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UNIVERSITY OF CALIFORNIA, SAN DIEGO

ER quality control: understanding misfolded protein recognition
and retrotranslocation

A Dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

in

Biology

by

Brian Keith Sato

Committee in charge:

Professor Randolph Hampton, Chair
Professor Elizabeth Komives
Professor Maho Niwa
Professor Suresh Subramani
Professor James Wilhelm

2008

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Chair

University of California, San Diego

2008

Dedication

This work is dedicated to my wife and family
and all others who have been kind enough
to impart their knowledge onto me.

Epigraph

“Equipped with his five senses, man explores the universe around him and calls the adventure Science.”

~Edwin Powell Hubble

“Science is a wonderful thing if one does not have to earn one’s living at it.”

~Albert Einstein

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Chapter 3 is a reprint of Sato, BK and Hampton RY. "Yeast Derlin Dfm1 interacts with Cdc48 and functions in ER homeostasis." *Yeast*. 2006. 23(14-15): 1053-64. I was the primary experimenter and Randolph Hampton and I wrote the manuscript.

Chapter 4 is a manuscript in preparation that will be submitted for publication. Renee Garza performed the following experiments: Figure 4-4, Figure 4-5, Figure 4-10 and Figure 4-20. Randolph Hampton wrote the manuscript and I edited it for use in this chapter.

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PUBLICATIONS

- Sato, BK and Hampton RY. "Yeast Derlin Dfm1 interacts with Cdc48 and functions in ER homeostasis." *Yeast*. 2006. 23(14-15): 1053-64.
- Sato BK, Schulz D and Hampton RY. "The Hrd1p ubiquitin ligase specifies recognition of integral membrane ERAD substrates." manuscript in preparation.
- Garza RM, Sato BK, Hampton RY. "In vitro analysis of Hrd1p-mediated retrotranslocation of its natural substrate HMG-CoA reductase." manuscript in preparation.

ABSTRACT OF THE DISSERTATION

ER quality control: understanding misfolded protein recognition
and retrotranslocation

by

Brian Keith Sato

Doctor of Philosophy in Biology

University of California, San Diego, 2008

Professor Randolph Hampton, Chair

As the site of folding for proteins of the secretory pathway, the endoplasmic reticulum (ER) must be equipped with an efficient quality control machinery. Through ER associated degradation (ERAD) misfolded luminal and membrane-bound proteins are tagged with poly-ubiquitin chains and degraded in a proteasome-dependent manner. ERAD is a multi-step process which is initiated by the identification of the misfolded protein. While luminal substrates degraded by the ERAD-L pathway appear to be recognized by a number of factors, it is unknown how misfolded membrane proteins are

identified by the ERAD-M pathway. As a rate-limiting factor for ERAD, and a specificity factor as a ubiquitin ligase, we tested whether Hrd1p plays a role in the identification of misfolded proteins. By mutating a number of conserved and hydrophobic residues within the Hrd1p transmembrane domain, we illustrated that Hrd1p can distinguish between misfolded luminal and membrane proteins, and that specific residues were important for the degradation of specific membrane proteins. Thus, in addition to Hrd1p's role as a ubiquitin ligase, it appears that this protein plays a role in the recognition of misfolded membrane proteins as well. Our studies also demonstrated that substrate binding does not appear to be sufficient to initiate ubiquitination, and that some type of signal is required following binding in order to activate the RING domain of Hrd1p. Following substrate ubiquitination, the protein must be removed from the ER in a process known as retrotranslocation, as the proteasome is located in the cytoplasm. To study retrotranslocation, we established an *in vitro* retrotranslocation assay, which we demonstrated was capable of retrotranslocating a natural ERAD-M substrate, Hmg2p. Retrotranslocated Hmg2p was soluble and full-length, and the process was dependent on Cdc48. With this assay, we were able to test the current model of retrotranslocation, in order to determine the core set of proteins which are essential for this process. This uncovered a role for the factors Ubx2p and Rad23p/Dsk2p in retrotranslocation, while demonstrating that the putative retrotranslocons Sec61p, Der1p/Dfm1p, and Hrd1p are dispensible for Hmg2p retrotranslocation. These studies have greatly increased our understanding of HRD-dependent degradation.

Chapter 1

Protein quality control

Protein quality control

It has been demonstrated that up to 30% of newly synthesized proteins cannot achieve their functional conformation (Schubert et al. 2000). In order to handle this load of misfolded or damaged proteins, the cell has established quality control pathways in a number of different compartments. This includes the endoplasmic reticulum, nucleus, mitochondria, and cytoplasm (Hampton 2002; Gardner et al. 2005; Park et al. 2007; Radke et al. 2008). Cells also have mechanisms to supplement quality control pathways during times of extreme stress, including the heat shock response and the unfolded protein response (Sorger 1991; Malhotra and Kaufman 2007). These pathways increase the transcription of genes required to handle an excess of unfolded proteins including chaperones. In addition to misfolded proteins, quality control pathways are responsible for degrading regulated proteins, including those involved in sterol synthesis and cell differentiation (Hampton et al. 1996; Swanson et al. 2001)

A common theme throughout the quality control pathways is that misfolded substrates are targeted for degradation through the attachment of a poly-ubiquitin chain. Ubiquitin chains are formed through the cooperative efforts of three different enzymes (Figure 1-1, (Pickart 2000; Pickart and Eddins 2004)). First, an E1 ubiquitin activating enzyme forms a covalent bond with ubiquitin in an ATP dependent process. This ubiquitin molecule is then transferred from the E1 to a conserved cysteine residue on an E2 ubiquitin conjugating enzyme. Finally, the ubiquitin molecule is targeted to a lysine residue on the misfolded protein with the assistance of a ubiquitin ligase (E3). There are two main classes of E3 proteins, RING E3s and HECT domain ligases (Jackson et al. 2000). RING domain containing ubiquitin ligases do not form a ubiquitin binding

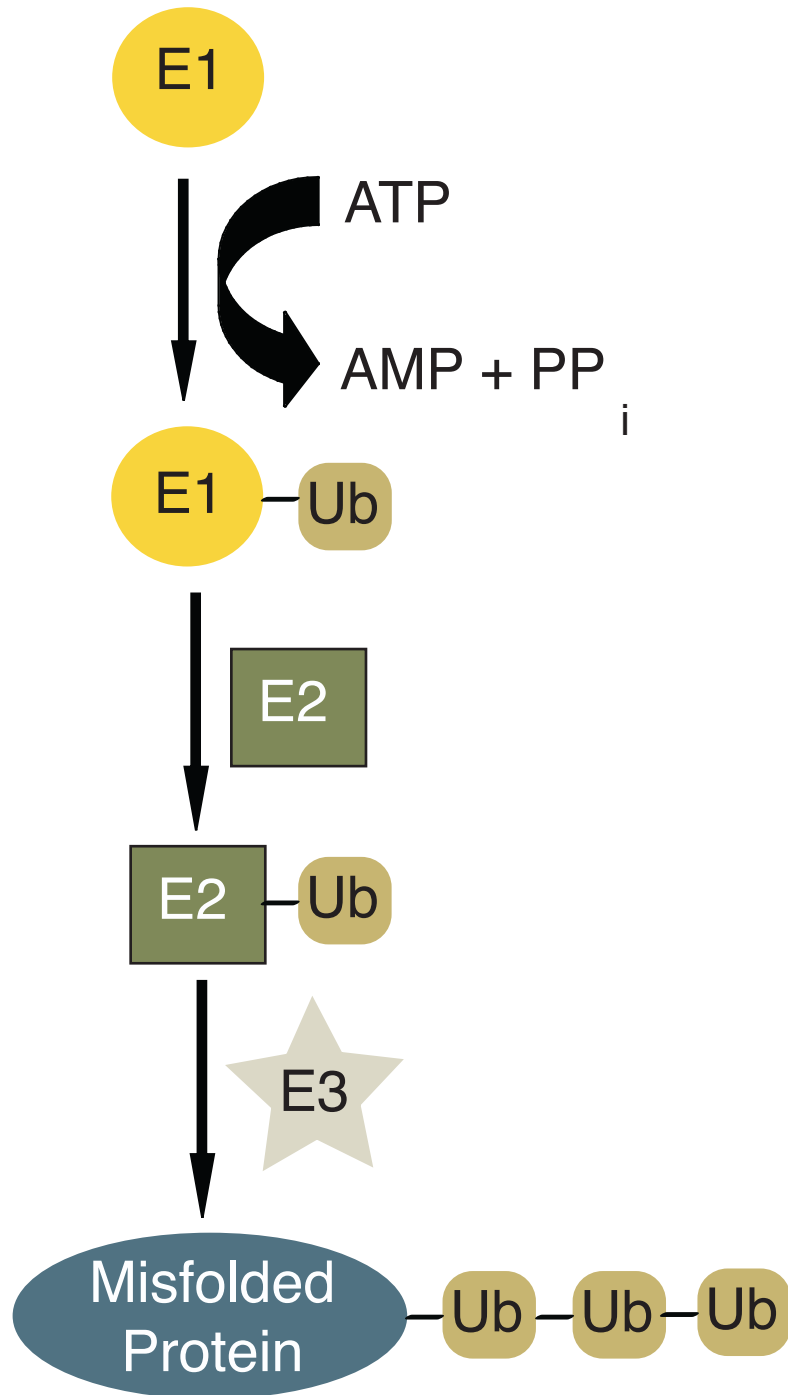


Figure 1-1 Enzyme cascade responsible for misfolded protein poly-ubiquitination

The E1 enzyme binds to ubiquitin in an ATP-dependent manner. This ubiquitin molecule is transferred to an E2 and with the assistance of an E3, the misfolded protein is tagged. The cycle repeats itself multiple times in order to form a poly-ubiquitin chain.

intermediate prior to attachment of ubiquitin onto the substrate, they act by bringing the substrate and the ubiquitin conjugating enzyme into close proximity. HECT domain ligases, on the other hand, are directly bound to ubiquitin on a conserved cysteine residue, after which this molecule is attached to a substrate protein (Pickart and Eddins 2004). One reason for this multi-step pathway is to increase the specificity of the ubiquitination reaction from one enzyme to the next. *Saccharomyces cerevisiae* expresses only one E1 enzyme and 11 E2 enzymes, while there are over 50 ubiquitin ligases (Hicke et al. 2005). In this way, each enzyme further down the ubiquitin cascade is responsible for a smaller subset of target proteins, thus increasing the regulation of ubiquitin attachment.

Ubiquitin molecules are capable of forming distinct poly-ubiquitin chains depending on the lysine residue upon which they are built. *In vivo*, it has been demonstrated that it is possible to form multi-ubiquitin chains with seven different lysines found on the ubiquitin molecule (Peng et al. 2003). The most common form of poly-ubiquitin chain is one linked through lysine 48 of ubiquitin, which directs tagged substrates to the proteasome for degradation (Finley et al. 1994). In order for K48-linked chains to be recognized by the proteasome, they must be at least four ubiquitin molecules long, and longer chains increase proteasomal recognition due to a greater number of tetraubiquitin units (Thrower et al. 2000).

All poly-ubiquitinated degradation substrates of the various quality control pathways converge at the proteasome. The 26S proteasome is a large multi-protein complex that is composed of two main structures (Schmidt et al. 2005), a hollow core particle which is responsible for the proteolytic activity of the proteasome, and two

regulatory particles, one on each side of the core particle, that contain proteins with a variety of functions. The regulatory particle is further divided into the base and the lid. These two components are capable of associating with poly-ubiquitinated proteins, protein de-ubiquitination, and unfolding of proteins prior to entry into the proteolytic chamber (Braun et al. 1999; Verma et al. 2002; Schmidt et al. 2005).

ERAD

Protein translocation and folding for nearly all proteins of the secretory pathway must occur in the endoplasmic reticulum. To accommodate for the large amount of misfolded and damaged proteins in this compartment, the cell has established a quality control pathway known as endoplasmic reticulum associated degradation (ERAD) (Hampton 2002). ERAD is responsible for the degradation of both membrane bound and luminal misfolded proteins. Once these proteins are identified, they are tagged with poly-ubiquitin chains, removed from the ER in a process known as retrotranslocation, and degraded by the cytoplasmic proteasome.

Protein ubiquitination is controlled by two ubiquitin ligase complexes built around the E3s Hrd1p and Doa10p (Bays et al. 2001a; Swanson et al. 2001). Each complex is responsible for the degradation of a distinct set of substrates. Hrd1p was identified through a screen for mutants defective in the degradation of a regulated protein, Hmg2p, the yeast homologue of Hmg-CoA reductase (Hampton et al. 1996). Hrd1p is composed of six transmembrane spans (Deak and Wolf 2001) and a cytosolic RING domain and is involved in the ubiquitination of both misfolded membrane proteins (ERAD-M) and soluble proteins with misfolded luminal domains (ERAD-L) (Carvalho

et al. 2006). The core proteins involved in both pathways are identical. The factors involved in ERAD-M (Figure 1-2) consist of Hrd1p, Hrd3p, Ubc7p and Cue1p. Hrd3p contains a transmembrane span and a large luminal domain, which tightly associates with Hrd1p, and one of its functions is to stabilize the Hrd1p protein (Gardner et al. 2000). Ubc7p is the soluble E2 which works with Hrd1p to ubiquitinate ERD substrates (Hampton and Bhakta 1997) and it is anchored by the ER membrane protein Cue1p. In addition, Cue1p functions to activate Ubc7p (Bazirgan and Hampton 2008). A more recently identified factor, Usa1p, appears to be involved in the proper regulation of Hrd1p-dependent ubiquitination ((Carvalho et al. 2006), Carrol and Hampton, manuscript in progress). ERAD-M also depends on the putative Cdc48p receptor, Ubx2p (Neuber et al. 2005; Schuberth and Buchberger 2005) and the Cdc48 complex, which is composed of a hexamer of Cdc48 proteins, and the co-factors Ufd1p and Npl4p (Bays et al. 2001b; Ye et al. 2001). Cdc48 is a soluble AAA ATPase that is involved in numerous functions in addition to ERAD, including mitotic spindle disassembly, nuclear envelope formation, and homotypic membrane fusion (Patel et al. 1998; Cao et al. 2003; Ramadan et al. 2007). While its exact function in ERAD is unknown, structural studies appear to suggest a chaperone-like function in which Cdc48p maintains retrotranslocated proteins in a partially unfolded state (DeLaBarre et al. 2006).

Misfolded luminal proteins degraded by the ERAD-L pathway utilize the same proteins that are involved in ERAD-M, in addition to a number of other factors that have been proposed to act in the recognition of these substrates (Figure 1-3). These recognition factors include Htm1p and Yos9p, both of which are capable of binding to misfolded glycoproteins (Jakob et al. 2001; Kim et al. 2005). It has also been

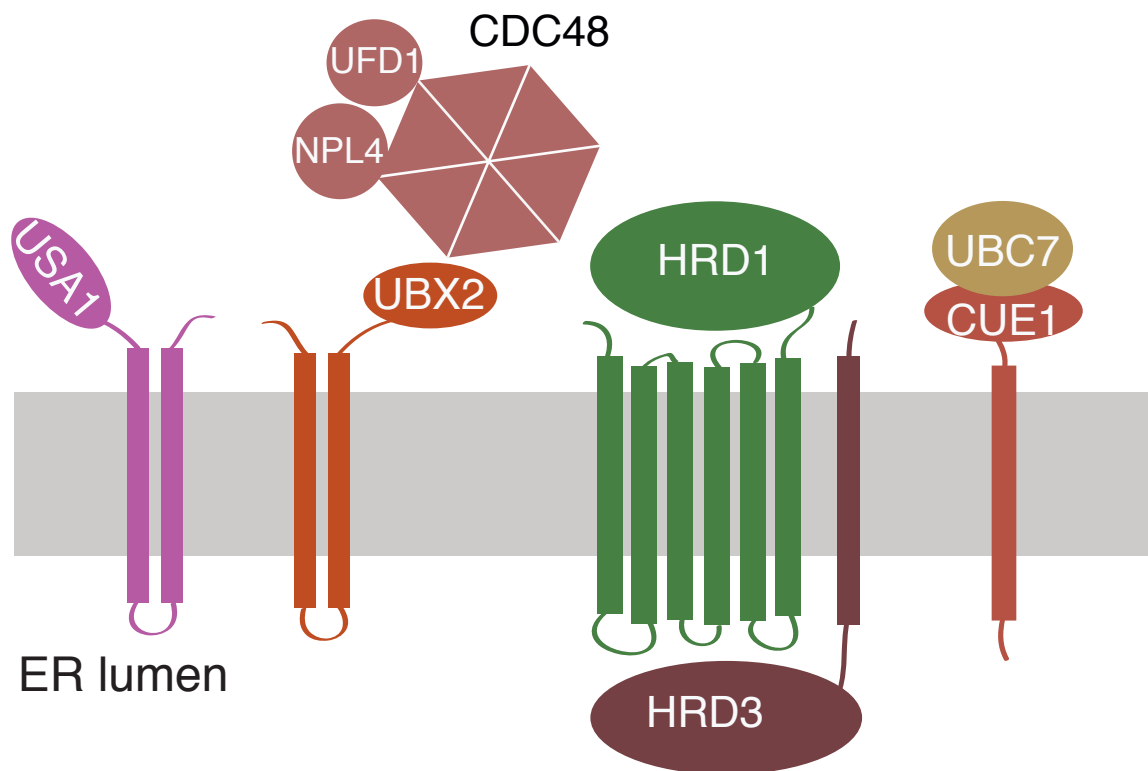


Figure 1-2 Factors involved in ERAD-M

Degradation of membrane-bound substrates involves protein ubiquitination through Hrd1p and Ubc7p. The Cdc48 complex then functions to retrotranslocate the misfolded protein. Ubx2p is proposed to act as a Cdc48p receptor, Usa1p is proposed to regulate Hrd1p activity, Cue1p acts as an anchor for Ubc7p and Hrd3p is involved in Hrd1p stability.

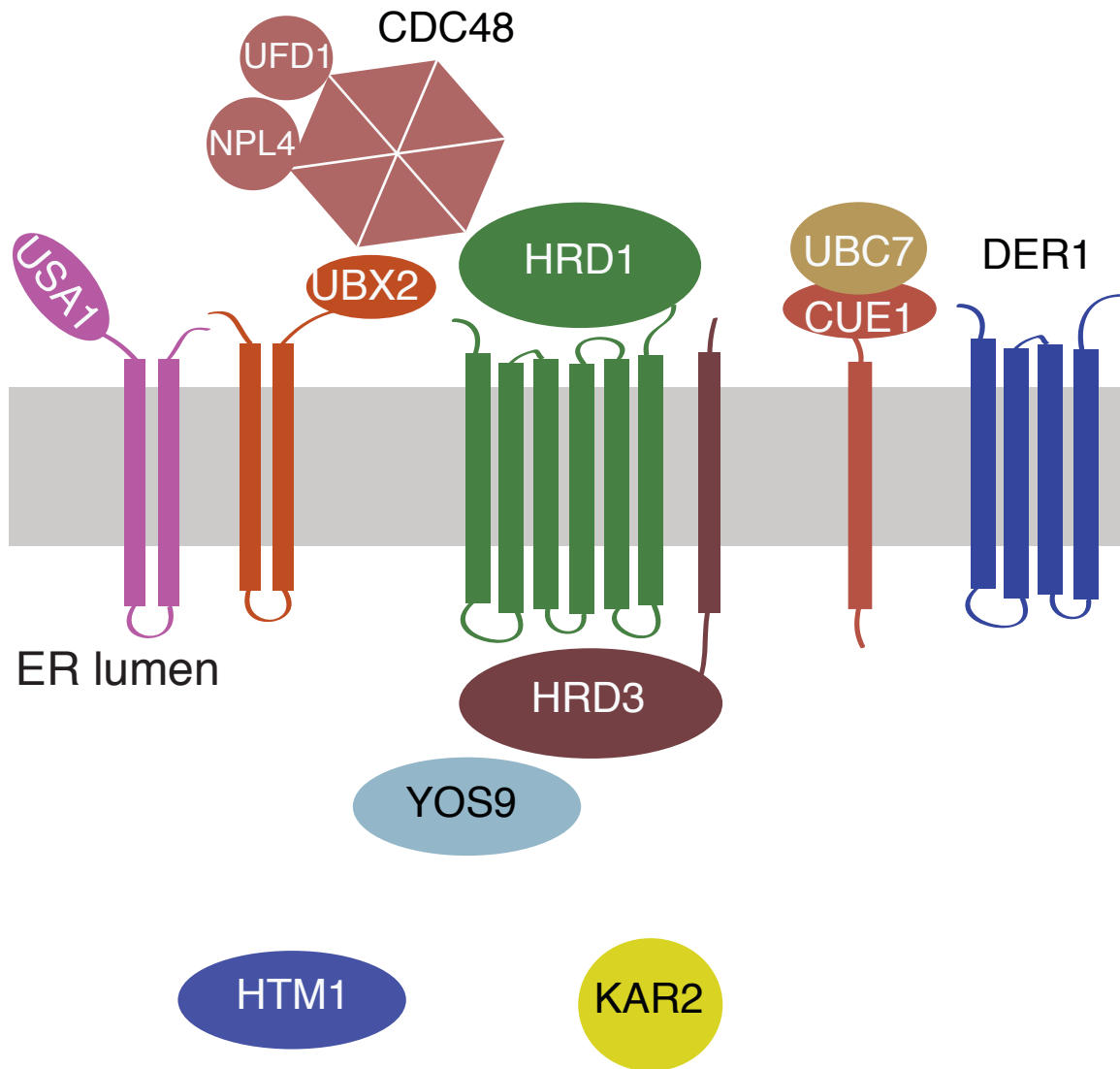


Figure 1-3 Factors involved in ERAD-L

Degradation of soluble, luminal substrates involves misfolded protein recognition through Htm1p, Kar2p, Yos9p and Hrd3p. Substrate ubiquitination requires Hrd1p and Ubc7p. The Cdc48p then functions to retrotranslocate the misfolded protein. Ubx2p is proposed to act as a Cdc48p receptor, Usa1p is proposed to regulate Hrd1p activity, Cue1p acts as an anchor for Ubc7p and Hrd3p is involved in Hrd1p stability. The function of Der1p is unknown.

demonstrated that Kar2p, the yeast homologue of BiP, and the luminal domain of Hrd3p are involved in misfolded protein interaction and delivery to Hrd1p for ubiquitination (Denic et al. 2006). Thus, while the ERAD-L pathway has a number of recognition factors required for protein degradation, ERAD-M appears to rely more heavily on Hrd1p for substrate recognition, as will be described in Chapter 2. Another ERAD-L core component is Der1p, one of the first identified ERAD factors (Knop et al. 1996). While its function is unknown, it is hypothesized to play a role in luminal misfolded protein retrotranslocation, although it has no function in the retrotranslocation or degradation of membrane substrates (Chapter 4).

A third branch of the ERAD pathway, ERAD-C, involves the degradation of substrates that contain a cytosolic misfolded domain (Figure 1-4). ERAD-C differs from the previously described pathways in that ubiquitination is dependent on Doa10p (Vashist and Ng 2004). Like Hrd1p, Doa10p is a RING E3 which contains multiple transmembrane spans and a cytosolic RING domain (Swanson et al. 2001; Kreft et al. 2006). Doa10p catalyzes substrate ubiquitination with the E2s Ubc6p, a single membrane-spanning ER protein, and Ubc7p. Like ERAD-L substrates, ERAD-C substrates have been demonstrated to require a bridging factor to bring substrates to the ubiquitin ligase. As the substrates contain misfolded cytosolic domains, Hsp70, a cytoplasmic chaperone, appears to deliver substrates to Doa10p. In the absence of this cytoplasmic chaperone, ubiquitination and binding of Ste6-166p, a multi-spanning membrane protein, by Doa10p is greatly inhibited (Nakatsukasa et al. 2008). Factors downstream of ubiquitination, including Ubx2p and the Cdc48 complex are also essential for the degradation of ERAD-C substrates.

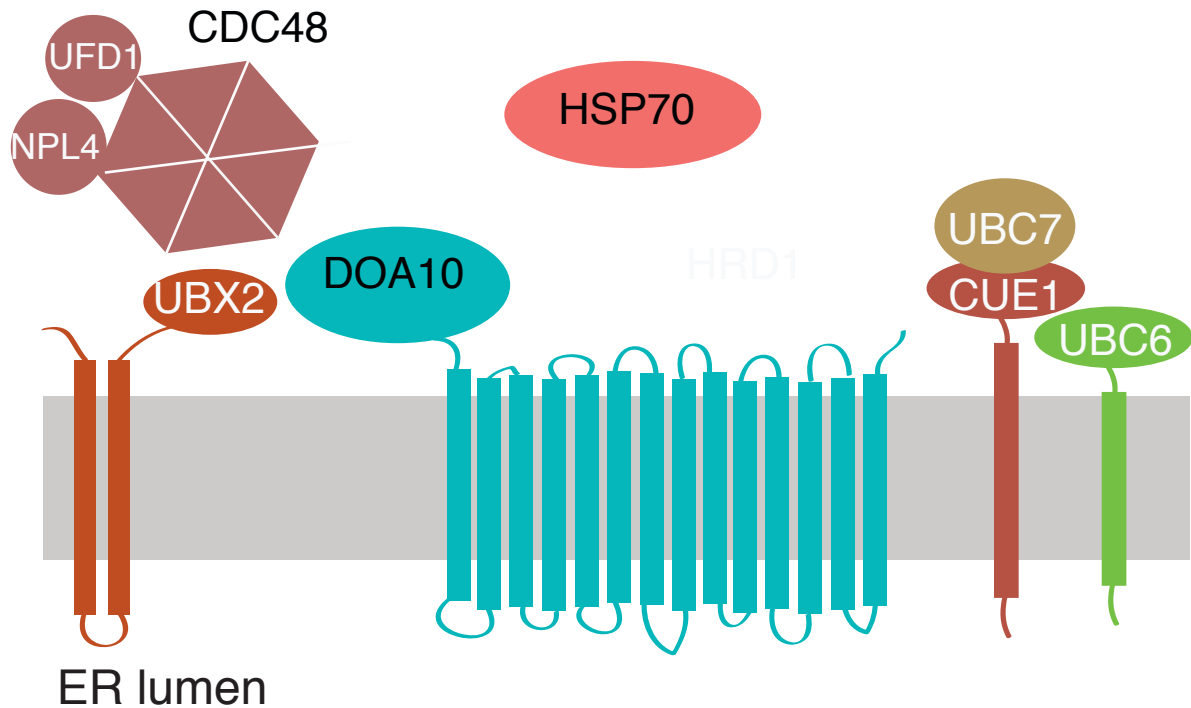


Figure 1-4 Factors involved in ERAD-C

Degradation of substrates with misfolded cytoplasmic domains involves protein ubiquitination through Doa10p, Ubc6p and Ubc7p. Substrate recruitment is assisted by the cytoplasmic chaperon Hsp70. The Cdc48 complex then functions to retrotranslocate the misfolded protein. Ubx2p is proposed to act as a Cdc48p receptor and Cue1p is a membrane anchor for Ubc7p.

The identity of these pre-retrotranslocation complexes is fairly well established through both genetic and biochemical means. On the other hand, the mechanism of retrotranslocation is filled with a number of unanswered questions. Retrotranslocation is the process by which an ERAD substrate is moved from the ER into the cytoplasm, and until recently in yeast had not been demonstrated for previously established ERAD substrates (Chapter 3, (Nakatsukasa et al. 2008)). Retrotranslocation in mammals has been studied, but the main assay has involved human cytomegalovirus-dependent ERAD (Shamu et al. 2001; Ye et al. 2001). While its identity is unknown, it is believed that retrotranslocation proceeds through a protein channel. This makes thermodynamic sense, as it is highly unfavorable to move a luminal protein or the luminal domains of a membrane protein through a hydrophobic bilayer. The first serious candidate for the retrotranslocon was Sec61p, the channel required for the passage of translated proteins from the cytoplasm into the ER (Deshaies and Schekman 1987). While some research has demonstrated impaired degradation in the presence of sec61 mutants (Plempner et al. 1997), there has been no definitive evidence to prove such a point. A more recent finding in mammals was the identification of the Derlin family of proteins (Lilley and Ploegh 2004; Ye et al. 2004). When expression of Derlin-1 is lowered, *in vitro* retrotranslocation of MHC-I molecules is inhibited. The derlins are homologous to the yeast ERAD factor, Der1p, one of the first isolated ERAD factors (Knop et al. 1996). In yeast, Der1p and the related factor Dfm1p, have been proposed to act as channels as well. This is unlikely to be the case, as Der1p is only required for ERAD-L and Dfm1p has no role in ERAD at all (Sato and Hampton 2006). Most recently, it has been hypothesized that the ubiquitin ligases, Hrd1p and Doa10p, themselves act as retrotranslocons. They both contain

multiple transmembrane domains and these transmembrane spans contain many hydrophilic residues which could form an aqueous pore. Both proteins also interact with luminal and cytoplasmic ERAD factors and are responsible for degradation of a wide variety of misfolded proteins. While this is an appealing idea, as of yet there is no strong evidence that these ligases also possess a retrotranslocation function.

Prior to proteasomal degradation, retrotranslocated proteins must first encounter a set of post-retrotranslocation factors. The first of these is Ufd2p, a ubiquitin ligase which has been named an E4, due to the fact that it acts by adding ubiquitin molecules to pre-existing ubiquitin chains (Richly et al. 2005). Ufd2p associates with Cdc48p and is a member of the Cdc48 ERAD complex. In the absence of Ufd2p, degradation substrates have much more low molecular weight poly-ubiquitination and their degradation is impaired. This is believed to be due to the fact that these lower molecular weight chains are less easily recognized by either the proteasome, or a set of proteasome delivery factors. These delivery factors, Rad23p and Dsk2p, contain a UBL domain, which is responsible for binding to the Cdc48 complex or the proteasome, and a UBA domain, which is capable of associating with poly-ubiquitin chains. It has been demonstrated that proteins with shorter poly-ubiquitin chains are less capable of interacting with Rad23p and Dsk2p. Since these two factors are capable of docking on the proteasome, the model is that Ufd2p extends the poly-ubiquitin chain of an ERAD substrate, which allows binding by Rad23p and Dskp, an action that results in movement of the misfolded protein to the proteasome. Once at the proteasome, it is de-ubiquitinated, unfolded, and degraded.

While much progress has been made regarding the mechanism by which misfolded proteins are degraded, there are still quite a few unknowns. Misfolded protein recognition appears to involve different chaperones for the ERAD-L and ERAD-C pathways, yet it is unknown how a misfolded membrane protein is identified. Also, protein ubiquitination is a fairly well understood process, but the details concerning the retrotranslocation of ERAD substrates, which occurs in conjunction with or following ubiquitination, are unknown. The identity of the retrotranslocon, or whether a retrotranslocon even exists, is a major question in the field. While the Cdc48p complex is essential for ERAD, its exact role has not been ascertained. In addition, the role of the proteasome in ERAD is not completely understood. Its role in protein proteolysis is well-established, but it is possible that this large complex or the many different factors which associate with it are involved in the steps upstream of degradation. Through the work in the following chapters, I have been able to answer many questions pertaining to our understanding of ERAD, specifically regarding the identification and retrotranslocation of misfolded membrane proteins.

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Chapter 2

The Hrd1p ubiquitin ligase specifies recognition of integral membrane ERAD substrates

Abstract

Hrd1p is the E3 ligase for the HRD pathway, which mediates ER-associated degradation (ERAD) of numerous misfolded or poorly assembled proteins in eukaryotes. HRD substrates fall into two broad classes: soluble luminal and membrane-anchored proteins, called ERAD-L and ERAD-M substrates. The degradation of membrane-anchored ERAD-M substrates can occur independently of the recognition factors implicated in ERAD-L, and Hrd1p is rate limiting for ERAD-M. Thus, Hrd1p plays a central role in the seemingly autonomous detection of this class of proteins. We wondered if the transmembrane domain was involved in detection of ERAD-M targets. The Hrd1p transmembrane domain is rich with hydrophilic residues, which would be appropriate for detection of misfolded domains in the intrabilayer milieu. Accordingly we mutated each of the hydrophilic residues of the Hrd1p transmembrane region, as well as residues that are highly conserved in the transmembrane domains of numerous Hrd1 homologues. From this collection of 77 mutants, we found Hrd1p variants that selectively stabilize ERAD-M substrates. Furthermore, we found Hrd1p mutants that strongly stabilize individual ERAD-M substrates with no effect on other ERAD-M or ERAD-L substrates. Thus, the Hrd1p transmembrane region bears determinants of high specificity in the ERAD-M pathway. Experiments focusing on the Hmg2-specific 3A-Hrd1p mutant support a model in which Hrd1p binding alone is not sufficient to promote ubiquitination. Instead, the folding state of the candidate proteins is assessed and specifically transmitted through the membrane domain to trigger ubiquitination. Thus, there appears to be an “allosteric” component to the activation of Hrd1p by ERAD-M substrates that is key to the high specificity of this branch of the HRD pathway.

Introduction

The endoplasmic reticulum associated degradation (ERAD) pathway mediates the destruction of numerous ER-localized proteins (Hampton 2002). ERAD functions mainly in the disposal of misfolded or unassembled proteins, but also participates in the physiological regulation of various functional proteins that reside in the ER (Hampton and Rine 1994; Ravid et al. 2006). The ERAD pathway has been implicated in a wide variety of normal and pathophysiological processes, including sterol synthesis, rheumatoid arthritis, fungal differentiation, cystic fibrosis, and several neurodegenerative diseases (Hampton and Rine 1994; Swanson et al. 2001; Zhang et al. 2002; Amano et al. 2003; Liang et al. 2006). Accordingly, there is great impetus to understand the molecular mechanisms that mediate this broadly important route of protein degradation.

ERAD proceeds by the ubiquitin-proteasome pathway, in which an ER-localized substrate is covalently modified by the addition of multiple copies of 7.6 kD ubiquitin in order to form a multi-ubiquitin chain that is recognized by the cytosolic 26S proteasome (Voges et al. 1999). Ubiquitin is added to the substrate by the successive action of three enzymes (Pickart 2001). The E1 ubiquitin activating enzyme uses ATP to covalently activate and then add ubiquitin to an E2 ubiquitin conjugating (UBC) enzyme. Ubiquitin is then transferred from the ubiquitin-charged E2 to the substrate or the growing ubiquitin chain by the action of an E3 ubiquitin ligase, resulting in a substrate-attached multi-ubiquitin chain that is recognized by the proteasome, leading to degradation of the ubiquitinated substrate.

The HRD pathway is one of the principal routes of ERAD in eukaryotes, being responsible for the degradation of both luminal and membrane-bound ER proteins (Vashist and Ng 2004). The HRD pathway E3 ligase is the highly conserved Hrd1p, which is rate-limiting for degradation of both classes of substrates (Bays et al. 2001a). Hrd1p is a multi-spanning ER membrane protein, consisting of an N-terminal membrane anchor linked to a soluble C-terminal domain with a RING-H2 domain characteristic of many E3 ligases (Figure 2-1, 2-2). The C-terminal region is responsible for catalyzing transfer of ubiquitin from the appropriate E2s to ERAD substrates, and this activity can be demonstrated *in vitro* (Bays et al. 2001a). However, successful degradation of ERAD substrates requires the presence of the Hrd1p membrane anchor, either as the full-length protein, or in some circumstances when expressed in trans with the active C-terminal region (Gardner et al. 2000). The multi-spanning Hrd1p membrane domain has numerous known functions, including binding to and communication with the luminal domain of Hrd3p, correct placement of the C-terminal ligase domain, and recruitment of ERAD factors for recognition of misfolded proteins, and for later steps in the pathway such as retrotranslocation (Gardner et al. 2000; Neuber et al. 2005; Bazirgan et al. 2006). The original substrate of the HRD pathway is Hmg2p, a yeast isozyme of the enzyme HMG-CoA reductase (HMGR), which is rate-limiting for sterol synthesis in eukaryotes, and the major clinical target for pharmaceutical management of cholesterol (Hampton et al. 1996a). Hmg2p undergoes regulated entry into the HRD pathway, so that when production of sterol pathway products is high, HRD-dependant degradation of Hmg2p is more rapid (Gardner and Hampton 1999). In this way, ERAD is employed as part of

feedback regulation of sterols, and a similar mechanism operates in mammals (Goldstein and Brown 1990).

HRD pathway substrates fall into two broad categories: soluble luminal proteins such as CPY*, or integral membrane proteins such as Pdr5* (Knop et al. 1996; Plemper et al. 1998). These prototype proteins are both misfolded mutants, but each substrate category is quite large, indicating that substrates are recognized by structural criteria that transcend the absence of any primary sequence relatedness between the various members of each group. In the case of luminal proteins, a variety of factors have been proposed to mediate the recognition of features of luminal ERAD substrates for presentation to the HRD machinery. The features appear to include both glycosylations that mark substrates as unfoldable, and structural aspects of misfolded proteins themselves, such as exposure of hydrophobic patches. The classic chaperone Kar2p, the luminal lectins Htm1p and Yos9p and the ER-anchored luminal domain of Hrd3p have all been implicated in recognition of luminal ERAD substrates (Jakob et al. 2001; Denic et al. 2006). However, Kar2p, Htm1p and Yos9p are not required for degradation of membrane-bound substrates such as Hmg2p, and Hrd3p is not required for membrane substrate degradation if sufficient Hrd1p is present. Similarly, the prototype derlin, Der1p, while absolutely required for degradation of luminal substrates, appears to play no role in the degradation of integral membrane substrates such as Hmg2p or Pdr5* (Plemper et al. 1998; Sato and Hampton 2006).

Because of these distinctions between luminal and membrane-anchored substrates, the degradation of each class of proteins is now referred to as ERAD-L for the luminal substrate pathway, and ERAD-M for the integral membrane pathway. In striking

contrast to the success of identifying factors for recognition of ERAD-L substrates, little is known about how membrane proteins are recognized as ERAD substrates.

In many cases in the ubiquitin pathway, the E3 ubiquitin ligase is the primary mediator of substrate recognition. Sometimes, a subunit of an E3 will directly bind to a substrate (Xia et al. 2008). In other cases, the substrate must be modified to allow recognition (Pickart and Eddins 2004). Thus, we wondered if the HRD ubiquitin ligase Hrd1p plays a direct role in ERAD-M substrate recognition. Although less is known about what features an aberrant, or “misfolded” membrane protein might possess, one simple idea is that those features would be presented within or near the lipid bilayer. A correctly folded and assembled integral membrane protein would be expected to present no free hydrophilic groups within the lipid region of the membrane, but rather would have each sequestered, or associated by hydrogen bonding with similar groups to maintain the energetically favorable isolation of such groups from the lipid environment. Binding to similar groups in an integral membrane E3 could result in detection of such “exposed hydrophiles”. With this idea in mind, the Hrd1p transmembrane anchor in fact has a high proportion of hydrophilic R groups in its six transmembrane spans that might serve such a detection function (Figure 2-2, and (Deak and Wolf 2001)).

As part of a systematic analysis of the Hrd1p transmembrane region, we have studied the effects of mutating these hydrophilic groups, along with other residues that are highly conserved in Hrd1p orthologues, to query the mechanisms of specific substrate recognition. By this approach, we have discovered a direct role for the Hrd1p transmembrane domain in the specific recognition of ERAD-M substrates, without the need for any of the factors that mediate the recognition and presentation of ERAD-L

substrates to the HRD pathway. Furthermore, a detailed analysis of one of our recognition-deficient mutants indicates a role for the transmembrane domain in modulation of the activity of the ligase upon encountering a substrate, consistent with our earlier studies on Hrd1p-substrate mechanisms. Thus, Hrd1p bears a code for detection of misfolded proteins in a membrane environment. Unraveling this code will have important implications in understanding the many processes that pertain to management of protein quality in normal and pathological cellular states.

Results

To gain a better understanding of Hrd1p's function in ERAD, we evaluated the role of specific transmembrane residues in Hrd1p-dependent degradation. The Hrd1p transmembrane region contains a large number of intra-bilayer hydrophilic amino acids, which we targeted for mutation (Figure 2-1, Figure 2-2). We also compared the sequence of the *Saccharomyces cerevisiae* transmembrane region to that of human Hrd1, human gp78, *Schizosaccharomyces pombe* Hrd1 and *Yarrowia lipolytica* Hrd1, to identify conserved residues, as they might be expected to have key roles in Hrd1 action. In total, over 70 distinct Hrd1 mutants were created in which single amino acid residues were changed to alanine.

We were particularly interested in exploring if Hrd1p participates in the specific recognition of distinct ERAD substrates. The Hrd1p loss of function mutants we have encountered previously, such as the C399S RING mutant, show a general inability to degrade ERAD substrates, and also result in strong stabilization of Hrd1p itself (Bays et al. 2001a). However, if Hrd1p participates in the specific detection of substrates, it should be possible to find mutants deficient in degradation of distinct classes of substrates, or perhaps even deficient in degradation of individual substrates.

For each of our Hrd1p mutants, we performed a series of tests to evaluate that residue's importance in Hrd1p-dependent ERAD. Specifically, we evaluated ERAD-M, ERAD-L, and Hrd1p's self-catalyzed degradation, since all three modes of HRD-dependent degradation have distinct rules and requirements (Gardner et al. 2000; Vashist and Ng 2004; Carvalho et al. 2006). To assess ERAD-M, we tested the degradation of the integral ER membrane protein Hmg2p by each Hrd1p variant. We used a non-

1 MVPENRRKQLAIFVVVVTYLLTFYCVYSATKTSVSFLQVTLKLN~~EGFNL~~MV
 51 LSIFILLNSTLLWQLLTKLLFGELRLIEHEHIFERLPFTIINTLFMSSLF
 101 HERYFFTVAFFGLLLYLKVFHWILKDRLEALLQSINDSTTMKTLIFSRF
 151 SFNLVLLAVVDYQIITRCISSIYTNQKSDIESTSLYLIQVMEFTMLLIDL
 201 LNLFQTCLNFWEFYRSQQSLSNENNHIHV

RING domain

Blue = intramembrane hydrophile

Red = conserved membrane domain

Figure 2-1 Amino acids of interest in the Hrd1p transmembrane domain

The amino acid sequence of the *HRD1* N-terminal transmembrane region. Underlined residues highlight the six transmembrane spans as defined by Deak and Wolf (2001). Residues in bold are those which we mutated to alanine in order to determine their role in Hrd1p-dependent degradation. Residues in blue are hydrophilic amino acids within the bilayer. Residues in red are conserved between different Hrd1p homologues. Circled residues are those which resulted in the phenotypes described in this work. Yellow residues when mutated result in an Hmg2p-specific defect. Turquoise residue when mutated resulted in a Pdr5*-specific defect.

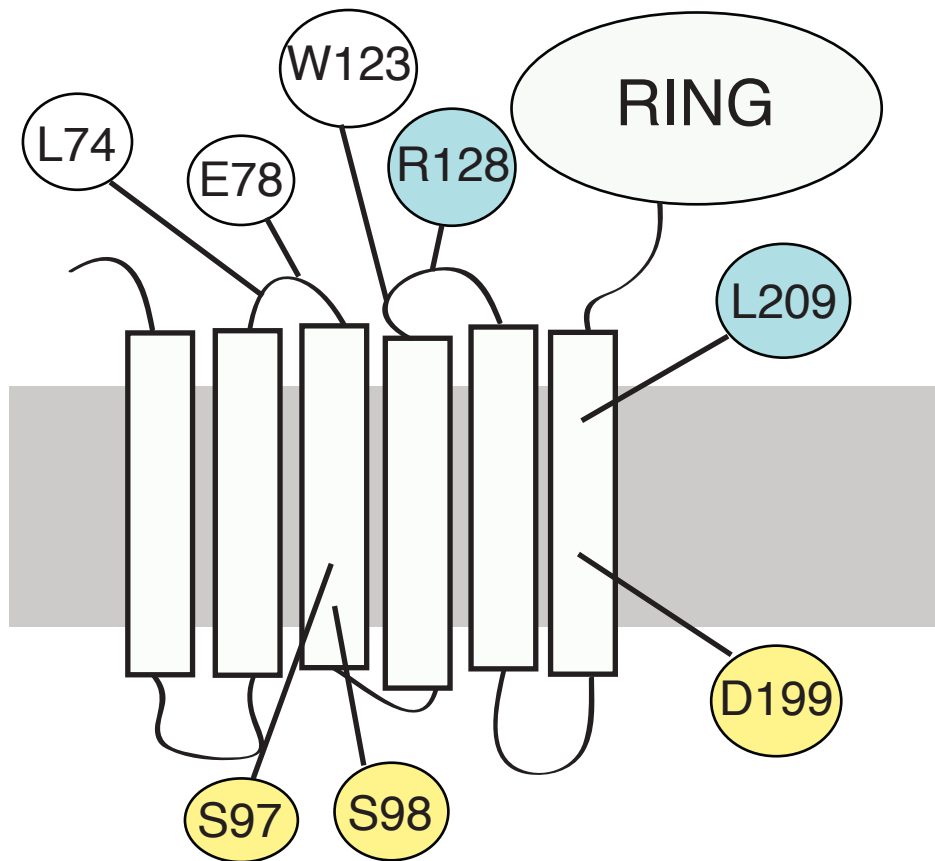


Figure 2-2 Hrd1p residues with ERAD defects when mutated

The image depicts the Hrd1p protein in the membrane and the relative positions of the residues of interest in the transmembrane region. Yellow residues when mutated result in an Hmg2p-specific defect. Turquoise residue when mutated results in a Pdr5*-specific defect.

catalytic Hmg2p-GFP, which allows evaluation of protein stability by flow cytometry or immunoblotting (Hampton et al. 1996b). To evaluate ERAD-L, each Hrd1p mutant was screened with KWW (Vashist and Ng 2004). KWW is an engineered substrate of viral origin, with a misfolded luminal domain, that enters the HRD pathway by ERAD-L. Finally, we tested each mutant Hrd1p for self-degradation. In the absence of Hrd3p, Hrd1p undergoes extremely rapid degradation catalyzed by its own RING domain (Gardner et al. 2000). This self-degradation has been posited to be important for Hrd1p regulation, and appears to be distinct from both ERAD-L and ERAD-M (our unpublished results, Carroll and Hampton manuscript in preparation). Thus, to test these aspects of Hrd1p function, each individual Hrd1p mutant was transformed into *hrd1Δ* strains expressing Hmg2p-GFP, KWW, or a *hrd3Δ* strain, allowing examination of the mutant's effects on ERAD-M, ERAD-L, and Hrd1p self-degradation, respectively. The effect of each Hrd1p mutant on substrate stability was assayed by cycloheximide chase, in which log phase cultures were treated with cycloheximide to stop protein synthesis, followed by flow cytometry or immunoblotting to determine substrate degradation rate. Interesting mutants were then studied further with other substrates and assays that addressed mechanistic features of ERAD.

One group of mutants showed a clear specificity for integral membrane, or ERAD-M, substrates. Hrd1p variants L74A Hrd1p, E78A Hrd1p and W123A Hrd1p were all impaired in the degradation of Hmg2p-GFP (Figure 2-3A). We next examined the degradation of two other ERAD-M substrates, 6myc-Hmg2p-GFP and Pdr5*, two other misfolded proteins that undergo HRD-pathway dependent degradation. 6myc-Hmg2p-GFP is a misfolded version of Hmg2p which does not respond to the degradation

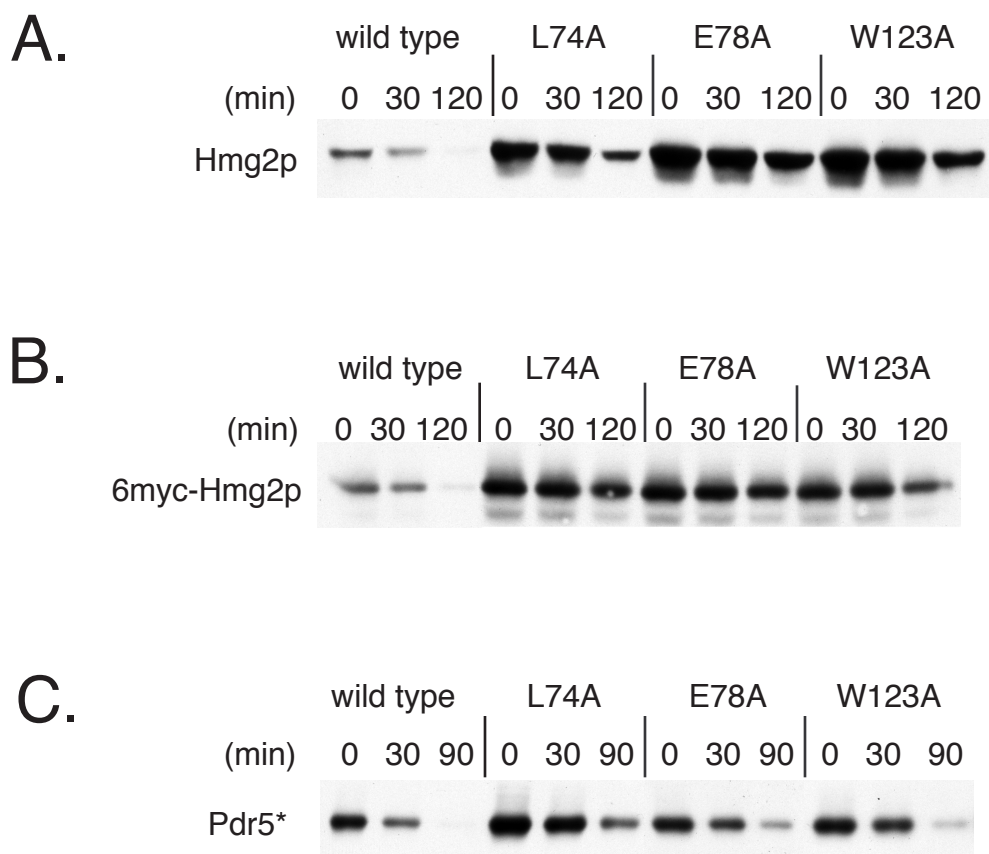


Figure 2-3 The above Hrd1p amino acids were important for the degradation of ERAD-M substrates

(A-C). Degradation of the tagged ERAD substrates was measured by cycloheximide chase in isogenic strains. Each isogenic *hrd1Δ* strain was transformed with the indicated version of *HRD1* or an empty vector in the case of *hrd1Δ* strains. Log phase cultures were grown and following cycloheximide addition, cells were lysed at various time points and analyzed by SDS-PAGE and immunoblotting. Total protein levels in each lane were equal as was verified by India ink staining (data not shown).

signals of the sterol pathway, and thus is constitutively degraded (Hampton et al. 1996a). All three Hrd1p mutants stabilized 6myc-Hmg2p-GFP and Pdr5* (Figure 2-3B, 2-3C). However, when we tested these three variants with ERAD-L substrates, we discovered that their deficiency was limited to only integral membrane substrates. We examined the degradation of both the prototype substrate CPY* and KWW and found that each mutant was fully competent for degradation of each substrate (Figure 2-4A, 2-4B). In all cases, the levels of each Hrd1p variant was identical to wild type, and each underwent normal, rapid degradation in the absence of Hrd3p (data not shown). Thus, the residues mutated in L74A Hrd1p, E78A Hrd1p and W123A Hrd1p were required for ERAD-M, yet were dispensable for the degradation of misfolded luminal substrates and Hrd1p self-degradation.

The above mutants show a selective deficiency for membrane-associated substrates, without any effect on luminal ones. In the next set of mutants, the specificity is even more striking, revealing different Hrd1p transmembrane determinants for recognition of different ERAD-M substrates. One of the most intriguing mutants we generated involved three intramembrane hydrophilic residues. Two distinct primary Hrd1p mutants, S97A/S98A, and D199A, were tested for their ability to degrade Hmg2p-GFP. Each of these mutants was partially defective in Hmg2p-GFP degradation (Figure 2-5A). When combined, the resulting triple mutant, S97A S98A D199A Hrd1p (which we will refer to as 3A-Hrd1p) showed a strong Hmg2p-GFP degradation block (Figure 2-5B). In fact, when compared to C399S Hrd1p, which contains a non-functional RING domain, the 3A-Hrd1p demonstrated a nearly identical degradation defect towards

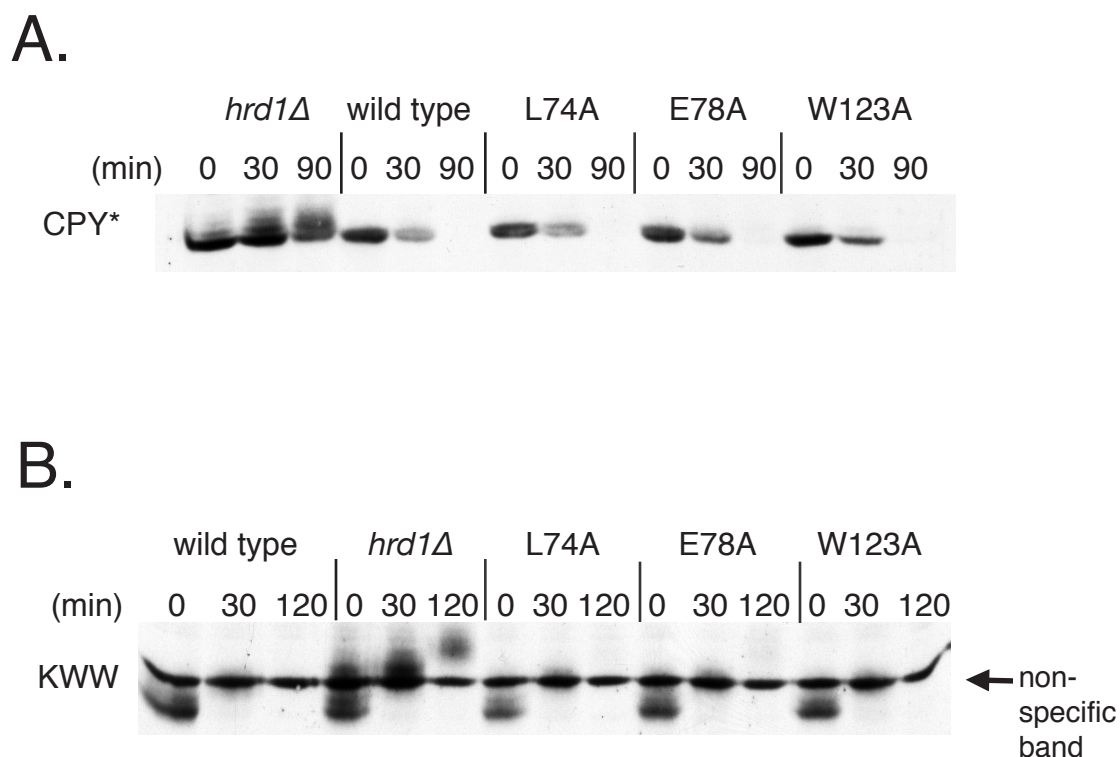


Figure 2-4 The above Hrd1p amino acids were dispensable for ERAD-L degradation

(A-B). Degradation of the tagged ERAD substrates was measured by cycloheximide chase in isogenic strains. Each isogenic *hrd1Δ* strain was transformed with the indicated version of *HRD1* or an empty vector in the case of *hrd1Δ* strains. Log phase cultures were grown and following cycloheximide addition, cells were lysed at various time points and analyzed by SDS-PAGE and immunoblotting. Total protein levels in each lane were equal as was verified by India ink staining (data not shown).

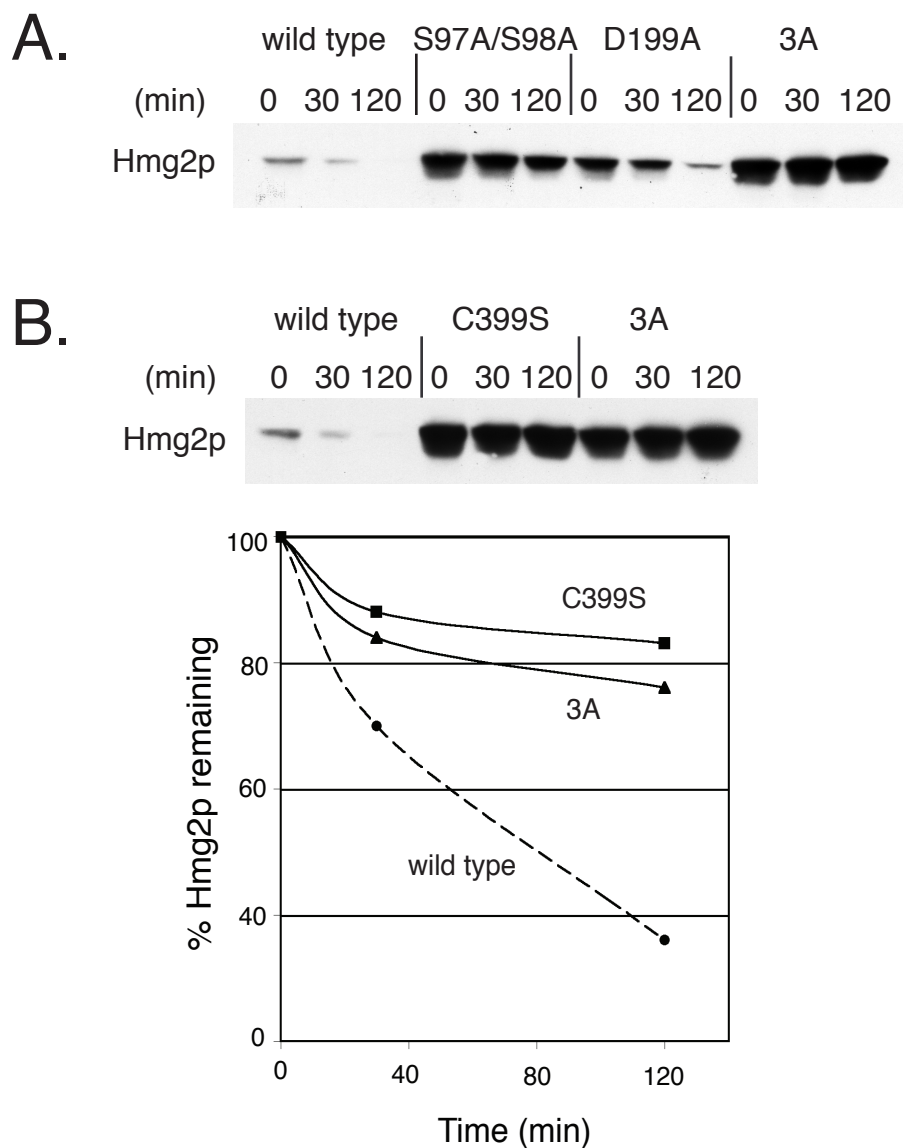


Figure 2-5 3A-Hrd1p was incapable of degrading Hmg2p-GFP

(A-B). Cycloheximide chases were performed as described in on isogenic *hrd1Δ* strains transformed with the indicated version of *HRD1*. 3A Hrd1p is S97A S98A D199A Hrd1p. (B). Hmg2p degradation was quantified using flow cytometry. Log phase cultures were grown and cycloheximide was added at varying time points to allow for simultaneous analysis of all cultures by a flow cytometer at the end of the experiment. Each point on the graph represents the mean fluorescence of 10,000 cells, with a standard error of the mean of $\pm 1\%$.

Hmg2p-GFP. 3A-Hrd1p showed a similarly strong defect in degradation of the related substrate 6myc-Hmg2p-GFP (Figure 2-6).

Hrd1p is an E3 ubiquitin ligase, but it is also in complex with components that mediate retrotranslocation (Carvalho et al. 2006). To evaluate where in the ERAD pathway 3A-Hrd1p-mediated stabilization of Hmg2p occurred, we directly tested the ability of this mutant to support Hmg2p ubiquitination using immunoprecipitation of Hmg2p-GFP followed by immunoblotting for Hmg2p or ubiquitin (Bays et al. 2001a). While Hmg2p-GFP was ubiquitinated in strains expressing wild type Hrd1p, there was a lack of ubiquitination in strains with the RING domain mutant, C399S Hrd1p, or 3A-Hrd1p (Figure 2-7). This defect was not alleviated by addition of zaragozic acid, which increases the physiological signal for Hmg2p degradation (Hampton and Bhakta 1997).

Alteration of these three specific amino acids may have produced a hypomorphic Hrd1p mutant. To test whether higher protein expression could complement the Hmg2p degradation deficiency, we overexpressed 3A-Hrd1p by placing it behind the strong TDH3 promoter. This resulted in an approximately twenty-fold increase in Hrd1p levels above the native promoter (data not shown). Despite the higher levels of 3A-Hrd1p when driven by the TDH3 promoter, Hmg2p-GFP degradation was still greatly impaired (Figure 2-8) compared to degradation by wild type Hrd1p. Thus, 3A-Hrd1p seemed to be intrinsically defective in Hmg2p degradation, even at high levels of this variant.

The 3A-Hrd1p mutant is a virtual null for degradation of Hmg2p and 6myc-Hmg2p. To test whether this was a general impairment for all ERAD substrates, we examined the degradation of a number of ERAD-L proteins by 3A-Hrd1p. In striking contrast to Hmg2p, the degradation of the ERAD-L substrates CPY*, KHN and KWW

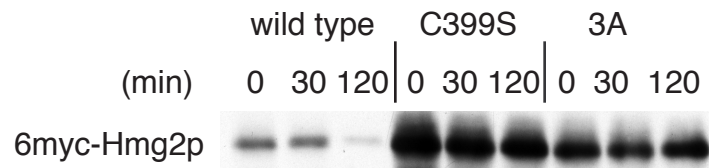


Figure 2-6 3A-Hrd1p was incapable of degrading 6myc-Hmg2p-GFP

Cycloheximide chases were performed as described on isogenic *hrd1Δ* strains transformed with the indicated version of *HRDI*. Total protein levels in each lane were equal as was verified by India ink staining (data not shown).

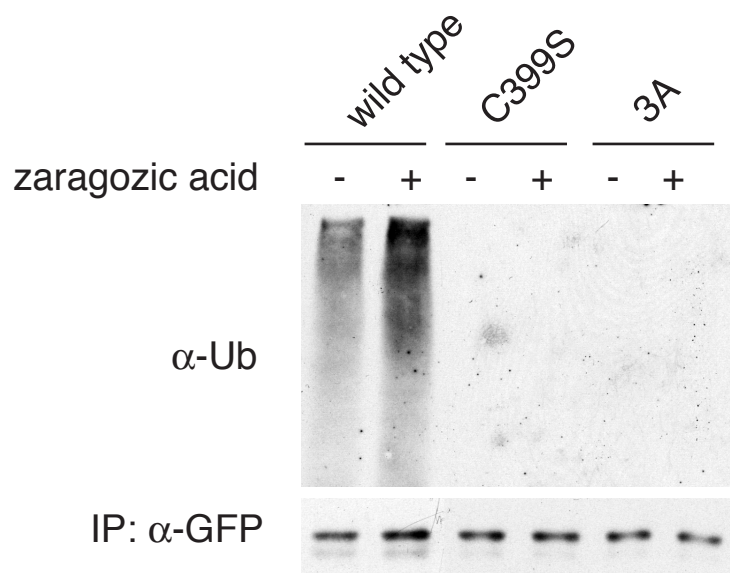


Figure 2-7 3A-Hrd1p was incapable of ubiquitinating Hmg2p-GFP

Hmg2p-GFP ubiquitination was assayed through a ubiquitin immunoprecipitation. Strains expressing the indicated versions of Hrd1p were grown to log phase and treated with DMSO or 10 μ g/ml zaragozic acid. They were then lysed and subjected to an anti-GFP immunoprecipitation. The resulting pull-down was analyzed by SDS-PAGE and immunoblotted with an anti-GFP or anti-ubiquitin antibody.

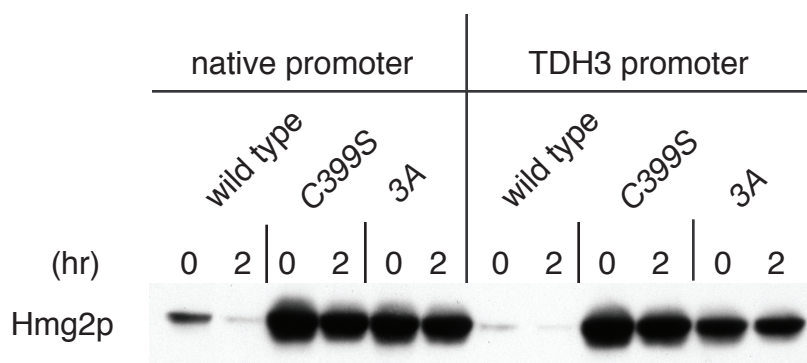


Figure 2-8 Overexpression of 3A-Hrd1p did not complement the Hmg2p-GFP degradation defect

Cycloheximide was added for two hours to isogenic *hrd1* Δ strains transformed with native promoter driven or TDH3-driven versions of *HRD1*. Lysates were analyzed as previously described.

were all degraded normally by 3A-Hrd1p, while showing the expected stabilization by C399S Hrd1p (Figure 2-9A, 2-9B, 2-9C). Thus, 3A-Hrd1p was completely competent for the degradation of luminal ERAD substrates, despite its near-null phenotype with Hmg2p degradation.

The Hmg2p degradation defect of 3A-Hrd1p led us to test other ERAD-M substrates, anticipating that they would show a similarly strong block in degradation. Surprisingly, Pdr5* was degraded identically in the presence of wild type or 3A-Hrd1p (Figure 2-10A), while it was stable in strains expressing C399S Hrd1p. We next tested the ability of 3A-Hrd1p to degrade Sec61-2p. *SEC61* is an essential gene, encoding the integral membrane protein Sec61p, which is responsible for translocation of nascent proteins into the ER. Strains with the *sec61-2* mutation are temperature sensitive, due to Hrd1p-mediated degradation that leads to lethally low levels of the Sec61-2 protein (Biederer et al. 1996). When Hrd1p is non-functional, *sec61-2* strains will grow at the normally non-permissive temperature 37°C. To test whether 3A-Hrd1p was capable of recognizing and degrading Sec61-2p, *sec61-2* strains expressing either wild type Hrd1p, C399S Hrd1p or 3A-Hrd1p were grown at 30°C and 37°C. All strains grew at similar rates at 30°C. *sec61-2* strains with wild type or 3A-Hrd1p were severely impaired for growth at elevated temperatures (Figure 2-10B), while those with the non-functional C399S Hrd1p showed robust growth at elevated temperatures, since the Sec61-2 protein could not be degraded (Bordallo et al. 1998). Thus, both wild type Hrd1p and 3A-Hrd1p were capable of Sec61-2p degradation. Finally, we evaluated the ability of 3A-Hrd1p to catalyze its own degradation. 3A-Hrd1p stability was tested both in the presence and absence of Hrd3p by cycloheximide chase. Like wild type Hrd1p, 3A-Hrd1p was stable

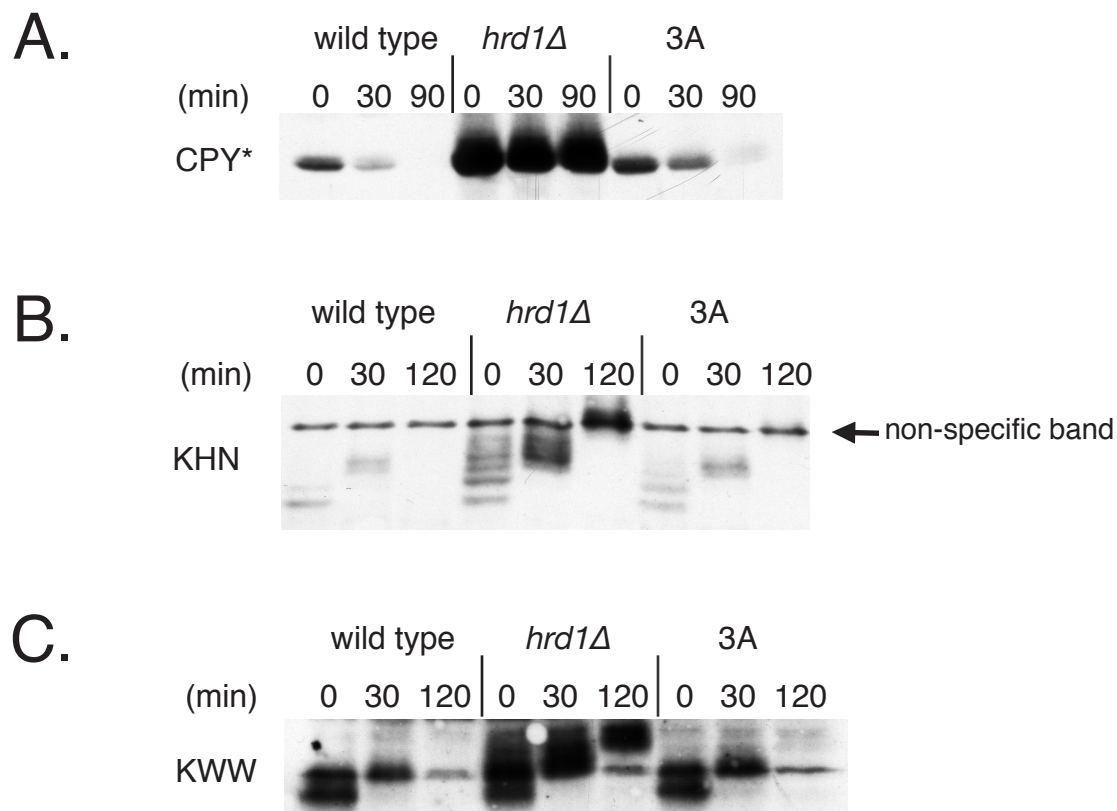


Figure 2-9 3A-Hrd1p was proficient in the degradation of ERAD-L substrates

(A-C). Cycloheximide chases were performed as described in Figure 2. Isogenic *hrd1Δ* strains were transformed with the indicated *HRD1* or an empty vector in *hrd1Δ* conditions. Total protein levels in each lane were equal as was verified by India ink staining (data not shown).

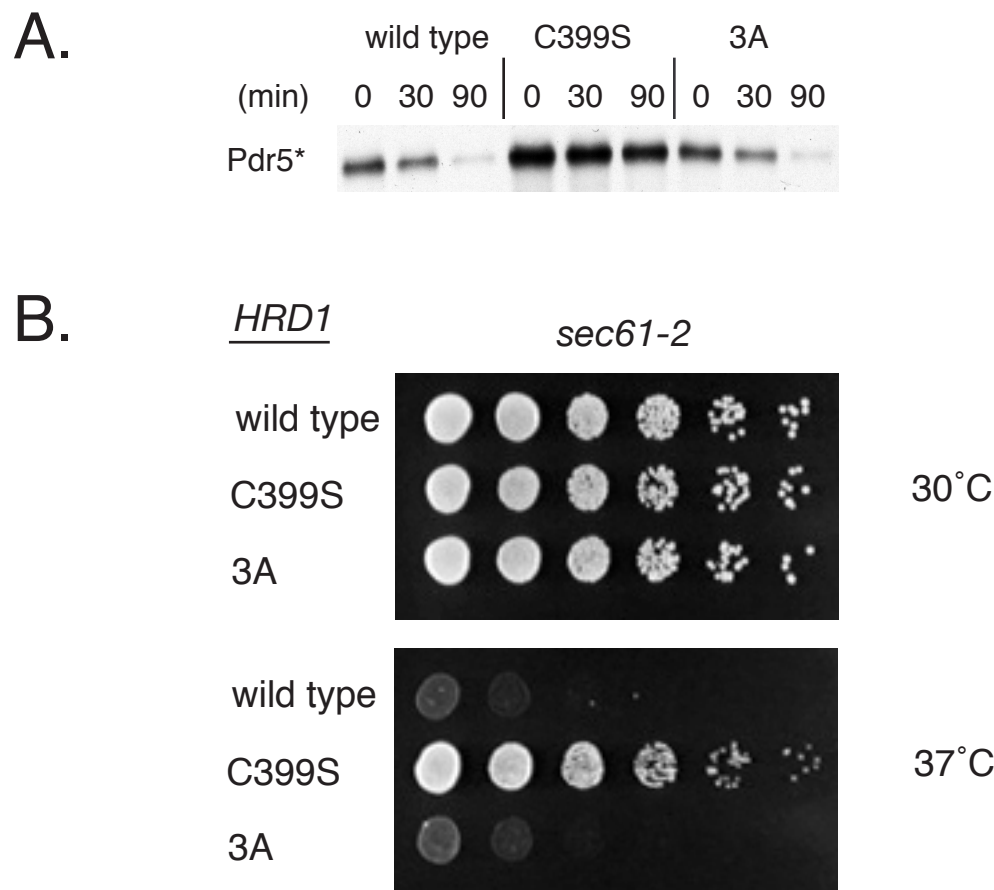


Figure 2-10 3A-Hrd1p was proficient in the degradation of non-Hmg2p related ERAD-M substrates

(A). Cycloheximide chases were performed as described. Isogenic *hrd1Δ* strains were transformed with the indicated *HRD1*. Total protein levels in each lane were equal as was verified by India ink staining (data not shown). (B). Degradation of Sec61-2p was assayed through a dilution assay examining the temperature sensitivity of *sec61-2* strains. A *sec61-2 hrd1Δ* strain was transformed with the indicated *HRD1*s. Cells were grown to log phase and spotted at 5-fold dilutions. Plates were grown at either 30°C or 37°C.

in the presence of Hrd3p, but underwent rapid degradation in a *hrd3Δ* strain (Figure 2-11A). In addition, this self-degradation was dependent on a functional RING domain (Figure 2-11B). Taken together, these data show that 3A-Hrd1p was impaired only in the degradation of Hmg2p-GFP or Hmg2p variants like 6myc-Hmg2p-GFP, but completely normal for other ERAD-M and ERAD-L substrates, as well as self-catalyzed degradation.

The 3A-Hrd1p mutant had a strikingly specific deficiency in ERAD function. To get a broader sense of the degree to which 3A-Hrd1p functions normally, we evaluated the unfolded protein response (UPR) in strains harboring the 3A mutant. In *hrd1Δ* or C399S Hrd1p strains, the lack of the HRD pathway causes a buildup of misfolded proteins and results in increased signaling through the UPR pathway (Friedlander et al. 2000). Using a GFP reporter for UPR (Bays et al. 2001b), we found that strains with the 3A-Hrd1p mutant had wild-type levels of UPR activity, while the C399S mutant strain had the expected increase in this signaling pathway (Figure 2-12). Thus, by this measure also, the 3A mutant showed normal function in an assay that requires recognition of what is presumably a wide variety of misfolded proteins that are typically generated during the course of normal ER function.

3A-Hrd1p has alterations in three hydrophilic amino acids that make it incapable of recognizing Hmg2p as a misfolded protein while maintaining essentially wild-type degradation of itself and all other ERAD substrates tested. The simplest interpretation of this observation is that distinct residues in the Hrd1p transmembrane domain mediate recognition of a given ERAD-M substrate, presumably through interactions with features of the protein that hallmark misfolding or aberrant assembly. If that is the case, perhaps other Hrd1p transmembrane mutants in our collection that degrade Hmg2p normally

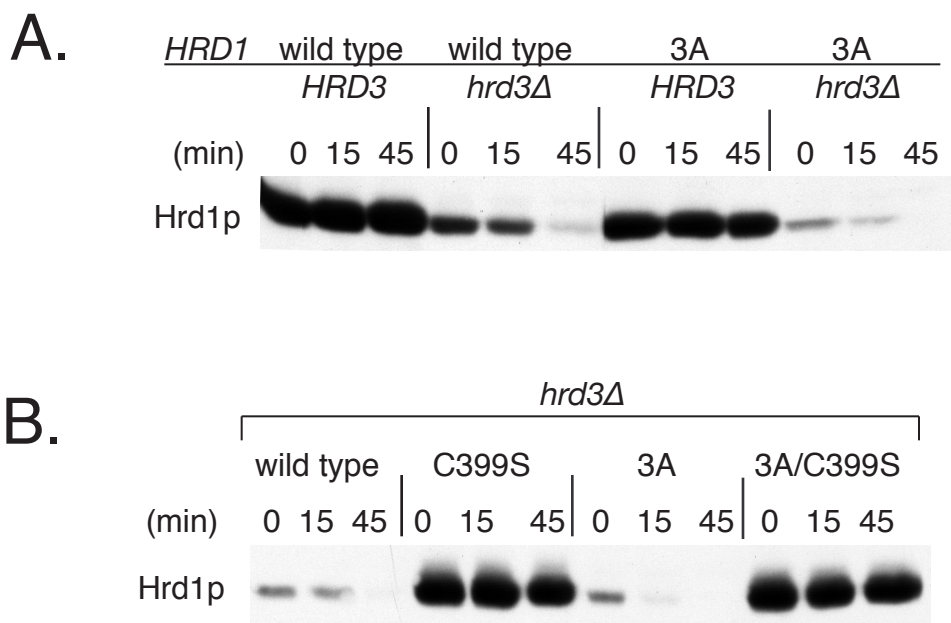


Figure 2-11 3A-Hrd1p was proficient in Hrd1p self-degradation

(A-B). Cycloheximide chases were performed as described. Isogenic *hrd1Δ* strains were transformed with the indicated *HRD1*. Total protein levels in each lane were equal as was verified by India ink staining (data not shown). 3A-Hrd1p experienced self-degradation in a C399S dependent manner. Hrd1p self-degradation was tested by cycloheximide chase of isogenic wild type and *hrd3Δ* strains expressing the indicated Hrd1p proteins.

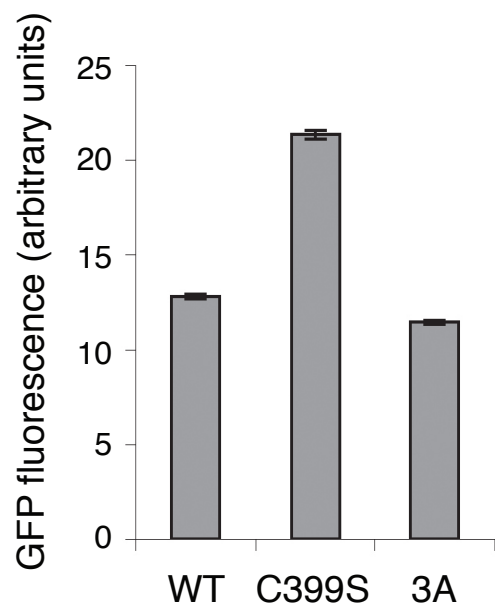


Figure 2-12 A strain expressing 3A-Hrd1p did not upregulate UPR

Strains expressing the UPRE4-GFP reporter and the indicated versions of Hrd1p were grown to log phase and the GFP fluorescence was measured by flow cytometry. Each bar represents 10,000 cells analyzed and the standard error of the mean is noted.

might have deficiencies in degradation of a distinct ERAD-M substrate, due to loss of residues needed for specific recognition of that substrate. Accordingly, we re-screened our collection of over 70 Hrd1p point mutants for the inability to degrade another ERAD-M substrate, Pdr5*. We found two such candidates. Strains expressing only R128A Hrd1p were fully proficient in Hmg2p degradation (Figure 2-13A), yet Pdr5* degradation was impaired (Figure 2-13B). Similarly, L209A showed a strong bias towards Pdr5* with a defect that rivaled C399S (Figure 2-14). In contrast, degradation of Hmg2p-GFP was slightly compromised, showing a small increase in steady-state levels but a wild-type degradation rate when quantified by flow cytometry (Figure 2-15). Sec61-2p degradation by L209A was also similar to wild type Hrd1p as measured by the growth phenotype of the *sec61-2* strain (Figure 2-16A). CPY* degradation and Hrd1p self-degradation were also only mildly slowed in the L209A mutant (Figure 2-16B, 2-16C). The L209A mutant has a specific lesion that is nearly the opposite of the 3A-Hrd1p mutant: Pdr5* is stabilized to the same extent as the C399S ring mutant, while Hmg2p degradation was only very slightly affected. Taken together, the diametric substrate specificities of 3A-Hrd1p on one hand, and L209A on the other, indicate that distinct residues of Hrd1p mediate recognition of distinct ERAD substrates.

The above studies show that the specificity for ERAD-M substrate recognition requires information in the transmembrane domain of Hrd1p. We focused our attention to the 3A-Hrd1p mutant to further understand the mechanism of ERAD-M substrate recognition mediated by the Hrd1p transmembrane domain.

In studies on the recognition of ERAD substrates, both Hrd3p and Yos9p have been implicated in binding to misfolded luminal proteins as an initial event in their

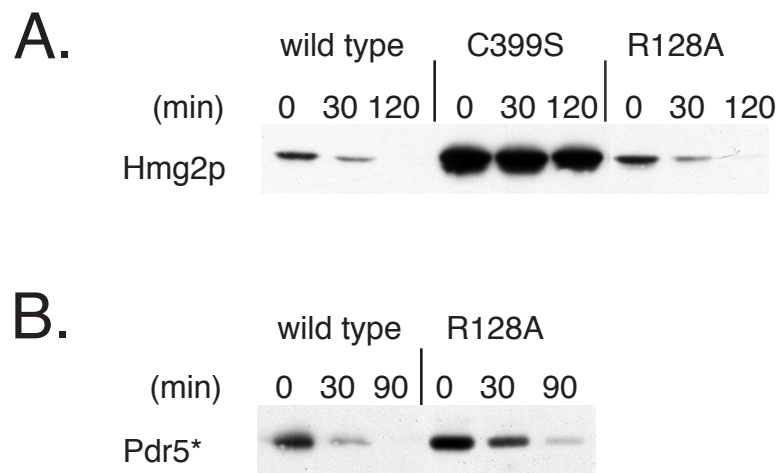


Figure 2-13 R128A Hrd1p demonstrated a specific defect for Pdr5* degradation

(A-B). Cycloheximide chases were performed as described on isogenic *hrd1Δ* strains transformed with the indicated Hrd1p protein. Total protein levels in each lane were equal as was verified by India ink staining (data not shown).

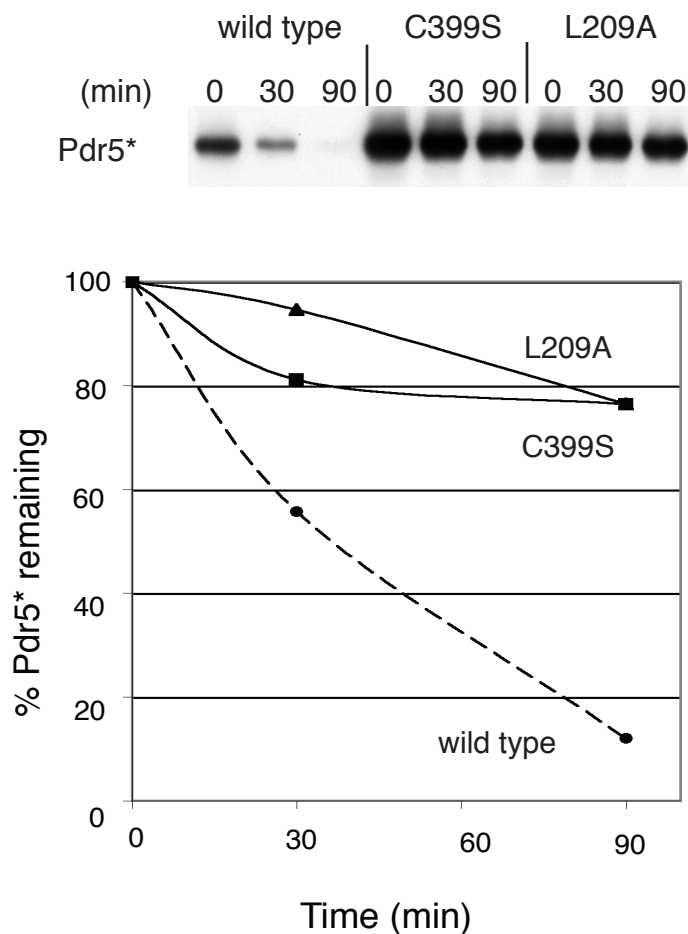


Figure 2-14 L209A Hrd1p had a null phenotype for Pdr5* degradation

Cycloheximide chases were performed as described on isogenic *hrd1Δ* strains transformed with the indicated Hrd1p protein. Total protein levels in each lane were equal as was verified by India ink staining (data not shown). Statistical analysis was obtained from the average of two experiments with a Typhoon 9400.

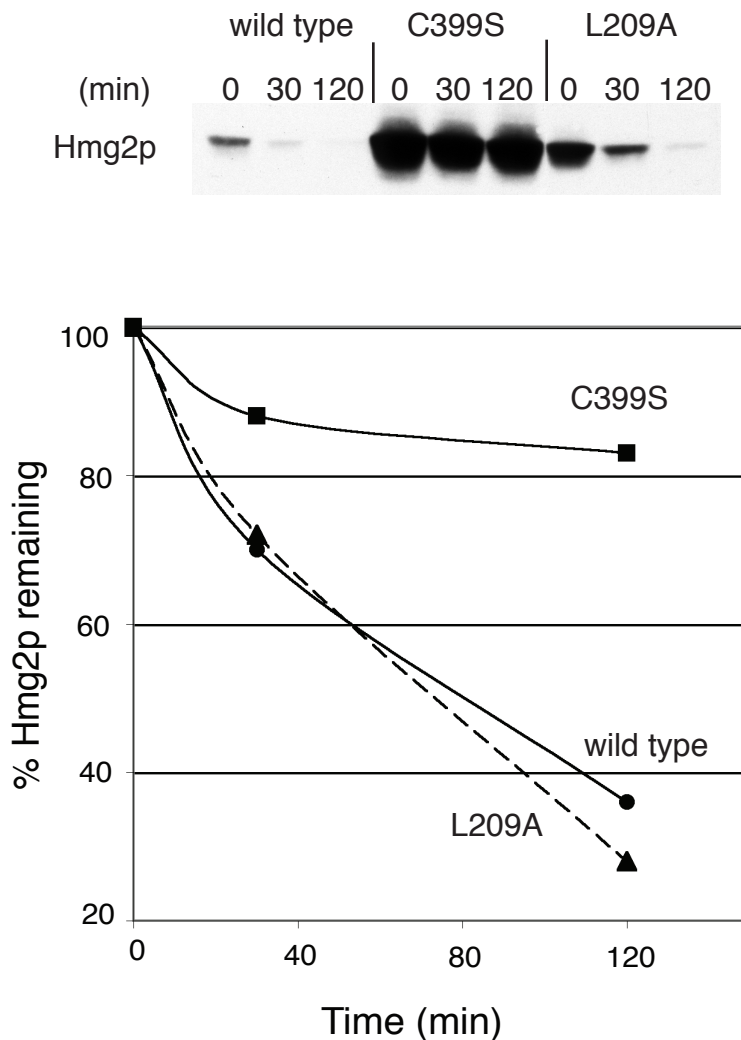


Figure 2-15 L209A Hrd1p degraded Hmg2p-GFP at a wild type rate

Cycloheximide chases were performed as described on isogenic *hrd1Δ* strains transformed with the indicated Hrd1p protein. Total protein levels in each lane were equal as was verified by India ink staining (data not shown). Statistical analysis was performed by flow cytometry with strains expressing Hmg2p-GFP. Each point represents 10,000 cells with a standard error of the mean of $\pm 1\%$.

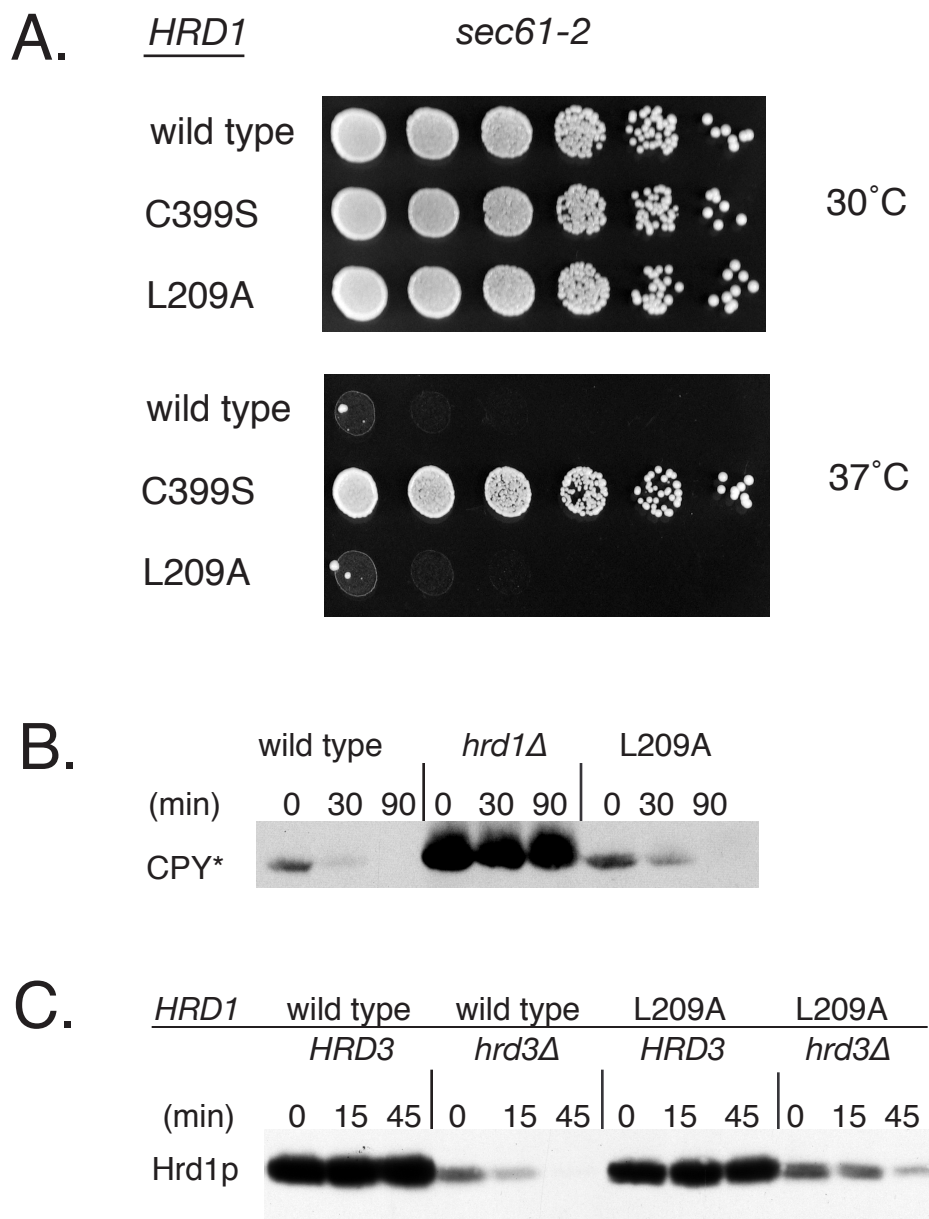


Figure 2-16 L209A Hrd1p degraded another ERAD-M substrate with wild type kinetics and had a minor defect in the degradation of other proteins

(A). Sec61-2p stability was assayed by dilution assay as described previously. (B). A cycloheximide chase utilizing strains expressing the indicated version of Hrd1p was performed as described. (C). Isogenic *hrd1Δ* or *hrd1Δhrd3Δ* strains transformed with the indicated *HRD1* were utilized to examine Hrd1p self-degradation.

presentation to the HRD complex. In contrast, Hrd1p-dependent degradation of membrane proteins can proceed in the absence of either of these proteins: Hmg2p degradation proceeds normally in a *yos9Δ* null (our unpublished data), and in the *hrd3Δ* null if levels of Hrd1p are sufficiently elevated to overcome its rapid degradation (Gardner et al. 2000). As a test of the autonomy of Hmg2p recognition mediated by the mutated 3A-Hrd1p residues, we compared the 3A mutant to wild-type Hrd1p, both in a *yos9Δ* and a *hrd3Δ* strain. In the *yos9Δ*, we compared the two Hrd1ps from the native promoter. In the *hrd3Δ* null, we overexpressed Hrd1p to overcome the drastic loss of either Hrd1p that occurs in the absence of Hrd3p. In both cases, the striking difference between Hrd1p and 3A-Hrd1p in degradation of Hmg2p-GFP was evident, and not dependent on either of these luminal substrate recognition factors (Figure 2-17, 2-18).

We next turned our attention to the role the residues defined by the 3A mutation played in Hmg2p recognition. In general, E3 ligases must bind a targeted substrate, and in some cases this appears to be sufficient to program ubiquitination (Bays et al. 2001a). However, it is not known if substrates need only to bind to ligases, or if in addition, the substrate must activate or transmit information to the ligase to bring about robust poly-ubiquitination. Our cross-linking studies indicate that for Hrd1p the latter model might be the case (Gardner et al. 2001). Hrd1p will cross-link degraded substrates Hmg2p and 6myc-Hmg2p, as well as the non-degraded K6R-Hmg2p, or the highly stable homologue Hmg1p with similar efficiencies (Gardner et al. 2001). In this case, substrate interaction is not sufficient for Hrd1p-mediated ubiquitination. Thus, we evaluated the 3A-Hrd1p mutant to determine whether the three residues were required for Hmg2p interaction or the subsequent transmission of structural information to the Hrd1p RING domain.

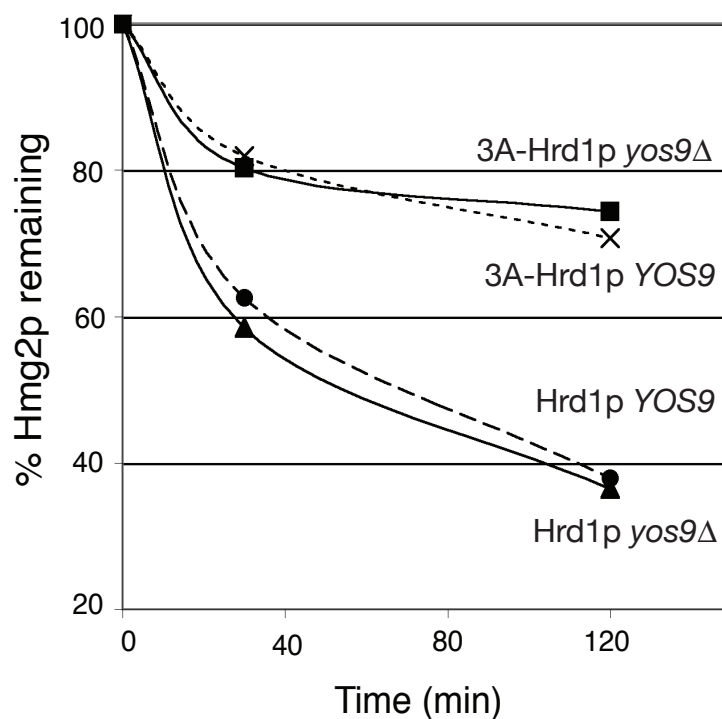
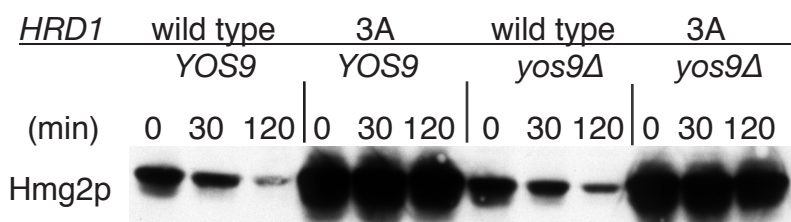


Figure 2-17 The Hmg2p-GFP degradation phenotypes of wild type and 3A-Hrd1p did not require the luminal recognition factor Yos9p

Isogenic *YOS9* and *yos9Δ* strains expressed either wild type or 3A-Hrd1p. These strains were tested for Hmg2p-GFP degradation upon the addition of cycloheximide as measured by both SDS-PAGE/immunoblotting and flow cytometry as previously described. Total protein levels in each lane were equal as was verified by India ink staining of the nitrocellulose (data not shown).

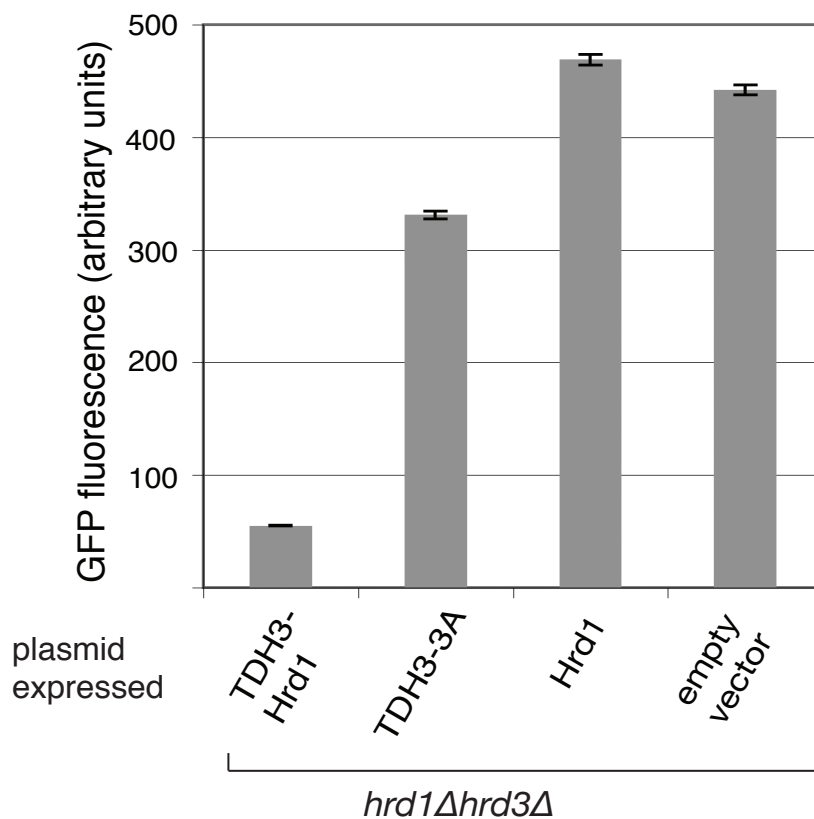


Figure 2-18 The Hmg2p-GFP degradation phenotypes of wild type and 3A-Hrd1p did not require the luminal recognition factor Hrd3p

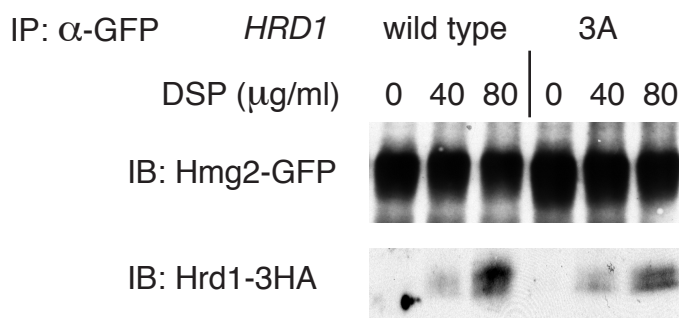
A *hrd1Δhrd3Δ* strain expressing Hmg2p-GFP was transformed with either TDH3 driven Hrd1p, TDH3 driven 3A-Hrd1p, native Hrd1p or an empty vector. Hmg2p-GFP steady state levels were obtained by a flow cytometer, with the standard error of the mean as indicated.

We tested whether Hmg2p-GFP was capable of interacting with 3A-Hrd1p. We utilized an *in vitro* crosslinking assay, in which ER-enriched microsomes were harvested from cells expressing Hmg2p-GFP and wild type or 3A-Hrd1p tagged with triple HA. A lipid-soluble crosslinker was added to the microsomes, followed by an anti-GFP immunoprecipitation. The resulting pull-down was then immunoblotted for Hmg2p-GFP and Hrd1p-3HA. From this experiment, it was clear that both wild type and 3A-Hrd1p associated with Hmg2p-GFP in a crosslinker-dependent manner (Figure 2-19A).

The crosslinking data implied that Hmg2p's interaction with 3A-Hrd1p was unaltered in 3A-Hrd1p expressing strains. We further evaluated this with an independent assay of Hmg2p-Hrd1p binding, a native co-immunoprecipitation. Again, microsomes were isolated and added to a 1.5% Tween-20 lysis buffer. An anti-GFP antibody was then added to the lysates in order to immunoprecipitate Hmg2p-GFP. Under these conditions, Hmg2p-GFP bound to wild type or 3A-Hrd1p with equal efficiency (Figure 2-19B). This interaction was specific, as a control immunoprecipitation with a strain lacking Hmg2p-GFP was unable to pull down Hrd1p. Thus, 3A-Hrd1p was capable of binding to Hmg2p-GFP.

The appropriate substrate interaction was intact in the microsomes isolated for the binding assays above. We used the same microsomes prepared for the native co-immunoprecipitation experiment to directly evaluate the ability of the 3A-Hrd1p to ubiquitinate Hmg2p-GFP in an *in vitro* ubiquitination assay. In this assay, microsome donor strains lacking Ubc7p and a cytosol donor strain with Ubc7p but lacking Hrd1p and Hmg2p-GFP were utilized as described (Flury et al. 2005). Microsomes, cytosol, and ATP were incubated for 1 hour at 30°C, producing substrate ubiquitination that is

A. Cross-linking



B. Native Co-Immunoprecipitation

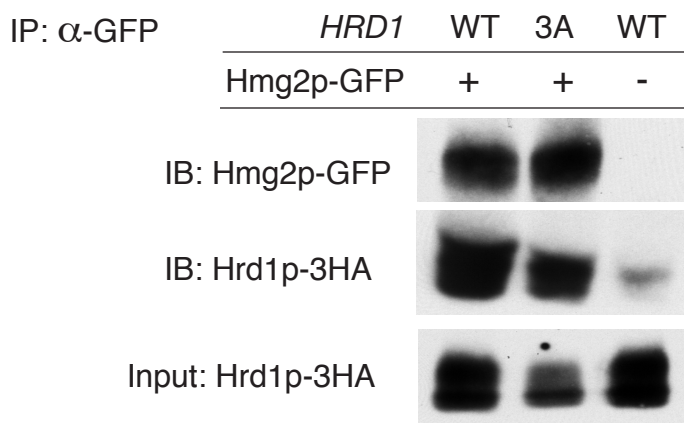


Figure 2-19 3A-Hrd1p association with its substrate Hmg2p-GFP was intact

(A). Isogenic strains expressing wild type or 3A-Hrd1p tagged with 3HA were grown to log phase. Microsomes were harvested from each strain and DMSO or increasing concentrations of the cross linker DSP was added. An anti-GFP immunoprecipitation was then performed and Hmg2-GFP and Hrd1-3A levels were measured by SDS-PAGE and immunoblotting. (B). A native co-immunoprecipitation was performed with microsomes isolated from strains expressing 3HA-tagged wild type or 3A-Hrd1p. Again, Hmg2p-GFP was immunoprecipitated with an anti-GFP antibody and the pull-down was immunoblotted for Hmg2p-GFP and Hrd1p-3HA levels.

both E2 and ATP dependent. Following the reaction, we performed an anti-GFP immunoprecipitation and immunoblotted for Hmg2p-GFP and ubiquitin. Only wild type Hrd1p was capable of Hmg2p-GFP ubiquitination *in vitro* (Figure 2-20A). This defect was specific for Hmg2p, as 3A-Hrd1p showed self-ubiquitination in the same *in vitro* reaction and was also capable of transferring ubiquitin to another substrate molecule, Pdr5* (Figure 2-20B, 2-21). Although Hrd1p and Hmg2p-GFP binding was unaffected by the introduction of the 3A mutations, 3A-Hrd1p could not ubiquitinate Hmg2p-GFP, indicating that substrate binding alone is insufficient to trigger Hrd1p-dependent ubiquitination. Thus, the 3A-defined residues were involved in transmission of specific structural information that allows processive ubiquitination.

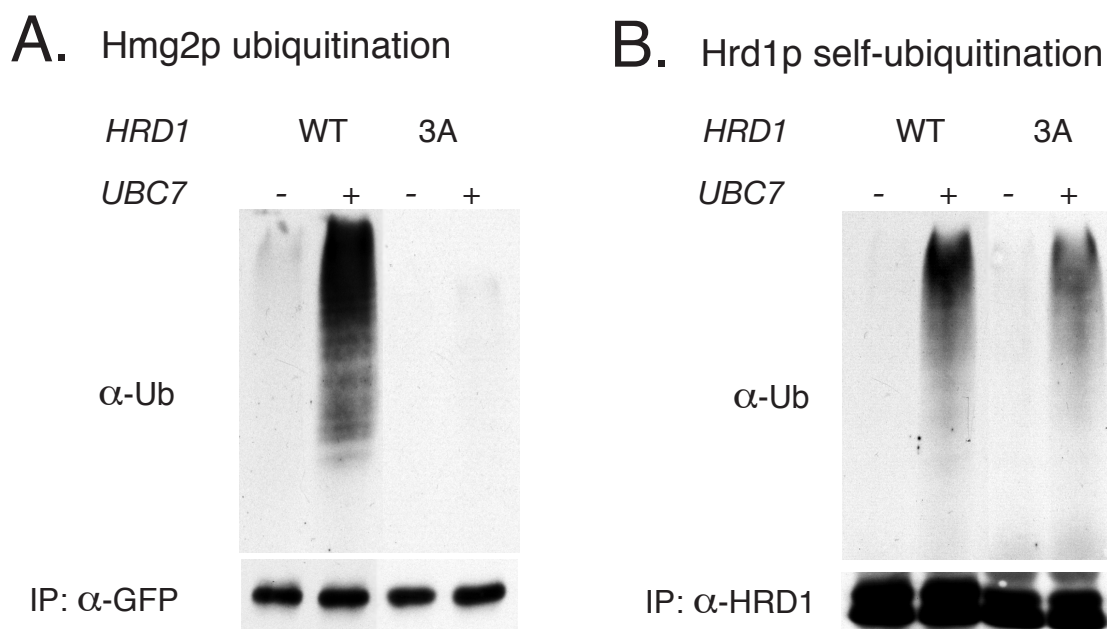


Figure 2-20 3A-Hrd1p is incapable of Hmg2p ubiquitination yet can catalyze self-ubiquitination *in vitro*

(A). The same microsomes utilized in the native co-IP experiment were added to cytosol from strains overexpressing Ubc7p to assay *in vitro* ubiquitination of Hmg2p-GFP. Microsomes, cytosol, and ATP were incubated for 1 hour at 30°C and then subjected to an anti-GFP immunoprecipitation. The immunoprecipitate was probed for ubiquitin and Hmg2p-GFP by SDS-PAGE and immunoblotting. The observed Hmg2p-GFP ubiquitination was absent when microsomes were incubated with *ubc7* Δ cytosol.

(B). The same experiment was performed as in (A) but an anti-Hrd1p antibody was used for the immunoprecipitation to examine the extent of Hrd1p self-ubiquitination *in vitro*.

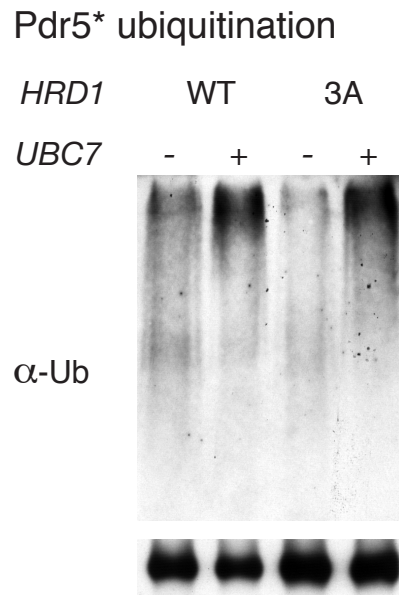


Figure 2-21 3A-Hrd1p can catalyze Pdr5* ubiquitination *in vitro*

Microsomes expressing Pdr5* were isolated and mixed with cytosol lacking or containing Ubc7p. Reactions were performed as previously described. Following the reaction, Pdr5* was immunoprecipitated with anti-HA antibodies.

Discussion

The recognition of misfolded proteins is a central and unresolved process in all protein quality control pathways. The diversity of substrates of a given pathway precludes using specific sequence motifs for substrate detection. Rather, it must be structural features that serve as criteria for substrate recruitment to degradative quality control pathways. Since E3 ligases are critical definers of specificity, misfolded substrate detection must include, in some manner, the ligase.

For Hrd1p, both luminal (ERAD-L) and integral membrane (ERAD-M) substrates are targets for degradation. Recognition of ERAD-L substrates appears to be relegated to factors that associate with the Hrd1p protein, such as Yos9p, Kar2p, and the luminal domain of Hrd3p (Denic et al. 2006). It is not clear if Hrd1p also contributes some intrinsic ability to recognize this class of substrates, but these ancillary factors are clearly of central importance in the recruitment of ERAD-L candidates.

Our studies with mutant versions of Hrd1p indicate that this ubiquitin ligase directly mediates the recognition of membrane-bound substrates, using information in the transmembrane domain to discern the appropriateness of a protein for HRD pathway ubiquitination. We first described Hrd1p mutants which possess selective defects in ERAD-M substrate targeting, without alteration in the destruction of ERAD-L substrates. Taken alone, this could simply mean that Hrd1p recruits different, unknown recognition factors for ERAD-M, and the various mutants were defective in recruitment or use of these factors. However, we further found mutants that were selective nulls for one ERAD-M substrate with little or no effect on another, and other mutants with orthogonal specificity. These substrate-specific ERAD-M mutants were also entirely normal for

ERAD-L recognition. Thus, extreme specificity for ERAD-M substrates lies in the transmembrane domain of Hrd1p, such that this part of the ligase determines the recognition of a specific substrate or a specific subset of proteins that display common features of misfolding that herald HRD pathway entry. It is worth noting that none of the nearly 80 mutants we made had any selective defects in ERAD-L. This implies that distinct rules govern recognition of ERAD-M substrates, although a more complete analysis of the Hrd1p membrane domain is needed to fully test the idea that ERAD-L recognition lies outside of the membrane anchor.

3A-Hrd1p is essentially a phenocopy of a C399S RING mutant, but only for Hmg2p-related substrates. We used our assays for Hrd1p ligase action on Hmg2p as a window into the manner that the Hrd1p transmembrane region participates in substrate recognition. We had previously demonstrated that Hrd1p interacts with Hmg2p by crosslinking (Gardner et al. 2001), and this interaction is preserved in the 3A mutant. Our new, independent native co-immunoprecipitation (co-IP) assay similarly showed that the Hrd1p-Hmg2p interaction was unaffected by the strongly stabilizing 3A mutations. However, *in vitro* Hrd1-dependent ubiquitination of Hmg2p, assayed in the same microsomes used for the co-IP experiments, was completely absent when 3A-Hrd1p was expressed, yet 3A-Hrd1p still functioned as a ligase, showing normal self-ubiquitination. Thus, the high specificity of the 3A mutation is not due to any measurable loss of interaction with Hmg2p, but rather to an inability of the still-active 3A-Hrd1p mutant to transfer ubiquitin to an Hmg2p that is in its proximity.

In our earlier interaction studies, we had noted that Hrd1p was able to interact with potential substrates in a fairly indiscriminant manner (Gardner et al. 2001). For

example, the highly stable Hmg1p isozyme, and mutants of Hmg2p that were similarly resistant to HRD-mediated degradation still showed crosslinking to Hrd1p that was indistinguishable from that observed with normally degraded Hmg2p. The model we proposed from these observations was that Hrd1p queries a variety of proteins by low-specificity interactions, and only when a given substrate has the appropriate structural features does ubiquitination occur. We believe that the residues modified in the 3A mutant transmit structural information about misfolded Hmg2p to the Hrd1p complex so that ubiquitination will proceed.

This model of ERAD-M substrate detection by Hrd1p pertains to a more general issue about ubiquitin ligases. One question that often arises is whether a ligase-substrate interaction is sufficient for ubiquitin transfer, or if there is some “allosteric” activation of the ligase complex that is additionally required for robust ubiquitination (Figure 2-22). There are cases in which imparting binding of a normally irrelevant protein to a ligase is sufficient for ubiquitination. For example, addition of an S-protein binding site to the Hrd1p or Doa10p RING domains allows specific ubiquitination of S-protein (Bays et al. 2001a; Swanson et al. 2001). However, in each of these cases, *in vitro* ubiquitination ceases after a few transfer reactions. In contrast, full-length membrane-bound Hrd1p ubiquitinates authentic substrates with great processivity *in vitro* (Figure 2-20A and (Flury et al. 2005)) and the difference between the engineered binding experiments and the processing of bone-fide substrates may be a demonstration of an informational or allosteric component in addition to substrate binding. At least for quality control substrates, this strategy of “general interaction-specific response” makes some teleological sense. A quality control ligase that is over-dedicated to interacting with only

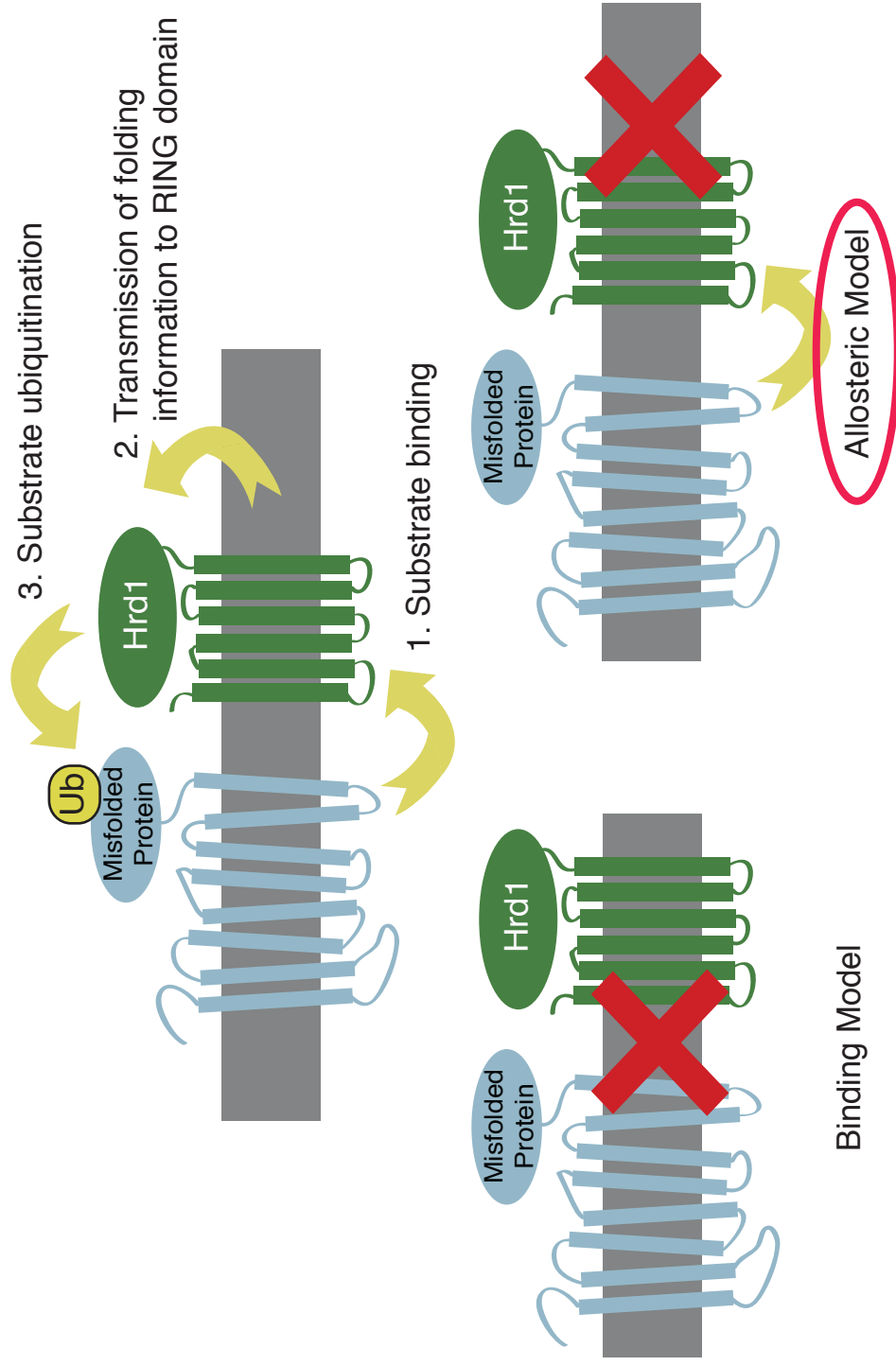


Figure 2-22 Hrd1p-dependent ubiquitination requires both binding and transmission of folding information to activate the RING domain

Binding alone is not sufficient to allow ubiquitination. Instead, it appears an allosteric component is also required in order for ubiquitination to occur.

a particular type of substrate would not be efficient in the general detection of the very large number of possible misfolded proteins that it might encounter. A useful analogy for such a ligase would be a garbage collection service that shows high specificity, removing only empty pizza boxes for disposal. The appropriate approach is instead to search for and remove all categories of garbage, including pizza boxes along with all other refuse.

The detection of ERAD-M substrates, that is, misfolded or unassembled membrane proteins, might be expected to follow rules distinct from those used to detect aqueous ERAD-L substrates. A misfolded aqueous protein would display a larger-than-normal proportion of surface hydrophobic residues, and indeed, proteins that detect misfolded soluble proteins, such as chaperones or UGGT (Dejgaard et al. 2004), have regions that can interact with exposed hydrophobic regions of their clients. Conversely, it is reasonable to imagine that misfolding of an integral membrane protein could result in inappropriate exposure of normally buried hydrophilic residues in the lipid region of the bilayer. Detection of these inappropriate residues could be accomplished by the ligase displaying membrane-embedded hydrophilic residues to interact with the inappropriately exposed lipid-phase hydrophilic residues of the substrate. Removal of these “detection” residues from the ligase would prohibit recognition of the misfolded substrate that signals through them. The 3A mutant of Hrd1p has three intramembrane residues changed from S, S and D to alanine, and the combined effect of these changes is a near-null for ubiquitination of only Hmg2p. Because the 3A mutant still interacts with Hmg2p in a manner indistinguishable from the wild-type Hrd1p, it would appear that these hydrophilic residues are involved in deciphering misfolding information, rather than binding of a misfolded substrate. In a similar manner, the R128A mutant had a specific,

albeit smaller, defect in Pdr5* degradation. Thus, it is likely that detection of uncomplexed hydrophilic residues is one broadly used strategy by which ERAD-M substrates are evaluated.

In a similar vein, it has been suggested that the TUL1 ubiquitin ligase recognizes unassembled membrane protein clients by use of hydrophilic intramembrane residues to detect uncomplexed substrate hydrophilic residues exposed by lack of a binding partner (Reggiori and Pelham 2002). It is interesting to note that the quality control ligases mammalian Hrd1, gp78, and yeast Doa10p each have a high density of intramembrane hydrophilic residues, as would be expected if hydrophilic scanning was a general strategy for membrane substrate evaluation. Thus, this simple strategy may be broadly employed in many circumstances that demand recognition of aberrant membrane proteins.

Direct loss of such “hydrophilic scanning” residues is probably not the only lesion in some of our mutants. The L209A mutant, which has slight general ERAD defects but is nearly null for the Pdr5* degradation has a missing intramembrane leucine, and presumably this alteration creates a structural change that specifically alters Pdr5* binding or evaluation. The three mutants, L74A, E78A, and W123A, that are selectively deficient for ERAD-M but not ERAD-L are all in short cytoplasmic loops. We would speculate that these mutants are deficient in actions that mediate ERAD-M specific processes common to all these substrates.

This study, in addition to a number of others, has demonstrated that the Hrd1p transmembrane domain plays a number of different roles. It acts as both a scaffold for a multi-protein ERAD complex as well as actively participates in the identification of misfolded proteins. Another reasonable role for the Hrd1p transmembrane region is as a

retrotranslocon, that is, a channel through which ERAD substrates are removed from the ER. It should be noted that none of the Hrd1p mutants highlighted above were defective in retrotranslocation as tested in an *in vitro* retrotranslocation assay (data not shown). We have also addressed this question in a current study characterizing this *in vitro* assay (Garza et al. manuscript in preparation). However, further analysis of Hrd1p mutants will help determine whether Hrd1p indeed plays a role in retrotranslocation.

Taken together, these results show that ERAD-M is directly and specifically mediated by the Hrd1p transmembrane domain. It appears that the transmembrane domain bears an “allosteric code” for detection of features that hallmark a degradation substrate, and this information appears to be discrete, so that the loss of recognition of a single substrate class can be observed in the appropriate mutant. Hopefully, the detailed structure of the Hrd1p transmembrane domain, and the complete collection of interaction partners for this protein will help us understand this code, and the rules by which misfolded proteins are invited to their destruction.

Materials and Methods

DNA manipulation and plasmid construction

All plasmids were constructed with standard molecular biology tools as has been described (Sato and Hampton 2006). The splicing by overlap elongation (SOEing) PCR technique (Horton et al. 1989) was utilized in the creation of Hrd1p point mutants.

Plasmid table available in supplemental materials (Table 2-1). Oligo sequences used for PCR are available upon request. The Pdr5* plasmid (pRH2312) was a generous gift from D. Wolf.

Yeast and Bacterial strains

Escherichia coli DH5 α were grown at 37°C in LB media with ampicillin (100 μ g/ml). Yeast strains were grown at 30°C unless otherwise noted in minimal media supplemented with dextrose and amino acids as previously described (Hampton and Rine 1994). The LiOAc method was utilized to transform yeast strains with plasmid DNA (Ito et al. 1983). Knock-outs were constructed by transforming yeast with the LiOAc method with a PCR product that encoded either G418 resistance or CloNAT/nourseothricin (Werner BioAgents, Jena, Germany) resistance and contained 50bp flanks homologous to the gene to be knocked out. (Baudin et al. 1993). Cells were allowed to grow on yeast peptone dextrose (YPD) for ~12 hours and then replica plated onto YPD plus 500 μ g/ml G418 or 200 μ g/ml nourseothricin.

All parent strains, the plasmids transformed into them, and the figures in which they were utilized are listed in Table 2-2.

Degradation assays and UPR measurements

Cyclohexamide chase degradation assays were performed as previously described (Sato and Hampton 2006) with SUME lysis buffer (1% SDS, 8M Urea, 10mM MOPS pH 6.8, 10mM EDTA). Flow cytometry was also undertaken as described (Sato and Hampton 2006). Data was obtained through a FACScalibur machine (Becton, Dickinson and Company, Franklin Lakes, NJ) and statistical analysis was performed with CellQuest software (Becton, Dickinson and Company, Franklin Lakes, NJ). Pdr5* quantitation for L209A Hrd1p was performed using a Typhoon 9400 (GE Healthcare, Piscataway, NJ).

Ubiquitin Immunoprecipitation

Ubiquitination of Hmg2p-GFP was examined as previously described (Bays et al. 2001a). Cells were grown into log phase and incubated with 10 μ g/ml zaragozic acid or a DMSO control for 7 minutes. Following this treatment, 3 OD of cells were pelleted. 100 μ l of SUME with protease inhibitors and N-ethyl maleimide (NEM) and 100 μ l of glass beads were added to lyse the cells. 1ml of IP buffer (150mM NaCl, 15mM Na₂HPO₄, 2% Triton-X100, 0.1% SDS, 0.5% DOC, 10mM EDTA, pH 7.5) with protease inhibitors and NEM was added to the cell extracts and the mix was centrifuged for 5 minutes at 16,000 x g. The supernatant was removed and 15 μ l of polyclonal anti-GFP antibody was added. The mix was incubated overnight at 4°C. 100 μ l of Protein-A sepharose (Amersham Biosciences) in IP buffer, (50% w/v) was added for 2 hours. Beads were washed once with IP buffer and once with IP wash buffer (50mM NaCl, 10mM Tris, pH 7.5) and incubated with 50 μ l of 2x Urea sample buffer (75mM MOPS

pH 6.8, 4% SDS, 200mM DTT, 0.2mg/ml bromophenol blue, 8M urea) for 10 minutes at 50°C. The samples were then loaded on a polyacrylamide gel. Following transfer to nitrocellulose, immunoblotting with an anti-ubiquitin or anti-GFP antibody was performed.

Crosslinking assay

Crosslinking was modified from that used by Gardner *et al.* (Gardner et al. 2000). Cells were grown to log phase and 5 OD were harvested. Cells were resuspended in B88 buffer (20mM Hepes pH6.8, 250mM sorbitol, 150mM KOAc, 5mM MgOAc) and vortexed for 6 minutes (1 minute on, 1 minute off) at 4°C. Various concentrations of the crosslinker DSP (Pierce Biotechnology, Rockford, IL) were added to the microsomes for 40 minutes at room temperature. The crosslinker was then quenched with the addition of 50µl of 1M Tris pH 7.5 for 10 minutes. Microsomes were centrifuged, then lysed in 300µl SUME lysis buffer plus 1% Triton-X100, 0.5% DOC, and protease inhibitors. 1ml of IP buffer was then added to each sample along with 15µl of anti-GFP antibody and the remainder of the immunoprecipitation was performed as above.

Native co-immunoprecipitation

The native co-immunoprecipitation (co-IP) assay was adapted from Gardner *et al.* (Gardner et al. 2000). Microsomes were harvested as in the crosslinking protocol, except it was performed in MF buffer (20mM Tris pH7, 100mM NaCl, 300mM sorbitol). Pelleted microsomes were resuspended in 1ml of Tween-20 buffer (500mM NaCl, 50mM Tris pH7.5, 100mM EDTA, 1.5% Tween-20) and incubated on ice for 15 minutes.

Lysates were then centrifuged for 30 minutes at 14,000 x g. The remainder of the IP was performed as above except the Tween-20 buffer was utilized for washes instead of IP buffer and IP wash buffer.

Dilution Assays

Growth of *sec61-2* strains was measure by dilution assay. Dilution assays were performed as previously described (Sato and Hampton 2006).

***In vitro* ubiquitination**

In vitro ubiquitination assays were performed as previously described (Flury et al. 2005). Briefly, microsome donor strains, containing TDH3-Hrd1-3HA (wild type or 3A), Hmg2p-GFP and *ubc7*Δ, were harvested. Microsomes were prepared identically as in the native co-immunoprecipitation experiments and finally resuspended in B88 buffer. Cytosol donor strains were centrifuged and underwent freeze-thaw lysis in B88 buffer and ultracentrifuged. For each ubiquitination reaction, a microsome strain was combined with 30mM ATP and cytosol that either did or did not express TDH3-Ubc7-2HA for 1 hour at 30°C. Immunoprecipitations were then performed as described above.

Acknowledgements

I would like to thank Dieter Wolf for plasmids, Maho Niwa for use of her Typhoon 9400 and Michael David for use of his flow cytometer.

Chapter 2 is a manuscript in preparation that will be submitted for publication. Daniel Schulz assisted me with screening the *hrd1* mutants for Pdr5* degradation and with the native co-immunoprecipitation experiments. I was the primary experimenter and Randolph Hampton and I wrote the manuscript.

Table 2-1 Plasmids used in Chapter 2

Plasmid	Gene expressed
pRH311	<i>YIp TRP1</i>
pRH313	<i>YIp URA3</i>
pRH469	<i>YIp URA3 pTDH3-HMG2-GFP</i>
pRH507	<i>YIp TRP1 pHRD1</i>
pRH642	<i>YIp TRP1 pHRD1-3HA</i>
pRH730	<i>YIp TRP1 pTDH3-HRD1-3HA</i>
pRH808	<i>YIp TRP1 pTDH3-HRD1</i>
pRH1122	<i>hrd1Δ::KanMX deletion cassette</i>
pRH1152	<i>YIp LEU2 pTDH3-UBC7-2HA</i>
pRH1209	<i>YIp URA3 p4XUPRE-GFP</i>
pRH1245	<i>YIp TRP1 pHRD1-3HA C399S</i>
pRH1377	<i>YCp URA3 pCPY*-HA</i>
pRH1694	<i>YIp URA3 TDH3-6MYC-HMG2-GFP</i>
pRH1718	<i>YIp TRP1 TDH3-HRD1-3HA C399S</i>
pRH1958	<i>YCp URA3 p3HA-KHnt</i>
pRH1960	<i>YCp URA3 p3HA-KWW</i>
pRH2213	<i>YIp pHRD1-3HA S97A S98A</i>
pRH2248	<i>YIp TRP1 pHRD1-3HA D199A</i>
pRH2269	<i>YIp TRP1 pHRD1-3HA S97A S98A D199A</i>
pRH2287	<i>YIp TRP1 pHRD1-3HA S97A S98A D199A C399S</i>

Table 2-1 continued Plasmids used in Chapter 2

pRH2288	<i>YIp TRP1 pHRD1 S97A S98A D199A</i>
pRH2312	<i>YCp HIS3 pHA-PDR5*</i>
pRH2344	<i>YIp TRP1 pHRD1-3HA L74A</i>
pRH2345	<i>YIp TRP1 pHRD1-3HA E78A</i>
pRH2350	<i>YIp TRP1 pHRD1-3HA W123A</i>
pRH2352	<i>YIp TRP1 pHRD1-3HA R128A</i>
pRH2360	<i>YIp TRP1 pHRD1-3HA L209A</i>
pRH2361	<i>YIp TRP1 pHRD1 L74A</i>
pRH2362	<i>YIp TRP1 pHRD1 E78A</i>
pRH2364	<i>YIp TRP1 pHRD1 W123A</i>
pRH2365	<i>YIp TRP1 pTDH3-HRD1-3HA S97A S98A D199A</i>
pRH2366	<i>YIp TRP1 pTDH3-HRD1 S97A S98A D199A</i>
pRH2398	<i>YIp TRP1 pHRD1 L209A</i>

Table 2-2 Strains used in Chapter 2

Strain	Genotype
RHY2814	<i>MATα ade2-101 ura3-52::URA3::HMGcd::HMG2-GFP met2 lys2-801 trp1::hisG leu2Δ his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 hrd1Δ::KanMX</i>
RHY2933	<i>MATα ade2-101 ura3-52::URA3::HMG2-GFP met2 LYS2 trp1::hisG leu2Δ his3Δ200 hmg2Δ::1MYC-HMG2 hrd1Δ::KanMX</i>
RHY2936	<i>Matα ade2-101 ura3-52::URA3::HMG2-GFP met2 lys-801 trp1::hisG leu2Δ his3Δ200::HIS3::pep4Δ hrd1Δ::KanMX ubc7Δ::LEU2</i>
RHY3005	<i>MATα ade2-101 ura3-52::URA3::HMGcd::HMG2-GFP met2 lys2-801 trp1::hisG leu2Δ his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 hrd1Δ::KanMX hrd3Δ::LEU2</i>
RHY4288	<i>MATα ade2-101 ura3-52 met2 lys2-801 trp1::hisG leu2Δ his3Δ200 HMG1 hmg2Δ::1MYC-HMG2 hrd1Δ::KanMX pep4Δ::HIS3 ubc7Δ::LEU2</i>
RHY4295	<i>MATα ade2-101 ura3-52 met2 lys2-801 trp1::hisG leu2Δ his3Δ200 HMG1 hmg2Δ::1MYC-HMG2 hrd1Δ::KanMX pep4Δ::HIS3 ubc7Δ::LEU2 TRP1::TDH3-UBC7-2HA</i>
RHY6152	<i>MATα ade2-101 ura3-52::HMGcd::HMG2-GFP met2 lys2-801 trp1::hisG leu2Δ his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 hrd1Δ::KanMX URA3::KWW-HA</i>
RHY6245	<i>Matα ade2-101 ura3-52::URA3 met2 lys-801 trp1::hisG leu2Δ his3Δ200::HIS3::pep4Δ hrd1Δ::KanMX ubc7Δ::LEU2 TRP1::TDH3-HRD1-3HA</i>
RHY6459	<i>MATα ade2-101 ura3-52::HMGcd::HMG2-GFP met2 lys2-801 trp1::hisG leu2Δ his3Δ200 hmg2Δ::HIS3 hrd1Δ::KanMX URA3::CPY*-HA</i>
RHY6561	<i>MATα ade2-101 MET2 LYS2 ura3-52 trp1::hisG leu2-3,112 HIS3 hrd1Δ::KanMX sec61-2</i>
RHY6576	<i>MATα ade2-101 ura3-52::URA3::HMG2-GFP met2 LYS2 trp1::hisG leu2Δ his3Δ200 hmg2Δ::1MYC-HMG2 hrd1Δ::KanMX HIS3::HA-Pdr5*</i>
RHY7098	<i>MATα ade2-101 ura3-52::URA3::HMGcd::UPRE4-GFP met2 lys2-801 trp1::hisG leu2Δ his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 hrd1Δ::KanMX</i>
RHY7099	<i>MATα ade2-101 ura3-52::URA3::HMGcd::6MYC-HMG2-GFP met2 lys2-801 trp1::hisG leu2Δ his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 hrd1Δ::KanMX</i>
RHY7510	<i>MATα ade2-101 ura3-52::HMGcd::HMG2-GFP met2 lys2-801 trp1::hisG leu2Δ his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 hrd1Δ::KanMX URA3::KHN-HA</i>
RHY7600	<i>MATα ade2-101 ura3-52::URA3::HMGcd::HMG2-GFP met2 lys2-801 trp1::hisG leu2Δ his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 hrd1Δ::KanMX yos9Δ::CloNAT</i>

Table 2-2 continued Strains used in Chapter 2

Parent Strain	Plasmid Expressed	Figure
RHY2814	pRH642 pRH730 pRH1245 pRH1718 pRH2213 pRH2248 pRH2269 pRH2344 pRH2345 pRH2350 pRH2352 pRH2360 pRH2365	2-3, 2-5, 2-7, 2-8, 2-11, 2-13, 2-15, 2-16, 2-17 2-8 2-5, 2-7, 2-8, 2-13, 2-15 2-8 2-5 2-5 2-5, 2-7, 2-8, 2-11, 2-17 2-3 2-3 2-3 2-13 2-15, 2-16 2-8
RHY2933	pRH642 pRH2269	2-19 2-19
RHY2936	pRH730 pRH808 pRH2365 pRH2366	2-19, 2-20 2-21 2-19, 2-20 2-21
RHY3005	pRH311 pRH642 pRH730 pRH1245 pRH2269 pRH2287 pRH2360 pRH2365	2-18 2-11, 2-16, 2-18 2-18 2-11 2-11 2-11 2-16 2-18
RHY4288		2-20, 2-21
RHY4295		2-20, 2-21
RHY6152	pRH311 pRH507 pRH2288 pRH2361 pRH2362 pRH2364	2-4, 2-9 2-4, 2-9 2-9 2-4 2-4 2-4
RHY6245		2-19

Table 2-2 continued Strains used in Chapter 2

RHY6459	pRH311 pRH507 pRH2288 pRH2361 pRH2362 pRH2364 pRH2398	2-4, 2-9, 2-16 2-4, 2-9, 2-16 2-9 2-4 2-4 2-4 2-16
RHY6561	pRH642 pRH1245 pRH2269 pRH2360	2-10, 2-16 2-10, 2-16 2-10 2-16
RHY6576	pRH642 pRH1245 pRH2269 pRH2344 pRH2345 pRH2350 pRH2352 pRH2360	2-3, 2-10, 2-13, 2-14 2-10, 2-14 2-10 2-3 2-3 2-3 2-13 2-14
RHY7098	pRH642 pRH1245 pRH2269	2-12 2-12 2-12
RHY7099	pRH642 pRH1245 pRH2269 pRH2344 pRH2345 pRH2350	2-3, 2-6 2-6 2-6 2-3 2-3 2-3
RHY7510	pRH311 pRH507 pRH2288	2-9 2-9 2-9
RHY7600	pRH642 pRH2269	2-17 2-17

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Chapter 3

Yeast Derlin Dfm1 binds Cdc48 and functions in ER homeostasis

Abstract

Recent studies have identified a protein called Derlin-1 that associates with the AAA-ATPase p97, and is implicated in late steps in the ER-associated protein degradation (ERAD) of MHC-I molecules. Derlin-1 has two *Saccharomyces cerevisiae* homologues, Der1p and Dfm1p. While Der1p has been studied extensively, little is known concerning Dfm1p. Accordingly we investigated the role of Dfm1p in ERAD, ER homeostasis, and interactions with the yeast p97 homologue Cdc48p. Dfm1p was not involved in the degradation of a number of Der1-dependent or independent ERAD substrates, nor was it redundant with either Der1p or Sec61p in ERAD. However, Dfm1p had a role in ER homeostasis, since both Dfm1p loss or overexpression could stimulate the unfolded protein response (UPR). Furthermore, Dfm1p interacted both genetically and physically with Cdc48p, the yeast p97 homologue, and this interaction is dependent on a novel 8-amino acid sequence found in the C-terminus of Dfm1p that we have termed a SHP box. Our genetic studies are consistent with the lack of a role for Dfm1p in ERAD, and indicate it participates in ER-related Cdc48p actions distinct from retrotranslocation. Finally, sequence analysis indicated that the UPR-related and Cdc48 interaction functions of Dfm1p could be separated, implying this protein has numerous functions in the cell. Thus, the interaction between derlins and p97 is conserved between yeast and mammals, although its function in ERAD is not. Furthermore, Dfm1p interacts with Cdc48p through its SHP boxes, and so defines a new motif for interaction with this widely-employed AAA ATPase.

Introduction

ER-associated degradation, or ERAD, refers to the ubiquitin-mediated degradation of both luminal and integral membrane proteins of the endoplasmic reticulum. This process is conserved from yeast to mammals, and is responsible for the destruction of diverse proteins that are often misfolded, unassembled or damaged. Many ERAD substrates have some or all of their sequence in the ER lumen, and these portions must be moved across the ER membrane in order for ubiquitin-mediated proteasomal hydrolysis to occur. This movement of proteins from the lumen to the cytosol is generally referred to as “retrotranslocation” or “dislocation” to distinguish it from the anterograde movement of cytoplasmically synthesized proteins into the lumen that is a normal part of ER protein kinesin.

One of the large open questions concerning ERAD is the mechanism for substrate retrotranslocation. Numerous studies have implicated the hexameric AAA-ATPases, mammalian p97 and its yeast homologue Cdc48p, in this step in their respective organisms, possibly supplying the motive force for protein extraction from the ER (Bays et al. 2001; Ye et al. 2001; Ye et al. 2003). p97/CDC48 has been reported to be involved in ERAD in a number of different species for substrates that are processed by distinct ligases (Bays et al. 2001; Ye et al. 2001; Hoyer et al. 2004). In addition, it is reasonably presumed that a protein pore mediates the actual transport of ERAD substrates out of the lumen, in a manner analogous to that used to move proteins across membranes in traditional secretion. Although some studies have suggested a role for the anterograde channel *SEC61* (Wiertz et al. 1996; Plemper et al. 1999), a new candidate family of proteins called the Derlins has received significant attention. This class of proteins was

independently discovered by two groups exploring virus-mediated ER degradation of MHC class I (Lilley and Ploegh 2004; Ye et al. 2004). Derlin-1 is a small multi-spanning membrane protein required for MHC-I degradation, and is named for its homology with yeast Der1p, one of the first ERAD factors characterized (Knop et al. 1996). In MHC-I degradation, Derlin-1 appears to function after ubiquitination, and forms a complex with mammalian p97. These features led the co-discoverers to propose that this protein may form or be part of the pore by which p97-mediated retrotranslocation proceeds (Lilley and Ploegh 2004; Ye et al. 2004).

Mammalian Derlins have homology to the prototype yeast ERAD factor Der1p (Figure 3-1A) (Lilley and Ploegh 2004; Ye et al. 2004). Like the Derlins, this small protein has multiple membrane spans and resides in the ER. Despite numerous studies involving Der1p (Knop et al. 1996; Hitt and Wolf 2004; Vashist and Ng 2004), the function of this protein is still unknown, but the connection with Derlin-1 leads to the idea that it is an integral participant in retrotranslocation. If Der1p were in fact a retrotranslocation factor, one would expect it to operate in concert with Cdc48p, which is known to function in retrotranslocation. This would be analogous to Derlin-1 and p97 since they likely function in a similar step in MHC-I ERAD (Lilley and Ploegh 2004; Ye et al. 2004). Previous work with Der1p does not appear to support this idea. As a retrotranslocation factor, Cdc48p is a universal degradation requirement, while Der1p functions in ERAD of a subset of substrates. However, Der1p is not the only yeast Derlin protein. There is a second homologue of Derlin-1 in the yeast genome, called *DFM1* (Hitt and Wolf 2004), for Der1-like family member 1 (Figure 3-1B). One model is that Dfm1p and Der1p operate together to carry out the role that Derlin-1 performs. Dfm1p is

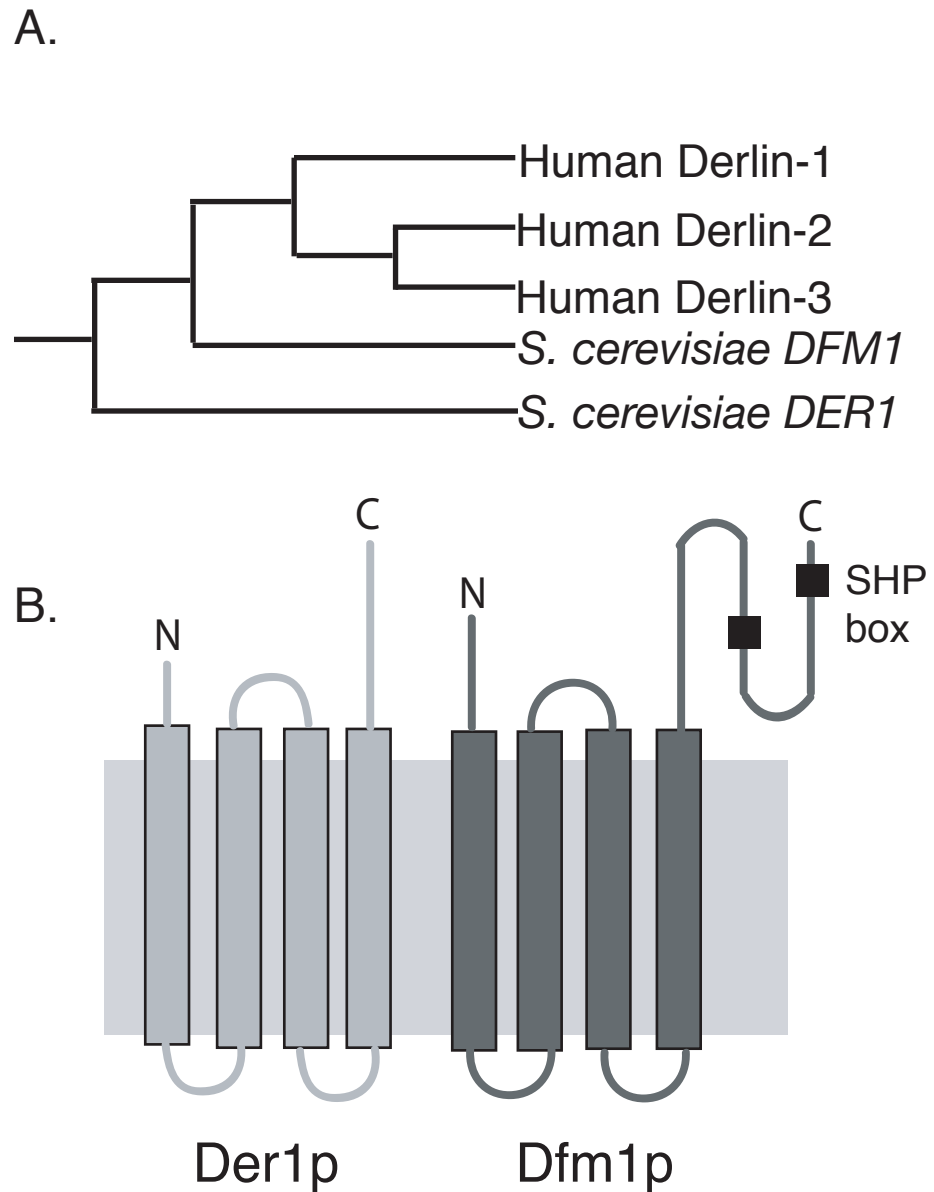


Figure 3-1 Models of Der1p and Dfm1p

(A). Phylogenetic tree of the human and yeast Derlin homologues. *DFM1* is more closely related to Derlin-1 than its yeast orthologue *DER1*. The tree was constructed as described by Lilley and Ploegh (Lilley and Ploegh 2004). (B). Der1p and Dfm1p are both ER-localized membrane proteins with four transmembrane domains. Dfm1p has an extended cytoplasmic tail which contains two 8 amino acid sequences that we have termed SHP boxes.

similar to Der1p, and has significant (in fact, slightly higher) homology to mammalian Derlin-1. Unlike Der1p, Dfm1p has an extended C-terminal cytoplasmic tail that contains two 8-amino acid sequences that we have termed SHP boxes. These sequences are found in a number of proteins that interact with Cdc48p, including Shp1p and Ufd1p. This leads to the appealing idea that perhaps Der1p and Dfm1p together mediate some aspect of ERAD related to Cdc48 that mirrors the relationship between Derlin-1 and p97. We have thus mounted a study of the Dfm1p protein, with an interest in evaluating its role in the ERAD of a number of substrates, its independence or redundancy with Der1p, its interaction with Cdc48p, and its involvement in ER homeostasis.

We have found that Dfm1p plays a role in the maintenance of ER homeostasis. Furthermore, Dfm1p interacts with Cdc48p both genetically and physically, and we have demonstrated that the SHP boxes, which appear to be novel Cdc48 binding sites, mediate this interaction. The effects of Dfm1p on ER stress and Cdc48 can be separated by molecular biological means. However, despite the roles in ER stress and the interaction with Cdc48p, Dfm1p did not function in ERAD by any measure we applied. Thus, Dfm1p's interaction with Cdc48p and role in ER homeostasis are distinct from ERAD, and will lead to a better understanding of the multiple functions of Cdc48 and the Derlins in the cell.

Results

Often, the loss of proteins involved in ER quality control causes an elevation of the unfolded protein response (UPR) (Friedlander et al. 2000; Travers et al. 2000), a signal transduction pathway activated upon the accumulation of unfolded proteins in the ER (Patil and Walter 2001). We first examined whether loss of the yeast Derlin Dfm1p would activate this signaling pathway, using the sensitive UPRE4-GFP reporter (Bays et al. 2001). This reporter is activated by the UPR, allowing measurement of pathway activity by flow cytometry. Loss of Der1p elevated UPR, while loss of Dfm1p alone did not cause a detectable change. Each of these results has been reported previously (Knop et al. 1996; Hitt and Wolf 2004). However, we found that addition of a *dfm1Δ* null mutation to an otherwise isogenic *der1Δ* strain produced a further elevation of the UPR above that caused by the *der1Δ* alone (Figure 3-2), indicating that Dfm1p had a hitherto undetected role in ER homeostasis.

Since loss of *DFM1* results in an increased UPR in a *der1Δ* strain, we felt compelled to further test if Dfm1p participates in ERAD, extending the work of Hitt *et al.* (Hitt and Wolf 2004) to a variety of substrates and genetic circumstances. As expected from that work, the loss of Dfm1p had no effect on the degradation of two soluble Der1p-dependent substrates CPY* and KHN, either alone or with a *der1Δ* (Figure 3-3A, 3-3B). This was also true of the membrane-spanning Der1p-dependant substrate KWW (Figure 3-3C). We further examined the effect of the *dfm1Δ* mutation on ERAD of two Der1p-independent substrates, Hmg2p and Ste6-166, both alone and in combination with the *der1Δ* mutation. The presence of the *dfm1Δ* had no effect on the

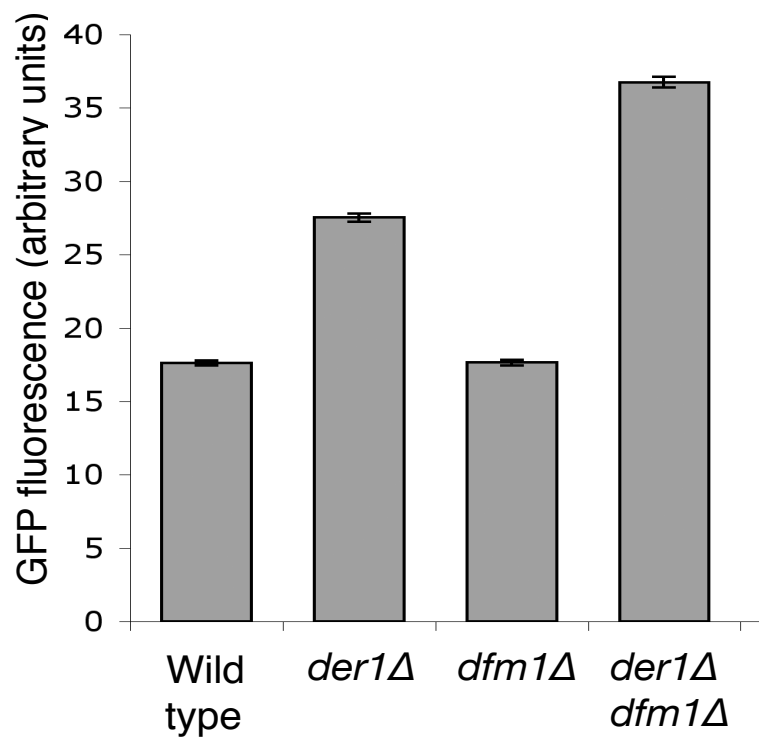


Figure 3-2 Elevated UPR caused by a *dfm1*Δ null allele relative to wild type and *der1*Δ

The indicated strains expressing the UPRE4-GFP reporter were grown in minimal media and the log phase GFP fluorescence was measured by flow cytometry. In this and subsequent flow cytometry experiments, 10,000 cells were analyzed for each experimental condition. Bars indicate the standard error of the mean.

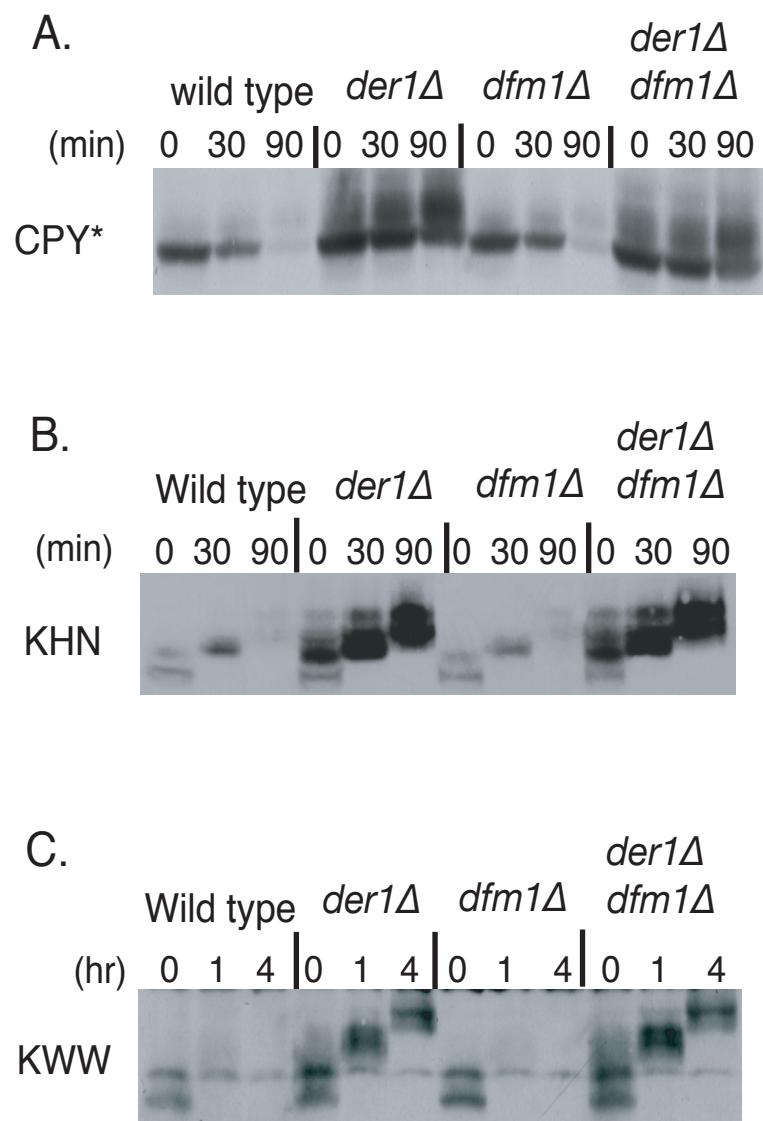


Figure 3-3 Dfm1p had no role in the degradation of Der1p-dependent ERAD

(A-C). Degradation of the indicated tagged ERAD substrates was measured by cycloheximide chase (CHX) in isogenic strains. After CHX addition, cells were lysed and analyzed by SDS-PAGE, immunoblotting for each substrate. Equal loading was verified by India ink staining (data not shown).

degradation of either of these substrates alone or in conjunction with *der1* Δ (Figure 3-4A, 3-4B), as also shown for Ste6-166 in recent work by Kreft *et al.* (Kreft *et al.* 2005).

The UPR experiment in figure 3-2 indicated that Dfm1p may have functions that are redundant with Der1p since its loss only had a discernable effect when *DER1* was also absent. Thus, we wondered if Dfm1p might be redundant for Der1p's ERAD function when expressed at sufficient levels. To test this, we made a Dfm1p plasmid with a strong *TDH3* promoter on a 2-micron plasmid to examine the effects of overexpressing this gene product. Despite the fact that this plasmid causes highly elevated levels of Dfm1p as indicated by immunoblotting (DNS) and had clear phenotypes (see below), there was no suppression of the *der1* Δ as measured by CPY* stabilization (Figure 3-5).

It has been hypothesized that the Derlins may be retrotranslocation factors that provide an exit route out of the ER. The translocon Sec61p has also been proposed to mediate transfer of ERAD substrates from the lumen to the cytosol. However, strains lacking *DFM1* and *DER1* do not stabilize several ERAD proteins, and a *sec61-2* mutant has only minor effects on the degradation of a number of substrates including Hmg2p. One possibility is that yeast Derlins and Sec61 mediate separate but overlapping exit strategies. Thus, we tested if the presence of the *der1* Δ *dfm1* Δ mutation would have an enhancing effect on the minor ERAD defect caused by the *sec61-2* mutation. In a *sec61-2* strain, the degradation of Hmg2p-GFP is slowed by a maximum of two-fold. This small difference is reproducible but cannot be enhanced by preincubation of the mutant cells at the non-permissive temperature for several hours prior to starting the degradation time course (DNS). However, stabilization of the ERAD substrate was not enhanced, and

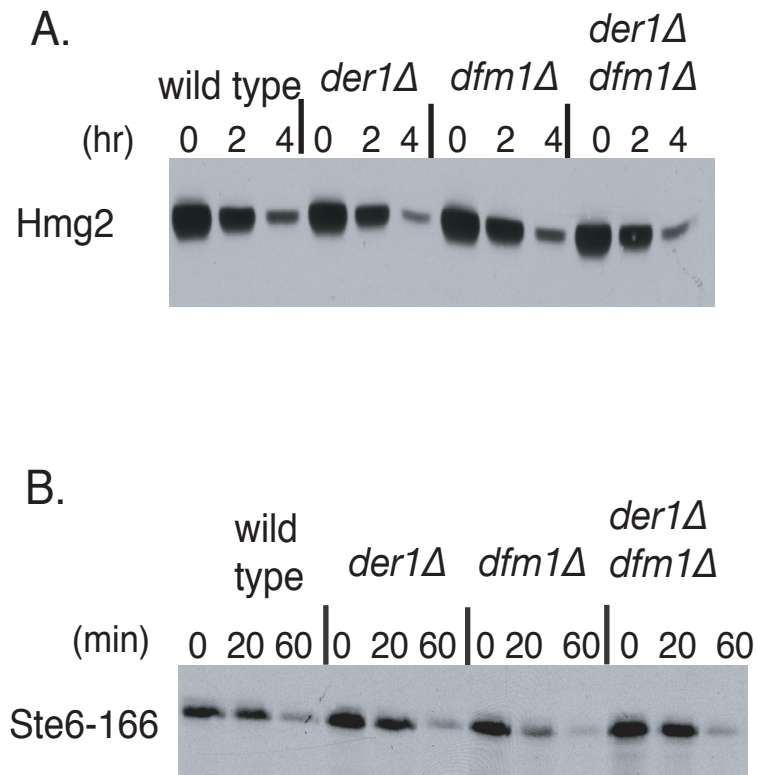


Figure 3-4 Dfm1p had no role in the degradation of Der1p-independent ERAD

(A-B). Degradation of the indicated tagged ERAD substrates was measured by cycloheximide chase (CHX) in isogenic strains. After CHX addition, cells were lysed and analyzed by SDS-PAGE, immunoblotting for each substrate. Equal loading was verified by India ink staining (data not shown).

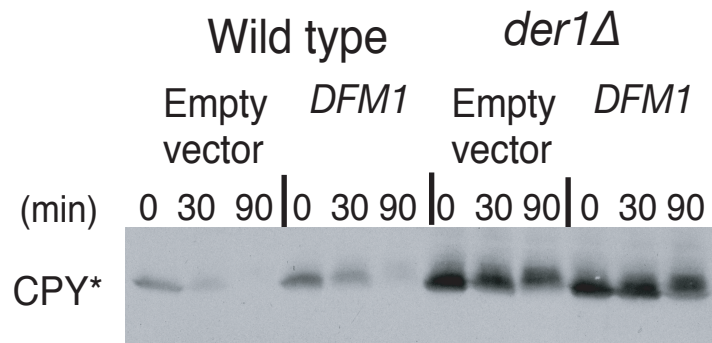


Figure 3-5 Overexpression of Dfm1p did not suppress a *der1*Δ mutant

Both wild type and *der1*Δ strains containing an empty vector plasmid or overexpressing *DFM1* plasmid were tested for CPY* degradation by cycloheximide chase. After CHX addition, cells were lysed and analyzed by SDS-PAGE, immunoblotting for each substrate. Equal loading was verified by India ink staining.

in fact the degradation of Hmg2p-GFP was slightly faster in the *sec61-2 der1Δdfm1Δ* triple mutant (Figure 3-6).

Taken together, these data indicate that Dfm1p does not participate in ERAD, either independently or in a redundant manner with Der1p or Sec61p. These observations extend the initial characterization of Dfm1p (Hitt and Wolf 2004), including direct tests of non Der1-dependent substrates, and confirm the idea that this protein, despite its homology to Der1p, is not an ERAD factor. In contrast to the previous studies, we found clear evidence that the Dfm1p protein plays a role in ER stress and homeostasis (Figure 3-2). We have followed up those observations, and show that the Dfm1p protein has a number of unique biological activities that distinguish it from Der1p and that it has a functional and physical interaction with Cdc48p.

We first examined whether there were phenotypes associated with overexpressing Dfm1. To our surprise, this reliably caused a significant increase in UPR signaling as measured with the UPRE4-GFP reporter (Figure 3-7). In contrast, Der1p caused minimal activation of the UPR response when expressed in the same manner, indicating that the UPR phenotype was specific for the Dfm1p protein. Generally, we have not observed any UPR induction upon strong expression of membrane proteins such as 6myc-Hmg2p (Hampton et al. 1996), or Hmg2p. With the thought that Dfm1p might cause the UPR effect by somehow interfering with ERAD, we also tested the effect of overexpressing Dfm1p in a *hrd1Δdoa10Δ* double mutant, which inhibits all known ERAD pathways. In this background, the Dfm1p protein was still able to strongly stimulate the UPR above the elevated background caused by loss of these two ERAD ubiquitin ligases (Figure 3-8).

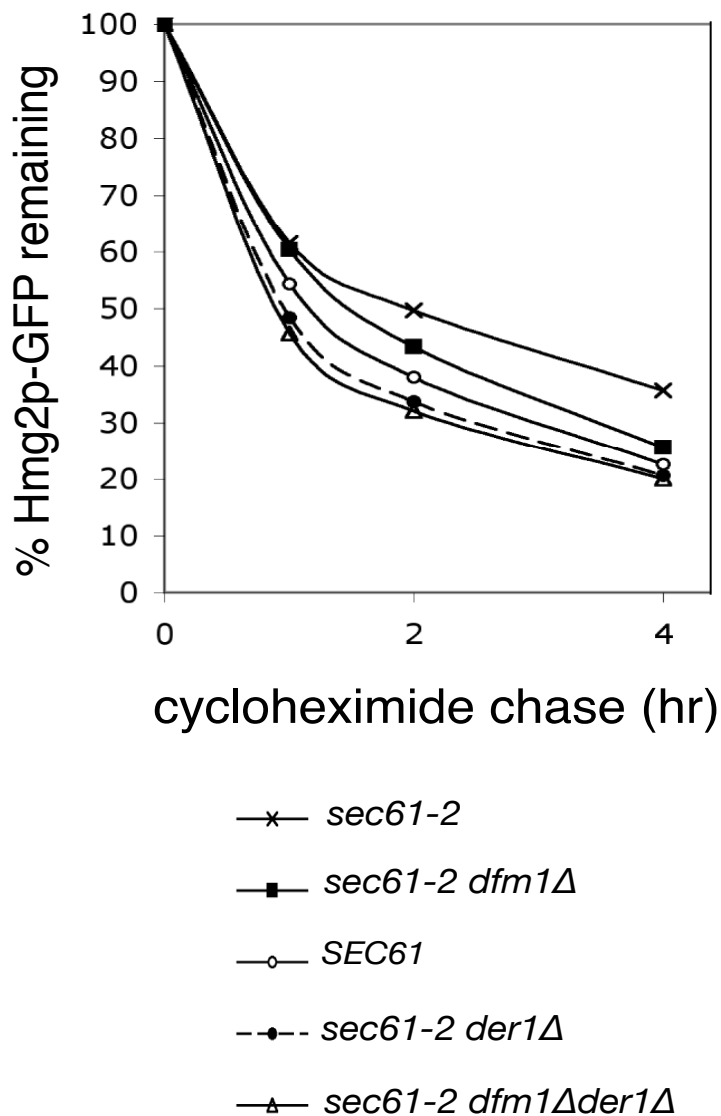


Figure 3-6 Null mutations of *DFM1* or *DER1* did not exacerbate the ERAD defect of a *sec61* mutant

The indicated strains expressing Hmg2p-GFP were grown into log phase and degradation was measured by a cycloheximide chase. CHX was added to isogenic cultures incubated at 30°C at the indicated times to allow for simultaneous analysis of all cultures by flow cytometry at the end of the experiment.

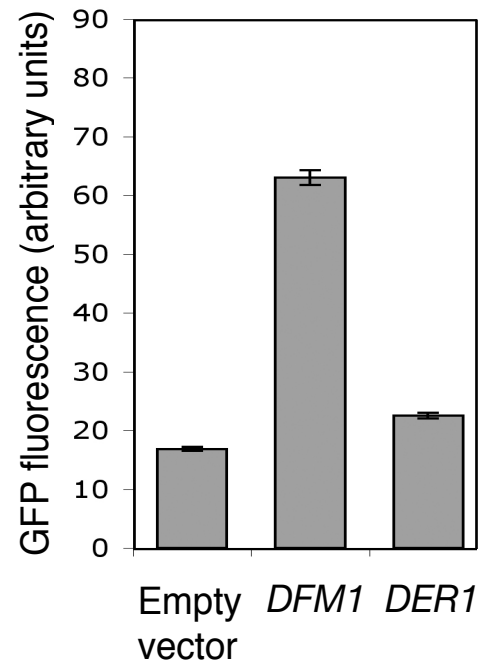


Figure 3-7 The unfolded protein response was upregulated by *DFM1* overexpression

A wild type strain expressing the UPR4-GFP reporter was transformed with empty vector or plasmids expressing *DFM1* or *DER1* driven by the strong TDH3 promoter. Cells were grown into log phase and then analyzed by flow cytometry for UPR levels.

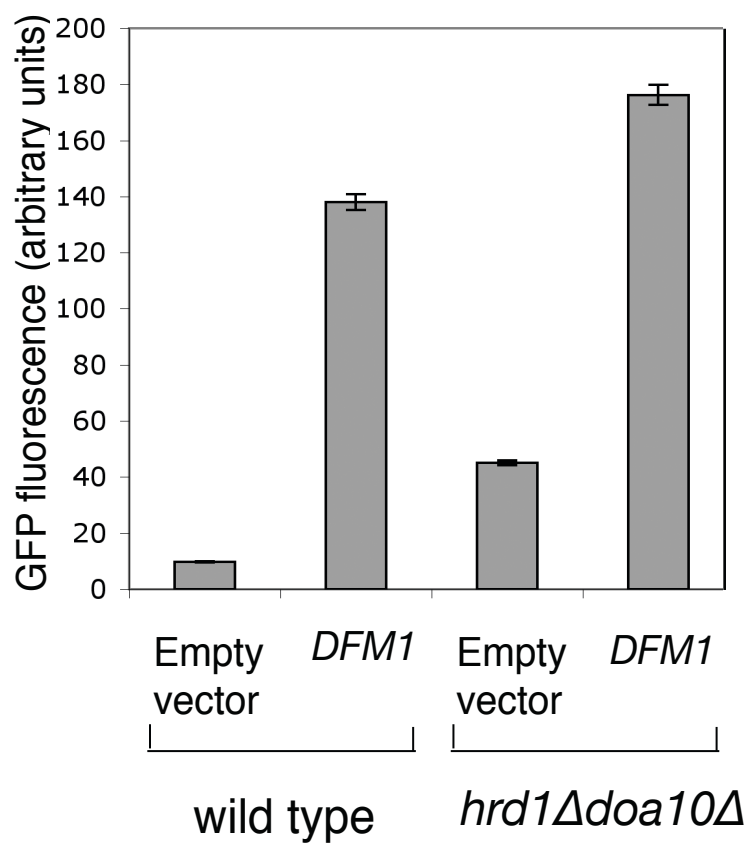


Figure 3-8 *DFM1*-stimulated UPR is not dependent on a functional ERAD pathway

Wild type and *hrd1Δdoa10Δ* strains with the UPRE-GFP reporter and harboring either empty vector or a *DFM1* overexpressing plasmid were compared for UPR by flow cytometry.

Because the Derlins are thought to work with p97, we next evaluated both genetic and physical interactions between Dfm1p and the yeast p97 homologue, Cdc48p. We found that overexpression of Dfm1 has very specific deleterious effects on a *cdc48* mutant (Figure 3-9). As shown in a dilution plating assay, when Dfm1p was strongly expressed, *cdc48-3* cells had an obvious growth defect at their normally permissive temperature that did not occur in an isogenic wild-type strain. This effect was only observed with the Dfm1p protein; similar expression of Der1p had no effect above that of the empty vector. The phenotype was not specific for the *cdc48-3* allele, as overexpression of Dfm1p had similar effects on a *cdc48-2* strain (DNS). However, the Dfm1-caused growth defect was also highly specific for *cdc48* loss of function, as Dfm1 overexpression had no effect on a temperature sensitive *cdc34* mutant (DNS).

We next asked whether this Dfm1 overexpression phenotype was similarly observable in an *npl4* mutant. Npl4p, along with a second co-factor Ufd1p, binds to Cdc48p to form a complex which functions in ERAD. These proteins are functionally related, as a mutation in any of the three produces similar ERAD defects (Bays et al. 2001). We reasoned that a similar genetic interaction would be evident between Dfm1 and Npl4. Surprisingly, the presence of overexpressed Dfm1 in an *npl4* mutant did not result in the killing phenotype observed with the *cdc48* mutant (Figure 3-10). Even when the overexpression experiment was performed only two degrees below the non-permissive temperature, addition of Dfm1p had no effect on the *npl4* mutant growth (Figure 3-10). Thus, it appears that the Dfm1p protein shows a very specific genetic interaction with Cdc48p. This action of Dfm1 appears to be unrelated to the

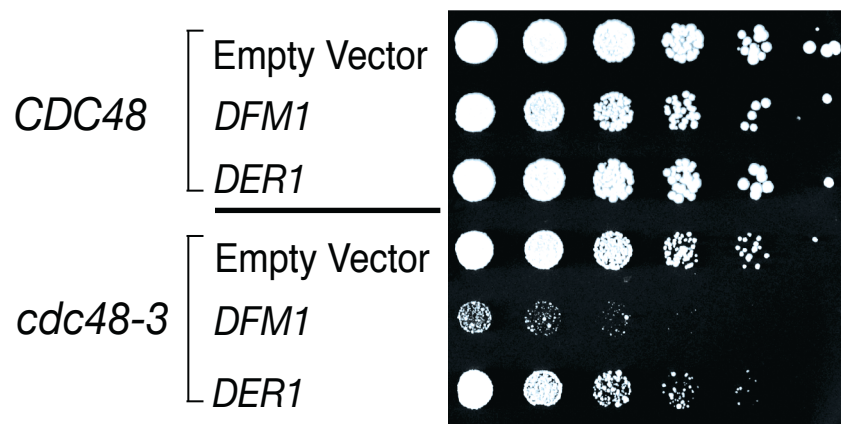


Figure 3-9 A genetic interaction between *DFM1* and *CDC48*

Wild type and *cdc48-3* strains with empty vector or plasmids overexpressing *DFM1* or *DER1* were compared for growth by dilution assay. Each strain was spotted at 5-fold dilutions on solid media, and plates were incubated at 32°C.

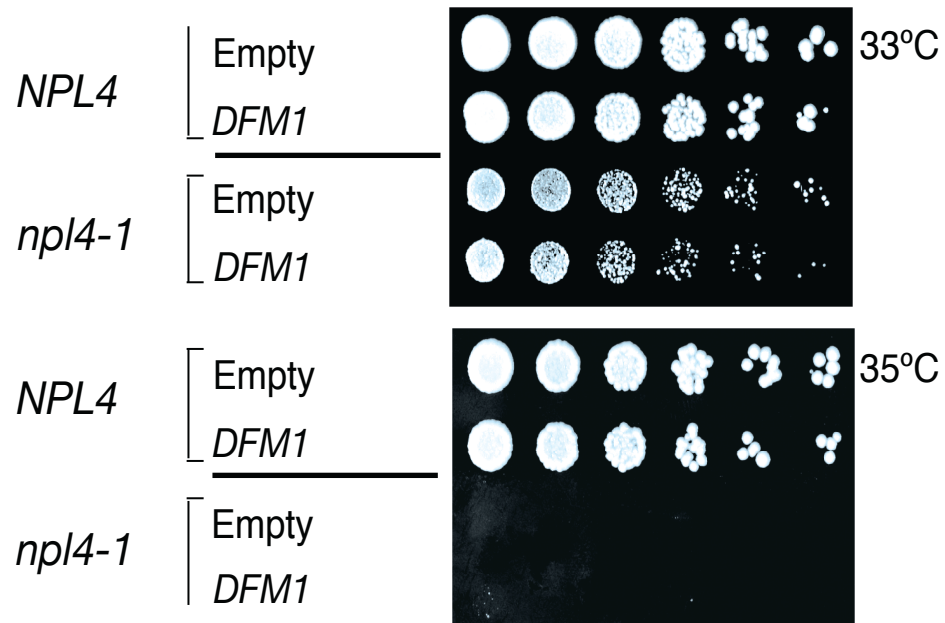


Figure 3-10 *DFM1* and *NPL4* do not interact genetically

A temperature sensitive *npl4-1* mutant was similarly tested for sensitivity to *DFM1* overexpression. Strains were grown as described in previous figures and plates were incubated at 33°C to show a lack of an effect of *DFM1* overexpression. An identical plate was incubated at 35°C to demonstrate the ts- phenotype of the *npl4-1*.

Cdc48/Npl4/Ufd1 complex, as similar effects were not observed between Dfm1p and an *npl4-1* mutant.

Cdc48 has numerous cellular functions (Latterich et al. 1995; Bays et al. 2001; Cao et al. 2003). The above data indicate that Dfm1p affects both Cdc48 and ER homeostasis, but plays no role in ERAD. Accordingly, we next tested the effect of Dfm1p overexpression on a mutant related to a non-ERAD function of Cdc48p, ER homotypic membrane fusion. This process requires the t-SNARE Ufe1 as well as Cdc48 (Latterich et al. 1995; Patel et al. 1998). We tested whether overexpression of Dfm1 produces the same phenotype in a temperature sensitive *ufe1* mutant that we observed in the *cdc48* mutant. As was the case for the *cdc48* mutant, we observed a growth defect in the *ufe1-1* strain overexpressing Dfm1 (Figure 3-11). This too was specific for Dfm1, as overexpression of Der1 had no effect on growth. Thus, in addition to a genetic interaction with *CDC48*, *DFM1* interacts genetically with *UFE1*.

We further studied the relationship between the Dfm1p and Cdc48p phenotypes through mapping of the sequence determinants for this phenotype. The Dfm1p killing of *cdc48* is specific for that paralogue; the Der1p protein did not cause *cdc48* lethality. The most salient difference between the two paralogues is the extended C-terminal tail on Dfm1p (Figure 3-1B). This portion of the protein is particularly interesting since it contains two SHP box sequences, found in the SEP domain of Shp1p. The SEP domain is also a feature of mammalian p47 and the *Drosophila eyes closed* gene, and has been demonstrated to act as an interaction site between p97 and p47 (Sang and Ready 2002; Yuan et al. 2004). We have analyzed the role of the Dfm1p C-terminal tail and the significance of the SHP box in the genetic interaction of Cdc48 with Dfm1.

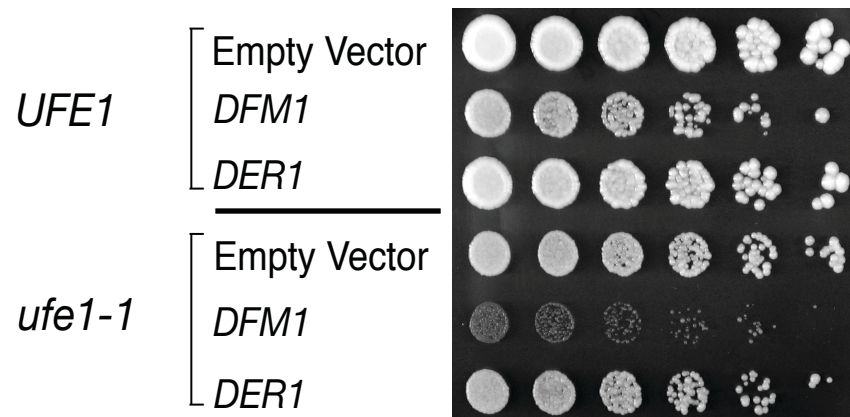


Figure 3-11 *DFM1* and *UFE1* interact genetically

A temperature sensitive *ufe1-1* mutant was similarly tested for sensitivity to *DFM1* overexpression. Strains were grown as described in previous figures and plates were incubated at 33°C.

We tested the importance of unique, C-terminal regions of Dfm1p, by making two *DFM1* mutants. We first mutated regions encoding the two SHP boxes of *DFM1*. The conserved sequence of FxGxGQRb (where x is a non-conserved amino acid and b is a basic amino acid) is present in Dfm1p at amino acids 284 (FSGRGQRL) and 324 (FQGRGQRV). We eliminated the SHP boxes by mutating all the conserved amino acids to alanine. Overexpression of this Dfm1 mutant lacking the SHP boxes (“*DFM1-5Ashp*”) had no effect on *cdc48* lethality, demonstrating that these sequences are necessary for the phenotype (Figure 3-12A). This lends credence to the idea that these motifs mediate an interaction with Cdc48p. Removal of the SHP box also blocked *DFM1*’s lethal effect on *ufe1* mutants (DNS). We then created a fusion gene that produces Der1p with the added Dfm1p C-terminal tail. Like wild type Der1p overexpression, Der1p with the added Dfm1 C-terminus had no effect on the growth of a *cdc48-3* strain (Figure 3-12B). Thus, the SHP box sequences are necessary for the Dfm1p dependant *cdc48* killing, but the C-terminal tail alone is not sufficient to cause this effect.

The genetic interaction between Dfm1p and Cdc48p implies that the two proteins physically interact in a SHP box dependant manner. We tested for a direct interaction with a co-immunoprecipitation experiment. We prepared strains expressing 3HA-Dfm1p alone or with a functional protA-Cdc48 fusion. The tagged Dfm1 construct caused the same overexpression phenotypes observed with untagged Dfm1 (DNS). Detergent lysates were prepared, and the protA-Cdc48p was precipitated from the lysates with IgG-coupled beads. The lysates were then immunoblotted with anti-Cdc48 antibodies or anti-HA to detect co-precipitated 3HA-Dfm1p. The Dfm1p was co-precipitated by the IgG

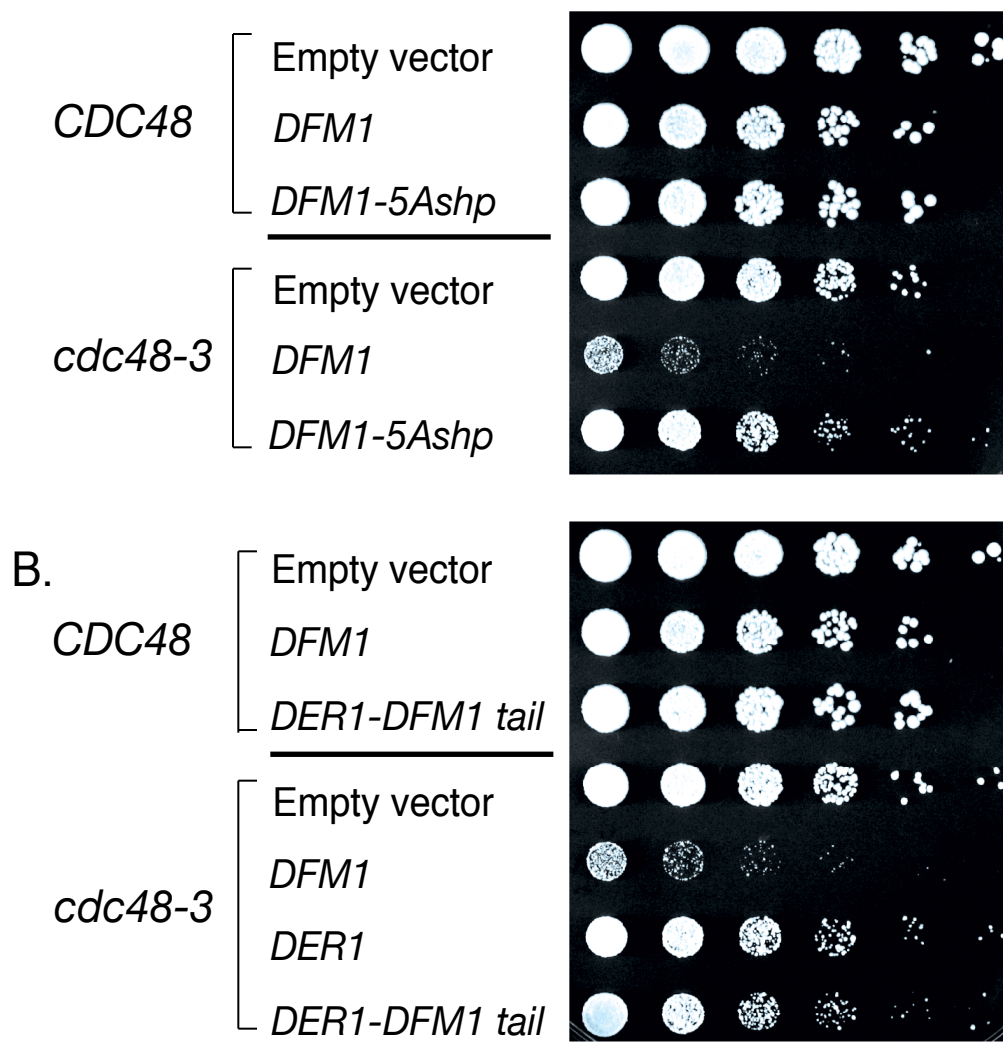


Figure 3-12 The Dfm1p C-terminal tail is necessary but not sufficient for the *cdc48-3* killing phenotype

(A-B). Wild type and *cdc48-3* strains expressing either empty vector, or plasmids overexpressing *DFM1*, *DER1*, *DER1-DFM1 tail*, or *DFM1-5Ashp* as indicated, were tested by dilution assay as previously described. Plates were incubated at 32°C.

beads, but only in strains that also expressed the protA-Cdc48 fusion (Figure 3-13). As expected from the overexpression studies above, the SHP boxes were required for co-precipitation of Dfm1p by Cdc48p. Dfm1p with mutant SHP boxes did not interact with Cdc48p, as indicated by the absence of any additional HA signal above background when protA-Cdc48 is present in the precipitation. Interestingly, the Dfm1p mutant missing the SHP boxes is somewhat more abundant than the wild type, so the loads were adjusted after precipitation to allow facile comparison with the strains expressing wild-type 3HA-Dfm1p.

Overexpressing Dfm1p had two phenotypes: UPR stimulation and killing of *cdc48* strains. We next evaluated the relationship between these two effects, using the Dfm1p variants generated above. It has been shown that *cdc48* mutants have an elevated UPR (Ye et al. 2001). Since Dfm1p and Cdc48p interact, one possibility was that Dfm1p overexpression caused sequestration of Cdc48p, thus elevating the UPR. This was not the case, as the two Dfm1p overexpression phenotypes showed entirely distinct sequence requirements. As shown above, elimination of the Dfm1 SHP boxes removed the *cdc48* killing phenotype. Conversely, overexpression of this *5Ashp* mutant still caused robust UPR that was, in fact, slightly higher than the upregulation caused by the wild type Dfm1p (Figure 3-14). Furthermore, overexpression of a Der1p fusion with the C-terminal tail of Dfm1p, which had no growth effect on *cdc48-3* (Figure 3-12), caused strongly upregulated UPR similar to wild type Dfm1p (Figure 3-14). It appears, in the context of a Der1p fusion, the Dfm1p C-terminal tail is sufficient to cause the UPR phenotype, whereas it is not sufficient to cause *cdc48* killing. Thus, the UPR effect and

cdc48 lethality have separable sequence requirements, indicating that the UPR upregulation is not due to Dfm1p sequestration of Cdc48p.

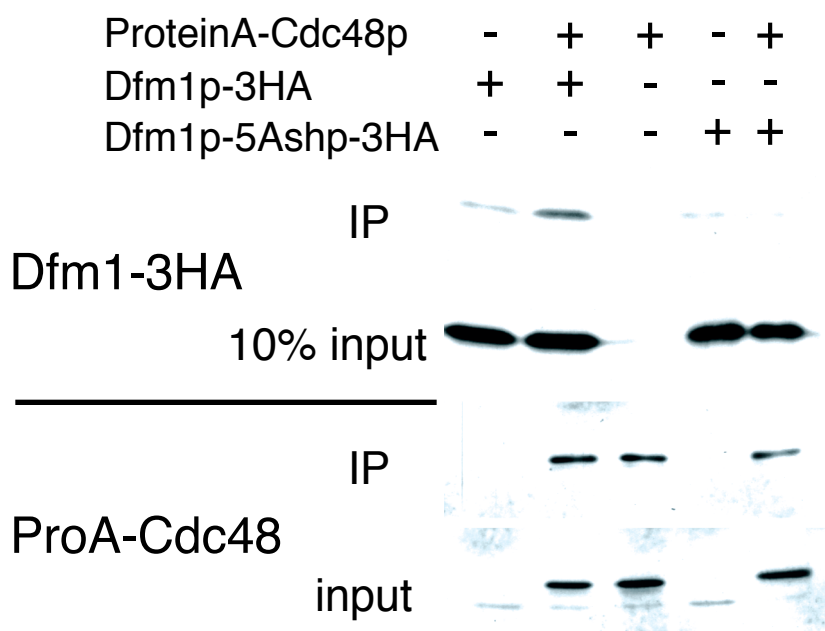


Figure 3-13 Dfm1p binds Cdc48p in a SHP box dependent manner

Strains expressing the indicated proteins were grown and equal amounts were harvested. Detergent lysate of ER-enriched microsomes were prepared as described and precipitated with IgG-beads to pull-down protein A-Cdc48. The precipitate were analyzed by SDS-PAGE and immunoblotted for either α -HA monoclonal antibody or α -CDC48 polyclonal antibody.

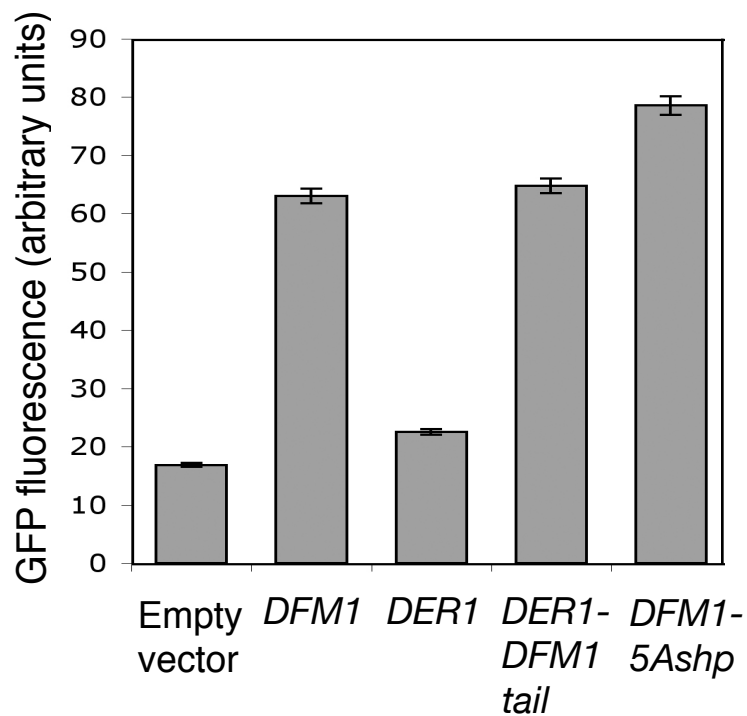


Figure 3-14 Sequence determinants of UPR induction by *DFM1*: the *Dfm1p* tail is sufficient for UPR induction

A strain expressing the UPR4-GFP reporter was transformed with the indicated overexpression constructs. Strains were grown into log phase and GFP fluorescence was measured by flow cytometry.

Discussion

The mammalian Derlins have generated great interest due to their observed role in ER degradation. Their participation in the p97-dependant part of the virally-mediated MHC-I degradation pathway implies they may have a general role in the retrotranslocation phase of ER degradation. Because many aspects of ERAD are conserved between yeast and mammals, we explored the function of the yeast Derlin homologue Dfm1, focusing both on its possible functions in ER homeostasis, and as an interaction partner for the yeast p97 homologue Cdc48.

Recent studies suggested that Dfm1p is not involved in ER degradation by direct analysis of several substrates or phenotypes (Hitt and Wolf 2004; Kreft et al. 2005). The Hitt *et al.* study also indicated that Dfm1p has no role in ER stress due to a lack of effects of the *dfm1Δ* single null. We found, however, that the *dfm1Δ* null allele enhanced the UPR of a *der1Δ* strain. Accordingly, we more fully investigated the roles of this protein in ERAD and other ER related-functions. Consistent with the earlier studies, *DFM1* was not required for the degradation of a number of Der1-dependant and independent substrates. Furthermore, Dfm1p was not redundant with Der1p or Sec61p. However, we found that altering the levels of Dfm1p clearly affected ER stress. In addition, we demonstrate both genetic and physical interaction between Dfm1p and Cdc48p, which is dependant on a novel Cdc48-binding motif found in the C-terminus of Dfm1p. Finally, the UPR and cdc48 interaction functions could be cleanly separated, indicating that Dfm1p plays a multifaceted role in non-ERAD, ER-related functions of the Cdc48 protein.

UPR signaling was sensitive to levels of Dfm1. Loss of Dfm1 exacerbated the UPR in a *der1Δ* null mutant, while overexpression caused significant UPR in a wild type strain. The overexpression phenotype was surprising since in our studies we have never observed this effect upon overexpression of numerous membrane proteins such as ERAD substrates. The Dfm1 result is comparable in intensity to the response caused by overexpression of CPY*, a misfolded ERAD substrate (Knop et al. 1996). However, we speculate that CPY* and Dfm1p overexpression may cause UPR by a different mechanism. CPY* is a misfolded protein, and its strong expression causes sustained ER stress due to its detection as a client protein (Knop et al. 1996). In contrast, we suspect Dfm1 alters UPR by engaging molecules from the signaling pathway itself, rather than by being an abundant, misfolded ER protein.

Because mammalian Derlin-1 is part of a complex that includes p97, we investigated interactions of Dfm1p with the p97 homologue Cdc48p. We found that Dfm1p displays highly specific genetic and physical interactions with Cdc48p. Overexpression of Dfm1p caused a drastic decrease in the viability of *cdc48* strains. This action was not caused by similar expression of the Der1p homologue and was dependent on the Dfm1 C-terminal SHP boxes. Consistent with the genetic interaction, we showed that Dfm1p directly binds Cdc48 in co-immunoprecipitation assays, and the binding was dependent on the intact SHP boxes. So, it would appear that a direct interaction between Dfm1p and Cdc48p is responsible for these genetic interactions. Importantly, while the SHP boxes are required for the genetic effects, they are not sufficient, since overexpression of a Der1p fusion with the SHP-containing C-terminal tail had no effect

on *cdc48* strains. Thus, the genetic interaction between Dfm1p and Cdc48 involves multiple parts of the molecule including the Cdc48-binding SHP motif.

These studies define the SHP box as a new Cdc48 interaction domain. This 8-amino acid sequence is found in other *S. cerevisiae* proteins as well, including Shp1p, Ufd1p and Rpn1p. Shp1p and Ufd1p are known Cdc48 binding proteins and Rpn1p is a proteasome subunit that could potentially interact with the Cdc48 complex. Studies performed by Ye et. al. (Ye et al. 2003) have identified the C-terminus of mammalian Ufd1 as a requirement for p97 binding. Not surprisingly, the SHP box in Ufd1p is located in the C-terminus as well. It will be interesting to learn whether interactions between Cdc48 and other SHP box containing proteins are similarly dependent on this motif.

Overexpression of Dfm1p has two distinct phenotypes: elevated UPR and killing of *cdc48* mutants. We were able to uncouple Dfm1's UPR and *cdc48* killing phenotypes by analyzing the sequence determinants for each. The lethal effects of *DFM1* on *cdc48* mutants required the C-terminal SHP boxes but could not be transferred to a fusion protein with the C-terminal tail. By contrast, Dfm1-caused UPR did not require SHP box function and was transferable with the Dfm1p C-terminal tail. Thus, the UPR is not merely a read-out of the Dfm1p-Cdc48p interaction, but rather indicates that Dfm1 interacts with a number of ER components through different regions of the protein.

Dfm1p does not appear to play a role in ERAD despite our and others' extensive efforts to discover such a function. Dfm1 does associate with Cdc48, but this action appears to occur independently of Cdc48's role in ERAD. It is possible that it has an ancillary ERAD function that we have yet to discover. Perhaps Dfm1p is not usually

required for ERAD in normal conditions, but becomes limiting under abnormal circumstances, such as stressful growth conditions. However, several hours of heat stress applied to *dfm1Δ* cells did not alter the degradation kinetics of CPY* or Hmg2p (DNS). Another possibility is that it is a negative regulator of ERAD in which case the null condition would not result in inhibition of substrate degradation. However, the *dfm1Δ* null mutant did not hasten degradation of any of the ERAD substrate studied above.

Dfm1p interacted with Cdc48 both genetically and physically, despite its non-participation in ERAD. This implies that Dfm1p participates in one of the several other functions of Cdc48p. Originally, *CDC48* was isolated in a screen for mutants that inhibit the cell cycle in yeast (Moir et al. 1982). It has been speculated that this cell cycle block is due to a role for Cdc48 in nuclear membrane fusion. In addition, both Cdc48 and p97 have been implicated in disassembly of mitotic spindles after anaphase (Cao et al. 2003). While Cdc48 has strong functional ties to cell cycle regulation, it is unlikely that Dfm1 has a positive action in these functions, since they are essential and loss of Dfm1p is not lethal. Alternatively, Dfm1p may play a modulatory role in one of these essential functions, and in this way exacerbate the phenotypes of *cdc48* hypomorphs upon overexpression.

Another function of Cdc48p is the mediation of ER homotypic fusion. Latterich *et al.* (Latterich et al. 1995) demonstrated that Cdc48 is required for ER homotypic membrane fusion. This process also requires Ufe1p, an ER localized t-SNARE. The current model is that Ufe1p binds to another Ufe1p molecule resulting in fusion. This Ufe1p complex is then dismantled through the actions of Cdc48 (Patel et al. 1998). The authors demonstrated that an *ufe1-1* mutant is severely inhibited for ER homotypic fusion

at 30°C, despite a lack of obvious growth defects. Although *ufe1*Δ strains are inviable due to the many roles Ufe1p plays, the possibility that ER homotypic membrane fusion is not essential makes it an appealing candidate for Dfm1p's function. Consistent with this idea, we observed that overexpression of Dfm1 inhibited the growth of a *ufe1-1* mutant. Furthermore, killing of *ufe1* mutants was also dependant on the SHP boxes of Dfm1p. This is in striking contrast to the lack of effect of Dfm1p overexpression on similarly temperature sensitive mutants of Npl4p, the protein that associates with Cdc48p and functions in ERAD. Taken together, the simplest model is that Dfm1p functions at the ER surface in conjunction with Cdc48p to perform or control a function that is distinct from ERAD, with the best candidate being homotypic fusion of the ER. We are currently testing the role of Dfm1p in this and other functions of Cdc48p.

How does the function of Dfm1p connect to the mammalian Derlins? Both form complexes with p97 homologues, and appear to function with these proteins. One possibility is that the two proteins are sufficiently diverged that their functional interactions with p97/Cdc48p are distinct; so that the yeast form is not involved in ERAD, while the mammalian form is. Alternatively, it may be that in both organisms, Derlins function in non-ERAD dependent functions of p97/Cdc48, and mammalian HCMV hijacks the p97-associated Derlin complex to employ it as a novel route of p97-mediated ERAD. There are other examples of virally-mediated rerouting of functions for targeted protein degradation. For example, human immunodeficiency virus type-1 (HIV) produces a protein, Vpu, that is involved ERAD of CD4 (Meusser and Sommer 2004). To effect this, Vpu recruits a cytoplasmic E3 ubiquitin ligase, β-TrCP that normally has no role in ERAD (Meusser and Sommer 2004). Similarly, the human papillomavirus E6

protein programs degradation of p53 by recruiting the E6AP E3 ligase which does not normally target p53 (Scheffner et al. 1993).

In any case, it is clear that the interaction of Derlins with p97/Cdc48 is a long-standing one, and could well be involved in a variety of actions of this essential and widely used AAA ATPase.

Materials and Methods

Plasmids and DNA methods

Polymerase chain reaction (PCR) reactions were performed as follows: Vent DNA polymerase (New England Biolabs, Ipswich, MA) was used in 100ul reactions (1x thermopol buffer, 400ng template, 1uM each oligo, 2% DMSO, 25mM Mg, 200uM dNTPs). The PCR reaction was carried out at 94°C for 2 min followed by 30 cycles of 94°C for 35 seconds, 55°C for 40 seconds and elongation at 72°C for varying times depending on product length. The reaction ended with a 7-minute incubation at 72°C. Oligo sequences used for PCR are available upon request. A table of all plasmids is available in supplemental materials (Table 3-1).

All plasmids were constructed with standard molecular biology techniques as has been described by Gardner *et al.* (Gardner et al. 1998). The splicing by overlap elongation (SOEing) PCR technique used therein was adapted from Horten *et al.* (Horton et al. 1989). The ProteinA-CDC48 plasmid (pRH2078) was a gift from M. Latterich (McGill University, Quebec). Plasmids expressing KHN (pRH1958) and KWW (pRH1960) were a gift from D. Ng (National University of Singapore). The Ste6-166 plasmid (pRH2058) was a gift from S. Michaelis (Johns Hopkins School of Medicine, MD).

Yeast and Bacterial strains

Escherichia coli DH5 α were grown at 37°C in LB media with ampicillin (100ug/ml). Yeast strains were grown at 30°C unless otherwise noted in minimal media

supplemented with dextrose and amino acids as previously described (Hampton and Rine 1994). The LiOAc method was utilized to transform yeast strains with plasmid DNA (Ito et al. 1983). Null alleles with coding regions replaced by selection markers were constructed by transforming yeast with the LiOAc method with a PCR product that encoded either G418 resistance or CloNAT/nourseothricin (Werner BioAgents, Jena, Germany) resistance and 5' and 3' 50bp flanks homologous to the gene to be disrupted (Baudin et al. 1993). Cells were allowed to grow on yeast peptone dextrose (YPD) for ~12 hours and then replica plated onto YPD plus 500ug/ml G418 or 200 µg/ml nourseothricin.

A table of all strains including genotypes is available in supplemental materials (Table 3-2). The *der1*Δ (RHY3604), *dfm1*Δ (RHY3689), and *der1*Δ*dfm1*Δ (RHY3690) strains as well as the corresponding wild type strain (RHY3688) were obtained through sporulation of strains 24247 and 23341 from the yeast deletion collection (ResGen/Invitrogen, Carlsbad, CA). Haploids of *der1*Δ and *dfm1*Δ strains were then crossed, sporulated, and dissected to obtain the double mutant. Gene knockout was confirmed through PCR. These strains were used for all substrate degradation studies as well as the indicated unfolded protein response flow cytometry experiments. *DER1* or *DFM1* were also disrupted in RHY471, an S288C derivative as described above. The *ufe1-1* and an isogenic wild type strain were a gift from H. Pelham (MRC Laboratory of Molecular Biology, Cambridge).

Degradation assays and UPR measurements

Cycloheximide chase degradation assays were performed as previously described (Gardner et al. 1998). Briefly, yeast strains were grown to log phase (<0.5 OD ABS=600) and cycloheximide was added to a final concentration of 50ug/ml. At each time point, a constant volume of culture was removed and lysed. Lysis began with the addition of 100ul of SUME (1% SDS, 8M Urea, 10mM MOPS pH6.8, 10mM EDTA) with protease inhibitors and 100ul of glass beads, followed by vortexing for 3 minutes, and finally the addition of 100ul of 2X USB (75mM MOPS pH6.8, 4% SDS, 200mM DTT, 0.2 mg/ml Bromophenol blue, 8M Urea) followed by a 10 minute incubation at 55°C. The resulting lysate was clarified by centrifugation and used for SDS-PAGE and immunoblotting.

Flow cytometry for GFP was also performed as described (Cronin and Hampton 1999). Cells were grown to an OD of less than 0.2. Data was obtained through a FACScalibur machine (Becton, Dickinson and Company, Franklin Lakes, NJ) and statistical analysis was performed with CellQuest software (Becton, Dickinson and Company, Franklin Lakes, NJ).

Dilution Assays

All strains were grown to an OD of less than 0.5 OD in supplemented minimal medium. A total of 0.35OD units were then harvested and resuspended in 1ml sterile water. Five-fold dilutions were then performed, and the serially diluted cultures were

spotted on medium with the appropriate supplements to select for plasmids in strains. Plates were then grown at indicated temperatures for three days.

Co-Immunoprecipitation Assays

The protocol was modified from that used by Grandi *et al.* (Grandi et al. 1993). Strains were grown to an OD of less than 0.5 OD, and 10OD units were then harvested by centrifugation. Microsomes were made according to Shearer *et al.* (Shearer and Hampton 2004). Briefly, cells were resuspended in 1.5ml of diluted XL buffer with protease inhibitors (AEBSF – 4-(2-(2-Aminoethyl)benzenesulphonyl fluoride), TPCK - Tosylphenylalanine chloromethyl ketone, leupeptin, pepstatin). An equal volume of glass beads was added and the resulting mixture was vortexed for 6 x 1 minute. The supernatant was then removed from the beads and the beads were washed once with 1ml of the same diluted XL buffer that was added to the supernatant. The supernatant was then centrifuged for thirty minutes at 21,000g. At this point, the microsome pellets were solubilized with an IP buffer containing a non-denaturing detergent to use in co-immunoprecipitation. A total of 300ul of this lysis buffer (2% Triton X-100, 20mM NaCl, 0.2mM MgCl₂, 20mM Tris-HCL pH8.0) and 100ul of glass beads were added to the microsomes followed by 3 x 1 minute of vortexing. The supernatant was removed to a new tube and the beads were washed once with IP buffer (15mM Na₂HPO₄, 150mM NaCl, 2% Triton X-100, 0.1% SDS, 0.5% deoxycholate, 10mM EDTA final pH7.5) with protease inhibitors. Supernatant was spun for 5 min at 16,000g to remove debris and then incubated with 100ul IgG-Sepharose beads (Amersham Biosciences) in IP buffer (10% w/v) at 4°C for 1 hour, followed by two 1ml washes with TST buffer (50mM Tris pH7.6,

150mM NaCl, 0.5% Tween-20), one 1ml wash with lysis buffer and two 0.5ml washes with 5mM NH₄Ac pH5.0. Each wash was followed by a ten second spin at less than 100g. Beads were aspirated to dryness and then 100ul of 2X USB was added followed by a 10 minute incubation at 55°C.

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Chapter 3 is a reprint of Sato, BK and Hampton RY. “Yeast Derlin Dfm1 interacts with Cdc48 and functions in ER homeostasis.” *Yeast*. 2006. 23(14-15): 1053-64. I was the primary experimenter and Randolph Hampton and I wrote the manuscript. The paper was edited to be properly formatted for this chapter.

Table 3-1 Plasmids used in Chapter 3

Plasmid	Genes expressed
pRH313	<i>YIp URA3</i>
pRH316	<i>CEN LEU2</i>
pRH469	<i>YIp URA3 pTDH3-HMG2-GFP</i>
PRH613	<i>YIp URA3 ADE2 pTDH3-HMG-GFP</i>
pRH728	<i>KanMX loxP-KanMX-loxP disruption cassette</i>
pRH1209	<i>YIp URA3 p4XUPRE-GFP</i>
pRH1236	<i>CEN LEU2 pSEC61</i>
pRH1377	<i>CEN URA3 pCPY*-HA</i>
pRH1429	<i>2μ LEU2 pTDH3 promoter</i>
pRH1619	<i>CEN ADE2</i>
PRH1625	<i>2μ ADE2</i>
pRH1838	<i>NatR loxP-CloNAT-loxP disruption cassette</i>
pRH1945	<i>YIp ADE2 URA3 p4XUPRE-GFP</i>
pRH1959	<i>CEN URA3 pKHN-3HA</i>
pRH1960	<i>CEN URA3 pKWW-3HA</i>
pRH1967	<i>2μ LEU2 pTDH3-DFM1</i>
pRH2038	<i>2μ LEU2 pTDH3-DER1</i>
pRH2039	<i>CEN ADE2 pDFM1-3HA</i>
pRH2045	<i>2μ LEU2 pTDH3-DER1-DFM1 tail</i>

Table 3-1 continued Plasmids used in Chapter 3

pRH2046	<i>2μ LEU2 pTDH3-DFM1-Δshp</i>
pRH2058	<i>2μ URA3 pPGK-STE6-166-3HA-GFP</i>
pRH2078	<i>CEN LEU2 pNOPPA-ProA-CDC48</i>
pRH2083	<i>2μ ADE2 pTDH3-DFM1</i>
pRH2087	<i>CEN ADE2 pDFM1-Δshp-3HA</i>
pRH2090	<i>CEN LEU2 pNOPPA-CDC48</i>
pRH2122	<i>2μ ADE2 pTDH3-DER1</i>

Table 3-2 Strains used in Chapter 3

Strain	Genotype
RHY3603	<i>MATa ADE2::ADE2::URA3::pTDH3-HMG2-GFP MET15 lys2Δ0 ura3Δ0 TRP1 leu2Δ0 his3Δ1</i>
RHY3604	<i>MATα ADE2::ADE2::URA3::pTDH3-HMG2-GFP MET15 lys2Δ0 ura3Δ0 TRP1 leu2Δ0 his3Δ1 Δder1::KanMX</i>
RHY3605	<i>MATa ADE2::ADE2::URA3::pTDH3-HMG2-GFP MET15 lys2Δ0 ura3Δ0 TRP1 leu2Δ0 his3Δ1 Δdfm1::KanMX</i>
RHY3606	<i>MATa ADE2::ADE2::URA3::pTDH3-HMG2-GFP MET15 lys2Δ0 ura3Δ0 TRP1 leu2Δ0 his3Δ1 Δder1:: KanMX Δdfm1::KanMX</i>
RHY3634	<i>MATa ade2-101 MET2 LYS2 ura3-52::URA3::pTDH3-HMG2-GFP TRP1 leu2-3,112 HIS3 CEN::LEU2 sec61-2</i>
RHY3635	<i>MATa ade2-101 MET2 LYS2 ura3-52::URA3::pTDH3-HMG2-GFP TRP1 leu2-3,112 HIS3 CEN::LEU2::pSEC61 sec61-2</i>
RHY3718	<i>MATa ADE2 MET15 lys2Δ0 ura3Δ0 TRP1 leu2Δ0 his3Δ1 CEN::URA3::pCPY*-HA</i>
RHY3719	<i>MATα ADE2 MET15 lys2Δ0 ura3Δ0 TRP1 leu2Δ0 his3Δ1 CEN::URA3::pCPY*-HA Δder1::KanMX</i>
RHY3720	<i>MATa ADE2 MET15 lys2Δ0 ura3Δ0 TRP1 leu2Δ0 his3Δ1 CEN::URA3::pCPY*-HA Δdfm1::KanMX</i>
RHY3721	<i>MATa ADE2 MET15 lys2Δ0 ura3Δ0 TRP1 leu2Δ0 his3Δ1 CEN::URA3::pCPY*-HA Δder1::KanMX Δdfm1::KanMX</i>
RHY3869	<i>MATa ADE2 MET15 lys2Δ0 ura3Δ0 TRP1 leu2Δ0 his3Δ1 CEN::URA3::pKHN-HA</i>
RHY3870	<i>MATα ADE2 MET15 lys2Δ0 ura3Δ0 TRP1 leu2Δ0 his3Δ1 CEN::URA3::pKHN-HA Δder1::KanMX</i>
RHY3871	<i>MATa ADE2 MET15 lys2Δ0 ura3Δ0 TRP1 leu2Δ0 his3Δ1 CEN::URA3::pKHN-HA Δdfm1::KanMX</i>
RHY3872	<i>MATa ADE2 MET15 lys2Δ0 ura3Δ0 TRP1 leu2Δ0 his3Δ1 CEN::URA3::pKHN-HA Δder1::KanMX Δdfm1::KanMX</i>
RHY3873	<i>MATa ADE2 MET15 lys2Δ0 ura3Δ0 TRP1 leu2Δ0 his3Δ1 CEN::URA3::pKWW-HA</i>
RHY3874	<i>MATα ADE2 MET15 lys2Δ0 ura3Δ0 TRP1 leu2Δ0 his3Δ1 CEN::URA3::pKWW-HA Δder1::KanMX</i>
RHY3875	<i>MATa ADE2 MET15 lys2Δ0 ura3Δ0 TRP1 leu2Δ0 his3Δ1 CEN::URA3::pKWW-HA Δdfm1::KanMX</i>
RHY3876	<i>MATa ADE2 MET15 lys2Δ0 ura3Δ0 TRP1 leu2Δ0 his3Δ1 CEN::URA3::pKWW-HA Δder1::KanMX Δdfm1::KanMX</i>

Table 3-2 continued Strains used in Chapter 3

RHY3923	<i>MATa ADE2::URA::UPRE::GFP MET15 lys2Δ0 ura3Δ0 TRP1 leu2Δ0 his3Δ1 2μ::LEU2::pTDH3-DFM1</i>
RHY3925	<i>MATa ADE2::URA::UPRE::GFP MET15 lys2Δ0 ura3Δ0 TRP1 leu2Δ0 his3Δ1 2μ::LEU2::pTDH3</i>
RHY3935	<i>MATα ade2-101 met2 lys2-801 ura3-52::URA3::UPRE-GFP TRP1 LEU2 his3Δ200</i>
RHY3936	<i>MATα ade2-101 met2 lys2-801 ura3-52::URA3::UPRE-GFP TRP1 LEU2 his3Δ200 Δder1::KanMX</i>
RHY3937	<i>MATα ade2-101 met2 lys2-801 ura3-52::URA3::UPRE-GFP TRP1 LEU2 his3Δ200 Δdfm1::CloNAT</i>
RHY3938	<i>MATα ade2-101 met2 lys2-801 ura3-52::URA3::UPRE-GFP TRP1 LEU2 his3Δ200 Δder1::KanMX Δdfm1::CloNAT</i>
RHY3964	<i>MATα ADE2 MET2 LYS2 URA3 TRP1 leu2-3,112 HIS3 2μ::LEU2::pTDH3 cdc48-3</i>
RHY3965	<i>MATα ADE2 MET2 LYS2 URA3 TRP1 leu2-3,112 HIS3 2μ::LEU2::pTDH3-DFM1 cdc48-3</i>
RHY4040	<i>MATa ADE2 MET2 LYS2 ura3-52::6MYC-HMG2 hmg1Δ::LYS2 hmg2Δ::HIS3 trp1::hisG leu2Δ HIS3 2μ::LEU2::pTDH3 npl4-1</i>
RHY4041	<i>MATa ADE2 MET2 LYS2 ura3-52::6MYC-HMG2 hmg1Δ::LYS2 hmg2Δ::HIS3 trp1::hisG leu2Δ HIS3 2μ::LEU2::pTDH3-DFM1 npl4-1</i>
RHY4128	<i>MATa ADE2::URA::UPRE::GFP MET15 lys2Δ0 ura3Δ0 TRP1 leu2Δ0 his3Δ1 2μ::LEU2::pTDH3-DER1</i>
RHY4327	<i>MATα ADE2 MET2 LYS2 URA3 TRP1 leu2-3,112 HIS3 2μ::LEU2::pTDH3-DER1 cdc48-3</i>
RHY4341	<i>MATa ADE2::URA::UPRE::GFP MET15 lys2Δ0 ura3Δ0 TRP1 leu2Δ0 his3Δ1 2μ::LEU2::pTDH3-DER1-DFM1 tail</i>
RHY4342	<i>MATa ADE2::URA::UPRE::GFP MET15 lys2Δ0 ura3Δ0 TRP1 leu2Δ0 his3Δ1 2μ::LEU2::pTDH3-DFM1-Δshp</i>
RHY4353	<i>MATa ADE2 MET2 LYS2 ura3-52::6MYC-HMG2 hmg1Δ::LYS2 hmg2Δ::HIS3 trp1::hisG leu2Δ HIS3 2μ::LEU2::pTDH3</i>
RHY4354	<i>MATa ADE2 MET2 LYS2 ura3-52::6MYC-HMG2 hmg1Δ::LYS2 hmg2Δ::HIS3 trp1::hisG leu2Δ HIS3 2μ::LEU2::pTDH3-DFM1</i>
RHY4357	<i>MATa ADE2 MET2 LYS2 ura3-52::URA3 TRP1 leu2-3,112 HIS3 2μ::LEU2::pTDH3</i>
RHY4358	<i>MATa ADE2 MET2 LYS2 ura3-52::URA3 TRP1 leu2-3,112 HIS3 2μ::LEU2::pTDH3-DFM1</i>

Table 3-2 continued Strains used in Chapter 3

RHY4359	<i>MATa ade2-101 MET2 LYS2 ura3-52::URA3::pTDH3-HMG2-GFP TRP1 leu2-3,112 HIS3 CEN::LEU2 Δder1::CloNAT sec61-2</i>
RHY4360	<i>MATa ade2-101 MET2 LYS2 ura3-52::URA3::pTDH3-HMG2-GFP TRP1 leu2-3,112 HIS3 CEN::LEU2 Δdfm1::CloNAT sec61-2</i>
RHY4361	<i>MATa ade2-101 MET2 LYS2 ura3-52::URA3::pTDH3-HMG2-GFP TRP1 leu2-3,112 HIS3 CEN::LEU2 Δder1::KanMX Δdfm1::CloNAT sec61-2</i>
RHY4396	<i>MATα ADE2 MET2 LYS2 ura3-52::URA3 TRP1 leu2-3,112 HIS3 2μ::LEU2::pTDH3-DER1</i>
RHY4397	<i>MATα ADE2 MET2 LYS2 ura3-52::URA3 TRP1 leu2-3,112 HIS3 2μ::LEU2::pTDH3-DER1-DFM1 tail</i>
RHY4398	<i>MATα ADE2 MET2 LYS2 ura3-52::URA3 TRP1 leu2-3,112 HIS3 2μ::LEU2::pTDH3-DFM1-Δshp</i>
RHY4399	<i>MATα ADE2 MET2 LYS2 URA3 TRP1 leu2-3,112 HIS3 2μ::LEU2::pTDH3-DER1-DFM1 tail cdc48-3</i>
RHY4478	<i>MATa ADE2 MET15 lys2Δ0 ura3Δ0 TRP1 leu2Δ0 his3Δ1 CEN::URA3::pPGK-STE6-166-HA-GFP</i>
RHY4479	<i>MATα ADE2 MET15 lys2Δ0 ura3Δ0 TRP1 leu2Δ0 his3Δ1 CEN::URA3:: pPGK-STE6-166-HA-GFP Δder1::KanMX</i>
RHY4480	<i>MATa ADE2 MET15 lys2Δ0 ura3Δ0 TRP1 leu2Δ0 his3Δ1 CEN::URA3:: pPGK-STE6-166-HA-GFP Δdfm1::KanMX</i>
RHY4481	<i>MATa ADE2 MET15 lys2Δ0 ura3Δ0 TRP1 leu2Δ0 his3Δ1 CEN::URA3:: pPGK-STE6-166-HA-GFP Δder1::KanMX Δdfm1::KanMX</i>
RHY4506	<i>MATa ADE2 MET15 lys2Δ0 ura3Δ0 TRP1 leu2Δ0 his3Δ1 CEN::URA3::pCPY*-HA 2μ::LEU2::pTDH3</i>
RHY4507	<i>MATa ADE2 MET15 lys2Δ0 ura3Δ0 TRP1 leu2Δ0 his3Δ1 CEN::URA3::pCPY*-HA 2μ::LEU2::pTDH3-DFM1</i>
RHY4508	<i>MATα ADE2 MET15 lys2Δ0 ura3Δ0 TRP1 leu2Δ0 his3Δ1 CEN::URA3::pCPY*-HA Δder1::KanMX 2μ::LEU2::pTDH3</i>
RHY4509	<i>MATα ADE2 MET15 lys2Δ0 ura3Δ0 TRP1 leu2Δ0 his3Δ1 CEN::URA3::pCPY*-HA Δder1::KanMX 2μ::LEU2::pTDH3-DFM1</i>
RHY4590	<i>MATa ADE2 MET15 lys2Δ0 ura3Δ0 TRP1 leu2Δ0 his3Δ1 CEN::URA3::pPGK-STE6-166-HA-GFP Δdoa10::CloNAT</i>
RHY4604	<i>MATa ade2-101 met2 lys2-801 ura3-52 trp1::hisG leu2Δ his3Δ200 CEN::ADE2::pDFM1-3HA CEN::LEU2::pNOPPA-ProA-CDC48</i>
RHY4685	<i>Mata ade2-1 MET2 LYS2 ura3-52 trp1-1 ufe1::TRP1 leu2-3,112 his3-11 CEN::LEU2::pTPI::UFE1 2μ::ADE2</i>

Table 3-2 continued Strains used in Chapter 3

RHY4686	<i>Mata ade2-1 MET2 LYS2 ura3-52 trp1-1 ufe1::TRP1 leu2-3,112 his3-11 CEN::LEU2::pTPI::UFE1 2μ::ADE2::pTDH3-DFM1</i>
RHY4687	<i>Mata ade2-1 MET2 LYS2 ura3-52 trp1-1 ufe1::TRP1 leu2-3,112 his3-11 CEN::LEU2::pTPI::ufe1-1 2μ::ADE2</i>
RHY4688	<i>Mata ade2-1 MET2 LYS2 ura3-52 trp1-1 ufe1::TRP1 leu2-3,112 his3-11 CEN::LEU2::pTPI::ufe1-1 2μ::ADE2::pTDH3-DFM1</i>
RHY4705	<i>MATa ade2-101 met2 lys2-801 ura3-52 trp1::hisG leu2Δ his3Δ200 CEN::ADE2::pDFM1-3HA CEN::LEU2::pNOPPA-CDC48</i>
RHY4706	<i>MATa ade2-101 met2 lys2-801 ura3-52 trp1::hisG leu2Δ his3Δ200 CEN::ADE2 CEN::LEU2::pNOPPA-ProA-CDC48</i>
RHY4716	<i>MATa ade2-101 met2 lys2-801 ura3-52 trp1::hisG leu2Δ his3Δ200 CEN::ADE2::pDFM1-Δshp-3HA CEN::LEU2::pNOPPA-ProA-CDC48</i>
RHY4718	<i>MATa ade2-101 met2 lys2-801 ura3-52 trp1::hisG leu2Δ his3Δ200 CEN::ADE2::pDFM1-Δshp-3HA CEN::LEU2::pNOPPA-CDC48</i>
RHY4959	<i>Mata ade2-1 MET2 LYS2 ura3-52 trp1-1 ufe1::TRP1 leu2-3,112 his3-11 CEN::LEU2::pTPI::UFE1 2μ::ADE2::pTDH3-DER1</i>
RHY4961	<i>Mata ade2-1 MET2 LYS2 ura3-52 trp1-1 ufe1::TRP1 leu2-3,112 his3-11 CEN::LEU2::pTPI::ufe1-1 2μ::ADE2::pTDH3-DER1</i>

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Chapter 4

In vitro analysis of Hrd1p-mediated retrotranslocation of its natural substrate HMG-CoA reductase

Abstract

Misfolded luminal and membrane proteins in the ER are targeted for degradation by the cytosolic proteasome. In order for this to occur, it is believed that these proteins must be removed from the ER in a process known as retrotranslocation. We have established an *in vitro* assay that reconstitutes the retrotranslocation of the 8 transmembrane-spanning ERAD-M substrate, Hmg2p. Our assay demonstrates that retrotranslocation of Hmg2p depends on the AAA-ATPase Cdc48p and results in the movement of full-length, soluble Hmg2p into the cytosol. Despite *in vivo* data which does not completely support a retrotranslocation role, the Cdc48-binding factor Ubx2p and the putative proteasome delivery proteins, Rad23p and Dsk2p, are key for this process to occur. We also examined the putative retrotranslocons Sec61p and Der1p/Dfm1p, neither of which had a role. The role of Hrd1p as the retrotranslocon was also tested through a self-destructive fusion protein that expressed the Hrd1p RING domain with a transmembrane domain of a normally stable protein. This protein underwent HRD-dependent degradation *in vivo* and retrotranslocation *in vitro*, both in the absence of Hrd1p.

Introduction

Endoplasmic reticulum associated degradation (ERAD) involves the degradation of misfolded proteins by the proteasome (Hampton 2002). These proteins are targeted for degradation through the attachment of a poly-ubiquitin chain, which is added on through the cooperative efforts of the ubiquitin enzymatic cascade. The ERAD pathway presents the cell with a topological conundrum as substrates are either sequestered in the lumen or embedded in the ER membrane with luminal portions, yet the proteasome, E2s and E3 active sites are all cytosolic. Thus, it was realized early on that a critical step in the ERAD pathway involves transfer of the ERAD substrate to the cytosol for proteasomal degradation by a process referred to as retrotranslocation or dislocation (Tsai et al. 2002). Retrotranslocation is energy-requiring, and the hexameric AAA-ATPase called Cdc48p in yeast and p97 in mammals has been proposed to drive transfer of ubiquitinated ERAD substrates across the ER membrane (Bays et al. 2001b; Ye et al. 2001). By analogy with the translocation of proteins into the ER membrane and lumen, it is thought that a protein channel mediates the passing of ER substrates exiting the membrane. Several candidate channels have been proposed, with the most prominent being the derlins (Lilley and Ploegh 2005; Ye et al. 2005). The derlins are found in ERAD E3 ligase complexes (Carvalho et al. 2006; Gauss et al. 2006), and are required in a reconstituted system for the movement of a model soluble ERAD substrate across the mammalian ER membrane (Lilley and Ploegh 2004; Ye et al. 2004). Other candidates include the Sec61 channel used in anterograde transfer of proteins, and the multispinning domains of the ER ligases themselves (Plempner et al. 1997; Kreft et al. 2006).

The yeast HRD pathway mediates ERAD of numerous misfolded ER proteins and the physiologically regulated degradation of normal HMG-CoA reductase (HMGR), a key enzyme in sterol synthesis (Hampton and Rine 1994). Specifically, the yeast HMGR isozyme Hmg2p undergoes HRD pathway-mediated degradation that is regulated in response to signals from the sterol pathway (Hampton et al. 1996). The integral membrane ER ligase Hrd1p, in conjunction with Hrd3p, mediates the recognition and ubiquitination of Hmg2p, which is an 8-spanning integral membrane ER protein. Ubiquitinated Hmg2p requires the Cdc48p/Ufd1p/Npl4p complex for efficient delivery to the proteasome, presumably by promoting retrotranslocation of ER-embedded Hmg2p, although this process has not been directly observed. Subsequent recognition of ubiquitinated Hmg2p and delivery to the proteasome has been proposed to be mediated by ubiquitin-binding adaptors Dsk2p and Rad23p (Richly et al. 2005). This model is the result of numerous laboratories using both *in vivo* degradation and assays of interaction to posit the role and order of action of these factors. The reconstitution of retrotranslocation will allow detailed testing of the model, and mechanistic analysis of the known and novel steps that lead an ERAD substrate to destruction.

We have been studying the HRD-dependent degradation of Hmg2p in order to understand both regulation of the sterol pathway and the molecular mechanisms of ERAD. As part of this effort, we have reconstituted Hrd1p-mediated ubiquitination and retrotranslocation of Hmg2p *in vitro* (Flury et al. 2005). We demonstrate that the entire 8-spanning Hmg2p protein is removed from the membrane by this process, remaining intact yet soluble after retrotranslocation. Using this assay we have discerned a core set

of proteins that can mediate the recognition and removal of Hmg2p from the ER, and addressed a number of questions pertinent to current models of ERAD.

Results

Hrd1p-mediated ubiquitination of Hmg2p involves both membrane-bound and soluble proteins. The E2 Ubc7p is soluble, as are ubiquitin, the proteasome, the Cdc48p complex and other coupling factors such as Rad23p and Dsk2p. Conversely, the E3 ligase, the substrate, and Cue1p, the required anchor for Ubc7p, are integral membrane proteins. Our assay uses two distinct strains as sources of ER membranes and cytosol that are mixed to initiate the *in vitro* reaction (Flury et al. 2005). The microsome strain expresses epitope-tagged ligase Hrd1p-3HA, substrate Hmg2p-GFP, Cue1p, and any other membrane-bound proteins required for the process. The microsome strain harbors a null mutation in *UBC7*, which encodes the principle E2 for Hrd-mediated ERAD (Bays et al. 2001a). The *ubc7Δ* null prohibits nearly all ERAD of Hmg2p-GFP while the microsome strain is intact. The cytosol strain is devoid of Hmg2p-GFP, and overexpresses Ubc7p from a strong TDH3 promoter to provide a pool of soluble E2 in the cytosol fraction that is available for the ERAD reaction when mixed with the microsomes.

The reaction is started by addition of Ubc7p-containing cytosol and ATP to separately prepared microsomes, followed by incubation at 30°C. Ubiquitin transfer is measured by solubilization of the entire reaction mix, immunoprecipitation of Hmg2p-GFP (or any other substrate being tested), and SDS-PAGE immunoblotting of the precipitates for Hmg2p-GFP itself or ubiquitin to detect the conjugates. The reaction as used in this work follows a number of biological criteria of specificity, including strict dependence on Hrd1p, the K6 lysine of Hmg2p, dependence on Ubc7p and Cue1p (Hampton et al. 1996; Gardner and Hampton 1999; Bays et al. 2001a). A typical

ubiquitination reaction is shown in Figure 4-1, demonstrating the dependence on Ubc7p and ATP.

In our assay, Hrd1p is expressed from the TDH3 promoter, producing levels sufficient to drive Hmg2p-GFP ubiquitination and retrotranslocation in the absence of a number of ERAD factors including Hrd3p (Gardner et al. 2000), Usa1p (Carroll and Hampton manuscript in preparation), and Yos9p (Carroll and Hampton unpublished observation). The assay thus defines the minimal components sufficient for successful retrotranslocation, providing the best avenue for complete reconstitution. In addition, Hrd1p at this level of expression drives Hmg2p ubiquitination independently of the level of sterol pathway signals required for Hmg2p degradation at lower levels of the ligase, thus obviating the need to preserve or supply the signal after lysis. Finally, this level of Hrd1p allows for the direct recovery and detection of the retrotranslocated substrate (see below), eventually allowing the study of the dislocated Hmg2p species and analysis of the partner molecules needed for solubilization of a multispinning membrane protein (see below).

To directly evaluate Hmg2p-GFP retrotranslocation, we fractionated the *in vitro* reaction mix to assess the amount of ubiquitinated Hmg2p-GFP present in the soluble phase. The reaction mix was divided into two equal volumes. One was processed without fractionation to evaluate total ubiquitination and the amount of Hmg2p-GFP present. The remaining half was centrifuged at 25,000 g. The supernatant was removed and the resulting membrane pellet was resuspended in reaction buffer to the same volume as the removed supernatant. All three equal volume samples were then analyzed for ubiquitinated Hmg2p-GFP. The results of this retrotranslocation assay are shown in

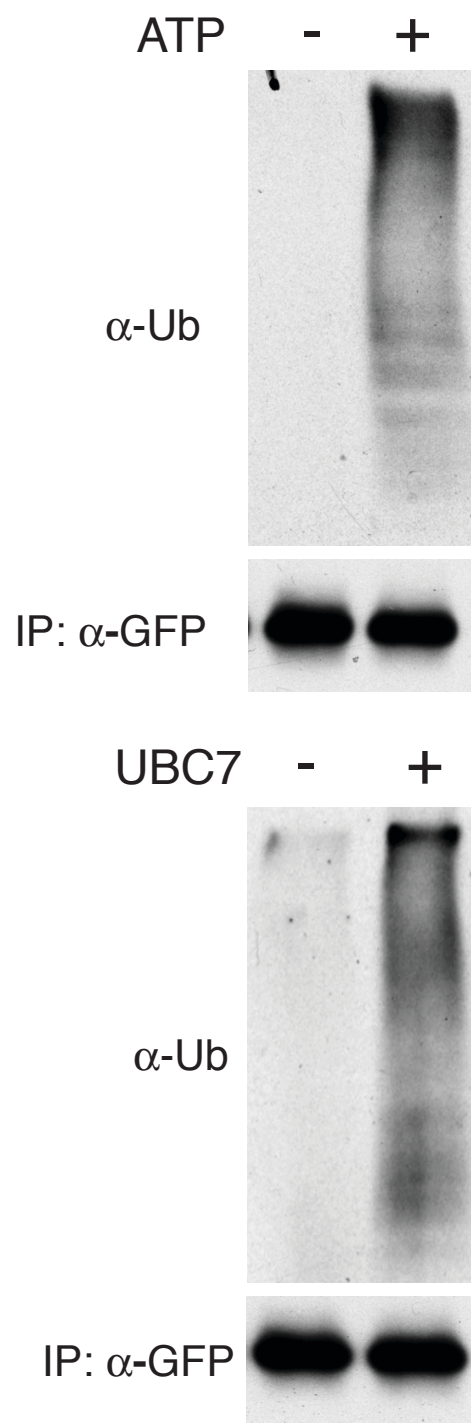


Figure 4-1 *In vitro* ubiquitination is ATP and Ubc7p dependent

In vitro ubiquitination is reconstituted when microsomes containing Hmg2p-GFP are combined with ATP and cytosol containing Ubc7p.

Figure 4-2, as three immunoblotting lanes labeled T (total lysate), S (supernatant) and P (pellet). Each lane shows both the ubiquitin immunoblot (for ubiquitinated Hmg2p-GFP) and the anti-GFP blot for unmodified Hmg2p-GFP. As can be seen, a significant portion of the ubiquitinated Hmg2p-GFP is in the supernatant, consistent with retrotranslocation. The completeness of the fractionation is indicated by the lack of native mobility Hmg2p-GFP in the S lane. Furthermore, essentially the same results are obtained with 100,000 g fractionation (data not shown). Immunoprecipitation of the supernatant with an irrelevant monoclonal (not shown) resulted in no anti-ubiquitin immunoreactivity, indicating that the ubiquitin immunoreactivity was from Hrd1p-modified Hmg2p-GFP generated in the reaction.

Retrotranslocation is thought to precede proteasomal degradation. Since there is abundant proteasome present in the cytosol fraction, we wondered if proteasomal degradation was occurring in the course of the assay. We repeated the retrotranslocation experiment in the presence of the proteasome inhibitor MG132 (Figure 4-3). With this treatment, the signal for retrotranslocated protein was more intense indicating the 20S core proteasome protease activity was contributing to lessened signal, presumably due to *in vitro* degradation of a fraction of the substrate. Thus, we used proteasome inhibitors in subsequent experiments.

As an 8-spanning, ER resident membrane protein, Hmg2p retrotranslocation is not energetically intuitive, although the possibility is predicted by current ERAD models. We next performed several tests to discern if the appearance of ubiquitinated Hmg2p-GFP was actual retrotranslocation, and thus a useful way to understand the post-ubiquitin phase of the HRD pathway.

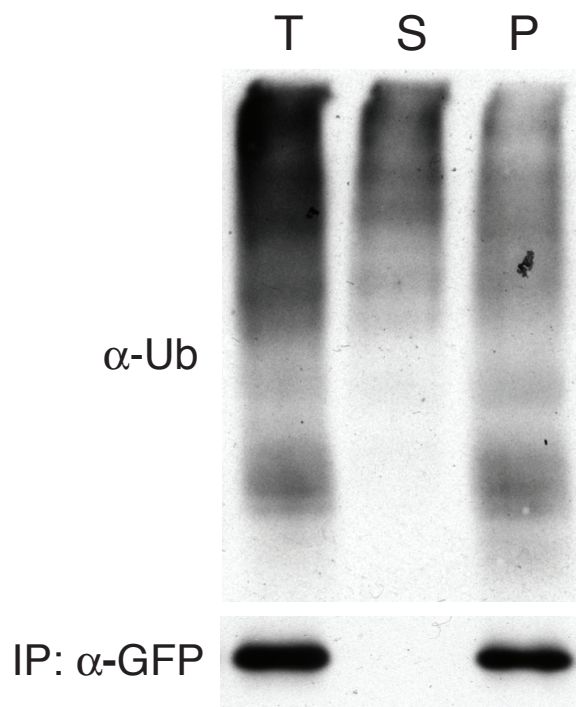


Figure 4-2 *In vitro* retrotranslocation

In vitro retrotranslocation is analyzed by centrifuging the *in vitro* ubiquitination reaction at 25,000 x g. The supernatant is removed to a new tube and the pellet fraction is resuspended in the same volume of fresh buffer. These fractions are compared to the non-separated total reaction.

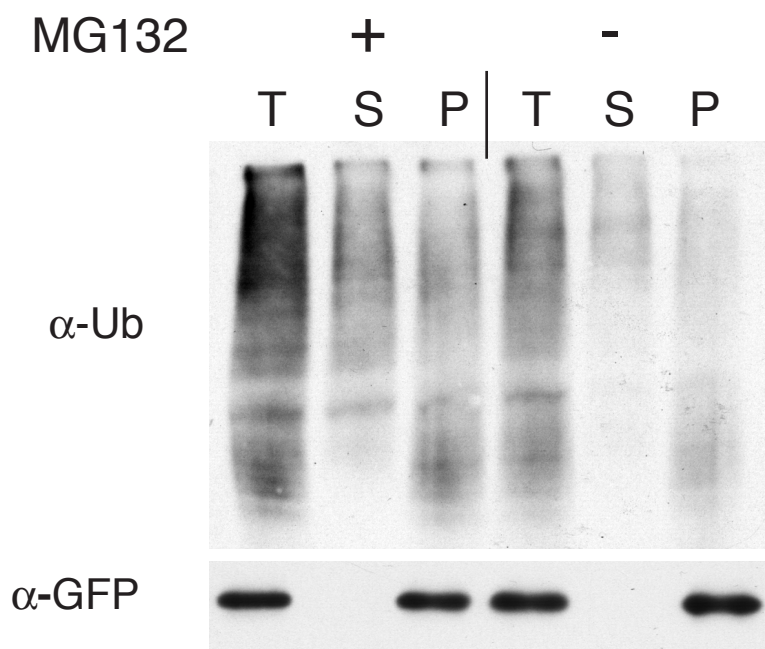


Figure 4-3 The amount of retrotranslocated protein is elevated in the presence of MG132

The *in vitro* retrotranslocation reaction was performed in the presence of 150 μ g/ml MG132 or DMSO as a control.

It has been suggested that membrane-embedded and luminal substrates can be divided into two sub-pathways of ERAD referred to as ERAD-M and ERAD-L, respectively. ERAD-L requires more factors, presumably because recognition, recruitment and exposure of the substrate to the cytosolic ubiquitination machinery requires separate processes that are not needed for integral membrane substrates. Some of these components include lectin/chaperones such as Yos9p (Friedmann et al. 2002; Szathmary et al. 2005) and traditional chaperones like Kar2p (Denic et al. 2006). These luminal proteins are not required for regulated degradation of Hmg2p. Furthermore, sufficient levels of Hrd1p will allow ERAD of both luminal and membrane-bound substrates in the absence of Hrd3p (Plempner et al. 1999; Gardner et al. 2000). Because Hrd3p serves as a protein link between the cytosol and the lumen (Gardner et al. 2000), we wondered if Hrd3p is required for the *in vitro* reactions described herein. Our suspicion was that Hrd3p would be dispensable for this reaction, since increasing Hrd1p above its genomic levels in the presence of wild type levels of Hrd3p, increases Hmg2p that ubiquitination and degradation (data not shown). Testing an otherwise isogenic *hrd3Δ* null strain confirmed this; the ubiquitination and retrotranslocation of Hmg2p was indistinguishable when compared to the normal *HRD3* strain (Figure 4-4). Thus, Hrd1p appears to play a central role in recognition and retrotranslocation of its natural substrate Hmg2p, as anticipated from its central role as defined by earlier genetic studies.

ER degradation of Hmg2p appears to be processive. A variety of tags have never revealed any fragments of this substrate produced by ERAD in our hands. This processivity is thought to be a common feature of ERAD and is consistent with wholesale removal of protein substrates by a retrotranslocation machinery. If this is the case, then

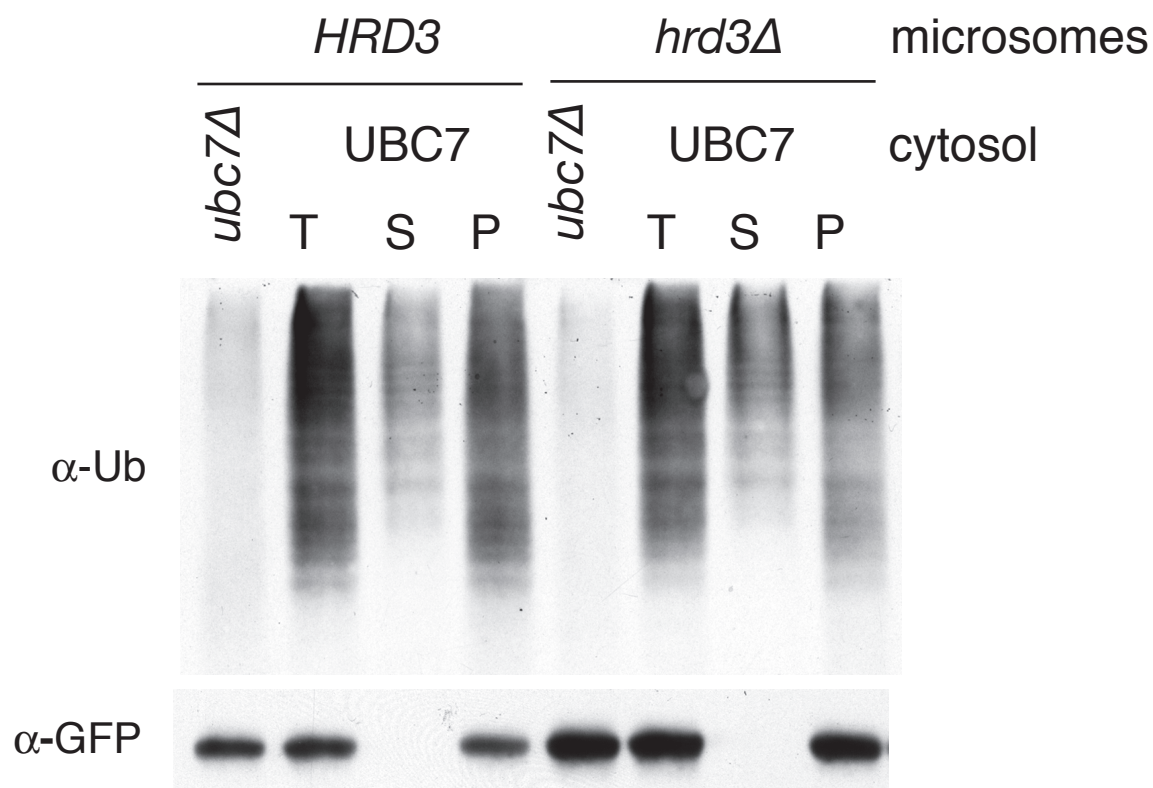


Figure 4-4 Retrotranslocation is proficient in the absence of Hrd3p

The *in vitro* retrotranslocation reaction was performed with wild type or *hrd3Δ* microsomes and wild type cytosol.

full-length Hmg2p-GFP would be predicted to be transported as a ubiquitinated molecule into the cytosolic fraction in the assay. Alternatively, the ubiquitin immunoreactivity that was precipitated with anti GFP antibodies could be GFP-containing products cleaved from the transmembrane region in the microsomal membrane. We employed an antibody raised against the last luminal loop (normally within the ER) to test if the luminal transmembrane domain determinants are present in the retrotranslocated ubiquitin-immunoreactive material. The results obtained with the anti-luminal antibodies were identical to those obtained with anti-GFP antibodies, indicating that both transmembrane domain and GFP epitopes are present in the soluble ubiquitinated substrate (Figure 4-5).

The Hmg2p-GFP N-terminal ER anchor has 8 transmembrane spans and is normally found only in membrane fractions. Accordingly, the presence of this intact molecule in the soluble fraction of the retrotranslocation assay was surprising, but consistent with current models of ERAD. We did a final experiment to confirm that the intact, ubiquitinated Hmg2p-GFP had been solubilized, by performing the immunoprecipitation of the cytosolic "S" fraction using an immunoprecipitation buffer without detergent (Figure 4-6). Parallel samples of the supernatant fraction were subjected to detergent-free immunoprecipitation with either pre-immune serum or an anti-GFP antibody. Although there is a higher background of non-specific ubiquitin immunoreactivity than seen with the normal IP procedure with detergent, there is a clear increase of ubiquitinated protein brought down by the anti-GFP serum, indicating that this ubiquitinated, polytopic membrane protein is present in the cytoplasmic fraction as a soluble protein, presumably in a complex with factors that mediate retrotranslocation.

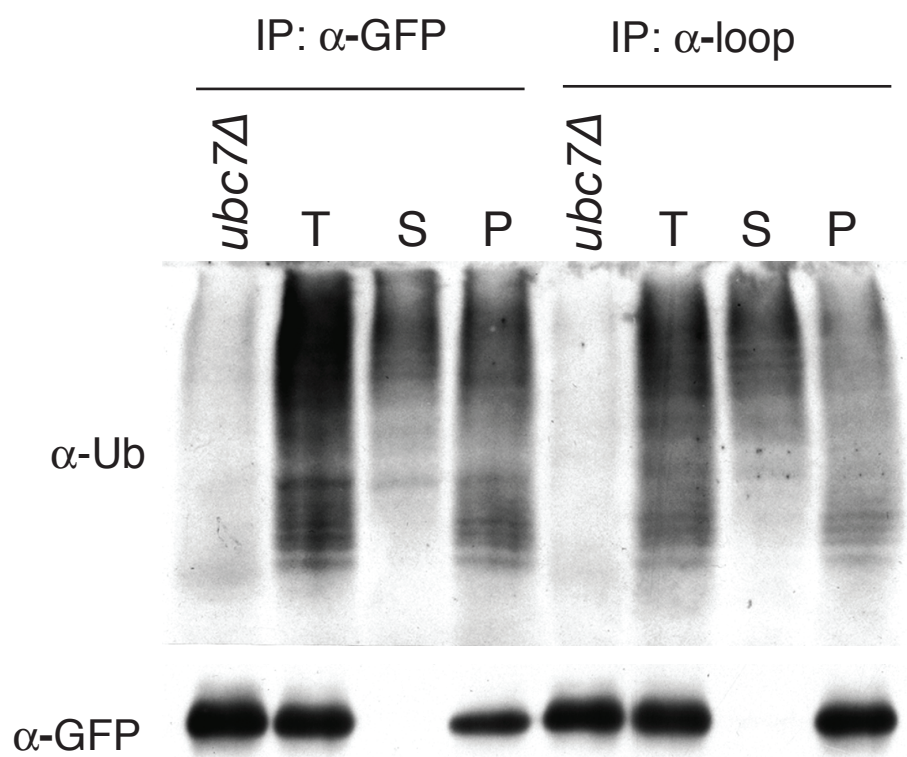


Figure 4-5 Retrotranslocation is identical with either anti-GFP or anti-Hmg2p loop antibodies

The *in vitro* retrotranslocation reaction was analyzed by immunoprecipitation with either an anti-GFP antibody or an antibody which localizes to a luminal loop of the Hmg2p transmembrane span.

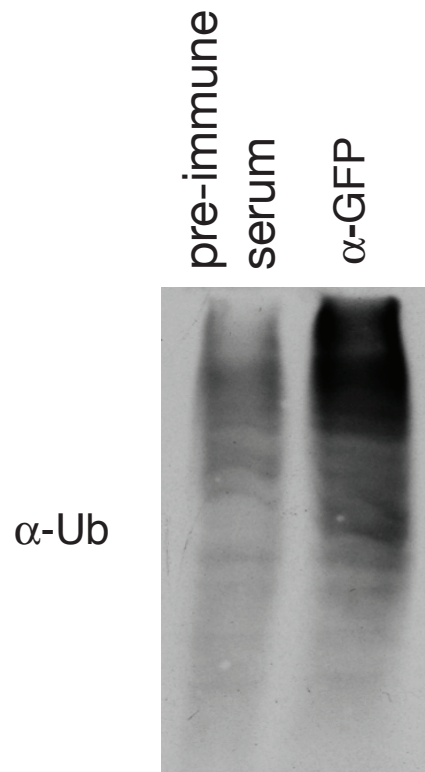


Figure 4-6 Retrotranslocated Hmg2p can be immunoprecipitated in a detergent-free IP

The supernatant fraction containing retrotranslocated Hmg2p was immunoprecipitated in the absence of SUME lysis buffer and with IP buffer that lacked all detergents. This was subjected to either pre-immune serum or an anti-GFP antibody.

The above experiments all indicate that ubiquitinated Hmg2p-GFP is moved to the soluble fraction intact. To directly test if full-length Hmg2p-GFP was retrotranslocated, we used recombinant Usp2, that efficiently strips ubiquitin from multi-ubiquitin chains on substrates (Ryu et al. 2006). Removal of ubiquitin from the immunoprecipitated protein allowed us to directly examine the molecular weight of the retrotranslocated GFP by immunoblotting. After immunoprecipitating the retrotranslocated, ubiquitinated protein, we divided the sample into two aliquots and treated one with buffer and the other with Usp2 (Figure 4-7). The upper panel shows an anti ubiquitin blot of the sample, and demonstrates the effect of the Usp2 "ubiquitin strippase". The lower panel shows the same sample immunoblotted with anti-GFP antibodies. Without ubiquitin stripping there is no detectable GFP, while in the stripped sample, the GFP immunoreactivity increases as a discrete band with the molecular weight of Hmg2p-GFP. Importantly, no other sizes of GFP immunoreactivity were liberated by Usp2; only the mobility of full-length Hmg2p-GFP was detected. This experiment directly demonstrates that intact Hmg2p-GFP is being moved to the cytosol as a multi-ubiquitinated protein, as indicated by the anti-loop antibody and as predicted by processive retrotranslocation. In this ubiquitin-stripping procedure, the immunoprecipitated protein from the "S" fraction is immunoblotted directly for GFP. Thus, the use of ubiquitin immunoblotting as an assay of retrotranslocated Hmg2p-GFP provides a way to examine this process without this background signal. Nevertheless, it was important to demonstrate that Hmg2p-GFP is moved in its entirety to the soluble fraction.

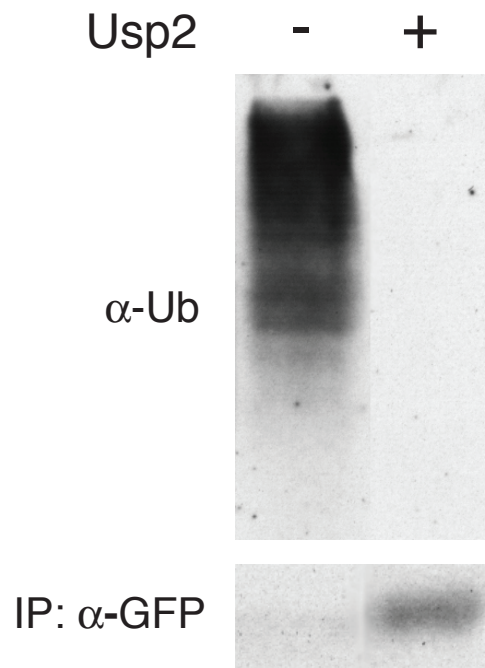


Figure 4-7 The ubiquitin signal in the supernatant fraction can be de-ubiquitinated which results in the appearance of an anti-GFP signal

The retrotranslocated fraction was incubated with or without the purified ubiquitin protease Usp2 for 1 hour at 37°C. Following this treatment, Hmg2p-GFP was immunoprecipitated with an anti-GFP antibody.

A unifying feature of many ERAD pathways is the proposed role of the hexameric AAA-ATPase Cdc48p (p97 in mammals) in retrotranslocation (Bays et al. 2001b; Ye et al. 2001; Jarosch et al. 2002). This essential complex, in conjunction with its binding partners Ufd1p and Npl4p, has been implicated in a post-ubiquitination step and has been directly shown in a permeabilized cell assay to mediate retrotranslocation of MHC-I molecules in the viral ERAD pathway promoted by the US2 and US11 proteins (Ye et al. 2003). Hmg2p is strongly stabilized by mutations in the Cdc48 complex, with a block that occurs after ubiquitination. We tested the role of Cdc48p in our *in vitro* retrotranslocation assay. We incorporated the *cdc48-2* allele in our assay strains (see methods), and simultaneously confirmed that this allele shows a strong *in vivo* block in Hmg2p-GFP degradation, even at the permissive temperature of 30°C (Figure 4-8A). The *cdc48-2* allele was evaluated *in vitro* when present in both the microsomal and cytoplasmic strains. Although Ubc7-dependent ubiquitination occurred, there was a nearly complete block in release of ubiquitinated Hmg2p-GFP into the cytoplasmic "S" fraction (Figure 4-8B). The block to ERAD occurs at the permissive growth temperature of 30°C, as did the block to retrotranslocation. It is interesting to note that the *in vitro* assay with *cdc48-2* shows a small but consistent decrease in Hmg2p ubiquitination.

The Cdc48 complex has binding sites for ubiquitin, located both on Ufd1p and Cdc48 itself (Flierman et al. 2003; Ye et al. 2003). The importance of these sites have been demonstrated for the case of virally-mediated MHC-I retrotranslocation by mammalian p97, and we wondered if the multi-ubiquitin chains served a similar "guiding" role in directing retrotranslocation with polytopic Hmg2p in yeast. p97-mediated recognition of multi-ubiquitin chains does not occur when bulky GST-Ub

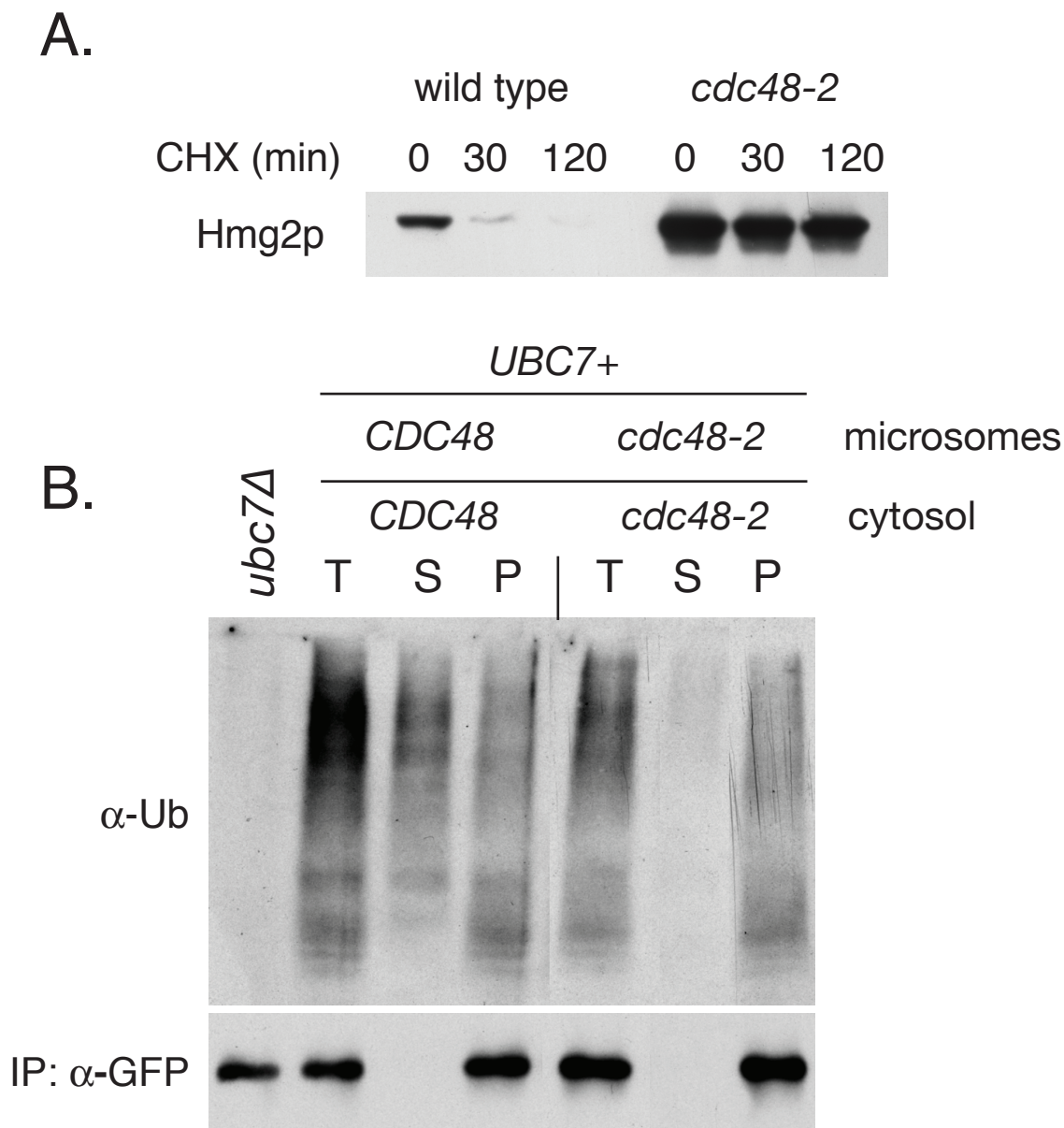


Figure 4-8 Cdc48p is required for Hmg2p degradation and retrotranslocation

(A). *In vivo* Hmg2p degradation was assayed by cycloheximide chase. Cycloheximide was added to the indicated strains and equal amounts of cells were harvested at certain time points. Lysates were analyzed by SDS-PAGE and immunoblotted with anti-GFP antibodies. Equal loading was verified by India ink staining. (B). *In vitro* retrotranslocation was analyzed by combining wild type or *cdc48-2* microsomes and cytosol. Ubiquitination did not occur when *cdc48-2* microsomes were combined with cytosol lacking Ubc7p (data not shown)

fusion is used instead of native Ub. Similarly, Hmg2p-GFP retrotranslocation was dependent on native ubiquitin. Use of the N-terminal GST-Ub fusion allowed *in vitro* ubiquitination to proceed producing large conjugates in the reaction mix (Figure 4-9). However there was little or no movement of the GST-Ub-tagged Hmg2p-GFP to the cytosol, underscoring the importance of the ubiquitin molecule in the retrotranslocation process. Because Hmg2p-GFP was effectively ubiquitinated, this also ruled out simple "ratcheting models" by which ubiquitin addition caused movement of the Hmg2p-GFP out of the ER due solely to that modification. Interestingly, a similar experiment with K6W ubiquitin, that blocks proteasomal degradation (Shang et al. 2005), did not inhibit the retrotranslocation assay (data not shown).

The Cdc48 complex is found both in the microsome fraction and in the cytoplasmic pool. In fact, recent work has shown that Cdc48 is bound to the HRD complex. Thus we wondered whether membrane-bound or soluble Cdc48p provided the activity for retrotranslocation. Because our assay requires cytoplasmic and membrane-bound components, we could examine this question by judicious use of *cdc48-2* or wild-type assay strains. We prepared microsomes and cytosol with either normal *CDC48* or the *cdc48-2* allele and ran the retrotranslocation assay with the various combinations of mutant or wild-type material, as indicated (Figure 4-10). Although the strongest effect on retrotranslocation was seen when both membrane and cytosol were *cdc48-2*, it was clear that the cytosol alone contributes the majority of the needed Cdc48 activity. This implies that soluble Cdc48 complexes are recruited from the aqueous medium for their role in retrotranslocation.

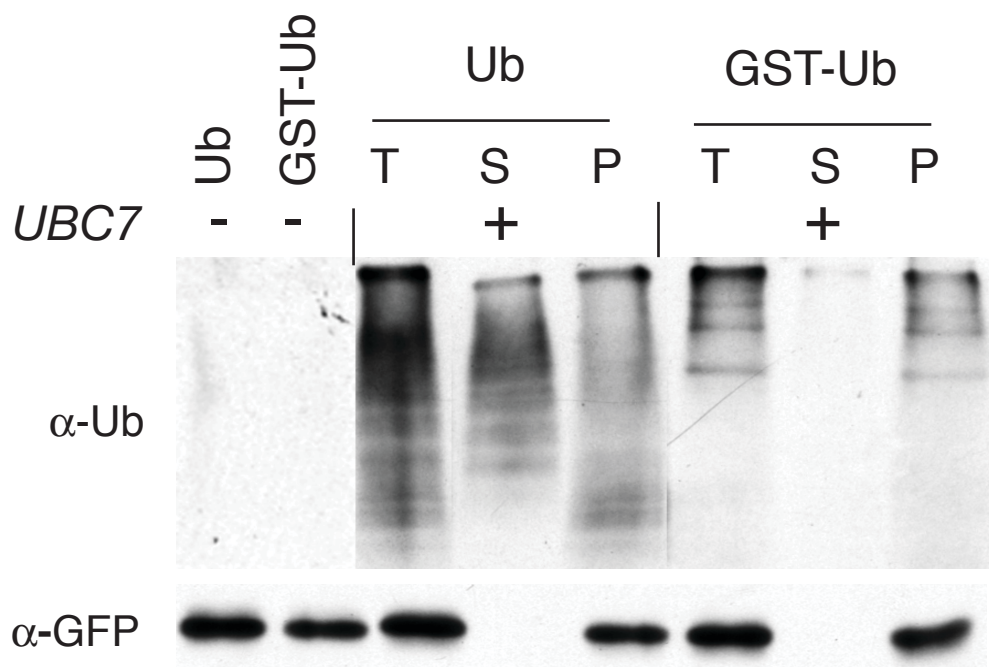


Figure 4-9 Poly-ubiquitin chains formed with GST-ubiquitin inhibit retrotranslocation of Hmg2p

The *in vitro* retrotranslocation reaction was performed in the presence of 20 μ M wild type ubiquitin or GST-ubiquitin.

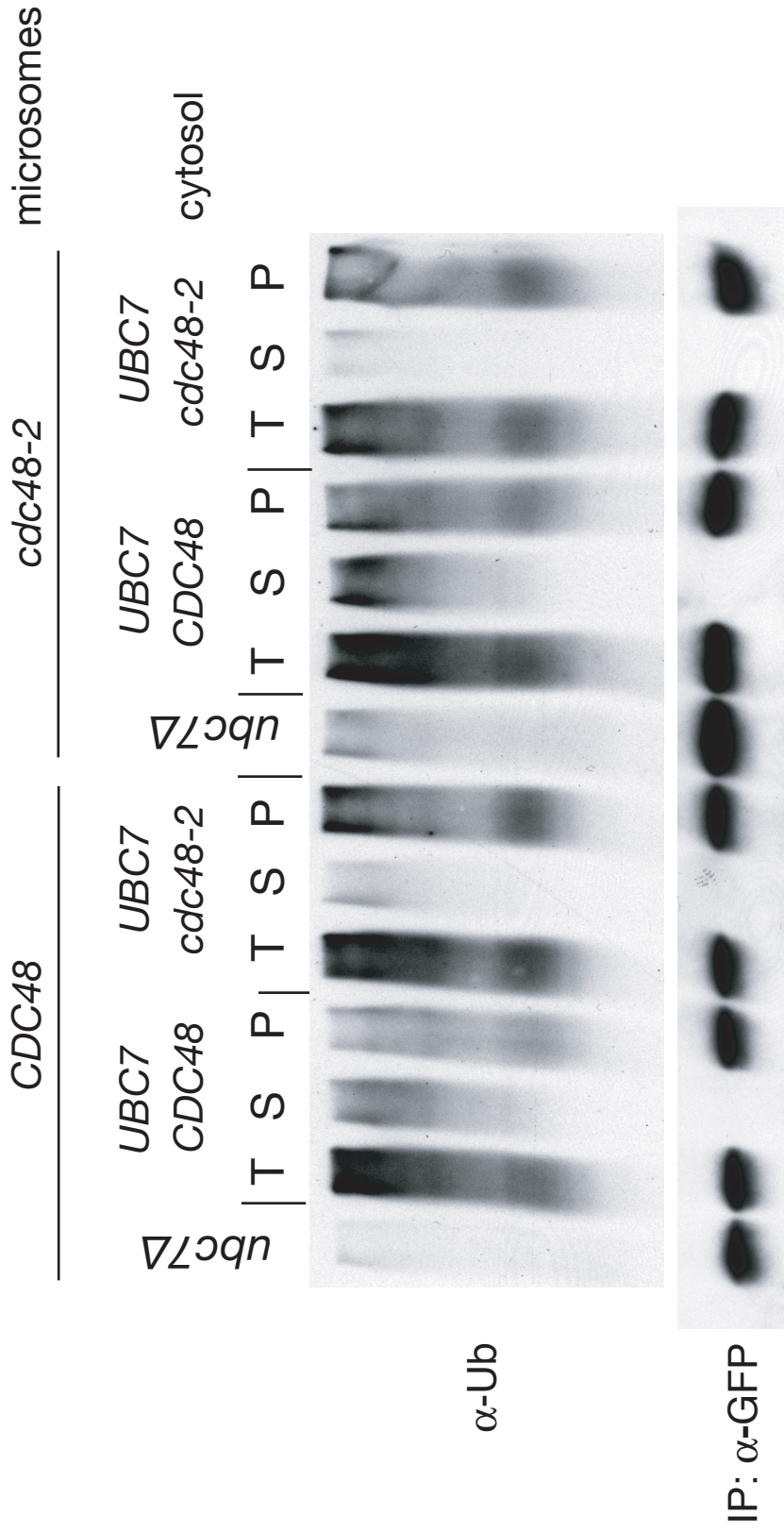


Figure 4-10 The cytosolic fraction of Cdc48p appears to supply the majority of the retrotranslocation function

In vitro retrotranslocation was analyzed by combining wild type or *cdc48-2* microsomes with the indicated versions of cytosol.

A significant portion of the Cdc48p complex is found in the cytosol and this fraction provides most of the Cdc48p involved in retrotranslocation, which leads naturally to the question of how it engages the HRD complex at the ER surface. It has been proposed from co-immunoprecipitation and *in vivo* experiments that the ER-localized integral membrane protein Ubx2p serves as an ER-localizing receptor or docking site for the Cdc48 complex (Neuber et al. 2005; Schuberth and Buchberger 2005). We used the retrotranslocation assay to directly test this idea. The *ubx2Δ* null allele was introduced into the microsomes strain. When the assay was run with *ubx2Δ* membranes, it was clear that retrotranslocation was completely abrogated, as shown by the complete lack of immunoreactivity in the soluble fraction (Figure 4-11). Not surprisingly, since Ubx2p is an integral membrane protein, cytosol from a wild-type *UBX2* strain did not allow retrotranslocation from *ubx2Δ* membranes (data not shown). However, the loss of microsomal Ubx2p also showed unexpected ubiquitination of Hmg2p-GFP in the absence of added Ubc7. This ubiquitination of Hmg2p is present in the microsomes fraction before the reaction is initiated, and was observed when the Hmg2p is directly immunoprecipitated from lysates of the *ubc7Δ* null microsomes strain (Appendix 2). This "pre-ubiquitination" is highly reproducible and implies that Ubx2 may have roles at other positions in the ERAD pathway, at least of Hmg2p. At present, we do not know how this process occurs, although further work conducted on Ubx2p can be found in Appendix 2.

We confirmed the generality of Ubx2p's requirement in retrotranslocation by examining Hrd1p itself as a substrate. Normally, Hrd1p is present in stoichiometric balance with Hrd3p, and is quite stable. In this assay, Hrd1p levels are sufficiently

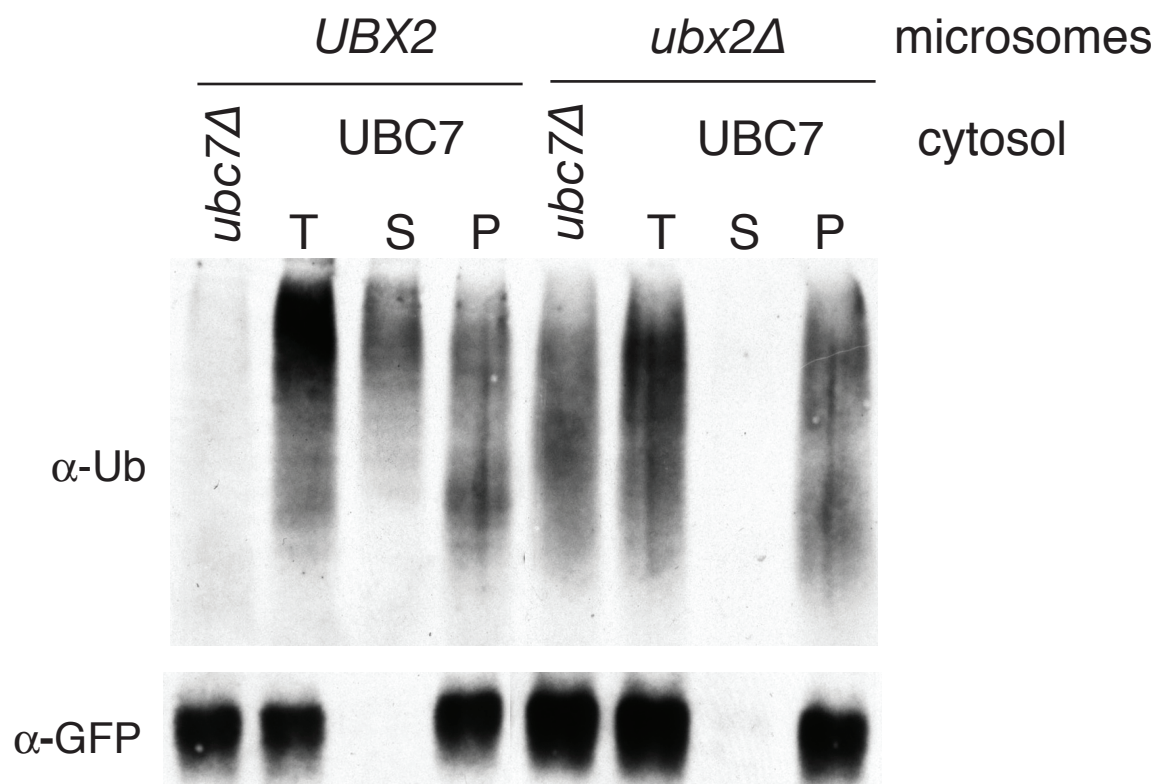


Figure 4-11 Retrotranslocation is inhibited in *ubx2Δ* microsomes

The *in vitro* retrotranslocation reaction was performed with wild type or *ubx2Δ* microsomes and wild type cytosol.

elevated to allow Hrd1p self-degradation, mediated by Hrd1p's own RING domain (Gardner et al. 2000). Hrd1p undergoes Ubc7-dependent self-ubiquitination *in vitro*. As with Hmg2p-GFP, a portion of the ubiquitinated Hrd1p is retrotranslocated (Figure 4-12) but this does not occur in the *ubx2Δ* microsomes. Curiously, Hrd1p does not show the pre-ubiquitination in the *ubx2Δ* microsomes that was seen with Hmg2p-GFP.

The block to retrotranslocation caused by *ubx2Δ* is strong with either Hmg2p or Hrd1p. However, it is important to note that *in vivo*, loss of Ubx2p only partially blocks ERAD. This is clear in previous reports (Neuber et al. 2005; Schuberth and Buchberger 2005), and is demonstrated in Figure 4-13, with Hmg2p-GFP. Flow cytometry profiles of cells expressing the substrate in wild-type or *ubx2Δ* null strains showed that there is an elevation of Hmg2p-GFP and a slowing of degradation, but not a complete block as was seen in the same strain harboring the hypomorphic *cdc48-2* allele. Clearly, there must be other ways for Cdc48p to get to the ERAD process, since a complete null of the Ubx2p receptor has a lesser effect on Hmg2p-GFP ERAD than a partially functioning hypomorph of *cdc48-2*. Nevertheless, the combination of the *in vitro* assay and *in vivo* analysis allows us to examine detailed mechanisms of known ERAD components and place their function in the broader context of multiple pathways and mechanisms of ERAD.

ERAD involves the transfer of ubiquitinated substrates to the proteasome from the ER surface. A number of genetic and biochemical studies have implicated a set of adaptor proteins that facilitate this transfer. These adaptors have both ubiquitin-binding UBA motifs and proteasome-binding UBL motifs, and thus appear well-suited for this job. Yeast has two of these factors, Dsk2 and Rad23, that have been previously

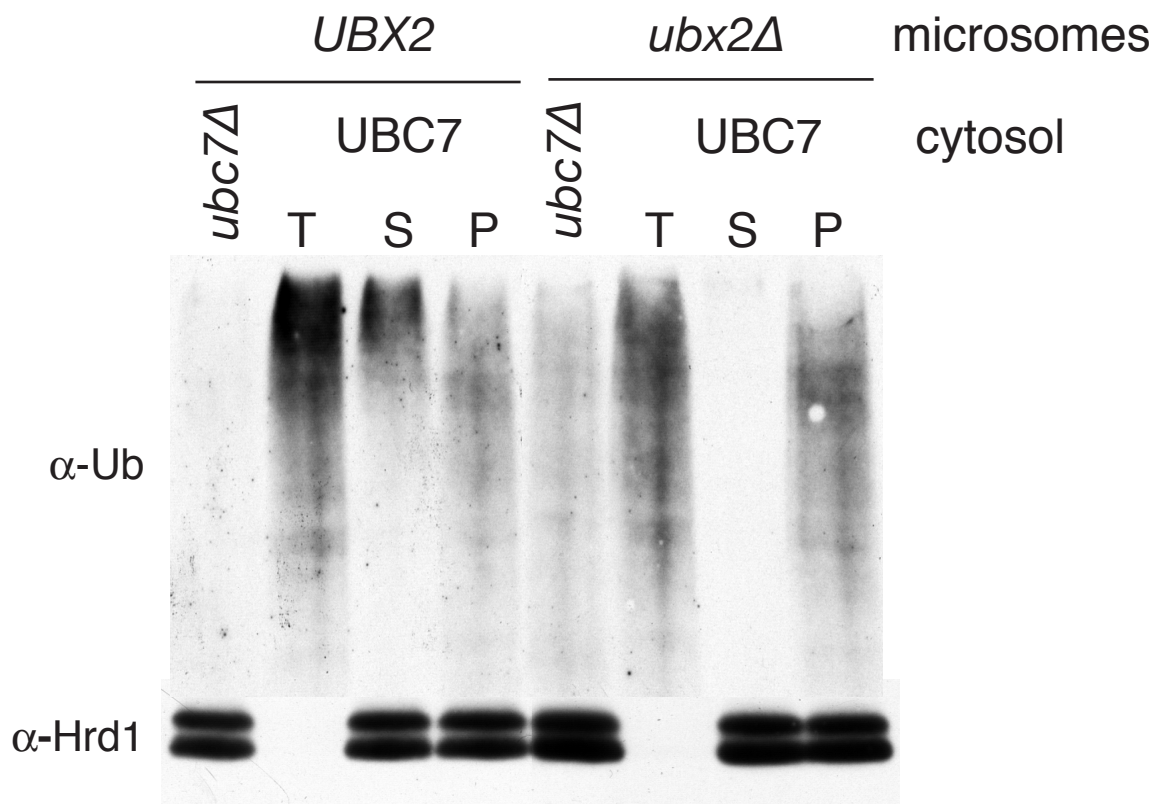


Figure 4-12 Hrd1p retrotranslocation is inhibited in *ubx2Δ* microsomes despite a lack of pre-ubiquitination

The *in vitro* retrotranslocation reaction was performed with wild type or *ubx2Δ* microsomes and wild type cytosol. Hrd1p was immunoprecipitated with anti-Hrd1p antibody and immunoblotted with anti-HA antibody.

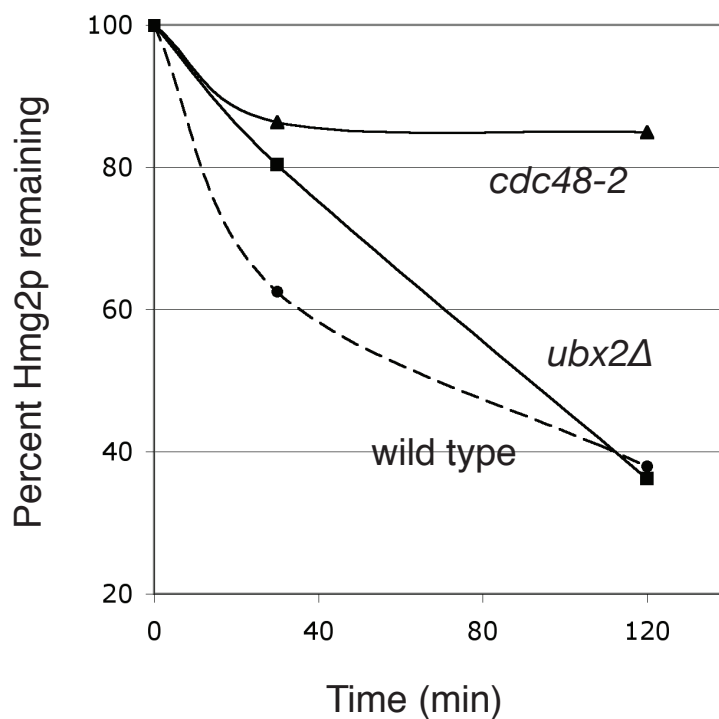
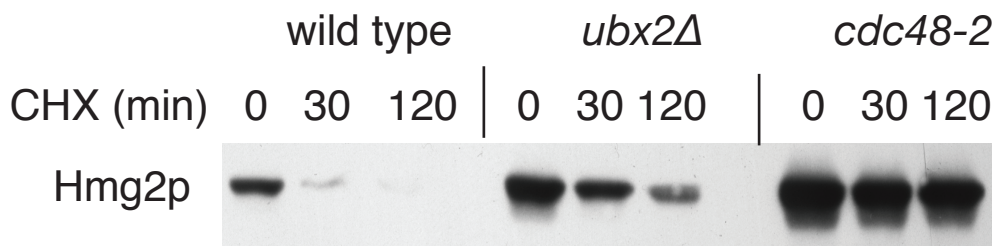


Figure 4-13 A *ubx2Δ* is not a phenocopy of a *cdc48* mutant

In vivo Hmg2p degradation was assayed by cycloheximide chase. Cycloheximide was added to the indicated strains and equal amounts of cells were harvested at certain time points. Lysates were analyzed by SDS-PAGE and immunoblotted with anti-GFP antibodies. Equal loading was verified by India ink staining. Quantitation was performed by flow cytometry as previously described (Chapter 2).

implicated in ERAD (Medicherla et al. 2004; Richly et al. 2005). They are sufficiently similar that the double null is required to observe an ERAD phenotype *in vivo*. By the simplest model, we would expect the *rad23Δdsk2Δ* double null to have no effect on retrotranslocation in our assay, since we use proteasome inhibitors. We first tested reactions in which both the cytosol and the microsomes were derived from double null strains. Surprisingly, the absence of these two adaptors had a strong effect on Hmg2p retrotranslocation (Figure 4-14).

We next determined whether the cytosol or microsome fraction had the greatest contribution of Rad23p and Dsk2p activity (Figure 4-14). Both Rad23p and Dsk2p are soluble proteins and thus, not surprisingly, the use of the *rad23Δdsk2Δ* double null cytosol had a significant effect on Hmg2p retrotranslocation. However, the combined use of both microsomes and cytosol from double null strains had the largest effect, implying that there might be a role for these adaptors at the microsome that is not the same as that provided by the soluble pool. Consistent with this, removal of Rad23p and Dsk2p in only the microsome fraction did not appear wild-type, and showed retrotranslocation of only very high molecular weight Hmg2p-GFP species. It is also clear from the figure that the extent of Hmg2p ubiquitination is lessened when these two proteins are missing from the cytosolic fraction. This is seen by comparing the total lysate lane from wild type with the other three examples. In all cases, the loss of Dsk2p and Rad23p causes a reproducible decrement of extent of Hmg2p ubiquitination, with the cytosolic pool having the larger role. Taken together, the results indicate that these ubiquitin-binding adaptors could be involved in retrotranslocation as well as delivery to the proteasome.

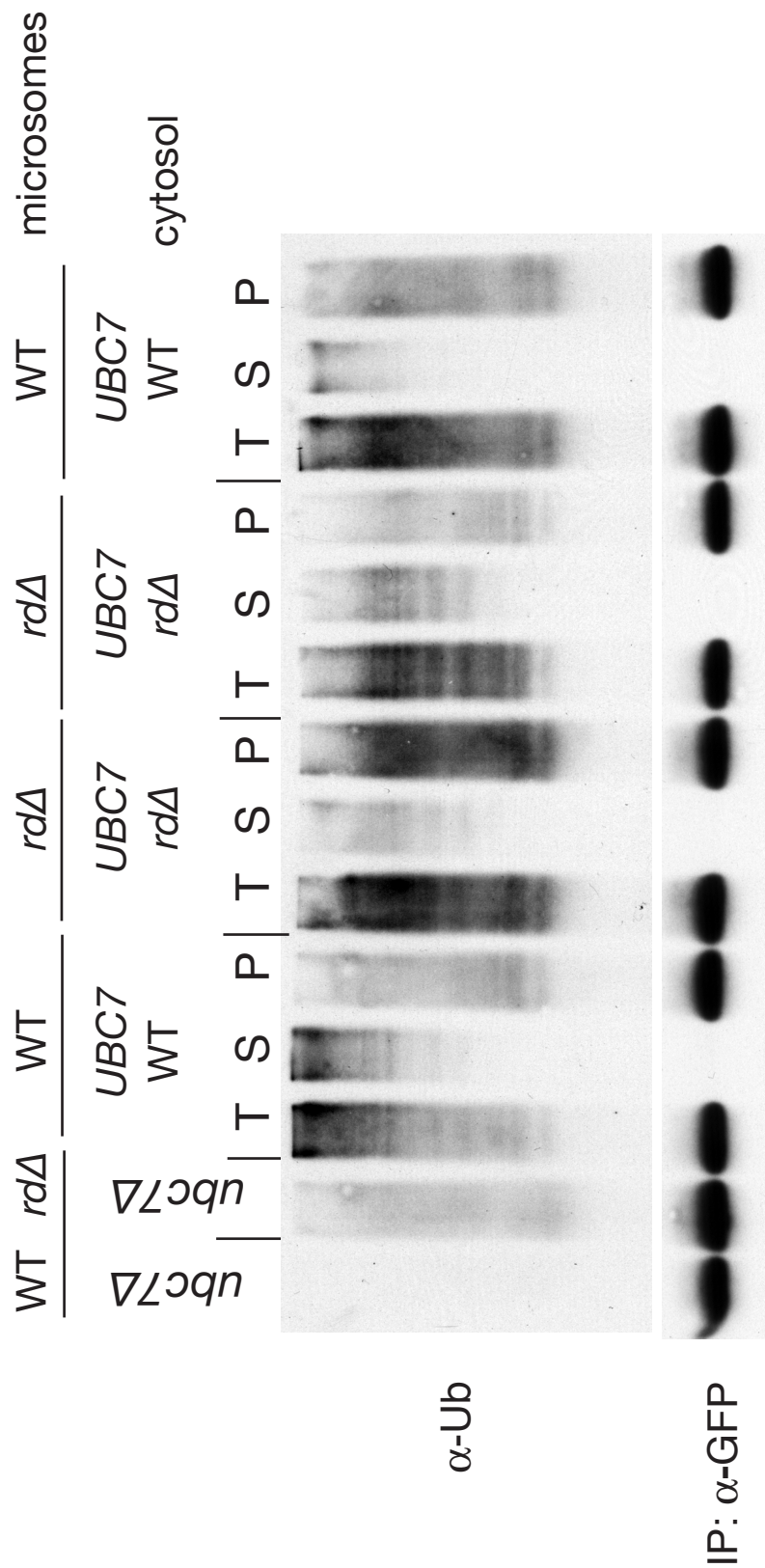


Figure 4-14 Rad23p and Dsk2p are required for Hmg2p retrotranslocation and both the cytosolic and membrane fractions appear to contribute to this function

In vitro retrotranslocation was analyzed by combining wild type or *rad23Δdsk2Δ* microsomes with the indicated versions of cytosol.

It is widely and reasonably thought that a pore or channel is required for the removal of ERAD substrates. In the case of Hmg2p, the intact molecule is removed to the cytosol, requiring the dislocation of 8 transmembrane spans and the four luminal loops which would present a substantial energy barrier for movement across the ER bilayer. The best candidates for such a pore include members of the derlin family (Lilley and Ploegh 2004; Ye et al. 2004), or the anterograde pore Sec61p (Plempner et al. 1997), both of which have been suggested to be broadly involved in dislocation with the strongest evidence favoring the derlins. Yeast has two members of this family, the original Der1p protein, and a homologous proteins Dfm1p that has demonstrable Cdc48p-binding activity (Knop et al. 1996; Sato and Hampton 2006). We tested membranes from a *der1Δdfm1Δ* double null strain in the retrotranslocation assay (Figure 4-15). We also tested membranes from the *sec61-2* strain, which has reproducible effects on the *in vivo* ERAD of Hmg2p (Sato and Hampton 2006), but they were fully proficient for retrotranslocation (Figure 4-16). In no case was there any effect of loss of these channel candidates on directly observed retrotranslocation of Hmg2p.

The centrality of Hrd1p in ERAD, and the lack of evidence for a role of putative channels in the retrotranslocation of Hmg2p led us to wonder if Hrd1p itself was providing channel function in addition to its role as the E3 ligase. This idea is structurally appealing, since Hrd1 has a multi-spanning membrane domain in addition to the cytosolic RING ligase domain. Furthermore, the Hrd1 N-terminal anchor has a substantial number of hydrophilic residues, which might be expected in a structure that forms an aqueous channel through which retrotranslocation substrates travel. However,

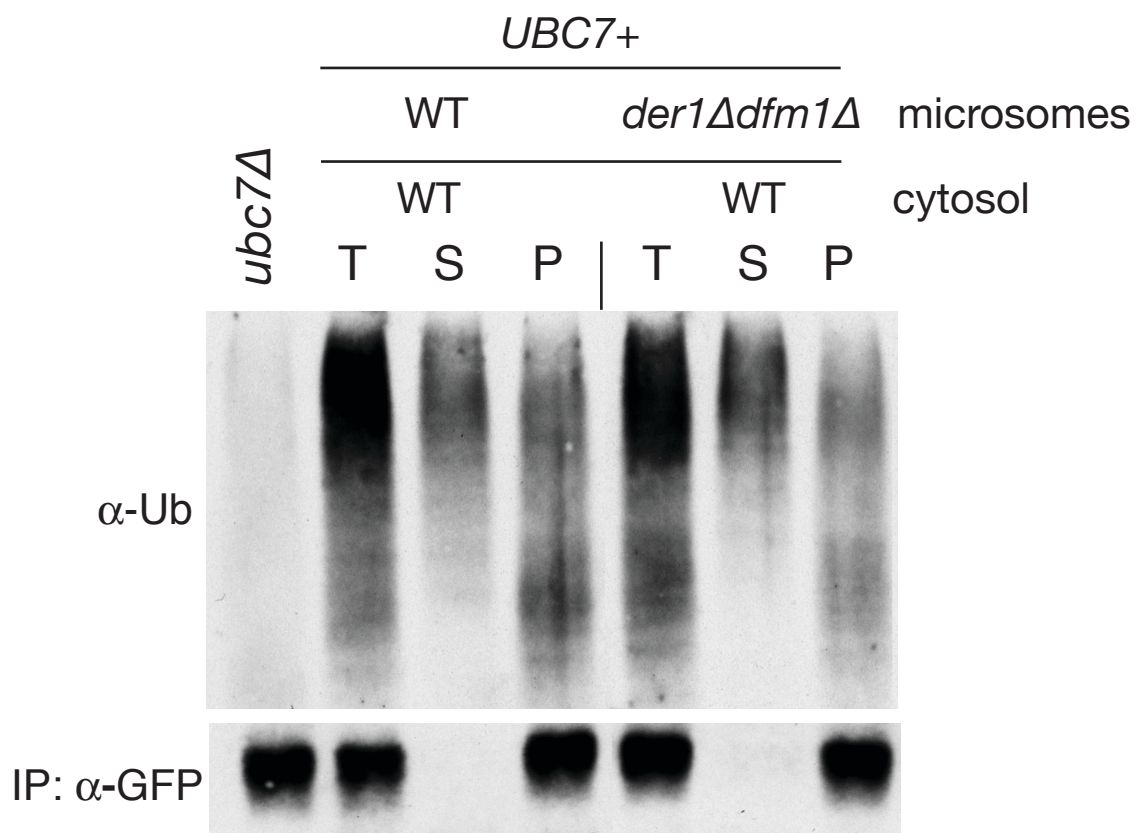


Figure 4-15 Der1p and Dfm1p have no role in Hmg2p retrotranslocation

In vitro retrotranslocation was analyzed by combining wild type or *der1Δdfm1Δ* microsomes and wild type cytosol. Ubiquitination did not occur when *der1Δdfm1Δ* microsomes were combined with cytosol lacking Ubc7p (data not shown)

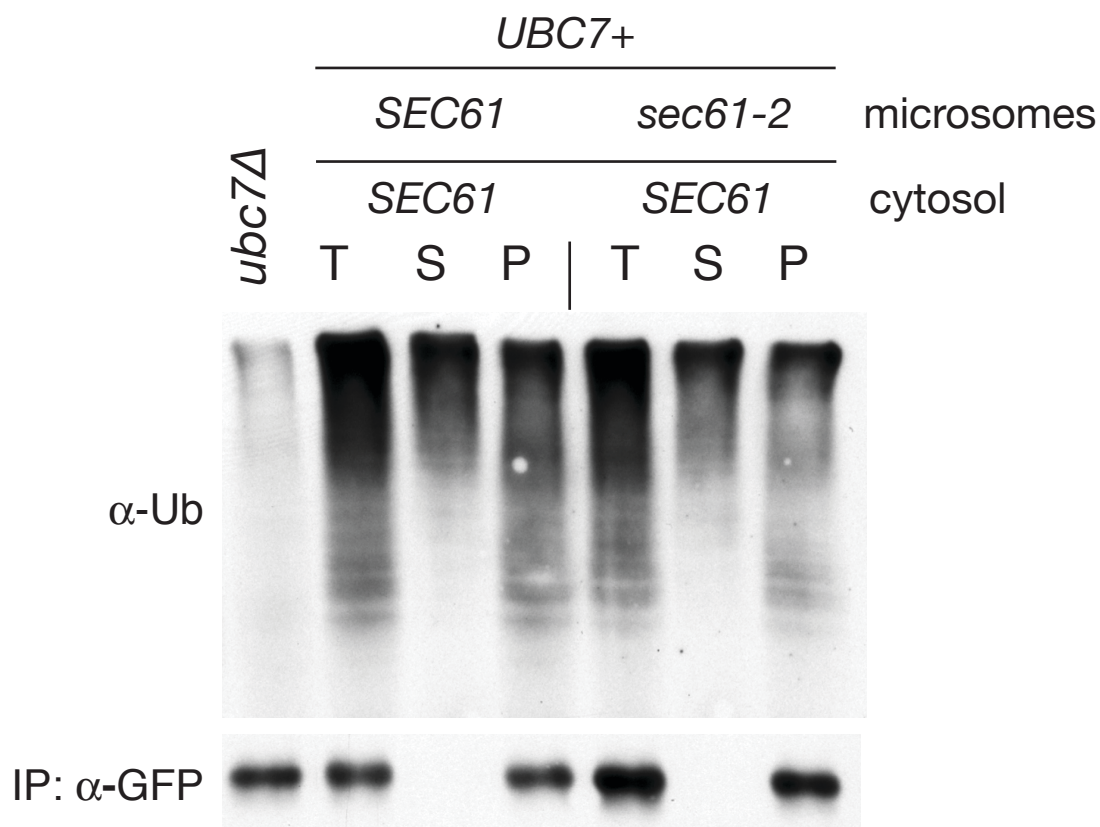


Figure 4-16 Sec61p has no role in Hmg2p retrotranslocation

In vitro retrotranslocation was analyzed by combining wild type or *sec61-2* microsomes and wild type cytosol. Ubiquitination did not occur when *sec61-2* microsomes were combined with cytosol lacking Ubc7p (data not shown)

the simple experiment of removing Hrd1 was not feasible because it also is necessary for Hmg2 ubiquitination, which is a prerequisite for subsequent removal by Cdc48.

To separate the Hrd1 ligase function from other possible activities, we devised a “self-destructive” substrate that employs the Hrd1p RING domain in the absence of its transmembrane region. We did this by fusing the Hrd1 cytosolic RING domain to the transmembrane domain of the normally highly stable Hmg1p HMG-CoA reductase isozyme, called Hmg1-Hrd1 (Figure 4-17A). *In vivo*, Hmg1-Hrd1 behaves in all ways like a HRD pathway substrate, but does not require Hrd1p’s presence due to the in-cis RING domain. Its degradation is very rapid, and strongly dependent on both Ubc7p and the intact RING domain (Figure 4-17B, 4-17C). Hmg1-Hrd1 degradation is also slowed by mutations in the RPN1 hypomorph *hrd2-1* and *cdc48-2* (Figure 4-18A, 4-18B). The degradation of Hmg1-Hrd1 occurs in a *hrd1Δ* null strain and in the *hrd1Δdoa10Δ* double null with very similar kinetics and extent as in a wild-type strain (Figure 4-18C). The rapid proteasomal degradation of Hmg1-Hrd1 implied that the autonomously degrading fusion protein undergoes retrotranslocation. We tested this directly *in vitro*, and indeed, ubiquitinated Hmg1-Hrd1 showed the expected movement to the soluble supernatant fraction (Figure 4-19). As predicted from the *in vivo* experiments, Hmg1-Hrd1 retrotranslocated with similar efficiency from microsomes derived from a *hrd1Δ* null strain. The *in vitro* retrotranslocation of the fusion was partially blocked in an assay run with both cytosol and supernatant from the *cdc48-2* mutant (Figure 4-19). Finally, we tested if intact Hmg1-Hrd1 retrotranslocation had occurred by again using the Usp2 “strippase” to demonstrate recovery of the full-length fusion protein from the retrotranslocated fraction (Figure 4-20). Thus, Cdc48-mediated retrotranslocation of this

HRD pathway substrate can occur in the complete absence of the Hrd1p transmembrane domain.

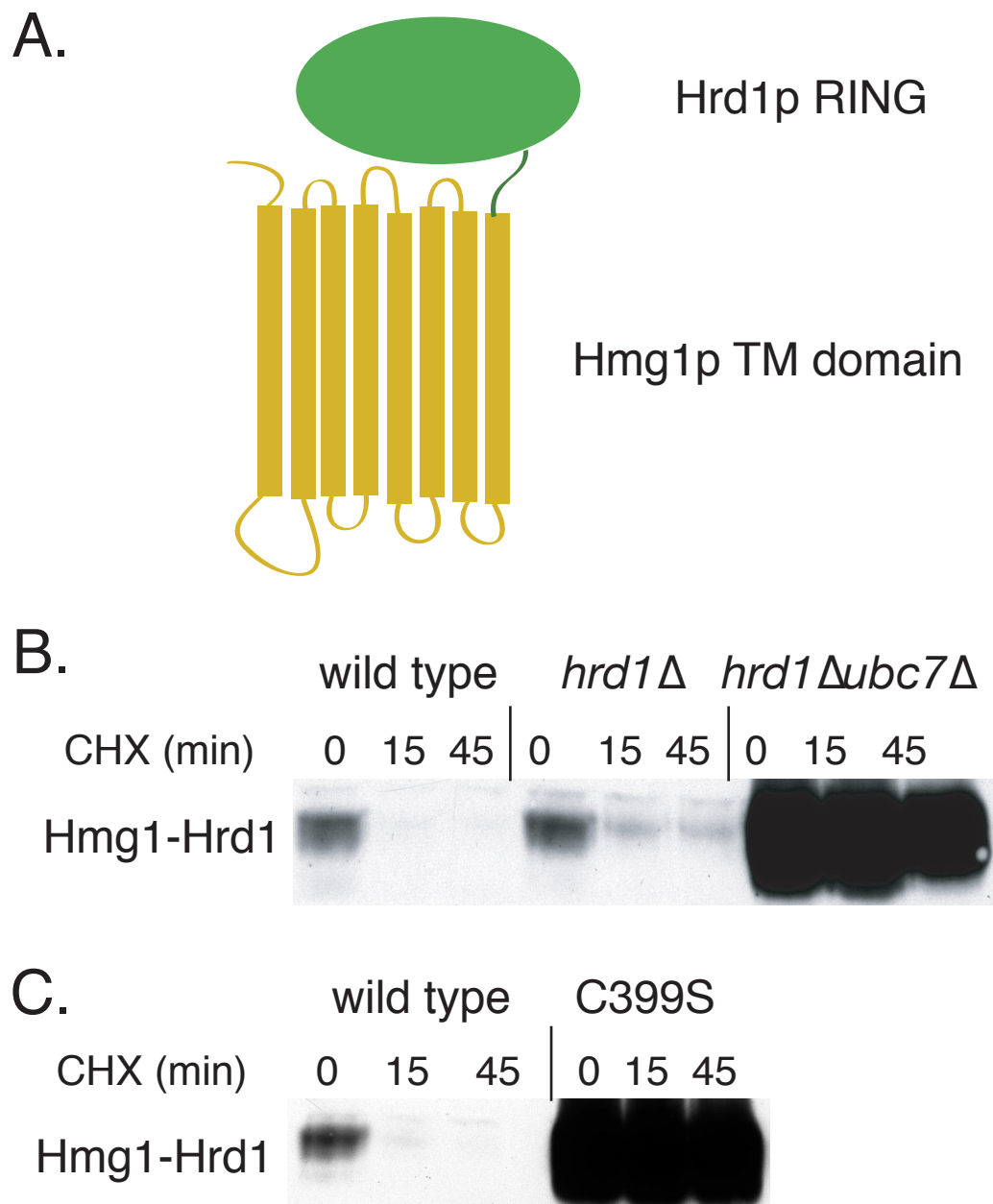


Figure 4-17 Hmg1-Hrd1 is a self-destructive substrate that is degraded in a Ubc7p and C399S dependent manner

(A). Hmg1-Hrd1 is created from the stable transmembrane domain of Hmg1p attached to the cytosolic RING domain of Hrd1p. (B). Cycloheximide chase of Hmg1p-Hrd1 in the indicated strains. CHX chases performed as previously described. (C). Cycloheximide chase with Hmg1-Hrd1 and Hmg1-Hrd1 with the C399S mutation of Hrd1p introduced.

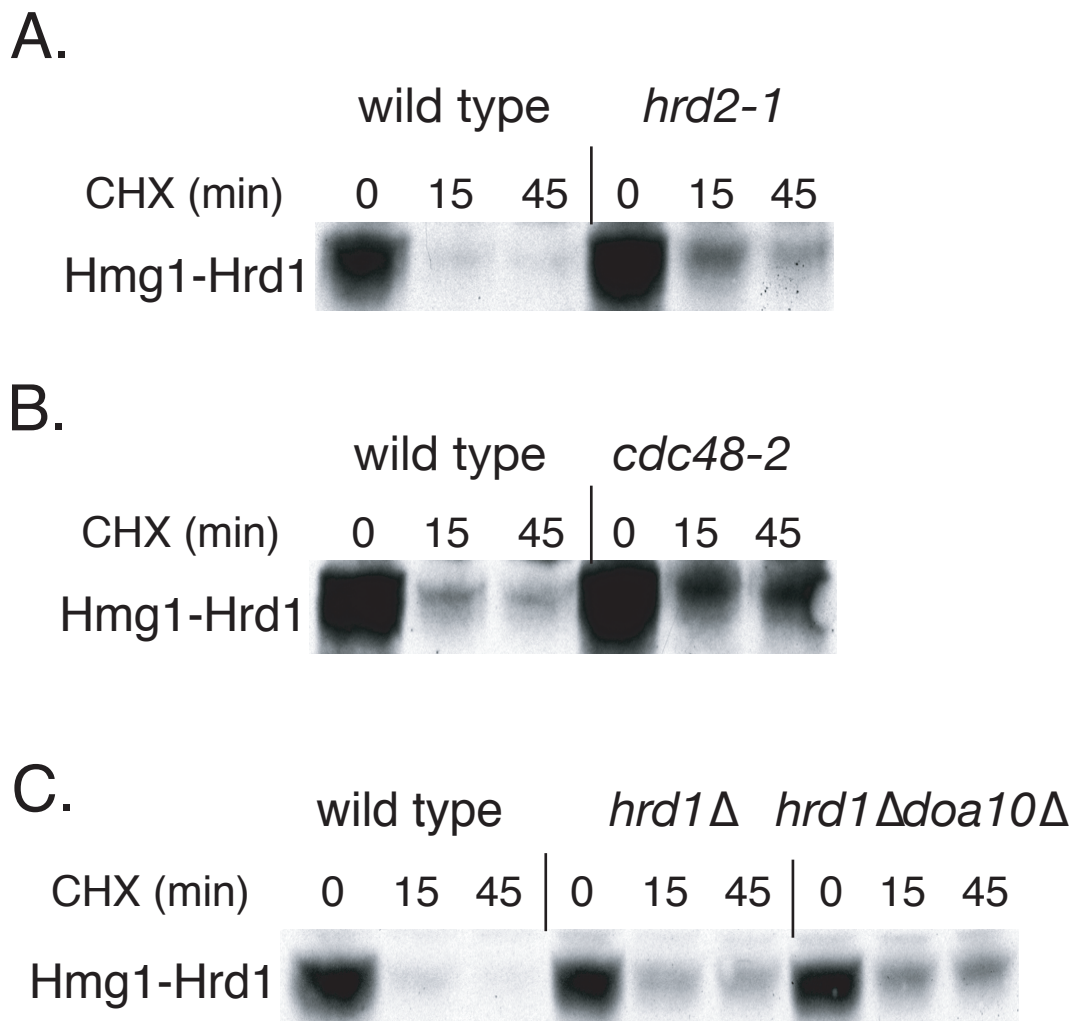


Figure 4-18 Hmg1-Hrd1 degradation occurs in a proteasome and Cdc48 dependent manner

(A-C). Cycloheximide chase of Hmg1p-Hrd1 in the indicated strains. CHX chases performed as previously described.

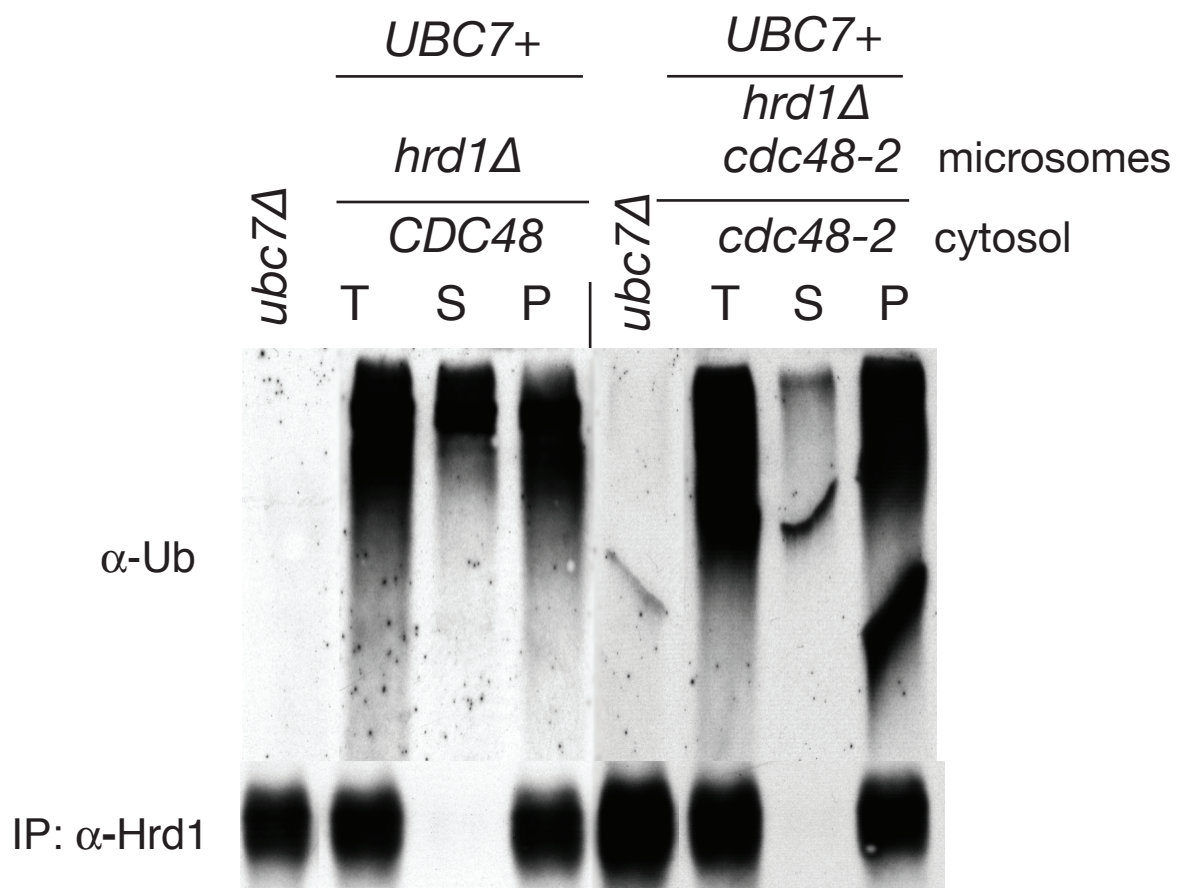


Figure 4-19 Hmg1-Hrd1 is retrotranslocated in the absence of Hrd1p in a Cdc48-dependent manner

In vitro retrotranslocation was analyzed by combining *hrd1Δ* microsomes with wild type cytosol and *hrd1Δcdc48-2* microsomes with *cdc48-2* microsomes. Reactions were performed for 45 minutes.

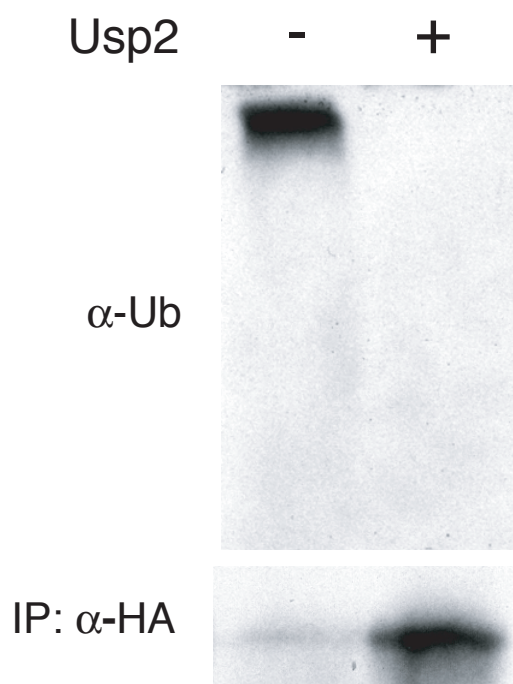


Figure 4-20 The ubiquitin signal in the supernatant fraction of Hmg1-Hrd1 can be de-ubiquitinated which results in the appearance of an anti-HA signal

The retrotranslocated fraction was incubated with or without the purified ubiquitin protease Usp2 for 1 hour at 37°C. Following this treatment, Hmg1-Hrd1 was immunoprecipitated with an anti-HA antibody.

Discussion

In this study we have successfully reconstituted Hrd1p-dependent retrotranslocation of a natural multi-spanning membrane substrate of the HRD pathway, Hmg2p. Once ubiquitinated, full-length Hmg2p-GFP is moved to the supernatant fraction, despite having 8 transmembrane spans and several luminal loops. We have shown this by several criteria, including: immunoprecipitation studies with both GFP and luminal-domain antibodies, the ability to capture ubiquitinated Hmg2p-GFP in the absence of detergent, and the direct recovery of full-length Hmg2p-GFP in the retrotranslocated fraction after removal of ubiquitin with Usp2. Thus, complete retrotranslocation occurs in our *in vitro* assay, opening the door to analysis and detailed mechanistic understanding of this event that seems to occur in all ERAD pathways. An important question that remains is whether completely retrotranslocated Hmg2p has a transient existence during its ERAD *in vivo*. Alternatively, proteasomal delivery could be sufficiently rapid to not allow buildup of this retrotranslocated intermediate. It appears that different substrates show different degrees of cytoplasmic buildup of fully retrotranslocated species during ERAD. Nevertheless, the ability to observe this process with Hmg2p both defines the capacity of this transfer mechanism and allows its intimate mechanistic study in ways that are not possible using intact cells.

In our approach, we have used sufficient Hrd1p levels to drive ubiquitination of Hmg2p without need for Hrd3p or Usa1p. Thus, we are examining the minimal requirements for *in vitro* ubiquitination and retrotranslocation. Importantly, *in vitro* ERAD of Hmg2p by this method recapitulates all the features of *in vivo* Hmg2p degradation. Ubc7p and its anchor Cue1p are required, as is the critical K6 lysine of

Hmg2p (Gardner and Hampton 1999; Bays et al. 2001a). Furthermore, Hrd1p-dependent ubiquitination of Hmg2p-GFP is specifically blocked by chemical chaperones as is *in vivo* degradation of this substrate (Shearer and Hampton 2004). Thus, information derived from our assay will be informative about the mechanism and energetics of retrotranslocation in the functioning *in vivo* HRD pathway. These results also reinforce the idea that Hrd1p is a central organizer of ERAD, consistent with multiple observations that Hrd1p alone can drive ERAD of membrane-anchored substrates (Gardner et al. 2000).

In both mammals and yeast, the AAA-ATPase Cdc48/p97 is a key component of the post-ubiquitination step in ERAD of multiple substrates. Elegant *in vitro* analyses of the virally-mediated degradation of MHC-I molecules by the US11 protein of cytomegalovirus clearly demonstrated the importance of this energy-consuming protein in the dislocation of that single-spanning substrate. Our studies above show that Cdc48p is similarly required for the complete removal of Hmg2p-GFP from the ER membrane. The requirement for ubiquitin is reasonable considering the ubiquitin-binding capacity of the Cdc48/Npl4/Ufd1 complex. Since our assay uses microsomes and cytosol prepared from separate yeast strains, we are able to evaluate the relative importance of cytosolic or membrane-bound Cdc48p in Hrd1p-dependent retrotranslocation, by using either mutant or wild type strains for each component. It was clear that the cytosolic pool of Cdc48p was the most important source of this activity: *cdc48-2* microsomes supported retrotranslocation only slightly lower than that in the all-wild type experiment, while *cdc48-2* cytosol with wild type microsomes resulted in almost no retrotranslocation of Hmg2-GFP. There is a pool of Cdc48p (as well as p97 in mammals) that is tightly bound

to microsomal membranes (Schuberth and Buchberger 2005), and that Cdc48p associates with Hrd1p, which taken alone might indicate that the bound pool is more important in ERAD. Nevertheless, the observed importance of soluble Cdc48p implies that movement from the cytosol to the surface of the ER occurs during the ERAD process. Perhaps Cdc48p plays a role in stabilizing the retrotranslocated Hmg2p, and so it must be replenished from the soluble pool as the reaction proceeds.

The participation of soluble Cdc48 in Hmg2p retrotranslocation strengthens the idea that there must be ways for Cdc48 to fruitfully interact with the ER membrane in order to effect its ERAD functions. A number of studies have implicated ER-localized Ubx2p as an ER-receptor for Cdc48. We directly tested this idea and found that Ubx2 is required for retrotranslocation of both Hmg2p and Hrd1 itself. Furthermore, this effect of a *ubx2Δ* null was only observed when the null mutation was present in the microsome strain. While the effects of Ubx2p are quite clear *in vitro*, in intact cells Hmg2p degradation is more strongly effected by the partial loss of function *cdc48-2* mutant than it is by a complete *ubx2Δ* null in otherwise isogenic strains (Figure 4-13). This genetic observation implies that there must be other ways that Cdc48 can engage the ERAD machinery in the absence of Ubx2p. This is consistent with the observation that loss of Ubx2p does not completely remove Cdc48 association from the ER membrane (Schuberth and Buchberger 2005). Nevertheless, our results are consistent with role for Ubx2p as a receptor for Cdc48p. Perhaps our assay is more sensitive due to the inevitable dilution of the cytosol fraction that occurs during its preparation, rendering the system more dependent on the ability of the Ubx2p microsomes to attract Cdc48p. It is important to note that the functions of Ubx2p are likely to be broader than only providing

efficient delivery of Cdc48p to the ERAD pathways. We have observed that a *ubx2Δ* null has a profound upregulation of the UPR that is much greater than that caused by strong ERAD-inhibiting *cdc48* mutant (unpublished observation), again indicating roles that transcend this receptor function. The *in vitro* assay is particularly useful when this is considered, since in intact cells, a *ubx2Δ* null mutant is causing ongoing strong regulatory responses that may cloud observation of its direct ERAD functions.

It appears that the entire ERAD pathway for Hmg2 can occur in our *in vitro* assay. Addition of proteasome inhibitors to our assay caused an increase in ubiquitinated Hmg2p, indicating the proteasomal destruction was occurring. Thus, we examined the involvement of the ubiquitin-binding, proteasome delivery adaptors Rad23p and Dsk2p *in vitro*. These proteins bind multi-ubiquitin chains and the proteasome by virtue of their UBA and UBL domains, respectively. Current models predict that these soluble adaptors would have a role after retrotranslocation, and thus would be expected to have no, or even enhancing, effect on the removal of ubiquitinated Hmg2p from the membrane. We observed that loss of these redundant factors in fact caused a strong block to retrotranslocation, implicating them in a step more upstream in the pathway. It could be that their ability to bind a ubiquitinated substrate contributes to the stabilization of the retrotranslocated substrate. It is also possible that they participate in formation of a complex that functions in several post-ubiquitination steps, including removal from the membrane and delivery to the proteasome. The Rad23p and Dsk2p adaptors are soluble proteins, and would be expected to function in the cytosolic fraction, and we saw the strongest effect when the cytosol fraction alone was prepared from the *rad23Δdsk2Δ* null. In addition, we noted that experiments using cytosol devoid of Rad23p and Dsk2p

resulted in a lessened extent of ubiquitination. This could be due to a role in recruiting the “E4” Ufd4, which has been posited to enhance ubiquitination of ERAD substrates including Hmg2p (Richly et al. 2005). We do not know if the effects on retrotranslocation are due to this lessening of ubiquitination extent. However, we think this is unlikely because in many experiments, we have observed that Hmg2p with a similar extent of ubiquitination in wild-type experiments does show robust retrotranslocation. Another intriguing possibility is that these ubiquitin binding, proteasome binding adaptors might recruit the proteasome to the newly ubiquitinated Hmg2p, and so provide additional impetus for retrotranslocation that works in conjunction with Cdc48. Whatever the mechanism, it is clear that these adaptors may function at multiple points in the ERAD pathway.

One of the challenging open questions concerning ERAD is the mechanism of exit from the ER membrane. Both luminal and membrane-bound substrates are expected to require a channel or pore for successful exit from the ER. In the case of Hmg2p, there is a significant luminal region. However, the identity of this channel remains unclear. There is evidence for members of the polytopic derlin family having a role in retrotranslocation of some proteins, and the derlins have a compelling association with the p97 in mammalian cells which both indicate a possible role in mediating dislocation of ERAD substrates. Furthermore, the anterograde pore Sec61 has not been ruled out, and can be found to have some role in lumen-to-cytosol movement of some test molecules (Simpson et al. 1999). We directly examined both the pair of derlins, Der1 and Dfm1, and Sec61 in our assay, and confirmed that neither the double mutant *der1Δdfm1Δ* nor the temperature-sensitive *sec61-2* mutant had any detectable effects on

retrotranslocation of Hmg2p-GFP. This is consistent with our *in vivo* studies which similarly showed a lack of effect of the *der1Δdfm1Δ* mutant, or even the *der1Δdfm1Δsec61-2* triple mutant on *in vivo* degradation of Hmg2p-GFP (Sato and Hampton 2006).

An appealing idea consistent with the lack of an obvious, separate channel is that the ligases themselves function as channels, in addition to their roles in ubiquitin transfer. Both Hrd1p and Doa10p, the two principle ERAD ligases in yeast, have large transmembrane regions (Deak and Wolf 2001; Kreft et al. 2006). The transmembrane spans of these ligases have a substantial proportion of relatively hydrophilic amino acids, as might be predicted for a membrane protein that forms an aqueous channel for transmission of peptide strands out of the ER. By this model, the ligases would mediate movement of substrates across the ER membrane and ubiquitinate them as they emerged. We tested this idea for Hrd1 by formation of a “self-destructive” fusion protein that had Hrd1p’s ubiquitination activity but lacked the Hrd1p transmembrane domain. When fused to the Hrd1p cytoplasmic domain, the normally stable Hmg1p underwent dramatically rapid degradation that required Ubc7p, the proteasome and Cdc48p. Appending the Hrd1p ligase domain to Hmg1p was sufficient for rapid ERAD that had many of the features of a typical HRD substrate. The resulting “self-destructive” Hmg1-Hrd1 fusion underwent *in vivo* degradation in both a *hrd1Δ* null and a *doa10Δhrd1Δ* double null, indicating that delivery to the proteasome could occur without either of the transmembrane regions being present in the cell. The retrotranslocation assay directly confirmed this: Hmg1-Hrd1 underwent Cdc48-dependent retrotranslocation, and like the Hmg2p substrate, was recovered intact upon removal of the ubiquitin with UBP2. The

retrotranslocation of this fusion occurred to the same extent in either a wild type or a *hrd1Δ* null, indicating that the Hrd1p transmembrane domain was not needed for retrotranslocation of this substrate from the ER membrane.

The result with the Hmg1-Hrd1 fusion indicated that the Hrd1 transmembrane domain is not required for removal of a HRD substrate from the ER membrane. There are three possibilities suggested from these and other results on ERAD retrotranslocation. Perhaps there is an as yet-undiscovered protein required for retrotranslocation of ERAD substrates that has evaded numerous screens and direct protein analyses that have been performed (Hampton et al. 1996; Knop et al. 1996; Carvalho et al. 2006; Denic et al. 2006). This factor could be essential to cells, or a poor genetic target, making its isolation by screening difficult. Alternatively, it may be that the bona fide ERAD channel is a composite of several proteins that can each function when a given component is removed. That is, there may an optimal channel of several proteins that can still function upon the removal of any one member. Finally, it is possible that retrotranslocation of Hmg2 occurs in a way that does not require a channel, but instead involves the recruitment of lipids to form a soluble intermediate. One version of this idea has been suggested in a recent essay (Ploegh 2007). While we find this possibility unlikely, it cannot be ruled out until the lack of a channel is understood by its discovery or the complete reconstitution of the process in a pure system devoid of channel candidates.

A striking result of this study is the observed movement of full-length Hmg2p into the cytosolic fraction by the combined action of Hrd1p and Cdc48. Recent work on the Ste6-166 transmembrane substrate implied that this multi-spanning membrane protein

was similarly moved to the soluble fraction, using arguments of molecular weight of the ubiquitinated intermediates (Nakatsukasa et al. 2008). The greater mass of Hmg2p moved by this immunoblotting assay allowed us to directly detect the retrotranslocated substrate. Combined, these results indicate that movement of entire transmembrane substrates is commonly occurring in ERAD, as has been suggested from *in vivo* studies with CFTR- Δ 508 in mammalian cells. It is not clear how the 8-spanning integral membrane protein Hmg2p remains soluble in the cytosol. It will be important to analyze the physical state and binding partners of this molecular species to gain insight into what processes and proteins are responsible for this heroic thermodynamic event.

A perplexing feature of our and other *in vitro* assays is the low extent of substrate conversion that occurs. While only a small fraction of Hmg2p is ubiquitinated, modification of a given Hmg2p molecule appears to be very efficient, such the small fraction of Hmg2p subjected to *in vitro* ubiquitination is modified with many copies of ubiquitin, as we have also seen *in vivo*. The lack of continued substrate processing appears to be a general feature of the several reported *in vitro* ER ubiquitination assays. Conversely, the retrotranslocation of Hmg2p, once ubiquitinated, is quite efficient. Our estimates, using the Usp2 stripping procedure of the total reaction mix vs. the supernatant fraction, is that about 30 %- 50% of the ubiquitinated Hmg2p is moved to the soluble fraction by retrotranslocation. Although we do not yet understand why such a small percentage of the Hmg2p is recruited for ubiquitination, it appears that once that occurs, the subsequent movement of it out of the ER is very efficient.

This and other *in vitro* approaches will allow detailed mechanistic analysis of the ERAD pathway. Our future directions will include the analysis of the nature and

composition of the retrotranslocated Hmg2p, and the role of the sterol regulatory signals that control Hmg2p degradation in coordinating this key step with the earlier events that are required for movement of substrates from the ER to their proteasomal destruction.

Materials and Methods

Yeast and Bacterial strains

Escherichia coli DH5 α were grown at 37°C in LB media with ampicillin (100 μ g/ml). Yeast strains were grown at 30°C unless otherwise noted in minimal media supplemented with dextrose and amino acids as previously described (Hampton and Rine 1994). The LiOAc method was utilized to transform yeast strains with plasmid DNA (Ito et al. 1983). Plasmids available in supplemental materials (Table 4-1). Knock-outs were constructed by transforming yeast with the LiOAc method with a PCR product that encoded either G418 resistance or CloNAT/nourseothricin (Werner BioAgents, Jena, Germany) resistance and contained 50bp flanks homologous to the gene to be knocked out. (Baudin et al. 1993). Cells were allowed to grow on yeast peptone dextrose (YPD) for ~12 hours and then replica plated onto YPD plus 500 μ g/ml G418 or 200 μ g/ml nourseothricin.

All microsome donor strains were derived from RHY2923 (*MAT α ade2-101 ura3-52::URA3::HMG2-GFP met2 lys2-801 trp1::hisG::TRP1::TDH3-HRD1-3HA leu2 Δ his3 Δ 200 HMG1 hmg2 Δ ::IMYC-HMG2 hrd1 Δ ::KanMX pep4 Δ ::HIS3 ubc7 Δ ::LEU2*). All cytosol donor strains were derived from 4288 (*MAT α ade2-101 ura3-52 met2 lys2-801 trp1::hisG leu2 Δ his3 Δ 200 HMG1 hmg2 Δ ::IMYC-HMG2 hrd1 Δ ::KanMX pep4 Δ ::HIS3 ubc7 Δ ::LEU2*). Degradation experiments were performed with strains derived from RHY853 (*MAT α ade2-101 ura3-52::URA3::HMGcd::HMG2-GFP met2 lys2-801 trp1::hisG leu2 Δ his3 Δ 200 hmg1 Δ ::LYS2 hmg2 Δ ::HIS3*).

Degradation assays

Cyclohexamide chase degradation assays were performed as previously described (Sato and Hampton 2006) with SUME lysis buffer (1% SDS, 8M Urea, 10mM MOPS pH 6.8, 10mM EDTA).

***In vitro* retrotranslocation assay**

In vitro retrotranslocation assays were performed as previously described (Flury et al. 2005). Microsome donor strains, containing TDH3-Hrd1-3HA, Hmg2p-GFP and *ubc7Δ*, were harvested by bead beating cells in MF buffer (20mM Tris pH 7, 100mM NaCl, 300mM sorbitol) for 6 minutes (1 minute on, 1 minute off), the centrifuging at 14,000 x g for 45 minutes after which they were resuspended in B88 buffer (20mM Hepes pH6.8, 250mM sorbitol, 150mM KOAc, 5mM MgOAc). Cytosol donor strains expressing TDH3-Ubc7-2HA were centrifuged and underwent freeze-thaw lysis in B88 buffer and ultracentrifuged. For each reaction, a microsome strain was combined with 30mM ATP and cytosol that either did or did not express TDH3-Ubc7-2HA for 1 hour at 30°C. Reactions were then centrifuged at 25,000 x g for 1 hour. The supernatant fraction was removed to a new tube and the pellet was resuspended in the same volumen with B88 buffer. Immunoprecipitations were then performed as follows. 200μl of SUME with protease inhibitors and N-ethyl maleimide (NEM) was added to each reaction followed by 600μl of IP buffer (150mM NaCl, 15mM Na₂HPO₄, 2% Triton-X100, 0.1% SDS, 0.5% DOC, 10mM EDTA, pH 7.5) with protease inhibitors and NEM. The supernatant was removed and 15μl of polyclonal anti-GFP, 30μl anti-loop antibody or 15μl anti-HRD1 antibody was added. The mix was incubated overnight at 4°C. 100μl

of Protein-A sepharose (Amersham Biosciences) in IP buffer, (50% w/v) was added for 2 hours. Beads were washed once with IP buffer and once with IP wash buffer (50mM NaCl, 10mM Tris, pH 7.5) and incubated with 55 μ l of 2x Urea sample buffer (75mM MOPS pH 6.8, 4% SDS, 200mM DTT, 0.2mg/ml bromophenol blue, 8M urea) for 10 minutes at 50°C. The samples were then loaded on a polyacrylamide gel. Following transfer to nitrocellulose, immunoblotting with an anti-ubiquitin or anti-GFP antibody was performed.

Ubiquitin stripping

Following the 25,000 g centrifugation of the retrotranslocation reaction, the supernatant from was incubated at 37°C for 1 hour with 4 μ g of purified Usp2. A control reaction was also incubated without any Usp2 added. Immunoprecipitations were performed as described above.

Non-detergent IP

Following the 25,000 g centrifugation of the retrotranslocation reaction, the supernatant fraction was incubated with a version of immunoprecipitation buffer lacking all detergents. In addition, SUME lysis buffer was not added. A control tube was incubated with pre-immune serum while the other with 15 μ l anti-GFP antibody. The remainder of the IP was performed as described above.

Self-destructive substrate

All work with the Hmg1-Hrd1 fusion protein was performed as with wild type Hmg2p, except the IP was performed with an anti-HRD1 antibody. In addition, the retrotranslocation reaction did not proceed for 1 hour as with Hmg2p, but instead for 40 minutes.

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Chapter 4 is a manuscript in preparation that will be submitted for publication. Renee Garza performed the following experiments: Figure 4-4, Figure 4-5, Figure 4-10 and Figure 4-20. Randolph Hampton wrote the manuscript and I edited it for use in this chapter.

Table 4-1 Plasmids used in Chapter 4

Plasmid	Gene expressed
pRH373	<i>YIp TRP1 pTDH3-UBC7-2HA</i>
pRH469	<i>YIp URA3 pTDH3-HMG2-GFP</i>
pRH728	<i>KanMX loxP-KanMX-loxP disruption cassette</i>
pRH730	<i>YIp TRP1 TDH3-HRD1-3HA</i>
pRH808	<i>YIp TRP1 TDH3-HRD1</i>
pRH1122	<i>hrd1Δ::KanMX</i>
pRH1152	<i>YIp LEU2 pTDH3-UBC7-2HA</i>
pRH1186	<i>ubc7Δ::LEU2</i>
pRH1788	<i>pep4Δ::HIS3</i>
pRH1838	<i>NatR loxP-CloNAT-loxP disruption cassette</i>
pRH2071	<i>YIp URA3 pTDH3-HMG1-HRD1</i>

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Chapter 5

Future Directions

Summary

ERAD is a multi-step process, and while we are continuously uncovering new information about this pathway, there is still much that needs to be learned. ERAD of both misfolded membrane and soluble proteins in the ER lumen requires the attachment of poly-ubiquitin chains, the retrotranslocation into the cytosol, and the degradation by the proteasome (Hampton et al. 1996; Ye et al. 2001; Hampton 2002). While this skeletal pathway has been established for years, the details governing it are still unknown.

My work has dealt with gaining a further understanding of ERAD. In Chapter 2, we sought to ascertain the role of the Hrd1p transmembrane domain in misfolded protein recognition. While ERAD-L appears to require a number of soluble factors (Denic et al. 2006) and ERAD-C involves cytoplasmic chaperones (Nishikawa et al. 2005), ERAD-M requires neither. In fact, degradation of ERAD-M substrates appears to require only Hrd1p and Hrd3p, and in the absence of Hrd3p, sufficient levels of Hrd1p are capable of ERAD-M degradation (Gardner et al. 2000). To determine whether the Hrd1p transmembrane domain plays a role in misfolded membrane protein recognition, we mutated a number of conserved and hydrophilic residues and assayed for defects in Hrd1p-dependent degradation. Through this work, we isolated mutants which were proficient in ERAD-L, yet were defective in the degradation of ERAD-M proteins. This specificity was then taken one-step further with versions of Hrd1p that were incapable in degrading only a single ERAD-M substrate. Further studies with one of these mutants illustrated that substrate binding was not sufficient to activate the Hrd1p RING domain. This implies that certain regions of the Hrd1p membrane domain are required for the

degradation of individual misfolded proteins and that Hrd1p binding is insufficient to trigger ubiquitination, an allosteric activation must occur following binding.

Chapters 3 and 4 covered more downstream aspects of the ERAD pathway, that of protein retrotranslocation. Retrotranslocation has been hypothesized to occur due to the fact that misfolded proteins are initially in the ER, yet are degraded by the cytoplasmic proteasome. It is also likely that this process requires an ER channel since these misfolded proteins contain hydrophilic domains that must pass through the hydrophobic bilayer. The identity of this channel still eludes us, yet much work has been done testing the role of Sec61p in the process (Plempner et al. 1997) as well as a recently identified mammalian protein, Derlin-1 (Lilley and Ploegh 2004; Ye et al. 2004). Derlin-1 was an especially intriguing protein, as two derlin homologues have been identified in yeast, Der1p and Dfm1p (Knop et al. 1996; Hitt and Wolf 2004). Chapter 3 involves the study of Dfm1p (Sato and Hampton 2006). Despite the homology to Derlin-1, Dfm1p plays no role in the ERAD of both Der1p-dependent and independent misfolded proteins, nor is it redundant with Der1p. Despite this lack of a role in ERAD, a *der1Δdfm1Δ* strain has an upregulated UPR compared to a *der1Δ* null and overexpression of Dfm1p highly upregulated the UPR. Dfm1p also interacts with Cdc48p as was demonstrated through a co-immunoprecipitation experiment, and overexpression of Dfm1p exacerbates the temperature sensitive phenotype of a *cdc48* mutant strain. In addition, Dfm1p may play a role in homotypic membrane fusion, as it also demonstrates a synthetic lethality with a mutant of Ufe1p, a t-SNARE that in conjunction with Cdc48p plays a role in this process (Lewis et al. 1997). While this has yet to be further explored, it is clear that unlike its mammalian counterpart, Dfm1p has no ERAD or retrotranslocation role.

Finally, Chapter 4 covered an *in vitro* retrotranslocation assay which recapitulated the retrotranslocation pathway of a membrane protein, Hmg2p. This retrotranslocation process depends on Cdc48p as has been previously demonstrated with p97 (Ye et al. 2003) and results in the removal of full-length, soluble Hmg2p into the cytosol. With this assay, we were able to test the current model of post-ubiquitination ERAD. We identified a role in retrotranslocation for both the putative Cdc48p receptor Ubx2p and the proposed proteasome delivery factors Rad23 and Dsk2p. *In vivo* analysis of *ubx2Δ* strains illustrated a minor defect in Hmg2p degradation, yet the retrotranslocation block was very strong. We also would not expect proteasome delivery factors to have a retrotranslocation function, but *rad23Δdsk2Δ* cytosol and microsomes were incapable of Hmg2p retrotranslocation, hinting that the proteasome could possibly play an active role in retrotranslocation. We also tested a number of putative retrotranslocons including Sec61p, Der1p, and Dfm1p, which had no retrotranslocation function. Hrd1p has also been proposed to act as a retrotranslocon, as it possesses six transmembrane spans (Deak and Wolf 2001) yet its ubiquitin ligase activity is localized to the cytoplasmic RING domain (Bays et al. 2001). To separate the Hrd1p ubiquitination function from a potential role in retrotranslocation, we utilized a fusion protein consisting of the stable Hmg1p transmembrane domain (Hampton and Rine 1994) attached to the Hrd1p RING domain. This protein was degraded in a Hrd1p-independent manner, yet relied on standard ERAD factors, including the proteasome, Ubc7p and Cdc48p. In our *in vitro* assay, the fusion protein was retrotranslocated, demonstrating that retrotranslocation can occur in the absence of Hrd1p. Thus, due to this assay we are able to answer a number of

questions regarding retrotranslocation and ERAD in a manner that cannot be accomplished with *in vivo* studies.

While the work in the preceding chapters has greatly increased our knowledge and understanding of the factors involved and the mechanism of ER quality control, there are still a number of pressing issues that need to be further addressed. Below I will highlight some of these questions.

How is the Hrd1p RING domain activated?

The work in Chapter 2 illustrated that specific residues in the Hrd1p transmembrane domain are capable of distinguishing between different types of misfolded proteins. 3A-Hrd1p could not degrade Hmg2p or Hmg2p-related proteins but was proficient in ERAD of all other substrates tested. This deficiency was not due to a lack of substrate interaction as 3A-Hrd1p was still capable of Hmg2p binding. Despite this association, Hmg2p was not ubiquitinated by 3A-Hrd1p, hinting to a model in which substrate binding produces an allosteric change in Hrd1p's structure (Figure 2-22). This change then activates the RING domain.

One question that we have yet to answer is whether 3A-Hrd1p is capable of bridging the interaction between Hmg2p and Ubc7p. Gardner et al. (Gardner et al. 2000) demonstrated that while Hrd1p binds to both stable and unstable proteins, Ubc7p only interacts with ERAD substrates. We would expect too that 3A-Hrd1p would be incapable of bridging the interaction between Ubc7p and Hmg2p. This would explain the discord between the substrate interaction and lack of ubiquitination.

To truly understand how Hrd1p catalyzes ubiquitination, we need to obtain the structure of the Hrd1p, Ubc7p and ERAD substrate complex. In this way, we could compare the Hrd1p structure in the presence of a misfolded protein versus a stable protein, or the structure of wild type Hrd1p to 3A-Hrd1p. It would also be possible to examine the position of the RING domain relative to the conformation of the transmembrane domain. It may be that an activated Hrd1p transmembrane domain always produces a certain conformational change in the RING domain, allowing interaction with Ubc7p. Hrd1p may have many different mechanisms (as can be seen in Chapter 2) to recognize misfolded proteins, but it is possible that each results in the same “active” form of Hrd1p. In this way, Hrd1p could switch between “active” and “inactive” states which would be accompanied by a corresponding shift of the RING domain. Another option is that Ubc7p also has the capacity to move between these “active” or “inactive” conformations, further increasing the level of specificity of the ubiquitination process.

Understanding how Hrd1p recognizes substrate proteins would be informative not only for ERAD, but for ubiquitin ligases in general. There are many known methods to activate ligases. In some cases, E3 proteins are part of larger protein structures, like the SCF complex, which have subunits that act to recruit substrates (Ang and Wade Harper 2005). Other E3s appear to have substrate binding pockets (Varshavsky 1997) while others bind only after a substrate is modified, such as through phosphorylation (Yoshida and Miki 2004). Gaining a greater knowledge about the specificity with which ubiquitin ligases choose their substrates is vital to understanding the ubiquitin pathway as well as the other cellular processes it regulates.

What is the function of Dfm1p?

While Dfm1p is not an ERAD factor, it appears to play some role in ER stress (Chapter 3). The fact that it interacts with Cdc48p makes it possible that it is involved in a Cdc48 function, which includes ERAD, cell cycle regulation, homotypic membrane fusion, and DNA repair and replication (Dreveny et al. 2004). Dfm1p also interacts genetically with Ufe1p, a t-SNARE involved in homotypic membrane fusion. A membrane fusion assay (Patel et al. 1998) utilizes microsomes from two different strains, one of which expresses a de-glycosylation enzyme. Microsomes from the other strain have a radiolabeled substrate translated inside and the two microsome populations are mixed. When membrane fusion occurs, the substrate protein is deglycosylated. While this is one method to test membrane fusion, we could also attempt to assay the process by mating two yeast strains, one expressing Hmg1p-RFP and the other expressing Hmg1p-GFP. Following mating, successful membrane fusion can be observed by the mixing of red and green ER membranes (Hampton lab unpublished observations). Uncovering a role in homotypic membrane fusion would be important for understanding Dfm1p's function, and would also tie into the larger question of how Dfm1p is capable of increasing ER stress.

What is the identity of the retrotranslocon?

Despite exhaustive efforts to identify the retrotranslocon (Plempner et al. 1997; Lilley and Ploegh 2004; Ye et al. 2004; Sato and Hampton 2006), there are still no obvious candidates. This could be due to a number of reasons. One is that many genetic screens for ERAD factors are performed with the yeast null collection, and thus any

essential genes would not be identified. Another is that the retrotranslocon could be composed of a variety of proteins, and that a lack of one factor would result in compensation by the others. It's also possible that retrotranslocation does not require a channel at all, and instead proceeds by a lipid-based mechanism (Ploegh 2007).

While genetic screens have been attempted, it might be worthwhile to merely assay the effect of all ER membrane proteins on degradation. While this seems cumbersome, this list is fairly manageable (Table 5-1). In this way, we can look for defects in both essential and non-essential proteins. If in fact the retrotranslocon exists, it must be a membrane protein and almost certainly must be localized in the ER.

Another possible way to identify the channel is with our *in vitro* retrotranslocation assay. At some point in the reconstitution of retrotranslocation, Hmg2p must be passing through the retrotranslocon. By stopping the reaction at this point, we could identify the proteins in complex with Hmg2p, one of which would be the retrotranslocon. It is possible to stop retrotranslocation of Hmg2p by using *cdc48-2* microsome and cytosol donor strains. Under these conditions, Hmg2p is ubiquitinated and in the membrane, implying that the protein is prepared to be retrotranslocated. As Cdc48p's role is likely downstream of the retrotranslocon, Hmg2p may be passing through the channel immediately before interacting with the Cdc48 complex. After allowing this to occur, we could add a membrane permeable crosslinker and immunoprecipitate (IP) Hmg2p. Following the IP, we could determine the Hmg2p-interacting factors through mass spectrometry analysis. The channel protein or proteins may be included in these factors. This would be a more direct manner of uncovering the retrotranslocon compared to further genetic screens.

What is the state of retrotranslocated Hmg2p?

The *in vitro* retrotranslocation assay results in the movement of full-length soluble Hmg2p into the cytoplasm. Hmg2p contains 8 transmembrane spans, so it is unlikely that it is floating alone in the cytoplasm. Such a scenario would result in protein aggregation, possibly inhibiting proteasomal degradation. To ascertain the factors bound to Hmg2p which are responsible for keeping it soluble, we can immunoprecipitate retrotranslocated Hmg2p and submit the product for mass spectrometry analysis. Factors identified from this study could include chaperones, the Cdc48 complex or proteasome delivery proteins. We also would learn the relative stoichiometry of Hmg2p and other members of this complex.

Density gradient centrifugation would also assist in our understanding of retrotranslocated Hmg2p. By performing this type of experiment, we could uncover whether lipids are associated with retrotranslocated protein. In addition, we would be able to determine the size of the Hmg2p post-retrotranslocation complex. The identity of the post-retrotranslocation complex could greatly increase our understanding of the mechanism of retrotranslocation and also could shed light on the steps between retrotranslocation and proteasomal degradation.

Table 5-1 Integral membrane ER proteins

Gene						
ACC1	CWH41	ERV46	HRD3	PMT1	SEC63	UFE1
AGP2	CYB5	FEN1	HSD1	PRM8	SEC66	USA1
ALG5	DER1	FPR2	ICE2	PRM9	SEC72	VMA21
ALG8	DFM1	GAB1	IZH1	RCE1	SED4	VMA22
ALG14	DIE2	GPI1	IZH3	RCR1	SHR3	VPH2
ASI1	DPM1	GPI8	JEM1	RFT1	SNL1	WBP1
ASI3	EMP24	GPI11	KAR5	ROT1	SOP4	WSC4
ATF2	EOS1	GPI12	LAS21	RSB1	SPF1	YDC1
AYR1	EPS1	GPI13	LEM3	RTN1	SPT23	YET1
BET1	ERD2	GPI14	LIP1	SAC1	SRP101	YKE4
BIG1	ERG25	GPI16	MCD4	SAY1	SRP102	YIF1
BOS1	ERG26	GPI17	MGA2	SBH1	SSH1	YIP1
BRL1	ERG27	GPI18	MID1	SCP160	SSM4	YPC1
CAX4	ERG28	GPI19	MNS1	SCS2	SSS1	YPT1
CHS7	ERF2	GSF2	MST27	SEC12	STE14	YOS1
CNE1	ERI1	HLJ1	MST28	SEC20	STE24	YSR3
CSG2	ERJ5	HMG1	NCE102	SEC59	SVP26	YJR015W
CSM4	ERV14	HMG2	OLE1	SEC61	UBC6	YLR301W
CUE1	ERV41	HRD1	PGA1	SEC62	UBX2	YNL194C

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Appendix 1

HRD mutant analysis

Introduction

A modified version of the original *HRD* screen (Hampton et al. 1996) was performed by Nathan Bays (see Nathan Bays' thesis and (Bays et al. 2001b)) in which the goal was to identify factors required for the degradation of the ERAD-M substrate 6myc-Hmg2p. The parent strain used in this screen contained two copies of *HRD1* and two copies of *HRD3*, in order to bias the screen against isolating mutations in those two genes. The parent strain was plated on media containing lovastatin, a specific inhibitor of the catalytic activity of HMG-CoA reductase. Thus, the only cells that can survive are those that have elevated levels of Hmg2. Through this analysis, Dr. Bays uncovered 21 different complementation groups, each representing a different gene required for Hmg2 degradation (Figure A1-1). A number of these complementation groups have been identified, including *HRD1*, *HRD2 (RPN1)*, *HRD3*, *HRD4 (NPL4)*, *HRD10 (UBC7)*, *HRD13 (CUE1)* and *HRD21 (RPN4)*. Still, there are a many unknown mutants. In an attempt to isolate factors required for Hmg2p retrotranslocation, I sought to determine whether each mutant was proficient for proteasome function and substrate ubiquitination. Those that were capable of both may possess a retrotranslocation defect. I would then clone these genes of interest. A table of the mutant strains (numbered with Nathan Bays' system), their complementation group, and their proteasome function as assayed with Δ ss-CPY* is included (Table A1-1).

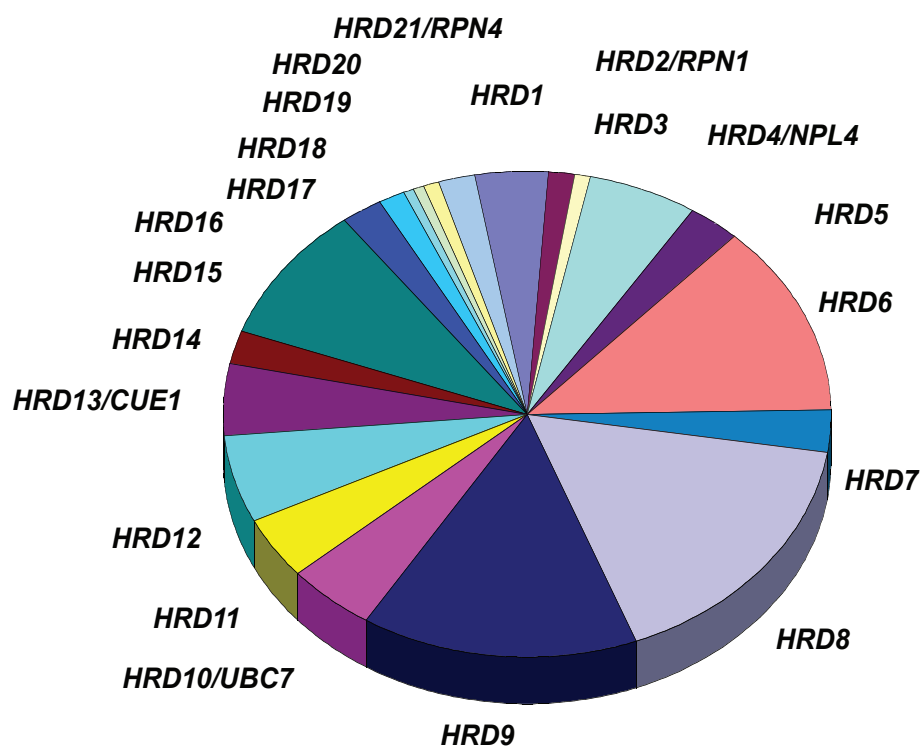


Figure A1-1 Representation of mutants isolated from the HRD screen performed by Nathan Bays

Twenty-one different complementation groups were identified which were impaired in the degradation of Hmg2p.

Test for lovastatin resistance and Hmg2p stabilization

Before further analysis of each complementation group, I wanted to determine which of the mutants isolated in the screen were still lovastatin resistant. I also tested whether this lovastatin resistance also resulted in stable Hmg2p-GFP. Interestingly enough, while the majority of the *HRD* mutants isolated were still lovastatin sensitive, very few of them stabilized Hmg2p-GFP. Those that did are included in Table A1-1.

Analysis for proteasome defects

While it would be difficult to classify every single mutant isolated in the screen, I chose representative strains from each complementation group and assayed whether the degradation defect was due to impaired proteasomal activity. To test for proteasome function, we utilized the cytoplasmic quality control substrate, Δ ss-CPY*-GFP, a version of the misfolded protein CPY* lacking the ER signal sequence (Medicherla et al. 2004). In strains wild type for proteasome function, this protein was degraded, and had low GFP fluorescence as measured by a flow cytometer. In contrast, a proteasome mutant had elevated levels of the protein (Figure A1-2). With this tool, we were able to quickly screen which of the *HRD* mutants were impaired in proteasome function and which were not. Initially, it appeared that a number of complementation groups were proficient for proteasome function, including *HRD8*, *HRD15*, *HRD17*, *HRD18* and *HRD19*. Due to this finding, and the fact that these mutants were capable of Hmg2p ubiquitination, I attempted to clone *HRD15* and *HRD17*. These both appeared to contain proteasome mutations from the complementation analysis, and so I re-screened all of my “non-proteasome” mutants. Unfortunately, all but *HRD8* appeared to be proteasome defective.

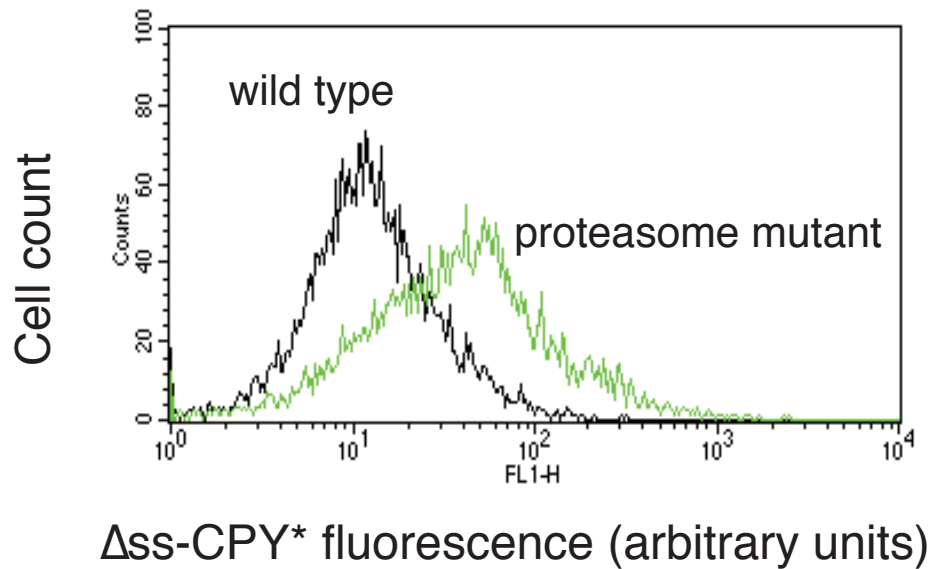


Figure A1-2 Δ ss-CPY* is stabilized in a proteasome mutant

Steady state levels of Δ ss-CPY* were measured in a wild type and proteasome (*hrd2-1*) mutant by flow cytometry. For each strain, 10,000 cells were measured.

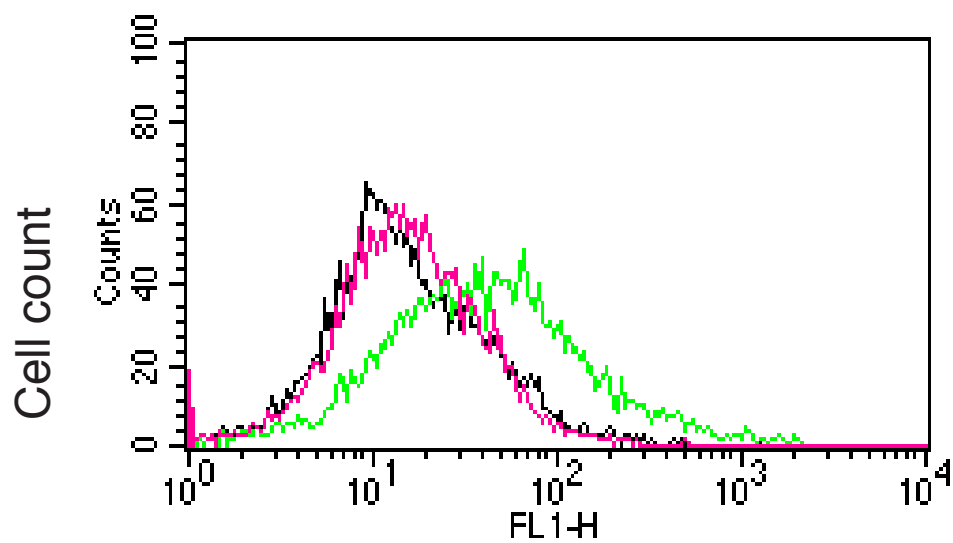
While I am unsure of the cause of this mistake, the initial analysis was performed by an undergraduate. Thus, it is possible that his inexperience led to the observed phenotypes.

***HRD8* characterization**

By the end of my analysis of the *HRD* mutant collection, *HRD8* was the only mutant that did not possess a proteasome defect (Figure A1-3A). *HRD8* was of special interest because Nathan Bays had previously shown that *HRD1* is a high-copy suppressor of *HRD8*. Despite this fact, complementation tests crossing *hrd1* Δ and *hrd8* mutant strains demonstrated that they were mutations in distinct genes (Nathan Bays' thesis). We demonstrated that *hrd8* mutants were strongly deficient in Hmg2p-GFP degradation and were proficient for Hmg2p ubiquitination (Figure A1-4, A1-5). We also asked whether *SEC61* or *CDC48* plasmids could complement the *hrd8* mutant but neither did (data not shown). Attempts to clone *HRD8* with the Rose library (Rose et al. 1987) and the Hirsch library (Engebrecht et al. 1990) were unsuccessful. Nathan Bays also tried numerous times to clone *HRD8* and also was unable to. It is possible that, like *DOA10*, *HRD8* DNA is toxic to bacteria and thus would not be found in a genomic library (Swanson et al. 2001). Whatever the case, it will be interesting to identify *HRD8* and its role in ERAD.

***HRD17* as RPT2**

hrd17 mutants initially appeared to be proficient for proteasome function (this later turned out to be not the case) and were lovastatin resistant. In addition, these strains grew more slowly than a wild type strain, a phenotype which I took advantage of for

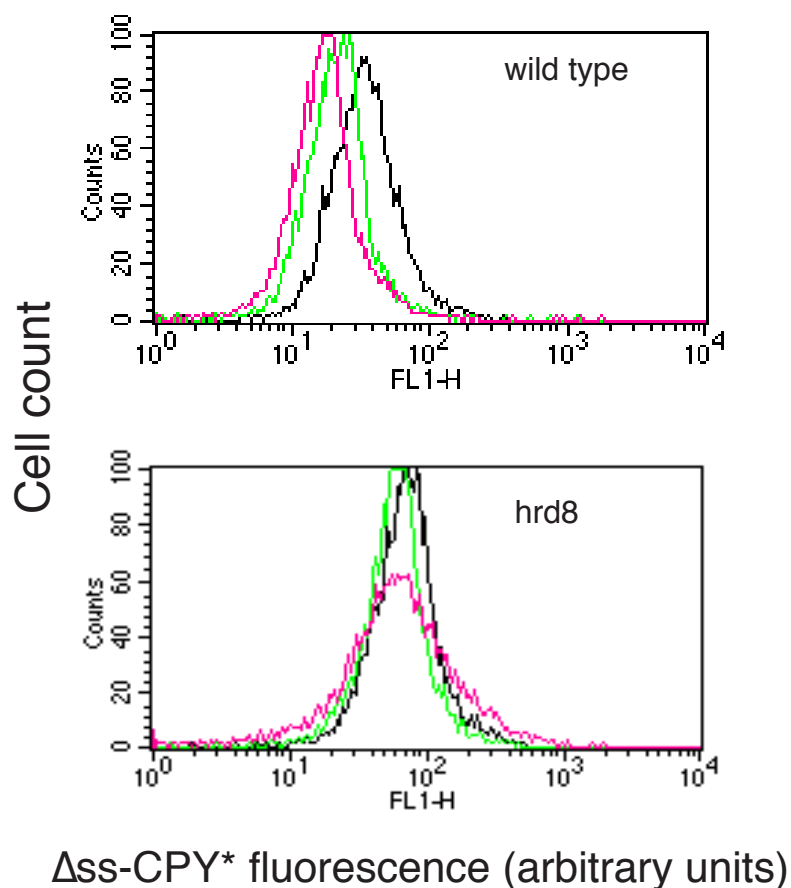


Δ ss-CPY* fluorescence (arbitrary units)

Black - wild type
Green - *hrd2-1*
Pink - *hrd8* mutant

Figure A1-3 A *hrd8* mutant does not possess a proteasomal defect

Steady state levels of Δ ss-CPY* were measured in a wild type, proteasome (*hrd2-1*) mutant and *hrd8* mutant (Nathan Bays 403) by flow cytometry. For each strain, 10,000 cells were measured.



Black - no drug
 Green - 1 hour CHX
 Pink - 3 hr CHX

Figure A1-4 A *hrd8* mutant is incapable of Hmg2p-GFP degradation

Hmg2p-GFP levels were measured in a cycloheximide chase in a wild type strain and *hrd8* mutant (Nathan Bays 403) by flow cytometry. For each strain, 10,000 cells were measured. Cycloheximide was added at $t = 0, 1,$ and 3 hours and all samples were analyzed at the same time.

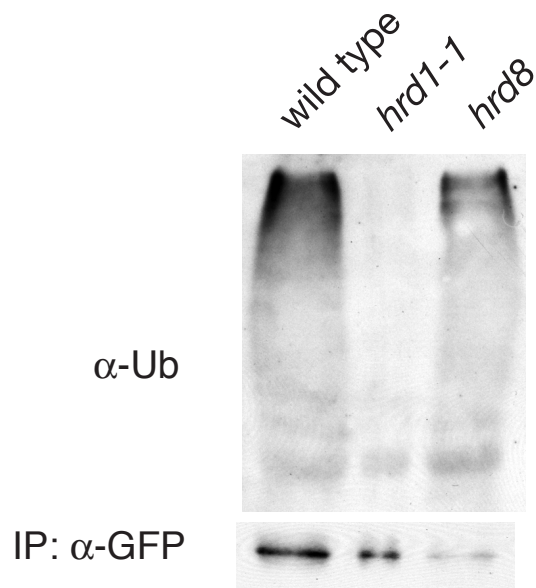


Figure A1-5 Hmg2p ubiquitination occurs in a hrd8 mutant strain

Hmg2p-GFP ubiquitination was assayed through a ubiquitin immunoprecipitation. Log phase growing strains were lysed and subjected to an anti-GFP immunoprecipitation. The resulting pull-down was analyzed by SDS-PAGE and immunoblotted with an anti-GFP or anti-ubiquitin antibody.

cloning of the mutant gene. To accomplish this, I used the Rose library (Rose et al. 1987). To clone *HRD17*, I transformed the mutant strain (Nathan Bays strain number 583) with DNA from the Rose library, plated the transformants and looked for those colonies which grew at a faster rate. From this analysis came a plasmid that expressed DNA from chromosome 4. This contained a number of ORFs including the proteasome subunit RPT2. To ensure that this was in fact *HRD17*, the *RPT2* gene was extracted from the complementating plasmid, and in fact, when this RPT2-less plasmid was introduced to *hrd17* mutants, the cells were again lovastatin resistant and slow growing, demonstrating that *RPT2* in fact is *HRD17*.

RPT2 is one of the members of the 19S proteasome (Rubin et al. 1998) and is one of the six AAA ATPases located in this complex. Rpt2p is proposed to be responsible for allowing substrate entry into the 20S proteolytic chamber (Nandi et al. 2006). While this is likely to be very important for Hmg2p degradation, it was not of immediate interest to us and was not studied any further.

HRD15 as PRE1?

hrd15 mutants also appeared to be non-proteasome mutants from the initial analysis (although this too would later be proven false). To clone *HRD15*, we utilized strain 162 (according to Nathan Bays' numbering system). This cloning was also performed with the Rose library (Rose et al. 1987) to test for complementation of the lovastatin resistant phenotype. Strains were transformed onto SC-URA plates and then later replica-plated onto lovastatin containing plates. One plasmid from the library which complemented the lovastatin-resistant phenotype of the *hrd15* mutant contained the

proteasome subunit *PRE1*. Unfortunately, attempts to remove the *PRE1* ORF by cloning were unsuccessful, so it was not confirmed whether or not *HRD15* is *PRE1*. This seems like a likely answer though, as *hrd15* mutants do demonstrate a proteasome defect.

PRE1 encodes an essential subunit of the 20S proteasome (Groll et al. 1997). It is one of the β subunits of the proteasome, and further analysis demonstrated that the Pre1p's peptidase activity is inactive in yeast (Groll et al. 1999; Nandi et al. 2006). While we do not know the specific role of Pre1p in Hmg2p degradation, it again highlights the role of the proteasome in ERAD.

Materials and Methods

DNA manipulation and plasmid construction

All plasmids were constructed with standard molecular biology tools as has been described (Sato and Hampton 2006). Plasmids used in these studies are listed in Table A1-2.

Yeast and Bacterial strains

Escherichia coli DH5 α were grown at 37°C in LB media with ampicillin (100 μ g/ml). Yeast strains were grown at 30°C unless otherwise noted in minimal media supplemented with dextrose and amino acids as previously described (Hampton and Rine 1994). The LiOAc method was utilized to transform yeast strains with plasmid DNA (Ito et al. 1983). Knock-outs were constructed by transforming yeast with the LiOAc method with a PCR product that encoded either G418 resistance or CloNAT/nourseothricin (Werner BioAgents, Jena, Germany) resistance and contained 50bp flanks homologous to the gene to be knocked out. (Baudin et al. 1993). Cells were allowed to grow on yeast peptone dextrose (YPD) for ~12 hours and then replica plated onto YPD plus 500 μ g/ml G418 or 200 μ g/ml nourseothricin.

Parent strain used in *HRD* mutant screen was RHY715 (*Mata, ade2-101, met2, lys2-801, ura3-52::6MYC-HMG2, hmg1 Δ ::LYS2, hmg2 Δ ::HIS3, trp1 Δ ::TRP1::HRD1, leu2 Δ ::LEU2::HRD3, his3 Δ 200*). *hrd8* mutant studies were performed with RHY4894

(*Mat α* , *ade2-101*, *met2*, *lys2-801*, *ura3-52::6MYC-HMG2*, *hmg1 Δ ::LYS2*, *hmg2 Δ ::HIS3*, *trp1::hisG*, *leu2 Δ* , *his3 Δ 200 hrd8*).

Degradation assays and UPR measurements

Cyclohexamide chase degradation assays were performed as previously described (Sato and Hampton 2006) with SUME lysis buffer (1% SDS, 8M Urea, 10mM MOPS pH 6.8, 10mM EDTA). Flow cytometry was also undertaken as described (Sato and Hampton 2006). Data was obtained through a FACScalibur machine (Becton, Dickinson and Company, Franklin Lakes, NJ) and statistical analysis was performed with CellQuest software (Becton, Dickinson and Company, Franklin Lakes, NJ).

Ubiquitin Immunoprecipitation

Ubiquitination of Hmg2p-GFP was examined as previously described (Bays et al. 2001a). Cells were grown into log phase and 3 OD were pelleted. 100 μ l of SUME with protease inhibitors and N-ethyl maleimide (NEM) and 100 μ l of glass beads were added to lyse the cells. 1ml of IP buffer (150mM NaCl, 15mM Na₂HPO₄, 2% Triton-X100, 0.1% SDS, 0.5% DOC, 10mM EDTA, pH 7.5) with protease inhibitors and NEM was added to the cell extracts and the mix was centrifuged for 5 minutes at 16,000 x g. The supernatant was removed and 15 μ l of polyclonal anti-GFP antibody was added. The mix was incubated overnight at 4°C. 100 μ l of Protein-A sepharose (Amersham Biosciences) in IP buffer, (50% w/v) was added for 2 hours. Beads were washed once with IP buffer and once with IP wash buffer (50mM NaCl, 10mM Tris, pH 7.5) and incubated with 50 μ l of 2x Urea sample buffer (75mM MOPS pH 6.8, 4% SDS, 200mM DTT, 0.2mg/ml

bromophenol blue, 8M urea) for 10 minutes at 50°C. The samples were then loaded on a polyacrylamide gel. Following transfer to nitrocellulose, immunoblotting with an anti-*ubiquitin* or anti-GFP antibody was performed.

Cloning of *hrd* mutant genes with genomic libraries

hrd mutants were transformed with either the Rose or Hirsch library and tested for complementation. For each mutant, we estimated for 20,000-30,000 cells to be transformed in an attempt to screen all the genes present in the library. When testing for lovastatin resistance, we first plated on dropout media. Once colonies were visible, we replica-plated onto a plate containing only minimal media without amino acids. This plate was then replica-plated onto a lovastatin plate, in order to sufficiently dilute the colony grown on lovastatin.

Acknowledgements

I would like to thank Carol Huang and Michael Imus for their efforts to characterize the many *HRD* mutants isolated by Nathan Bays.

Table A1-1 HRD mutant characterization

The following HRD mutants were lovastatin resistant and stabilized Hmg2p-GFP. Each mutant is labeled according to complementation group and number system created by Nathan Bays (NB).

NB number	Complementation group	Δ ss-CPY* stable?	Hmg2p stable?
403	<i>HRD8</i>	No	Yes
567	<i>HRD9</i>	Yes	Yes
56	<i>HRD14</i>	Yes	Yes
140	<i>HRD15</i>	Yes	Yes
162	<i>HRD15</i>	Yes	Yes
498	<i>HRD16</i>	Yes	Yes
583.3	<i>HRD17</i>	Yes	Yes
550	<i>HRD18</i>	Yes	Yes

Table A1-2 Plasmids used in Appendix 1

Plasmid	<i>Gene expressed</i>
pRH469	<i>YIp URA3 pTDH3-HMG2-GFP</i>
pRH507	<i>YIp TRP1 HRD1</i>
pRH508	<i>YIp LEU2 HRD3</i>
pRH1236	<i>YCp LEU2 SEC61</i>
pRH1941	<i>YCp URA3 Δss-CPY*</i>
pRH2078	<i>YCp LEU2 pNOPPA ProA-CDC48</i>
pRH2120	<i>YCp URA3 Rose library fragment RPT2 containing</i>
pRH2121	<i>YCp URA3 Rose library fragment RPT2 lacking</i>
pRH2131	<i>YCp URA3 Rose library fragment Pre1 containing</i>

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Appendix 2

UBX domain-containing proteins

Introduction

Cdc48p (p97 in mammals) is a AAA ATPase originally identified in a genetic screen for mutants with cell cycle defects (Hartwell et al. 1973). It is now known that Cdc48p plays a role in homotypic membrane fusion (Patel et al. 1998), mitotic spindle disassembly (Cao et al. 2003), nucleus reformation following mitosis (Ramadan et al. 2007), and ER quality control (Bays et al. 2001b; Ye et al. 2001). Cdc48p has been demonstrated to play a role in retrotranslocation as has been demonstrated from work in Chapter 4 and other labs (Ye et al. 2001; Jarosch et al. 2002). Due to the essential role of Cdc48p in ERAD, it is important to characterize Cdc48 binding proteins. The Cdc48p ERAD complex consists of the co-factors Npl4p and Ufd1p (Johnson et al. 1995; Bays et al. 2001b), and the E4 ligase Ufd2p (Richly et al. 2005). More recently, a novel Cdc48p binding motif has been identified, the UBX domain (Decottignies et al. 2004). It has been demonstrated that there are seven members of the UBX domain containing family in yeast, all of which are capable of interacting with Cdc48p (Schuberth et al. 2004). In addition, a number of these proteins appear to be involved in the degradation of Ub fusion proteins (Decottignies et al. 2004; Schuberth et al. 2004) as well as ERAD substrates (Neuber et al. 2005; Schuberth and Buchberger 2005; Wilson et al. 2006). One protein in particular, Ubx2p is proposed to act as an ER-localized Cdc48 receptor. Ubx2p interacts with both ERAD ligases Hrd1p and Doa10p, and a *ubx2Δ* strain has ERAD defects (Neuber et al. 2005; Schuberth and Buchberger 2005). In order to gain a better understanding of the role of UBX domain in ERAD, I tested for ERAD defects in the absence of each UBX domain containing protein.

***UBX2* and *UBX4* are involved in Hmg2p degradation**

To test for a role of UBX domain containing factors in ERAD, I examined Hmg2p degradation in *shp1Δ (ubx1Δ)*, and *ubx2Δ* through *ubx5Δ* strains (Renee Garza had previously examined *ubx6Δ* and *ubx7Δ* nulls which had no Hmg2p degradation defect). Of all tested, only *ubx2Δ* and *ubx4Δ* strains were defective in Hmg2p degradation (Figure A2-1). Both had similar phenotypes, with a higher Hmg2p steady state level although degradation still occurred. When combined together, the defects were additive, with the *ubx2Δubx4Δ* double null showing an even higher steady state level, although the protein was still not completely stable (Figure A2-1). While *ubx2Δ* strains were defective in the degradation of numerous substrates, including CPY* and Pdr5* (Figure A2-2A, A2-2B), *ubx4Δ* strains were wild type for both of these degradation substrates (Figure A2-2A, A2-2B), while the double null had an exacerbated defect when compared to the *ubx2Δ* single null. This led us to believe that Ubx2p and Ubx4p were redundant for some function, which would explain the greater defect in the double null. To test whether this was the case, we overexpressed Ubx4p in a *ubx2Δ* strain, to see whether the overexpressing plasmid could complement the Hmg2p degradation defect. Although the plasmid complemented a *ubx4Δ* strain, overexpression had no effect on *ubx2Δ*, demonstrating that Ubx2p and Ubx4p do not appear to have overlapping functions (Figure A2-3A, A2-3B, A2-3C). Thus, from the *in vivo* data collected, it appears that while Ubx2p plays a general role for all substrates tested, *ubx4Δ* strains are only impaired in the degradation of Hmg2p.

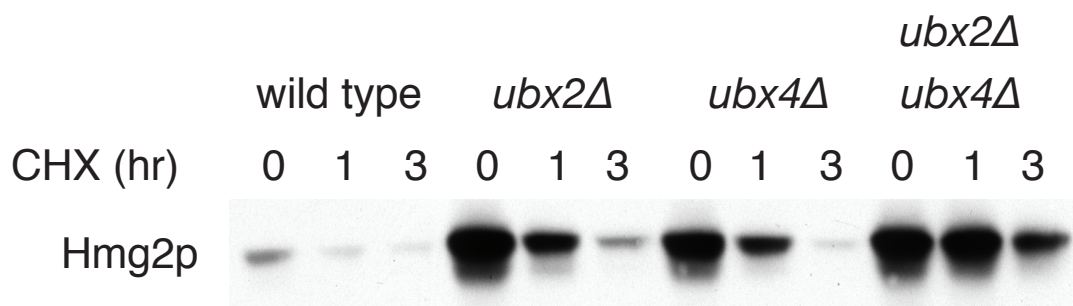


Figure A2-1 Ubx2p and Ubx4p are involved in Hmg2p degradation

In vivo Hmg2p degradation was assayed by cycloheximide chase. Cycloheximide was added to the indicated strains and equal amounts of cells were harvested at certain time points. Lysates were analyzed by SDS-PAGE and immunoblotted with anti-GFP antibodies. Equal loading was verified by India ink staining.

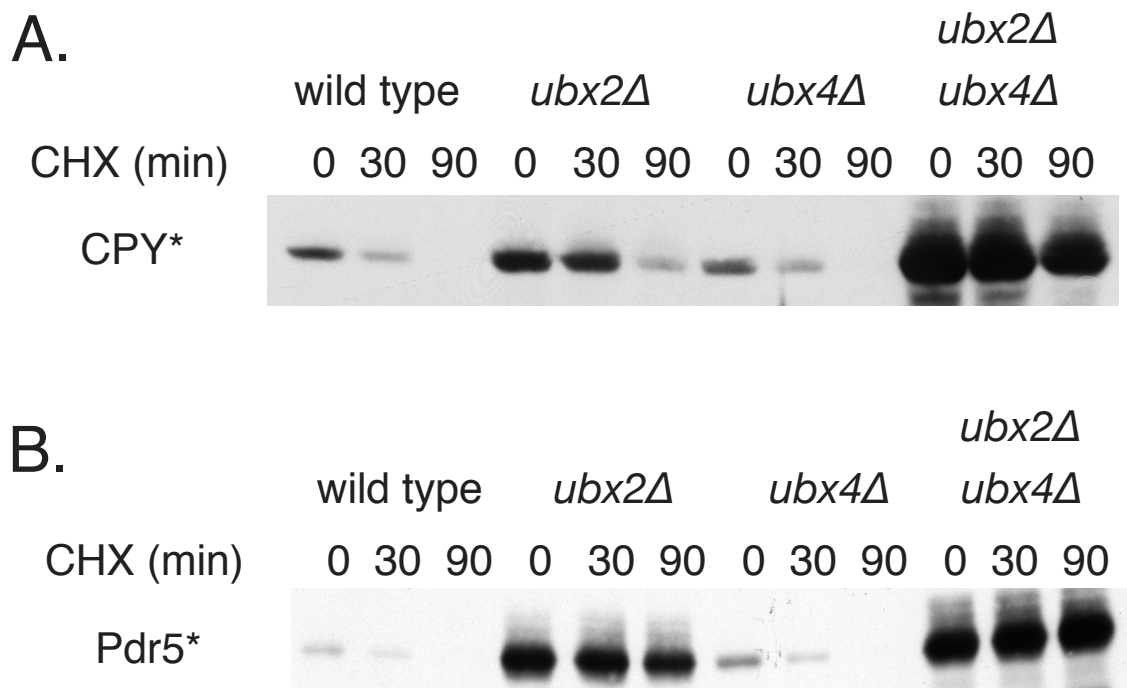


Figure A2-2 *ubx4Δ* exacerbates the *ubx2Δ* degradation defects of CPY* and Pdr5*

(A-B). *In vivo* degradation of CPY* and Pdr5* was assayed by cycloheximide chase. Cycloheximide was added to the indicated strains and equal amounts of cells were harvested at certain time points. Lysates were analyzed by SDS-PAGE and immunoblotted with anti-GFP antibodies. Equal loading was verified by India ink staining.

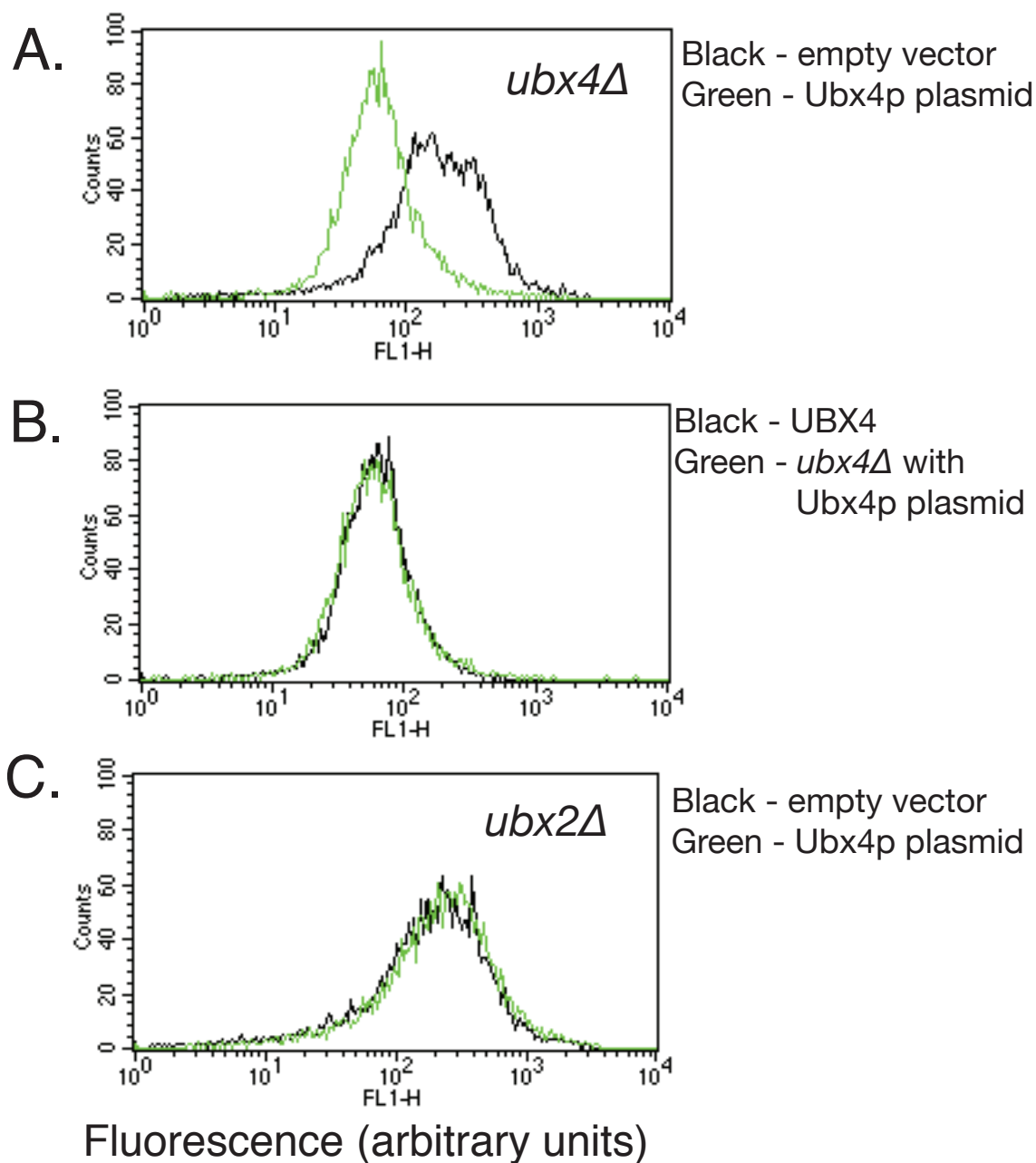


Figure A2-3 Overexpression of Ubx4p does not complement a *ubx2Δ* phenotype

Steady state levels of Hmg2 were obtained by flow cytometry. Each curve represents 10,000 cells. (A). The Ubx4p 2 μ plasmid complements a *ubx4Δ* strain. (B). A wild type strain is superimposable with a *ubx4Δ* strain expressing the Ubx4p plasmid. (C). A *ubx2Δ* strain with empty vector or Ubx4p plasmid has an identical Hmg2p degradation defect.

Unfolded Protein Response

In order to gain a better understanding of Ubx2p's function, we performed a number of other tests on *ubx2Δ* strains. One method to uncover the extent of a protein's role in ER quality control is to examine whether the unfolded protein response (UPR) is elevated in that protein's absence. The unfolded protein response is activated when there is an excess of misfolded proteins in the ER, and results in the upregulation of chaperones and ERAD factors, and the downregulation of general protein translation (Mori et al. 1992). Surprisingly, a *ubx2Δ* strain had a UPR that was elevated 30-40 times higher than a wild type strain (Figure A2-4). We have seen a UPR this high only when cells are treated with drugs that produce massive protein unfolding, such as DTT or tunicamycin (our unpublished results). The fact that the ER is so massively stressed in this scenario demonstrates that Ubx2p likely plays a much larger role in ER homeostasis than only as a Cdc48p receptor. On the other hand, a *ubx4Δ* strain had no elevated UPR (Figure A2-4).

Ubiquitination and Retrotranslocation phenotypes

If in fact Ubx2p does act as a Cdc48 receptor, we would expect a *ubx2Δ* null to have a similar degradation defect as a *cdc48* mutant. Since this is not the case (Figure 4-13), we wondered whether Ubx2p played a role in retrotranslocation. To test this, we examined retrotranslocation of Hmg2p in microsomes derived from a *ubx2Δ* strain. This result showed a striking defect in retrotranslocation (Figure 4-11) illustrating the necessity of Ubx2p in retrotranslocation. Interestingly, we observed ubiquitination of Hmg2p in *ubx2Δ* microsomes that were incubated with cytosol that lacked Ubc7p. In

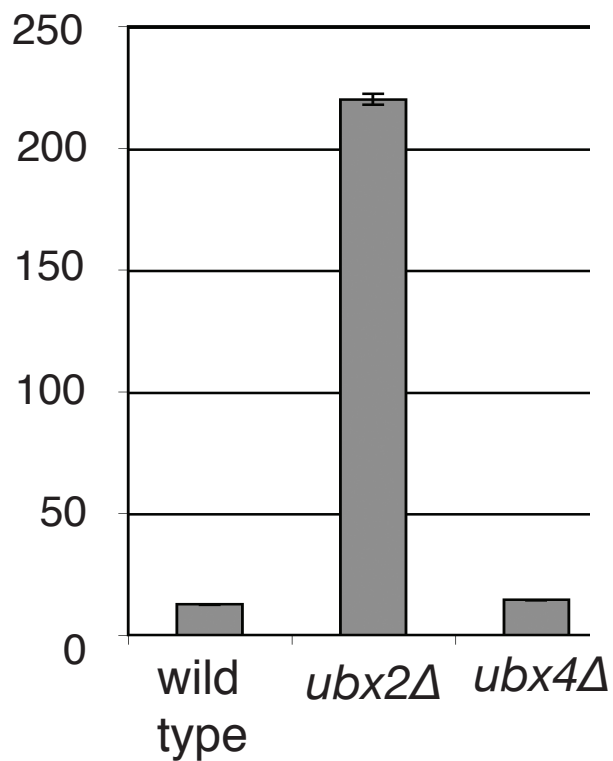


Figure A2-4 *ubx2Δ* strains have a highly elevated unfolded protein response

The unfolded protein response was measured in strains expressing the 4xUPRE::GFP expression plasmid. Fluorescence was measured by flow cytometry. For each strain, 10,000 cells were analyzed. The standard error of the mean is as indicated.

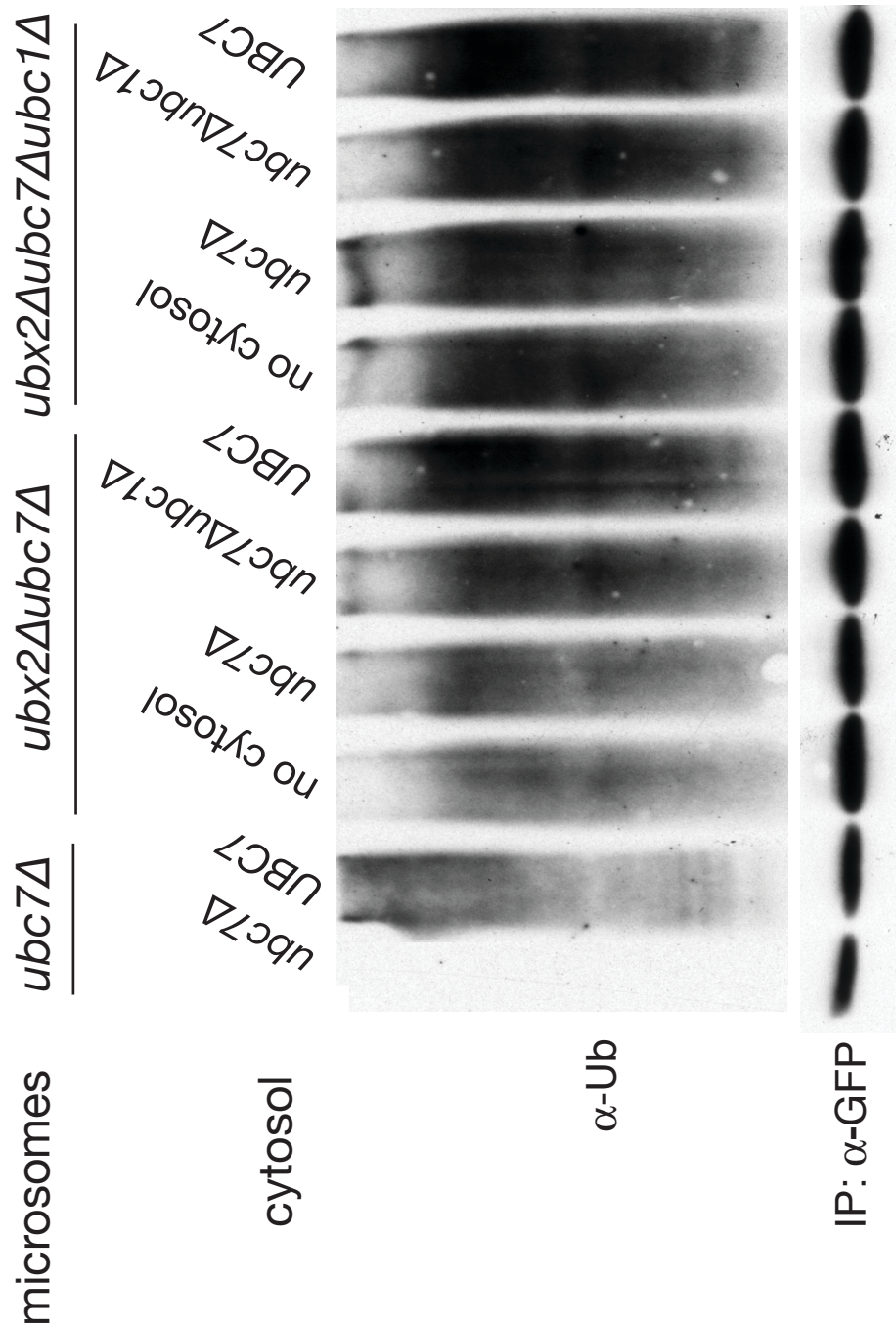


Figure A2-5 Pre-ubiquitination of Hmg2p occurs in *ubx2Δ* strains in a Ubc7p and Ubc1p independent manner

In vitro ubiquitination reactions were performed with the indicated microsomal and cytosol strains. Following the reaction, Hmg2p was immunoprecipitated with anti-GFP antibody and the resulting pull-down was analyzed with anti-Ub and anti-GFP antibodies.

fact, this ubiquitination occurred prior to the reaction, which we refer to as pre-ubiquitination (Figure A2-5). Not only was the pre-ubiquitination Ubc7p independent, it also occurred in *ubc7Δubc1Δ* microsomes (Figure A2-5). Since Ubc7p and Ubc1p have been demonstrated to be the only ubiquitin conjugating enzymes involved in Hmg2p ubiquitination (Bays et al. 2001a), we wondered what other abnormal properties this pre-ubiquitination possessed. Previously, our lab has shown that two lysine residues in the Hmg2p transmembrane domain, K6R and K357R are required for Hmg2p ubiquitination and degradation (Gardner and Hampton 1999). These mutations also prevent *in vitro* ubiquitination as well (Renee Garza, unpublished observations). In *ubx2Δ* microsomes though, K6R Hmg2p was still ubiquitinated, demonstrating another manner in which this pre-ubiquitination is unusual (Figure A2-6). This implies that Ubx2p may have a direct or indirect role in the proper regulation of Hrd1p. All of these pre-ubiquitination phenotypes are Hrd1p-dependent. In the absence of Hrd1p, Hmg2p is not pre-ubiquitinated (Figure A2-7). To ensure that this was not only an effect of overexpressing Hrd1p (which is required for *in vitro* ubiquitination), I tested whether Hmg2p was ubiquitinated in a *ubc7Δubx2Δ* with native Hrd1p. In fact, this was the case, and like our previous data, native Hrd1p was capable of ubiquitinating K6R Hmg2p as well (Figure A2-8).

In contrast to the *ubx2Δ* phenotype, *ubx4Δ* microsomes and cytosol had no retrotranslocation defect, nor did we observe pre-ubiquitination of Hmg2p in the absence of Ubc7p (Figure A2-9).

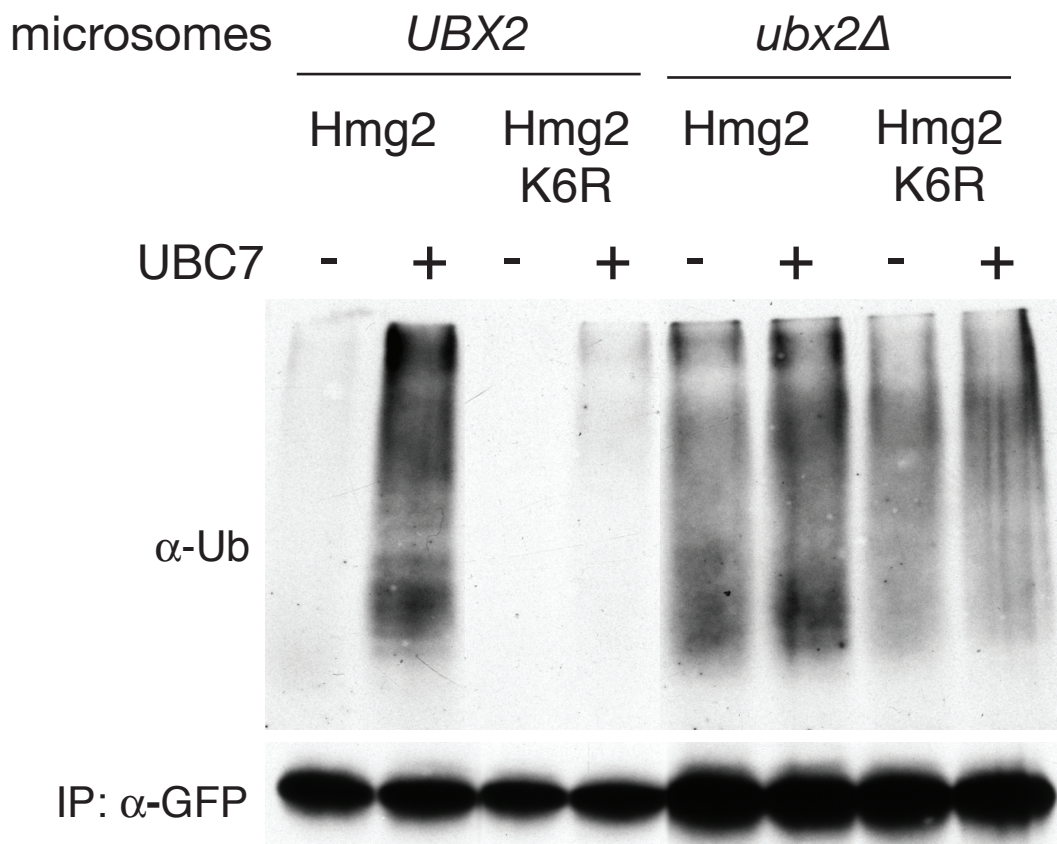


Figure A2-6 Pre-ubiquitination of Hmg2p occurs in *ubx2Δ* strains in a K6 independent manner

In vitro ubiquitination reactions were performed with the indicated microsomes and cytosol strains. Following the reaction, Hmg2p was immunoprecipitated with anti-GFP antibody and the resulting pull-down was analyzed with anti-Ub and anti-GFP antibodies.

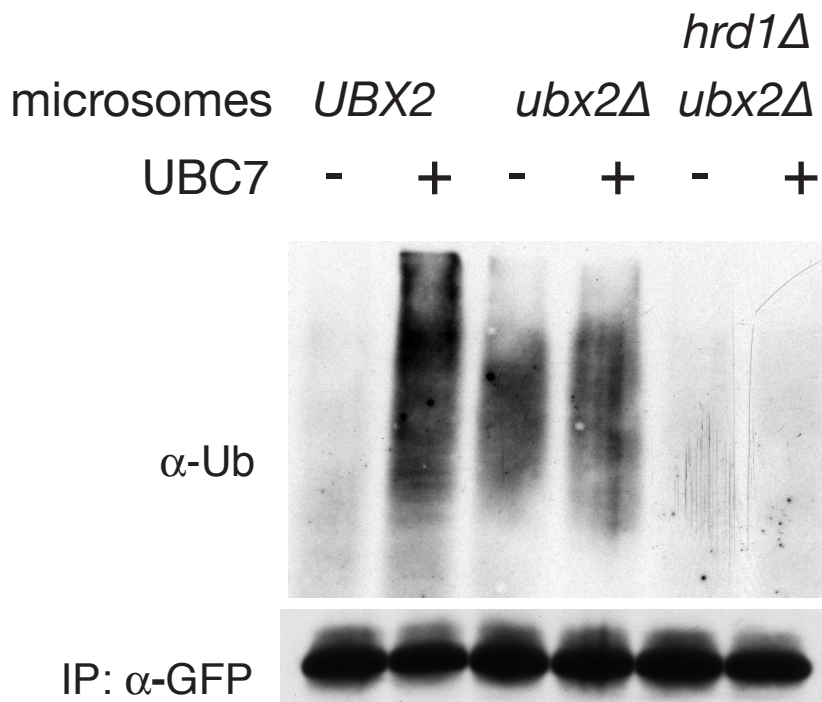


Figure A2-7 Pre-ubiquitination of Hmg2p is Hrd1p dependent

In vitro ubiquitination reactions were performed with the indicated microsome and cytosol strains.

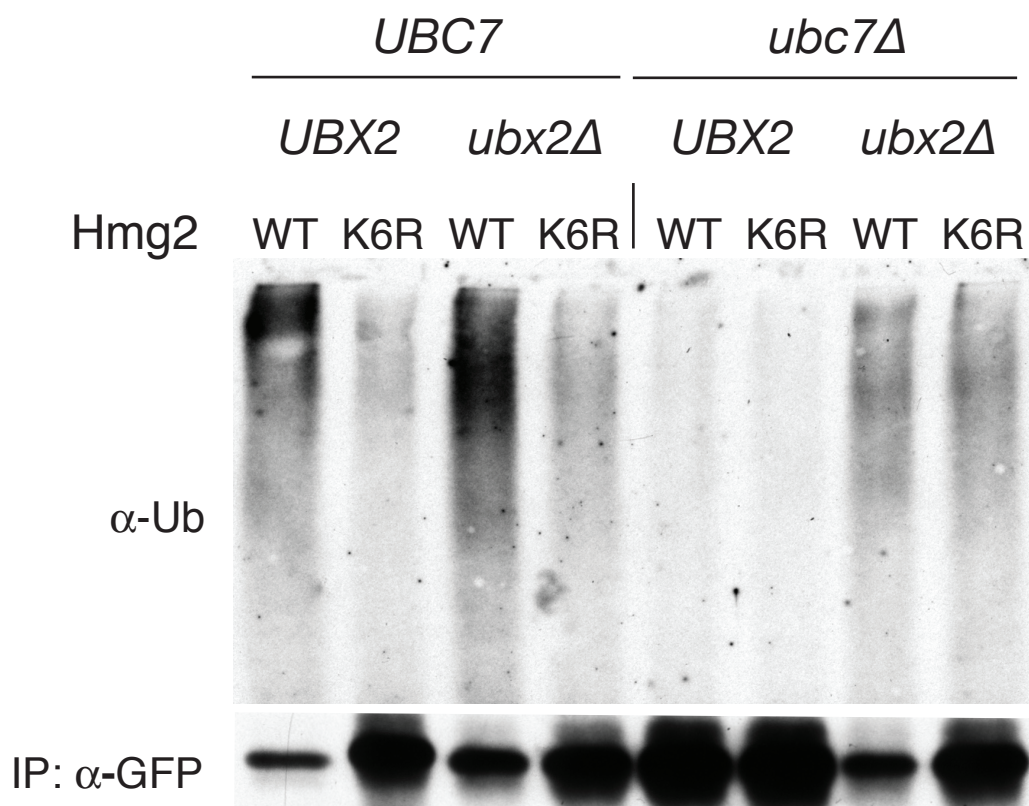


Figure A2-8 Pre-ubiquitination of Hmg2p occurs *in vivo* in the absence of Ubc7p

Strains expressing the indicated version of Hmg2p were tested for Hmg2p ubiquitination in the presence or absence of Ubc7p and Ubx2p. Hmg2p was immunoprecipitated with anti-GFP antibody and the resulting pull-down was analyzed with anti-Ub and anti-GFP antibodies.

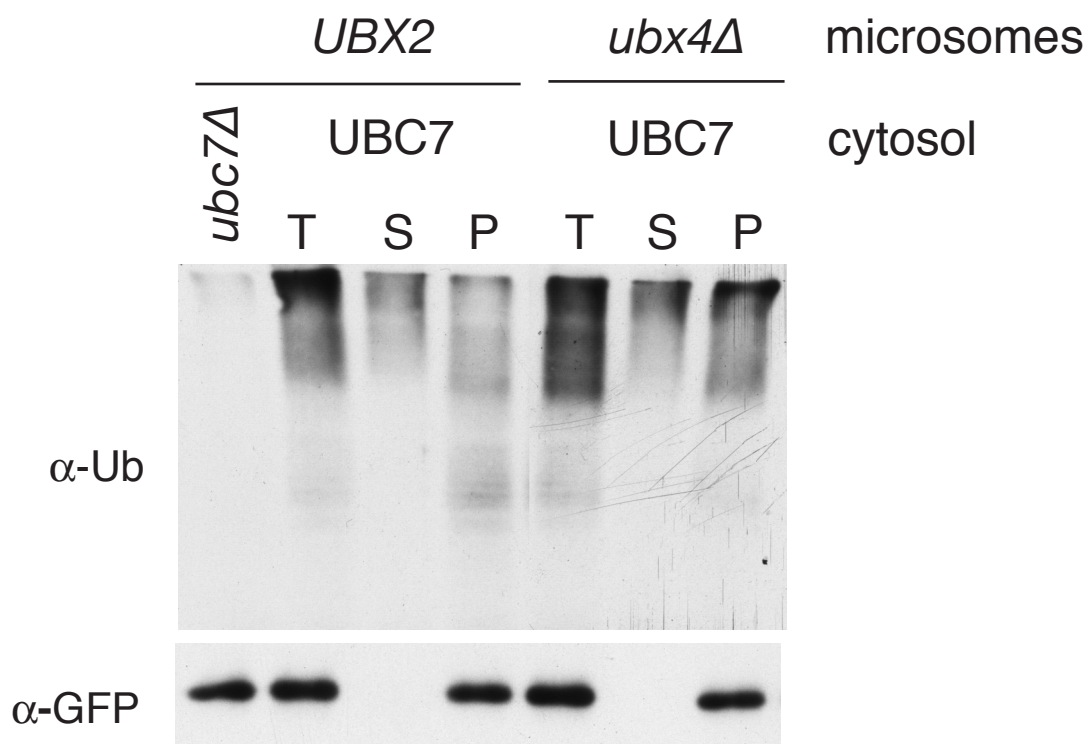


Figure A2-9 Retrotranslocation is proficient in *ubx4Δ* microsomes

The *in vitro* retrotranslocation reaction was performed with wild type or *ubx4Δ* microsomes and wild type cytosol. Hmg2p is not ubiquitinated in *ubx4Δ* microsomes when incubated in *ubc7Δ* strains (data not shown).

Future Directions

These results imply that Ubx2p may be regulating Hrd1p's ubiquitination function. In the absence of Ubx2p, Hrd1p is capable of Hmg2p ubiquitination that is independent of Ubc7p, Ubc1p and K6 of Hmg2p. As would be expected from the lack of retrotranslocation *in vitro*, Hmg2p degradation is impaired in a *ubx2Δubc7Δ* strain *in vivo*, demonstrating that the pre-ubiquitination is not sufficient to promote degradation (Figure A2-10). Thus, formation of "normal" ubiquitin chains may be necessary for retrotranslocation. While it is known that K48 lysine chains are required for proteasomal degradation (Pickart and Eddins 2004), it is unknown whether it is also a requirement for retrotranslocation. One possibility is that factors such as Ufd1p and Npl4p can only bind to certain poly-ubiquitin chains. To examine this, we first need to identify the E2(s) responsible for the Hmg2p pre-ubiquitination. Once this is discovered, we could inhibit pre-ubiquitination by creating a null of that E2 in the presence of *ubx2Δ*. With these microsomes, we could then add back K48 only (all other lysine residues mutated to R) ubiquitin, and ask whether Hrd1p in the absence of Ubx2p is capable of forming K48-linked ubiquitin chains. Another possibility is to immunoprecipitate pre-ubiquitinated Hmg2p and submit the precipitated protein for mass spectrometry analysis. This would then be compared to Hmg2p which has been ubiquitinated in a wild type strain.

In addition to Ubx2p, it would be interesting to uncover the function of Ubx4p. The fact that it is specifically required for Hmg2p degradation is unusual and implies that it may be involved in Hmg2p regulation rather than the ERAD pathway. This is unlikely though as *ubx4Δ* strains are also defective in 6myc-Hmg2p degradation (Figure A2-11)

and also exacerbates the *ubx2Δ* degradation defect of other non-Hmg2p proteins (as illustrated above). The fact that it possesses a UBX domain also implies that it would have a more global ER quality control function, as Cdc48p is essential for ERAD of all classes of substrates. Clearly, further investigation is required to determine Ubx4p's function.

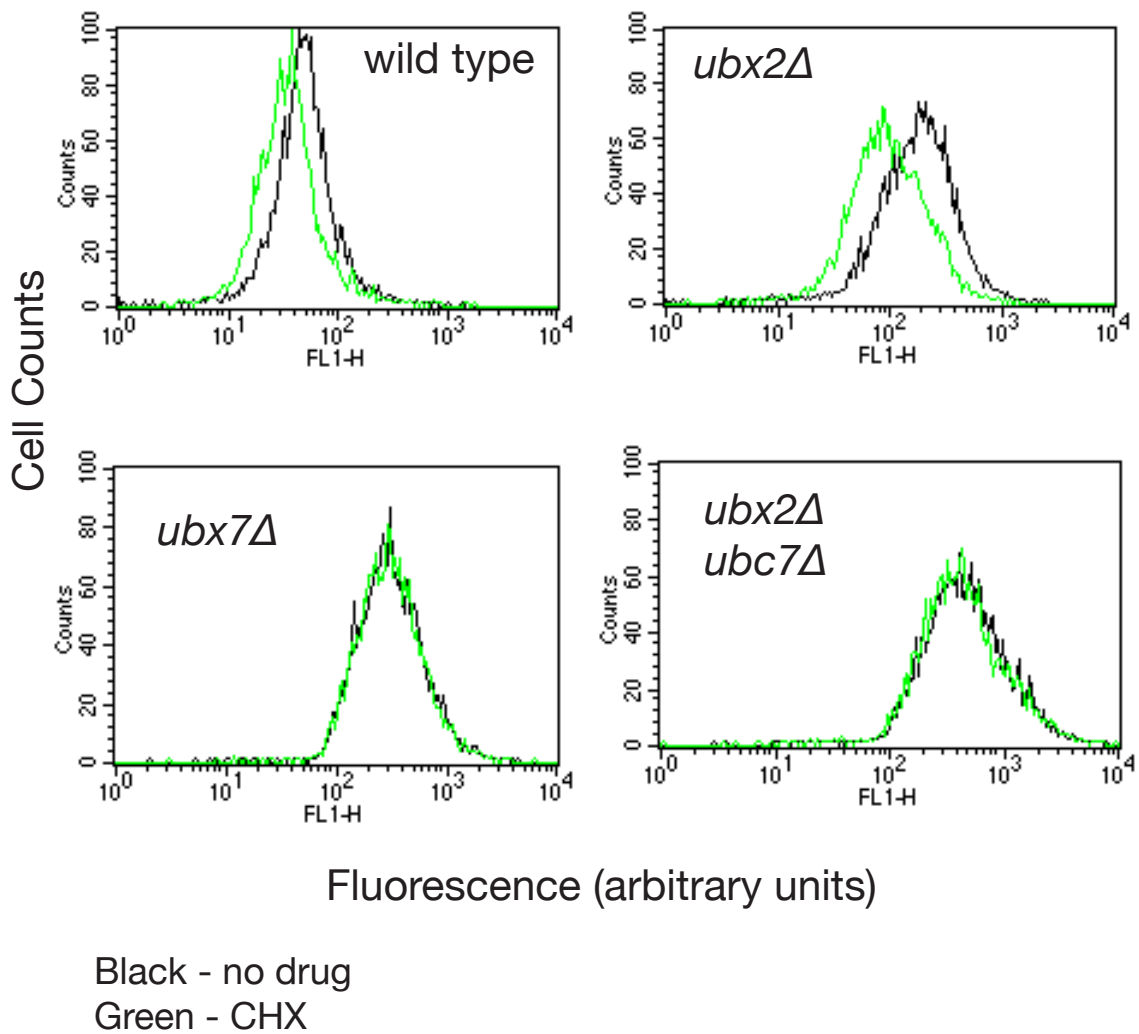


Figure A2-10 Despite pre-ubiquitination, Hmg2p-GFP is stable in *ubx2Δubc7Δ* strains

Steady state levels of Hmg2 were obtained by flow cytometry. Each curve represents 10,000 cells. Cells treated with cycloheximide for 2 hours were compared to cells without drug added.

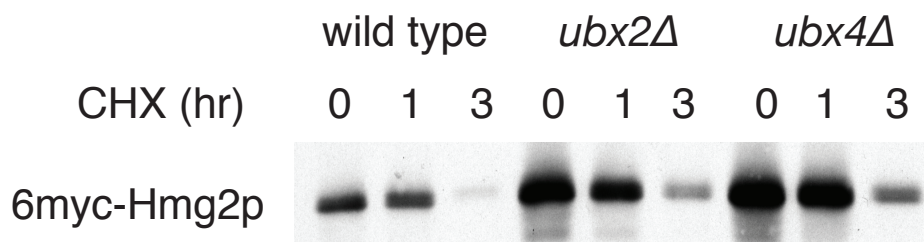


Figure A2-11 Ubx2p and Ubx4p are involved in 6myc-Hmg2p degradation

In vivo 6-myc-Hmg2p degradation was assayed by cycloheximide chase. Cycloheximide was added to the indicated strains and equal amounts of cells were harvested at certain time points. Lysates were analyzed by SDS-PAGE and immunoblotted with anti-GFP antibodies. Equal loading was verified by India ink staining.

Materials and Methods

DNA manipulation and plasmid construction

All plasmids were constructed with standard molecular biology tools as has been described (Sato and Hampton 2006). Plasmids used in these studies are listed in Table A1-2.

Yeast and Bacterial strains

Escherichia coli DH5 α were grown at 37°C in LB media with ampicillin (100 μ g/ml). Yeast strains were grown at 30°C unless otherwise noted in minimal media supplemented with dextrose and amino acids as previously described (Hampton and Rine 1994). The LiOAc method was utilized to transform yeast strains with plasmid DNA (Ito et al. 1983). Knock-outs were constructed by transforming yeast with the LiOAc method with a PCR product that encoded either G418 resistance or CloNAT/nourseothricin (Werner BioAgents, Jena, Germany) resistance and contained 50bp flanks homologous to the gene to be knocked out. (Baudin et al. 1993). Cells were allowed to grow on yeast peptone dextrose (YPD) for ~12 hours and then replica plated onto YPD plus 500 μ g/ml G418 or 200 μ g/ml nourseothricin.

Strains used for all degradation experiments were derived from RHY853 (*MAT α ade2-101 ura3-52::URA3::HMGcd::HMG2-GFP met2 lys2-801 trp1::hisG leu2 Δ his3 Δ 200 hmg1 Δ ::LYS2 hmg2 Δ ::HIS3*). *In vitro* retrotranslocation experiments were performed with the following strains. All microsome donor strains were derived from RHY2923 (*MAT α ade2-101 ura3-52::URA3::HMG2-GFP met2 lys2-801*

trp1::hisG::TRP1::TDH3-HRD1-3HA leu2Δ his3Δ200 HMG1 hmg2Δ::1MYC-HMG2 hrd1Δ::KanMX pep4Δ::HIS3 ubc7Δ::LEU2). All cytosol donor strains were derived from 4288 (*MATα ade2-101 ura3-52 met2 lys2-801 trp1::hisG leu2Δ his3Δ200 HMG1 hmg2Δ::1MYC-HMG2 hrd1Δ::KanMX pep4Δ::HIS3 ubc7Δ::LEU2*).

Degradation assays and UPR measurements

Cyclohexamide chase degradation assays were performed as previously described (Sato and Hampton 2006) with SUME lysis buffer (1% SDS, 8M Urea, 10mM MOPS pH 6.8, 10mM EDTA). Flow cytometry was also undertaken as described (Sato and Hampton 2006). Data was obtained through a FACScalibur machine (Becton, Dickinson and Company, Franklin Lakes, NJ) and statistical analysis was performed with CellQuest software (Becton, Dickinson and Company, Franklin Lakes, NJ).

***In vitro* retrotranslocation assay**

In vitro retrotranslocation assays were performed as previously described (Flury et al. 2005). Microsome donor strains, containing TDH3-Hrd1-3HA, Hmg2p-GFP and *ubc7Δ*, were harvested by bead beating cells in MF buffer (20mM Tris pH 7, 100mM NaCl, 300mM sorbitol) for 6 minutes (1 minute on, 1 minute off), the centrifuging at 14,000 x g for 45 minutes after which they were resuspended in B88 buffer (20mM Hepes pH6.8, 250mM sorbitol, 150mM KOAc, 5mM MgOAc). Cytosol donor strains expressing TDH3-Ubc7-2HA were centrifuged and underwent freeze-thaw lysis in B88 buffer and ultracentrifuged. For each reaction, a microsome strain was combined with 30mM ATP and cytosol that either did or did not express TDH3-Ubc7-2HA for 1 hour at

30°C. Reactions were then centrifuged at 25,000 x g for 1 hour. The supernatant fraction was removed to a new tube and the pellet was resuspended in the same volumen with B88 buffer. Immunoprecipitations were then performed as follows. 200µl of SUMO with protease inhibitors and N-ethyl maleimide (NEM) was added to each reaction followed by 600µl of IP buffer (150mM NaCl, 15mM Na₂HPO₄, 2% Triton-X100, 0.1% SDS, 0.5% DOC, 10mM EDTA, pH 7.5) with protease inhibitors and NEM. The supernatant was removed and 15µl of polyclonal anti-GFP, 30µl anti-loop antibody or 15µl anti-HRD1 antibody was added. The mix was incubated overnight at 4°C. 100µl of Protein-A sepharose (Amersham Biosciences) in IP buffer, (50% w/v) was added for 2 hours. Beads were washed once with IP buffer and once with IP wash buffer (50mM NaCl, 10mM Tris, pH 7.5) and incubated with 55µl of 2x Urea sample buffer (75mM MOPS pH 6.8, 4% SDS, 200mM DTT, 0.2mg/ml bromophenol blue, 8M urea) for 10 minutes at 50°C. The samples were then loaded on a polyacrylamide gel. Following transfer to nitrocellulose, immunoblotting with an anti-ubiquitin or anti-GFP antibody was performed.

Table A2-1 Plasmids used in Appendix 2

Plasmid	<i>Genotype</i>
pRH373	<i>YIp TRP1 pTDH3-UBC7-2HA</i>
pRH469	<i>YIp URA3 pTDH3-HMG2-GFP</i>
pRH671	<i>YIp URA3 TDH3-HMG2 K6R-GFP</i>
pRH728	<i>KanMX loxP-KanMX-loxP disruption cassette</i>
pRH730	<i>YIp TRP1 TDH3-HRD1-3HA</i>
pRH808	<i>YIp TRP1 TDH3-HRD1</i>
pRH1122	<i>hrd1Δ::KanMX</i>
pRH1152	<i>YIp LEU2 pTDH3-UBC7-2HA</i>
pRH1186	<i>ubc7Δ::LEU2</i>
pRH1209	<i>YIp URA3 UPRE-GFP</i>
pRH1377	<i>YCp URA3 CPY*-HA</i>
pRH1694	<i>YIp URA3 TDH3-6MYC-HMG2-GFP</i>
pRH1788	<i>pep4Δ::HIS3</i>
pRH1838	<i>NatR loxP-CloNAT-loxP disruption cassette</i>
pRH2071	<i>YIp URA3 pTDH3-HMG1-HRD1</i>
pRH2312	<i>YCp HIS3 HA-Pdr5*</i>

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