not clear yet. Copper binding was initially proposed as a substrate recognition step followed by transfer of copper(I) to the translocation pore. Consistently, several disease causing mutations within WND have been mapped to Atx1 binding sites (59,60). However, site-directed mutagenesis and deletion of MBDs also suggest a regulatory role for these domains in the catalytic activities of P_{1B}-type ATPases. Recently, it was proposed that the metallochaperone can transfer a metal substrate directly to the trans-membrane metal-binding site for

translocation (61). Collectively, while significant progress has been made in the metal binding properties of MBDs, their functional roles in activity and/or regulation of P_{1B}-type ATPases remain elusive.

1.2.3. Regulation. Several modes of regulation control expression levels, subcelluar localization, and activities of P_{1B}-type ATPases. Transcriptional control in response to metal ions appears to be a major regulatory mechanism in bacteria and plants (29,32,33,45). However, in mammals metal mediated regulation of Copper(I)-transporting P_{1B}-type ATPases mainly relies on alterations of their subcelluar trafficking (59,62-65). In a low copper medium, the human MNK and WND proteins are localized to the *trans*-Golgi network where they transport copper for incorporation into secretory proteins. When copper levels are elevated, MNK is mobilized to the plasma membrane in a reversible manner. The biological significance of this trafficking event is that MNK participates in copper extrusion to protect cells from toxicity. In the liver, excess copper mobilizes WND from the trans-Golgi network to a cytoplasmic vesicle-like compartment associated with the canicular membrane of bile ducts. The biological significance appears to be an important mechanism for excretion of excess copper into bile to maintain systemic copper homeostasis. However, it is not known exactly what molecular changes (e.g. conformational change, phosphorylation, disulfide bond formation, and/or interaction with other proteins) trigger trafficking of MNK and WND in response to copper.

1.3. Subcellular Trafficking of Membrane Proteins in Yeast

Membrane proteins including P_{1B}-type ATPases traffic through the secretory pathway to their final destination. In *S. cerevisiae*, as many as 282 membrane solute transporters have been identified of which a minimum of 125 reside at the cell surface (66). A number of these transporters have been reported to be subject to strict regulation in context of their trafficking and degradation according to environmental conditions. Many of the same principles which govern the regulation of transporters appear to be conserved in plants and animals making yeast a powerful system for dissecting these mechanisms in detail.

1.3.1. Folding, Maturation and Assembly at the Endoplasmic Reticulum. The endoplasmic reticulum (ER) is the gateway for entry into the secretory pathway. Newly synthesized membrane proteins are cotranslationally integrated into the ER through a narrow cytosolic channel formed by the heterotrimeric Sec61p complex. Most transmembrane proteins do not possess a cleavable signal sequence but instead are targeted to the ER by the first hydrophobic segment which functions as an internal signal sequence (67). Translocation is halted by hydrophobic stop-transfer segments within the primary amino acid sequence where charged residues flanking the hydrophobic segment are believed to determine the orientation of the segment across the membrane (68). During or after translocation protein folding is assisted by various chaperones. In some instances proteins acquire N-linked glycosylation, disulfide bond formation or must oligomerize into higher order structures before being deemed fully mature (69). Correct protein folding and assembly is often inefficient and can be hindered by genetic error and

cellular stress. The accumulation of unfolded proteins induces the unfolded protein response (UPR) pathway which alleviates ER stress by inhibiting protein translation and up-regulating protein folding and degradation pathways (70) .

1.3.2. ER to Golgi Trafficking. After passing all quality control checkpoints, proteins are packaged into transport vesicles destined for the Golgi prior to sorting into the secretory pathway. Intracellular sorting between compartments requires vesicle carriers that are released from a donor to an acceptor compartment following vesicle fusion (71). Correctly folded and assembled cargo proteins are packaged into COPII-coated vesicles and are transferred to the Golgi network. For some cargo proteins, packaging requires a diacidic motif which is recognized by the COPII protein, Sec24 (72). Cargo receptors and components of the membrane fusion machinery are returned to the ER by COPI coated vesicles (Figure 1.4.).

1.3.3. Golgi to Plasma Membrane Trafficking. The mechanism of cell surface targeting of plasma membrane proteins is largely unknown. Once at the Golgi apparatus membrane proteins are sorted to the plasma membrane or other secretory compartments via secretory vesicles (SV). At least two different routes, involving vesicle populations of different densities, low-density secretory vesicles (LDSV) or high density secretory vesicles (HDSV) carry distinct exocytic cargo *en route* to the plasma membrane (73). Association with membrane lipid rafts is required for proper trafficking of some membrane proteins to the plasma membrane (74).

1.3.4. Ubiquitin Dependent Endocytosis and Vacuolar Degradation. Proteins are often marked for degradation by post-translational conjugation to ubiquitin, a 76

amino acid protein that is conserved from yeast to humans (75). The first step in ubiquitination is the activation of the C-terminal α carboxyl group of ubiquitin by ATP resulting in an adenyl intermediate followed by the subsequent formation of a thiol ester bond with a cysteine of an (E1) ubiquitin-activating enzyme. Ubiquitin is then transferred to a cysteine of a ubiquitin-conjugating enzyme (E2). Ubiquitin loaded E2s bind specifically to ubiquitin ligases (E3) that bind directly to protein substrates. The E3s themselves can be conjugated with ubiquitin through a thioester bond before substrate attachment, or act as a bridge between the activated E2 and its substrate. Ubiquitin is usually attached through an isopeptide bond to the ε-amino group of a lysine residue of the substrate.

Several cell surface transporters are down-regulated by ubiquitin-dependent endocytosis followed by vacuolar targeting via endosomal compartments. A key factor in this process is the ubiquitin E3 ligase Rsp5 along with other adaptor proteins which can mediate client specific ubiquitination of substrates (76).

The ubiquitin moiety acts as an internalization signal which is recognized by ubiquitin binding proteins that contain variable effector domains which recruit clathrin proteins that function in membrane bending and invagination. Internalization is also thought to require correct organization of the actin cytoskeleton as several transporters accumulate as ubiquitin conjugates in mutants impaired in cytoskeleton formation (77). Following vesicle budding and internalization, ubiquitinated cargos are transported into endocytic compartments called early endosomes (EE). At this holding station, endocytic vesicles can be recycled back to the plasma membrane or can fuse with the limiting

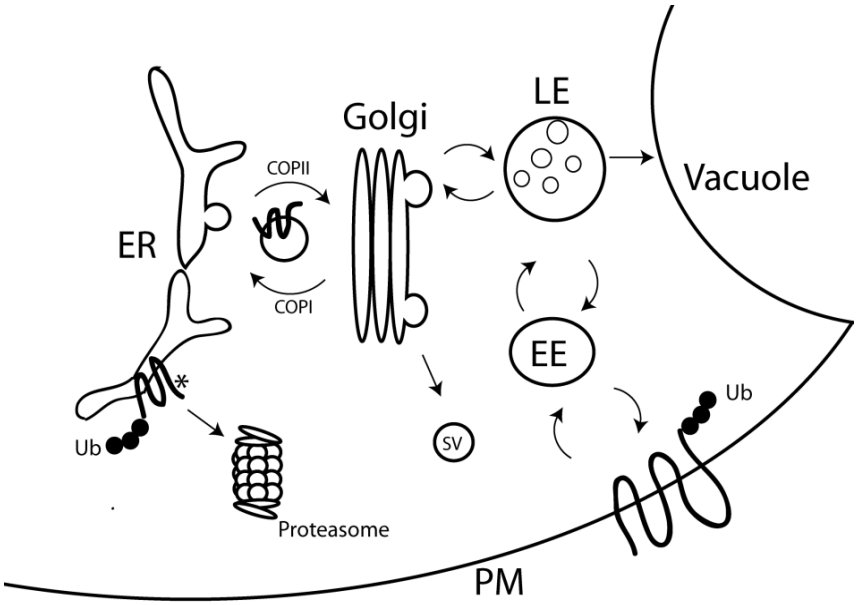

Figure 1.4. Trafficking of membrane proteins in yeast. Correctly folded and assembled cargo proteins are packaged into COPII-coated vesicles and are transported to the Golgi network. Terminally misfolded proteins (*) are retranslocated to the cytosol for proteasome dependent degradation. COPI proteins induce formation of return vesicles that retrieve cargo receptors and components of membrane fusion machinery to the ER. Cell surface proteins are transported from the trans-Golgi network to the plasma membrane (PM) via secretory vesicles (SV). Ubiquitin dependent endocytosis targets some plasma membrane proteins to the early endosome (EE). Internalized substrates can be recycled back to the plasma membrane or can proceed to late endosomes/multivesiclar body (LE/MVB) where ubiquitinated cargo is internalized into vesicles. Fusion of the mature LE/MVB with the vacuole delivers internalized contents for vacuolar degradation. Under certain conditions some plasma membrane proteins are directly sorted to the vacuole for degradation.

membrane of the late endosome/multivesiclar body (LE/MVB). At the limiting membrane of the LE/MVB ubiquitinated substrates are internalized into vesicles. Subsequent fusion with the vacuole delivers the internal vesicles carrying ubiquitinated cargos into the vacuolar lumen for degradation by vacuolar proteases and lipases. This process allows for discrimination in the sorting of proteins to the vacuole limiting membrane or to the lumen of this organelle for degradation. In some instances, newly synthesized membrane proteins are ubiquitinated at the Golgi where they are diverted directly to the vacuole for degradation without ever reaching the plasma membrane (Figure 1.4.).

1.3.5. Physiological Regulation of Transporter Trafficking. Organisms must meet the demands of changing conditions within the environment. Several nutritional transporters are down-regulated by targeting to the vacuolar degradation pathway in response to specific stimuli. The general amino acid permease, Gap1, is active under conditions of limiting nitrogen supply. Under these conditions the *GAP1* gene is transcribed and newly synthesized protein is targeted directly to the plasma membrane. When a preferred nitrogen source is available, such as ammonia, the *GAP1* gene is repressed and pre-synthesized Gap1 is rapidly removed from the plasma membrane through ubiquitin dependent endocytosis and is sorted into the MVB/vacuolar pathway for degradation (78). When cells are grown with glutamate as the sole nitrogen source, newly synthesized Gap1 is ubiquitinated by Rsp5 and is directly targeted to the MVB/vacuolar pathway without ever reaching the plasma membrane (79).

Metal transporters are also tightly regulated due to the requirement of nutritional metals at specific concentrations which is critical to avoid potential toxicity. Zrt1, a high-affinity Zn transporter, is rapidly internalized by Rsp5-dependent ubiquitination within minutes of exposure to high Zn levels to prevent toxicity (43). The Mn transporters, Smf1 and Smf2, are targeted to the vacuole by Rsp5 dependent ubiquitination in response to excess Mn (80). Interestingly, mutations that abolish Smf1 transport activity affect trafficking which suggests that conformational states during metal transport are recognized by the ubiquitination machinery (81).

There is growing evidence that direct substrate binding to transporters plays a pivotal role in controlling membrane trafficking. The nucleotide transporter Fur4 undergoes Rsp5 dependent ubiquitination and internalization in the presence of excess uracil. If synthesized in the presence of its uracil substrate, Fur4 undergoes Rps5 mediated ubiquitination at the Golgi and is directly sorted to the vacuolar degradation pathway (82,83). A Fur4 mutant, which has a very low affinity for uracil, circumvents vacuolar sorting and instead localizes to the plasma membrane despite the presence of intracellular uracil. This observation suggests that binding of uracil to Fur4 leads to conformational changes that facilitate ubiquitination.

Perhaps the best illustration of trafficking regulation by substrate binding is that of the Arn1 ferrichrome (FC) transporters. *S. cerevisiae* can uptake iron bound to siderophores by two separate systems, one of which requires the ARN family of iron-siderophore transporters. In the absence of its FC substrate Arn1 localizes to endosome-like intracellular vesicles. It has been proposed that low concentrations of endocytosed

FC substrate a sensed by a high affinity binding site of the Arn1 transporter which promotes sorting from endosomes to the cell surface for iron-siderophore uptake. While at the cell surface, excess amounts of FC bind to a low affinity binding site of Arn1, which induces internalization and targeting to the MVB/vacuolar pathway for degradation thus preventing excess FC uptake (84,85).

1.4. Quality Control and Regulated Degradation at the ER. Misfolded proteins which do not pass ER quality control specifications are transported back across the ER membrane into the cytosol where they are almost always ubiquitinated before degradation by the 26S proteasome (86-89). This process has been referred to as ER-associated degradation (ERAD) (90). Inefficiencies in ERAD result in accumulation of malfolded proteins and cell death leading to various diseases such as diabetes, inflammation and neurological disorders including Alzheimer's disease, Parkinson's disease and bipolar disorders (91)

1.4.1. Recognition of soluble luminal substrates

Hsp70 chaperones, including mammalian BiP or yeast Kar2, bind exposed residues with overall hydrophobic character which would be normally buried in a properly folded protein (68). Hsp70 chaperones bind and release substrates in an ATP dependent manner. Hsp70s like BiP and its co-chaperones are essential for maintaining solubility of newly translocated proteins which in turn facilitates proper folding (92). It is then logical that luminal chaperones are also important for delivery and/or targeting of terminally mis-folded proteins for ERAD (93). Indeed Kar2 is required for efficient degradation of several soluble luminal ERAD substrates in yeast (94).

Instead of binding avidly to hydrophobic peptides, some ER luminal chaperones known as lectins preferentially interact with distinct sugar residues which are appended to secretory proteins by ER resident oligosaccharyltransferases (95-97). A core Glc_3-Man_9-$GlcNAc_2$ glycan (where Glc is glucose, Man is mannose and GlcNac is N-acetylglucosamine) is added to asparagine residues. The trimming and re-addition of

glucose residues act as a folding "timer". The monoglucosylated species has a high affinity for calnexin and calreticulin, which are integral membrane and soluble lectins located in the ER, respectively. Both calnexin and calreticulin interact with ERp57, a member of the PDI family which forms transient disulfide bonds with calnexin and calreticulin-bound proteins to assist in folding (98,99). This "timer" is controlled by mannosidase I, which slowly cleaves the α1,2-linked mannose residue from the middle branch generating a $Man_8GlcNAc_2$ glycan which leads to association with ER degradation-enhancing 1,2-mannosidase-like protein (EDEM) or Htm1p/Mnl1p in yeast (100-102). EDEM then diverts misfolded proteins for dislocation into the cytosol and proteasomal degradation.

1.4.2. Recognition of Integral Membrane ERAD Substrates. Little is known about how misfolded integral membrane proteins are recognized and sorted into the ERAD pathway but like luminal substrates this process likely involves the exposure of hydrophobic residues which are normally buried in a native state. Since the ER membrane forms a barrier between the ER lumen and the cytosol, misfolded lesions present in luminal domains or cytosolic domains of integral membrane proteins are spatially separated and require different recognition factors. Integral membrane substrates can be classified according to where misfolding occurs within the protein. Integral membrane proteins with lesions that occur within cytoplasmic domains utilize the ERAD-C pathway, whereas misfolding that occurs in luminal or transmembrane domains follow the ERAD-L and ERAD-M pathways, respectively (Figure 1.5.).

Cytosolic Hsp70 chaperones and Hsp40 co-chaperones have been proposed to play roles in the recognition of ERAD-C substrates by binding exposed hydrophobic residues (93). However it is uncertain whether cytoplasmic chaperones maintain protein solubility by preventing aggregation of exposed hydrophobic residues or whether they play an active role in substrate recognition and recruitment to the ERAD machinery. ER resident E3 ubiquitin ligases may also play a role in recognition of ERAD-C substrates.

Integral membrane proteins with luminal lesions utilize many of the same factors involved in the recognition of misfolded soluble proteins that reside in the ER lumen. These include luminal Hsp70/40 chaperones (Kar2/Jem1 and Scj1), protein disulfide isomerase (PDI), and putative carbohydrate binding lectins (Yos9 and Htm1/Mnl1) (93). Factors involved in the recognition of lesions within the transmembrane domain (ERAD-M) are largely unknown. It has been proposed that they can be directly recognized by ER-membrane localized E3 ligases.

1.4.3. Retranslocation. Proteasomal degradation requires retrograde transport out of the ER to the cytosol. It remains unknown how exactly this occurs. It has been postulated that retranslocation occurs through Sec61, which is the major component of the translocation channel that imports polypeptides into the ER (103,104). Nevertheless, Sec61 cannot be the only retranslocation channel as integral membrane ERAD substrates are degraded proficiently in *sec61* mutants.

In most cases ubiquitination plays an integral part in the retranslocation process and has been proposed to serve as a molecular ratchet by preventing the polypeptide from slipping back through the retranslocation channel. Since the catalytic domains of ER

resident E3 ubiquitin ligases are located in the cytoplasm, luminal ERAD substrates must be retranslocated to the cytosolic face of the ER prior to ubiquitination. However, for integral membrane ERAD substrates containing cytosolic domains it is uncertain if retranslocation precedes ubiquitination.

1.4.4. Ubiquitination of ERAD Substrates. Nearly all ERAD substrates are polyubiquitinated prior to degradation by the proteasome (88). In *S. cerevisiae*, the ubiquitination of ERAD substrates is mediated by two ER resident RING member ubiquitin E3 ligases, Hrd1 and Doa10 (105) (106). The catalytic RING domains of both Hrd1 and Doa10 are located on the cytoplasmic face of the ER. Doa10 also resides within the nuclear envelope where it catalyzes ubiquitination of nuclear substrates (107). Hrd1 is stabilized by formation of a complex with Hrd3, another ER resident membrane protein. Hrd3 contains a large luminal domain which is thought to be involved in substrate sensing of luminal lesions and is required for ERAD of both soluble and polytopic membrane proteins (108). With the E2 ubiquitin conjugating enzyme, Ubc7 which is tethered to the ER membrane via Cue1, Hrd1 catalyzes the ubiquitination of ERAD substrates with membrane or luminal lesions (109). Ubc1, another E2 ubiquitin conjugating enzyme, has also been implicated in Hrd1 catalyzed ubiquitination of the mutant vacuolar enzyme, carboxy-peptidase yscY-S255R, commonly known as CPY* (110). On the other hand, ERAD substrates with cytoplasmic lesions, or unassembled subunits of oligomeric complexes are ubiquitinated by Doa10. Doa10 dependent ubiquitination requires the Ubc7/Cue complex and to a lesser extent the tail anchored Ubc6 (111). One of the best studied Doa10 substrates is a truncated mutant of the Ste6

ATP-binding cassette (ABC) transporter of the pheromone, alpha factor, denoted as Ste6*. Normally, wildtype Ste6 is slowly internalized from the cell surface and targeted for vacuolar degradation by ubiquitin-mediated endocytosis (112), however a truncation of the Ste6 C-terminal cytosolic tail results in rapid degradation by ERAD with a half-life of less than 10 min (113).

According to sequence homology, two human Hrd1 homologues (HRD1 and gp78) and one Doa10 homologue (TEB4) have been identified. The mammalian homologues of yeast Ubc6 and Ubc7 are MmUbc6 and MmUbc7, respectively. Like yeast Ubc6, MmUbc6 is anchored to the ER by a C-terminal membrane domain. However a Cue1 homologue has not been identified in mammals. Instead MmUbc7 is recruited to the ER by a Cue domain already present within gp78 (114).

1.4.5. Substrate Extraction and Delivery to the Proteasome. Following ubiquitination, a series of ubiquitin-binding proteins escort modified substrates from the ER to the proteasome. One of these factors is the AAA ATPase, Cdc48 (p97 in mammals) which along with its cofactors (Ufd1 and NpI4) is involved in substrate extraction and escort of ubiquitinated substrates to the proteasome. Cdc48 is recruited to the ER membrane by the integral membrane protein, Ubx2 (115). The Cdc48 complex binds oligo-ubiquitinated substrates as well as Ufd2, an E4 multiubiquitination enzyme which further extends ubiquitin chains to aid in proteasomal recognition (116). Polyubiquitinated substrates are recognized by the ubiquitin chain receptors, Rad23 and Dsk2, which also bind Ufd2 in a Cdc48 dependent manner. Rad23 and Dsk2 share overlapping properties since ERAD defects are only apparent in a Δ*dsk2* Δ*rad23* double

mutant. Png1p, an N-glycanase that discriminates between correctly folded and mis-folded glycoproteins is also recruited to the proteasome via Rad23 in order to remove glycans from glycosylated ERAD substrates prior to proteasomal degradation (117). In some instances the proteasome itself has been attributed to extraction of substrates from the ER. The 19S regulatory particle contains six AAA-ATPases that could also provide the energy for extraction of some ERAD substrates. For example in yeast a mutant of pro-alpha factor (pαF*) is not ubiquitinated and the 19S regulatory particle is the only cytosolic factor required for export from the ER *in vitro* (88*)*. A significant fraction of proteasomes in the cell are associated with the ER suggesting that extraction and degradation is a tightly coupled process (118).

1.4.6. Regulation of Protein Expression by the ERAD Pathway. In addition to performing a strict quality control role, ERAD can be harnessed for regulated degradation of select proteins in response to cellular signals. While quality control mechanisms recognize common structural hallmarks of non-native proteins, short target sequences called degrons can target naturally expressed proteins for degradation. Under certain conditions, proteins may display degrons that are recognized by the ER quality control system and are degraded. These can be masked upon signal sensing which allows escape from ERAD. Degrons which are recognized by the ERAD machinery have been described for cytosolic, ER resident and secretory proteins in both yeast and mammals.

In yeast ubiquitination of the cytosolic/nuclear localized transcriptional repressor MATα2 is dependent upon the ER-resident Ubc6 and Ubc7 E2 conjugating enzymes and the Doa10 E3 ubiquitin ligase (119,120). A transplantable 67 amino acid degradation

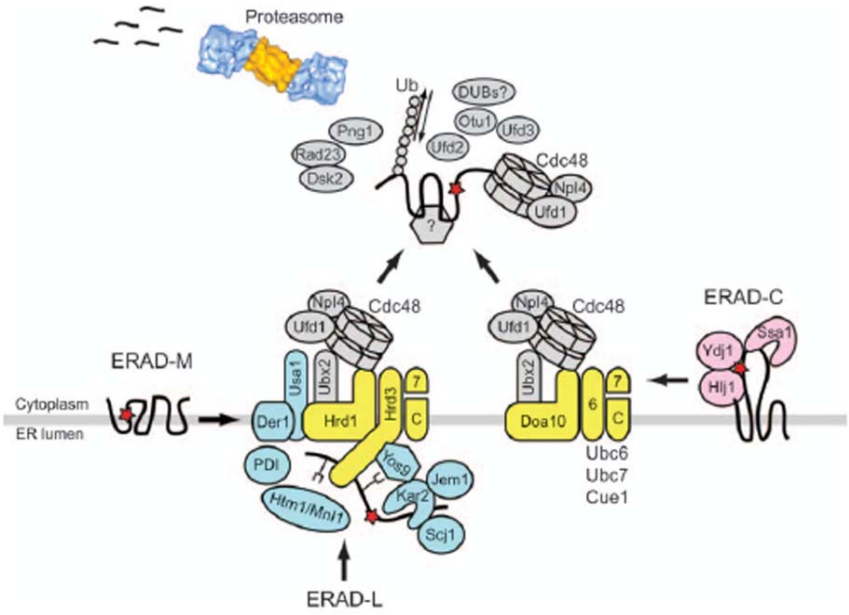

Figure 1.5. Molecular Factors Involved in Yeast ERAD. ERAD-L substrates are recognized by ER lumenal Hsp70/Hsp40 chaperones (Kar2, Jem1 and Scj1), PDI, putative lectins (Yos9 and Htm1/Mnl1) and the lumenal domain of Hrd3. ERAD-C substrates are recognized by cytosolic Hsp70/Hsp40 chaperones (Ssa1, Ydj1 and Hlj1) and the Doa10 E3 ligase. ERAD-M substrates may be directly recognized by the Hrd1 E3 ligase. Ubiquitination of ERAD-L and ERAD-M substrates require Hrd1 while Doa10 is the ubiquitin ligase of ERAD-C substrates. The Ufd2 E4 ligase may be involved ubiquitin chain extension of some substrates. Extraction and proteasome delivery of membrane substrates requires the AAA ATPase, Cdc48 and its Ubx2 membrane anchor. The ubiquitin binding proteins Rad23 and Dsk2 play a role in proteasomal recruitment. Ubiquitin moieties are removed by a series of de-ubiquitinating enzymes (DUBs) that may include Rpn1, and/or Otu1. Glycans are removed from glycosylated substrates by Png1. Red star depicts misfolding or degradation signals that lead to entry into the ERAD degradation pathway. Figure adapted from (93).

signal designated Deg1, targets MATα2 for Doa10 ubiquitination and proteasomal degradation. A ~19 residue determinant within Deg1 appears to be crucial for recognition and degradation. Deg1 is predicted to form an amphipathic helix in which a hydrophobic face appears to be the most critical recognition determinant (121). Heterodimerization of

MATα2 with its binding partner, MATa1 masks the Deg1 signal resulting in its stabilization.

A series of Doa10 recognized degrons were identified in a selection of genomic sequences which destabilized the stable cytosolic protein, Ura3 (122). One of these artificial degrons identified is CL1, a 16 amino acid sequence (ACKNWFSSLSHGVIHL) also predicted to form an amphipathic helix and like Deg1 requires patches of bulky hydrophobic residues (123). A similar amphipathic helical structure targets serum and glucocorticoid-induced kinase 1 (SGK1) to an ER-resident E3 ligase for ubiquitination and degradation in mammals (124). Together these observations suggest that surface exposed hydrophobic residues which are normally buried in native proteins can serve as a recognition motif for ERAD.

In mammals, secretion of the cholesterol and triglyceride carrier protein, apoliprotein B100 (ApoB) is also regulated by components of ERAD pathway. A significant portion of newly synthesized ApoB is degraded by both proteasomal and non-proteasomal pathways, which are regulated by metabolic factors. When conditions are not favorable for ApoB assembly with lipids, ApoB is ubiquitinated and degraded by the proteasome (125). This process is facilitated by cytosolic chaperones of the hsp70 and hsp90 class during ER translocation (125). It has been proposed that ApoB is targeted to the ERAD machinery co-translationally while still bound to the ribosome. In this model efficient translocation of ApoB into the ER lumen is dependent on microsomal triglyceride transfer protein (MTP) dependent lipid transfer to the nascent polypeptide (126). The stalling of translocation leads to prolonged exposure of hydrophobic domains

within ApoB to the cytosol for recognition by components of the Ub-proteasome pathway.

The ER resident HMG-CoA reductase (HMGR) is the rate limiting enzyme of the mevalonate pathway by which cholesterol and a variety of isoprenoids are synthesized. The degradation of HMGR and its yeast homologue, Hmg2, are physiologically regulated by sterols (127-130). When sterol flux is high, HMGR is targeted for ERAD in order to decrease enzyme levels. When flux is low, HMGR circumvents ERAD, and enzyme levels increase. Hrd1 (HMG-CoA reductase degradation) with its E2 partners Ubc1 and Ubc7 mediate the ubiquitination and degradation of Hmg2 in yeast (131,132). Gp78, the mammalian orthologue of Hrd1 was later identified as the ubiquitin ligase of HMGR (133). It is not surprising that ER resident proteins like HMGR/Hmg2 are degraded by ERAD machinery that also resides in the ER. The molecular signal for yeast Hmg2 degradation is derived from farnesyl pyrophosphate (FPP), an intermediate in the mevalonic acid pathway. In the presence of FPP, Hmg2 acquires structural features of a misfolded protein as indicated by increased susceptibility to proteolysis which is reversed by chemical chaperones such as glycerol (134). The N-terminal transmembrane domain of Hmg2 is both necessary and sufficient for sterol mediated regulation and fusion of this domain can impart regulation of other stable proteins (135). The details regarding the nature of the recognition signal within Hmg2 are not known but mapping studies have uncovered a distributed degron where structural information required for recognition is dispersed throughout the first N-terminal transmembrane domain (135). More recently, two yeast ER membrane anchored chaperone proteins, NSG1 and NSG2 were shown to

be involved in the stabilization of Hmg2 through interaction with the sterol sensing N-terminal domain (136). Further studies of the human orthologue, Insig-1, demonstrated that sterol induced binding of Insig-1 to the sterol sensing domain of HGMR, leads to recruitment to the ubiquitin ligase, gp78 (133). Thus, it appears that other adaptor proteins can contribute to the specificity of ER resident ubiquitin ligases.

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Chapter 2

A Cadmium-transporting P_{1B}-type ATPase in Yeast *Saccharomyces cerevisiae*

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2.1. Abstract

Detoxification and homeostatic acquisition of metal ions are vital for all living organisms. We have identified PCA1 in yeast S. cerevisiae as an overexpression suppressor of Cu toxicity. PCA1 possesses signatures of a P1B-type heavy metal transporting ATPase which is widely distributed from bacteria to humans. Cu resistance conferred by PCA1 is not dependent on catalytic activity, but it appears that a cysteine-rich region located in the amino-terminus sequesters Cu. Unexpectedly, when compared to two independent natural isolates and an industrial S. cerevisiae strain, the PCA1 allele of common laboratory strains we have examined possesses a missense mutation in a predicted ATP-binding residue conserved in P1B-type ATPases. Consistent to a previous report which identifies an equivalent mutation in a Cu transporting P1B-type ATPase of a Wilson disease patient, the PCA1 allele found in laboratory yeast strains is non-functional. Overexpression or deletion of the functional allele in yeast demonstrates that PCA1 is a Cd efflux pump. Cd as well as Cu and Ag, but not other metals examined, dramatically increase PCA1 protein expression through post-transcriptional regulation and promote subcellular localization to the plasma membrane. Our study has revealed a novel metal detoxification mechanism in yeast mediated by a P1B-type ATPase that is unique in structure, substrate specificity and mode of regulation.

2.2. Introduction.

Excretion and detoxification of non-physiological metals, homeostatic absorption and utilization of nutritional yet toxic metals are fundamental biological processes. Metal toxicity and deficiency resulting from defects in metabolism and excess accumulation through environmental contamination are implicated in a number of disorders, including failure in normal growth and development, and initiation and progression of degenerative diseases (1-7). For instance, a genetic defect in copper (Cu) excretion in the liver leads to Wilson disease exhibiting hepatic failure and neuronal dysfunctions (7). Cadmium (Cd) is a well-known industrial and environmental pollutant implicated in cancer, neurological disorders, reproductive defects, and endocrine disruption (8-12). Cd exerts its toxic effects by the perturbation of cellular redox balance, inhibition of DNA repair, disruption of metal ion homeostasis, and alterations in signal transduction (8-12). Mechanistic insights into metal metabolism in living organisms would facilitate prevention and treatment of metal-related disorders and to develop methods for efficient remediation of toxic metals from the environment.

Organisms have evolved defense mechanisms to combat the toxic effects of heavy metal ions. The P_{1B}-type ATPase family of heavy metal transporters that are distributed from bacteria to humans extrude toxic metal ions such as Cu, Ag, Zn, Co, Pb and/or Cd from the cell (13-18). Functional characterization of this family of transporters has supported the conclusion that efflux mechanisms play critical roles in metal detoxification in bacterial cells (13-15). Cu-specific P_{1B}-type ATPases have been identified in eukaryotes as well (2,7,16,17). Cloning of genes defective in Menkes and

Wilson disease have revealed that two P_{1B}-type ATPases (ATP7a and ATP7b) play essential roles in Cu acquisition and excretion in humans (2,7,16). Plants express Cu, Zn, Co, Pb and/or Cd-translocating ATPases that appear to be involved in distribution and compartmentalization of these metal ions (17,18).

While metal efflux systems have begun to be characterized (16-24), it has been suggested that in eukaryotes metallothionine (MT), a Cys-rich low-molecular-weight protein, and glutathione (GSH)-mediated sequestration appears to be the major mechanism in neutralizing toxic metals (25-29). Ycf1 in *S. cerevisiae*, a vacuolar membrane ATP-binding cassette (ABC) transporter, sequesters glutathione-conjugated Cd (30). Phytochelatin, a GSH polymer synthesized in plants and yeast *S. pombe* also detoxifies heavy metals (27). Metal-responsive transcription factor 1 (MTF-1) regulates basal and metal-inducible expression of MTs and other genes in mammals, fruit fly, and fish (31-34). In contrast to mammalian genes encoding MT, Cu but not Cd and Zn induces the Cup1 gene in *S. cerevisiae* through the *ACE1* transcription regulator (35,36). Consistent to their critical roles in induction of the genes involved in metal detoxification, deletion of MTF1 in mice and *ACE1* in *S. cerevisiae*, results in enhanced metal sensitivity (32,35).

In order to gain better insights into the mechanisms of heavy metal metabolism, we have selected *S. cerevisiae* cDNAs that suppress Cu sensitivity of the *ace1*Δ yeast strain. One of the cDNAs identified encodes the putative P_{1B}-type ATPase, *PCA1*, which has been suggested to be involved in Cu and/or Fe homeostasis (37,38). Previously, the Cd resistance of a mutant yeast strain selected on toxic concentrations of Cd was mapped

to the *PCA1* gene which contained multiple mutations (39). However, the role and mechanisms of action of PCA1 in metal metabolism have remained elusive. Here we show that PCA1-mediated Cu resistance is dependent on its Cys-rich amino terminal domain. The PCA1 allele in all laboratory yeast strains examined carries a missense mutation in a conserved residue resulting in loss of function. The wild-type PCA1 allele confers Cd resistance by an efflux mechanism accompanied by a novel mode of metal-dependent post-transcriptional regulation.

2.3. Experimental Procedures

2.3.1. Yeast Strains, Media, and Phenotypic Tests. Strain BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and null mutants *ace1Δ*::KanMX6, *pca1Δ*::KanMX6 were purchased from Open Biosystems. Strain RM11-1a (40) (*MATa leu2Δ0 ura3Δ0 HO::KanMX6*) was kindly provided by Leonid Kruglyak (Fred Hutchison Cancer Research Center, Seattle, WA). RM11-1a *pca1Δ*::URA3 and BY4741 *ace1Δ*::KanMX6 Δ*pca1*:HIS3 were generated by PCR based homologous recombination (41). For consistency and simultaneous comparisons of Cu and Cd sensitivity, all experiments unless indicated otherwise were performed using the BY4741 *ace1Δ* strain. Plasmids were transformed into yeast using the lithium acetate procedure (42). Yeast cells were grown on synthetic complete (SC) media (2 % dextrose or galactose, 0.67 % yeast nitrogen base, and 0.2 % dropout mixture for plasmid selection). For phenotypical analysis, ~5 μl of yeast cells (OD_{600} 1.0) were spotted on selective media (1.5 % agar) supplemented with the indicated concentrations of Cd ($CdCl_2$) or Cu ($CuCl_2$) (Sigma) and incubated at 30 °C for 2 days.

2.3.2. Selection of cDNAs Conferring Cu Resistance. Strain BY4741 *ace1*Δ strain was transformed with a yeast cDNA library (43). Transformants (~$1x10^6$ colonies) growing on SC-Ura media were collected by re-suspending in sterilized distilled water, diluted, and plated on galactose-containing SC-Ura media supplemented with Cu (0.1 mM $CuCl_2$). Plasmids containing cDNA were isolated from yeast colonies growing on selection media and identified by sequencing. cDNA-dependent Cu resistance was confirmed by re-transformation of the isolated plasmid into *ace1*Δ yeast cells.

2.3.3. **Plasmid Construction.** The *PCA1* ORF (open reading frame) was PCR amplified from yeast genomic DNA and cloned into *BamHI* and *XhoI* restriction sites of a single-copy yeast expression vector for GPD (p413 GPD) (44) or GAL1 (p413 GAL1) (45) gene promoter-mediated constitutive expression. The coding sequence of the PCA1 N-terminal domains (aa 1-392 and 1-452) were PCR amplified with a primer set containing a start codon and artificially generated stop codon, and subcloned into *BamHI* and *EcoRI* sites of p413-GPD. The PCA1 truncation mutant (aa 393-1216) was generated by PCR amplification of the sequences including start and stop codons, and was subcloned into p413 GPD at *BamHI* and *XhoI* restriction sites. PCA1-GFP and PCA1-Flag were constructed by generation of a *NotI* restriction site after the start codon for insertion of *NotI* flanked GFP (green fluorescent protein) without start and stop codons or two tandem Flag epitopes, respectively. For natural promoter driven expression, the *PCA1* ORF including 810 bp upstream of the start position was cloned into *SacI* and *XhoI* restriction sites of plasmid pRS413 (46). Site-directed mutagenesis was conducted by the primer overlap extension method (47). All constructs were confirmed by sequencing.

2.3.4. Fluorescence Microscopy. Yeast cells were transformed with PCA1-GFP or PCA1-WT-GFP expression plasmids and cultured in SC-His media at 30 °C with agitation until mid-log phase. Metals were added to the culture media for 15 min to 2 hrs prior to imaging. Cells were collected, washed in phosphate-buffered saline (PBS) and imaged on a confocal microscope (Olympus FV500).

2.3.5. Metal Measurements. Yeast cells were cultured until mid-log phase and metal ions were added to the culture media for 6 hours. Cells were collected in 2 mL

aliquots and washed two times in PBS containing 10 mM EDTA. Cell pellets were dissolved in 70 % nitric acid at 60 °C for 30 min and diluted to 10 % nitric acid. To measure Cd excretion, cells were cultured with 5 mM $CdCl_2$ for 30 min, washed two times in PBS containing 10 mM EDTA, and re-suspended in fresh media prior to sample collection at the indicated time points. Total cellular metal levels were measured by inductively coupled plasma mass spectrometry (ICPMS) at the Department of Geological Sciences, University of Michigan.

2.3.6. Immunoblotting. Total protein extracts were prepared from yeast cells using glass beads and triton X-100 (1 %) as described previously (48). Protein concentrations were measured by the BCA method (Pierce) according to the manufacturer's specifications. Protein samples were resolved by reducing SDS-PAGE and transferred to a nitrocellulose membrane. PCA1-GFP was detected by chemiluminescence using a primary rabbit anti-GFP polyclonal antibody (1:2000) (Santa Cruz Biotechnology INC.) and secondary goat anti-rabbit HRP-conjugated antibody (1:5000) (Santa Cruz Biotechnology INC.). PCA1-Flag was probed with primary mouse anti-Flag M2 monoclonal antibody (1:1000) (Sigma) and secondary sheep anti-mouse IgG HRP-conjugated antibody (Amersham) (1:5000). Loading control, phosphoglycerate kinase (PGK), was detected by chemiluminescence using mouse monoclonal anti-PGK antibodies (1:4,000) (Molecular Probes) and secondary sheep anti-mouse IgG HRP-conjugated antibody (1:5,000) (Amersham).

2.3.7. Northern Blotting. Yeast cells were cultured until mid-log phase and supplemented with either 50 μM $CdCl_2$ or 50 μM $CuCl_2$ for 15 and 60 min. Total RNA

was extracted from cells and 25 ug was separated on an RNA gel (0.75 % agarose and 2 % formaldehyde) and transferred to a nitrocellulose membrane (Protran). Gene specific DNA probes labeled with [α-^{32}P]dCTP (Amersham) were generated using the random primer labeling system (Invitrogen). Hybridization of radio-labeled probes with RNA transcripts was performed by previously described methods (49). Relative mRNA levels were detected by autoradiography.

2.4. Results

2.4.1. The PCA1 N-terminal domain confers Cu resistance in yeast. To identify new factors involved in heavy metal defense, we carried out a selection of *S. cerevisiae* cDNAs that suppress lethality of the *ace1*Δ yeast strain on toxic Cu media. A cDNA encoding *PCA1*, one of two P_{1B}-type ATPases in the *S. cerevisiae* genome (37,50), was identified. Yeast GPD gene promoter-driven constitutive expression of *PCA1* confers resistance in the *ace1*Δ strain to Cu (0.1 mM $CuCl_2$) compared to empty vector transformed control cells (Fig. 1A). Deletion of *PCA1* in the *ace1*Δ strain resulted in a slight increase in sensitivity to Cu toxicity (Fig. 2.1A). However, in a wild-type strain, Cu resistance was not easily discernable upon either over-expression or deletion of *PCA1* (data not shown).

PCA1 contains all of the conserved features of the P_{1B}-type ATPase family which is specific for heavy metal transport (16-18,51) (Fig. 2.1B). These features include eight predicted transmembrane domains, where an intramembranous metal-transporting CPx motif is located in the sixth trans-membrane domain (Fig. 2.1B). The catalytic domains reside within cytosolic loops which include the nucleotide binding domain (N-domain) and the phosphorylation domain (P-domain) (Fig. 2.1B). PCA1 contains a single CxxC heavy metal binding motif located within the N-terminal region. An intriguing feature of PCA1 compared with other $P1_B$-type ATPases is a Cys-rich N-terminal extension ~550 amino acids before the first trans-membrane domain (Fig. 2.1B). In order to ascertain whether the observed Cu resistance by PCA1 is dependent on ATPase function, we carried out site-directed mutagenesis of conserved residues of this family of proteins and

assayed for Cu sensitivity. However, Cu resistance remained unchanged in all mutants (Fig. 2.1C), suggesting that Cu resistance is not dependent on metal translocation. Given that the N-terminus contains several cysteine residues (33 Cys), which could serve as Cu ligands, we tested whether this domain could independently confer resistance. A peptide corresponding to amino acid residues 1-452 which includes the CxxC motif (Fig.2.1B) was expressed in the *ace1*Δ strain. Expression of this peptide resulted in Cu resistance above that of full-length PCA1, presumably by chelating Cu ions in a manner analogous to MT (Fig. 2.1D). Cu resistance is maintained even after mutation of the conserved CxxC motif (C421A, C423A) (Fig. 2.1D) implying the existence of other Cu coordination site(s) in addition to this well-characterized metal-binding motif.

2.4.2. Common laboratory yeast strains possess a G970R mutation in PCA1. S288c is a commonly used *S. cerevisiae* haploid strain in which its entire genome has been sequenced (52). Comparisons of the *PCA1* sequence of S288c with natural isolates, RM11-1a (40) and YJM789 (53), using BLAST (NCBI, http://www.ncbi.nlm.nih.gov/blast/) revealed that the S288c strain carries a single nucleotide change in the *PCA1* gene, which results in a G970R substitution. (Fig. 2.2). Our sequencing results of *PCA1* cloned from the RM11-1a strain as well as an industrial baker's yeast strain (Fleischmann's) confirmed the G970R substitution. In order to determine if this mutation was unique to the S288c strain, we sequenced the *PCA1* ORF of several laboratory yeast strains, BY4741 (Open Biosystems), DTY1 (54), EG103 (55), SKY9 (56), and YPH98 (46). All contained the G970R mutation (data not shown), which suggests a common lineage among theses strains. Furthermore, sequence alignments of

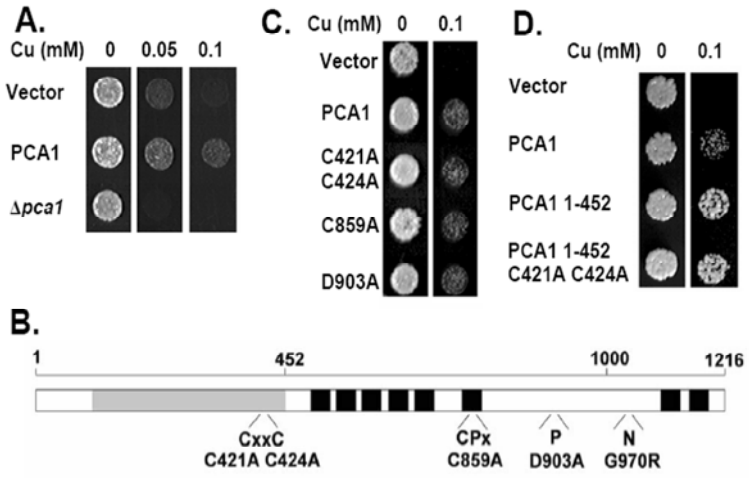

Figure 2.1. Cu resistance is mediated by the Cys-rich N-terminal domain of PCA1. *A*, PCA1 over-expression confers Cu resistance. BY4741 *ace1*Δ transformed with empty vector (p413-GPD) or PCA1 expression vector and BY4741 *ace1*Δ *pca1*Δ yeast cells were cultured in SC (SC-His) media and spotted on solid media supplemented with Cu ($CuCl_2$) at the indicated concentrations. *B*, Schematic depiction of PCA1 sequence features. PCA1 contains the signatures of a P_{1B}-type ATPase, eight predicted transmembrane domains (black boxes), intra-membranous CPx motif within the sixth transmembrane domain, a CxxC metal binding domain, a phosphorylation domain (P), and a nucleotide-binding domain (N). The N-terminal Cys-rich domain is shaded gray. Conserved residues that have been mutated for characterization are indicated under each domain. *C*, Cu resistance conferred by PCA1 is not dependent on metal translocation. BY4741 *ace1*Δ cells transformed with empty vector, a PCA1 expression vector or PCA1 with mutations in the CxxC motif (C421A and C424A), the CPx motif (C859A), or the aspartyl phosphorylation residue (D903A) were spotted on SC-His media with or without Cu supplementation. *D*, Cu resistance is conferred by the PCA1 N-terminus. Cells expressing empty vector, PCA1, PCA1 residues 1-452, or PCA1 residues 1-452 with site-directed mutations of the predicted metal-binding cysteines (C421A, C424A) were spotted on SC-His media with or without Cu supplementation.

prokaryotic, yeast, plant and human P_{1B}-Type ATPases show that this Gly residue is invariantly conserved among this family of proteins (Fig. 2.2). Recently, structural studies of the nucleotide binding domain (N-domain) of two P_{1B}-type ATPases (CopA and ATP7b) places this Gly residue within an ATP binding pocket (57,58). The functional importance of this residue is further underscored by an equivalent G1101R substitution in ATP7b previously reported in patients with Wilson disease (59). Together, our data along with recent reports strongly support the conclusion that many laboratory *S. cerevisiae* strains contain a mutation of a critical residue in PCA1 that may play essential roles in function, expression, and/or regulation.

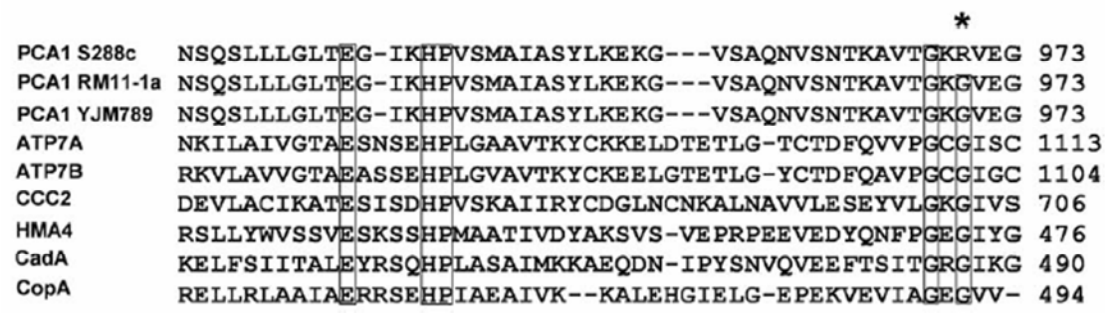

Figure 2.2. Yeast laboratory strains contain a G970R mutation in PCA1. A region of the predicted nucleotide binding domain (N-domain) of PCA1 from strains S288c, RM11-1a and YJM789 were aligned with the corresponding region of other P_{1B}-type ATPases, including human Cu-transporting ATP7a and ATP7b, *S. cerevisiae* CCC2, *A. thaliana* HMA4, *S. auereous* CadA, and *A. fulgidus* CopA. Boxes highlight conserved residues. Asterisk marks G970R substitution identified in the S288c and other laboratory *S. cerevisiae* strains. Sequences were obtained from data bases at The National Center for Biotechnological Information (NCBI) and alignments were performed with ClustalX (1.81).

2.4.3. PCA1 natural allele confers hyper-resistance to Cd. To characterize the function of PCA1, we expressed the natural allele (PCA1-WT) and the G970R mutant allele (PCA1) in a laboratory yeast strain and examined metal tolerance. Indeed, expression of PCA1-WT allows cells to grow on media containing a Cd concentration over ten-fold higher compared to vector and PCA1 carrying the G970R mutation. (Fig.2.3A). However, growth on media supplemented with Cu was indistinguishable, which is consistent for Cu resistance being dependent on metal binding rather than ATPase activity **(**Fig. 2.3A). No growth advantages in cells expressing PCA1-WT were observed when toxic concentrations of Zn, Mn, Co or Fe were added to growth media (data not shown), suggesting specificity in metal binding and translocation by PCA1-WT.

Since the RM11-1a strain contains a functional *PCA1* allele, it would be predicted that this strain is more resistant to Cd compared to laboratory strains. Cd sensitivity was compared between the RM11-1a strain and our BY4741 laboratory strain. Indeed, the BY4741 strain is much more sensitive to Cd (Fig 2.3B). Deletion of *PCA1*-WT in the RM11-1a strain reduces Cd resistance compared to control cells (Fig. 2.3B) underscoring its significance in Cd defense. Cd tolerance returns to wild-type levels in the RM11-1a *pca1Δ* strain transformed with a natural promoter driven *PCA1*-WT plasmid confirming that Cd sensitivity was specific for deletion of *PCA1*-WT (Fig. 2.3B). As expected deletion of the non-functional *PCA1* in the BY4741 strain did not significantly affect Cd resistance. However, introduction of *PCA1*-WT under control of its natural promoter rescued growth on Cd media (Fig. 2.3B). Interestingly, the RM11-1a strain displayed a

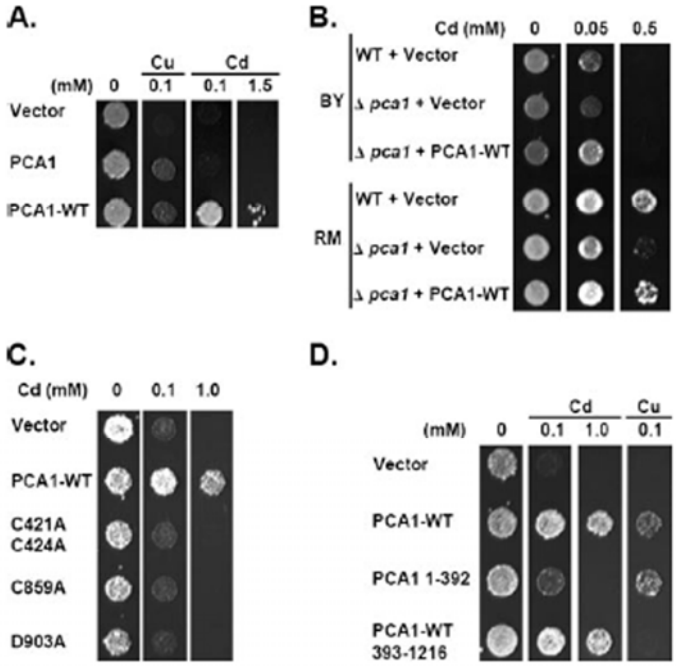

Figure 2.3. PCA1 natural allele (PCA1-WT) plays a critical role in Cd defense. *A*, PCA1-WT confers resistance to Cd toxicity. BY4741 *ace1*Δ cells expressing PCA1 cloned from a laboratory strain (PCA1) or natural isolate (PCA1-WT) under control of a constitutive promoter of the GPD gene were spotted on SC-His media supplemented with $CuCl_2$ or $CdCl_2$ at the indicated concentrations. *B*, PCA1-WT plays a physiological role in Cd defense. Cd resistance of laboratory strain BY4741 (BY) was compared to natural isolate RM11-1a (RM). Wild type BY and RM strains or *Δpca1* null mutants were transformed with empty vector or PCA1-WT under control of its natural promoter. Cells were spotted on SC-Leu media supplemented with $CdCl_2$ at indicated concentrations. *C*, Cd resistance conferred by PCA1-WT is dependent on P_{1B}-type ATPase catalytic activity. Amino acid substitutions in PCA1-WT were made in the amino terminal CxxC motif (C421A and C424A), the CPx motif (C859A), or the aspartyl phosphorylation residue (D903A). Cells expressing PCA1-WT or mutants were spotted on SC-His media supplemented with indicated concentrations of $CdCl_2$. *D*, The Cys-rich N-terminus of PCA1-WT is not essential for Cd resistance. BY4741 *ace1*Δ cells expressing empty vector, PCA1-WT, PCA1 (1-392) and truncated PCA1-WT (393-1216) were spotted on SC-His solid media supplemented with the indicated concentrations of $CuCl_2$ or $CdCl_2$.

Cd growth advantage over the BY4741 strain when *PCA1*-WT is null-mutated, implying that there may be other factor(s) contributing to Cd resistance (Fig. 2.3B).

We then asked whether Cd resistance is coupled with active metal transport of a P_{1B}-type ATPase. Site-directed mutagenesis of conserved amino acids among P_{1B}-type ATPases, including the CxxC motif (C421, C424), the CPx motif (C859), and the phosphorylation site (D903), completely abolished Cd resistance (Fig. 2.3C). These results suggest that Cd resistance is dependent on an active metal transport mechanism.

2.4.4. The Cys-rich N-terminal domain of PCA1 is not essential for Cd resistance. PCA1 carries a Cys-rich N-terminal domain that does not have significant sequence homology with any characterized metal-binding protein. We examined the role of this domain in Cd resistance by expressing the N-terminal 392 amino acid domain and a truncation PCA1-WT allele lacking this domain. The truncated PCA1-WT allele (aa 393-1216) includes the CxxC motif since it is critical for Cd resistance (Fig. 2.3C). Cells expressing the N-terminal 392 amino acid peptide are more resistant to both Cd and Cu compared with empty vector-transformed cells (Fig. 2.3D). Truncation of this N-terminal domain did not significantly affect Cd resistance as compared to full length PCA1-WT (Fig. 2.3D). These data suggest that the Cys-rich N-terminal domain itself reduces Cu and Cd toxicity, but is not critical for Cd transport. In contrast, Cu resistance conferred by PCA1-WT was abolished in the truncated mutant (Fig. 2.3D), which further supports the conclusion that Cu resistance conferred by PCA1 is not dependent on ATPase activity but rather metal sequestration by this domain.

2.4.5. PCA1-mediated Cd export. We addressed three different possible mechanisms governing PCA1-WT mediated Cd resistance. First, PCA1-WT could be involved in Cd sequestration and/or compartmentalization to intracellular organelles. To test this possibility, total cellular Cd levels were measured by inductively coupled plasma mass spectrometry (ICPMS) of cells expressing empty vector, PCA1 or PCA1-WT when cultured in Cd supplemented media. Cells expressing PCA1-WT as compared to empty vector and PCA1 displayed a marked decrease in total cellular Cd accumulation (Fig. 2.4A). These results indicate that PCA1-WT either reduces Cd uptake or enhances efflux. Second, to address the possibility that PCA1-WT may indirectly reduce cellular Cd uptake, yeast cells expressing empty vector or *GAL1* promoter-mediated expression of PCA1-WT were pre-treated with toxic amounts of Cd under glucose repression. Cells were then washed to remove extracellular Cd and spotted on solid SC media containing either glucose or galactose. Empty vector-transformed cells and PCA1-WT displayed similar growth under glucose repression (Fig. 4B). However, cells expressing PCA1-WT displayed an obvious growth advantage on galactose media in which PCA1-WT is expressed (Fig. 2.4B). Since PCA1-WT expression was repressed during Cd exposure its role in Cd defense must occur after Cd has already entered the cell. Third, to conclusively demonstrate an active efflux role, yeast cells were pre-treated with Cd, washed to remove extracellular Cd, re-suspended in fresh media, and collected at 30 min intervals to determine cellular Cd content. Cd levels in cells expressing empty vector remained relatively constant for 60 min after Cd removal (Fig. 2.4C and inset). In contrast, Cd

levels rapidly declined in cells expressing PCA1-WT (Fig. 2.4C and inset). All together, these data strongly support the conclusion that PCA1-WT functions as a Cd efflux pump.

2.4.6. Metal-dependent regulation of PCA1 expression. Since Cd concentration in the environment likely fluctuates, it is reasonable to predict that Cd regulates PCA1 expression. However, Northern blot analysis did not indicate any induction of the *PCA1* gene after the addition of either Cd or Cu to culture media (Fig. 2.5A). Both Cd and Cu induced *GSH1* encoding an enzyme required for GSH synthesis under the same experimental conditions (Fig. 2.5A). We next examined the possibility of post-transcriptional regulation. Total protein extracts from cells expressing N-terminal Flag epitope-tagged PCA1-WT were subjected to Western blot analysis. PCA1-WT-Flag and non-tagged PCA1-WT exhibit indistinguishable Cd resistance indicating that epitope tagging does not perturb function (data not shown). Although PCA1-WT expression was mediated by a relatively strong GPD gene promoter, PCA1-WT-Flag was hardly detectable. However, Cd supplementation to culture media dramatically enhanced PCA1-WT-Flag protein levels in a time and dose-dependent manner (Fig. 2.5B). Not only Cd but also Cu and Ag specifically increased PCA1-WT-Flag protein levels (Fig. 2.5C). Since the coding sequences of PCA1-WT were inserted into a vector for constitutive expression by a GPD gene promoter and CYC1 terminator, it is likely that Cd, Cu, and Ag stabilize PCA1 rather than regulate translation. CCC2, a Cu-transporting P_{1B}-type ATPase, inserted into the same vector, was not regulated by metal ions supporting the conclusion that the observed metal regulation of PCA1 is independent of the GPD promoter and/or CYC1 terminator (data not shown).

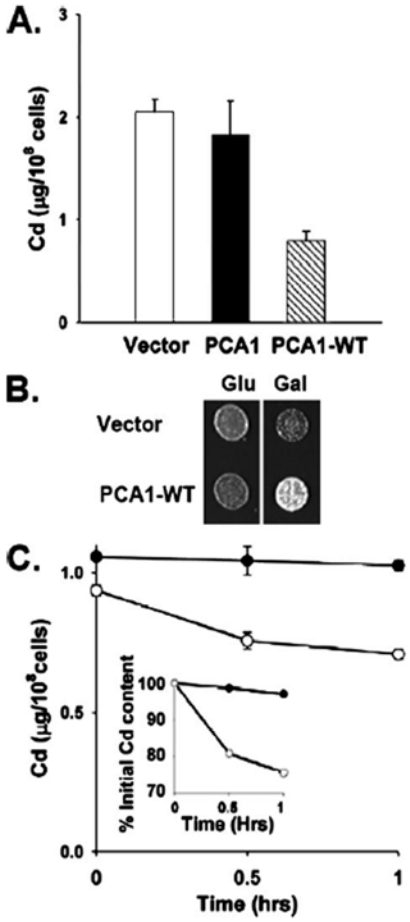

Figure 2.4. PCA1-WT Cd resistance is dependent on an efflux mechanism. *A*, Over-expression of PCA1-WT reduces total cellular Cd content. BY4741 *ace1*Δ cells transformed with empty vector (p413-GPD) or PCA1 or PCA1-WT expression vectors were cultured in SC-His media supplemented with Cd (50 µM $CdCl_2$) for six hours. Cells were collected, washed, and total cellular Cd levels were measured by ICPMS. Bar graphs represent the average ±S.D. of four independent measurements. *B*, BY4741 *ace1*Δ cells were transformed with an expression plasmid of PCA1-WT under control of the *GAL1* gene promoter or control plasmid. Cells were cultured in SC-His media supplemented with 1mM $CdCl_2$ for 1 hr under glucose repression, washed, and spotted on SC-His media containing either glucose (Glu) or galactose (Gal). *C*, PCA1-WT enhances Cd efflux. BY4741 *ace1*Δ cells transformed with empty vector (closed circles) or a GPD promoter-mediated PCA1-WT expression vector (open circles). Cells were cultured in SC-His media supplemented with 0.5mM $CdCl_2$ for 30 min, washed and re-suspended in fresh media. Aliquots were collected at indicated time points and Cd levels were measured. Each data point represents the average ±S.D. of four independent samples. Inset indicates percent of initial Cd content.

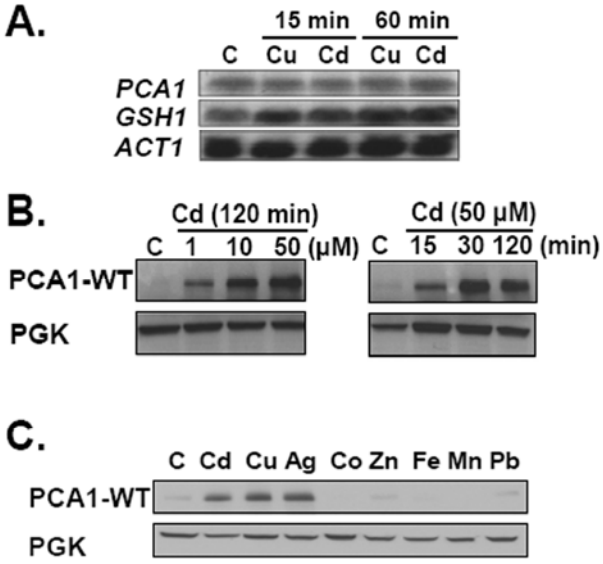

Figure 2.5. Metal-dependent regulation of PCA1 expression. *A*, Cd and Cu do not induce transcription of *PCA1*. BY4741 *ace1*Δ cells were cultured in SC media supplemented with Cd (50 μM $CdCl_2$) or Cu (50 μM $CuCl_2$) for 15 or 60 min prior to total RNA extraction. Transcripts of *PCA1*, *GSH1* (a positive control), and *ACT1* (a loading control) were detected by Northern blot analysis using gene-specific ^{32}P-labeled probes. *B*, Post-transcriptional regulation of PCA1-WT. BY4741 *ace1*Δ cells transformed with PCA1-WT-Flag were cultured in SC-His media supplemented with Cd (1, 10 or 50 μM, $CdCl_2$) for 120 min (left panel) or 50 μM $CdCl_2$ (15, 30 or 120 min) (right panel). PCA1-WT-Flag expression was detected by Western blot analysis. PGK (phosphoglycerate kinase) was probed as a loading control. *C*, Metal specific post-transcriptional regulation of PCA1-WT. BY4741 *ace1*Δ cells transformed with a PCA1-WT-Flag expression construct were cultured for 120 min in SC-His media supplemented with Cd (50 uM $CdCl_2$), Cu (50 uM $CuCl_2$), Ag (25 μM $AgNO_3$), Co (4 mM $CoSO_4$), Zn (15 mM $ZnCl_2$), Fe (2 mM $FeC_6H_5O_7HCl$), Mn (15 mM $MnCl_2$), and Pb (0.2 mM $Pb(NO_3)_2HCl$). PCA1-WT-Flag expression was analyzed by Western blot using anti-Flag antibodies. PGK was probed as a loading control.

2.4.7. Subcellular localization of PCA1-WT. The function of PCA1-WT as a Cd efflux pump suggests that it is localized at the plasma membrane. To test this prediction, both PCA1-WT and PCA1 fused with GFP at the N-terminus were localized by confocal fluorescence microscopy. The Cd resistance conferred by tagged PCA1-WT-GFP and untagged PCA1-WT were indistinguishable (data not shown). Under normal conditions both PCA1 and PCA1-WT display faint fluorescent signals distributed throughout the cytoplasm (Fig. 2.6A). In accordance with the enhanced protein expression determined by Western blotting (Fig. 2.5B and 2.5C), the addition of Cd (Fig. 2.6A) as well as Cu and Ag (data not shown) to culture media dramatically enhanced fluorescent signal in cells. Furthermore, consistent with a predicted role in Cd efflux, PCA1-WT-GFP is primarily localized at the plasma membrane in the presence of Cd (Fig. 2.6A) as well as Cu and Ag (data not shown). Addition of Zn, Fe, Co, Pb, and Mn to the culture media had no significant effect on fluorescent signals for PCA1-WT-GFP or PCA1-GFP (data not shown). Expression of the non-functional PCA1-GFP was enhanced by Cd, however it did not properly localize to the plasma membrane (Fig. 2.6A, 2.6B), suggesting that the G970R mutation perturbs trafficking. Western blot analysis of PCA1-GFP and PCA1-WT-GFP detected a product of the predicted size (Fig. 2.6B), indicating that the GFP signal reflects full-length PCA1 protein. These data demonstrate that metal ions regulate PCA1-WT protein levels and stimulate plasma membrane localization.

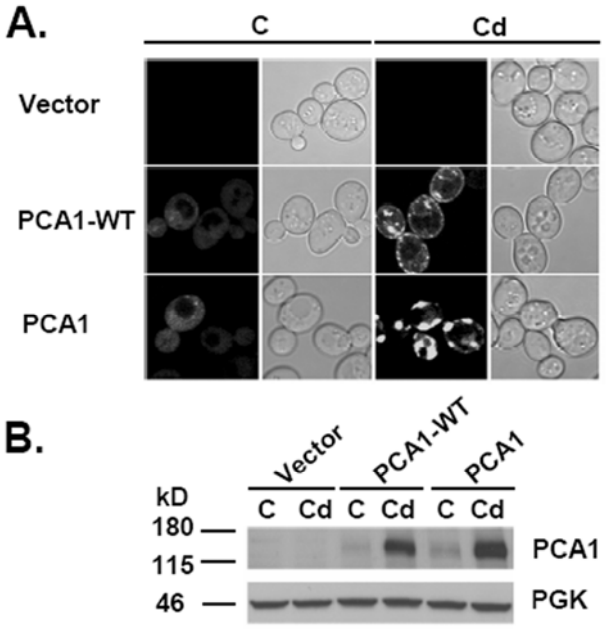

Figure 2.6. Metal and functionality-dependent changes in subcellular distribution of PCA1. *A*, Subcellular localization of wild-type and nonfunctional PCA1. BY4741 *ace1*Δ cells transformed with empty vector, PCA1-WT-GFP or PCA1-GFP were cultured to mid-log phase with or without Cd (50μM $CdCl_2$) supplementation for 120 min. Cells were washed and fluorescence was visualized by confocal microscopy (left panels) and differential interference contrast (right panels). *B,* Western analysis of PCA1-GFP fusion proteins. Total protein extracts were prepared from cells with or without Cd supplementation to the culture media and PCA1-WT-GFP or PCA1-GFP were detected by Western blot analysis using anti-GFP antibodies. PGK (phosphoglycerate kinase) was probed as a loading control.

2.5. Discussion

The P-type ATPase family of integral membrane proteins translocates various ions and phospholipids across membranes using the chemical energy of ATP hydrolysis (15-18,51,60-62). Virtually all organisms rely on these transporters for maintaining a transmembrane gradient of various ions, which are vital for nutrient uptake, neurotransmission, signaling, and excretion of toxic metals (15-18,51,60-62). The P1B-type ATPase specifically transports soft metals, such as Cu, Cd, Zn and Pb (13-18). Our data presented herein support the conclusion that PCA1, one of the two P1B-type ATPases in the S. cerevisiae genome, is a Cd efflux pump which is non-functional in all five laboratory yeast strains that we have examined. Metal-induced stabilization and distribution of PCA1 at the plasma membrane is likely an important mechanism of rapid response to excess Cd. This is a novel mode of regulation identified in this family of proteins. Function, structural features and metal-dependent regulation patterns of PCA1 revealed important new insights into the classification, substrate specificities, mechanisms of action, and regulation of P1B-type ATPases. Moreover, this study also uncovered that S. cerevisiae laboratory strains that have been used widely as an outstanding model system in biomedical sciences does not mirror, at least in cellular Cd defense, the strains living in natural habitats.

PCA1 has been proposed and classified as a Cu-transporting P1B-type ATPase (37). However our data does not support a Cu-transporting function. Instead the Cys-rich N-terminal domain appears to be the contributing factor involved in Cu resistance. The abundant Cys residues may serve as ligands for Cu binding. Expression of

this domain itself confers Cu tolerance in yeast, which supports the hypothesis that these abundant Cys residues participate in metal coordination. However, detoxification of Cu-generated reactive oxygen species (4) by this domain can not be excluded as superoxide dismutase activity has been reported for the similar Cys-rich MT (63). It is interesting to note that AtHMA2 and AtHMA4, two P1B-type ATPases of A. thaliana, possess a similar Cys/His-rich domain at the C-terminus (19,21,22). However, these domains do not show significant sequence identity with each other or with any other known metal-binding proteins. It appears that this region is not essential for AtHMA2/4 function (64,65). Consistently, truncation of the Cys-rich N-terminal domain, excluding the CxxC motif, does not alter PCA1-WT mediated Cd resistance. This region may play regulatory roles through interaction with metals and/or with other proteins. Several Ca++-transporting P-type ATPases were shown to carry autoinhibitory domains (51,60). Binding of regulators and phosphorylation of C-teminal residues of H+-transporting ATPases in plants has also been reported (51,61,66). We are currently testing the regulatory roles of the Cys-rich N-terminal domain of PCA1.

A conserved signature of P1B-type ATPase family is the one to six CxxC motifs located within the N-terminal region (67). For instance, human ATP7a and ATP7b contain six such motifs. The two Cys residues bind a single Cu (Cu+) ion in a linear bicoordinate manner (68). While Cu binding to these motifs is firmly established by several methods, their roles in the function and regulation of ATP7a and ATP7b remain unclear. These motifs have been suggested to influence Cu translocation and/or subcellular trafficking of ATP7a and ATP7b (67). Site-directed mutagenesis of the CxxC

motif in PCA1 showed that the two Cys residues are necessary for Cd resistance, which suggests Cd binding to this domain is critical for PCA1 function. Cd coordination unlikely occurs through two Cys residues but rather with multiple ligands. Future metal binding studies and structural characterization of the Cys-rich domain would reveal how PCA1 coordinates Cd or other metal ions. Distinct structural features for metal coordination of this domain may explain substrate specificity of P1B-type ATPases. Given that the cytosolic Cu carrier, Atox1, directly interacts with the metal binding domain of ATP7a and ATP7b, PCA1 may acquire Cd from a carrier molecule. Since Cd binds thiol groups (25-29), glutathione and/or other cytosolic Cd carriers may specifically interact with the PCA1 metal binding domain to transfer Cd. For instance, YCF1, a vacuolar ABC transporter utilizes bis(glutathionato)Cd as its substrate (30).

P1B-type ATPases are categorized into several subfamilies depending on substrates and structural features (69), although the molecular determinant(s) for the substrate specificities is not known. Given that PCA1 deletion or over-expression specifically alters cellular accumulation and resistance of Cd, PCA1 appears to be the first Cd-specific P1B-type ATPase. It is possible that PCA1 may efflux other heavy metal ions but since they do not enhance protein expression, substrate specificity can not be deciphered by metal resistance. We have addressed this issue by the generation of a functional PCA1 mutant which is constitutively expressed even in the absence of Cd (D. Adle and J. Lee, unpublished data). Additionally, in vitro biochemical ATPase assays would further define PCA1 substrate specificity.

Sequence analysis of the PCA1 ORF of two independent natural strains, and an industrial strain clearly identified a G970R mutation to exist in all examined laboratory strains. Although a few other polymorphisms exist, all carried the G970R substitution. It is likely that the ancestral S. cerevisiae laboratory strain carrying the original G970R mutation was propagated to several research laboratories across the world. Interestingly, a PCA1 allele containing several (27 amino acid) missense mutations was identified in a yeast strain that was selected on media containing lethal concentrations of Cd (39). Among these mutations, an R970G mutation was implicated in Cd resistance conferred by the mutant PCA1 (39). Thus, it appears that in this report a non-functional PCA1 mutant was reverted to the functional allele under Cd stress. The non-functional PCA1 allele, at least partially, explains Cd sensitivity of a laboratory strain (BY4741) compared with a natural isolate (RM11-1a). The RM11-1a strain was still hyper-resistant compared to BY4741 even when PCA1 was deleted in these strains, implicating the existence of other contributing factors involved in Cd defense. Elucidation of the underlying reason of Cd hyper-resistance in the RM11-1a strain would lead to a better understanding of mechanisms involved in Cd detoxification.

Regulation of subcellular distribution and transcription of P1B-type ATPases have been previously reported, suggesting the significance of active regulation of this family of transporters. ATP7a and ATP7b localize to the ER-golgi membrane where they transport Cu across the membrane for incorporation into proteins in the secretory pathway (7,16,50,67). Excess cellular accumulation of Cu induces trafficking of ATP7a to the plasma membrane and ATP7b to cytoplasmic vesicles (67). In addition to post-

translational regulation, several examples of metal-dependent transcriptional regulation of genes encoding P1B-type ATPases have been reported in plant, yeast, and bacteria (19,21,70-73). PCA1 mRNA levels are not regulated by Cd or Cu. However, Cd dramatically regulates PCA1 protein levels and promotes localization to the plasma membrane, which is a unique mode of regulation for this family of transporters. Interestingly, Cu and Ag also regulate PCA1 protein expression, although PCA1 does not transport these metals. Since the N-terminus of PCA1 possesses a Cys-rich region which likely binds Cu, it is plausible that metal binding to this domain controls turnover of PCA1.

Given that the Gly970 residue in PCA1 is conserved among this family of proteins and has been positioned in an ATP binding pocket of two independent structures of P1B-type ATPases (58,74), it is likely that this residue is involved in ATP binding. Interestingly, the G970R mutant exhibits a distinct localization pattern compared with that of PCA1-WT. An equivalent mutation identified in ATP7b of Wilson disease patients might also lead to defects in subcellular distribution. A specific conformation resulting from ATP binding and metal translocation may determine proper trafficking of PCA1. This may occur through PCA1 conformation-dependent attraction of secretory and/or endocytosis machinery. We are currently exploring these possibilities.

Cd is one of the most toxic environmental pollutants. It is removed slowly from the body, which may be due to high affinity binding to cellular ligands, such as thiol groups in proteins, glutathione, methallothionein, and cysteine. Cd transporters in hepatocytes and renal epithelial cells, such as ATP-binding cassette, multi-drug resistance

protein, GSH-Cd complex transporter, have been proposed to efflux GSH or Cys-conjugated Cd (23,24). However, no report has identified other P1B-type ATPases in the human genome in addition to ATP7a and ATP7b. Further characterization of Cd efflux systems in higher eukaryotes would advance our ability to combat Cd-related disorders and develop methods for remediation of toxic heavy metals.

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Chapter 3

Expressional Control of a Cadmium-Transporting P_{1B}-Type ATPase by a Metal Sensing Degradation Signal

Note: The results described in this chapter have been published

The authors and title of the paper are:

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3.1. Abstract

Cadmium is a highly toxic environmental contaminant implicated in various diseases. Our previous data demonstrated that Pca1, a P1B-type ATPase, plays a critical role in cadmium resistance in yeast S. cerevisiae by extruding intracellular cadmium. This illustrates the first cadmium-specific efflux pump in eukaryotes. In response to cadmium, yeast cells rapidly enhance expression of Pca1 by a post-transcriptional mechanism. To gain mechanistic insights into the cadmium-dependent control of Pca1 expression, we have characterized the pathway for Pca1 turnover and the mechanism of cadmium sensing that leads to up-regulation of Pca1. Pca1 is a short-lived protein ($t_{1/2}<5$ min) and is subject to ubiquitination when cells are growing in media lacking cadmium. Distinct from many plasma membrane transporters targeted to the vacuole for degradation via endocytosis, cells defective in this pathway did not stabilize Pca1. Rather, Pca1 turnover was dependent on the proteasome. These data suggest that, in the absence of cadmium, Pca1 is targeted for degradation before reaching the plasma membrane. Mapping of the N-terminus of Pca1 identified a metal-responding degradation signal encompassing amino acids 250 to 350. Fusion of this domain to a stable protein demonstrated that it functions autonomously in a metal-responsive manner. Cadmium sensing by cysteine residues within this domain circumvents ubiquitination and degradation of Pca1. These data reveal a new mechanism for substrate-mediated control of P1B-type ATPase expression. Cells have likely evolved this mode of regulation for a rapid and specific cellular response to cadmium.

3.2. Introduction

While several heavy metals are essential micronutrients, many of them are toxic environmental contaminants with no physiological role. Genetic disorders in metal metabolism (e.g., hemochromatosis and Wilson disease) in which excess iron and copper are accumulated in the liver, respectively (1-3), provide striking evidence for the toxicity of nutritional metal ions. Cadmium is a highly toxic environmental contaminant linked to a series of diseases in humans and animals, including renal disease, cancer, reproductive defects, and endocrine disruption (4-5). Human exposure to cadmium through diet, smoking, pollution, and occupation is widespread (4,5). Cadmium released into the environment enters the food chain through its accumulation in plants, fish, shellfish, and other animals (4-7). Cadmium concentrations in plants usually range from 0.5 to 5 μM (6); however, in some plants, it can reach more than 100 μM (8). High levels of cadmium can be accumulated in soil due to pollution, abandoned mines, or geological features. The content of cadmium and acidity of soils determine cadmium levels in grains and vegetables produced from the land (4,7,8).

Cadmium has a high affinity for sulfhydryl (–SH) groups in proteins and glutathione, a major cellular reducing buffer, which results in inactivation of proteins and cellular oxidative stress (4,9,10). A recent report showed that cadmium markedly increases mutation rates by specifically inhibiting the DNA mismatch repair system (11). Cadmium exposure during pregnancy is associated with low birth weight and increased incidence of spontaneous abortion (4,7,11-14). Cadmium affects steroid synthesis pathways and secretion of progesterone, a hormone that plays critical roles in maintaining

pregnancy (12). Cadmium induces uterine hyperplasia, mammary gland development, and early onset of puberty in rat models (13,14). These estrogenic effects lead to infertility and cancer (14). Moreover, cadmium could exhibit synergistic estrogenic effects with other xenoestrogens, such as phytoestrogens in soybeans and vegetables (14).

Organisms have evolved systems for excretion and detoxification of non-physiological metals and homeostatic acquisition of nutritional yet toxic metals. Metal transporters, cytosolic delivery molecules, and chelators have been identified in many organisms ranging from bacteria to humans (1,3,26-28). Given that cadmium is not a nutritional metal ion, organisms do not actively acquire cadmium. Rather, cadmium enters the body through non-specific mechanisms and/or transporters involved in the acquisition of essential metal ions. DMT1 (DCT1, Nramp2) iron transporter, zinc transporters, and calcium channels are all involved in cadmium absorption (15-19). Metallothionein (MT), a cysteine-rich low molecular weight metal-chelating peptide, and glutathione (GSH), a cysteine-containing tripeptide, sequester metal ions. In eukaryotes, this is considered the major mechanism for neutralizing toxic metals, including cadmium (20-23). Ace1 is a transcription factor inducing expression of copper defense genes in *S. cerevisiae*, including *CUP1* and *CRS5* encoding metal chelators, and *SOD1* encoding a superoxide dismutase (24). Ycf1, a vacuolar membrane ATP-binding cassette (ABC) transporter, sequesters glutathione-conjugated cadmium and other metals into the vacuole (25). Thus, Δ*ace1* and Δ*ycf1* strains are sensitive to copper and cadmium, respectively. Phytochelatin, a GSH polymer synthesized in plants and fission yeast, also chelates heavy metals (21). Several heavy metal efflux pumps, including P_{1B}-type ATPases, ABC

transporters, and cation diffusion facilitators play roles for extruding toxic heavy metals (3,26-28). These metal-transporters play vital roles for uptake, compartmentalization, and extrusion of nutritional and non-physiological heavy metals. In bacterial cells, P_{1B}-type ATPases are vital for extrusion of metals, including cadmium, copper, and lead (26, 27). However, the cadmium efflux systems in eukaryotes remain uncharacterized.

Consistent to the fact that cellular defense systems respond to metal levels, several modes of regulation control expression, subcellular localization, and activities of P_{1B}-type ATPases. Transcriptional control appears to be a major regulatory mechanism in bacteria and plants (26-28). In mammals copper controls subcellular trafficking of copper-transporting P_{1B}-type ATPases. In a low copper medium, ATP7a and ATP7b are localized in the *trans*-Golgi network where they transport copper for its incorporation into secretory proteins (3,29). When copper levels are elevated, ATP7a is mobilized to the plasma membrane in a reversible manner (3,29). The biological significance of this molecular event is that ATP7a participates in copper uptake at the intestine and protects cells from copper toxicity. Excess copper mobilizes ATP7b from the *trans*-Golgi network to cytoplasmic vesicle-like compartments in the liver (3,29), which appears to be an important mechanism for excretion of excess copper into bile to maintain systemic copper homeostasis.

In a previous study, we demonstrated that Pca1, a predicted P_{1B}-type metal-transporting ATPase, is critical for cadmium defense in yeast *S. cerevisiae* (30). Pca1 is rapidly up-regulated in response to cadmium without any change in its transcript levels (30), which suggests a new mode of substrate-mediated regulation for this family of

proteins. To elucidate the mechanisms underlying the expressional control of Pca1 in response to cadmium, we have characterized the pathways for turnover of Pca1 and identified a metal-sensing *cis*-acting element that controls turnover of Pca1. Our data demonstrate that a degradation signal within the cysteine-rich N-terminus rapidly targets Pca1 for ubiquitination and degradation by the proteasome. This occurs before Pca1 reaches the plasma membrane where it extrudes cadmium. However, direct cadmium sensing by the degradation signal prevents turnover, which leads to enhanced expression of Pca1. Cells have likely evolved this mode of regulation for a rapid and specific response against cadmium. Together, we have identified a new metal binding domain and revealed a unique mode of regulation for a P_{1B}-type ATPase by its own substrate.

3.3. Experimental Procedures

3.3.1. Yeast Strains, Media, and Growth Conditions. A BY4741 haploid *S. cerevisiae* strain (*MATa his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*) and null mutants, including *ace1*::KanMX6, *ycf1*:: KanMX6, *pep4*:: KanMX6, *vps4*::KanMX6 and *pdr5*:: KanMX6 strains, were purchased from OpenBiosystems. Gene deletion in each strain was confirmed by PCR using gene specific primer sets. We received *end4-1* (31), *npi1* (32), and *pre1-1pre2-1* (33) strains from Drs. Caroline Philpott, (National Institute of Health), Bruno André, (Universite Libré de Bruxelles), and Dieter Wolf (Universität Stuttgart), respectively. Yeast cells were cultured with synthetic complete (SC) media (2% dextrose, 0.2% amino acid mixture, and 0.67% yeast nitrogen base) lacking specific amino acids for plasmid selection. If no temperature is specified, cells were cultured at 30 ^{o}C. For phenotypical analysis on solid media (1.5% agar), 5 μl of yeast cells (OD_{600} 1.0) were spotted on selective media supplemented with the indicated concentrations of cadmium ($CdCl_2$) or copper ($CuCl_2$) and incubated at 30 °C for 2 days before photography. Cycloheximide chases were performed by the addition of 100 ug/mL cycloheximide to logarithmically growing cultures prior to collection into equal volumes of ice-cold kill buffer (phosphate buffered saline (PBS) containing 15 mM NaN_3) at the indicated time points. For proteasome inhibition, a Δ*pdr5* strain that blocks proteasome inhibitor efflux (34), was cultured 5 hrs in the presence of 40 μM MG132 (Calbiochem) in 0.1% dimethyl sulfoxide (Sigma).

3.3.2. Plasmid Construction and Manipulation. If no expression vector is specified, single copy yeast vectors (p416-GPD) (35) were used for glyceradehyde-3-

phosphate dehydrogenase (GPD) gene promoter-driven constitutive expression of Pca1, N-terminal truncated Pca1, CaCRP, Pca1-CaCRP fusion, N-terminal 250-350 peptide, Fet3 iron oxidase, CL1 degradation signal peptide, and Gap1 general amino acid permease. For the green fluorescent protein (GFP) or hemagglutinin (HA) epitope fusion, *NotI* flanked GFP or three tandem HA epitopes were inserted into the *NotI* site which was artificially generated after ATG translation start codon by PCR. Expression plasmids of CL1 artificial degradation signal fused with GFP were constructed as described (36,45). Site-directed mutagenesis was conducted by the primer overlap extension method (37). We sequenced all constructs to confirm correct sequence and in frame fusion. Common molecular biology techniques, including plasmid amplification using *Escherichia coli*, and purification, followed previously established methods (38). Plasmid transformation into yeast was performed using the lithium acetate method (39).

3.3.3. Fluorescence Microscopy. Yeast cells were cultured in SC selective media at 30 °C with agitation until mid-log phase. When indicated, metals ($CdCl_2$ or $CuCl_2$) were added to the culture media prior to imaging. Cells were collected, re-suspended in PBS and imaged on a confocal microscope (Olympus FV500) as described (30).

3.3.4. Immunoblotting. Total protein extracts were prepared by breaking cells with glass beads in PBS containing protease inhibitor cocktail (Complete Mini) (Roche) and 1% triton X-100. Protein concentrations were measured using the BCA kit (Pierce) according to the manufacturer's specifications. Lysates were denatured in SDS sample buffer containing dithiothreitol (DTT) (25 mM) for 15 min at 37 °C and resolved by SDS-PAGE and transferred to a nitrocellulose membrane. GFP fusion proteins were

detected by rabbit anti-GFP polyclonal antibodies (Santa Cruz Biotechnology INC) and secondary goat anti-rabbit IgG antibodies conjugated to horseradish peroxidase (HRP) (Santa Cruz Biotechnology INC). HA-tagged proteins were probed with rabbit anti-HA polyclonal antibodies (Rockland) and secondary goat anti-rabbit IgG antibodies conjugated to HRP (Santa Cruz Biotechnology INC). Loading control, 3-phosphoglycerate kinase (PGK), was detected with mouse monoclonal anti-PGK antibodies (Molecular Probes) and secondary sheep anti-mouse IgG antibodies conjugated to HRP (Amersham). Signals were captured on film (Midwest Scientific) by chemiluminescence (SuperSignal West Pico) (Pierce).

3.3.5. Detection of Ubiquitination. Cells were broken by glass beads in PBS containing protease inhibitors (Complete Mini) (Roche), 5 mM N-ethylmaleimide, 1 mM phenyl-methylsulfonyl fluoride, and 1% Triton X100. HA-tagged proteins were immunoprecipitated with an HA-Tag IP/Co-IP kit (Pierce) according to manufacturer specifications. Proteins were eluted by incubation of immobilized anti-HA agarose beads in 2XSDS sample buffer at 37 °C for 15 min and reduced in the presence of 0.1 M DTT for an additional 15 min at 37 °C. Ubiquitin-conjugated proteins were detected by immunoblotting using mouse monoclonal antibodies against ubiquitin (Covance).

3.4. Results

3.4.1. Cadmium Rapidly Induces Pca1 Expression by Increasing Stability. A unique feature of Pca1 is a cytosolic cysteine-rich N-terminal extension of nearly 400 residues (Fig. 3.1A). To investigate the mechanism underlying the post-transcriptional regulation and role of the N-terminal extension in turnover of Pca1, we have constructed expression plasmids of full length Pca1 and Pca1 with truncation of the first 392 amino acids. A GFP or triple HA epitope was fused at the N-terminus. All Pca1 constructs were functional in conferring cadmium resistance (Fig. 3.1B). Western blot analysis of Pca1 showed that Pca1 is a short-lived protein ($t_{1/2}$ < 5min) (Fig. 3.1C). However, up-regulation of Pca1 is clearly visible 5 min after addition of cadmium to culture media (Fig. 3.1D), indicating a rapid cellular response to cadmium. Pca1 tagged with the GFP or c-myc epitope at the N or C-terminus, respectively, also showed similar regulation by cadmium (data not shown), suggesting that cadmium's effect on Pca1 expression is independent of epitope or its location. This expression control by cadmium can be explained by enhanced translation or stability of Pca1 protein. To decipher these two possibilities, Pca1 turnover rate was examined by cycloheximide chases in the absence or presence of cadmium in culture media. Indeed, stability of Pca1 is dramatically increased in the presence of cadmium (Fig. 3.1E). These data suggest that the induction of Pca1 occurs through cadmium's ability to prevent the rapid turnover of Pca1.

3.4.2. Pca1 Is Not Stabilized in a Strain Defective in Endocytosis. In yeast, several plasma membrane proteins are internalized into endocytic vesicles that enter the early

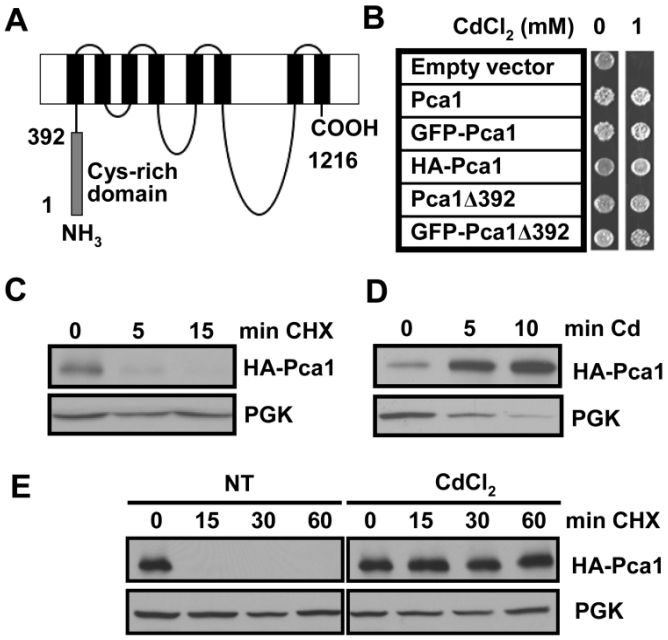

Figure 3.1. Cadmium rapidly enhances Pca1 expression by increasing stability. *A,* Schematic depiction of the Pca1 P_{1B}-type ATPase. Gray and black rectangles represent the N-terminal cysteine-rich cytosolic domain encompassing the first 392 amino acids and eight predicted transmembrane domains, respectively. *B,* Cadmium resistance in cells expressing Pca1 alleles. Pca1, N-terminal GFP-fused Pca1 (GFP-Pca1), three tandem HA epitope-tagged Pca1 (HA-Pca1), and Pca1 deleted of the N-terminal cysteine-rich domain (Pca1Δ392) with or without N-terminal GFP fusion were expressed in a BY4741 yeast strain in which endogenous Pca1 is non-functional (30). Cells (5 µl, OD_{600} 1.0) were spotted on SC solid media containing the indicated concentration of $CdCl_2$, and then cell growth was assessed after two days. *C*, *D*, and *E*, HA-Pca1 expression was detected by Western blotting using anti-HA antibodies. The same blot was probed for PGK to determine equal loading. *C,* Cycloheximide chase of yeast cells expressing HA-Pca1. Exponentially growing cells were co-cultured with cycloheximide (100 µg/mL) and then collected at the indicated time points for Western blot analysis. *D,* Rapid up-regulation of Pca1 in response to cadmium. Cadmium (50 µM $CdCl_2$) was added to exponentially growing yeast cultures, and then cells were collected into ice-cold kill buffer (15 mM NaN3 PBS) at the indicated time points. *E,* Cadmium-induced stabilization of Pca1 determined by cycloheximide chase. Yeast cells pre-cultured for 15 min without (NT) or with cadmium (20 µM $CdCl_2$) were collected at the indicated time points. Cycloheximide (100 µg/mL) was added to the culture media after collection of time zero. Total protein extracts were subject to Western blot analysis.

endosome where they can be recycled back to the surface or are delivered to the vacuole for degradation (40,41). We considered that Pca1 might be transiently localized at the cell surface before being rapidly internalized and degraded. In this pattern of trafficking, cadmium sensing would shift the distribution of Pca1 to the cell surface for cadmium efflux. If the internalization step were to be blocked then Pca1 would be expected to accumulate at the plasma membrane in the absence of cadmium. To test this possibility, we investigated the subcellular localization of Pca1 fused with GFP (Fig. 3.1B) in a strain harboring a temperature-sensitive allele of *END4*. This strain exhibits conditional defects in both fluid phase and receptor-mediated endocytosis at the restrictive temperature (31). Confocal microscopy revealed that GFP-Pca1 expressed in the *end4-1* mutant at the restrictive temperature did not accumulate at the plasma membrane (data not shown) and remained unstable as demonstrated by cycloheximide chase (Fig. 3.2A). The defect in endocytosis was confirmed by inhibition of internalization of the membrane impermeable dye, Lucifer Yellow CH (31) in the *end4-1* strain at the restrictive temperature (data not shown). These results suggest that Pca1 turnover in the absence of cadmium occurs either at the plasma membrane or before sorting to the plasma membrane.

3.4.3. Pca1 Degradation Is Dependent on the Proteasome But Not Vacuolar Proteases. Plasma membrane proteins can be directly sorted to the vacuole for degradation. For instance the Gap1 amino acid permease is directly targeted to the vacuole when preferred nitrogen sources are available (42). Perhaps, in an analogous manner Pca1 may sense cadmium before trafficking to the plasma membrane or is otherwise degraded by the vacuole. To test this, we examined localization and turnover

rate of Pca1 in a Δ*pep4* strain that is deficient in vacuolar protease activity (43). GFP-Pca1 did not accumulate in the vacuole of the Δ*pep4* strain, which was distinct from Fet3-GFP, a cell surface iron oxidase, previously shown to undergo vacuolar degradation (44) (data not shown). A cycloheximide chase confirmed that Pca1 undergoes degradation at a similar rate in the Δ*pep4* background compared to an isogenic wild-type strain (Fig. 3.2B), suggesting no significant role for vacuolar proteases in degradation of Pca1. The dependence of Pca1 degradation through the vacuolar/endosomal pathway was further examined in a Δ*vps4* null mutant, which is defective in both anterograde (late endosome-to-vacuole) and retrograde (late endosome-to-Golgi) trafficking. Resident proteins of the late endosome are typically trapped in pre-vacuolar compartments/multivesicular bodies in *vps4* mutants. As shown in figure 3.2C, GFP-Pca1 does not accumulate in such compartments, unlike Fet4-GFP. We conclude that Pca1 degradation is not dependent on the vacuolar degradation pathway.

Given that Pca1 turnover is not dependent on vacuolar proteases, we questioned whether the rapid degradation of Pca1 was dependent upon the proteasome. Indeed, treatment of cells with the protease inhibitor MG132 led to a significant increase in Pca1 expression compared to vehicle-treated controls (Fig. 3.2D). This experiment was performed in a Δ*pdr5* strain, previously shown to limit the efflux of protease inhibitors (34). We also examined the steady state expression of Pca1 in a *pre1-1 pre2-1* double mutant, which is defective in proteasomal activity (33). Consistently, Pca1 is stabilized in this strain although to a much lesser extent, which is likely due to some remaining proteolytic activities in the *pre1-1pre2-1* strain that participates in Pca1 degradation (Fig.

3.2D). Consistent to the fact that ubiquitination is a pre-requisite for proteasomal targeting, ubiquitination of Pca1 was readily detectible (absence of ubiquitin ladder (Fig. 3.2F). However, it is diminished to non-detectable levels after cells are exposed to cadmium (Fig. 3.2F). This observation hints toward a mechanistic explanation for cadmium's role in preventing the degradation of its own exporter. Rsp5 is an E3 ubiquitin-protein ligase that is known to be involved in regulation of several plasma membrane proteins, such as metal dependent down regulation of the Znt1 zinc transporter and Smf1 manganese transporter (40). However, an Rsp5-deficient strain did not stabilize Pca1 (data not shown), suggesting no role for this ubiquitin-protein ligase in Pca1 turnover.

To exclude the possibility that stabilization of Pca1 by cadmium may reflect toxic effects of cadmium on the cellular systems for ubiquitination and proteasomal degradation, we tested the effects of cadmium on the expression of GFP-CL1 that has been utilized as a reporter for determining the activities of the ubiquitination-proteasome pathway (45). As shown in Fig. 3.2G, cadmium exposure at the same concentrations that markedly stabilize Pca1 (Fig. 3.1E) had no significant influence on the expression levels of GFP-CL1. Thus, cadmium's inhibition of proteasomal degradation of Pca1 is specific. Our previous data also showed that Pca1 protein expression is enhanced when yeast cells are exposed to cadmium as low as 1 μM concentrations (30) which are similar to those found in plants where natural yeast cells grow (6).

3.4.4. The Cysteine-rich Cytosolic Extension Contains a Degradation Signal. We postulated that Pca1's cysteine-rich N-terminal extension might contain a metal-

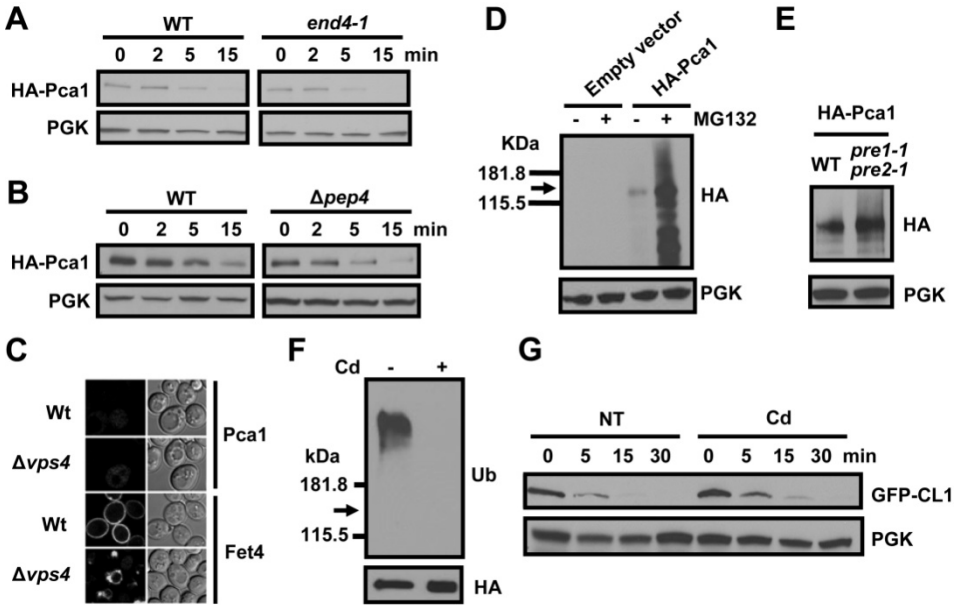

Figure 3.2. Proteasome-dependent degradation of Pca1 and prevention of ubiquitination by cadmium. *A,* Cycloheximide chase and Western blotting of HA-Pca1 expressed in *end4-1* strain and isogenic parental strain (WT). Cells were cultured at 37 ^{o}C to block endocytosis and collected at the indicated time points for Western blot analysis. *B,* Cycloheximide chase of HA-Pca1 expressed in Δ*pep4* strain and isogenic parental (WT) strain. *C,* Confocal imaging of GFP-Pca1 or Fet4-GFP expressed in Δ*vps4* or isogenic parental strain. *D*, Empty vector or HA-Pca1 was expressed in Δ*pdr5* cells in which proteasome inhibitor efflux is reduced (34). Cells were cultured in the presence (+) or absence (-) of proteasome inhibitor MG132 (40 μM, 5 hrs). Total protein extracts were subject to Western blot analysis using anti-HA antibodies. *E,* HA-Pca1 was expressed in a temperature-sensitive *pre1-1pre2-1* proteasome defective mutant or isogenic parental strain (WT) at restrictive temperature (37^{o}C) for two hours. Relative expression of HA-Pca1 was detected by anti-HA immunoblotting. *F,* Cadmium inhibits Pca1 ubiquitination. Cells expressing HA-Pca1 were cultured with (+) or without (-) cadmium (50 μM $CdCl_2$, 2 hrs). HA-Pca1 in total protein extracts was immunoprecipitated with beads conjugated with anti-HA antibodies. HA-Pca1 and ubiquitin-attached HA-Pca1 were detected by immunoblotting using anti-ubiquitin (Ub) and anti-HA antibodies. Arrow indicates the expected migration of non-ubiquitinated HA-Pca1. *G,* Cadmium does not stabilize a model substrate of the ubiquitination-proteasome degradation pathway. Expression levels of GFP-CL1 were detected in cells pre-cultured for 1 hr without (NT) or with cadmium (50 μM $CdCl_2$). Cycloheximide (100μg/mL) was added to the culture media after collection of time zero. Total protein extracts prepared from cells collected at the indicated time points were subject to Western blot analysis using anti-GFP antibodies. PGK was probed to determine equal loading.

absence of copper or cadmium, deletion of this domain (Pca1Δ392) resulted in constitutive expression at the cell surface even in the absence of cadmium (Fig. 3.3A and 3.3B). These data strongly suggest that Pca1 turnover occurs via this domain. Consistent with the idea that Pca1 ubiquitination is dependent on a degradation signal within the N-terminal domain, ubiquitination was not detected in Pca1Δ392 (Fig. 3.3C). While the Pca1(1-392) domain is necessary for Pca1 turnover, co-expression of this domain with Pca1Δ392 separately did not lead to enhanced turnover of Pca1Δ392 (data not shown). This suggests that in order to serve as a degradation signal for Pca1 this domain should be associated with the entire protein.

To determine whether the N-terminal regulatory domain is sufficient for controlling the expression of another plasma membrane transporter, we fused the peptide comprising amino acid residues 1 to 392 of Pca1 to the N-terminus of a copper-transporting P_{1B}-type ATPase (CaCRP) identified from *C. albicans* (46,47) and then expressed in *S. cerevisiae*. The Pca1(1-392)-CaCRP fusion was fully functional in conferring copper tolerance to yeast cells indicating correct folding and trafficking (Fig. 3.4A). Confocal microscopy (Fig. 3.4B) and Western blot analysis (Fig. 3.4C) demonstrated that residues 1 to 392 in Pca1 control CaCRP expression specifically in a cadmium and copper dependent manner. Hence, this domain functions as an autonomous degradation signal that can be masked when cells are growing in media supplemented with cadmium or copper.

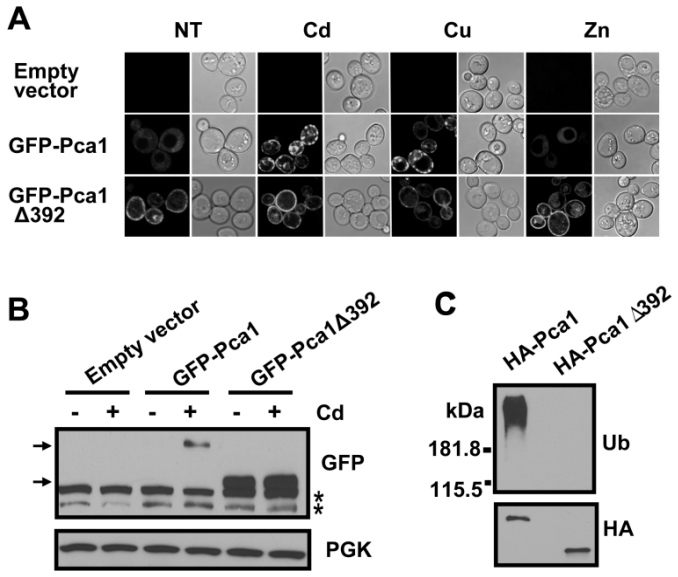

Figure 3.3. The cysteine-rich N-terminus of Pca1 contains a degradation signal. *A,* Metal-dependent expression of GFP-Pca1 or GFP-Pca1Δ392. Cells expressing GFP-Pca1 or GFP-Pca1Δ392 were cultured in the absence (NT) or presence of metal ions (50 μM $CuCl_2$, 50 μM $CdCl_2$, or 15 mM $ZnCl_2$ for 1 hr). GFP fluorescence in cells was visualized by confocal microscopy. Copper sensitive Δ*ace1* strain (24) was used for these experiments presented in both *A* and *B*. *B,* Western blotting of GFP-Pca1 and GFP-Pca1Δ392 in cells cultured with (+) or without (-) cadmium (50 μM $CdCl_2$ for 2 hrs) using anti-GFP antibodies. Asterisk indicates non-specific bands. Top arrow indicates full-length GFP-Pca1 and bottom arrow indicates GFP-Pca1Δ392. *C,* Ubiquitination of Pca1 is dependent on the N-terminal domain. HA-Pca1 and HA-Pca1Δ392 were immunoprecipitated and then detected by Western blotting using anti-ubiquitin (Ub) and anti-HA antibodies.

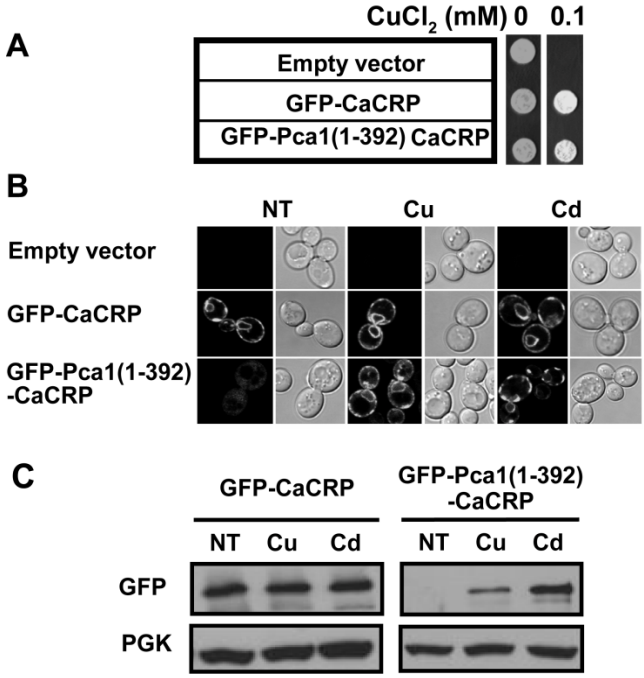

Figure 3.4. The N-terminal cytosolic extension of Pca1 is an auto-regulatory domain that can be masked by cadmium and copper. *A,* Yeast cells expressing GFP-tagged wild-type CaCRP or CaCRP fused with the first 392 amino acids of the N-terminus of Pca1 (Pca1(1-392)) were spotted on solid media containing toxic copper ($CuCl_2$, 0.1 mM). *B,* Expression of GFP-CaCRP and GFP-Pca1(1-392)-CaCRP in cells cultured with $CuCl_2$ or $CdCl_2$ (50 μM, 2 hrs) were determined by visualization of GFP fluorescence with confocal microscopy. *C,* Western blot analysis of cells cultured as described above (*B*) using anti-GFP antibodies. A copper sensitive Δ*ace1* strain (24) was used for these experiments.

We aligned this domain with characterized P_{1B}-type ATPases and other proteins. However, the Pca1 N-terminal domain does not possess any significant sequence similarity to known proteins. Therefore, we employed a mapping strategy to define a signal sequence of metal-dependent degradation. Serial deletions of the N-terminus and fusion of various Pca1 N-terminal fragments to CaCRP were made (Fig. 3.5A) and then expressed in yeast cells. Characterization of expression levels and metal-dependent stabilization using confocal microscopy revealed that a region encompassing residues 250 to 350 (Pca1(250-350)) is necessary and sufficient for both rapid turnover and cadmium or copper- dependent stabilization (Fig. 3.5A and 3.5B). Since the Pca1(250-350) degradation signal likely interacts with cellular factor(s) necessary for Pca1 degradation, we hypothesized that excess expression of this domain would compete for degradation of full-length Pca1. Indeed, co-expression of the Pca1(250-350) peptide with Pca1 leads to enhanced expression of Pca1 (Fig. 3.5C). Collectively, these data indicate that this Pca1(250-350) contains a degradation signal that can be masked by metal sensing.

3.4.5. Cysteine Residues within the Pca1 Regulatory Domain Are Required for Metal Sensing. This 250-350 amino acid peptide contains seven cysteine residues (Fig. 3.6A). Given that cysteine is a known metal ligand, we predicted metal ion binding to this domain. A Pca1(250-350) peptide tagged with three tandem copies of HA epitope (HA-Pca1(250-350)) was expressed in Δ*ace1* or Δ*ycf1* strains that are sensitive to copper or cadmium, respectively (24,25). We hypothesized that this peptide would bind and sequester metal ions like metallothionein (20), which would confer resistance to copper and cadmium. Growth assays on media containing copper or cadmium at toxic levels

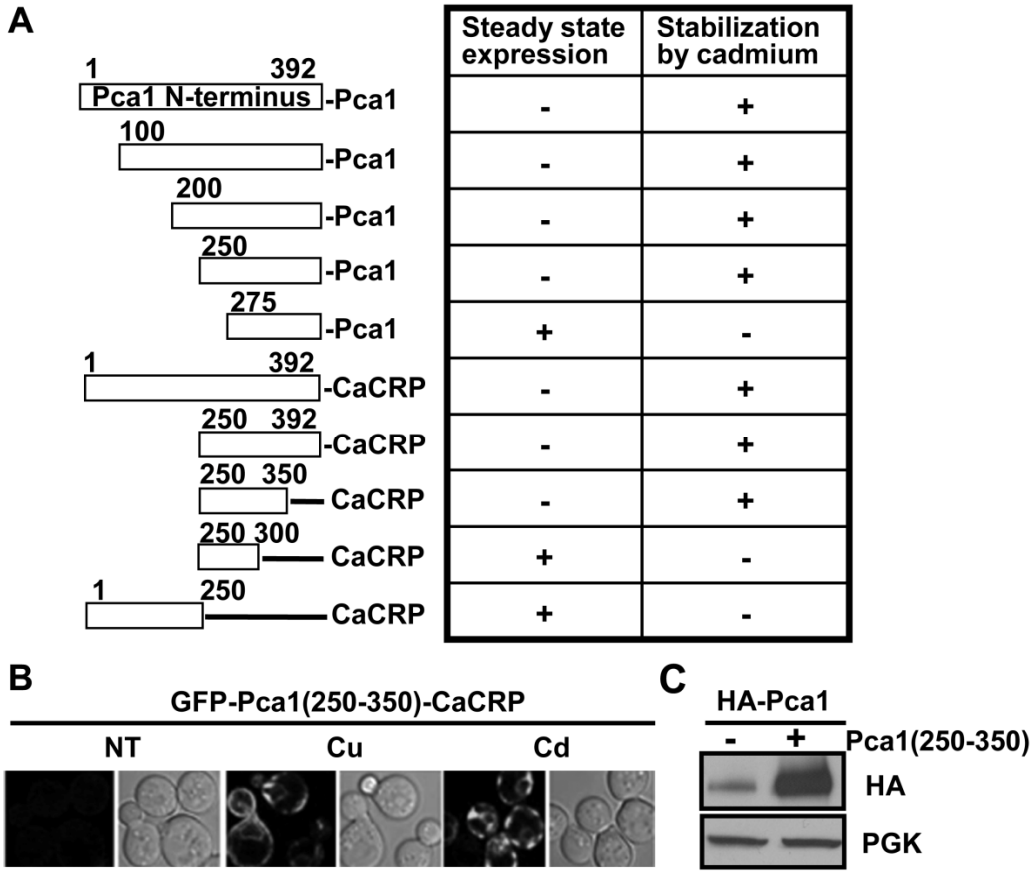

Figure 3.5. The N-terminal domain encompassing amino acids 250 to 350 is necessary and sufficient for the control of Pca1 and CaCRP expression in a metal-dependent manner. *A,* Pca1 with or without N-terminal truncations and Pca1 N-terminal fragments fused with CaCRP were expressed in yeast. A GFP was tagged in frame at the N-terminus of these expression constructs for visualization. Steady state GFP fluorescence in cells growing exponentially was scored as weak (-) or strong (+) by confocal microscopy. Cadmium (50 μM $CdCl_2$ for 2 hrs) dependent enhancement (+) of GFP fluorescence was determined. *B,* Confocal microscopy of GFP-Pca1(250-350)-CaCRP fusion in cells cultured in the absence (NT) or presence of metals (50 μM $CuCl_2$ or $CdCl_2$ for 2 hrs). *C,* Determination of HA-Pca1 expression levels in cells with (+) or without (-) co-expression of the Pca1(250-350) peptide. HA-Pca1 was detected by immunoblotting with anti-HA antibodies. PGK in the same blot was probed as a loading control. A copper sensitive Δ*ace1* strain (24) was used for these experiments.

showed that cells expressing HA-Pca1(250-350) displayed cadmium and copper resistance compared with empty vector transformed cells (Fig. 3.6B). However, this peptide did not confer zinc tolerance in a zinc-sensitive strain (data not shown). These data are in accordance with that both cadmium and copper, but not zinc, stabilized Pca1 (Fig. 3.3A) and CaCRP fused with this regulatory domain (data not shown). The same peptide in which all cysteine residues are substituted to alanine (ΔCys), did not confer metal resistance (Fig. 3.6B), supporting roles for cysteine residues in metal binding. To ensure equal or greater levels relative to the wild-type peptide (Fig. 3.6D), we expressed HA-Pca1(250-350)ΔCys peptide using a high-copy plasmid.

We next examined which cysteine residues within this domain play roles for conferring resistance to cadmium and/or copper. Site-directed mutation of the CxC motif (C298A, C300A) abolished copper resistance but not cadmium resistance (Fig. 3.6C). Conversely, mutation of the CC motif (C311A, C312A) abolished cadmium resistance yet retained the ability to confer copper resistance (Fig. 3.6C). These data support distinct roles for these cysteine residues in copper or cadmium coordination.

We predicted that cysteine mutations would compromise metal-dependent masking of the degradation signal. Indeed, cadmium or copper could not stabilize CaCRP fused with HA epitope-tagged Pca1(250-350) where all seven cysteine residues were substituted to alanine (ΔCys) (Fig. 3.6E upper panel, fifth and sixth lanes). However, steady state expression levels without metal addition to culture media (NT) were not significantly different between control (WT) and cysteine mutant (ΔCys) (Fig. 3.6E upper panel, first lane versus fourth lane) suggesting that cysteine residues are specifically

necessary for cadmium or copper-induced stabilization but not turnover. Collectively, these data suggest that cysteine-based metal sensing within a *cis*-acting degradation signal serves as a molecular switch for rapid up-regulation of Pca1.

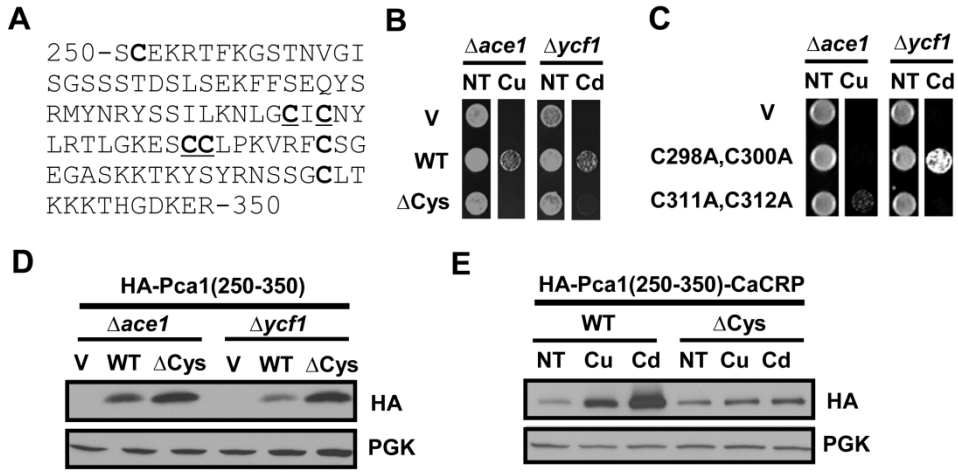

Figure 3.6. Cysteine residues within the regulatory domain of Pca1 are required for metal sensing. *A,* Sequence of a metal-responsive degradation signal encompassing amino acids 250 to 350 in the N-terminus of Pca1. Seven cysteine (C) residues are in boldface. C298, C300, C311, and C312 are underlined. *B,* Empty vector (V), a single-copy expression vector of N-terminal triple HA-tagged Pca1(250-350) (HA-Pca1(250-350), WT), and a multi-copy expression vector of HA-Pca1(250-350) with substitution of all seven cysteine residues to alanine (ΔCys) were transformed into copper sensitive Δ*ace1* (24) and cadmium sensitive Δ*ycf1* (25) strains. Cells growing exponentially were spotted on SC media without (NT) or with supplementation of toxic copper or cadmium (50 μM $CuCl_2$ or $CdCl_2$). Cell growth was assayed after two days. *C,* Empty vector (V) and an expression vector of HA-Pca1(250-350) possessing specific cysteine mutations as indicated were transformed into cells. Cell growth was determined as described above (*B*). *D,* Western blotting of the HA-Pca1(250-350) peptides in total protein extracts of cells described above (*B*) with anti-HA antibodies. PGK was detected to determine equal loading. *E,* CaCRP fused with HA-Pca1(250-350) at the N-terminus without (WT) or with site-directed mutagenesis of all seven cysteine residues to alanine (ΔCys) were expressed in a Δ*ace1* strain. Total protein extracts of cells cultured without (NT) or with copper or cadmium (20 μM $CuCl_2$ or $CdCl_2$ for 1 hr) were subject to Western blot analysis using anti-HA and anti-PGK antibodies.

3.5. Discussion

Our data reveal a new mode of expressional control of a metal-transporting P_{1B}-type ATPase. In the absence of cadmium, Pca1 is rapidly targeted for degradation at the proteasome via an N-terminal degradation signal. However, cadmium sensing allows Pca1 to circumvent degradation, which is likely attributed to conformational changes within the degradation signal induced by metal binding. Hence, cells are able to rapidly express Pca1 at the cell surface only when Pca1 is required for cadmium efflux.

Since cadmium is extremely toxic and cadmium levels in the environment fluctuate, transcriptional or translational regulation of a cadmium detoxification system may not be fast enough for cell protection. The constant synthesis of Pca1 would be an obvious advantage in coping with this problem. The underlying reason for rapid degradation of Pca1 could be explained by the fact that Pca1 expression is not necessary when cells are growing without cadmium stress. However, other factors may be important for keeping Pca1 levels at a minimum. For example, Pca1 could play another role other than cadmium efflux (e.g., efflux of nutritional metals), which would be deleterious in the absence of cadmium. Indeed, it was shown that cells over-expressing Pca1 grow slowly (48). If Pca1 transports nutritional metal ions due to broad substrate specificity, as shown for several other metal-transporting P-type ATPases (26-28), expression of Pca1 only when cadmium is in excess could prevent loss of essential metals that are limiting for growth. While our previous data showed that Pca1 does not change cellular accumulation of zinc, manganese, cobalt, or iron (30), Pca1 may transport these or other metal ions under specific growth conditions. Secondly, it is not known what

form(s) of cadmium and other metals are substrates of P_{1B}-type ATPases. Ycf1, a vacuolar cadmium ABC transporter in yeast, transports both glutathione-conjugated cadmium and glutathione itself (25,49). A recent report showed that cysteine dramatically increases the activities of CopA, ZntA and HMA2 P_{1B}-type ATPases (50). Pca1 may extrude cysteine, glutathione, or other ions such as sulfur with or without conjugation of cadmium. If so, constitutive extrusion of these cellular factors would be deleterious, since they are critical for redox homeostasis and metabolism (51,52). Alternatively, cysteine or other cytosolic molecules may activate Pca1 as shown for ZntA and HMA2 (50), resulting in constitutive ATP hydrolysis in the absence of cadmium.

Internalization followed by degradation at the vacuole is a major pathway for turnover of plasma membrane proteins. For example, Zrt1 zinc transporter, Fet3/Ftr1 iron transporter complex, and Smf1 manganese transporter are subject to this mode of posttranslational regulation (40,44,53,54). Given that these transporters play roles in metal ion uptake, they are down regulated when cells are exposed to excess metal ions. Substrate-dependent ubiquitination of membrane transporters is often a signal that triggers turnover of these transporters (40). However, Pca1 regulation by cadmium is unique when compared with these other metal ion transporters in that it is rapidly up-regulated by cadmium-mediated rescuing from degradation. Moreover, degradation of Pca1 occurs at the proteasome in an endocytosis-independent manner. It is interesting to note that cellular trafficking of Arn1, a transporter of an iron-siderophore complex, is regulated by its substrate at several steps. Substrate sensing triggers plasma membrane sorting of Arn1, and its cycling between the plasma membrane and endosomes is induced

by substrate binding at the cell surface (55,56). Pca1 sorting to the cell surface versus degradation at the proteasome may be controlled by a similar mechanism as characterized for Arn1. We are currently pursuing the identification of molecular factors that are involved in Pca1 turnover and the subcellular compartments where cells determine the fate of Pca1.

Most P_{1B}-type ATPases possess N-terminal metal binding domains (MBDs) that are thought to play roles in catalysis and/or regulation. The conserved MBD (~70 amino acids, βαββαβ fold) contains a GMXCXXC sequence in which two cysteine residues coordinate a metal ion (3,29). While this domain is conserved in many P_{1B}-type ATPases, the numbers of it vary, ranging from one to six. The N-terminus of Pca1 contains only one conserved MBD in which two cysteine residues are essential for function (30). Human copper transporting ATPases possess six MBDs at the N-terminus. The Atx1 metallochaperone delivers copper through direct docking to MDBs with different preferences among them (57-59), suggesting distinct functions of these domains. The structure and metal coordination of MBDs are relatively well characterized; however, the roles for these domains in ATPase function are not yet clear. Copper binding was initially proposed as a substrate recognition step followed by transfer of metal to the translocation pore. However, site-directed mutagenesis and deletion of MBDs suggest a regulatory role. For example, the 5^{th} or 6^{th} MDB of human ATP7a is necessary and sufficient for copper-induced redistribution (60). An inhibitory role of the N-terminus of a copper transporting ATPase has been proposed as well (61). Interestingly, a recent report (62) suggested the presence of either additional regulatory copper-binding site(s) within the

protein or another unknown mechanism for copper sensing. Collectively, while significant progress in the characterization of conserved MBDs in N-terminus of P_{1B}-type ATPases has been made, the functional roles of these domains remain elusive.

We have identified a distinct MBD in the N-terminus of Pca1 that is necessary for extremely efficient control of Pca1 expression in response to cadmium. Several atypical MBDs have been identified in other P_{1B}-type ATPases as well. The core sequence of N-terminal MBD's of *A. thaliana* HMA2-4 is substituted by a degenerate sequence, GICC(T/S)SE (63). HMA2 and HMA4 zinc ATPases possess C-terminal MBDs (several di-cysteine pairs and an eleven histidine stretch) that regulate enzyme activities (63,64). A CHHC predicted metal binding sequence was identified at the C-terminus of the CopA copper ATPase in *Archaeoglobus fulgidus* (65). The histidine-rich N-terminal metal binding domain of the CopB copper ATPase in *A. fulgidus* appears to have a regulatory role affecting metal transport rates by controlling metal release (66). In addition to heavy metal-transporting P_{1B}-type ATPases, it was also shown that Ca^{2+}-transporting P-type ATPases carry inhibitory domains (67). Cytosolic regulators and phosphorylation of C-terminal residues control activities of H^{+}-transporting ATPases in plants (68). Hence, auto-regulatory mechanisms may be widespread in P-type ATPases.

We propose that direct metal binding to the Pca1(250-350) domain induces conformational changes that would mask a signal recognized by the protein degradation machinery. Expression of Pca1(250-350) itself confers both cadmium and copper tolerance, which supports the conclusion that this domain directly binds metals. Substitution of cysteine residues within this domain abolished metal resistance suggesting

a role for cysteines in metal coordination. Site-directed mutation of cysteine residues suggests that binding site(s) of cadmium and copper do not overlap directly. Given that Pca1 does not transport copper (30), the physiological significance of the stabilization of Pca1 by copper is not obvious at this moment. It is necessary to point out that in a copper-sensitive Δ*ace1* strain Pca1 stabilization by copper is observed at similar concentrations to that of cadmium; however, in wild-type cells relatively high copper concentration (~2 mM) is necessary for Pca1 stabilization (data not shown). *CUP1* and *CRS5*-encoded metal chelators that are up-regulated by Ace1 likely have higher affinity to copper than the N-terminus of Pca1.

Fusion of the Pca1(250-350) domain with a stable protein leads to a rapid turnover and metal-dependent stabilization, demonstrating a role as a autonomous and metal-responsive degradation signal. However, this domain does not carry significant sequence identity with characterized degradation signals or known proteins. Given that Pca1(250-350) is located in the middle of the N-terminus, the N-end rule pathway of protein degradation is unlikely involved in Pca1 degradation (69). Pro, Glu, Ser, and Thr amino acid enriched sequence (PEST sequence) is a proteolytic signal (70). However, Pca1 250-350 is not predicted to contain a PEST signal (https;//embl.bcc.univie.ac.at/). It has been proposed that exposure of hydrophobic residues could recruit molecular chaperones, which results in re-folding to a functional protein or targeting for degradation. Structural characterization of this domain in apo and cadmium-bound forms would reveal what characteristics lead to degradation of Pca1 and how metal binding masks this degradation signal. We are currently pursuing characterizations of structure

and metal binding of this domain in detail; however, purification of ample quantities of the Pca1(250-350) peptide has been a technical challenge (Adle and Lee, unpublished data).

It is interesting to note that the N-terminus of a human copper ATPase interacts with several proteins, although the physiological significance of these interactions remains to be elucidated. For example, MURR1/COMMD1 binds with the N-terminus of ATP7b, which decreases stability of newly synthesized ATP7b (71). A hepatocytic isoform of PLZF (promyelocytic leukemia zinc finger) protein interacts with the C-terminus of ATP7b, which positively regulates ERK signal transduction (72). Glutaredoxin interacts with the N-terminus of ATP7a and ATP7b (76), and the dynactin subunit p62 interacts with the N-terminus of ATP7b (73). These protein-protein interactions may play critical roles for the function, regulation of catalytic activities, and/or subcellular trafficking of ATP7b and possibly other P_{1B}-type ATPases. The regulatory domain identified at the N-terminus of Pca1 may interact with accessory protein(s) that target Pca1 to the ubiquitin-proteasome pathway.

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Chapter 4

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

4.1. Abstract

Cadmium is a highly toxic environmental contaminant implicated in various disorders. A major mechanism for cadmium detoxification in yeast *Saccharomyces cerevisiae* relies on extrusion via Pca1, a P-type ATPase. While an N-terminal degron targets Pca1 for degradation before its secretion to the plasma membrane, cadmium in the growth media rapidly up regulates Pca1 by preventing its turnover. Here we show that the endoplasmic reticulum-associated degradation (ERAD) system, known for its role in protein quality control of secretory proteins, is unexpectedly responsible for the regulation of Pca1 expression by cadmium. Direct cadmium sensing at the ER leads to conformational changes of this degron, which suggests how recognition of Pca1 for ERAD is prevented. This regulated conversion of an ERAD substrate to a secretory competent state in response to a cellular need illustrates a novel mechanism for expressional control of a plasma membrane protein. Yeast has likely evolved this mode of regulation for a rapid response against cadmium toxicity at the expense of constant synthesis and degradation of Pca1. ERAD of a portion of secretory proteins might occur via signal-dependent regulatory mechanisms as demonstrated for Pca1.

4.2. Introduction

Metal ions are highly toxic, although several metals such as iron, copper and zinc are essential micronutrients. Organisms possess delicate systems for detoxification and excretion of nonphysiological metals and homeostatic metabolism of nutritional yet toxic metals (1,2). Pca1 in *S. cerevisiae* is a multi-spanning transmembrane protein that belongs to the family of P_{1B}-type ATPase heavy metal transporters widely distributed from bacteria to humans (3). Pca1 functions in the efflux of cadmium across the plasma membrane (4), an extremely toxic environmental pollutant that causes various human diseases, such as cancer, kidney disease and endocrine disruption (5).

We have previously demonstrated that Pca1 is a short-lived protein that is targeted for ubiquitination and proteasomal degradation before reaching the cell surface (6). However, in the presence of cadmium, Pca1 is rapidly up regulated due to the prevention of its degradation (6). An autonomous degron encompassing amino acid residues 250-350 within the N-terminal cytosolic domain is necessary and sufficient for both degradation and metal sensing (6). Given that the cell surface expression of several plasma membrane proteins is regulated by ubiquitin-mediated endocytosis followed by vacuolar degradation, Pca1 turnover and cadmium-responsive degradation represents an interesting mode of expressional control in which subcellular trafficking and stability are determined by its substrate during secretion.

To uncover molecular factors involved in the expressional control of Pca1, we have devised a genetic screen to identify mutants that are defective in Pca1 degradation in the absence of cadmium. Unexpectedly, our data presented herein demonstrate that

components of the ER associated degradation (ERAD) system target Pca1 for proteasomal degradation.

Secretory proteins in eukaryotes are inserted into the ER lumen or membrane where correct folding and maturation occurs. Numerous factors, such as molecular chaperones, glycosylation enzymes, and protein disulfide isomerases, are involved in this process. Genetic mutations and environmental stresses (e.g., heat, oxidative stress) enhance the probability of failure of protein folding and maturation. The unfolded protein response (UPR) pathway enhances the capability of protein folding when cells are challenged by these stresses (7). The ERAD system eliminates terminally misfolded or unassembled proteins, which is critical for the prevention of toxic accumulation of aberrant proteins (8, 9). Several mutant proteins, unassembled subunits of secretory proteins, and heterologously expressed proteins have all been identified as ERAD substrates (8, 9). However, Pca1 is a naturally expressed and functional protein and there is no evidence of multimer assembly for pca1.

Cadmium is extremely toxic and its levels fluctuate in the environment. Thus, transcriptional and/or translational control of defense factors may not be fast enough for cell protection. Our results suggest that yeast cells have evolved to harness the ERAD system for rapid up-regulation of Pca1 cell surface expression by direct sensing of intracellular cadmium at the ER.

4.3. Experimental Procedures

4.3.1. Yeast strains, media and growth conditions. Yeast strains used in this study are listed in Table 4S1. Most strains are from the Saccharomyces Genome Deletion Project (10) which was purchased from Open Biosystems. Double knockouts were generated by PCR-based gene deletion using pFA6a-His3MX6 as a template for homologous recombination (11). Diagnostic PCR using gene-specific primer sets confirmed correct replacement of target genes by the His3MX6 cassette. Yeast cells were cultured in YPD or synthetic complete (SC) media as previously described (4).

4.3.2. Construction of expression plasmids and transformation. A single copy yeast vector was used for glyceraldehyde-3-phosphate dehydrogenase (GPD) gene promoter-mediated constitutive expression unless specified (12). For the generation of expression plasmids of green fluorescent protein (GFP) or triple hemagglutinin (HA) epitope-fusion proteins, NotI restriction enzyme site-flanked GFP or three tandem HA epitopes were inserted into a NotI site artificially generated at the N or C-terminal end. A plasmid containing four tandem copies of the 22bp Unfolded Protein Response Element (UPRE) inserted upstream of a disabled CYC1 promoter lacZ gene fusion (13) was kindly provided by Dr. Peter Walter (University of California, School of Medicine, San Francisco, California). A Ste6* expression plasmid (14) was generously provided by Dr. Susan Michaelis (John Hopkins School of Medicine, Baltimore, Maryland). Site-directed mutagenesis was conducted by the primer overlap extension method (15). Plasmids were transformed into yeast using the lithium acetate procedure (16). Yeast cells were grown at 30°C on synthetic complete (SC) media for plasmid selection.

Table 4S.1. Yeast *Saccharomyces cerevisiae* strains used in this study.

Strain	Genotype	Reference
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems
BY4741 Δcue1	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 cue1::KanMX*	Open Biosystems
BY4741 Δdoa10	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 doa10::KanMX*	Open Biosystems
BY4741 Δhrd1	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hrd1::KanMX*	Open Biosystems
BY4741Δhrd1 Δdoa10	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hrd1::KanMX doa10::HIS3*	This study
BY4741 Δubc6	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ubc6::HIS3*	(42)
BY4741 Δubc7	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ubc7::KanMX*	Open Biosystems
BY4741 Δubc6 Δubc7	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ubc6::HIS3 ubc7::NatMX*	(42)
SEC23	*MATα ura3-52 leu2-3,-112*	(25)
sec23-1	*MATα ura3-52 his4-619 sec23-1*	(25)
CDC48	*MATa ura3-52 his3Δ200 leu2Δ1 trpΔ63*	(43)
cdc48-1	*MATa ura3-52 his3Δ200 leu2Δ1 trpΔ63 cdc48-3*	(43)
W303-1b	*MATα ade2 his3 leu2 ura3 trp1 can1-100*	(19)
KNY111	*MATα ade2 his3 leu2 ura3 trp1 can1-100 DOA10-13MYC-KANMX*	(19)

4.3.3. Selection of yeast mutants constitutively expressing Pca1. A functional Pca1 fused with green fluorescent protein (GFP) at the N-terminus was expressed in a yeast *S. cerevisiae* deletion pool (Open Biosystems) containing individual deletions of all nonessential genes. Highly fluorescent cells (top 1% among the collected ~400,000 transformants) were sorted by flow cytometry. Positive candidates were confirmed by confocal microscopy and gene knockouts were identified using specific primer sets for PCR amplification and sequencing of a unique 20 base "tag" sequence (10).

4.3.4. Preparation of cell extracts and immunoblotting. All immunoblotting was performed essentially as described previously (4). Briefly, total protein extracts were prepared by vortexing yeast cells with glass beads in PBS containing protease inhibitor cocktail (Complete Mini) (Roche) and 1% triton X-100. Lysates were denatured in SDS sample buffer containing 25mM DTT for 15min at 37°C and resolved by SDS-PAGE prior to transfer to nitrocellulose membrane and antibody probing.

4.3.5. Cycloheximide chase analysis. Protein synthesis was inhibited by the addition of 100 μg/mL cycloheximide to logarithmically growing cultures. Cells (A_{600}=15) were collected into equal volumes of ice-cold kill buffer (PBS containing 15 mM NaN_3) at the indicated time points. For zero time points, cells were collected before addition of cycloheximide. Cells were pelleted by centrifugation and stored at -80°C until preparation of cell extracts for SDS-PAGE and immunoblotting (6). Pca1 was detected by chemiluminescence using anti-HA antibodies and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibodies. Phosphoglycerate kinase (PGK) was probed as a loading control using anti-PGK antibodies and HRP-conjugated anti-mouse IgG

antibodies. Signals were quantified using Total Lab TL100 software and were normalized to PGK.

4.3.6. ^{64}Cu blotting and autoradiography. Hemagglutinin (HA) epitope-tagged Pca1(250-350) or Pca1(250-350)ΔCys where all cysteine residues were mutated to alanine were immunoprecipitated from Δ*doa10* cell lysates using anti-HA antibody-conjugated beads (Pierce). Samples were subjected to SDS-PAGE and electroblotted onto a nitrocellulose membrane. Membranes were equilibrated in metal binding buffer (17), probed with ~10 μCi of ^{64}Cu (~1μM $CuCl_2$) (Isotrace Technique) for 1hr, and then washed extensively prior to autoradiography and protein staining (MemCode) (Pierce). Competition with ^{64}Cu binding was determined in the presence of 5-fold excess of non-radioactive competitor ions.

4.3.7. Limited trypsin proteolysis of Pca1(250-350). Cytosolic fractions were obtained from Δ*doa10* cells expressing triple HA epitope-tagged Pca1(250-350) or Pca1(250-350)ΔCys cultured with or without cadmium (50μM $CdCl_2$, 1hr). Lysates were incubated with trypsin (Sigma) for 10min on ice before addition of 0.2μg/mL soybean trypsin inhibitor (Fluka BioChemika) for an additional 15min on ice. Proteolysis patterns were visualized by anti-HA immunoblotting.

4.3.8. β-Galactosidase reporter assay. Cells were assayed for β-galactosidase expression as described previously (18). Briefly, cells (A_{600}=1) were permeabilized with chloroform and 0.1% SDS in 1mL of Z buffer and incubated with 0.2 mL of 4 mg/mL ONPG (*ortho*-Nitrophenyl-β-galactoside) dissolved in Z buffer. Reactions were quenched

by adding 0.5 mL of Na_2CO_3. β-Galactosidase levels were converted to Miller units $[(A_{420})/(T_{min})(V_{mL})(A_{600})]$.

4.3.9. Microsome preparation and chemical cross-linking. Microsome preparation was performed as previously described with modifications (19). Cells (A_{600}=30) were disrupted by glass bead vortexing in 300µL lysis buffer (20mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 50mM potassium acetate (KoAc), 2mM ethylenediaminetetraacetic acid (EDTA**)**, 0.1M sorbitol, 1mM dithiothreitol (DTT), 1mM phenylmethanesulphonylfluoride (PMSF) and HALT protease inhibitor cocktail (Pierce) for ten 30sec intervals alternating with incubation on ice. An additional 250µL buffer 88 (20mM HEPES, 50mM KOAc, 250mM sorbitol and 5mM magnesium acetate (MgOAc) was added. Unbroken cells and glass beads were cleared by low speed centrifugation (3,000rpm for 5min) and then repeated with a second rinse with buffer 88. The supernatant fractions were pooled and centrifuged 18,000g for 20min at 4°C. The supernatant was decanted and the final microsomal membrane pellet was washed once and re-suspended in 150µL of 0.2M triethanolamine, pH 8.0 and incubated on ice for 1hr with 100µg/mL dimethyl 3,3'-dithiobispropionimidate (DTBP) a water soluble, membrane permeable and thiol reversible cross-linker. Cross-linking reactions were quenched by addition of 40uL of 1M Tris (pH 7.5) and incubated on ice for an additional 20min. Cell pellets were collected by centrifugation at 18,000g for 15min at 4°C and washed with 400µL PBS. For membrane solubilization, cell pellets were resuspended in 100µL of 1% SDS in PBS and incubated at 37°C for 30min. Triton X-100 was added for a total concentration of 1.5% (400µL total volume) and incubated on ice

for an additional 30min. From this lysate, HA-tagged proteins were immunoprecipitated with anti-HA antibody-conjugated sepharose beads (Pierce) overnight at 4°C according to manufacturer. Immobilized proteins were eluted with 2XSDS sample buffer at 37°C for 15min. Chemical cross-linking was reversed by incubation with 0.1M DTT for an additional 15min at 37°C before SDS-PAGE and immunoblotting.

4.3.10. Oligomycin resistance assay. Wild type and Δ*doa10* strains expressing empty vector, Yor1-GFP or Pca1(1-392)-Yor1-GFP were cultured in SC selective media to mid-log phase. Cells (~5 μL, A_{600}=0.1) were spotted on solid YPEG media (2% Bacto-Peptone, 1% yeast extract, 2% ethanol, 3% glycerol and 1.5% agar), prepared with or without the addition of cadmium (10μM $CdCl_2$) and oligomycin (2.5μg/mL). Plates were incubated at 30°C for two days before photography.

4.3.11. Immunoprecipitation and detection of ubiquitin. Cells were broken by glass beads vortexing in PBS containing protease inhibitors (Complete Mini) (Roche), 5mM N-ethylmaleimide, 1mM PMSF and 1% Triton X-100. HA epitope tagged proteins were immunoprecipitated with an HA-Tag IP/Co-IP kit (Pierce) according to manufacturer specifications. Proteins were eluted by incubation of immobilized anti-HA agarose beads in 2XSDS sample buffer at 37°C for 15min and reduced in the presence of 0.1M DTT for an additional 15min at 37°C. Ubiquitin conjugated proteins were detected by immunoblotting using mouse monoclonal antibodies against ubiquitin (Covance).

4.4. Results

4.4.1. Pca1 is targeted for ER-associated degradation in the absence of cadmium. In order to identify molecular factors involved in Pca1 turnover, we selected yeast mutants exhibiting defects in Pca1 degradation. An expression construct of a fully functional Pca1 fused with green fluorescent protein (GFP-Pca1) was transformed into a collection of mutant yeast strains, each lacking a single nonessential gene (10). Cells emitting strong GFP signal (as a consequence of a defect in degradation of the GFP-Pca1 fusion) were selected using a flow cytometer, and gene deletions resulting in high Pca1 expression were identified (Fig. 4.S1A).

Unexpectedly, Pca1 is highly expressed in cells with deletions of the *CUE1* gene (Fig. 4.S1B and 4.S1C). *CUE1* encodes an ER membrane-bound protein that recruits Ubc7, a cytosolic ubiquitin-conjugating enzyme, to the ER surface (20). Given that Cue1 is a crucial factor involved in ERAD, we further examined Pca1 stabilization in yeast strains bearing deletions in factors required for ERAD, including Ubc7 (21) and Ubc6 ubiquitin-conjugating enzymes (22), and ER resident Doa10 (23) and Hrd1 (24) ubiquitin ligases. In yeast, the recognition of integral membrane ERAD substrates is governed by the location of the misfolded domain. Lesions in soluble domains on the cytosolic face of the ER belong to the ERAD-C (cytoplasmic pathway) in which their ubiquitination and degradation are largely dependent on Doa10 (8,9). On the other hand, Hrd1 is involved in ubiquitination and degradation of substrates with misfolded regions in soluble luminal domains (ERAD-L) or within transmembrane domains (ERAD-M) (8,9). Pca1 stabilization was evident in Δ*ubc7* or Δ*doa10* but not Δ*hrd1* strains (Fig. 4.1A).

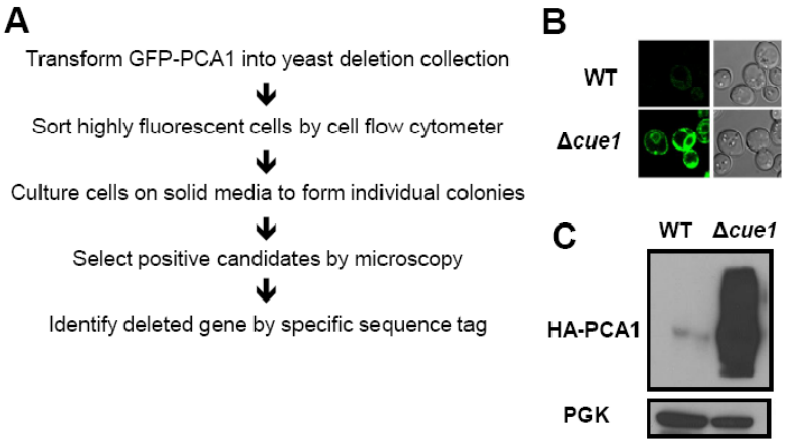

Figure 4.S1. Genetic Screen conducted to identify factors involved in Pca1 degradation. *A,* Schematic of genetic screen strategy. *B,* Confocal fluorescence microscopy of wildtype or Δ*cue1* cells expressing GFP-fused Pca1. *C,* Western blot of HA-Pca1 expressed in Δ*cue1* background. PGK was probed as a loading control.

Cycloheximide chase of HA-Pca1 further confirmed no significant role for Hrd1 in Pca1 turnover (Fig. 4.1B), indicating the specificity of Doa10 in Pca1 turnover. Consistent with ubiquitination by this E3 ligase, Pca1 ubiquitination was dramatically reduced in the Δ*doa10* strain (Fig. 4.1C). Pca1 turnover was also decelerated in Δ*ubc6* strain, but to a lesser extent than in the Δ*ubc7* strain (Fig. 4.S2). Collectively, these data indicated that in the absence of its cadmium substrate, Pca1 was rapidly degraded through the ERAD pathway. Given that we could only select *CUE1* null mutants multiple times despite several ERAD components are involved in Pca1 turnover, our selection of mutants was not saturated.

4.4.2. Degradation of Pca1 and cadmium sensing occur at the ER. Given that Pca1 degradation and ubiquitination required Doa10, an ER-resident ubiquitin ligase, we reasoned that Pca1 degradation occurs before its secretion from the ER. To demonstrate

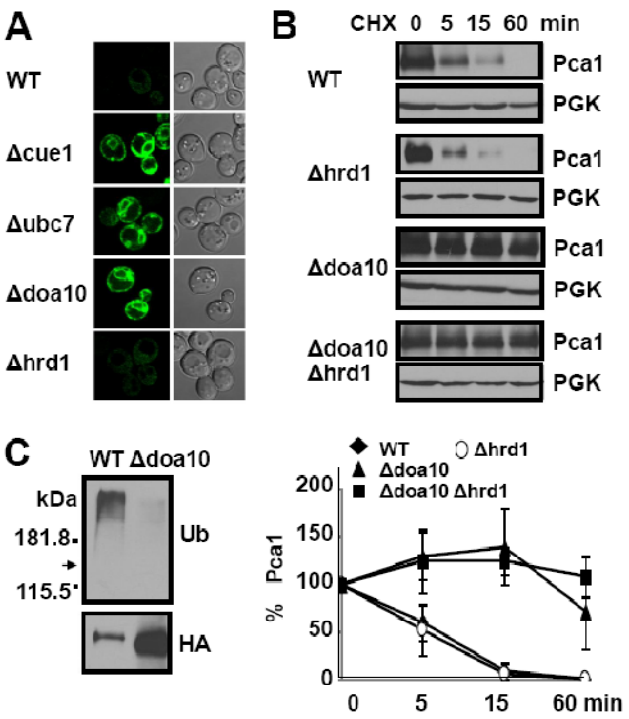

Figure 4.1. Pca1 is degraded through the ERAD pathway. (*A*) Confocal microscopy of GFP-fused Pca1 expressed in wildtype (WT), Δ*cue1*, Δ*ubc7,* Δ*doa10* or Δ*hrd1* strains. (*B*) Cycloheximide chase and immunoblotting of HA epitope tagged Pca1 (HA-Pca1) expressed in wildtype (WT), Δ*hrd1*, Δ*doa10* or Δ*doa10* Δ*hrd1* strains at the indicated time points. Each blot was probed for phosphoglycerate kinase (PGK) to determine equal loading. For quantification, pixel densities of HA-Pca1 were normalized to those of PGK. Average ±S.D. of three independent experiments is graphed (*lower panel*). (*C*) Detection of ubiquitin-conjugated Pca1. Immunoprecipitation of HA-Pca1 in wildtype (WT) or Δ*doa10* cells followed by Western blot with anti-Ub or HA antibodies. Arrow indicates expected migration of HA-Pca1.

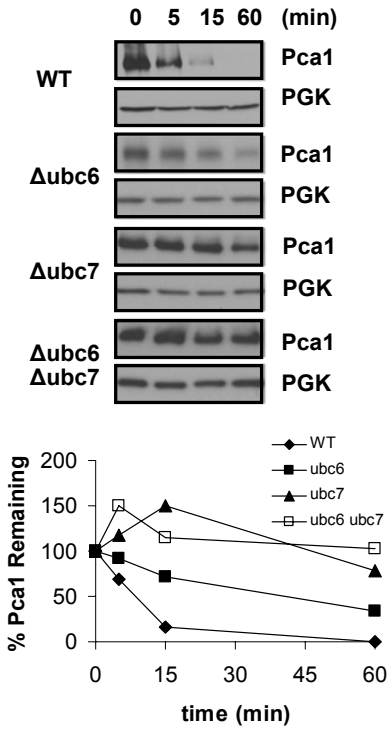

Figure 4.S2. Roles for Ubc7 and Ubc6 ubiquitin conjugating enzymes in Pca1 turnover. (*A*) Cycloheximide chase and immunoblotting of HA epitope tagged Pca1 expressed in wildtype (WT), Δ*ubc6*, Δ*ubc7,* or Δ *ubc6* Δ*ubc7* strains at the indicated time points. Each blot was probed for phosphoglycerate kinase (PGK) to determine equal loading. Representative data from three independent experiments is shown. (*B*) For quantification, pixel densities of Pca1 were normalized to those of PGK.

this hypothesis, we first examined the fate of Pca1 turnover in a *sec23-1* mutant which exhibits a temperature sensitive defect in COPII vesicle dependent ER to Golgi trafficking (25). As shown in Fig. 4.2A, trapping Pca1 in the ER had no significant effect on turnover rate, demonstrating that Pca1 degradation does not require its exit from the ER. Second, we determined the stability of Pca1 in a temperature sensitive mutant of the Cdc48/Np14p/Ufd1 AAA-ATPase, which is required for efficient ER extraction and degradation of integral membrane ERAD-C substrates such as Ste6*, a mutant plasma

membrane protein (26). Consistent with a role for Cdc48 in Pca1 turnover, stabilization of Pca1 is apparent in the *cdc48-3* strain compared to its isogenic wild-type strain at restrictive temperature (Fig. 4.2B). Since Pca1 degradation occurs at the ER, it would be intuitive that cadmium sensing which blocks degradation would also occur at the ER. As expected, addition of cadmium to cell cultures prevented Pca1 degradation when ER exit is blocked (Fig. 4.2C).

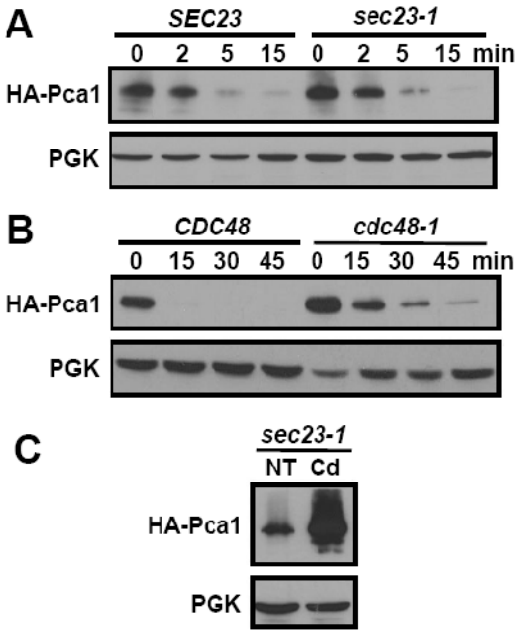

Figure 4.2. Pca1 degradation and cadmium sensing occur at the ER. (*A*) HA-ragged Pca1 was expressed in *SEC23* and a *sec23-1* temperature sensitive mutant. To block ER to Golgi transport, cells were cultured at restrictive temperature (37°C for 30min). After cycloheximide addition to culture media, cells were collected at the indicated time points. Pca1 was detected by Western blotting using anti-HA antibodies. (*B*) Cycloheximide chase and Western blotting of HA-Pca1 expressed in *CDC48* and a *cdc48-3* temperature sensitive mutant cultured at restrictive temperature (37°C for 30min). (*C*) Cadmium enhances Pca1 expression when ER to Golgi transport is blocked. A *sec23-1* strain expressing HA-Pca1 was cultured at restrictive temperature (37°C for 30min) prior to the addition of cadmium (50μM $CdCl_2$) for an additional 30min at restrictive temperature before collection for immunoblotting. Each blot was probed for PGK to determine equal loading.

4.4.3. Inhibition of Pca1 ERAD by cadmium is specific. To confirm if the inhibitory effect of cadmium on ERAD is specific to Pca1, we addressed possible toxic effects of cadmium on ER functions, such as inhibition of ERAD machinery, or saturation of ERAD capacity by excess accumulation of damaged and/or misfolded proteins. Accumulation of misfolded proteins in the ER generally leads to induction of the unfolded protein response (UPR) (27). The overall status of ER function was monitored with a UPR reporter construct (UPRE-LacZ) (13). Despite being an ERAD substrate, constitutive expression of Pca1 did not lead to any significant increase in reporter gene expression over basal levels (Fig. 4.3A). Cadmium concentrations that induce robust expression of Pca1 (Fig. 4.3B) led to a minimal UPR response (Fig. 4.3A), whereas DTT strongly enhanced reporter levels (Fig. 4.3A) without affecting Pca1 expression (Fig. 4.3B). Furthermore, cadmium did not stabilize Ste6*, a well-studied Doa10 substrate (Fig. 4.3C). Collectively, these data indicated that the cadmium dependent stabilization of Pca1 is specific and was not due to over-accumulation of misfolded protein intermediates or the inactivation of ERAD machinery.

4.4.4. The N-terminus of Pca1 contains a targeting signal for ERAD. Aberrant secretory proteins typically engage quality control checkpoints, which prevents their exit from the ER. For instance, when the ERAD pathway is inactivated, some substrates are trapped in the ER rather than being secreted to the plasma membrane (26,28). We took advantage of the ability of the Yor1 ATP-binding cassette (ABC) transporter to extrude oligomycin, a mitochondrial toxin (29). If the Pca1 degron prevents ER exit, then Yor1 fused with this degron would be retained in the ER and no oligomycin resistance would

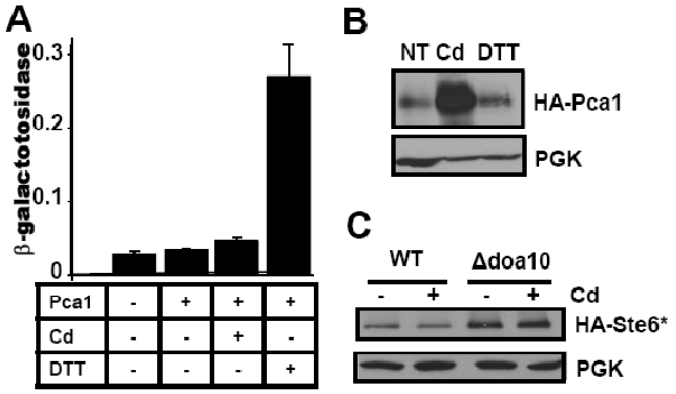

Figure 4.3. Cadmium-dependent inhibition of Pca1 ERAD is specific. (*A*) Unfolded protein response (UPR) determined by a UPRE-LacZ reporter construct (13). Empty vector (-) or an expression construct of HA epitope tagged Pca1 (+) was co-transformed with a UPRE-LacZ reporter construct in a wild-type (WT) yeast strain. Cells were cultured in SC selective media with (+) or without (-) supplementation of cadmium (Cd) (50μM $CdCl_2$, 1hr). As a positive control of UPR response, cells were cultured with DTT (2mM, 1hr) (13). Each bar represents the average ± S.D. of β-galactosidase activities of four different samples. (*B*) Western blot analysis of HA-Pca1 expressed in non-treated cells (NT) or cells cultured in media supplemented with cadmium (Cd) (50μM $CdCl_2$) or DTT (2mM) for 1hr. (*C*) Western blot analysis of HA epitope tagged Ste6* in WT or Δ*doa10* cells cultured without (-) or with (+) supplementation of cadmium (Cd) (50μM $CdCl_2$, 1hr) to culture media. Each blot was PGK to determine equal loading.

be conferred. Fusion of Pca1(1-392) converted the normally stable Yor1 transporter to an unstable substrate which is stabilized by cadmium in culture media or deletion of *DOA10* (Fig. 4.S3). Consistent with its rapid proteolysis, in the absence of cadmium the Pca1(1-392)-Yor1-GFP fusion did not confer resistance to oligomycin (Fig. 4.4A, third row, middle panel). However, upon supplementation of cadmium (Fig. 4.4A, third row, right panel) or expression in a Δ*doa10* strain, (Fig. 4.4A, fourth row, middle panel) the fusion protein confers oligomycin resistance. These results demonstrate that the Pca1(1-392)-Yor1-GFP fusion reaches the cell surface for oligomycin efflux when its degradation is

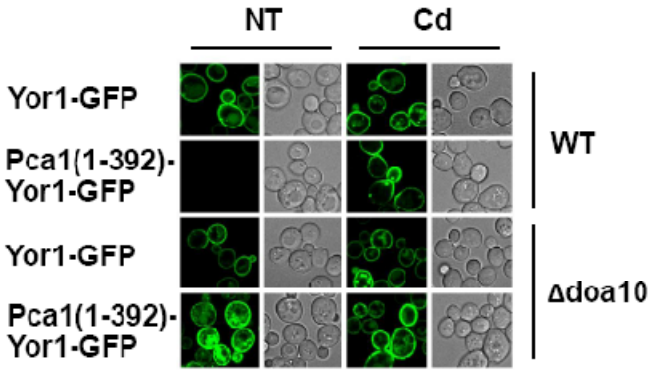

Figure S4.3. Cadmium and Doa10 dependent regulation of Pca1(1-392)-Yor1-GFP
Confocal fluorescent microscopy of Yor1-GFP and Pca1(1-392)-Yor1-GFP expressed in a WT and Δ*doa10* cells non-treated (NT) or with addition of cadmium (50μM $CdCl_2$, 1hr) .

prevented. Oligomycin resistance in the Δ*doa10* strain expressing the Pca1(1-392)-Yor1-GFP fusion without cadmium suggests that metal sensing is not a prerequisite for ER exit. This is also in agreement with our previous observation where deletion of residues 1-392 did not perturb Pca1 trafficking or function (6).

We next examined whether the Pca1 degron can target a cytosolic protein for ERAD. Our previous mapping of the Pca1 N-terminus identified a minimal region encompassing amino acids 250-350 to be sufficient for rapid degradation (6). Expression of a soluble GFP reporter C-terminally fused to Pca1 residues 250-350 (GFP-Pca1(250-350)) was regulated in a cadmium and Doa10 dependent manner (Fig. 4.4B). However, cadmium did not prevent turnover of GFP fused with CL1, an artificial Doa10 degron (30) (Fig. 4.4B). These data indicate that the Pca1 degron can also target a cytosolic protein for ERAD. We conclude that the Pca1 degron does not function as either an ER

exit or retention signal but rather serves as a targeting signal for Doa10 dependent ERAD.

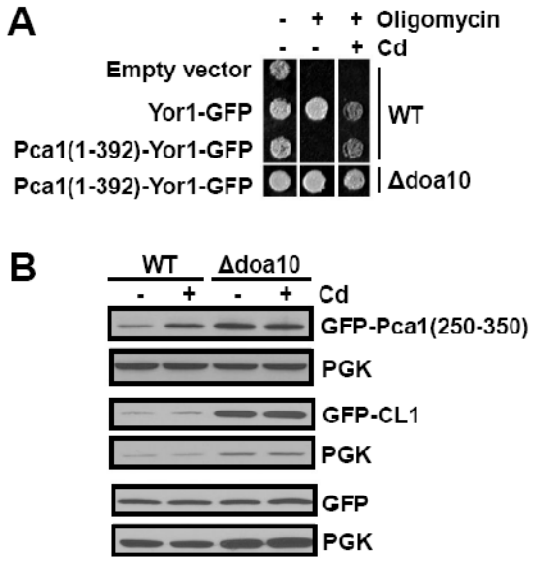

Figure. 4.4. A degron in the N-terminus of Pca1 is not an ER retention signal. *A*, Cell growth on media containing oligomycin. Cells (5µl of cells diluted to A_{600}= 0.1) were spotted on YPEG solid media that was made with (+) or without (-) the addition of oligomycin (2.5µg/mL) and/or cadmium (10µM $CdCl_2$). Cell growth was monitored after 3 days. *B,* GFP without or with fusion of Pca1(250-350) or the CL1 artificial degron were expressed in WT and Δ*doa10* strains. Cells were cultured in media with (+) or without (-) supplementation of cadmium (50µM $CdCl_2$, 1hr). GFP levels in total protein extracts were measured by Western blotting using anti-GFP antibodies. Each blot was probed for phosphoglycerate kinase (PGK) to determine equal loading.

4.4.5. Physical interaction between Pca1 and Doa10 via the N-terminal degron. We hypothesized that the N-terminus of Pca1 functions as a Doa10 recognition motif. Pca1 and Doa10 interaction was determined by chemical crosslinking. Microsomes were prepared from cells expressing thirteen c-myc epitope-tagged Doa10 (Doa10-myc) (19) and a fully functional HA-tagged Pca1 (HA-Pca1), or with deletion of

the N-terminal regulatory domain (HA-Pca1Δ392) (6). Ste6* served as a positive control for physical interaction between an ERAD substrate and Doa10 (19). Microsomes were treated with dimethyl dithiobispropionimidate (DTBP), a thiol reversible and membrane permeable cross-linker, and HA-tagged proteins were immunoprecipitated by anti-HA conjugated sepharose beads. Doa10 was co-precipitated from cells expressing Pca1 or Ste6* but not Pca1Δ392 which lacks the N-terminal degron (Fig. 4.5 first panel). This data demonstrated that the N-terminus is required for either direct or indirect interaction of Pca1 with Doa10.

4.4.6. Metal binding and conformational change of the Pca1 degron. Given that cadmium or copper rescued Pca1 from degradation and that the Pca1 N-terminus contains potential metal binding residues (6), we predicted that metal binding to this region would mask a degradation signal. Since attempts to purify adequate amounts of this protein have been a technical challenge, we utilized a (^{64}Cu(II)) blotting assay (17) in which HA-Pca1(250-350) was immunoprecipitated using anti-HA antibody conjugated beads and transferred to a nitrocellulose membrane. Autoradiography of blots incubated with ^{64}Cu showed specific binding of radioactive copper to HA-Pca1(250-350) as no signal was observed from bands corresponding to the same peptide in which all seven cysteine residues were mutated to alanine (HA-Pca1(250-350)ΔCys) (Fig. 4.6A). Cu(II) or Cd(II) but not Zn(II) effectively competed for ^{64}Cu(II) binding implying that copper and cadmium can compete for the same binding site(s) (Fig. 4.6B). Cd(II) appeared to compete more effectively than copper at the same concentration (Fig. 4.6B), suggesting higher

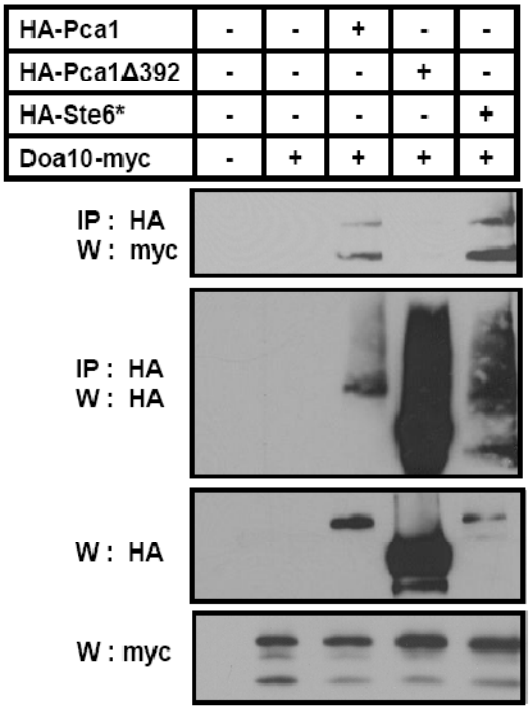

Figure 4.5. The N-terminal regulatory domain is required for physical interaction between Pca1 and Doa10. Expression constructs of HA-Pca1, Pca1 deleted of amino acid residues 1-392 (HA-Pca1Δ392), or Ste6* (HA-Ste6*) were expressed in a strain containing a chromosomal integration of 13 c-myc epitope tagged Doa10 (Doa10-myc). Chemical cross-linking was performed using microsomes prepared from these cells (See Materials and Methods). HA tagged proteins were immunoprecipitated (IP) and denatured under reducing conditions to break crosslinking. Samples were subjected to SDS-PAGE and Western blotting (W) using anti-myc antibodies (IP:HA, W:myc), and then the same blot was stripped and re-probed with anti-HA antibodies (IP:HA, W:HA). Total protein extracts were resolved on SDS-PAGE and analyzed by Western blotting using anti-HA (W:HA) or anti-myc (W:myc) antibodies.

binding affinity. These metal binding specificities were in accordance with previously observed cadmium and copper specific regulation of Pca1 stability (4).

To examine conformational changes associated with metal binding, we employed a limited trypsin proteolysis assay of the Pca1 degron (HA-Pca1-(250-350)). Western

blotting indicates that this domain is more resistant to proteolysis when cells were pre-cultured with cadmium (Fig. 4.6C upper panel). No protection was observed in the same peptide containing site-directed mutations of cysteine residues (Fig. 4.6C lower panel). These data suggest that cadmium binding to cysteine residues within the Pca1(250-350) degron induces a conformational change, which likely acts as a molecular switch allowing Pca1 to circumvent ERAD.

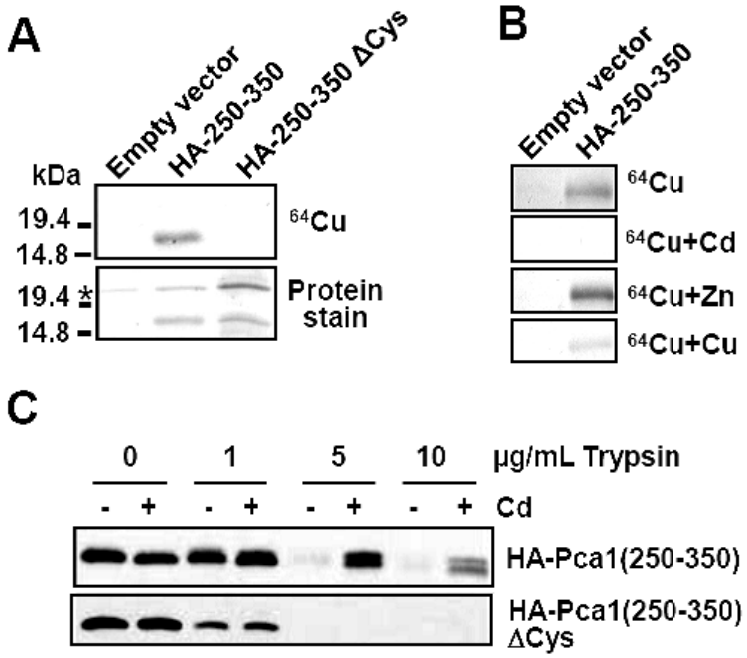

Figure. 4.6 Metal binding and conformational changes within the Pca1 degron. Pca1 amino acid residues 250-350 with N-terminal triple HA epitope tagging (HA-Pca1(250-350)) and HA-Pca1(250-350)ΔCys where all seven cysteine residues were substituted to alanine were expressed in a Δ*doa10* strain. (*A*) Immunoprecipitated HA-Pca1(250-350) and HA-Pca1(250-350)ΔCys were subjected to ^{64}Cu blotting (1 μM $CuCl_2$, ~10 μCi) followed by autoradiography (See Material and Methods). Asterisk indicates non-specifically precipitated bands. (*B*) ^{64}Cu blotting was performed in the presence of 5-fold excess non-radioactive competitor ions. (*C*) Cell lysates prepared from cells cultured with (+) or without (-) cadmium ($CdCl_2$ 50 μM, 1hr) were subjected to increasing concentrations of trypsin proteolysis followed by anti-HA immunoblotting.

4.5. Discussion

Our data supports a model (Fig. 4.7) illustrating an unanticipated regulatory mechanism by which cells control the expression of a plasma membrane protein. In the absence of cadmium, newly synthesized Pca1 is targeted for ERAD by an N-terminal degron resulting in rapid turnover at the proteasome. Cadmium sensing by this degron induces a conformational change, which prevents the recognition of Pca1 by the ERAD machinery. Hence, cells are able to rapidly elevate Pca1 expression in response to cadmium.

Given that cadmium is an extremely toxic metal of which environmental levels fluctuate, the constant synthesis of a cadmium exporter would be advantageous for cell survival. ERAD of Pca1 at the early step of synthesis keeps Pca1 levels at a minimum, since expression is not necessary when cells are growing without cadmium stress. It is also possible that Pca1 could play a role other than cadmium efflux, which would be deleterious in the absence of cadmium. For instance, if Pca1 transports nutritional metal ions due to low substrate specificity as shown for other P_{1B}-type ATPases (31), the constant degradation of Pca1 would prevent the loss of essential metals that are limiting for growth.

ERAD eliminates misfolded or unassembled proteins, which is necessary for the prevention of toxic accumulation of aberrant proteins (8, 9). At least 30% of newly synthesized proteins are rapidly degraded by proteasomes (32). It has been suggested that this observation represents the degradation of misfolded proteins and defective ribosomal products resulting from errors in translation. This pool of peptides also provides both host

and viral antigenic peptides to be displayed by MHC class I molecules (33). Our study presented herein suggests another unexplored role for ERAD, which occurs in a degron-dependent manner and would allow for fine tuning of secretory protein expression in response to cellular cues.

Regulation of Pca1 expression at the ER may represent a conserved and largely uncharacterized system by which cells control the expression levels of secretory proteins. Consistent with this argument, molecular factors in the ERAD pathway are involved in regulated destruction of proteins. Sterol metabolic status regulates ERAD of ER-resident HMG-CoA reductase, the rate-limiting enzyme of sterol synthesis (34). Co-translational degradation of apolipoprotein B at the ER is enhanced when lipid efflux from the liver is not favored (35). In yeast, the Doa10 ubiquitin ligase also targets the cytoplasmic MATα2 repressor for degradation in the absence of its MATa1 binding partner (36). However, Pca1 is the first plasma membrane protein that is targeted to the ERAD pathway by a degron in a substrate-dependent manner.

There is accumulating evidence suggesting expressional control of plasma membrane transporters and ion channels early in the secretory pathway, although the implication of ERAD in this process has not been firmly established. For instance, it was shown that ubiquitination and proteasome-dependent degradation affects the expression of a variety of cell surface proteins such as aquaporin (37), acetylcholine receptor (38), ATP sensitive K^+ channels (39), and opioid receptors (40). Intriguingly, under normal conditions only 40% of newly synthesized human δ opioid receptors ever reach the cell surface; however membrane-permeable opioid ligands facilitate maturation and ER

export (41). It is of interest to determine whether this opioid dependent degradation of its receptor occurs by a similar mechanism that we have established for Pca1.

It is unknown whether Doa10 directly recognizes the Pca1 degron or whether other accessory factor(s) recruit Pca1 for ubiquitination. The requirement of the Pca1 N-terminus in the co-precipitation of Doa10 suggests a physical interaction between the Pca1 degron and Doa10, although it is not certain yet whether this interaction is mediated by other factor(s). In the case of Ste6*, cytosolic Hsp70 chaperones aid in this process (19). However, our preliminary data did not indicate any significant role for Hsp70 chaperones in Pca1 ERAD.

The recognition determinants of the Pca1 degron and that of other ERAD substrates remain unknown. The current hypothesis is that misfolded proteins display normally buried hydrophobic residues to the cytosolic surface, which serves as a targeting signal for degradation. The Deg1 degron of MATα2 repressor is predicted to form an amphipathic helix of which hydrophobic residues are crucial for its instability (36). However, there is no obvious similarity in the primary sequence of Deg1 and the Pca1 degron. Our preliminary mutagenesis studies have revealed that single amino acid substitutions of hydrophobic residues hinder ERAD recognition of the Pca1 degron. These observations suggest that specific characteristics within this degron are recognized by the ERAD machinery rather than a general misfolded structure as has been postulated for other ERAD substrates. Structural characterization of the Pca1 metal sensing degron in apo and metal-bound forms would define what features attract the ERAD machinery. This study would also provide useful information for the identification of other proteins

that may possess a similar type of degradation signal. While the Pca1 degron does not have significant sequence identity with known proteins, we could identify potential metal binding cysteine and histidine-rich extensions in predicted Pca1 family members of plants (31) and fungi. We are currently testing whether expression of these proteins or if other proteins in yeast, plants and mammals are also regulated by ERAD.

The identification of the molecular factors involved in Pca1 turnover reveals a novel regulatory mechanism by which yeast cells selectively control the expression of a plasma membrane protein at an early step of synthesis. Small molecules, including substrates or metabolites, or signaling pathways may actively regulate protein secretion at the ER in a target specific manner as demonstrated for Pca1.

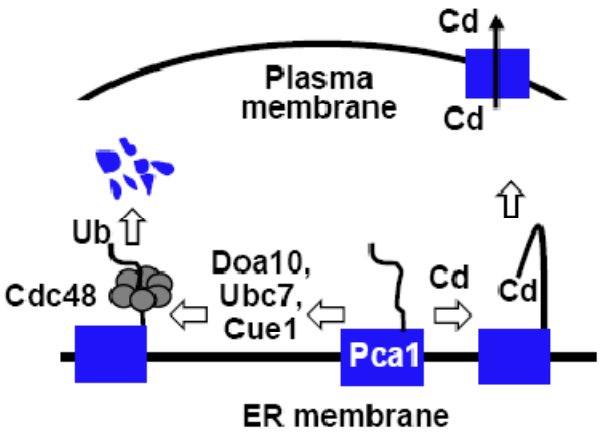

Figure 4. 7 Model of cadmium-regulated expression of Pca1. Cadmium sensing occurs at the ER by metal binding to the cytosolic N-terminal domain of Pca1 followed by conformational changes which prevents recognition for ubiquitination by ERAD factors, including Cue1, Ubc7, and Doa10. Pca1 extraction from the ER is facilitated by Cdc48 after ubiquitination. Black squares indicate Pca1 trans-membrane domains. Black arrow indicates cadmium efflux via Pca1 at the plasma membrane.

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Chapter 5

Significance and Future Directions

5.1. Identification of a New Metal-transporting P-type ATPase

Cadmium is a serious environmental contaminant implicated in various disorders. Human exposure to cadmium is unavoidable and widespread. In the context of these public health concerns, understanding the mechanisms of heavy metal metabolism represents an important research avenue. Pca1 represents the first cadmium-specific efflux system. Knowledge gained from this study may lead to the development of new strategies for prevention and treatment of cadmium related disorders.

P_{1B}-type ATPases are divided into different subclasses based on structural features and substrate specificity. Sequence alignments which have identified conserved metal binding residues have been used to predict substrate specificity, although the structural determinants of substrate specificity for P_{1B}-type ATPases have not been firmly established. According to these criteria, Pca1 is currently classified as a copper (I) translocating P_{1B}-type ATPase; however, functional characterization of Pca1 supports cadmium selectivity. Given that all known cadmium translocating P_{1B}-type ATPases in plants and bacteria also transport other divalent heavy metals (e.g. zinc (II), lead (II), cobalt (II), mercury (II)), Pca1 appears to be a new P_{1B}-type ATPase subtype. Since, a mechanistic understanding of the determinants of substrate specificity are lacking, Pca1 will be an important tool for understanding ion selectivity of P_{1B}-type ATPases.

Our BLAST searches have revealed several potential Pca1 homologues from fungi that have high sequence identity and similarity with Pca1 (Figure 5.1). Cloning and functional characterization of these genes would establish a new cadmium-specific subtype of a P_{1B}-type ATPase

It does not appear that a cadmium-transporting P_{1B}-type ATPase exists in the human genome in addition to copper-transporting ATP7a and ATP7b. However, several lines of evidence suggest that mammals possess cadmium export mechanism(s). Identification of cadmium exporters in humans would lead to better understanding of the mechanisms of cadmium detoxification and open up a new research avenue for combating cadmium-related disorders.

Organism	**% identity**	**% similarity**
G .zea	45.0	62.5
P .janthinellum	45.6	60.9
A. fumigatus	43.0	59.8
A.clavatus	42.4	58.5
C. immitus	33.7	51.8
B.fuckeliana	33.5	46.9

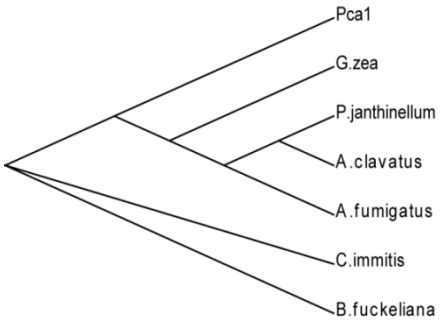

Figure 5.1. Sequence comparisons and phylogenetic tree of possible functional homologues of Pca1 from other fungi. Protein sequences were retrieved from the NCBI data base. Alignments were produced by Clustal W.

5.2. Genetic Variations in Molecular Factors Involved in Cadmium Detoxification

Sequence comparisons of Pca1 revealed a missense mutation in a critical residue, which leads to a non-functional Pca1 allele. This mutation exists in several laboratory strains but not in natural isolates. The most plausible explanation is that the ancestral strain harboring the G970R mutation was propagated to research laboratories across the world. The non-functional Pca1 allele at least in part explains the cadmium hypersensitivity of laboratory yeast strains compared to natural strains. It is interesting to note that a natural strain deleted of Pca1 was still more resistant to cadmium than a laboratory yeast strain (unpublished data), which suggests that other cadmium resistance factors remain to be discovered. Metal concentrations in the environment are variable and can rapidly fluctuate, imposing a strong selection pressure for cadmium defense genes. Population bottlenecks and decreased selection pressure in a laboratory environment have likely resulted in the accumulation of deleterious mutations in genes involved in cadmium resistance. The genetic variation of cadmium resistance among yeast strains provides an interesting research model to identify new cadmium resistance genes and explore gene-environment interactions associated with evolution.

5.3. A Novel Mode of Expressional Control of a Plasma Membrane Protein

For the first time, our work has definitively demonstrated that the expression levels of a plasma membrane protein can be regulated at the ER in response to cellular needs. Importantly, we have also isolated a specific response element and have offered a mechanistic explanation for ligand dependent masking of an ERAD signal. Several layers of evidence have been presented which support these claims. First, Pca1 is rapidly degraded via the ubiquitination-proteasome pathway; however in the presence of its cadmium substrate, ubiquitination is inhibited, resulting in Pca1 stabilization. Second, Pca1 degradation requires components of the ERAD pathway, including ER resident E2 and E3 ubiquitin ligases. Third, cysteine dependent metal binding to the Pca1(250-350) domain result in conformational changes, which is believed to be a mechanism of degradation signal masking.

It is of interest to determine whether any other cell surface proteins are regulated at the ER in a similar manner as has been demonstrated for Pca1. Indeed, the ubiquitin proteasome system has been implicated in modulation of a variety of cell surface proteins including, aquaporin (1), ACh receptor (2), ATP sensitive K^+ channels (3), and opioid receptors (4). Intriguingly, under normal conditions only 40% of newly synthesized human δ opioid receptors ever reach the cell surface. However, membrane-permeable opioid ligands can facilitate their maturation and ER export (5). Ligand and/or cellular signal-dependent escape of ERAD by secretory proteins may be a prevalent but uncharacterized regulatory mechanism determining protein expression levels in response to cellular needs.

5.4. A New Protein Degradation Signal

A fundamental question that remains unanswered is whether the mode of Pca1 regulation and its degradation signal are conserved in other P_{1B}-type ATPases or other unrelated proteins. Sequence alignments of the degradation signal motif in Pca1 with close homologues in fungi have revealed a consensus sequence (Figure 5.2). Interestingly, cysteine residues implicated in copper or cadmium binding are completely conserved. Selective mutagenesis of other conserved residues and cloning and characterization of these Pca1 homologues will determine if cadmium-regulated ERAD is conserved among these proteins.

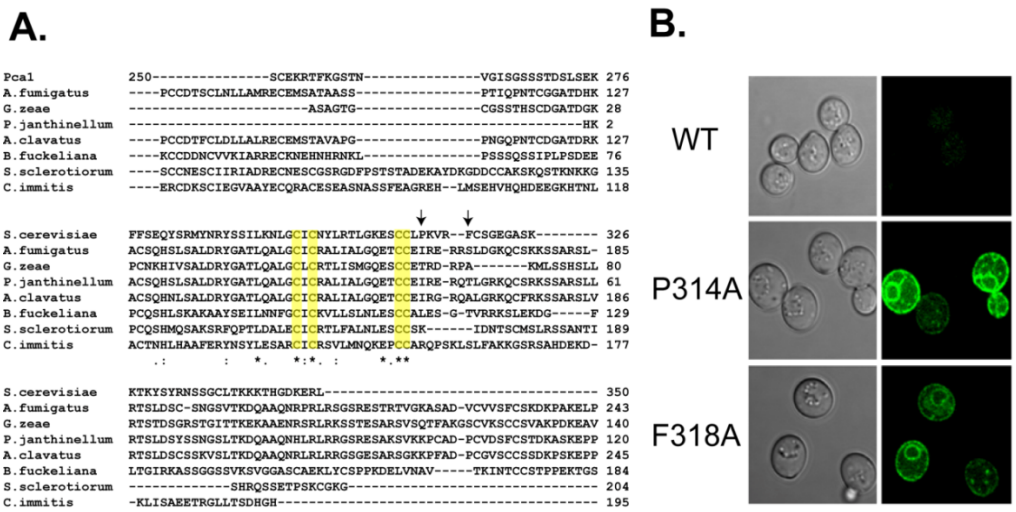

Figure 5.2. Determinants of the Pca1 Degron. (A) Sequence alignment of the Pca1(250-350) domain with putative Pca1 homologues. Cysteine residues previously implicated in metal binding are highlighted. Arrow indicates P314 and F318. (B) Confocal fluorescent microscopy of GFP-Pca1 or mutant alleles expressed in a wild-type yeast strain.

5.5. Does the ERAD Machinery Recognize a Specific Signal Rather Than General Misfolding?

The structural features of ERAD substrates and how they are recognized for delivery to the ER degradation machinery remain unknown. The current hypothesis is that degrons display characteristics of misfolded proteins in which normally buried hydrophobic residues are exposed to the cytosolic surface. Due to a lack of structural data, the only insight into the degradation requirements of a natural degron come from mutational analysis of the Deg1 signal identified from yeast MATα repressor. A similar approach will identify key determinants for recognition of the Pca1 degron. Our preliminary mutagenesis studies have identified key bulky hydrophobic residues (P314 and F318) that are major determinants of the Pca1 degron (Figure 5.2). Although these residues do not appear to be conserved, they are in close proximity to the C311 and C312 residues which were previously implicated in cadmium binding (Chapter 3). Ultimately, structural characterization of the Pca1 metal sensing degron in apo and metal-bound forms would, for the first time, define what features constitute an ERAD signal and how they can be masked by ligand binding.

It is unknown whether the Doa10 E3 ligase directly recognizes the Pca1 degron or whether other accessory factor(s) recruit Pca1 for ubiquitination. There is some evidence that cytosolic Hsp70 chaperones aid in this process, as is the case for Ste6* (6). However, our preliminary data do not indicate any significant role for Hsp70 chaperones in Pca1 ERAD (unpublished observations). The requirement for the Pca1 N-terminus in the co-immunoprecipitation of Doa10 suggests an interaction between the Pca1 degron and

Doa10. However, it is not certain yet whether this interaction is mediated by other factor(s). Pull down of interacting proteins followed by tandem MS/MS peptide sequencing would be a strategy for identification of these factors.

5.6. Broad Impact of this Study in Biology and Medicine

The unanticipated discovery of Pca1 regulation by ERAD has significant implications in understanding cellular regulatory mechanisms of protein expression. For some time it has been known that a large fraction of newly synthesized proteins is rapidly degraded by proteasomes (7). The current view is that this observation most likely represents the degradation of misfolded proteins and defective ribosomal products that result from errors in translation. It is becoming increasingly evident, especially after the elucidation of Pca1 regulation at the ER, that the degradation of such a large pool of newly synthesized proteins may be part of a regulatory process. This mode of regulation allows cells to rapidly respond to cellular cues. Ligand binding and/or post-translational regulation (e.g., phosphorylation, protein-protein interactions) followed by conformational masking of ERAD signals may be a common but largely uncharacterized cellular phenomenon. Perhaps a large portion of the secretory proteome is regulated in this manner. Characterization of conserved features in degrons like those found in the N-terminal domain of Pca1 could lead to the identification of other proteins in which ERAD plays a regulatory role in protein expression. Structural characterization of these degrons may make it one day possible to design small molecules to control the expression or degradation of a select protein through the ERAD pathway as a therapeutic strategy.

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