UC San Diego

UC San Diego Electronic Theses and Dissertations

Title

ERAD Substrate Recognition by the HRD1 Transmembrane Domain : : A Molecular Genetic Analysis

Permalink

https://escholarship.org/uc/item/1hk9720k

Author

Ma, Mengxiao

Publication Date

2013

Peer reviewed|Thesis/dissertation

eScholarship.org Powered by the California Digital Library
University of California

UNIVERSITY OF CALIFORNIA, SAN DIEGO

ERAD Substrate Recognition by the HRD1 Transmembrane Domain: A Molecular Genetic Analysis

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

Mengxiao Ma

Committee in charge:

Professor Randolph Hampton, Chair
Professor James Wilhelm
Professor James Kadonaga

2013

Copyright

Mengxiao Ma, 2013

All rights reserved.

The Thesis of Mengxiao Ma is approved and it is acceptable in quality and form for publication on microfilm and electronically:

_____

_____

_____

Chair

University of California, San Diego

2013

TABLE OF CONTENTS

Signature Page……..…iii

Table of Contents…….…….…..iv

List of Figures…….……..…v

List of Tables……...vii

Acknowledgments……...…….....viii

Abstract……..……..ix

Introduction……..…….……1

Results……...…6

Discussion…….……...…15

Figures…….21

Materials and Methods…….……...36

References……...44

LIST OF FIGURES

Figure 1: Stability of S215A-Hmg2p-GFP……..……22

Figure 2: Expected phenotype of overactive
Hrd1p mutants……..……23

Figure 3: Hrd1p levels of isolated candidates ……...……...……...……..……24

Figure 4: Mutation mapping of the transmembrane
domain of overactive Hrd1p mutants ……..……..……..…25

Figure 5: Depiction of overactive mutation….……...……...……...……..……28

Figure 6: Degradation of Hmg2p SSD
mutants by V108E-Hrd1p ……..…..……29

Figure 7: Degradation of Hmg2p ubiquitination
mutants by V108E-Hrd1p …….……..……..……..…30

Figure 8: Degradation of Hmg2p by V108E-Hrd1p
is unregulated ……...…….……...…...……..……...…31

Figure 9: Degradation of other ERAD substrates by V108E-Hrd1p ……...…..……...…...……..……32

Figure 10: Degradation of Hmg2p and S215A by different V108 mutants ……..…....……33

Figure 11: V108E-Hrd1p does not have altered stability ……...…..……...……....……..……34

Figure 12: Allosteric model of Hrd1p substrate recognition ……..…....…….……35

LIST OF TABLES

Table 1: Plasmids……40

Table 2: Strains……41

Table 3: Oligos……43

ACKNOWLEDGEMENTS

I would first like to express my greatest appreciation to Professor Randolph Hampton for providing me the opportunity to join his lab and for his guidance and support as the chair of my committee. Professor Hampton has always been extremely encouraging and helpful, always challenging me to think scientifically.

I would also like to thank Chandra Theesfeld, a former post-doc, who taught me how to pipette and mentored me for my first year in the lab. Chandra was extremely patient and has taught me most of the bench skills that I know now.

I would also like to acknowledge William Partlo, who, as an "undergraduate researcher" made the V108E-Hrd1p ARS/CEN construct (pRH2621) during his first cloning attempt and is now a graduate student at UCSD.

Finally, I would like to thank everyone in the Hampton lab for all of the help and discussions they shared with me and especially their troubleshooting expertise when experiments went wrong.

ABSTRACT OF THE THESIS

ERAD Substrate Recognition by the HRD1 Transmembrane Domain: A Molecular Genetic Analysis

by

Mengxiao Ma

Master of Science in Biology

University of California, San Diego, 2013

Professor Randolph Hampton, Chair

Endoplasmic Reticulum Associated Degradation (ERAD) is a conserved pathway that is responsible for the degradation of aberrant proteins in the ER. These proteins are first tagged with ubiquitin through the concerted action of E1, E2, and E3 ubiquitination enzymes and then degraded by the proteasome. Hrd1p is one of the principle E3 ubiquitin ligases involved in ERAD. It is an integral ER membrane protein with six transmembrane spans and a cytosolic RING domain and is responsible for the recognition and degradation of a wide variety of ER-localized misfolded proteins,

including lumenal (ERAD-L) and integral membrane (ERAD-M) substrates. Previous studies have suggested that Hrd1p substrate recognition function as an “allosteric” model and that the Hrd1p transmembrane domain is a key site for substrate selectivity. To further explore this idea, we have conducted a screen to isolate Hrd1p mutants that show increased activity toward substrates of the HRD pathway. We have isolated a single point mutation in the transmembrane domain of Hrd1p that imparts more effective degradation of an ERAD-M substrate. Further analysis of this mutation revealed that it causes selective increased activity toward a single substrate and degrades all other substrates normally. This selective increase in activity supports the idea that different regions of the transmembrane domain are responsible for the recognition of different Hrd1p substrates.

Introduction

One of the many functions of the endoplasmic reticulum (ER) is to maintain protein quality control through ubiquitin-mediated degradation of aberrant proteins. Misfolded or nonnative proteins in the ER are recognized and degraded by a set of factors that make up the ER-associated degradation (ERAD) pathway (Hampton 2002a; Sommer and Wolf 1997). Proteins that enter the secretory pathway are first translocated into and folded in the ER. The ER must be able to identify and destroy proteins that are misfolded before they are sent out to the rest of the cell; therefore, it plays a crucial role in maintaining protein integrity of the cell. The major pathway that has evolved to regulate ER protein quality control is the ERAD pathway (Brodsky 2012). Destruction of proteins by ERAD is carried out via the ubiquitin-proteasome route (Hampton 2002). This involves covalently attaching ubiquitin onto the targeted substrate and shuttling the poly-ubiquitinated substrate to the cytosolic proteasome for proteolysis. Attachment of ubiquitin requires the concerted action of three enzymes: E1, E2, and E3. An E1 ubiquitin-activating enzyme first activates ubiquitin in a two-step process using ATP. Then it transfers the ubiquitin to an E2 ubiquitin-conjugating enzyme, which allows for covalent attachment of the activated ubiquitin onto the substrate by the action of an E3 ubiquitin-protein ligase. In this process, the E3 ubiquitin ligase determines which proteins undergo ubiquitination; however, the mechanism by which the E3 is able to recognize these proteins is still unclear.

The two E3 ubiquitin ligases involved in ERAD are Hrd1p and Doa10p (Bays et al. 2001; Swanson et al. 2001). Each ligase controls the degradation of a distinct set of substrates with minimal overlap. Substrates of the HRD pathway fall into two distinct categories, misfolded soluble lumenal proteins, designated ERAD-L and misfolded

membrane proteins, designated ERAD-M. However, while both types of substrates require the activity of Hrd1p in order to be degraded, Hrd1p seems to play a bigger role in substrate recognition of ERAD-M substrates (Sato et al. 2009).

A well-studied ERAD-M pathway substrate is Hmg2p, a yeast isozyme of the mammalian 3-hydroxy 3-methylglutaryl coenzyme A reductase (HMG-CoA reductase, HMGR) (Hampton 2002b; Hampton and Garza 2009). Hmg2p catalyzes the rate-limiting step in sterol biosynthesis and undergoes regulated degradation by Hrd1p (Hampton and Rine 1994; Hampton et al. 1996a). Another ERAD-M substrate is Pdr5*, a mutant version of the pleiotropic drug resistance gene Pdr5 that is retained in the ER instead of transported to the plasma membrane after entering the secretory pathway (Plemper et al. 1998). A classic ERAD-L pathway substrate is CPY*, a mutant form of carboxypeptidase Y (CPY) that is retained in the ER lumen and degraded by the HRD pathway (Wolf and Schäfer 2005).

Hrd1p was identified in a screen for genes required for degradation of Hmg2p (Hampton et al. 1996a). Hrd1p is a resident ER membrane protein that contains six transmembrane spans and a cytosolic RING domain that catalyzes ubiquitination of ERAD-M and ERAD-L substrates (Bays et al 2001; Deak and Wolf 2001; Carvalho et al. 2006). Hrd1p functions in a complex with several other proteins in order to target substrates for ubiquitination and degradation. Through proteomic studies, the Hrd1p complex is known to consist of Hrd1p, Hrd3p, Usa1p, Yos9p, Der1p, Ubx2p, Kar2p, and the Cdc48-Npl4-Ufd1 complex (Denic et al. 2006).

Hrd1p's transmembrane domain, aside from having a structural function, has been implicated in the recognition of ERAD-M substrates (Sato et al. 2009). In a

previous study, conserved and hydrophilic amino acids in Hrd1p's transmembrane domain were mutated to alanine to examine the importance of these residues in substrate recognition. Some of these mutants showed surprisingly selective defects for degradation of individual ERAD-M substrates, but none of these mutants were defective for ERAD-L substrates. The Hrd1p mutants defective for degradation of specific ERAD-M substrates, however, still showed normal interaction with those substrates, supporting the idea that substrate selectivity follows an "allosteric" model, in which recognition of a substrate's folding state is a separate phenomenon from substrate interaction, and that the two events may possibly be uncoupled. In these Hrd1p mutants, there was decreased recognition of the specified ERAD substrates, leading to less degradation of these substrates. To further explore these ideas, we have conducted a genetic screen for overactive mutants of Hrd1p that show increased degradation of ERAD substrates.

In this screen, an untagged Hrd1p expressed off the native promoter was used in order to ensure that any interaction between overexpression and/or the tag and any mutations will not result in a false positive. This plasmid was also an ARS/CEN plasmid to allow for *in vivo* recombination cloning and straightforward yeast transformations. A stable variant of Hmg2p was used as an optical reporter to determine increased activity of Hrd1p mutants. This variant has a point mutation, S215A, and is completely stable in the presence of wild-type Hrd1p (Figure 1). S215A-Hmg2p-GFP is degraded only when Hrd1p is overexpressed using a strong promoter, TDH3 (Figure 2). In other words, TDH3-Hrd1 is essentially a phenocopy of what we expect overactive Hrd1p mutants to look like. This allows us to easily distinguish between wild-type and

overactive Hrd1p phenotypes during the screen. After the candidates are isolated, preliminary analysis will be conducted to determine if the initial phenotypes should be pursued.

Results

Development of a screening technique to look for overactive Hrd1p mutants

To explore the idea of whether Hrd1p could be made to be more active in degrading its normal substrates, we took a genetic approach and conducted a screen to isolate Hrd1p mutants that show increased degradation of S215A-Hmg2p-GFP. We began by creating a library of mutagenized Hrd1p plasmids through chemical mutagenesis using hydroxylamine. We then transformed these Hrd1p plasmids into a strain expressing S215A-Hmg2p-GFP, an Hmg2p variant that is completely stable in the presence of native levels of Hrd1p but degraded when Hrd1p is overexpressed (Figure 2). This difference in the amount of GFP present is clearly distinguishable on plates using an optical screening technique developed by our lab. When freshly grown yeast colonies are viewed using a yellow filter under blue light, those expressing less GFP can be easily distinguished (Cronin and Hampton 1999). A difference in fluorescence of less than two-fold can be easily seen. We utilized this as a screening tool to isolate possible candidates that have increased Hrd1p activity. Using this technique, we were able to screen over 250,000 colonies.

After candidates were isolated using the plate assay described above, we then cured the plasmid to make sure the dark phenotype seen on plates was Hrd1p-dependent. To do this, each candidate isolated from the screen was grown overnight in YPD. This ensured that a proportion of the cells lost the Hrd1p plasmid. We confirmed this by plating a small amount of the culture on agar plates that do not select for the Hrd1p plasmid and then replica plating onto agar plates that do select for the Hrd1p plasmid. There should be significantly less colonies that appear on the replica plate. If the dark phenotype was Hrd1p-dependent, both bright and dark colonies should appear on the

first plate but only dark colonies appear on the replica plate. If the dark phenotype was not caused by Hrd1p, then only dark colonies should appear on both plates. During this process, we also analyzed and quantified the fluorescence of the candidates by flow cytometry.

Using hydroxylamine mutagenesis, we only found false positives that appeared dark but not Hrd1p-dependent. The plasmid curing showed that these colonies remained dark even after Hrd1p was removed and quantification using flow cytometry showed that these colonies were as dark as a no-GFP control.

Since chemical mutagenesis did not yield any mutants, we decided to try a different mutagenesis approach. In this approach, only the transmembrane region of Hrd1p was targeted instead of the entire plasmid. We created a library of mutated Hrd1p transmembrane domains using error-prone PCR. This library was then recombined with the rest of the Hrd1p plasmid *in vivo* in yeast. There was about 150-base pairs that overlapped on each side. This technique allowed us to immediately screen for mutants without a separate cloning step.

Most of the colonies showed a wild type Hrd1p phenotype and a fraction of the colonies appeared darker. These colonies were isolated and plasmid-cured as described above to make sure that the dark phenotype was Hrd1p-dependent.

After this initial analysis, the candidates that showed Hrd1p-dependent phenotypes were isolated and their Hrdp1 plasmids were extracted. These plasmids were reintroduced into the screening strain to confirm that the phenotype was mutant Hrd1p plasmid-dependent. Since the Hrd1p transmembrane domain is at the N-terminus, part of the Hrd1p promoter was inevitably included during PCR mutagenesis. Because

of this, mutations in the promoter region may have potentially altered the expression of Hrd1p, resulting in an overexpression phenotype. To test this, we performed a Western blot to make sure that the candidates expressed Hrd1p levels comparable to strains expressing unmutagenized Hrd1p (Figure 3).

Sequence analysis of overactive Hrd1p mutants

After preliminary analysis to isolate the overactive candidates, we sequenced the Hrd1p transmembrane region of these plasmids. We isolated a total of fourteen Hrd1p candidates that were able to degrade S215A-Hmg2p-GFP to varying degrees. Each candidate had an average of five mutations in the transmembrane domain. We mapped out the mutations in each of the mutants and found that there were several mutations that were present in more than one candidate (Figure 4). From sequence analysis of these isolated mutants, we discovered that a single residue change, V108E, was sufficient to lower the steady state levels of S215A-Hmg2p-GFP. This mutation was present in almost half of the Hrd1p mutants obtained from the screen and when present by itself, showed one of the strongest phenotypes seen in the candidates. V108E is located on the fourth transmembrane segment in the Hrd1p transmembrane domain closer to the ER lumenal side (Figure 5).

Next, we began to examine V108E-Hrd1p's increased activity against other ERAD substrates.

V108E-Hrd1p shows increased, unregulated activity toward Hmg2p-related substrates

Since the screen was conducted using S215A-Hmg2p-GFP, a stable mutant of Hmg2p-GFP, we wanted to test other variants of Hmg2p, including wild-type Hmg2p-GFP. Hmg2p-GFP is a fusion of the transmembrane domain of Hmg2p at the N-terminus and GFP at the C-terminus that responds to regulatory signals of the mevalonate pathway just like native Hmg2p (Hampton et al. 1996b). It is subject to degradation by wild-type Hrd1p and strongly destabilized when Hrd1p is overexpressed. Similar to overexpressed Hrd1p, V108E-Hrd1p also lowered the steady-state levels of Hmg2-GFP more so than wild type Hrd1p (Figure 6).

We then tested another stable mutant, L219F-Hmg2p-GFP. Analogous to S215A-Hmg2p-GFP, L219F is completely stable in the presence of normal Hrd1p and does not undergo regulated degradation (Theesfeld et al. 2011). Both of these Hmg2p mutants have a mutation in the sterol sensing domain, rendering them unable to respond to degradation signals. The sterol sensing domain (SSD) is a conserved region found in several mammalian proteins and plays a regulatory role in sterol-related pathways (Kuwabara and Labouesse 2002). Similarly to S215A-Hmg2p-GFP, V108E-Hrd1p also lowered steady-state levels of L219F-Hmg2p-GFP (Figure 6).

Another category of Hmg2p-related substrates we decided to test was ubiquitination mutants. These Hmg2p mutants have either lysine6 or lysine357 changed to arginine, preventing the covalent attachment of ubiquitin by Hrd1p's RING domain at the mutated position. When either K6R or K357R is present in an otherwise normal Hmg2p-GFP, it becomes stabilized and unable to undergo complete degradation.

K357R is slightly less stable than K6R but both mutants show significantly decreased ubiquitination (Gardner and Hampton 1999). When these Hmg2p mutants are expressed in the presence of the overactive mutant V108E-Hrd1p, both K6R and K357R are significantly destabilized compared to wild-type Hrd1p (Figure 7). Perhaps V108E-Hrd1p is more active in binding K6R and K357R and thus increasing the likelihood of ubiquitination at the nonmutated lysine position.

Hrd1p-dependent degradation of normal Hmg2p *in vivo* is regulated by a farnesyl pyrophosphate (FPP)-derived signal that is a downstream product of the sterol biosynthesis pathway, geranylgeranyl pyrophosphate (GGPP) (Garza et al. 2009). When cellular GGPP is abundant, Hrd1p-dependent Hmg2p degradation is accelerated. Surprisingly, this molecule can be added to intact cells, and the increase in Hmg2-GFP degradation can be detected by flow cytometry. In contrast, the drug lovastatin causes a decrease in GGPP, and consequently Hmg2p-GFP degradation that can be detected by increased fluorescence. We decided to examine the effect of lovastatin and GGPP on Hmg2p-GFP degradation mediated by V108E-Hrd1p. Since our screening substrate S215A-Hmg2p-GFP is not a regulated Hrd1p substrate, we decided to use normal Hmg2p-GFP to test V108E-Hrd1p's response to sterol degradation signals. Lovastatin and GGPP were added to separate cultures of yeast expressing both Hmg2p-GFP and V108E-Hrd1p or wild-type Hrd1p and incubated for four hours, allowing enough incubation time for the drugs to reach full effect. When expressed alongside wild-type Hrd1p, Hmg2p-GFP became stabilized with the addition of lovastatin and destabilized with the addition of GGPP as expected. However, in the presence of V108E-Hrd1p,

both lovastatin and GGPP produced very little effect on the steady-state levels of Hmg2p-GFP (Figure 8).

Taken together, these data show that V108E-Hrd1p is significantly overactive in degrading Hmg2p and its related substrates and shows constitutive degradation of Hmg2p.

Other Hrd1p substrates are not subject to V108E-Hrd1p's increased activity

To gain a better understanding of the specificity of V108E-Hrd1p's activity, we tested several other HRD pathway substrates that are not Hmg2p-related. We examined the degradation of another ERAD-M substrate, Pdr5*, a mutant version of Pdr5 that is retained in the ER and degraded by the HRD pathway (Plemper et al. 1998). In order to examine the degradation of Pdr5*, we constructed an integrating version of V108E-Hrd1p, thus ensuring a single copy. Surprisingly, Pdr5* was degraded normally by V108E-Hrd1p (Figure 9). The degradation rate of Pdr5* as seen by a cycloheximide chase assay was equal for both wild-type Hrd1p and V108E-Hrd1p.

We then examined the degradation of an ERAD-L substrate, CPY*, by V108E-Hrd1p. CPY* is a mutant version of CPY that is retained in the lumen of the ER and degraded via the HRD pathway (Wolf and Schäfer 2005). In a cycloheximide chase assay, the degradation rate of CPY* in the presence of V108E-Hrd1p is similar to wild-type Hrd1p (Figure 9). Previous studies have shown that the Hrd1p transmembrane domain had a larger effect on ERAD-M substrate recognition compared to ERAD-L; thus, it was not surprising that the overactive Hrd1p mutant would show no increase in the degradation rate of CPY*.

Therefore, V108E-Hrd1p's increased activity seems to be substrate-specific for only Hmg2p-related substrates and degrades all other substrates normally.

V108E-Hrd1p's increased activity is glutamate specific

In order to test the importance of the specific residue change in V108E-Hrd1p's increased activity toward Hmg2p, we made several other V108 Hrd1p variants. We wanted to understand the characteristics of V108E-Hrd1p that allowed increased activity toward Hmg2p. We first decided to mimic the negative charge produced by glutamate in V108E-Hrd1p by constructing V108D-Hrd1p. We anticipated that the change from a neutral residue to a negatively charged residue inside the membrane is largely responsible for V108E-Hrd1p's ability to increase degradation of Hmg2p. When V108D was expressed in the presence of Hmg2p-GFP and S215A-Hmg2p-GFP, both substrates showed lower steady-state levels as expected. However, V108D showed a much weaker phenotype than V108E, lowering the steady-state levels of S215A-Hmg2p-GFP by not even two-fold (Figure 10).

We then constructed V108K-Hrd1p to examine the effect that a positive charge in this position would have on Hmg2p degradation. Interestingly, V108K was also one of the mutations found in a candidate with multiple mutations isolated from the screen. However, the lone mutation V108K was not able to decrease the steady-state levels of S215A-Hmg2p-GFP but was able to increase degradation of Hmg2p-GFP (Figure 10). Even though V108K was unable to overcome the stability of S215A-Hmg2p-GFP, it still showed increased degradation of Hmg2p-GFP compared to wild-type Hrd1p. Changing valine to a residue with either a positive or negative charge seems to make

Hrd1p at least slightly better at degrading Hmg2p-GFP and the same as wild-type at degrading S215A-Hmg2p-GFP. Perhaps introducing a charge into the transmembrane span allows a change in structure that promotes increased interaction with the substrate.

Next, we wanted to see if a glutamate-like residue without the negative charge contributed to V108E-Hrd1p's phenotype. However, similar to V108K, V108Q was unable to degrade S215A-Hmg2p-GFP but was able to increase the degradation of normal Hmg2p slightly compared to wild-type Hrd1p.

Lastly, we decided to examine the effect on Hrd1p activity if we replaced valine with a residue very similar to valine structurally. We expect that this residue change will not result in any change in Hrd1p's activity. To do this, we constructed the variant V108L. Leucine has the same branched structure as valine but its side chain is slightly longer. As expected, V108L functioned as wild-type in degrading both S215A-Hmg2p-GFP and normal Hmg2p-GFP.

Since all the V108 variants, except for V108L, showed at least a slight decrease in Hmg2p-GFP steady-state levels, we believe that the loss of a neutral residue is most likely a contributing factor to V108E-Hrd1p's overactive phenotype. However, most of the increased activity seems to be due to the specific presence of glutamate, even though replacing the neutral valine or leucine with either a charged residue or glutamate-like residue also contributes to increased degradation of Hmg2p.

Even though none of the other V108 mutants showed as strong a phenotype compared to wild-type Hrd1p as V108E-Hrd1p, it is also important to realize that none of these mutants were defective in degrading Hmg2p-GFP or S215A-Hmg2p-GFP compared to normal Hrd1p.

Discussion

Distinct classes of overactive Hrd1p mutants

Given the nature of this screen, we speculate that four different possible classes of mutants might arise. The first class includes mutants that are only overactive toward S215A-Hmg2p-GFP. Since this was the only substrate that we screened with, it is reasonable to assume that some overactive Hrd1ps that we isolated would be specific for only S215A.

The second class of mutants that we anticipated, and actually found, were mutants that have increased activity toward all Hmg2p-related substrates. Since all Hmg2p substrates are structurally similar, it would make sense that a mutant Hrd1p that showed increased activity toward S215A-Hmg2p-GFP would also have increased activity toward other Hmg2p variants. The mutant V108E-Hrd1p is part of this second class of mutants. From all of the tests that we had conducted using V108E-Hrd1p, we demonstrated that V108E-Hrd1p had increased degradation toward stable Hmg2p SSD mutants, stable ubiquitination mutants, and wild-type Hmg2p-GFP. However, toward non-Hmg2p-related substrates such as Pdr5* (ERAD-M substrate) and CPY* (ERAD-L substrate), V108E-Hrd1p showed normal degradation.

The third possible class of mutants would have increased activity toward many different or all ERAD substrates. Perhaps some of the candidates that we isolated with multiple mutations fit into this class. Using the allosteric model of substrate recognition, there seems to be two possible ways to get a mutant that is overactive toward unrelated ERAD substrates. One way is if multiple interaction sites in Hrd1p are mutated and made better able to interact with the different substrates that these different interaction sites targeted. A second way is if the region of the transmembrane domain that is

responsible for activation of the Hrd1p RING domain is made more active, then there would be increased ubiquitination of all recognized substrates.

The last class of mutants that we had anticipated was Hrd1ps that are able to degrade non-substrates as well as normal substrates. These mutants were the least likely to be discovered by our screen because 4-6 residue changes, which is the average using our PCR mutagenesis protocol, seems unlikely to be able to change Hrd1p enough for it to develop a different function. In order to find such a phenotype, perhaps other factors in the Hrd1p complex should be targeted as well.

Other characteristics of V108E-Hrd1p

When an enzyme is mutated to show increased activity toward one subset of substrates, the resulting variant may show decreased or normal activity toward its other substrates. In the case of V108E-Hrd1p, we do not see any compromised activity toward other non-Hmg2p related ERAD substrates.

Another target of Hrd1p-mediated degradation is Hrd1p itself. Hrd1p self-degradation is catalyzed by its own RING domain and seems to be a distinct process from both ERAD-L and ERAD-M (Carroll and Hampton 2010). In strains expressing wild-type Hrd1p, Hrd1p is very stable, with a half-life of over 30 minutes. Compared to wild-type Hrd1p, V108E-Hrd1p does not show altered stability or self-degradation (Figure 11).

In our screen, we were looking for candidates that were essentially phenocopies of Hrd1p overexpression for S215A-Hmg2p-GFP degradation. As seen in Figure 1, TDH3-Hrd1 significantly lowered the steady-state levels of S215A-Hmg2p-GFP and

this phenotype was the basis that we used to screen for overactive Hrd1p mutants. Another characteristic of overexpressed Hrd1p is increased cell toxicity in our lab strain, S288C. When Hrd1p is overexpressed using the strong promoter TDH3 in our strain, there is a significant increase in doubling time and an increased tendency for the cells to lose the Hrd1p plasmid. These cells are clearly sicker than the cells that have native levels of Hrd1p or no Hrd1p. These characteristics seem to be alleviated when the cells are grown at room temperature. However, V108E-Hrd1p does not induce any cell toxicity in our lab strain even though it has increased activity. Although we did not specifically test for toxicity of V108E-Hrd1p in our lab strain, we were able to grow these strains under the same conditions and temperature as wild-type Hrd1p strains and saw no difference in doubling time or viability.

Hrd1p transmembrane domain plays a large role in substrate selectivity

V108E-Hrd1p demonstrated a very substrate-specific gain-of-function phenotype, supporting the idea that different regions of the Hrd1p transmembrane domain are responsible for the recognition of different substrates. Earlier interaction assays have shown that while Hrd1p interacts indiscriminately with many proteins, it targets only a subset of those for degradation (Gardner et al. 2001). Previous studies with Hrd1p deficient mutants have already suggested that certain conserved residues of the Hrd1p transmembrane domain are important for the degradation of different ERAD substrates. When these residues are mutated to alanine, Hrd1p is unable to degrade certain ERAD-M substrates but is still shown to interact with them (Sato et al. 2009).

Similarly in this study, V108E-Hrd1p's increased ability to degrade only Hmg2p-related substrates provides additional support for the hypothesis that substrate specificity is strongly determined by different regions of the transmembrane domain. A possible explanation for V108E-Hrd1p's Hmg2p-specific phenotype is that the valine to glutamate mutation may have resulted in a structural change that locked Hrd1p into the "recognition conformation" for Hmg2p-related substrates. In a normal Hrd1p, this conformation would only result when Hmg2p is recognized as a substrate. However, V108E-Hrd1p is already preconditioned to target that substrate for ubiquitination. It is less likely that the residue change resulted only in increased substrate interaction. If the structural change only promoted increased interaction but no change to recognition, then we would have seen increased degradation of normal Hmg2 but still no degradation of normally stable Hmg2p-related substrates, such as S215A-Hmg2p-GFP. This idea is an incorporation of the allosteric model illustrated in Figure 12.

Future Directions/Suggestions

The next step in answering the question of Hrd1p substrate recognition is to look for Hrd1p mutants that are either globally overactive or ERAD-M or ERAD-L specific. Instead of screening for increased activity toward a single ERAD substrate, we may utilize two distinct substrates and look for the lowering of steady-state levels of both substrates. In order to do this, we would need to first create a fusion protein using another ERAD substrate and a fluorescent protein other than GFP. Using two different channels, we would be able to screen for candidates that show decreased fluorescence for both fluorescent reporters. Another way to look for globally active Hrd1p mutants is

to take advantage of the toxicity of overexpressing Hrd1p in our lab strain. This can be used in combination with an optical reporter, such as S215A-Hmg2p-GFP. Overactive candidates will show decreased fluorescence and temperature sensitivity.

The resulting mutants may have single point mutations or multiple mutations. Whichever the case, we will be able to better understand the layout of the Hrd1p transmembrane domain in regards to substrate selectivity. Perhaps several substrate-specific point mutations expressed together are necessary to produce a globally overactive Hrd1p or a single mutation will be sufficient to create a globally overactive Hrd1p. There is also a possibility that such mutants do not even exist and this will also help us better understand the mechanism of substrate recognition by Hrd1p. Unraveling this mystery of substrate recognition will allow us to better understand ER quality control.

Figures

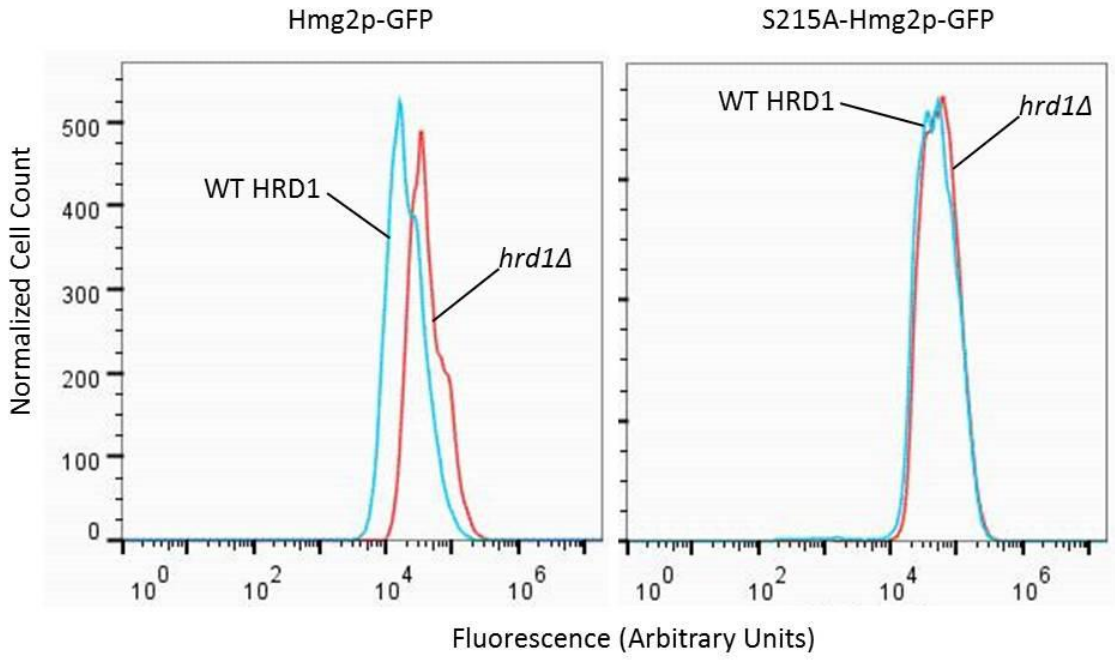


Figure 1. Stability of S215A-Hmg2p-GFP

S215A-Hmg2p-GFP is completely stable in the presence of wild-type Hrd1p and was used as the screening substrate to isolate overactive Hrd1p mutants

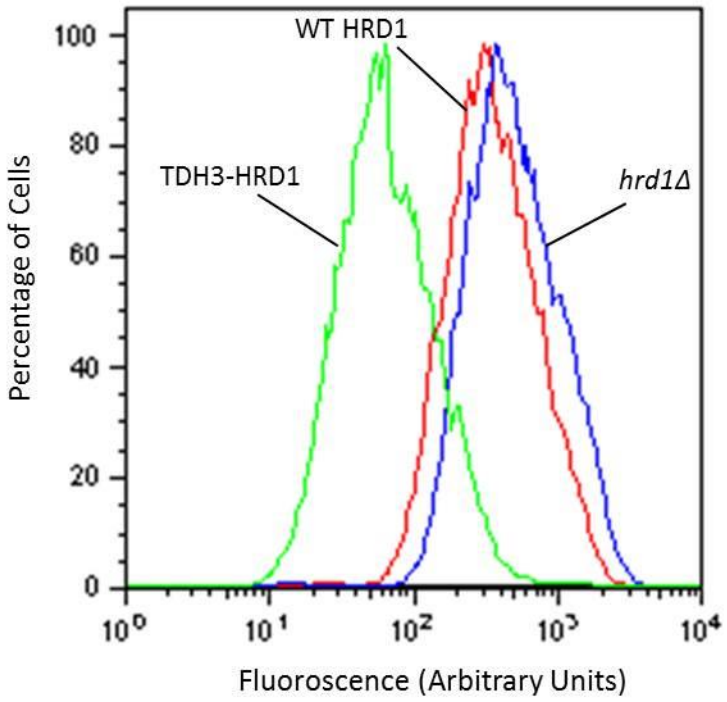


Figure 2. Expected phenotype of overactive Hrd1p mutants

Overactive Hrd1p mutants are expected to produce steady-state levels of S215A-Hmg2p-GFP comparable to overexpression of native Hrd1p.

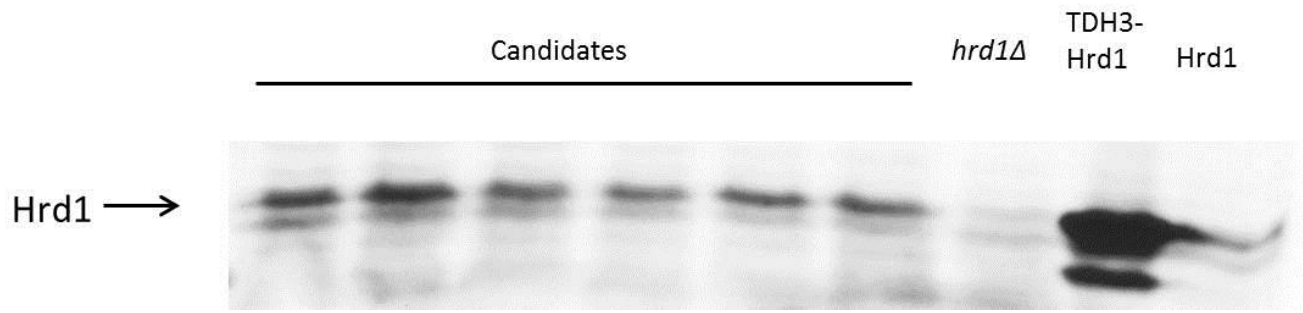


Figure 3. Hrd1p levels of isolated candidates

Steady-state levels of Hrd1p in overactive candidates is measured to ensure that the phenotype is not due to overexpression of Hrd1p. Log phase cultures of each candidate was harvested and the samples analyzed by SDS-PAGE and Western Blot. To blot for Hrd1p, anti-Hrd1 was prepared from Hrd1p antiserum adsorbed against a *hrd1Δ* blot. Overexpression of Hrd1p showed a significant increase in Hrd1p levels as detected by Western blot. The isolated candidates had Hrd1p levels comparable to native Hrd1p. The total protein level in each lane was verified to be equal by India ink staining (data not shown).



Figure 4. Mutation mapping of the transmembrane domain of overactive Hrd1p mutants

This is a comparison of all the mutations found in the isolated overactive Hrd1p mutants. As noted, there are ten amino acid positions that are mutated in more than one candidate. These mutations are highlighted in gray. (TM=transmembrane span; C=cytosolic loop; L=lumenal loop)

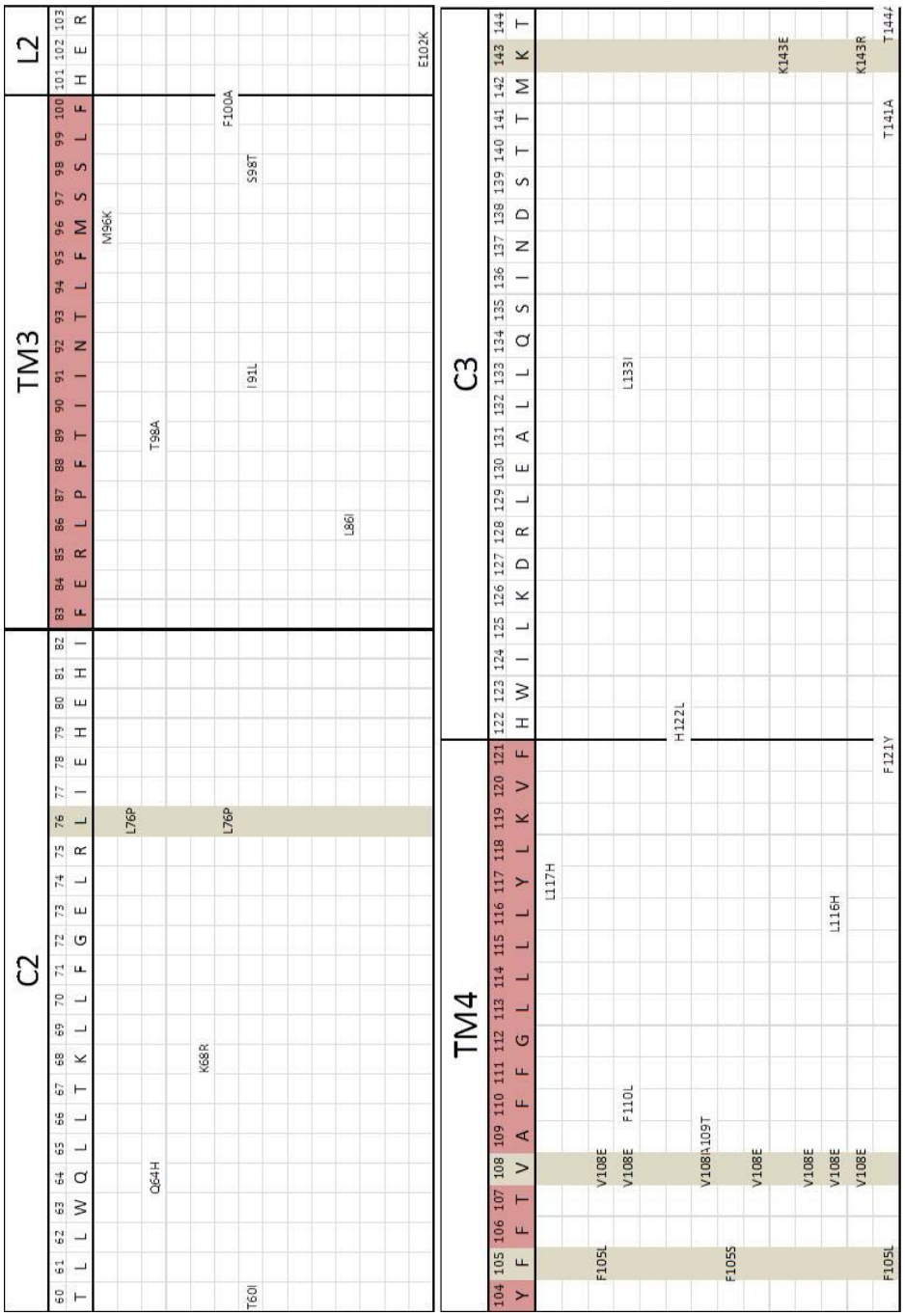


Figure 4 continued.

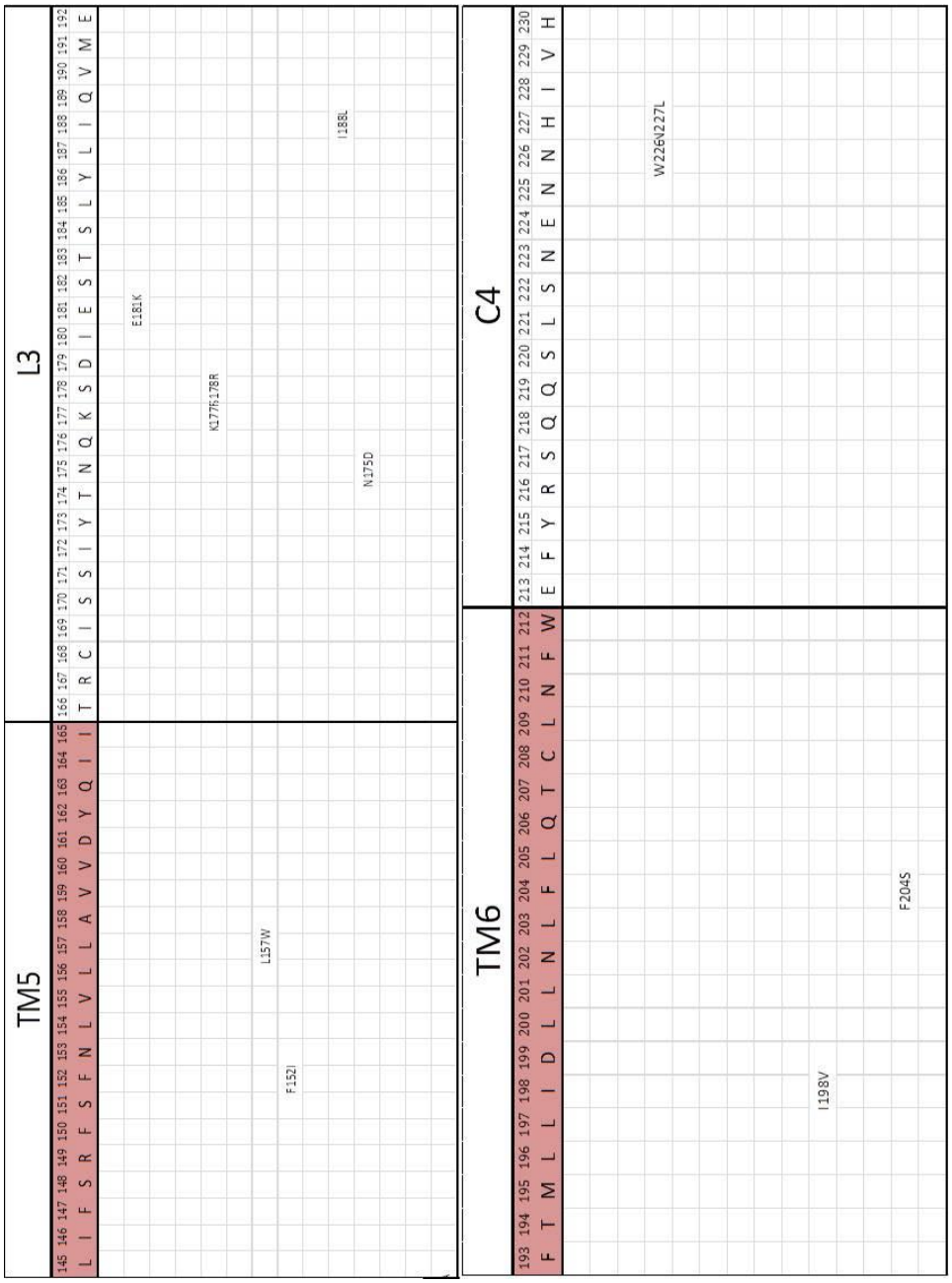


Figure 4 continued.

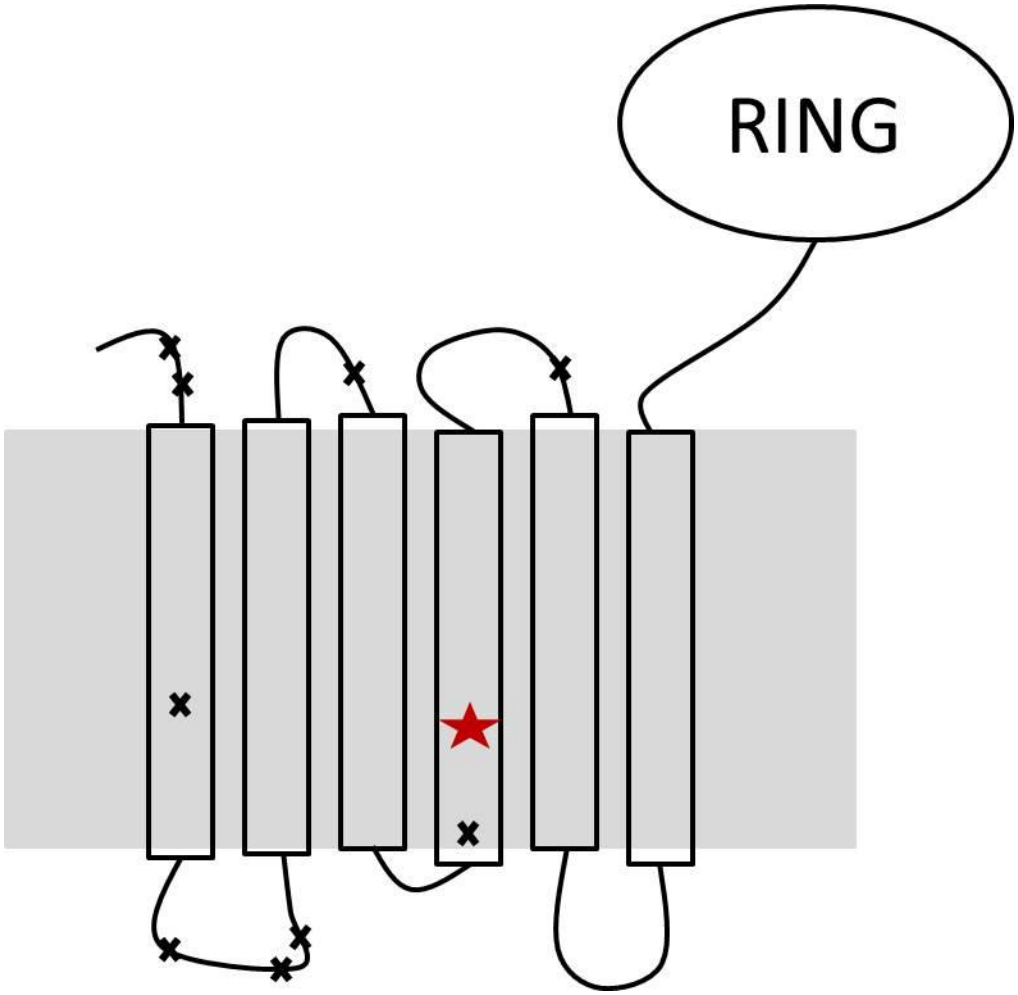


Figure 5. Depiction of overactive mutation

Hrd1p with the predicted transmembrane segments. The position of the overactive mutation, V108E, is labeled with a red star. The positions of other mutations found in multiple candidates are labeled with X.

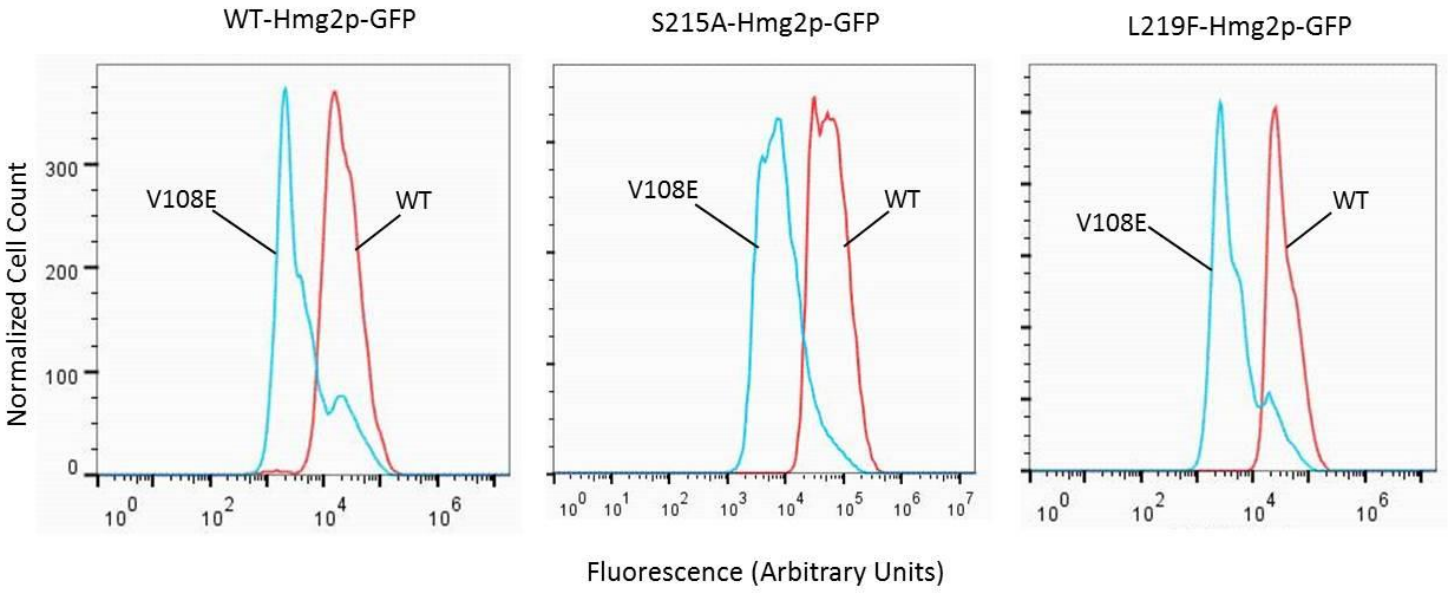


Figure 6. Degradation of Hmg2p SSD mutants by V108E-Hrd1p

Steady-state levels of Hmg2p-GFP and its stable variants S215A and L219F as measured by flow cytometry. As determined by the optical screen, V108E-Hrd1p decreases the steady-state levels of S215A- Hmg2p-GFP compared to WT-Hrd1p. Similarly, V108E also decreases the steady-state levels of WT-Hmg2p-GFP and L219F-Hmg2p-GFP, another stable SSD mutant. WT=wild-type Hrd1; V108E=V108E-Hrd1p.

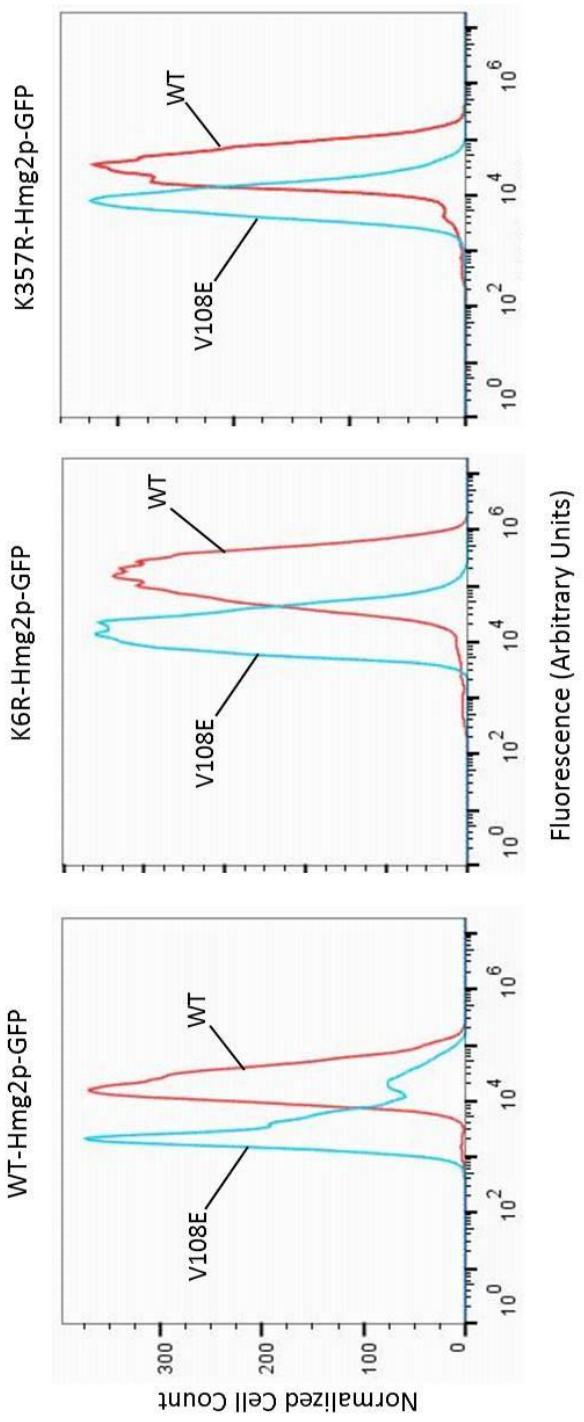


Figure 7. Degradation of Hmg2p ubiquitination mutants by V108E-Hrd1p

Steady-state levels of normal Hmg2p-GFP and its stable variants K6R and K357R as measured by flow cytometry. WT=wild-type Hrd1; V108E=V108E-Hrd1p.

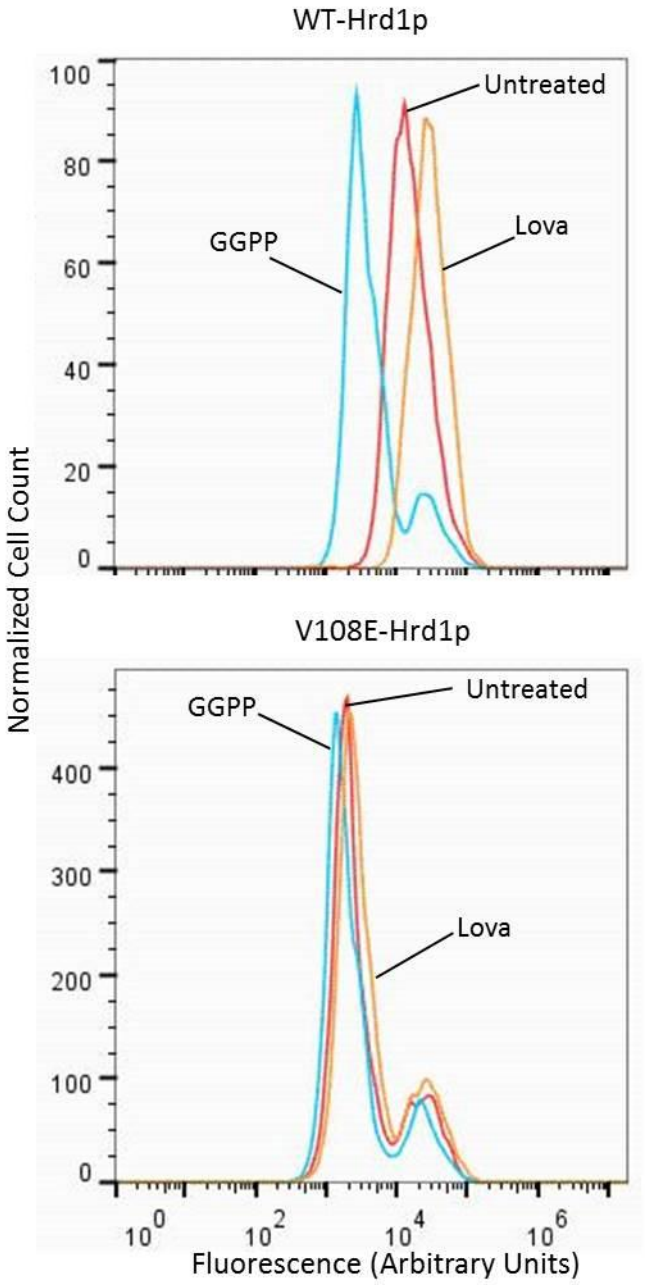


Figure 8. Degradation of Hmg2p by V108E-Hrd1p is unregulated

Lovastatin and GGPP were added to log phase cultures and incubated for four hours at 30˚C. A 4-hour treatment with lovastatin or GGPP produced the maximum effect that can be seen by flow cytometer.

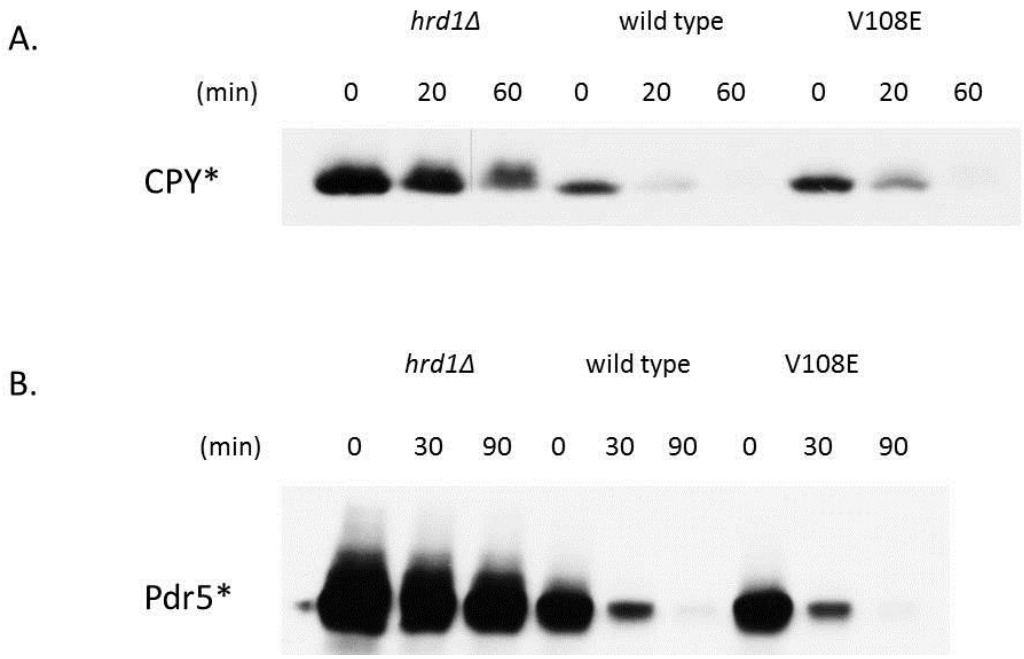


Figure 9. Degradation of other ERAD substrates by V108E-Hrd1p

Cycloheximide chase assay of CPY* (A) and Pdr5* (B) in the presence of V108E-Hrd1p. Each isogenic *hrd1Δ* strain was transformed with empty vector, wild-type HRD1, or V108E-HRD1. Log phase cultures were grown and cells were lysed for the 0 min time-point. After adding cycloheximide, cells were lysed at the next stated time-points and analyzed by SDS-PAGE and Western blot. The total protein level in each lane was verified to be equal by India ink staining (data not shown).

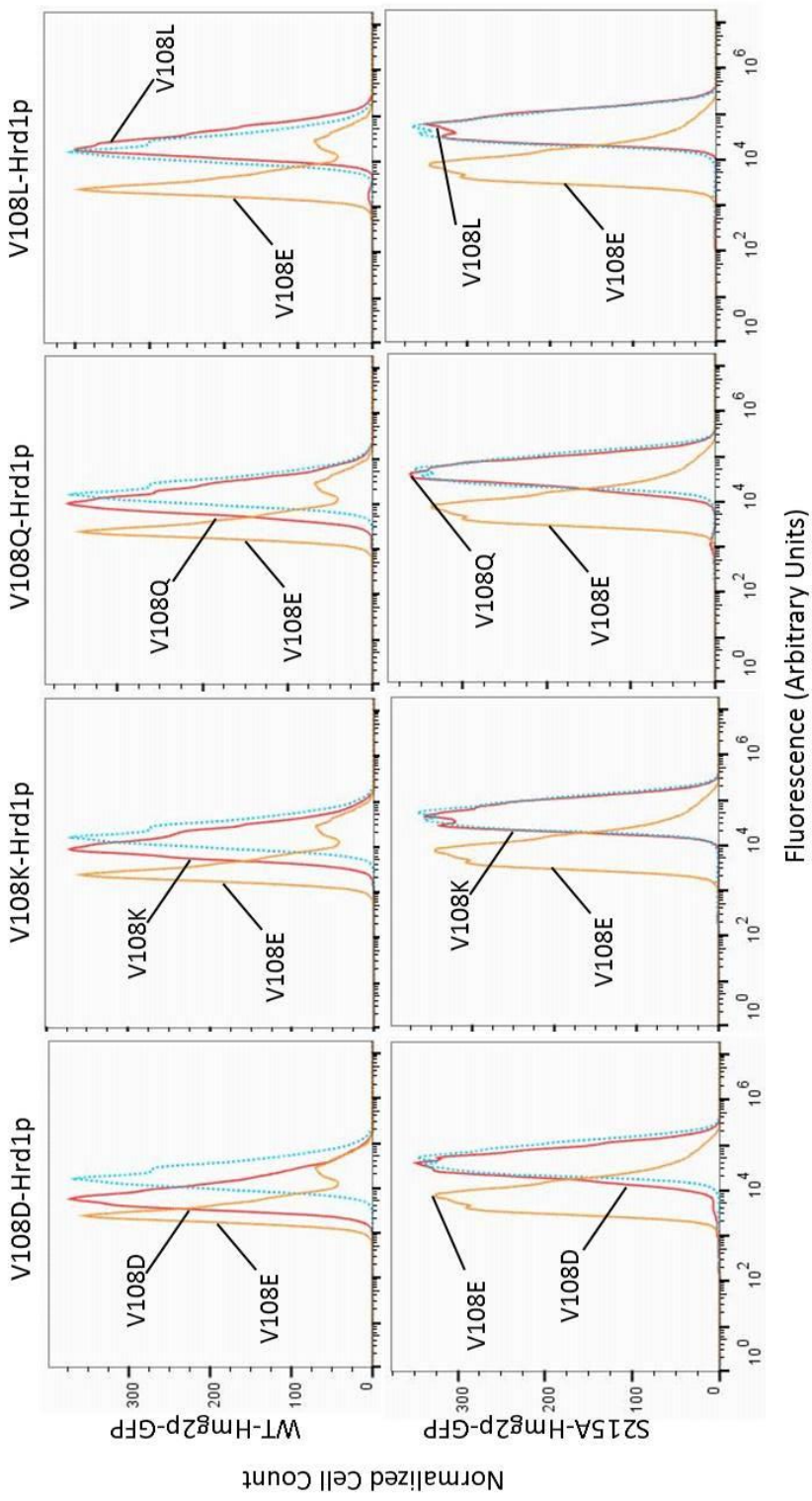


Figure 10. Degradation of Hmg2p and S215A by different V108 mutants

V108E shows the strongest phenotype in both WT-Hmg2p and S215A. The rest of the V108 mutants show weak phenotypes and are not much better than WT-Hrd1p at degrading Hmg2p and S215A, if at all. However, it is also important to note that none of the V108 mutants have decreased ability to degrade Hmg2p and S215A. Dotted blue is normal Hrd1p; red is the V108 mutant being tested; orange is V108E.

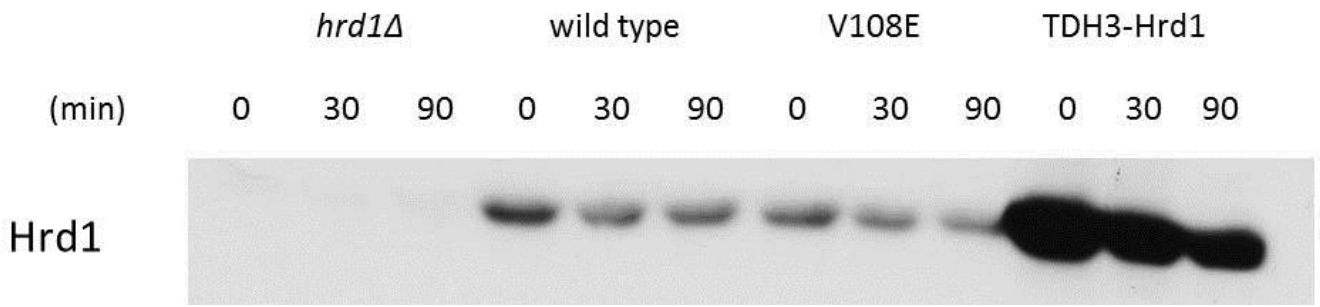


Figure 11. V108E-Hrd1p does not have altered stability

Cycloheximide chase assay of Hrd1p. Each isogenic *hrd1Δ* strain was transformed with empty vector, wild-type HRD1, V108E-HRD1, or TDH3-HRD1. The samples were analyzed by SDS-PAGE and Western Blot with Hrd1p antiserum as prepared in Figure 2. The total protein level in each lane was verified to be equal by India ink staining (data not shown).

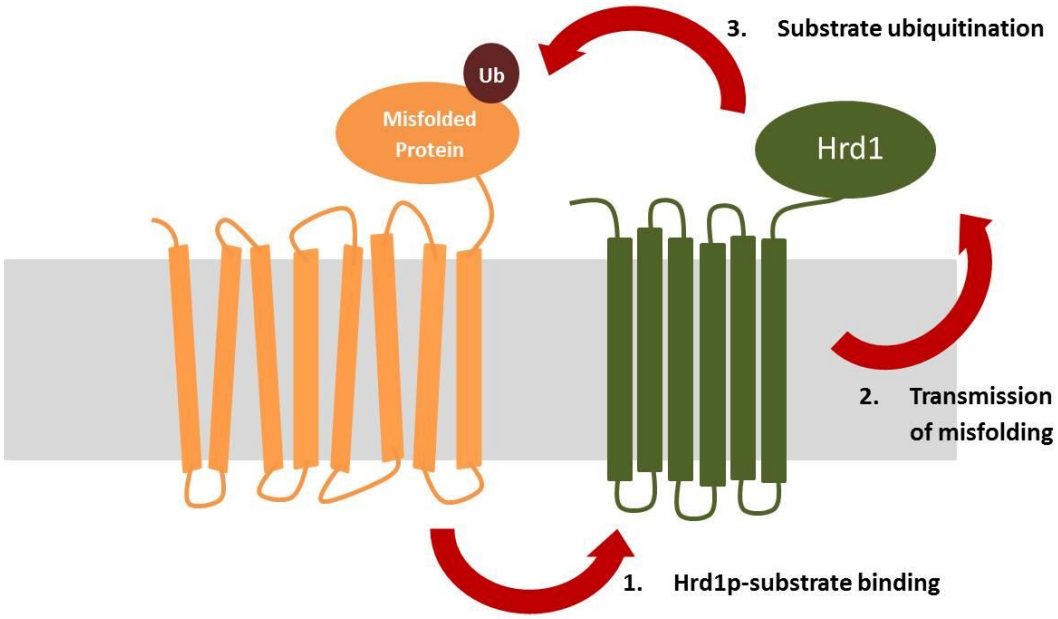


Figure 12. Allosteric model of Hrd1p substrate recognition

Hrd1p first interacts with misfolded substrate, allowing transmission of information about the substrate's folding from the transmembrane domain to the RING domain. This is then followed by substrate ubiquitination. V108E-Hrd1p is hypothesized to lock Hrd1p in a "recognition conformation" for Hmg2p-related substrates.

Materials and Methods

Yeast and Bacterial Strains

Yeast strains were grown at 30˚C in minimal media supplemented with amino acids and 2% dextrose unless otherwise noted. *Escherichia coli* DH5α were grown at 37˚C in LB media with ampicillin.

Plasmid Construction and DNA Methods

The PCR mutagenized plasmid library was constructed by *in vivo* homologous recombination in the screening strain RHY 8856. The insert is the transmembrane domain of Hrd1p and was created using error-prone PCR. The vector was created by restriction digest of pRH 2556 using Sal1 and Nco1 to cut out the Hrd1p transmembrane domain. These two pieces of DNA were co-transformed into yeast using the LiOAc method (Ito et al. 1983). The amount of insert and vector used was a 3 (insert): 1 (vector) molar ratio. Oligos for the PCR mutagenesis were designed so that the insert and vector have about 150 base pairs overlapping on each side during co-transformation.

The V108 variants were made using the splicing by overlap elongation (SOEing) PCR technique (Horton et al. 1989) and recombined *in vivo* in yeast as described above. In the first round of PCR, separate reaction mixtures containing Primers A and B and Primers C and D created two overlapping fragments containing the V108 mutations. In the second round of PCR, Primers A and D were used to create a long fragment from the two overlapping fragments resulting in the insert used in recombination cloning as described above.

Hrd1p Plasmid Mutagenesis

Chemical mutagenesis of Hrd1p with hydroxylamine was done by incubating the 10ug of Hrd1p plasmid (pRH2556) in 500uL of 1M hydroxylamine hydrochloride (Sigma-Aldrich) for 20 hours at 37˚C. The DNA was then purified by ethanol precipitation and used directly for transforming yeast.

PCR mutagenesis of Hrd1p was used to target the Hrd1p transmembrane domain. *Taq* polymerase from NEB was used with the provided ThermoPol buffer and supplemented with additional MgCl2 and MnCl2. The mutagenic mixture contained a total of 7 mM MgCl2, 0.5 mM MnCl2, 5 units of *Taq* polymerase, 0.2 mM dGTP and dATP, 1 mM dCTP and dTTP, 20 fmoles of template DNA, and 30 pmoles of each primer. The reaction ran 30 cycles of 94˚C for 1 min, 45˚C for 1 min, and 72˚C for 1 min. These alterations were adapted from Cadwell and Joyce's paper on PCR mutagenesis to promote errors during PCR (Cadwell and Joyce 1992).

Degradation assays

Cyclohexamide (CHX) chase degradation assays were performed using overnight yeast cultures grown in synthetic complete media minus uracil and histidine. CHX was used at 50 μg/ml (Sigma-Aldrich) and added just after the 0 min time point for CPY* and Pdr5* CHX chase assays. Cells taken at the indicated time points were resuspended in SUME lysis buffer (1% SDS, 8M Urea, 10mM MOPS pH 6.8, 10mM EDTA) with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 100 mM leupeptin hemisulfate, 76 mM pepstatin A, and 142 mM TPCK) and lysed by vortexing with 0.5 mm acid-washed glass beads for 4 minutes. An equal volume of 2X urea sample buffer (75mM MOPS pH 6.8, 4% SDS, 200mM DTT, 0.2mg/ml

bromophenol blue, 8M urea) was then added and the sample was incubated at 55˚C for 5 minutes. Proteins were resolved by 8% SDS-PAGE and transferred to nitrocellulose. 5% nonfat-dried milk in TBSHT (10 mm Tris, pH 8.0, 150 mm NaCl, 0.45% Tween 20) was used to block and 2% nonfat-dried milk in TBSHT was used in all antibody incubations except for Hrd1p antiserum. HA-Pdr5* and CPY*-HA were detected using Anti-hemagglutinin (HA) obtained as an ascites fluid from Covance, Inc. HRP-conjugated goat anti-mouse recognized the primary antibody (Jackson ImmunoResearch, West Grove, PA). Anti-Hrd1 was generated by incubating 2.5 μL of 2.7mg/mL affinity-purified anti-Hrd1 overnight at 4˚C in 10mL TBSHT with a *hrd1Δ* blot. Horseradish Peroxidase (HRP)-conjugated goat anti-rabbit recognized the Hrd1 antibody (Zymed). Western Lightning ECLplus reagents were used for immunodetection (PerkinElmer Life Sciences).

Flow cyometry

The GFP fluorescence of cells was measured with a BD Biosciences Accuri C6 flow cytometer. Cultures were grown in selective media overnight to ~0.2 OD and following the treatment of drugs, the fluorescence of 10,000 cells in each sample was measured. Lovastatin (25 μg/ml) and GGPP (22 μM) were added separately to cultures and incubated for four hours at 30˚C. For steady-state measurements, cultures were grown to ~0.2 OD overnight and without any additional treatment, the fluorescence of each sample was measured. FlowJo software was used to analyze the data and plot fluorescence vs. cell count histograms.

Table 1. Plasmids

Plasmid	**Gene Expressed**
pRH311	*YIp TRP1*
pRH313	*YIp URA3*
pRH314	*YCp HIS3*
pRH469	*YIp URA3 pTDH3-HMG2-GFP*
pRH507	*YIp TRP1 pHRD1*
pRH808	*YIp TRP1 pTDH3-HRD1*
pRH997	*YIp URA3 pTDH3-K6R-HMG2-GFP*
pRH1377	*YCp URA3 pCPY*-HA*
pRH1692	*YIp URA3 pTDH3-K357R-HMG2-GFP*
pRH2177	*YIp URA3 pTDH3-S215A-1myc-HMG2-GFP*
pRH2183	*YIp URA3 pTDH3-L219F-1myc-HMG2-GFP*
pRH2556	*YCp HIS3 pHRD1*
pRH2621	*YCp URA3 pHRD1 V108E*
pRH2627	*YCp LEU2 pHA-PDR5**
pRH2678	*YCp URA3 pHRD1 V108D*
pRH2679	*YCp URA3 pHRD1 V108K*
pRH2680	*YCp URA3 pHRD1 V108Q*
pRH2681	*YCp URA3 pHRD1 V108L*
pRH2682	*YIp TRP1 pHRD1 V108E*

Table 2. Strains

Strain	Genotype
RHY8856	*Matα ade2-1 ura3-1::URA3::pTDH3-S215A-1MYC-HMG2-GFP MET2 LYS2 trp1-1 leu2-3,112 his3-11,15 hrd1Δ::KanMX*
RHY8937	*Matα ade2-1 ura3-1::URA3::pTDH3-S215A-1MYC-HMG2-GFP MET2 LYS2 trp1-1 leu2-3,112 his3-11,15 hrd1Δ::KanMX HIS3::HRD1*
RHY8937	*Matα ade2-1 ura3-1::URA3::pTDH3-S215A-1MYC-HMG2-GFP MET2 LYS2 trp1-1 leu2-3,112 his3-11,15 hrd1Δ::KanMX TRP1::pTDH3-HRD1*
RHY9281	*Matα ade2-1 ura3-1::URA3::pTDH3-K6R-HMG2-GFP MET2 LYS2 trp1-1 leu2-3,112 his3-11,15 hrd1Δ::KanMX HIS3*
RHY9282	*Matα ade2-1 ura3-1::URA3::pTDH3-K6R-HMG2-GFP MET2 LYS2 trp1-1 leu2-3,112 his3-11,15 hrd1Δ::KanMX HIS3::HRD1*
RHY9284	*Matα ade2-1 ura3-1::URA3::pTDH3-K6R-HMG2-GFP MET2 LYS2 trp1-1 leu2-3,112 his3-11,15 hrd1Δ::KanMX HIS3::V108E-HRD1*
RHY9285	*Matα ade2-1 ura3-1::URA3::pTDH3-K357R-HMG2-GFP MET2 LYS2 trp1-1 leu2-3,112 his3-11,15 hrd1Δ::KanMX HIS3*
RHY9286	*Matα ade2-1 ura3-1::URA3::pTDH3-K357R-HMG2-GFP MET2 LYS2 trp1-1 leu2-3,112 his3-11,15 hrd1Δ::KanMX HIS3::HRD1*
RHY9287	*Matα ade2-1 ura3-1::URA3::pTDH3-K357R-HMG2-GFP MET2 LYS2 trp1-1 leu2-3,112 his3-11,15 hrd1Δ::KanMX HIS3::V108E-HRD1*
RHY9347	*Matα ade2-1 ura3-1::URA3::pTDH3-S215A-1MYC-HMG2-GFP MET2 LYS2 trp1-1 leu2-3,112 his3-11,15 hrd1Δ::KanMX HIS3::V108E-HRD1*
RHY9348	*Matα ade2-1 ura3-1::URA3::pTDH3-S215A-1MYC-HMG2-GFP MET2 LYS2 trp1-1 leu2-3,112 his3-11,15 hrd1Δ::KanMX HIS3::V108D-HRD1*
RHY9349	*Matα ade2-1 ura3-1::URA3::pTDH3-S215A-1MYC-HMG2-GFP MET2 LYS2 trp1-1 leu2-3,112 his3-11,15 hrd1Δ::KanMX HIS3::V108K-HRD1*
RHY9350	*Matα ade2-1 ura3-1::URA3::pTDH3-S215A-1MYC-HMG2-GFP MET2 LYS2 trp1-1 leu2-3,112 his3-11,15 hrd1Δ::KanMX HIS3::V108Q-HRD1*
RHY9351	*Matα ade2-1 ura3-1::URA3::pTDH3-S215A-1MYC-HMG2-GFP MET2 LYS2 trp1-1 leu2-3,112 his3-11,15 hrd1Δ::KanMX HIS3::V108L-HRD1*

RHY9352	*Matα ade2-101 ura3-52::URA3::HMG2-GFP met2 lys2-801 TRP1 LEU2 his3Δ200 hrd1Δ::KanMX HIS3::HRD1*
RHY9353	*Matα ade2-101 ura3-52::URA3::HMG2-GFP met2 lys2-801 TRP1 LEU2 his3Δ200 hrd1Δ::KanMX HIS3::V108E-HRD1*
RHY9354	*Matα ade2-101 ura3-52::URA3::HMG2-GFP met2 lys2-801 TRP1 LEU2 his3Δ200 hrd1Δ::KanMX HIS3::V108D-HRD1*
RHY9355	*Matα ade2-101 ura3-52::URA3::HMG2-GFP met2 lys2-801 TRP1 LEU2 his3Δ200 hrd1Δ::KanMX HIS3::V108K-HRD1*
RHY9356	*Matα ade2-101 ura3-52::URA3::HMG2-GFP met2 lys2-801 TRP1 LEU2 his3Δ200 hrd1Δ::KanMX HIS3::V108Q-HRD1*
RHY9357	*Matα ade2-101 ura3-52::URA3::HMG2-GFP met2 lys2-801 TRP1 LEU2 his3Δ200 hrd1Δ::KanMX HIS3::V108L-HRD1*
RHY9345	*Mata ade2-101 ura3-52::URA3::pTDH3-L219F-1MYC-HMG2-GFP met2 lys2-801 trp1::hisG::TRP1 leu2Δ his3Δ200 HIS3::HRD1*
RHY9346	*Mata ade2-101 ura3-52::URA3::pTDH3-L219F-1MYC-HMG2-GFP met2 lys2-801 trp1::hisG::TRP1 leu2Δ his3Δ200 HIS3::V108E-HRD1*
RHY9359	*Matα ade2 ura3-52 met2 lys2-801 trp1::hisG leu2Δ his3Δ200 hrd1Δ::KanMX URA3::CPY*-HA HIS3*
RHY9360	*Matα ade2 ura3-52 met2 lys2-801 trp1::hisG leu2Δ his3Δ200 hrd1Δ::KanMX URA3::CPY*-HA HIS3::HRD1*
RHY9361	*Matα ade2 ura3-52 met2 lys2-801 trp1::hisG leu2Δ his3Δ200 hrd1Δ::KanMX URA3::CPY*-HA HIS3::V108E-HRD1*
RHY9363	*Matα ade2 ura3-52 met2 lys2-801 trp1::hisG leu2Δ his3Δ200 hrd1Δ::KanMX LEU2::HA-Pdr5* HIS3*
RHY9364	*Matα ade2 ura3-52 met2 lys2-801 trp1::hisG leu2Δ his3Δ200 hrd1Δ::KanMX LEU2::HA-Pdr5* HIS3::HRD1*
RHY9365	*Matα ade2 ura3-52 met2 lys2-801 trp1::hisG leu2Δ his3Δ200 hrd1Δ::KanMX LEU2::HA-Pdr5* HIS3::V108E-HRD1*

Table 3. Oligos

Name	Description	Sequence
oRH4308	Forward primer for PCR mutagenesis	GACACCTACAAAAGTACACTG
oRH4309	Reverse primer for PCR mutagenesis	CCTCCAGGCCGGTAAATTG
oRH4333	Primer A for V108 SOEing reaction	GTTGAACTTTGCATACCCGG
oRH4334	Primer D for V108 SOEing reaction	CATAGACAAATGAAGTGCCG
oRH4378	Primer B for V108K	CCAAAAAATGCCTTTGTGAAAAAATACC
oRH4379	Primer C for V108K	GGTATTTTTTCACAAAGGCATTTTTTGG
oRH4380	Primer B for V108L	CCAAAAAATGCCAATGTGAAAAAATACC
oRH4381	Primer C for V108L	GGTATTTTTTCACATTGGCATTTTTTGG
oRH4382	Primer B for V108D	CCAAAAAATGCATCTGTGAAAAAATACC
oRH4383	Primer C for V108D	GGTATTTTTTCACAGATGCATTTTTTGG
oRH4384	Primer B for V108Q	CCAAAAAATGCTTGTGTGAAAAAATACC
oRH4385	Primer C for V108Q	GGTATTTTTTCACACAAGCATTTTTTGG

References

Bays, N.W., Gardner, R.G., Seelig, L.P., Joazeiro, C.A., and Hampton, R.Y. 2001. Hrd1p/Der3p is a membrane-anchored ubiquitin ligase required for ER-associated degradation. *Nature cell biology* **3**(1): 24-29.

Brodsky, J.L. 2012. Cleaning up: ER-associated degradation to the rescue. *Cell* **151**(6): 1163-1167.

Cadwell, R.C., and Joyce, G.F. 1992. Randomization of genes by PCR mutagenesis. *Genome Research* **2**: 29-30.

Carroll, S.M., and Hampton, R.Y. 2010. Usa1p is required for optimal function and regulation of the Hrd1p endoplasmic reticulum-associated degradation ubiquitin ligase. *The Journal of biological chemistry* **285**(8): 5146-5156

Carvalho, P., Goder, V., and Rapoport, T.A. 2006. Distinct ubiquitin-ligase complexes define convergent pathways for the degradation of ER proteins. *Cell* **126**(2): 361-373.

Cronin, S.R., and Hampton, R.Y. 1999. Measuring protein degradation with green fluorescent protein. *Methods in Enzymology* **302**: 58-73.

Deak, P.M. and Wolf, D.H. 2001. Membrane topology and function of Der3/Hrd1p as a ubiquitin-protein ligase (E3) involved in endoplasmic reticulum degradation. *The Journal of biological chemistry* **276**(14): 10663-10669.

Denic, V., Quan, E.M., and Weissman, J.S. 2006. A luminal surveillance complex that selects misfolded glycoproteins for ER-associated degradation. *Cell* **126**(2): 349-359.

Gardner, R.G., Shearer, A.G., and Hampton, R.Y. 2001. In vivo action of the HRD ubiquitin ligase complex: mechanisms of endoplasmic reticulum quality control and sterol regulation. *Molecular and cellular biology* **21**(13): 4276-4291.

Gardner, R.G., and Hampton, R.Y. 1999. A “distributed degron” allows regulated entry into the ER degradation pathway. *EMBO J* **18**(21): 5994-6004.

Garza, R.M., Tran, P.N, and Hampton, R.Y., 2009. Geranylgeranyl Pyrophosphate is a potent regulator of HRD-dependent 3-hydroxy-3-methylglutaryl-CoA reductase degradation in yeast. *The Journal of biological chemistry* **284**(51): 35368-35380.

Hampton, R.Y. 2002a. ER-associated degradation in protein quality control and cellular regulation. *Current Opinion in Cell Biology* **14**(4): 476-482.

Hampton, R.Y. 2002b. Proteolysis and sterol regulation. *Annual review of cell and developmental biology* **18**: 345-378

Hampton, R.Y., Gardner, R.G., and Rine, J. 1996a. Role of 26S proteasome and HRD genes in the degradation of 3-hydroxy-3-methylglutaryl-CoA reductase, an integral endoplasmic reticulum membrane protein. *Molecular biology of the cell* **7**(12): 2029-2044.

Hampton, R.Y., and Garza, R.M. 2009. Protein quality control as a strategy for cellular regulation: lessons from ubiquitin-mediated regulation of the sterol pathway. *Chemical Reviews* **109**(4): 1561-1574.

Hampton, R.Y., Koning, A., Wright, R., and Rine, J. 1996b. In vivo examination of membrane protein localization and degradation with green fluorescent protein. *Proceedings of the National Academy of Sciences of the United States of America* **93**(2): 828-833.

Hampton, R.Y. and Rine, J. 1994. Regulated degradation of HMG-CoA reductase, an integral membrane protein of the endoplasmic reticulum, in yeast. *The Journal of cell biology* **125**(2): 299-312.

Horton, R.M., Hunt, H.D., Ho, S.N., Pullen, J.K., and Pease, L.R. 1989. Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene* **77**(1): 61-68.

Ito, H., Fukuda, Y., Murata, K., and Kimura, A. 1983. Transformation of intact yeast cells treated with alkali cations. *Journal of bacteriology* **153**(1): 163-168.

Kuwabara, P.E., and Labouesse, M. 2002. The sterol-sensing domain: multiple families, a unique role? *Trends in Genetics* **18**(4): 193-201.

Plemper, R.K., Egner, R., Kuchler, K., and Wolf, D.H. 1998. Endoplasmic reticulum degradation of a mutated ATP-binding cassette transporter Pdr5 proceeds in a concerted action of Sec61 and the proteasome. *The Journal of biological chemistry* **273**(49): 32848-32856.

Sato, B.K., Schulz, D., Do, P.H., and Hampton, R.Y. 2009. Misfolded membrane proteins are specifically recognized by the transmembrane domain of the Hrd1p ubiquitin ligase. *Molecular Cell* **34**(2): 212–222.

Sommer, T., and Wolf, D.H. 1997. Endoplasmic reticulum degradation: reverse protein flow of no return. *FASEB J* **11**(14): 1227-1233.

Swanson, R., Locher, M., and Hochstrasser, M. 2001. A conserved ubiquitin ligase of the nuclear envelope/endoplasmic reticulum that functions in both ER-associated and Matalpha2 repressor degradation. *Genes & development* **15**(20): 2660-2674.

Theesfeld, C.L., Pourmand D., Davis T., Garza, R.M., and Hampton, R.Y. 2011. The sterol-sensing domain (SSD) directly mediates signal-regulated endoplasmic reticulum-associated degradation (ERAD) of 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase isozyme Hmg2. *The Journal of biological chemistry* **286**(30): 26298-307

Wolf, D.H., and Schäfer A. 2005. CPY* and the power of yeast genetics in the elucidation of quality control and associated protein degradation of the endoplasmic reticulum. *Current topics in microbiology and immunology* **300**: 41-56.