UNIVERSITY OF CALIFORNIA, SAN DIEGO

# Functional Analysis of the Ubiquitin Ligase Hrd1p with the Ubiquitin-Conjugating Enzyme Ubc7p

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

Doctor of Philosophy

in

Biology

by

Omar Al-Kasim Bazirgan

Committee in charge:

Professor Randolph Y. Hampton, Chair
Professor Scott D. Emr
Professor Elizabeth A. Komives
Professor Maho Niwa
Professor Michael P. Yaffe

2007

The Dissertation of Omar Al-Kasim Bazirgan is approved, and it is acceptable in quality and form for publication on microfilm:

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Chair

University of California, San Diego

2007

*This work is dedicated to, and was made possible by,*

*the many extraordinary teachers and mentors I have been privileged to learn from,*

*foremost among whom are my mother and father.*

Happy is he who gets to know the reasons for things.

*Virgil*

The joy of discovery is certainly the liveliest that the mind of man can ever feel.

*Claude Bernard*

I am among those who think that science has great beauty. A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena, which impress him like a fairy tale.

*Marie Curie*

Science is not about control. It is about cultivating a perpetual condition of wonder in the face of something that forever grows one step richer and subtler than our latest theory about it. It is about reverence, not mastery.

*Richard Powers*

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opportunities a priority. They taught me to cherish learning and knowledge by example, and embraced and encouraged my interests no matter where they led. Most significantly, they spared me any pressure to please them by constantly showering me with love and affection. They told me I was capable of anything, and that they would be pleased with me no matter what I did. For these many gifts, and thousands more, I will always be grateful.

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Chapter 4 is a manuscript in preparation that will be submitted for publication.

# Vita

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|---|---|
| 1994 | International Baccalaureate Diploma, Mira Loma High School |
| 1998 | Bachelor of Science, University of California, Davis |
| 1998-1999 | Post-graduate Researcher, Department of Molecular and Cellular Biology, University of California, Davis |
| 2007 | Doctor of Philosophy, University of California, San Diego |

## PUBLICATIONS

**Bazirgan O.A.**, Garza R.M., Hampton R.Y. Determinants of RING-E2 fidelity for Hrd1p, a membrane-anchored ubiquitin ligase. Journal of Biological Chemistry. Oct. 11 2006; PMID: 17035235

**Bazirgan O.A.**, Hampton R.Y. Cdc48-Ufd2-Rad23: the road less ubiquitinated? Nature Cell Biology. 2005 Mar;7(3):207-9. PMID: 15738968

Srayko M, Buster D.W., **Bazirgan O.A.**, McNally F.J., Mains P.E. MEI-1/MEI-2 katanin-like microtubule severing activity is required for Caenorhabditis elegans meiosis. Genes and Development. 2000 May 1;14(9):1072-84. PMID: 10809666

McNally K.P., **Bazirgan O.A.**, McNally F.J. Two domains of p80 katanin regulate microtubule severing and spindle pole targeting by p60 katanin. Journal of Cell Science. 2000 May;113 ( Pt 9):1623-33. PMID: 10751153

ABSTRACT OF THE DISSERTATION

Functional Analysis of the Ubiquitin Ligase Hrd1p with
the Ubiquitin-Conjugating Enzyme Ubc7p.

by

Omar Al-Kasim Bazirgan

Doctor of Philosophy in Biology

University of California, San Diego, 2007

Professor Randolph Y. Hampton, Chair

Ubiquitin is a covalent protein tag that alters the stability or behavior of a growing list of proteins. Covalent attachment of ubiquitin to target proteins occurs through a cascade of enzymes: Ubiquitin is charged by a ubiquitin-activating enzyme (E1), and transferred to a ubiquitin-conjugating enzyme (E2). Then, transfer of ubiquitin from E2 to a target protein is brokered by a ubiquitin ligase (E3). A critical aspect of E3 function is the selection of a particular E2 to accomplish ubiquitination of a substrate. We examined the requirements for correct E2-E3 specificity in the RING-

H2 ubiquitin ligase Hrd1p, an ER-localized protein known to use primarily Ubc7p for its function. Versions of Hrd1p containing the RING motif from homologous E3s were unable to carry out Hrd1p function, revealing a requirement for the specific Hrd1p RING motif *in vivo*. An *in vitro* assay revealed that these RING motifs were sufficient to function as ubiquitin ligases, but that they did not display the E2 specificity predicted from *in vivo* results. We further refined the *in vitro* assay of Hrd1p function by demanding not only ubiquitin ligase activity, but also specific activity that recapitulated both the E2 specificity and RING selectivity observed *in vivo*. Doing so revealed that correct E2 engagement by Hrd1p required the presence of portions of the Hrd1p soluble cytoplasmic domain outside the RING motif, the placement of the Hrd1p ubiquitin ligase in the ER membrane, and presentation of Ubc7p in the cytosolic context. We confirmed that these conditions supported the ubiquitination of Hrd1p itself, and the transfer of ubiquitin to the prototype substrate Hmg2p-GFP, validating Hrd1p self-ubiquitination as a viable assay of ligase function. During these studies we observed enhanced Ubc7p-dependent ubiquitination in the presence of soluble Cue1p, which interacts with Ubc7p *in vivo*. Soluble Cue1p promoted the transfer of ubiquitin from Ubc7p to other ubiquitin molecules in solution. We also observed that this stimulation of Ubc7p by Cue1p and the anchoring of Ubc7p to the ER membrane by Cue1p were both necessary for Ubc7p function *in vivo*. Ubc7p activation by Cue1p was observed at the ER and with Ubc7p relocated to a cytosolic E3. In total, these studies have substantially improved and expanded our understanding of how Hrd1p functions to degrade proteins in the ER.

# Chapter 1:

# A ubiquitous system

It is clear from the more than 200 cell types in humans that a single genome can give rise to a diverse array of cellular forms and functions. This is accomplished by carefully controlling which proteins, and how much of them, are present in each cell type. Cells also require dynamic control of their proteome to grow, divide, and respond to changing conditions. While regulation of transcription and translation have long been known to play a critical role in controlling and modifying the proteome, protein degradation has emerged as an equally important axis to regulate and modify cell function. Importantly, protein degradation also plays a vital role in ridding the cell of misfolded and damaged proteins, preventing the deleterious consequences of their accumulation. Proteins in the cell are degraded by several mechanisms, including intra-mitochondrial AAA proteases, entry into the vacuole/lysosome, or targeting to the proteasome (Arnold and Langer, 2002; Klionsky and Emr, 2000; Yu, et al., 2004; Zwickl, et al., 1999).

The 26S proteasome is an evolutionarily conserved macromolecular protein degradation complex. It is comprised of two major subcomplexes: a core particle with proteolytic activity, and a regulatory cap that controls access to the core (Figure 1-1) (Smalle and Vierstra, 2004; Zwickl, et al., 1999). The 20S core particle is a barrel formed by 28 subunits arranged in 4 heptameric rings, with proteolytic active sites facing an inner cavity away from the cytosol (Voges, et al., 1999). The 19S regulatory cap is comprised of approximately 18 subunits (the number varies by species) that collaborate to control the entry of proteins into the proteolytic chamber of the 20S core particle. Regulatory cap subunits recruit proteins targeted for proteasome-mediated

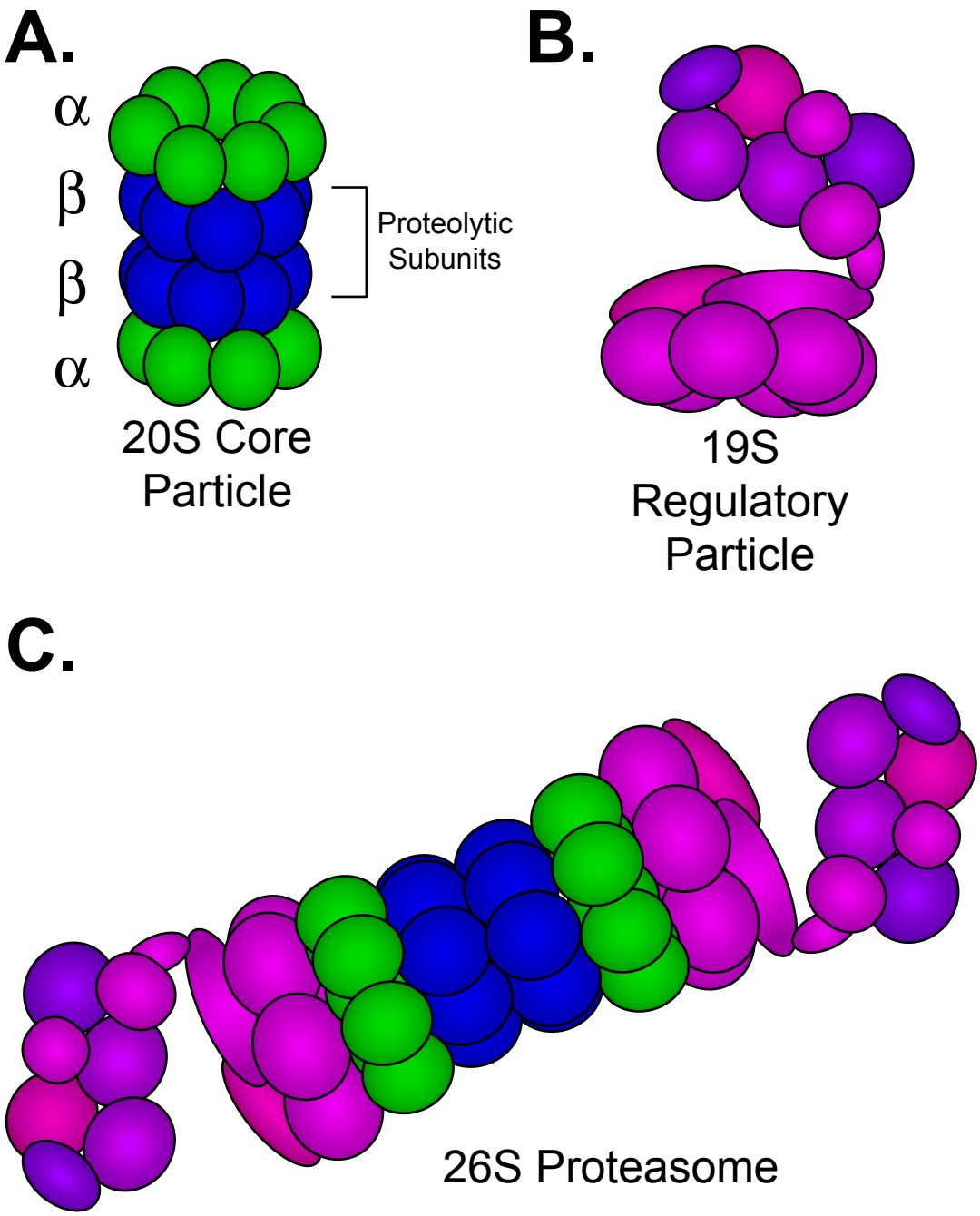


**Figure 1-1: Schematic representation of the proteasome.**

**A.** 14 α and 14 β subunits assemble into seven-membered rings to build the 20S core particle. Proteolytic activity is on the inner face of 3 β subunits in each ring. **B.** The 19S regulatory particle is comprised of 18 subunits that control access to the core particle **C.** Two regulatory particles arrange themselves on each end of the 20S core to produce the 26S proteasome.

degradation, unfold these targeted proteins, and translocate them into the core particle for proteolysis (Leggett, et al., 2002; Wolf and Hilt, 2004). The proteasome is the executioner, not the judge or jury. It does not decide which proteins to degrade. Rather, it senses the outcome of this decision-making process. Proteins targeted for proteasome-mediated degradation are labeled with a specific covalent post-translational modification that is recognized by 19S regulatory cap subunits, directing the targeted protein to the proteasome for degradation (Glickman and Ciechanover, 2002; Hershko and Ciechanover, 1998; Mayer, et al., 2005; Thrower, et al., 2000).

This covalent modification is made from a highly conserved 8kD protein named ubiquitin. The carboxy-terminus of ubiquitin is linked to the target protein through a primary amine; usually the ε-amino group of a lysine residue, or occasionally the amino-terminus itself (Goldknopf and Busch, 1977). Ubiquitin bound to a target-protein can itself be modified with ubiquitin, which in turn can receive ubiquitin, and so on, forming a polyubiquitin chain (Hershko and Heller, 1985). A specific type of polyubiquitin chain, linked only using lysine 48 residues of ubiquitin, is recognized by the proteasome (Thrower, et al., 2000). Highlighting the importance of this mechanism, yeast cannot grow when expressing only ubiquitin whose lysine 48 has been mutated to arginine (Finley, et al., 1994).

Ubiquitination of a protein is accomplished by a cascade of enzymes (Figure 1-2) (Hershko and Ciechanover, 1998; Hershko, et al., 1983; Pickart, 2001). First, ubiquitin activating enzyme (E1) consumes ATP to form a high energy thioester bond between the c-terminus of ubiquitin and a cysteine residue of the E1. This ubiquitin-

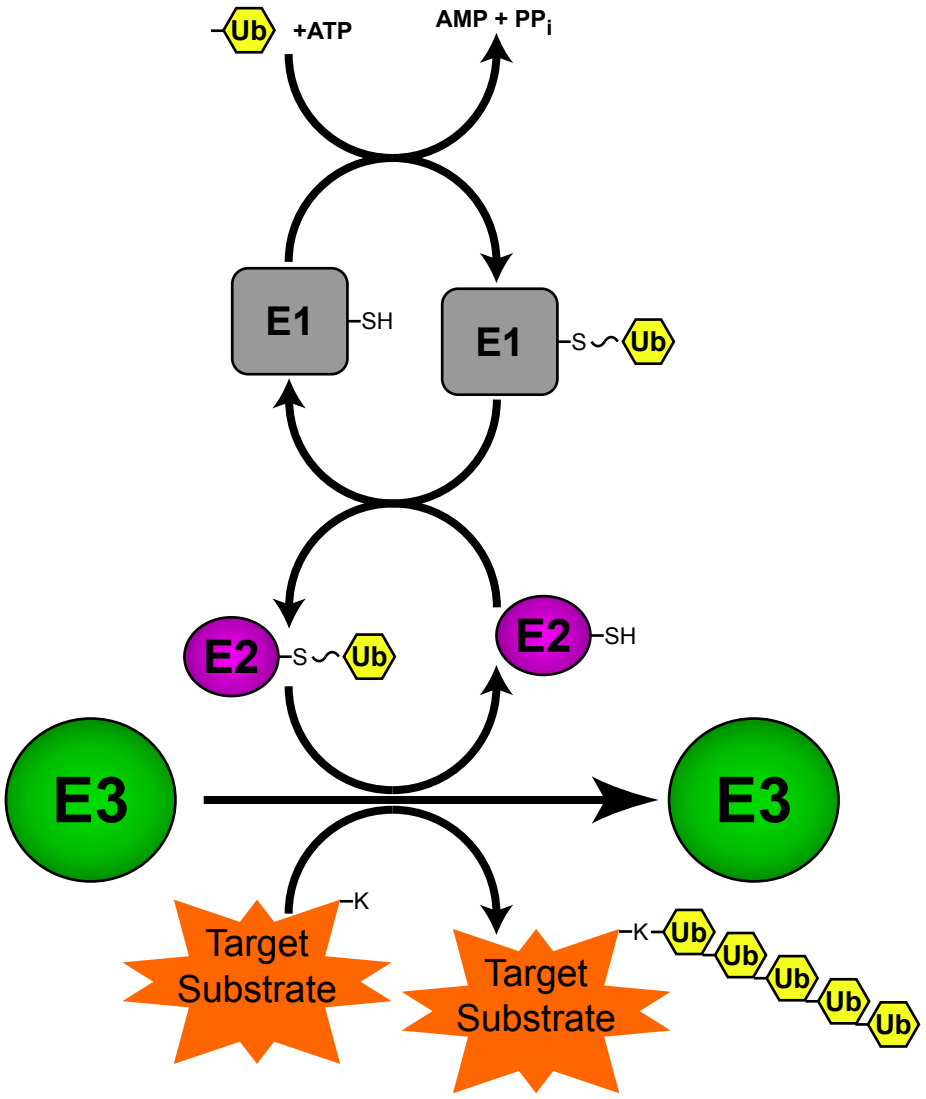


**Figure 1-2: The enzymes that facilitate ubiquitination: E1, E2, E3.**

Ubiquitin (Ub) and ATP interact with ubiquitin activating enzyme (E1), consuming ATP to form a high-energy thioester bond between E1 and ubiquitin. E1 then transfers ubiquitin to a ubiquitin activating enzyme (E2), forming another high energy thioester on the E2. A ubiquitin ligase (E3) coordinates the transfer of ubiquitin from the thioester of the E2, to a lysine residue (K) on the target substrate. Continued ubiquitination on ubiquitin itself assembles a polyubiquitin chain.

E1 adduct then encounters a ubiquitin-conjugating enzyme (E2), and ubiquitin is transferred to the E2, again as a thioester. The ubiquitin-E2 then interacts with a ubiquitin ligase (E3) that can also interact with target substrates. This E3 then facilitates transfer of ubiquitin to the substrate. This cascade of enzymes is hierarchical: the *S. cerevisiae* genome encodes only one E1, which activates ubiquitin for all 11 E2s. Each E2 interacts with a subset of the scores of E3s that must, in turn, specify all the cell's ubiquitination substrates. The *C. elegans*, *D. melanogaster*, and human genomes each encode at least 20-25 E2s, and what could be hundreds of E3s (Jones, et al., 2002; Pickart and Eddins, 2004). This hierarchical scheme allows tremendous variability in E3s, permitting the selective ubiquitination and degradation of a wide variety of substrates while completely conserving the chemistry of ubiquitin conjugation and the mechanism of ubiquitin recognition by the proteasome.

Consequently, ubiquitin-proteasome dependent degradation controls a wide variety of important cellular processes. At various points in the cell cycle, coordinated proteasomal degradation is required to remove mitotic cyclins, inhibitory kinases like Sic1p, transcription factors such as c-Myc, and to release sister chromatids at anaphase (Thornton and Toczyski, 2006) (Nakayama and Nakayama, 2006) (Fung and Poon, 2005). Transcription is also manipulated by proteasomal degradation of transcription factors such as HIF-1α (Huang, et al., 1998), MyoD (Abu Hatoum, et al., 1998), Gcn4 (Kornitzer, et al., 1994; Meimoun, et al., 2000) or transcriptional repressors like yeast MATα2 (Hochstrasser, et al., 1991; Hochstrasser and Varshavsky, 1990). In signal transduction, IκB is ubiquitinated and degraded upon phosphorylation, and the Ras-

GTP exchange factor Ras-GRF2 is degraded upon Ras binding (Chen, 2005) (de Hoog, et al., 2001). Also, metabolic pathways are subject to regulation by proteasome activity. In yeast, high glucose levels lead to fructose-1,6-bisphosphatase degradation (Schork, et al., 1994; Schork, et al., 1995). The rate limiting enzyme in cholesterol synthesis, Hmg co-A reductase, is degraded by the proteasome to modulate sterol biosynthesis in yeast and mammals (Hampton and Rine, 1994; Nakanishi, et al., 1988; Sever, et al., 2003; Song and DeBose-Boyd, 2004).

In addition to the specific targeting of individual proteins to precisely control a specific biological process, the proteasome is also involved in more generalized degradation of cell proteins. Muscle tissues are found to liberate amino acids during starvation or denervation atrophy using skeletal muscle proteasomes (Medina, et al., 1995; Wing and Goldberg, 1993; Wing, et al., 1995). The proteasome also receives proteins that are misfolded or damaged. The endoplasmic reticulum (ER) has protein quality control mechanisms that function through a ubiquitin- and proteasome-mediated mechanism (Hampton, 2002; McCracken and Brodsky, 1996; McCracken and Brodsky, 2003; Werner, et al., 1996). Proteasome-dependent quality control pathways have also been identified in the cytosol (Meacham, et al., 2001; Murata, et al., 2001; Xu, et al., 2002), and recently in the nucleus (Gardner, et al., 2005). Protein quality control, particularly in the ER is a focus of this work, and will be discussed in more detail below.

This brief survey of proteasomal degradation targets is not comprehensive, and is presented to highlight the breadth of biological functions that the ubiquitin-

proteasome system impacts. This is a testament to the diversity among E3 proteins in the hierarchical E1, E2, E3 ubiquitination scheme. Based on sequence homology of known E3s, four classes of E3 have emerged: HECT, RING, U-box and PHD domain E3s (Bottomley, et al., 2005; Pickart and Eddins, 2004). The HECT-domain E3s (an acronym for their **H**omology to the **E**6-AP protein's **C**-**T**erminus) have a conserved cysteine that receives charged-ubiquitin from the E2 before transfer to the target substrate (Scheffner, et al., 1995). The RING-motif E3s, and the more recently discovered U-box and PHD E3s, act as a scaffold to bring E2 and substrate protein into proximity (Bottomley, et al., 2005; Pickart and Eddins, 2004). Some RING motif ligases are part of a larger multi-subunit E3 complex, such as the anaphase promoting complex (APC) or the Skp1-Cul1-F-box (SCF) E3 complex (King, et al., 1995; Seol, et al., 1999). The associated proteins in these complexes allow for further regulation or substrate diversification by a single RING motif E3 protein (Kipreos and Pagano, 2000; Murray, 2004).

In addition to the diversity of substrates targeted and morphological diversity among the E3s, the ubiquitin system exhibits functional diversity in the variety of ubiquitin modifications that can be attached to proteins in the cell. Only lysine 48-linked polyubiquitin chains will target a protein to the proteasome. Yet, proteasome-independent functions of ubiquitin modification have emerged. Lysine-63 linked polyubiquitin chains modify PCNA during DNA repair and this is required for DNA damage tolerance (Chiu, et al., 2006; Hoege, et al., 2002; Spence, et al., 1995). TRAF6 is an E3 that, in response to proinflamatory cytokines, activates IκB kinase by

adding a lysine-63 linked polyubiquitin chain (Deng, et al., 2000; Kanayama, et al., 2004). Lysine-63 polyubiquitin is found linked to the L28 ribosomal subunit in a cell-cycle dependent manner (Spence, et al., 2000). In each of these cases, turnover of the polyubiquitinated protein is not observed. These processes use Mms2/Ubc13, an E2 devoted to making lysine-63 linkages (Hofmann and Pickart, 1999; Hofmann and Pickart, 2001). In addition to lysines 48 and 63, ubiquitin has 5 other lysines, and all seven varieties of polyubiquitin linkage have been detected *in vivo* (Peng, et al., 2003). Ubiquitin can also modify a protein without building a polymer. Ubiquitin's ability to covalently modify protein was discovered in analyses of histone H2B (Goldknopf and Busch, 1977). Only recent has it become clear that mono-ubiquitination on H2B affects methylation on histone H3 with consequences for gene silencing (Daniel, et al., 2004; Sun and Allis, 2002). Monoubiquitination of transmembrane proteins is also required for their sorting into the multivesicular body for degradation in the vacuole (Dunn, et al., 2004; Katzmann, et al., 2004).

E3 ubiquitin ligases govern not only substrate recognition, but also E2 recruitment and the type of ubiquitin modification a substrate will receive. Thus, E3s are at the nexus of several decisions that are made before a protein is modified with ubiquitin. Understanding these subtleties of E3 function will be essential for predicting how the many uncharacterized E2s and E3s will behave with one another, and for designing customized E2s or E3s as experimental tools or therapeutics. We want to understand how E3s and E2s recognize one another, how these proteins achieve specific ubiquitination of a degradation target, and how a polyubiquitin chain

is formed. We approach these global questions from the perspective of a specific E3, and our studies of its function in the ER.

Nascent secretory proteins are translocated into the ER and must fold into their correct conformation within the lumen of the ER before progressing through the secretory pathway. Misfolded proteins are detected and removed by what is termed ER-associated degradation (ERAD), a mechanism that *erad*icates a variety of luminal and transmembrane-spanning proteins in a proteasome-dependent manner. Increases in the burden of misfolded ER proteins are sensed by a conserved signaling mechanism called the unfolded protein response (UPR), which boosts production of ERAD proteins, as well as chaperones, lipid biosynthetic proteins, glycosylation enzymes, and others (Travers, et al., 2000). Cells that are defective in ERAD have increased UPR, and cells that are defective in both ERAD and this transcriptional response have synthetic lethal phenotypes and are extremely sensitive to stress (Friedlander, et al., 2000; Travers, et al., 2000). These intertwined responses regulate each other to balance ER stress.

Imbalances in ER stress, both too much and too little, have been implicated in various diseases of ageing. In pancreatic beta cells, which produce insulin, elevated ER stress and the resulting apoptosis has been implicated in type II diabetes onset (Haynes, et al., 2004; Ozawa, et al., 2005; Ozcan, et al., 2006). In contrast, too little ER stress can also be problematic. Patients with rheumatoid arthritis have overgrowth of synovial cells in their joints. Cells from these patients were found to have high levels of a human ERAD protein, which inhibited their apoptosis. Overexpression of

the ERAD protein allowed overgrowth of synovial cells through reduced ER stress (Amano, et al., 2003). There is also a potential theraputic benefit to circumventing ERAD in the case of patients with cystic fibrosis. The most common disease allele of the cystic fibrosis transmembrane conductance regulator (CFTR) still functions normally as an ion channel, but never reaches the plasma membrane due to detection by the ERAD surveillance apparatus (Younger, et al., 2006). When cells are manipulated to suppress ERAD of CFTR, some mutant protein is delivered to the plasmas membrane, restoring function (Zhang, et al., 2003).

This important ER-localized mechanism of ubiquitination and proteasomal degradation poses several interesting challenges for the cell. How are luminal ER proteins ubiquitinated if they are sequestered away from ubiquitin by the ER membrane? How are these proteins moved across the membrane to be ubiquitinated and delivered to the proteasome? How does the cell sense misfolded proteins, and how are these different from correctly folded proteins in the ER? In addition to these questions that apply specifically to ERAD, our general questions about the ubiquitin-proteasome system still hold. How do E2s and E3s recognize one another in a specific manner? How is polyubiquitination by the ubiquitin ligase coordinated with the substrate? To better understand this mechanism of protein quality control, and learn how the cell overcomes these challenges, we have studied ERAD in *Saccharomyces cerevisiae* (Hampton, 2002; McCracken and Brodsky, 2003).

The Hrd1p ubiquitin ligase is one of several E3s that mediate ERAD (Bays, et al., 2001; Haynes, et al., 2002; Swanson, et al., 2001). Hrd1p homologs have also

been implicated in mammalian ERAD (Amano, et al., 2003; Fang, et al., 2001). Hrd1p is responsible for the degradation of the yeast HMG-CoA reductase isozyme Hmg2p (Hampton, et al., 1996). Other Hrd1p-dependent ERAD substrates have been identified, including the Prc1-1p mutant of carboxypeptidase Y (CPY*), Vph1p (when absent its binding partner Vma21p), and Sec61-2p (Bordallo, et al., 1998; Plemper, et al., 1998; Wilhovsky, et al., 2000). Hrd1p consists of an N-terminal multispanning membrane anchor and a cytoplasmic C-terminal region bearing a RING-H2 motif (Figure 1-3). The cytoplasmic portion is required for Hrd1p-dependent ERAD *in vivo* (Gardner, et al., 2000) and functions autonomously as a ubiquitin ligase *in vitro* (Bays, et al., 2001). Hrd1p resides in a complex with an accessory protein, the ER integral membrane protein Hrd3p (Gardner, et al., 2000). The cytoplasmic C-terminal RING-H2 domain of Hrd1p is exposed to numerous E2s; yet, Hrd1p only employs Ubc7p, and to a much lesser extent Ubc1p, in the execution of its function (Bays, et al., 2001). However, the Hrd1p RING domain functions *in vitro* with E2s that are not used *in vivo* (Bays, et al., 2001), leading us to wonder which features of Hrd1p contribute to its high selectivity for Ubc7p *in vivo*.

We reasoned that careful study of Hrd1p would help us understand how misfolded proteins are recognized, how E2 interaction is governed, and how polyubiquitination is coordinated. Using an *in vitro* assay of misfolded protein ubiquitination with a recombinant cytosolic portion of Hrd1p, we hoped to identify a misfolded protein interacting domain that could explain how Hrd1p distinguishes misfolded proteins from the others in the ER. With facile *in vivo* assays of Hrd1p

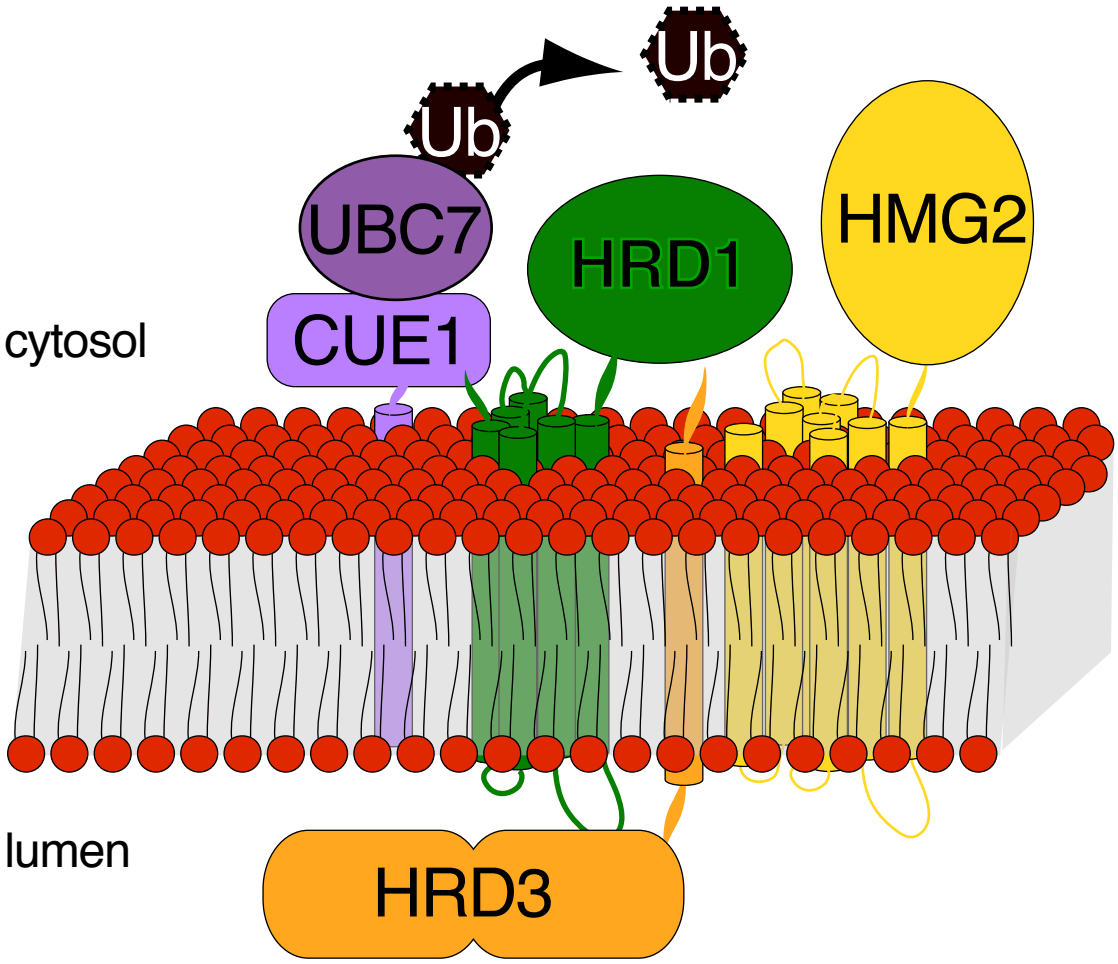


**Figure 1-3: Hrd1p and other ERAD proteins in the ER membrane.**

Hrd1p has a soluble cytosolic domain and an ER-membrane anchor with six transmembrane spans. The RING motif and E3 activity lie in the cytosolic domain. Hrd3p is predominantly a lumenal protein with one transmembrane span, and forms a complex with Hrd1p. Ubc7p is the E2 most favored by Hrd1p, and is shown anchored to the membrane through Cue1p, which is also required for ERAD. Hrd1p is depicted coordinating the transfer of ubiquitination from Ubc7p to the Hrd1p-dependent ERAD substrate, Hmg2p.

function, we were able to test the predictions suggested by *in vitro* results. In doing so, we learned that our *in vitro* assay did not recapitulate the more complicated function of Hrd1p *in vivo*. We then refined a different assay that would allow exploration of how specific E2-E3 pairing is determined by Hrd1p, and how this pairing differed with Hrd1p *in vitro* and *in vivo*. We discovered several criteria for Hrd1p to produce polyubiquitination with its native E2 Ubc7p, that suggested both a critical role for the Hrd1p RING motif, and a requirement for other portions of Hrd1p as well. Through this, we defined *in vitro* conditions allowing further analyses of Hrd1p functions. We were also able to ask questions about the relationship between Hrd1p and Hrd3p. Portions of Hrd1p were identified to be necessary for its regulation by Hrd3p. We also identified a new activity for an ERAD factor required for Hrd1p function. This activity has implications for the mechanism by which Hrd1p coordinates the processive addition of ubiquitin to its substrates.

## References:


1. Abu Hatoum, O., Gross-Mesilaty, S., Breitschopf, K., Hoffman, A., Gonen, H., Ciechanover, A. and Bengal, E. (1998). Degradation of myogenic transcription factor MyoD by the ubiquitin pathway in vivo and in vitro: regulation by specific DNA binding. *Mol Cell Biol* **18** (10) 5670-5677.

2. Amano, T., Yamasaki, S., Yagishita, N., Tsuchimochi, K., Shin, H., Kawahara, K., Aratani, S., Fujita, H., Zhang, L., Ikeda, R., Fujii, R., Miura, N., Komiya, S., Nishioka, K., Maruyama, I., Fukamizu, A. and Nakajima, T. (2003). Synoviolin/Hrd1, an E3 ubiquitin ligase, as a novel pathogenic factor for arthropathy. *Genes Dev* **17** (19) 2436-2449.

3. Arnold, I. and Langer, T. (2002). Membrane protein degradation by AAA proteases in mitochondria. *Biochim Biophys Acta* **1592** (1) 89-96.

4. 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. *Nat Cell Biol* **3** (1) 24-29.

5. Bordallo, J., Plemper, R. K., Finger, A. and Wolf, D. H. (1998). Der3p/Hrd1p is required for endoplasmic reticulum-associated degradation of misfolded lumenal and integral membrane proteins. *Mol Biol Cell* **9** (1) 209-222.

6. Bottomley, M. J., Stier, G., Pennacchini, D., Legube, G., Simon, B., Akhtar, A., Sattler, M. and Musco, G. (2005). NMR structure of the first PHD finger of autoimmune regulator protein (AIRE1). Insights into autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) disease. *J Biol Chem* **280** (12) 11505-11512.

7. Chen, Z. J. (2005). Ubiquitin signalling in the NF-kappaB pathway. *Nat Cell Biol* **7** (8) 758-765.

8. Chiu, R. K., Brun, J., Ramaekers, C., Theys, J., Weng, L., Lambin, P., Gray, D. A. and Wouters, B. G. (2006). Lysine 63-polyubiquitination guards against translesion synthesis-induced mutations. *PLoS Genet* **2** (7) e116.

9. Daniel, J. A., Torok, M. S., Sun, Z. W., Schieltz, D., Allis, C. D., Yates, J. R., 3rd and Grant, P. A. (2004). Deubiquitination of histone H2B by a yeast acetyltransferase complex regulates transcription. *J Biol Chem* **279** (3) 1867-1871.

10. de Hoog, C. L., Koehler, J. A., Goldstein, M. D., Taylor, P., Figeys, D. and Moran, M. F. (2001). Ras binding triggers ubiquitination of the Ras exchange factor Ras-GRF2. *Mol Cell Biol* **21** (6) 2107-2117.

11. Deng, L., Wang, C., Spencer, E., Yang, L., Braun, A., You, J., Slaughter, C., Pickart, C. and Chen, Z. J. (2000). Activation of the IkappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. *Cell* **103** (2) 351-361.

12. Dunn, R., Klos, D. A., Adler, A. S. and Hicke, L. (2004). The C2 domain of the Rsp5 ubiquitin ligase binds membrane phosphoinositides and directs ubiquitination of endosomal cargo. *J Cell Biol* **165** (1) 135-144.

13. Fang, S., Ferrone, M., Yang, C., Jensen, J. P., Tiwari, S. and Weissman, A. M. (2001). The tumor autocrine motility factor receptor, gp78, is a ubiquitin protein ligase implicated in degradation from the endoplasmic reticulum. *Proc Natl Acad Sci U S A* **98** (25) 14422-14427.

14. Finley, D., Sadis, S., Monia, B. P., Boucher, P., Ecker, D. J., Crooke, S. T. and Chau, V. (1994). Inhibition of proteolysis and cell cycle progression in a multiubiquitination-deficient yeast mutant. *Mol Cell Biol* **14** (8) 5501-5509.

15. Friedlander, R., Jarosch, E., Urban, J., Volkwein, C. and Sommer, T. (2000). A regulatory link between ER-associated protein degradation and the unfolded-protein response. *Nat Cell Biol* **2** (7) 379-384.

16. Fung, T. K. and Poon, R. Y. (2005). A roller coaster ride with the mitotic cyclins. *Semin Cell Dev Biol* **16** (3) 335-342.

17. Gardner, R. G., Nelson, Z. W. and Gottschling, D. E. (2005). Degradation-mediated protein quality control in the nucleus. *Cell* **120** (6) 803-815.

18. Gardner, R. G., Swarbrick, G. M., Bays, N. W., Cronin, S. R., Wilhovsky, S., Seelig, L., Kim, C. and Hampton, R. Y. (2000). Endoplasmic reticulum degradation requires lumen to cytosol signaling. Transmembrane control of Hrd1p by Hrd3p. *J Cell Biol* **151** (1) 69-82.

19. Glickman, M. H. and Ciechanover, A. (2002). The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. *Physiol Rev* **82** (2) 373-428.

20. Goldknopf, I. L. and Busch, H. (1977). Isopeptide linkage between nonhistone and histone 2A polypeptides of chromosomal conjugate-protein A24. *Proc Natl Acad Sci U S A* **74** (3) 864-868.

21. Hampton, R. Y. (2002). Proteolysis and sterol regulation. *Annu Rev Cell Dev Biol* **18** 345-378.

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

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

24. Haynes, C. M., Caldwell, S. and Cooper, A. A. (2002). An HRD/DER-independent ER quality control mechanism involves Rsp5p-dependent ubiquitination and ER-Golgi transport. *J Cell Biol* **158** (1) 91-101.

25. Haynes, C. M., Titus, E. A. and Cooper, A. A. (2004). Degradation of misfolded proteins prevents ER-derived oxidative stress and cell death. *Mol Cell* **15** (5) 767-776.

26. Hershko, A. and Ciechanover, A. (1998). The ubiquitin system. *Annu Rev Biochem* **67** 425-479.

27. Hershko, A. and Heller, H. (1985). Occurrence of a polyubiquitin structure in ubiquitin-protein conjugates. *Biochem Biophys Res Commun* **128** (3) 1079-1086.

28. Hershko, A., Heller, H., Elias, S. and Ciechanover, A. (1983). Components of ubiquitin-protein ligase system. Resolution, affinity purification, and role in protein breakdown. *J Biol Chem* **258** (13) 8206-8214.

29. Hochstrasser, M., Ellison, M. J., Chau, V. and Varshavsky, A. (1991). The short-lived MAT alpha 2 transcriptional regulator is ubiquitinated in vivo. *Proc Natl Acad Sci U S A* **88** (11) 4606-4610.

30. Hochstrasser, M. and Varshavsky, A. (1990). In vivo degradation of a transcriptional regulator: the yeast alpha 2 repressor. *Cell* **61** (4) 697-708.

31. Hoege, C., Pfander, B., Moldovan, G. L., Pyrowolakis, G. and Jentsch, S. (2002). RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. *Nature* **419** (6903) 135-141.

32. Hofmann, R. M. and Pickart, C. M. (1999). Noncanonical MMS2-encoded ubiquitin-conjugating enzyme functions in assembly of novel polyubiquitin chains for DNA repair. *Cell* **96** (5) 645-653.

33. Hofmann, R. M. and Pickart, C. M. (2001). In vitro assembly and recognition of Lys-63 polyubiquitin chains. *J Biol Chem* **276** (30) 27936-27943.

34. Huang, L. E., Gu, J., Schau, M. and Bunn, H. F. (1998). Regulation of hypoxia-inducible factor 1alpha is mediated by an O2-dependent degradation domain via the ubiquitin-proteasome pathway. *Proc Natl Acad Sci U S A* **95** (14) 7987-7992.

35. Jones, D., Crowe, E., Stevens, T. A. and Candido, E. P. (2002). Functional and phylogenetic analysis of the ubiquitylation system in Caenorhabditis elegans: ubiquitin-conjugating enzymes, ubiquitin-activating enzymes, and ubiquitin-like proteins. *Genome Biol* **3** (1) RESEARCH0002.

36. Kanayama, A., Seth, R. B., Sun, L., Ea, C. K., Hong, M., Shaito, A., Chiu, Y. H., Deng, L. and Chen, Z. J. (2004). TAB2 and TAB3 activate the NF-kappaB pathway through binding to polyubiquitin chains. *Mol Cell* **15** (4) 535-548.

37. Katzmann, D. J., Sarkar, S., Chu, T., Audhya, A. and Emr, S. D. (2004). Multivesicular body sorting: ubiquitin ligase Rsp5 is required for the modification and sorting of carboxypeptidase S. *Mol Biol Cell* **15** (2) 468-480.

38. King, R. W., Peters, J. M., Tugendreich, S., Rolfe, M., Hieter, P. and Kirschner, M. W. (1995). A 20S complex containing CDC27 and CDC16 catalyzes the mitosis-specific conjugation of ubiquitin to cyclin B. *Cell* **81** (2) 279-288.

39. Kipreos, E. T. and Pagano, M. (2000). The F-box protein family. *Genome Biol* **1** (5) REVIEWS3002.

40. Klionsky, D. J. and Emr, S. D. (2000). Autophagy as a regulated pathway of cellular degradation. *Science* **290** (5497) 1717-1721.

41. Kornitzer, D., Raboy, B., Kulka, R. G. and Fink, G. R. (1994). Regulated degradation of the transcription factor Gcn4. *Embo J* **13** (24) 6021-6030.

42. Leggett, D. S., Hanna, J., Borodovsky, A., Crosas, B., Schmidt, M., Baker, R. T., Walz, T., Ploegh, H. and Finley, D. (2002). Multiple associated proteins regulate proteasome structure and function. *Mol Cell* **10** (3) 495-507.

43. Mayer, J., Ciechanover, A. and Rechsteiner, M. (Eds) (2005). *Protein Degradation: Ubiquitin and the Chemistry of Life*, Wiley-VCH.

44. McCracken, A. A. and Brodsky, J. L. (1996). Assembly of ER-associated protein degradation in vitro: dependence on cytosol, calnexin, and ATP. *J Cell Biol* **132** (3) 291-298.

45. McCracken, A. A. and Brodsky, J. L. (2003). Evolving questions and paradigm shifts in endoplasmic-reticulum-associated degradation (ERAD). *Bioessays* **25** (9) 868-877.

46. Meacham, G. C., Patterson, C., Zhang, W., Younger, J. M. and Cyr, D. M. (2001). The Hsc70 co-chaperone CHIP targets immature CFTR for proteasomal degradation. *Nat Cell Biol* **3** (1) 100-105.

47. Medina, R., Wing, S. S. and Goldberg, A. L. (1995). Increase in levels of polyubiquitin and proteasome mRNA in skeletal muscle during starvation and denervation atrophy. *Biochem J* **307 ( Pt 3)** 631-637.

48. Meimoun, A., Holtzman, T., Weissman, Z., McBride, H. J., Stillman, D. J., Fink, G. R. and Kornitzer, D. (2000). Degradation of the transcription factor Gcn4 requires the kinase Pho85 and the SCF(CDC4) ubiquitin-ligase complex. *Mol Biol Cell* **11** (3) 915-927.

49. Murata, S., Minami, Y., Minami, M., Chiba, T. and Tanaka, K. (2001). CHIP is a chaperone-dependent E3 ligase that ubiquitylates unfolded protein. *EMBO Rep* **2** (12) 1133-1138.

50. Murray, A. W. (2004). Recycling the cell cycle: cyclins revisited. *Cell* **116** (2) 221-234.

51. Nakanishi, M., Goldstein, J. L. and Brown, M. S. (1988). Multivalent control of 3-hydroxy-3-methylglutaryl coenzyme A reductase. Mevalonate-derived product inhibits translation of mRNA and accelerates degradation of enzyme. *J Biol Chem* **263** (18) 8929-8937.

52. Nakayama, K. I. and Nakayama, K. (2006). Ubiquitin ligases: cell-cycle control and cancer. *Nat Rev Cancer* **6** (5) 369-381.

53. Ozawa, K., Miyazaki, M., Matsuhisa, M., Takano, K., Nakatani, Y., Hatazaki, M., Tamatani, T., Yamagata, K., Miyagawa, J., Kitao, Y., Hori, O., Yamasaki, Y. and Ogawa, S. (2005). The endoplasmic reticulum chaperone improves insulin resistance in type 2 diabetes. *Diabetes* **54** (3) 657-663.

54. Ozcan, U., Yilmaz, E., Ozcan, L., Furuhashi, M., Vaillancourt, E., Smith, R. O., Gorgun, C. Z. and Hotamisligil, G. S. (2006). Chemical chaperones reduce ER

stress and restore glucose homeostasis in a mouse model of type 2 diabetes. *Science* **313** (5790) 1137-1140.

55. Peng, J., Schwartz, D., Elias, J. E., Thoreen, C. C., Cheng, D., Marsischky, G., Roelofs, J., Finley, D. and Gygi, S. P. (2003). A proteomics approach to understanding protein ubiquitination. *Nat Biotechnol* **21** (8) 921-926.

56. Pickart, C. M. (2001). Mechanisms underlying ubiquitination. *Annu Rev Biochem* **70** 503-533.

57. Pickart, C. M. and Eddins, M. J. (2004). Ubiquitin: structures, functions, mechanisms. *Biochim Biophys Acta* **1695** (1-3) 55-72.

58. 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. *J Biol Chem* **273** (49) 32848-32856.

59. Scheffner, M., Nuber, U. and Huibregtse, J. M. (1995). Protein ubiquitination involving an E1-E2-E3 enzyme ubiquitin thioester cascade. *Nature* **373** (6509) 81-83.

60. Schork, S. M., Bee, G., Thumm, M. and Wolf, D. H. (1994). Catabolite inactivation of fructose-1,6-bisphosphatase in yeast is mediated by the proteasome. *FEBS Lett* **349** (2) 270-274.

61. Schork, S. M., Thumm, M. and Wolf, D. H. (1995). Catabolite inactivation of fructose-1,6-bisphosphatase of Saccharomyces cerevisiae. Degradation occurs via the ubiquitin pathway. *J Biol Chem* **270** (44) 26446-26450.

62. Seol, J. H., Feldman, R. M., Zachariae, W., Shevchenko, A., Correll, C. C., Lyapina, S., Chi, Y., Galova, M., Claypool, J., Sandmeyer, S., Nasmyth, K. and Deshaies, R. J. (1999). Cdc53/cullin and the essential Hrt1 RING-H2 subunit of SCF define a ubiquitin ligase module that activates the E2 enzyme Cdc34. *Genes Dev* **13** (12) 1614-1626.

63. Sever, N., Song, B. L., Yabe, D., Goldstein, J. L., Brown, M. S. and DeBose-Boyd, R. A. (2003). Insig-dependent ubiquitination and degradation of mammalian 3-hydroxy-3-methylglutaryl-CoA reductase stimulated by sterols and geranylgeraniol. *J Biol Chem* **278** (52) 52479-52490.

64. Smalle, J. and Vierstra, R. D. (2004). The ubiquitin 26S proteasome proteolytic pathway. *Annu Rev Plant Biol* **55** 555-590.

65. Song, B. L. and DeBose-Boyd, R. A. (2004). Ubiquitination of 3-hydroxy-3-methylglutaryl-CoA reductase in permeabilized cells mediated by cytosolic E1 and a putative membrane-bound ubiquitin ligase. *J Biol Chem* **279** (27) 28798-28806.

66. Spence, J., Gali, R. R., Dittmar, G., Sherman, F., Karin, M. and Finley, D. (2000). Cell cycle-regulated modification of the ribosome by a variant multiubiquitin chain. *Cell* **102** (1) 67-76.

67. Spence, J., Sadis, S., Haas, A. L. and Finley, D. (1995). A ubiquitin mutant with specific defects in DNA repair and multiubiquitination. *Mol Cell Biol* **15** (3) 1265-1273.

68. Sun, Z. W. and Allis, C. D. (2002). Ubiquitination of histone H2B regulates H3 methylation and gene silencing in yeast. *Nature* **418** (6893) 104-108.

69. 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 Dev* **15** (20) 2660-2674.

70. Thornton, B. R. and Toczyski, D. P. (2006). Precise destruction: an emerging picture of the APC. *Genes Dev* **20** (22) 3069-3078.

71. Thrower, J. S., Hoffman, L., Rechsteiner, M. and Pickart, C. M. (2000). Recognition of the polyubiquitin proteolytic signal. *Embo J* **19** (1) 94-102.

72. Travers, K. J., Patil, C. K., Wodicka, L., Lockhart, D. J., Weissman, J. S. and Walter, P. (2000). Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER-associated degradation. *Cell* **101** (3) 249-258.

73. Voges, D., Zwickl, P. and Baumeister, W. (1999). The 26S proteasome: a molecular machine designed for controlled proteolysis. *Annu Rev Biochem* **68** 1015-1068.

74. Werner, E. D., Brodsky, J. L. and McCracken, A. A. (1996). Proteasome-dependent endoplasmic reticulum-associated protein degradation: an unconventional route to a familiar fate. *Proc Natl Acad Sci U S A* **93** (24) 13797-13801.

75. Wilhovsky, S., Gardner, R. and Hampton, R. (2000). HRD gene dependence of endoplasmic reticulum-associated degradation. *Mol Biol Cell* **11** (5) 1697-1708.

76. Wing, S. S. and Goldberg, A. L. (1993). Glucocorticoids activate the ATP-ubiquitin-dependent proteolytic system in skeletal muscle during fasting. *Am J Physiol* **264** (4 Pt 1) E668-676.

77. Wing, S. S., Haas, A. L. and Goldberg, A. L. (1995). Increase in ubiquitin-protein conjugates concomitant with the increase in proteolysis in rat skeletal muscle during starvation and atrophy denervation. *Biochem J* **307 ( Pt 3)** 639-645.

78. Wolf, D. H. and Hilt, W. (2004). The proteasome: a proteolytic nanomachine of cell regulation and waste disposal. *Biochim Biophys Acta* **1695** (1-3) 19-31.

79. Xu, W., Marcu, M., Yuan, X., Mimnaugh, E., Patterson, C. and Neckers, L. (2002). Chaperone-dependent E3 ubiquitin ligase CHIP mediates a degradative pathway for c-ErbB2/Neu. *Proc Natl Acad Sci U S A* **99** (20) 12847-12852.

80. Younger, J. M., Chen, L., Ren, H. Y., Rosser, M. F., Turnbull, E. L., Fan, C. Y., Patterson, C. and Cyr, D. M. (2006). Sequential quality-control checkpoints triage misfolded cystic fibrosis transmembrane conductance regulator. *Cell* **126** (3) 571-582.

81. Yu, L., Lenardo, M. J. and Baehrecke, E. H. (2004). Autophagy and caspases: a new cell death program. *Cell Cycle* **3** (9) 1124-1126.

82. Zhang, X. M., Wang, X. T., Yue, H., Leung, S. W., Thibodeau, P. H., Thomas, P. J. and Guggino, S. E. (2003). Organic solutes rescue the functional defect in delta F508 cystic fibrosis transmembrane conductance regulator. *J Biol Chem* **278** (51) 51232-51242.

83. Zwickl, P., Voges, D. and Baumeister, W. (1999). The proteasome: a macromolecular assembly designed for controlled proteolysis. *Philos Trans R Soc Lond B Biol Sci* **354** (1389) 1501-1511.

# Chapter 2:

# Misfolded protein recognition

## **Introduction**:

The ubiquitin-proteasome system is a widely used mechanism for modulating proteins in the cell (Huang and D'Andrea, 2006; Nakayama and Nakayama, 2006; Staub and Rotin, 2006). A cascade of enzymes is employed in a hierarchical manner to catalyze the ubiquitination of target proteins (Hershko and Ciechanover, 1998; Pickart, 2001). A ubiquitin activating enzyme (E1) consumes ATP to form a thioester bond between the c-terminus of ubiquitin and a cysteine residue on the E1. Ubiquitin is transferred from E1 to a conserved a ubiquitin conjugating enzyme (E2), again forming a thioester on a conserved E2 cysteine. Then, a ubiquitin ligase (E3) mediates ubiquitin transfer to its substrate, resulting in covalent attachment of ubiquitin by its c-terminus to a primary amine on the target protein. Unlike the HECT class of E3, which receives ubiquitin directly on a conserved cysteine residue before ubiquitinating its target, the RING motif E3s do not covalently bind ubiquitin (Joazeiro and Weissman, 2000). The RING motif-containing ligases are thus thought to mediate the proximity of E2 and substrate (Gardner, et al., 2001; Pickart and Eddins, 2004; VanDemark and Hill, 2002).

Many proteins, such as cyclins, transcription factors, cell-signaling kinases, and metabolic enzymes, are degraded by the ubiquitin-proteasome system in response to specific metabolic or cell-cycle cues (Glickman and Ciechanover, 2002). Ubiquitination also targets improperly folded proteins in the secretory pathway for eradication by the proteasome through ER-associated degradation (ERAD) (Meusser, et al., 2005; Werner, et al., 1996). Hrd1p is a membrane-localized ubiquitin ligase

(E3) required for degradation of Hmg2p (Hampton, et al., 1996b) and other quality-control ERAD substrates *in vivo* (Bordallo, et al., 1998; Wilhovsky, et al., 2000). Hrd1p is anchored in the endoplasmic reticulum with six transmembrane spans, and has a cytosolic domain containing the conserved RING motif (Figure 1-3). *In vitro* studies found that a RING motif-containing portion of Hrd1p exhibits ubiquitin ligase activity (Bays, et al., 2001). Hrd1p catalyzes formation of high molecular weight ubiquitin chains, and transfers ubiquitin to a heterologous protein in a binding-dependent manner (Bays, et al., 2001). Most interestingly, the soluble portion of Hrd1p tested *in vitro* prefers to ubiquitinate a denatured substrate (Bays, et al., 2001). It appeared that the *in vivo* quality-control scanning function of Hrd1p was manifest in a tractable *in vitro* assay. We wanted to further study this aspect of Hrd1p function to determine if this *in vitro* activity is involved in Hrd1p quality control function. It is thought that E3s like Hrd1p facilitate ubiquitination by bringing E2 and substrate into proximity (Gardner, et al., 2001; VanDemark and Hill, 2002). For Hrd1p to be involved in the degradation of many different proteins having no apparent sequence homology to one another, we reasoned that Hrd1p can participate in specifically recognizing misfolded proteins. We sought to understand if the preference for misfolded proteins by the soluble portion of Hrd1p tested *in vitro* was part of this recognition process.

To better understand the role Hrd1p plays in misfolded protein recognition, we tested multiple substrates to expand the breadth of misfolded substrates amenable to this assay. We also tested a portion of Hrd1p implicated in misfolded protein

recognition for its requirement in substrate ubiquitination. Using truncated constructs of Hrd1p in both *in vitro* and *in vivo* assays of Hrd1p function, we determined this portion of Hrd1p was not necessary for substrate recognition *in vitro* or for Hmg2p-GFP degradation *in vivo*. Most importantly, we observed that the ubiquitination of denatured substrates *in vitro* was not a property specific to the Hrd1p RING, but rather a property shared by another E3 whose known function is not in protein quality-control like Hrd1p, but in recognizing a specific protein. Thus, we determined that this *in vitro* assay of Hrd1p function might not accurately recapitulate physiologically relevant aspects of Hrd1p substrate recognition and ubiquitination, emphasizing the need for a new approach to studying Hrd1p function.

## Results:

Earlier *in vitro* studies using a soluble portion of the Hrd1p ubiquitin ligase (E3) suggested that misfolded proteins might be a preferred target of ubiquitination for Hrd1p (Bays, et al., 2001). Because Hrd1p targets misfolded proteins for ubiquitination *in vivo* (Bordallo, et al., 1998; Plemper, et al., 1998; Wilhovsky, et al., 2000), we thought Hrd1p might have an intrinsic ability to recognize misfolded proteins. We hoped to explore this property of Hrd1p using assays of *in vitro* ubiquitination activity to understand how Hrd1p might accomplish this, and to determine which portion of Hrd1p is involved in specific recognition of misfolded substrates.

Though full-length Hrd1p is membrane localized, a soluble portion of the cytosolic domain of Hrd1p was fused to GST (GST-Hrd1p) and affinity-purified. *In vitro* ubiquitination reactions were run as described (Bays, et al., 2001), by combining ATP, ubiquitin, E1, E2, affinity-purified GST-Hrd1p, and heat-denatured bovine serum albumin (BSA) as a model quality control substrate. Biotinylated BSA was incubated for 5 minutes in either a 100˚C water bath (boiled) or a room temperature water bath (unboiled) before addition to the *in vitro* reactions, which were then incubated at 30˚C for 2 hours. These reactions were stopped with denaturing solutions, then incubated with a biotin-binding resin to capture the BSA-biotin substrate. The resin was washed to remove non-biotinylated reaction proteins, aspirated, and heated in sample buffer to release the BSA-biotin. SDS-PAGE and ubiquitin immunoblotting revealed the extent to which native or heat-denatured BSA-

biotin was ubiquitinated by the GST-Hrd1p fusion. As expected, no BSA-biotin and unboiled BSA-biotin reactions showed weak ubiquitination (Figure 2-1, lanes 1 and 2) in comparison to an identical reaction with boiled BSA-biotin (Figure 2-1, lane 3). In the absence of ubiquitin or GST-Hrd1p (Figure 2-1, lanes 4 and 5) the high molecular weight immunoreactivity was nearly absent. As a control for equal precipitation of BSA-biotin from the *in vitro* reactions, horseradish-peroxidase-conjugated streptavidin (streptavidin-HRP) was used to directly detect BSA-biotin on western blots (Figure 2-1, lower panel). From these blots we could also see the change in molecular weight of a portion of the BSA-biotin in the assay, confirming that BSA-biotin itself was ubiquitinated, and that this ubiquitination was strongest with boiled BSA-biotin. This suggested GST-Hrd1p had the intrinsic ability to recognize and ubiquitinate a misfolded protein *in vitro*, as previously observed (Bays, et al., 2001). Figure 2-1 also highlights the sensitivity of anti-ubiquitin immunoblots. Although the blots for BSA-biotin showed only a small fraction of the BSA was ubiquitinated and shifted to a higher molecular weight, there was strong ubiquitin immunoreactivity, especially at high molecular weights, because each additional ubiquitin molecule is also an additional epitope for the ubiquitin antibodies. It is noteworthy that while the ubiquitination in these *in vitro* reactions was highly processive resulting in ubiquitin immunoreactivity at very high molecular weight, only a small fraction of the BSA substrate was shifted from the major BSA-biotin band by addition of ubiquitin.

Hrd1p degrades a variety of misfolded proteins *in vivo* (Bordallo, et al., 1998; Hampton, et al., 1996b; Plemper, et al., 1998; Wilhovsky, et al., 2000), but it is not

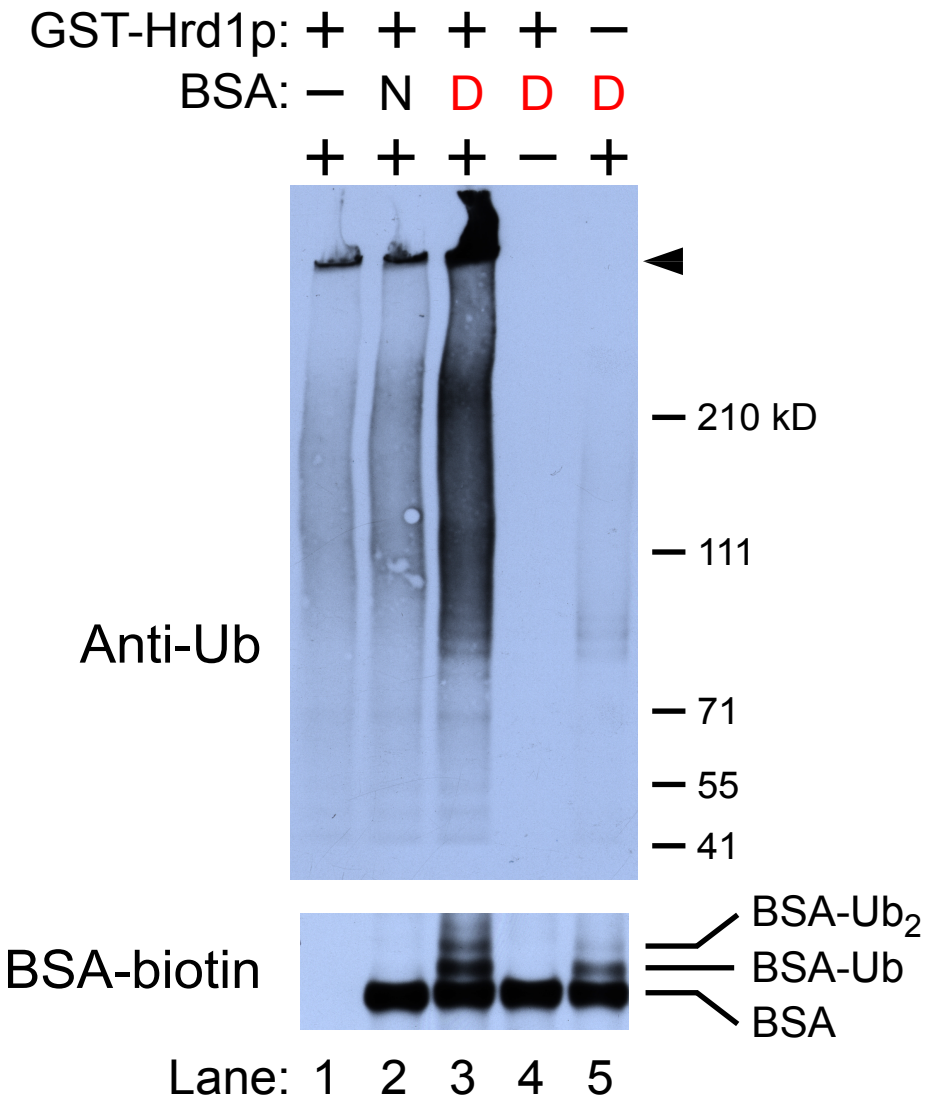


**Figure 2-1: Hrd1p prefers to ubiquitinate a denatured substrate.**

*In vitro* ubiquitination reactions with GST-Hrd1p were run with native (N) or denatured (D) BSA-biotin. Reactions were stopped and BSA-biotin was captured using a biotin-binding resin. Ubiquitin immunoblotting (upper panel) revealed the formation of high molecular weight ubiquitin chains. Blotting with horseradish peroxidase-conguated streptavidin to detect BSA-biotin (lower panel) allowed detection of BSA-biotin (BSA), and the higher-mobility ubiquitinated BSA-biotin (BSA-Ub). Proteins were resolved on 3-8% gradient SDS-PAGE gels (Nupage). Arrowhead indicates bottom of sample-loading wells.

known how these proteins are recognized, and how they are distinguished from the pool of correctly folded proteins. Although the BSA-biotin in our *in vitro* assay is not a native substrate of yeast Hrd1p, it is a secreted protein that must first traverse the ER before exiting the cell. Its denaturation-dependent ubiquitination suggested that this model misfolded substrate might display hallmarks of misfolded proteins detected by Hrd1p *in vivo*. We hoped to use this assay to better understand how the Hrd1p ubiquitin ligase recognizes its misfolded protein targets.

Since Hrd1p can detect a variety of quality-control substrates *in vivo*, we thought Hrd1p might also ubiquitinate many different proteins in our *in vitro* ubiquitination assay. We obtained biotinylated preparations of BSA, ß-galactosidase, concanavalin A, lactoperoxidase, and transferrin, and subjected them to thermal denaturation as before. To examine the solubility of these proteins after the denaturing heat treatment, we subjected the heat-treated proteins to centrifugation at 20,000 x g. Supernatant (S) and pellet (P) fractions were compared to unboiled supernatants representing total protein (T), and SDS-PAGE and Coomassie staining revealed the relative solubility of each protein after heat treatment. Most of these biotinylated proteins were not soluble after heat treatment (Figure 2-2). While BSA and transferrin had much more protein in the supernatant than pellet fractions, ß-galactosidase, concanavalin A, and lactoperoxidase were poorly soluble, with most protein in the pellet fraction. The lack of solubility of these proteins made them poor candidates for testing ubiquitination by Hrd1p in our soluble *in vitro* ubiquitination assay.

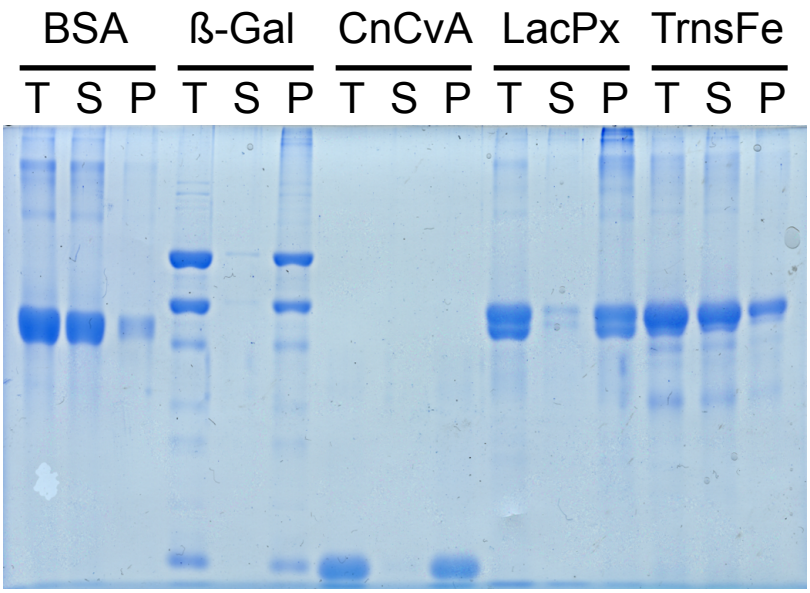


**Figure 2-2: Solubility of Heat-denatured biotinylated proteins.**

Biotinylated preparations of bovine serum albumin (BSA), ß-galactosidase (ß-Gal), concanavalin-A (CnCvA), lactoperoxidase (LacPx), and transferrin (TrnsFe) at 1mg/ml in HDB buffer were heated in 100˚C water bath for 5 min. Supernatant (S) and pellet (P) fractions of heat-treated biotinylated proteins were compared to total soluble fraction of unheated protein (T). Proteins were resolved by SDS-PAGE (8%) and Coomassie stained.

Like BSA-biotin, biotinylated transferrin (TrFe-biotin) was largely soluble after heat treatment, so we prepared ubiquitination reactions with boiled or unboiled TrFe-biotin as substrate for GST-Hrd1p. These reactions were stopped with denaturing solutions, then incubated with a biotin-binding resin to capture the TrFe-biotin substrate. As before, the resin was washed to remove non-biotinylated reaction components, aspirated, and heated in sample buffer to release the substrate for SDS-PAGE and ubiquitin immunoblotting. The TrFe-biotin retrieved from the *in vitro* reactions showed high molecular weight ubiquitin immunoreactivity that required both Hrd1p and ubiquitin (Figure 2-3). However, ubiquitination of TrFe-biotin was not improved by heating the substrate, as was observed with BSA-biotin. Thus, denaturation-dependent ubiquitination by Hrd1p was observed with BSA-biotin as substrate, but not TrFe-biotin. Although biotinylated ß-galactosidase, concanavalin A, and lactoperoxidase were largely insoluble upon heat treatment as observed in Figure 2-2, we tested these substrates in the *in vitro* substrate ubiquitination assay (data not shown). We observed some ubiquitination of both heated and unheated substrate proteins but the unequal solubility of these unboiled and boiled substrates made meaningful comparisons of the relative ubiquitination intensity between boiled and unboiled substrate difficult. Although Hrd1p targets multiple proteins for degradation *in vivo*, among the few test substrates examined *in vitro*, strong denaturation-dependent substrate ubiquitination was only observed with BSA-biotin. Using this biotinylated BSA substrate, we proceeded to examine the requirements of the Hrd1p protein for ubiquitination of BSA-biotin.

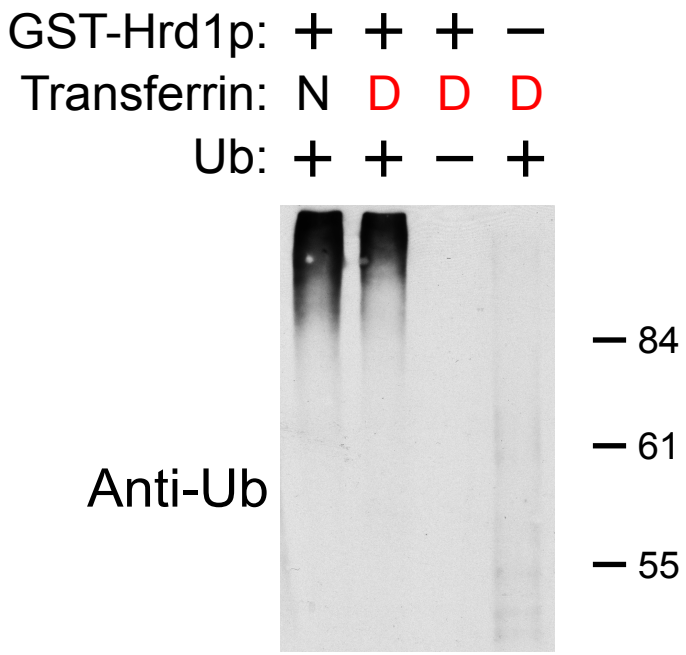


**Figure 2-3: Hrd1p shows equal preference for denatured and un-heated transferrin.**

*In vitro* ubiquitination reactions with GST-Hrd1p were prepared with native (N) or denatured (D) transferrin-biotin. Reactions were stopped and transferrin-biotin was captured with biotin-binding resin. Ubiquitin immunoblotting indicated transferrin-biotin is not preferentially ubiquitinated by Hrd1p upon denaturation. Proteins were resolved by SDS-PAGE (8%).

Using the *in vitro* denaturation-dependent substrate ubiquitination assay, we sought to identify the portion of Hrd1p responsible for misfolded protein recognition. The GST-Hrd1p construct used in these *in vitro* assays contained only the 204 c-terminal amino acids of Hrd1p, which includes the complete RING motif (Figure 2-4). We reasoned that if the soluble cytoplasmic domain of Hrd1p did indeed have a misfolded protein recognition module, that it must be in this portion of Hrd1p. To test this idea, we generated two truncations removing c-terminal sequence from GST-Hrd1p to examine the requirement of these sequences for Hrd1p function *in vitro*. The truncation constructs, GST-Hrd1p-Tr1 and GST-Hrd1p-Tr2 (Figure 2-4), were expressed in bacteria and purified in the same manner as GST-Hrd1p, and the purified proteins were tested *in vitro* for ubiquitin ligase activity and for substrate ubiquitination.

First we used these proteins as E3 in the substrate-independent ubiquitination assay. *In vitro* ubiquitination reactions were run as before by combining ATP, ubiquitin, E1, E2, and the appropriate affinity-purified GST-Hrd1p construct. Total reactions were resolved by SDS-PAGE, and ubiquitin immunoblotting revealed the formation of polyubiquitin chains. Although they were weaker than the GST-Hrd1p, the high molecular weight ubiquitin immunoreactivity observed with both truncations confirmed that GST-Hrd1p-Tr1 and GST-Hrd1pTr2 were active E3s (Figure 2-5). The E3 activity of the truncations was much better than the inactive C399S point mutant of Hrd1p or the no E3 control. We then wanted to use these constructs of

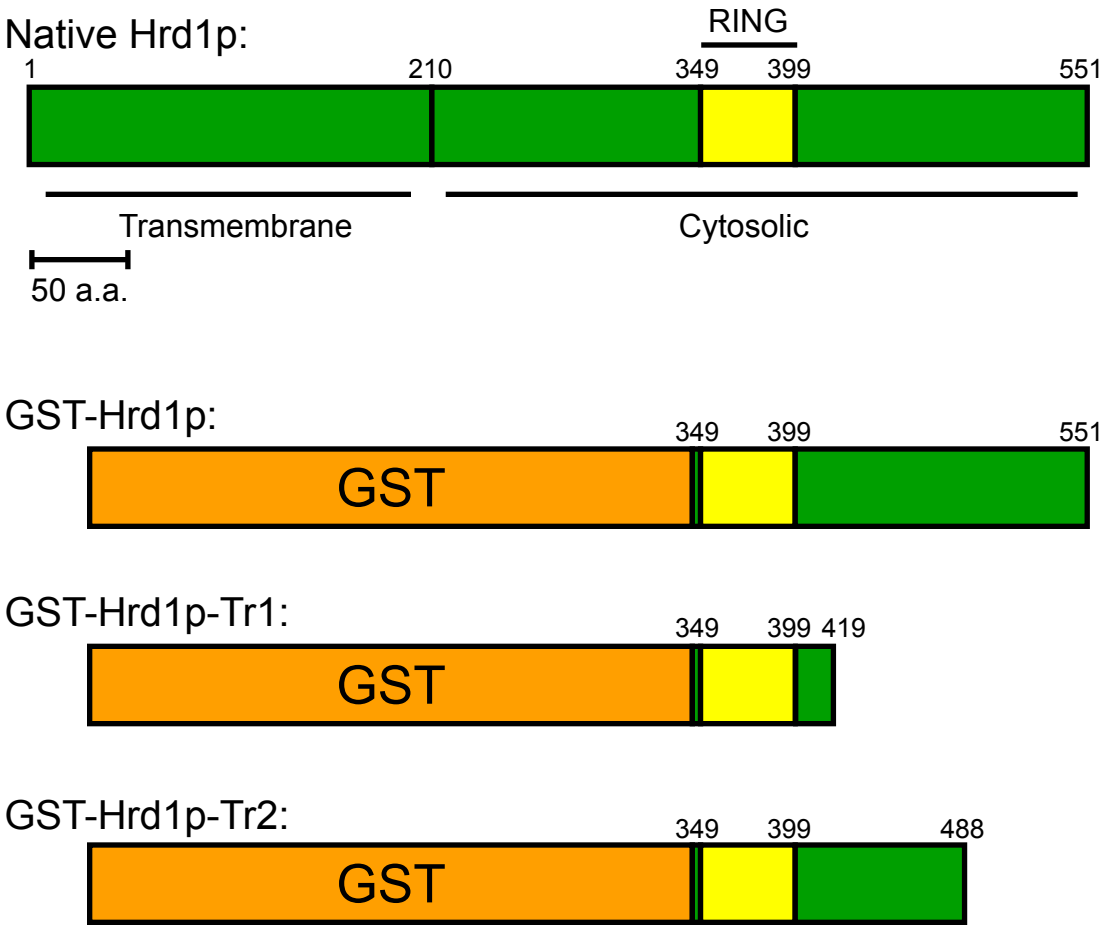


**Figure 2-4: Schematic of Hrd1p and GST-Hrd1p constructs.**

**A.** A representation of Hrd1p primary sequence, highlighting the transmembrane region, cytosolic region, and RING motif, drawn to the scale indicated. **B.** GST-Hrd1p and truncations of the Hrd1p c-terminus (GST-Hrd1p-Tr1 and GST-Hrd1p-Tr2), drawn to scale as in A, with glutathione-s-transferase (GST) fused to portions of Hrd1p as indicated.

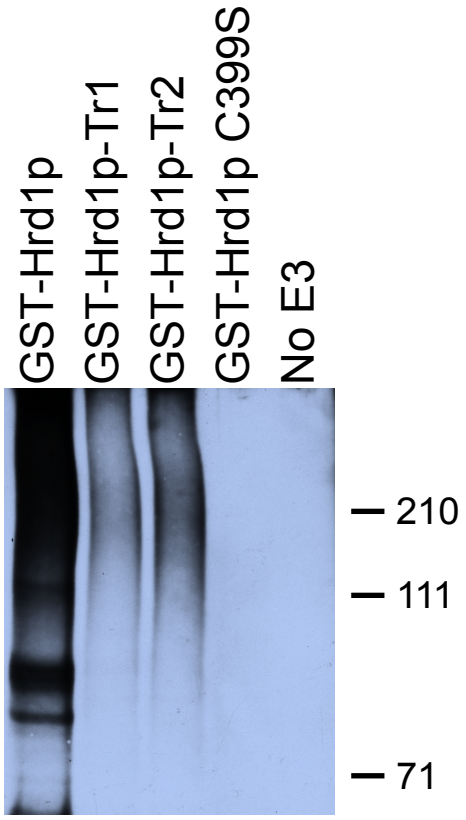


**Figure 2-5: Truncations of Hrd1p are active ubiquitin ligases.**

The formation of polyubiquitin chains in substrate-independent *in vitro* ubiquitination reactions was evaluated with the indicated Hrd1p constructs described in Fig 2-4. Reaction mixes were resolved by SDS-PAGE (8%) and ubiquitin immunoblotting.

Hrd1p to examine what role, if any, the c-terminal portion of Hrd1p was playing in the recognition of misfolded substrates.

Since the E3 activity of GST-Hrd1p-Tr1 was similar to GST-Hrd1p-Tr2, we tested the more severe GST-Hrd1p-Tr1 truncation in the BSA substrate ubiquitination assay to ask if Hrd1p sequence c-terminal to the RING motif was necessary for recognition of denatured BSA-biotin. As before, reaction were run with unboiled or boiled BSA, incubated with resin to capture the biotinylated BSA substrate, then resolved by SDS-PAGE and immunoblotted with ubiquitin antibodies. Both the GST-Hrd1p and GST-Hrd1p-Tr1 were able to ubiquitinate BSA-biotin in a denaturation-dependent manner (Figure 2-6). This was detected both by increased high molecular weight ubiquitin immunoreactivity (upper panel) and the mobility-shift of BSA-biotin (lower panel). GST-Hrd1p-Tr1 showed less ubiquitination activity than GST-Hrd1p with both unboiled and boiled BSA-biotin, consistent with the reduced E3 activity of GST-Hrd1p-Tr1 seen in Figure 2-5. Nonetheless, both GST-Hrd1p and the truncated GST-Hrd1p-Tr1 showed enhanced ubiquitination of boiled BSA-biotin compared to unboiled BSA-biotin, suggesting that both E3s retained the capacity to recognize the misfolded substrate. Results obtained with GST-Hrd1p-Tr2 were similar to those with GST-Hrd1p-Tr1 (data not shown). These results suggested that the c-terminal portion of Hrd1p was not involved in the recognition of misfolded protein in the *in vitro* assay.

Our goal for studying Hrd1p *in vitro* was to learn how Hrd1p recognizes misfolded-protein targets *in vivo*. If the *in vitro* assay was making physiologically-relevant predictions of Hrd1p function, then the c-terminus of Hrd1p should be

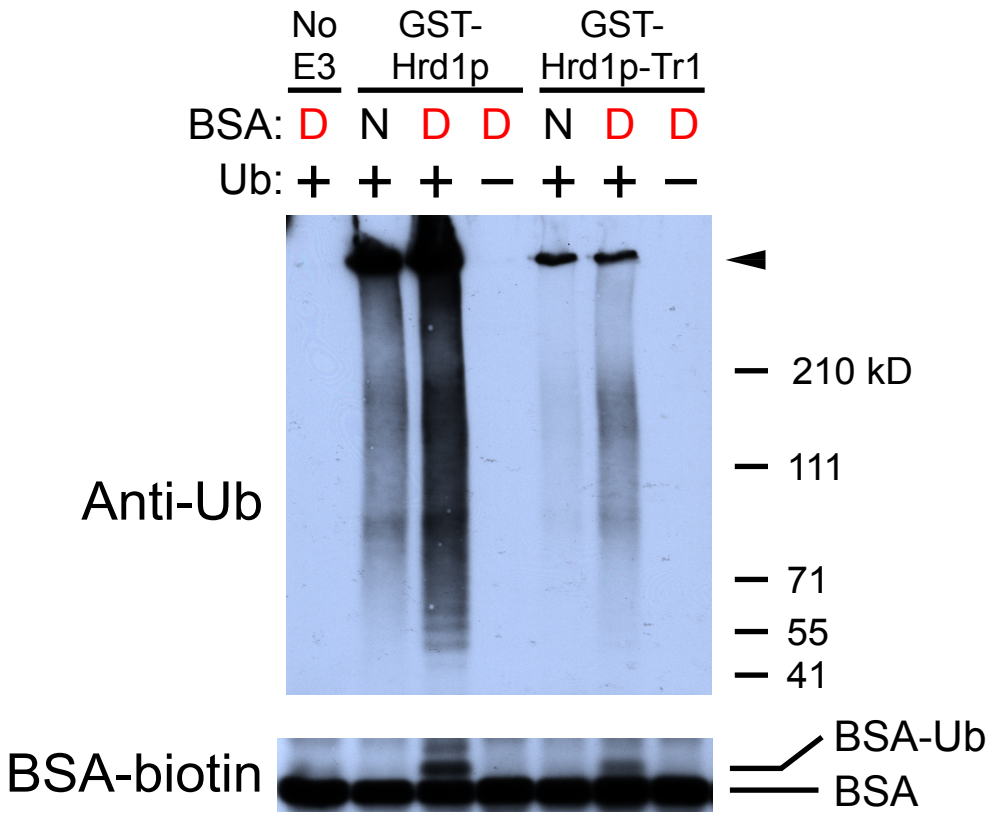


**Figure 2-6: The Hrd1p c-terminus is not necessary for denatured-substrate ubiquitination.**

*In vitro* ubiquitination reactions with GST-Hrd1p or the GST-Hrd1p-Tr1 c-terminal truncation as E3 were prepared with native (N) or denatured (D) BSA-biotin. Reactions were stopped and BSA-biotin was captured using a biotin-binding resin. Ubiquitin immunoblotting (upper panel) and horseradish-peroxidase-conguated streptavidin blotting (lower panel) indicate that denature BSA-biotin was preferentially ubiquitinated by both GST-Hrd1p and GST-Hrd1p-Tr1. Proteins were resolved on 3-8% gradient SDS-PAGE gels (Nupage). Arrowhead indicates bottom of sample-loading wells.

dispensable for the recognition of degradation substrates *in vivo*. To test this prediction, we assayed c-terminal truncations of Hrd1p using an *in vivo* assay of Hrd1p function. As Hrd1p is required for the degradation of Hmg2p-GFP, *hrd1*Δ null allele strains have increased Hmg2p-GFP and thus, increased fluorescence. Introduction of a Hrd1p-encoding plasmid complements the *hrd1*Δ mutation, restoring degradation of Hmg2p-GFP and reducing GFP fluorescence in the strain. These differences in Hmg2p-GFP fluorescence can be measured in living cells by flow microfluorimetry. Thus, a *hrd1*Δ Hmg2p-GFP-expressing test strain allows facile measurement of the *in vivo* Hrd1p activity of any version of Hrd1p expressed in it (Bazirgan, et al., 2006; Gardner, et al., 2000). HA epitope-tagged full-length Hrd1p (Hrd1p-WT) or versions with the c-terminal truncations depicted in Figure 2-7A (Hrd1p-Tr1 and Hrd1p-Tr2) were expressed from the native *HRD1* promoter in the assay strain described above and subjected to flow cytometry. Histograms plotting the number of cells (y-axis) having a given arbitrary fluorescence (x-axis) were generated for each strain, indicating the Hmg2p-GFP level in the presence of each Hrd1p variant (Figure 2-7B, left panel). The histogram of the strain with empty vector is shifted to the right, because Hmg2p-GFP levels are high in this *hrd1*Δ strain. The identical strain expressing Hrd1p-WT has much lower levels of Hmg2p-GFP, and a histogram shifted to the left, because Hmg2p-GFP can be degraded in this strain (Figure 2-7B, left panel). Both Hrd1p-Tr1 and Hrd1p-Tr2 partially complemented the *hrd1*Δ null allele as measured by the partial reduction in Hmg2p-GFP fluorescence compared to empty vector and Hrd1p-WT (Figure 2-7B, left panel). Both c-terminal truncations of

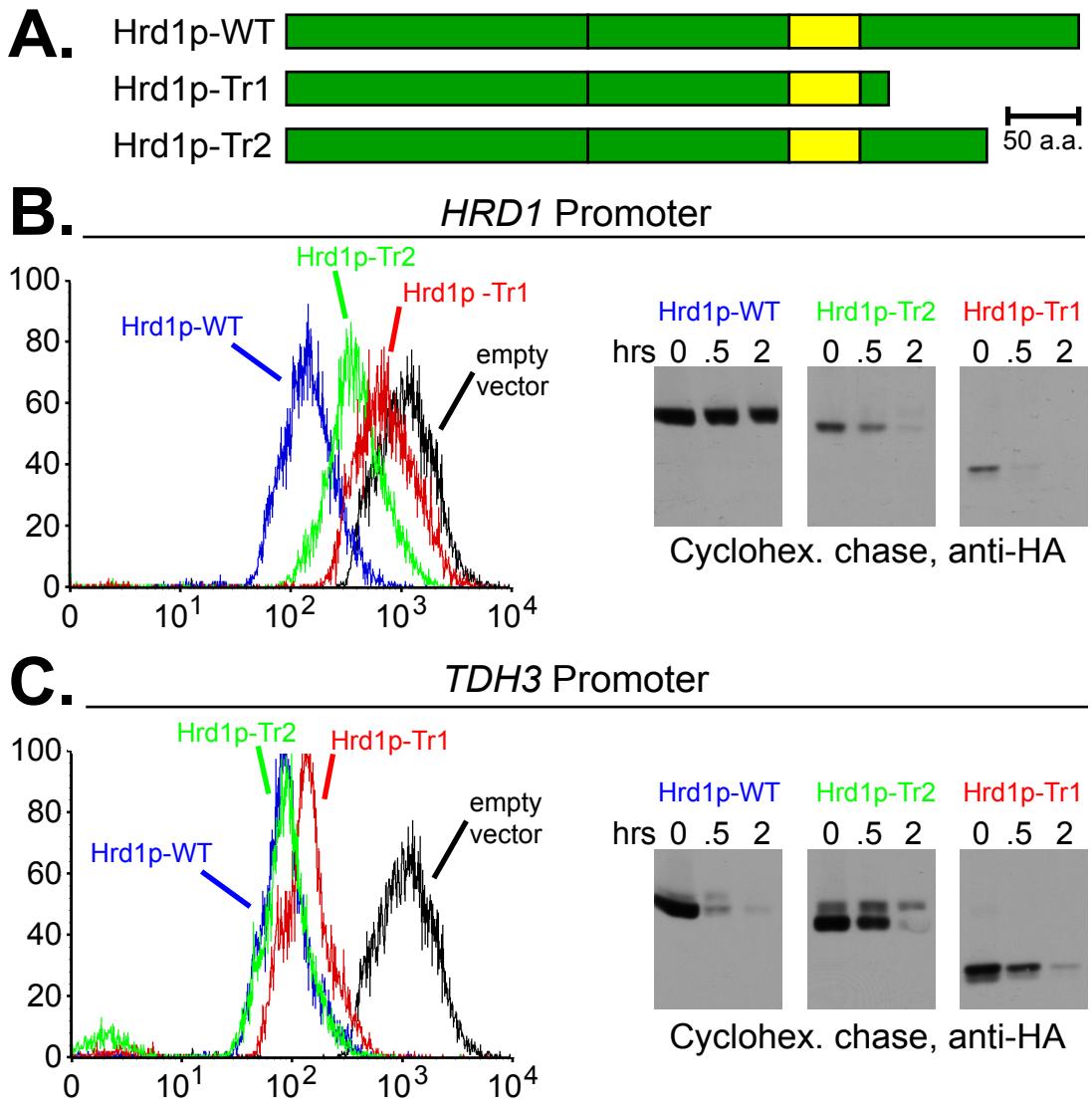


**Figure 2-7: The Hrd1p c-terminus is not necessary for ERAD *in vivo*.**

**A.** Schematic of Hrd1p and c-terminal truncations of Hrd1p expressed *in vivo*, drawn to the indicated scale. **B.** Hrd1p and c-terminal truncations expressed from the native promoter show partial complementation of the *hrd1*Δ allele. Hmg2p-GFP levels were measured in *hrd1*Δ strains expressing either empty vector or the indicated Hrd1p construct (left panel). Histograms plot the number of cells (y-axis) having a given arbitrary fluorescence (x-axis). Expression and stability of HA-epitope tagged Hrd1p constructs *in vivo* was examined by cycloheximide chase assays with HA-epitope immunoblotting (right panels). **C.** Hrd1p and c-terminal truncations expressed from the strong *TDH3* promoter showed complete complementation of the *hrd1*Δ allele. Hmg2p-GFP levels were measured (left panel) and cycloheximide chase assays performed (right panels) as in B.

Hrd1p showed some capacity to degrade Hmg2p-GFP, suggesting the c-terminus of Hrd1p was not required for substrate recognition. However, if these truncation constructs were able to degrade Hmg2p, why were they not fully cable to do so like full-length Hrd1p?

We used the identical HA-epitope tags in Hrd1p-WT, Hrd1p-Tr1, and Hrd1p-Tr2 to examine their *in vivo* expression levels. Although expressed from the same promoter, we thought the truncated proteins might be less stable *in vivo* than full-length Hrd1p, so we examined the stability of each Hrd1p construct using a cycloheximide chase degradation assay (Hampton, et al., 1996c). Treatment of growing cells with cycloheximide inhibits protein translation while allowing degradation of already expressed proteins. Whole-cell lysates from strains incubated in cycloheximide for the indicated times were resolved by SDS-PAGE and immunoblotted with HA-epitope antibodies. As expected, full-length Hrd1p-WT was completely stable (Figure 2-7B, right panels) (Bazirgan, et al., 2006; Gardner, et al., 2000). The Tr1 and Tr2 truncations of Hrd1p had reduced steady state levels, and the Hrd1p constructs underwent degradation in the cycloheximide chase assay (Figure 2-7B, right panels). Interestingly, the level of Hrd1p-variant *in vivo* correlated with the extent of Hmg2p-GFP degradation: Hrd1p-Tr2 was more abundant than Hrd1p-Tr1, and more Hmg2p-GFP degradation occurred in the presence of Hrd1p-Tr2 than Hrd1p-Tr1. Full-length Hrd1p was more abundant than either truncation, and promoted the most Hmg2p-GFP degradation. Despite the unequal expression levels *in vivo* of these Hrd1p constructs, the partial complementation of the *hrd1*Δ null allele

observed with both truncations implied that the c-terminus of Hrd1p was not required for ERAD of Hmg2p-GFP.

If incomplete complementation of *hrd1*Δ by Hrd1p-Tr2 and Hrd1p-Tr1 was due only to their insufficient expression compared to Hrd1p-WT, and the c-terminus of Hrd1p was not important for the degradation of Hmg2p-GFP, then we would predict overexpression of these constructs would improve Hmg2p-GFP degradation. Hrd1p, Hrd1p-Tr1 and Hrd1p-Tr2 were expressed from the strong *TDH3* promoter in the same *hrd1*Δ strain with Hmg2p-GFP as above, and assayed for Hmg2p-degradation by flow cytometry. Both Hrd1p-Tr1 and Hrd1p-Tr2 showed strong stimulation of Hmg2p-GFP degradation when overexpressed (Figure 2-7C, left panel). Strains overexpressing Hrd1p-Tr2 and full-length Hrd1p-WT had superimposable fluorescence histograms. Overexpressed Hrd1p-Tr1 also showed substantial reduction of Hmg2p-GFP levels. Interestingly, overexpressed Hrd1p-Tr1 levels were similar to native-promoter Hrd1p-WT levels, and these proteins showed similar capacity to degrade Hmg2p-GFP. Thus, sufficient expression of the most severe Hrd1p c-terminal truncation still allowed normal degradation of Hmg2p-GFP, suggesting that the portion of Hrd1p absent in Hrd1p-Tr1 (amino acids 420-551) play no role in recognizing degradation substrates of Hrd1p. These *in vivo* results were consistent with the results observed *in vitro*, where the equivalent c-terminal truncations of GST-Hrd1p were also able to ubiquitinate heat-denatured BSA-biotin.

We were surprised that the GST-Hrd1p-Tr1 construct, containing little more sequence of Hrd1p than the RING motif (Figure 2-4), was able to ubiquitinate the

model denatured BSA-biotin substrate *in vitro*. Finding that the Hrd1p c-terminus was not required for ERAD *in vivo*, we wondered if recognition of misfolded proteins by Hrd1p might be an inherent property of the Hrd1p RING motif. To test this idea we could not simply remove the RING motif, because the RING is required for E3 activity, which is necessary for our assays of substrate recognition and ubiquitination. We could, however, compare the activity of the Hrd1p RING with the RING motif from another protein. We designed two GST fusions that were identical except for their RING motif sequence, and chose the Praja1 protein to supply the comparison RING motif. Praja1 is a mammalian E3 that degrades Dlxin-1, a regulator of the homeodomain transcription factor Dlx5 (Sasaki, et al., 2002). The Praja1 ubiquitin ligase plays no known role in protein quality control, but like Hrd1p, it stimulates polyubiquitin chain formation in substrate-independent *in vitro* ubiquitination assays (Lorick, et al., 1999). GST-Praja1-RING is identical to GST-Hrd1p-RING except for precise replacement of the Hrd1p RING motif (amino acids 349-399) with the Praja1 RING motif (Figure 2-8A). To confirm the E3 activity of GST-Hrd1p-RING and GST-Praja1-RING, *in vitro* ubiquitination reactions were run as before by combining ATP, ubiquitin, E1, E2, and a GST-E3 construct. Total reactions were resolved by SDS-PAGE, and ubiquitin immunoblotting revealed the formation of polyubiquitin chains. GST-Hrd1p, GST-Hrd1p-RING, and GST-Praja1-RING all showed strong ubiquitin ligase activity in comparison to the GST-Hrd1p-RING(C399S) negative control, which has an inactivating point-mutation in the RING motif (Figure 2-8B).

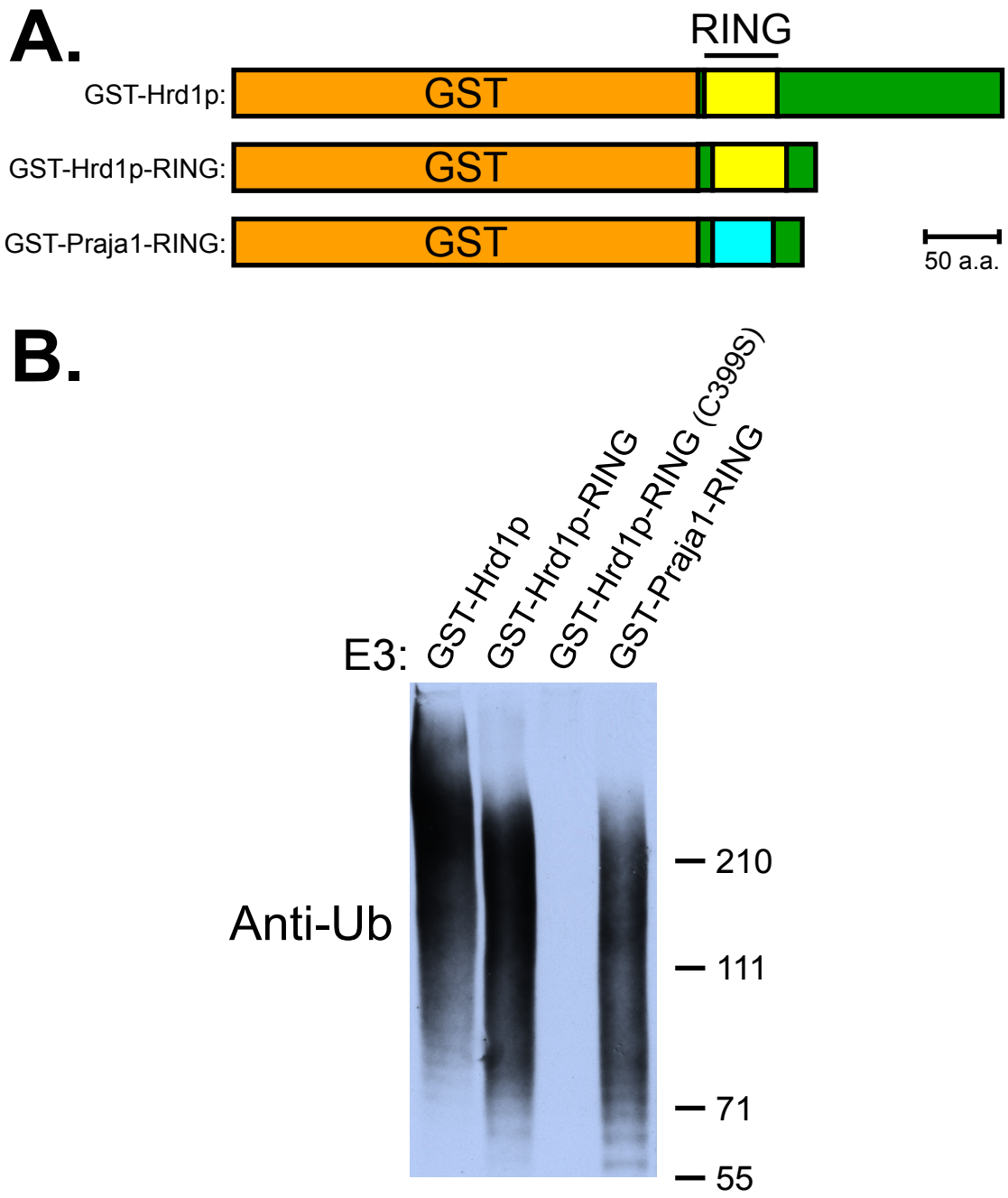


**Figure 2-8: GST-Hrd1p-RING and GST-Praja1-RING have E3 activity.**

**A.** Schematic of the GST-RING constructs tested *in vitro*. **B.** The formation of polyubiquitin chains in substrate-independent *in vitro* ubiquitination reactions was evaluated with the RING motif-containing proteins in A. After incubation, reaction mixes were resolved by 3-8% gradient SDS-PAGE and ubiquitin immunoblotting. GST-Hrd1p-RING and GST-Praja1-RING were slightly less active than GST-Hrd1p, suggesting little more than the RING motif was required for E3 activity.

GST-Hrd1p-RING and GST-Praja1-RING showed comparable E3 activity, suggesting they are similarly potent E3s.

Using this pair of GST-RING E3s, we could ask whether the Hrd1p RING motif had a unique capacity to seek out misfolded proteins *in vitro* by comparing GST-Hrd1p-RING to GST-Praja1-RING in the denatured substrate ubiquitination assay. As before, ATP, ubiquitin, E1, E2, and a GST-RING construct were combined with unboiled or boiled BSA-biotin, and incubated at 30˚C for 2 hours. The reactions were stopped, incubated with biotin-binding resin to capture the BSA-biotin substrate, and the resin-bound protein was resolved by SDS-PAGE and immunoblotted with ubiquitin antibodies. To our surprise, both GST-Hrd1p-RING and GST-Praja1-RING showed more ubiquitination with boiled BSA-biotin than unboiled BSA-biotin, as did the GST-Hrd1p control (Figure 2-9). Apparently, the preference of denatured BSA over native BSA was not only a property of the Hrd1p RING motif, but also of the Praja1 RING motif as well.

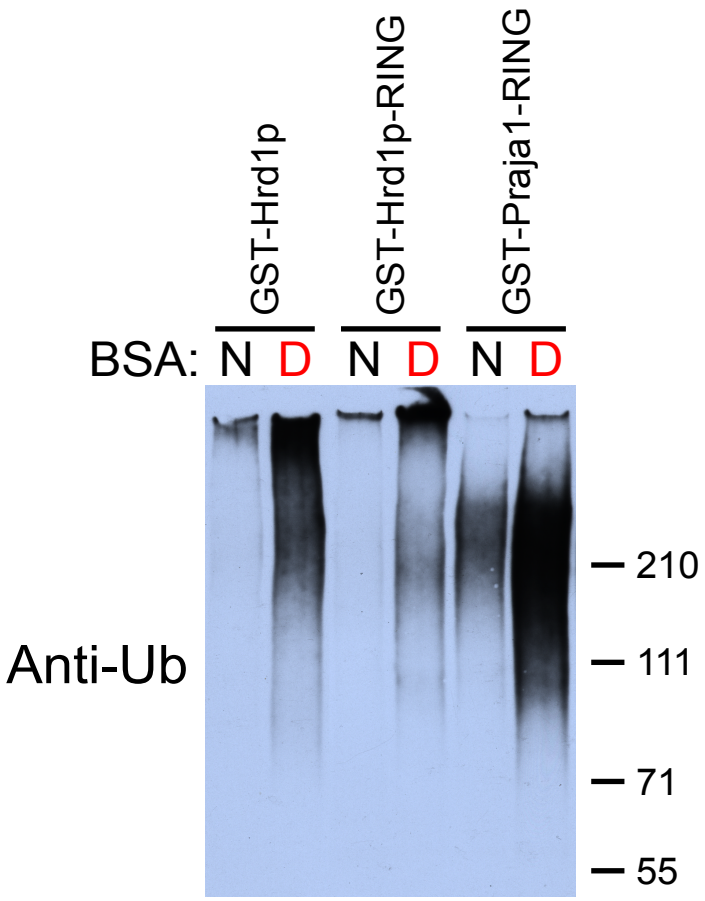


**Figure 2-9: The preference for a denatured substrate was not specific to the Hrd1p RING.**

*In vitro* ubiquitination reactions with GST-Hrd1p, GST-Hrd1p-RING or GST-Praja1-RING as E3 were prepared with native (N) or denatured (D) BSA-biotin. Ubiquitin immunoblotting indicated that denatured BSA-biotin was preferentially ubiquitinated by each of the E3s tested. Reactions were stopped and BSA-biotin was captured using a biotin-binding resin. Proteins were resolved on 3-8% gradient SDS-PAGE gels (Nupage). Arrowhead indicates bottom of sample-loading wells.

## Discussion:

Ubiquitin ligases are thought to facilitate the interaction of ubiquitin-conjugating enzymes (E2s) with a specific target for ubiquitination. The observation that Hrd1p preferred to ubiquitinate a misfolded substrate (Figure 2-1 and Bays, et. al., 2001) seemed consistent with the known function of Hrd1p as a quality-control ubiquitin ligase. Hrd1p works in concert with an accessory protein Hrd3p to form a HRD complex (Gardner, et al., 2000). In the absence of Hrd3p, ERAD is restored by overexpression of Hrd1p, suggesting that Hrd1p is the key component of HRD-dependent ERAD (Gardner, et al., 2000; Plemper, et al., 1999). Although recent findings have suggested there are more protein components in the HRD complex that could play the role of misfolded protein recognition, they had not been reported when these studies were conducted (Carvalho, et al., 2006; Denic, et al., 2006). While it still remains possible that Hrd1p recognizes misfolded proteins, there are important reasons why the *in vitro* assays described here did not demonstrate this.

In the substrate-independent ubiquitination assays, total reactions could be resolved by SDS-PAGE and immunoblotted to assess the formation of high molecular weight ubiquitin chains, which were readily produced if E1, E2, E3, ubiquitin, and ATP were present. These reactions are termed substrate-independent for their lack of an exogenous substrate, but the polyubiquitin chains do not appear to form spontaneously in solution without linkage to a target protein. In the substrate-independent ubiquitination reactions, ubiquitin immunoreactivity always appeared to begin at a molecular weight consistent with the RING motif-fusion plus one ubiquitin,

suggesting that ubiquitin chains were built on the E3 protein. Hrd1p self-degradation is observed *in vivo*: in the absence of Hrd3p, Hrd1p is degraded, keeping Hrd1p and Hrd3p in stoichiometric equivalence (Gardner, et al., 2000). The *in vivo* degradation of Hrd1p requires an intact RING motif and the E2 Ubc7p. Thus, it is possible that the substrate-independent ubiquitination is a manifestation of this natural property of Hrd1p. Alternatively, an *Arabidopsis* RING motif E3 that forms high molecular weight ubiquitin chains *in vitro* was found to form these chains on the affinity purification domain to which the RING motif was fused (Matsuda, et al., 2001). This suggests that substrate-independent ubiquitination, which is observed with many RING E3s, happens because the affinity-purification domain fused to the E3 is essentially a covalently attached artificial substrate. In either case, substrate-independent ubiquitination is a useful and facile assay of E3 activity *in vitro*.

However, this tendency for substrate-independent ubiquitination made the assay of substrate ubiquitination more challenging. We could not detect the ubiquitination of BSA without purification of the substrate away from the other reaction components, because substrate-independent ubiquitination was also occurring in these reactions. Except for the addition of substrate, the denatured-substrate assays and substrate-independent assays of ubiquitination contained the same reaction components. Consequently, the same high molecular weight ubiquitin chains formed in both reactions, obfuscating the weaker substrate ubiquitination. Thus, the substrate had to be isolated from the reaction mix to detect its ubiquitination. We chose to use biotinylated substrates to achieve this post-reaction purification, because biotin would

survive the denaturing conditions used to stop the reactions, and its high affinity for streptavidin would tolerate more stringent washing to remove the other polyubiquitinated reaction proteins. Although this seemed like a promising strategy, we later determined that the commercially-prepared biotinylated proteins were produced by linking biotin to primary amines— the same chemical group sought by ubiquitin's c-terminus for covalent attachment to substrates. The biotinylated substrates were essentially pre-blocked against ubiquitin linkage. We thought that the increased ubiquitination after heat treatment was due to improved Hrd1p interaction, but the heat-denaturation could have improved ubiquitination of BSA-biotin by increasing the solvent accessibility of unblocked lysine residues that were not exposed to biotin-conjugation chemistry before denaturation. However, it is difficult to understand why the same trend was not observed with transferrin-biotin, which was also biotinylated on primary amines. Transferrin was ubiquitinated both with and without heat treatment. It is possible that the biotinylation of transferrin was less extensive than that of BSA-biotin, and that BSA-biotin had nearly complete blockage of primary amines, but we did not examine this. At the concentrations of protein used in these *in vitro* ubiquitination assays, RING motifs may have non-specific avidity for proteins that allows transient interaction and ubiquitination. Of the five biotinylated substrates tested, only BSA-biotin was resistant to ubiquitination before heat treatment. We think non-denatured BSA-biotin was uniquely protected from ubiquitination by the extent of its biotinylation in comparison to the other substrates tested.

The *in vitro* ubiquitination assays were performed with the E2 HUBC4, a human E2 described previously and used widely to test E3 activity *in vitro* (Bays, et al., 2001; Joazeiro, et al., 1999; Mori, et al., 1997; Swanson, et al., 2001). HUBC4 is derived from the human E2 now termed Ubch5b, and its closest yeast homologs are the UBC4/UBC5 pair. In yeast, Hrd1p ubiquitination is accomplished through Ubc7p (Hampton and Bhakta, 1997), and to a much lesser extent Ubc1p (Bays, et al., 2001). It is possible that Hrd1p and Ubc7p *in vivo* cooperate to identify misfolded proteins in a way that Hrd1p and HUBC4 cannot. It is also possible that the few heat-denatured substrate proteins we tested *in vitro* were inherently different from substrates of Hrd1p in a way that we do not understand. Though there are several known substrates of Hrd1p, they are a small fraction of all the proteins in the ER, and the ER has other ubiquitin ligases for quality control in addition to Hrd1p (Swanson, et al., 2001). It might be possible to purify a native substrate of Hrd1p to test in the substrate ubiquitination assay. A point mutant of the gene encoding carboxypeptidase Y (CPY) expresses a misfolded protein (CPY*) that is retained in the ER and degraded by Hrd1p (Bordallo, et al., 1998). The native protein folds correctly and proceeds through the secretory pathway. Versions of CPY and CPY* could be purified and tested in the substrate ubiquitination assay, and existing antibodies to CPY could be used instead of biotin-binding resin to separate the substrate from other ubiquitinated proteins in the *in vitro* reactions.

Although there were difficulties with the *in vitro* experiments described, the results *in vivo* were much more conclusive. It was clear from these experiments that

the c-terminal truncations of Hrd1p were still able to facilitate Hmg2p-GFP ubiquitination, indicating that the c-terminal portion of Hrd1p was not necessary for recognition of this substrate protein. Although the truncations of Hrd1p were unstable proteins, sufficient expression allowed full ERAD function with Hmg2p-GFP. Hrd1p is normally a stable protein, but becomes unstable in the absence or Hrd3p. The destabilization of the Hrd1p truncations suggests that the c-terminal portion of Hrd1p was involved in sensing the presence of Hrd3p. Consistent with this, Hrd1p-Tr1 was degraded at a rate similar to full-length Hrd1p in the absence of Hrd3p.

It was surprising that little more than the RING motif, absent most of the sequence from the ubiquitin ligase, could support self-ubiquitination and transfer of ubiquitin to substrates in these *in vitro* assays. However, the *in vitro* denaturation-dependent ubiquitination of BSA-biotin was not specific to the Hrd1p RING, making it unlikely that this assay was reflecting the misfolded-protein scavenging function we presume for Hrd1p *in vivo*. Alternatively, if Praja1 is found to have a function in protein quality control, it might be worth revisiting this assay. These *in vitro* assay conditions have been used to demonstrate the ubiquitin ligase activity of several RING motif-containing proteins (Bays, et al., 2001; Joazeiro, et al., 1999; Kikkert, et al., 2004; Lorick, et al., 1999; Swanson, et al., 2001). While useful for testing the presence or absence of E3 activity, it is possible that more subtle aspects of Hrd1p function may be difficult to study in these conditions. Moreover, this assay was conducted with HUBC4, a different E2 than Hrd1p normally uses in the cell. Because ligases exhibit strong E2 preference *in vivo*, we wanted to examine Hrd1p ubiquitin

ligase activity with its native E2, Ubc7p. In the next chapter we examine Hrd1p function with Ubc7p *in vitro* and *in vivo*, using extensive comparisons between Hrd1p function *in vivo* and the *in vitro* assay activity to assure that the predictions of the new *in vitro* system are physiologically relevant to Hrd1p biology.

## Methods:

*Recombinant DNA* - All DNA segments synthesized by PCR were verified by sequencing. All GST-fusion proteins were expressed from the pET42b(+) bacterial expression plasmid (Novagen). GST-Hrd1p and GST-Hrd1p-C399S were expressed from the previously described pRH1466 and pRH1468, respectively (Bays, et al., 2001). Sequences encoding the c-terminal residues of Hrd1p were removed from pRH1466 using a PCR SOEing approach (Horton, et al., 1989) to produce GST-Hrd1p-Tr1 (pRH1590) and GST-Hrd1p-Tr2 (pRH1591). The production of the Hrd1p-3HA coding region was described (Gardner, et al., 2000), and this sequence was positioned behind the native *HRD1* promoter in pRH1650 to express Hrd1p-WT. Sequences encoding the c-terminal residues of Hrd1p were removed from pRH1650 using a PCR SOEing approach to produce Hrd1p-Tr1 (pRH11651) and Hrd1p-Tr2 (pRH1652). The HA-epitope-tagged Hrd1p-encoding sequences were placed behind the strong *TDH3* promoter to overexpress Hrd1p-WT (pRH1653), Hrd1p-Tr1 (pRH1654), or Hrd1p-Tr2 (pRH1655). GST-Hrd1p-RING was made by PCR SOEing a portion of *HRD1* into the pET42b(+) bacterial expression plasmid to produce pRH1749. GST-Hrd1p-RING(C399S) was made by PCR SOEing a portion of *HRD1* bearing the C399S mutation into the pET42b(+) bacterial expression plasmid to produce pRH1750. GST-Praja1-RING was made by replacing the RING motif sequence of Hrd1p in pRH1749 with that from Praja1, using a PCR SOEing approach (pRH1751).

*Strains and Media* - Yeast were cultured at 30˚C as described, in minimal media with 2% glucose and amino acid supplements, and all yeast strains were derived from the same genetic background used in our previous work (Hampton, et al., 1996c; Hampton and Rine, 1994). Strains for evaluating the *in vivo* degradation of Hmg2p-GFP were derived from the previously described RHY853 (Gardner, et al., 2000), expressing Hmg2-GFP and the independently-expressed catalytic domain of Hmg2p as its sole source of HMG-CoA reductase. *HRD1* was replaced in RHY853 with the G418-resistance marker kanMX (Guldener, et al., 1996) to produce RHY2814, an assay strain to test Hrd1p function *in vivo* by observing degradation of Hmg2p-GFP (Bazirgan, et al., 2006). All Hrd1p expressing plasmids described above were linearized with *PmlI* and integrated into RHY2814 at the *TRP1* locus. RHY2940 was the empty vector control strain. RHY2949, RHY2951, and RHY2952 expressed Hrd1p-WT, Hrd1p-Tr1, and Hrd1p-Tr2, respectively, from the native *HRD1* promoter. RHY2953, RHY2954, and RHY2955 expressed Hrd1p-WT, Hrd1p-Tr1, and Hrd1p-Tr2, respectively, from the strong *TDH3* promoter.

*Protein Purification* - All recombinant proteins were expressed in Rosetta (DE3) *E. coli* (Novagen) grown in LB with appropriate antibiotics. 0.6 O.D./ml cultures were induced with 0.5mM IPTG for 12-16 hours at 15˚C. Bacterial pellets were harvested, washed in normal saline (0.9M NaCl), and frozen at –80˚C. Pellets were thawed and resuspended in extraction buffers as described below, and lysed using a Branson Sonifier 450 (VWR) with six rounds of 30s sonication/30s ice incubation. After affinity column purification, elution, and concentration, proteins

were dialyzed into buffer HDB containing 10% glycerol. Single-use aliquots were flash-frozen with liquid nitrogen and stored at –80˚C. Recombinant protein concentrations were determined by Coomassie staining of SDS-PAGE-resolved samples and comparison to BSA.

His-Tag purification of E1 and HUBC4: - Bacterial pellets from 2L cultures expressing 6-His mouse UBA1 or HUBC4 were resuspended in 40mls of His-Extraction Buffer (50mM sodium phosphate pH 7.4, 300mM NaCl, 0.5% Nonidet NP-40, 5% glycerol) with protease inhibitors (13 μM AEBSF, 3.6 μM TPCK, 2.6μM leupeptin, 1.8 μM pepstatin, 0.56mM 6-aminohexanoic acid, 0.56mM benzamidine, and 2.5mM 2-mercaptoethanol), sonicated as above, and centrifuged at 12,000 x g for 20minutes in an SS34 rotor. The supernatant was transferred to a new tube with 0.5ml Talon Cell-Thru resin (BD Biosciences) equilibrated in His-Extraction Buffer, and gently nutated for 20min at room temperature. The resin was centrifuged (3000g) and washed with 10mls His-Extraction Buffer and protease inhibitors above for 10min at RT two times. The washed resin was then transferred to a 1cm diameter column and washed with 30mls His-Wash Buffer (50mM sodium phosphate pH 7.8, 300mM NaCl, 5mM imidazole, 5% glycerol, 10mM 2-mercaptoethanol). His-tagged E1 was eluted from the resin with 3mls of His Wash Buffer + 150mM imidazole, and was collected in 500ul fractions and analyzed by Bradford assay with BSA standard. Fractions with more than 0.1 mg/ml protein were pooled and concentrated with Amicon Ultra-15 5,000 MWCO filters (Millipore). Concentrated protein was dialyzed over 24 hours with 3x 1L HDBG (25mM HEPES, 0.7mM sodium phosphate, 137mM

NaCl, 5mM KCl, pH 7.4, 10% glycerol) in a 3ml 10,000 MWCO Slide-A-Lyzer cassette (Pierce).

GST Protein Purification: - Each bacterial pellet from 1L of culture expressing a GST protein was resuspended in 25mls of buffer HDB (25mM HEPES, 0.7mM sodium phosphate, 137mM NaCl, 5mM KCl, pH 7.4) + protease inhibitors (7.5 mM EDTA, 1.5 mM PMSF, 10μM leupeptin, 7μM pepstatin, 28 μM TPCK, 130 μM AEBSF, 2.5 mM 6-aminohexanoic acid, 2.5mM benzamidine, and 7.5mM DTT), and sonicated as above. To this was added 6ml 2.5M NaCl, 600ul 1M sodium phosphate pH7.4, and 1.2ml 25% Triton X-100. This was nutated 20min at 4˚C, and centrifuged for 1hr at 40,000g in SS34 rotor. Buffer HDBW (HDB + 20mM sodium phosphate pH7.4, 10 mM 2-mercaptoethanol, 1% Triton X-100.) was added to the supernatant to a final volume of 50ml along with 1ml of glutathione-Sepharose-4B resin (Pharmacia) and nutated for 1hr at 4˚C. The resin was transferred to a 1cm diameter column and washed with 10 mls of each of the following buffers: HDBW with 1.25mM PMSF and 5mM EDTA; HDBW with 1.25mM PMSF, 5mM EDTA, and 0.5M NaCl; HDBW with 0.5% deoxycholate; HDBW with 0.5M NaCl; and Final Wash (40mM Tris pH8.0, 50mM NaCl, 10mM 2-mercaptoethanol). Protein was eluted in 1ml fractions of Elution Buffer (20mM Tris pH 8, 10mM 2-mercaptoethanol, 20mM reduced glutathione) incubated with column resin for 10min before recovery. Fractions were analyzed by Bradford assay with BSA standard. Fractions with more than 0.1 mg/ml protein were pooled and concentrated with Amicon Ultra-15 5,000 MWCO filters (Millipore). Concentrated protein was dialyzed for 24 hours with 3x 1L HDBG

(25mM HEPES, 0.7mM sodium phosphate, 137mM NaCl, 5mM KCl, pH 7.4, 10% glycerol) in a 0.5ml 3,000 MWCO Slide-A-Lyzer cassette (Pierce).

*Substrate-independent In Vitro Ubiquitination* - Ubiquitin was resuspended from lyophilized powder in Ubiquitin Storage Buffer (50mM Tris pH7.5, 50mM NaCl, 10% glycerol) and frozen. Reactions were performed in 1x Ubiquitination Buffer (50 mM Tris pH 7.5, 2.5 mM $MgCl_2$, 0.5 mM DTT) with 3mM ATP, 170 µg/ml ubiquitin, 10 µg/ml E1, 20 µg/ml E2, and 30 µg/ml of E3 (GST-fusion) in a total volume of 15µl. Reactions mixtures were prepared on ice, then incubated at 30˚C for 2 hours, and stopped with an equal volume of 2x sample buffer (4% SDS (w/v), 8M urea, 75mM MOPS pH6.8, 200mM DTT, 0.2 mg/ml bromphenol blue) and analyzed by SDS-PAGE and anti-ubiquitin immunoblotting.

*Denaturation-dependent In Vitro Substrate Ubiquitination* – A solution of HDB with 1mg/ml BSA-biotin (Sigma) was heated in a 100˚C water bath for 5 minutes (boiled) or incubated at room temperature (unboiled). *In vitro* ubiquitination reactions were prepared as above, and incubated as above with the addition of 1 µl of 1 mg/ml BSA-biotin, to a concentration of 70 µg/ml. Reactions were stopped with 100ul of SUME (1%w/v SDS, 8M urea, 10mM MOPS pH6.8, 10mM EDTA) and vortexed. 300µl of PC100 (20mM HEPES pH 7.9, 100mM KCl, 0.2mM EDTA, 5mM MgCl, 0.1% Triton X-100, 20% glycerol) with 5mg/ml BSA was added to each reaction. Neutravidin-agarose (Pierce), a resin-conjugated streptavidin variant, was pre-equilibrated in PC100 with 5mg/ml BSA. 60µl of 50 % slurry was added to each reaction tube, which were then nutated at room temperature for 2 hours. The

Neutravidin-agarose resin was pelleted by microcentrifugation at 1000 rpm for 30 seconds, washed twice with PC100+ (PC100 + 5mg/ml BSA), twice with PC100det (PC100 + 0.5% deoxycholate, 0.5% Triton X-100), once with PC100, and aspirated to dryness. Resin-bound protein was liberated with 50ul of 2x sample buffer and analyzed by SDS-PAGE and immunoblotting with anti-ubiquitin antibody or blotting with horseradish-peroxidase-conjugated streptavidin.

*Flow Cytometry* - Log phase cultures ($O.D._{600} < 0.5$) grown in minimal medium at 30˚C were transferred to flow cytometer sample tubes and measured with a Becton Dickinson FACScalibur instrument. Flow microfluorimetric data were analyzed and histograms plotting the number of cells (y-axis) with a given arbitrary fluorescence (x-axis) were generated using CellQuest flow cytometry software. In all cases, histograms represented 10,000 individual cells.

*Cycloheximide-Chase Degradation Assay* - This assay was performed as described (Hampton, et al., 1996a). Briefly, log phase cultures of cells expressing HA-epitope tagged Hrd1p or RING variants were treated with 50µg/ml cycloheximide to arrest protein synthesis. At indicated times, 1 O.D. of log phase cells were harvested, lysed, and 0.1 O.D. equivalents were resolved by SDS-PAGE and immunoblotted for epitope-tagged protein.

**Table 2-1. Strains used in chapter two.**

| **Strain** | ***Genotype*** | **Figure** |
|---|---|---|
| RHY2940 | *MATα ade2-101 met2 lys2-801 his3Δ200 leu2Δ hmg1Δ::LYS2 hmg2D::HIS3 ura3-52::URA3::pTDH3-HMGcd::pTDH3-HMG2-GFP hrd1Δ::KanMX trp1::hisG::TRP1(pRH311)* | 2-7 |
| RHY2941 | *MATα ade2-101 met2 lys2-801 his3Δ200 leu2Δ hmg1Δ::LYS2 hmg2D::HIS3 ura3-52::URA3::pTDH3-HMGcd::pTDH3-HMG2-GFP hrd1Δ::KanMX trp1::hisG::TRP1::HRD1-3HA(pRH642)* | 2-7 |
| RHY2951 | *MATα ade2-101 met2 lys2-801 his3Δ200 leu2Δ hmg1Δ::LYS2 hmg2D::HIS3 ura3-52::URA3::pTDH3-HMGcd::pTDH3-HMG2-GFP hrd1Δ::KanMX trp1::hisG::TRP1::HRD1-Tr1-3HA(pRH1651)* | 2-7 |
| RHY2952 | *MATα ade2-101 met2 lys2-801 his3Δ200 leu2Δ hmg1Δ::LYS2 hmg2D::HIS3 ura3-52::URA3::pTDH3-HMGcd::pTDH3-HMG2-GFP hrd1Δ::KanMX trp1::hisG::TRP1::HRD1-Tr2-3HA(pRH1652)* | 2-7 |
| RHY2953 | *MATα ade2-101 met2 lys2-801 his3Δ200 leu2Δ hmg1Δ::LYS2 hmg2D::HIS3 ura3-52::URA3::pTDH3-HMGcd::pTDH3-HMG2-GFP hrd1Δ::KanMX trp1::hisG::TRP1:: pTDH3-HRD1-3HA(pRH1653)* | 2-7 |
| RHY2954 | *MATα ade2-101 met2 lys2-801 his3Δ200 leu2Δ hmg1Δ::LYS2 hmg2D::HIS3 ura3-52::URA3::pTDH3-HMGcd::pTDH3-HMG2-GFP hrd1Δ::KanMX trp1::hisG::TRP1:: pTDH3-HRD1-Tr1-3HA(pRH1654)* | 2-7 |
| RHY2955 | *MATα ade2-101 met2 lys2-801 his3Δ200 leu2Δ hmg1Δ::LYS2 hmg2D::HIS3 ura3-52::URA3::pTDH3-HMGcd::pTDH3-HMG2-GFP hrd1Δ::KanMX trp1::hisG::TRP1:: pTDH3-HRD1-Tr2-3HA(pRH1655)* | 2-7 |

**Table 2-2. Plasmids used in chapter two.**

| Plasmid | Construction |
|---|---|
| pRH1289 | 6HIS-E1 (mouse UBA1) Mori, S. et al. *European Journal of Biochemistry* **247**, 1190-6 (1997). |
| pRH1290 | 6HIS-HUBC4 Mori, S. et al. *European Journal of Biochemistry* **247**, 1190-6 (1997). |
| pRH1466 | GST-Hrd1p, GST fused to the C-terminal 203 amino acids of Hrd1p in pET42b(+)<br>Bays, NCB |
| pRH1468 | GST-Hrd1p C399S, GST fused to the C-terminal 203 amino acids of Hrd1p in pET42b(+) with C399S mutation<br>Bays, NCB |
| pRH1590 | GST-Hrd1p-Tr1, GST fused to amino acids 348 to 419 of Hrd1p. |
| pRH1591 | GST-Hrd1p-Tr2, GST fused to amino acids 348 to 488 of Hrd1p. |
| pRH311 | pRS404, *TRP1*/YIp<br>Genetics 122: 19-27 (May, 1989) |
| pRH642 | $P_{HRD1}$-Hrd1p-3HA. *TRP1*/YIp<br>Native promoter Hrd1p from *hrd1-1* complementing plasmid was subcloned into pRS404 to make pRH507. PCR SOEing was used to add the 3HA tag. |
| pRH1650 | Native promoter Hrd1p-3HA. *TRP1/YIp* |
| pRH1651 | Native promoter Hrd1p-Tr1-3HA. *TRP1/YIp*<br>NsiI/SphI fragment of pRH1608 cloned into pRH642 opened with NsiI/SphI. |
| pRH1652 | Native promoter Hrd1p-Tr2-3HA. *TRP1/YIp*<br>NsiI/SphI fragment of pRH1609 cloned into pRH642 opened with NsiI/SphI. |
| pRH1653 | *TDH3* promoter Hrd1p-3HA. *TRP1/YIp*<br>NsiI/SphI fragment of pRH1600 cloned into pRH730 opened with NsiI/SphI. |
| pRH1654 | *TDH3* promoter Hrd1p-Tr1-3HA. *TRP1/YIp*<br>NsiI/SphI fragment of pRH1608 cloned into pRH730 opened with NsiI/SphI. |
| pRH1655 | *TDH3* promoter Hrd1p-Tr2-3HA. *TRP1/YIp*<br>NsiI/SphI fragment of pRH1609 cloned into pRH730 opened with NsiI/SphI. |
| pRH1749 | GST-Hrd1p-RING<br>The Hrd1p RING was amplified by PCR from pRH642 and subcloned into pET42b(+) with NcoI and EcoRI. |

**Table 2-2 continued. Plasmids used in chapter two.**

| Plasmid | Construction |
|---|---|
| pRH1750 | GST-C399S-Hrd1p-RING<br>The C399S-Hrd1p RING was amplified by PCR from pRH1245 and subcloned into pET42b(+) with NcoI and EcoRI. |
| pRH1751 | GST-Praja1-RING<br>The Praja1 RING was amplified by PCR from pRH1644 and subcloned into pET42b(+) with NcoI and EcoRI. |

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Allan M. Weissman provided a Praja1 plasmid used as PCR template to construct the Praja1 RING-containing GST fusion. I thank Nathan Bays for sharing his expertise in *in vitro* ubiquitination. I also thank Randy Hampton for his critical reading of this chapter.

## References:


1. 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. *Nat Cell Biol* **3** (1) 24-29.

2. Bazirgan, O. A., Garza, R. M. and Hampton, R. Y. (2006). Determinants of RING-E2 Fidelity for Hrd1p, a Membrane-anchored Ubiquitin Ligase. *J Biol Chem* **281** (51) 38989-39001.

3. Bordallo, J., Plemper, R. K., Finger, A. and Wolf, D. H. (1998). Der3p/Hrd1p is required for endoplasmic reticulum-associated degradation of misfolded lumenal and integral membrane proteins. *Mol Biol Cell* **9** (1) 209-222.

4. 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.

5. 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.

6. 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. *Mol Cell Biol* **21** (13) 4276-4291.

7. Gardner, R. G., Swarbrick, G. M., Bays, N. W., Cronin, S. R., Wilhovsky, S., Seelig, L., Kim, C. and Hampton, R. Y. (2000). Endoplasmic reticulum degradation requires lumen to cytosol signaling. Transmembrane control of Hrd1p by Hrd3p. *J Cell Biol* **151** (1) 69-82.

8. Glickman, M. H. and Ciechanover, A. (2002). The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. *Physiol Rev* **82** (2) 373-428.

9. Guldener, U., Heck, S., Fielder, T., Beinhauer, J. and Hegemann, J. H. (1996). A new efficient gene disruption cassette for repeated use in budding yeast. *Nucleic Acids Res* **24** (13) 2519-2524.

10. Hampton, R., Dimster-Denk, D. and Rine, J. (1996a). The biology of HMG-CoA reductase: the pros of contra-regulation. *Trends Biochem Sci* **21** (4) 140-145.

11. Hampton, R. Y. and Bhakta, H. (1997). Ubiquitin-mediated regulation of 3-hydroxy-3-methylglutaryl-CoA reductase. *Proc Natl Acad Sci U S A* **94** (24) 12944-12948.

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

13. Hampton, R. Y., Koning, A., Wright, R. and Rine, J. (1996c). In vivo examination of membrane protein localization and degradation with green fluorescent protein. *Proc Natl Acad Sci U S A* **93** (2) 828-833.

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

15. Hershko, A. and Ciechanover, A. (1998). The ubiquitin system. *Annu Rev Biochem* **67** 425-479.

16. 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.

17. Huang, T. T. and D'Andrea, A. D. (2006). Regulation of DNA repair by ubiquitylation. *Nat Rev Mol Cell Biol* **7** (5) 323-334.

18. Joazeiro, C. A. and Weissman, A. M. (2000). RING finger proteins: mediators of ubiquitin ligase activity. *Cell* **102** (5) 549-552.

19. Joazeiro, C. A., Wing, S. S., Huang, H., Leverson, J. D., Hunter, T. and Liu, Y. C. (1999). The tyrosine kinase negative regulator c-Cbl as a RING-type, E2-dependent ubiquitin-protein ligase. *Science* **286** (5438) 309-312.

20. Kikkert, M., Doolman, R., Dai, M., Avner, R., Hassink, G., van Voorden, S., Thanedar, S., Roitelman, J., Chau, V. and Wiertz, E. (2004). Human HRD1 is an E3 ubiquitin ligase involved in degradation of proteins from the endoplasmic reticulum. *J Biol Chem* **279** (5) 3525-3534.

21. Lorick, K. L., Jensen, J. P., Fang, S., Ong, A. M., Hatakeyama, S. and Weissman, A. M. (1999). RING fingers mediate ubiquitin-conjugating enzyme (E2)-dependent ubiquitination. *Proc Natl Acad Sci U S A* **96** (20) 11364-11369.

22. Matsuda, N., Suzuki, T., Tanaka, K. and Nakano, A. (2001). Rma1, a novel type of RING finger protein conserved from Arabidopsis to human, is a membrane-bound ubiquitin ligase. *J Cell Sci* **114** (Pt 10) 1949-1957.

23. Meusser, B., Hirsch, C., Jarosch, E. and Sommer, T. (2005). ERAD: the long road to destruction. *Nat Cell Biol* **7** (8) 766-772.

24. Mori, S., Tanaka, K., Kanaki, H., Nakao, M., Anan, T., Yokote, K., Tamura, K. and Saito, Y. (1997). Identification of an ubiquitin-ligation system for the epidermal-growth-factor receptor--herbimycin A induces in vitro ubiquitination in rabbit-reticulocyte lysate. *Eur J Biochem* **247** (3) 1190-1196.

25. Nakayama, K. I. and Nakayama, K. (2006). Ubiquitin ligases: cell-cycle control and cancer. *Nat Rev Cancer* **6** (5) 369-381.

26. Pickart, C. M. (2001). Mechanisms underlying ubiquitination. *Annu Rev Biochem* **70** 503-533.

27. Pickart, C. M. and Eddins, M. J. (2004). Ubiquitin: structures, functions, mechanisms. *Biochim Biophys Acta* **1695** (1-3) 55-72.

28. Plemper, R. K., Bordallo, J., Deak, P. M., Taxis, C., Hitt, R. and Wolf, D. H. (1999). Genetic interactions of Hrd3p and Der3p/Hrd1p with Sec61p suggest a retro-translocation complex mediating protein transport for ER degradation. *J Cell Sci* **112 ( Pt 22)** 4123-4134.

29. 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. *J Biol Chem* **273** (49) 32848-32856.

30. Sasaki, A., Masuda, Y., Iwai, K., Ikeda, K. and Watanabe, K. (2002). A RING finger protein Praja1 regulates Dlx5-dependent transcription through its ubiquitin ligase activity for the Dlx/Msx-interacting MAGE/Necdin family protein, Dlxin-1. *J Biol Chem* **277** (25) 22541-22546.

31. Staub, O. and Rotin, D. (2006). Role of ubiquitylation in cellular membrane transport. *Physiol Rev* **86** (2) 669-707.

32. 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 Dev* **15** (20) 2660-2674.

33. VanDemark, A. P. and Hill, C. P. (2002). Structural basis of ubiquitylation. *Curr Opin Struct Biol* **12** (6) 822-830.

34. Werner, E. D., Brodsky, J. L. and McCracken, A. A. (1996). Proteasome-dependent endoplasmic reticulum-associated protein degradation: an unconventional route to a familiar fate. *Proc Natl Acad Sci U S A* **93** (24) 13797-13801.

35. Wilhovsky, S., Gardner, R. and Hampton, R. (2000). HRD gene dependence of endoplasmic reticulum-associated degradation. *Mol Biol Cell* **11** (5) 1697-1708.

# Chapter 3:

# Determinants of RING-E2 fidelity for Hrd1p, a membrane-anchored ubiquitin ligase

## Abstract:

A critical aspect of E3 ubiquitin ligase function is the selection of a particular E2 ubiquitin conjugating enzyme to accomplish ubiquitination of a substrate. We examined the requirements for correct E2-E3 specificity in the RING-H2 ubiquitin ligase Hrd1p, an ER-localized protein known to use primarily Ubc7p for its function. Versions of Hrd1p containing the RING motif from homologous E3s were unable to carry out Hrd1p function, revealing a requirement for the specific Hrd1p RING motif *in vivo*. An *in vitro* assay revealed that these RING motifs were sufficient to function as ubiquitin ligases, but that they did not display the E2 specificity predicted from *in vivo* results. We further refined the *in vitro* assay of Hrd1p function by demanding not only ubiquitin ligase activity, but also specific activity that recapitulated both the E2 specificity and RING selectivity observed *in vivo*. Doing so revealed that correct E2 engagement by Hrd1p required the presence of portions of the Hrd1p soluble cytoplasmic domain outside the RING motif, the placement of the Hrd1p ubiquitin ligase in the ER membrane, and presentation of Ubc7p in the cytosolic context. We confirmed that these conditions supported the ubiquitination of Hrd1p itself, and the transfer of ubiquitin to the prototype substrate Hmg2p-GFP, validating Hrd1p self-ubiquitination as a viable assay of ligase function.

## Introduction:

Ubiquitin is a covalent protein tag that alters the stability or behavior of a growing list of proteins (Huang and D'Andrea, 2006; Nakayama and Nakayama, 2006; Petroski and Deshaies, 2005; Staub and Rotin, 2006). Covalent attachment of ubiquitin to target proteins occurs by a cascade of enzymes, beginning with a ubiquitin activating enzyme (E1) hydrolyzing ATP to form a thioester-linked ubiquitin-bound intermediate. The E1 next passes its ubiquitin to a ubiquitin conjugating enzyme (E2), again as a thioester-linked intermediate. Finally, ubiquitination of the target protein is brokered by a ubiquitin ligase (E3) that facilitates transfer of ubiquitin from the E2 to a lysine on the target protein (or a previously added ubiquitin) to form an isopeptide bond. *In vivo* the ubiquitin ligase activity of a given E3 is not universally supported by all E2s (Hershko and Ciechanover, 1998; Hochstrasser, 1996). A typical E3 will function with only one or two of many E2s *in vivo* (Bays, et al., 2001; Gardner, et al., 2005; Pickart, 2001; Seol, et al., 1999; Swanson, et al., 2001). Thus, the compatibility between E3 and E2 is a critical aspect of this enzyme cascade.

Many E3s share a zinc-binding sequence called the RING motif (Fang, et al., 2003). This characteristic sequence, along with several variants (Hatakeyama, et al., 2001; Zhang, et al., 2005), is found in a large number of known or putative E3s, where it is required for ubiquitin ligase activity both *in vivo* and *in vitro* (Bays, et al., 2001; Chen, et al., 2006; Gardner, et al., 2005; Gardner, et al., 2000; Lorick, et al., 1999; Seol, et al., 1999). Unlike the HECT domain ligases, the RING ligases and their variants do not form a covalent adduct with ubiquitin during catalysis (Pickart and

Eddins, 2004; Weissman, 2001). Despite the prevalence of RING motifs among the growing number of ubiquitin ligases, and their necessity for ligase function in these proteins, the role of the RING motif in promoting E2 specificity and ubiquitin transfer is not fully understood. Structural analyses suggest that residues in the RING motif make contact with E2s, but so too do some residues outside the RING motif (Brzovic, et al., 2003; Katoh, et al., 2003; Zheng, et al., 2002; Zheng, et al., 2000). It is not clear whether the RING motif alone is sufficient to specifically engage an E2 and stimulate ubiquitination activity, or whether other *in cis* determinants outside the RING motif contribute to E2 selectivity. We have addressed these questions for the case of the ubiquitin ligase Hrd1p.

A significant component of protein degradation in eukaryotes occurs at the surface of the ER and is generally referred to as ERAD, for ER-associated degradation. ERAD is responsible for degradation of a variety of integral membrane and luminal proteins in the ER (Brodsky and McCracken, 1999). The Hrd1p ubiquitin ligase in *Saccharomyces cerevisiae* is one of several E3s that mediate ERAD (Bays, et al., 2001; Haynes, et al., 2002; Swanson, et al., 2001), with homologs in all eukaryotes (Amano, et al., 2003; Fang, et al., 2001). Hrd1p is responsible for the degradation of the yeast HMG-CoA reductase isozyme Hmg2p (Hampton, et al., 1996), and a variety of misfolded ER proteins (Bordallo, et al., 1998; Plemper, et al., 1998; Wilhovsky, et al., 2000). Hrd1p consists of an N-terminal multispanning membrane anchor and a cytoplasmic C-terminal region bearing a RING-H2 motif. The cytoplasmic portion is required for Hrd1p-dependent ERAD *in vivo* (Gardner, et al., 2000) and functions

autonomously as a ubiquitin ligase *in vitro* (Bays, et al., 2001). Hrd1p is complexed with the ER integral membrane protein Hrd3p. One function of Hrd3p is to promote Hrd1p stability. In the absence of Hrd3p, Hrd1p undergoes rapid degradation mediated by the Hrd1p RING-H2 motif resulting in Hrd1p levels too low to sustain ERAD (Gardner, et al., 2000). If Hrd1p levels are elevated in the absence of Hrd3p, ERAD will proceed, indicating that Hrd1p is a key protein of HRD pathway ubiquitination (Gardner, et al., 2000).

The cytoplasmic C-terminal RING-H2 domain of Hrd1p is exposed to numerous ubiquitin E2s; yet, Hrd1p only employs Ubc7p, and to a much lesser extent Ubc1p, in the execution of its function (Bays, et al., 2001). This same E2 specificity is observed for Hrd1p self-ubiquitination in the absence of Hrd3p (Gardner, et al., 2000). However, the Hrd1p RING domain will function *in vitro* with E2s that are not used *in vivo* (Bays, et al., 2001), leading us to wonder which features of Hrd1p contribute to its high selectivity for Ubc7p *in vivo*. In particular, we were interested in developing biochemical approaches for studying Hrd1p action that would faithfully recapitulate this selectivity in biochemically tractable conditions. We have determined that *cis*-acting portions of Hrd1p, the membrane anchoring protein Cue1p, and the placement of Hrd1p in the ER membrane bilayer are all critical to reconstituting *in vitro* the function of Ubc7p with Hrd1p.

## Results:

Removal or mutation of the Hrd1p RING motif eliminates Hrd1p function (Bays, et al., 2001; Gardner, et al., 2000). In order to more fully understand the role of the Hrd1p RING in E2 selection we created versions of the *HRD1* coding region expressing Hrd1p with RING motifs from other ubiquitin ligases. We chose the RING sequences from gp78, hsHrd1/synoviolin, and Praja1 (Figure 3-1). gp78 is a ubiquitin ligase involved in ERAD that engages a mammalian homolog of Ubc7p (Ube2G2) (Chen, et al., 2006; Song, et al., 2005; Zhong, et al., 2004). hsHrd1 is another mammalian ligase implicated in ERAD (Allen, et al., 2004; Amano, et al., 2003; Kaneko, et al., 2002; Kikkert, et al., 2004), and both gp78 and hsHrd1 are the nearest mammalian homologs to Hrd1p. Praja1 is a ligase with no known function in ERAD (Saha, et al., 2006; Sasaki, et al., 2002), but like the others has been observed to form ubiquitin chains *in vitro* (Lorick, et al., 1999). To test these RING motifs *in vivo*, 3HA-tagged constructs of Hrd1p with replaced RING sequences or a wild-type Hrd1p RING control were expressed from the native promoter as single copy integrants in a *hrd1Δ* null strain and tested for ability to degrade Hmg2p-GFP, which can be assayed both biochemically and by flow cytometry (Bays, et al., 2001; Cronin, et al., 2000; Gardner, et al., 1998).

When Hmg2p-GFP is expressed in a *hrd1Δ* null, its steady-state levels are high because it cannot be degraded (Gardner, et al., 2000). The increased fluorescence of *hrd1*Δ cells is revealed by a fluorescence histogram strongly shifted to the right (Figure 3-2*A*, "empty vector"). Expression of wild type *HRD1* restores degradation of

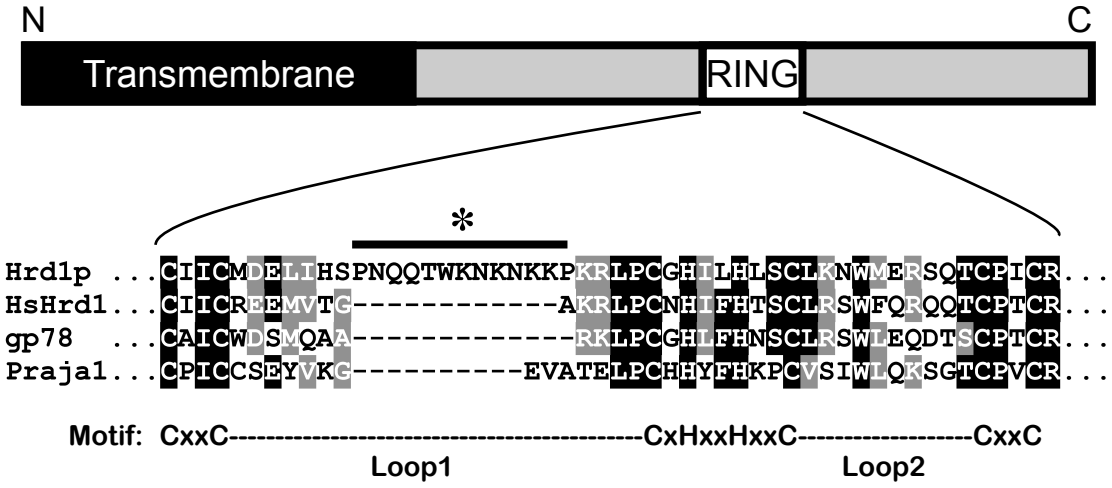


**Figure 3-1: RING motifs used to replace the native RING in Hrd1p.**

Schematic depiction of Hrd1p, and sequence alignment of RING-H2 motifs used to replace Hrd1p RING sequence in otherwise full-length Hrd1p. Loop1 and Loop2 represent variable sequences in between conserved cysteine/histidine residues that define the RING motif. Asterisk (*) indicates sequence of Hrd1p in Loop1 that was removed in the Hrd1p-Δpro mutation.

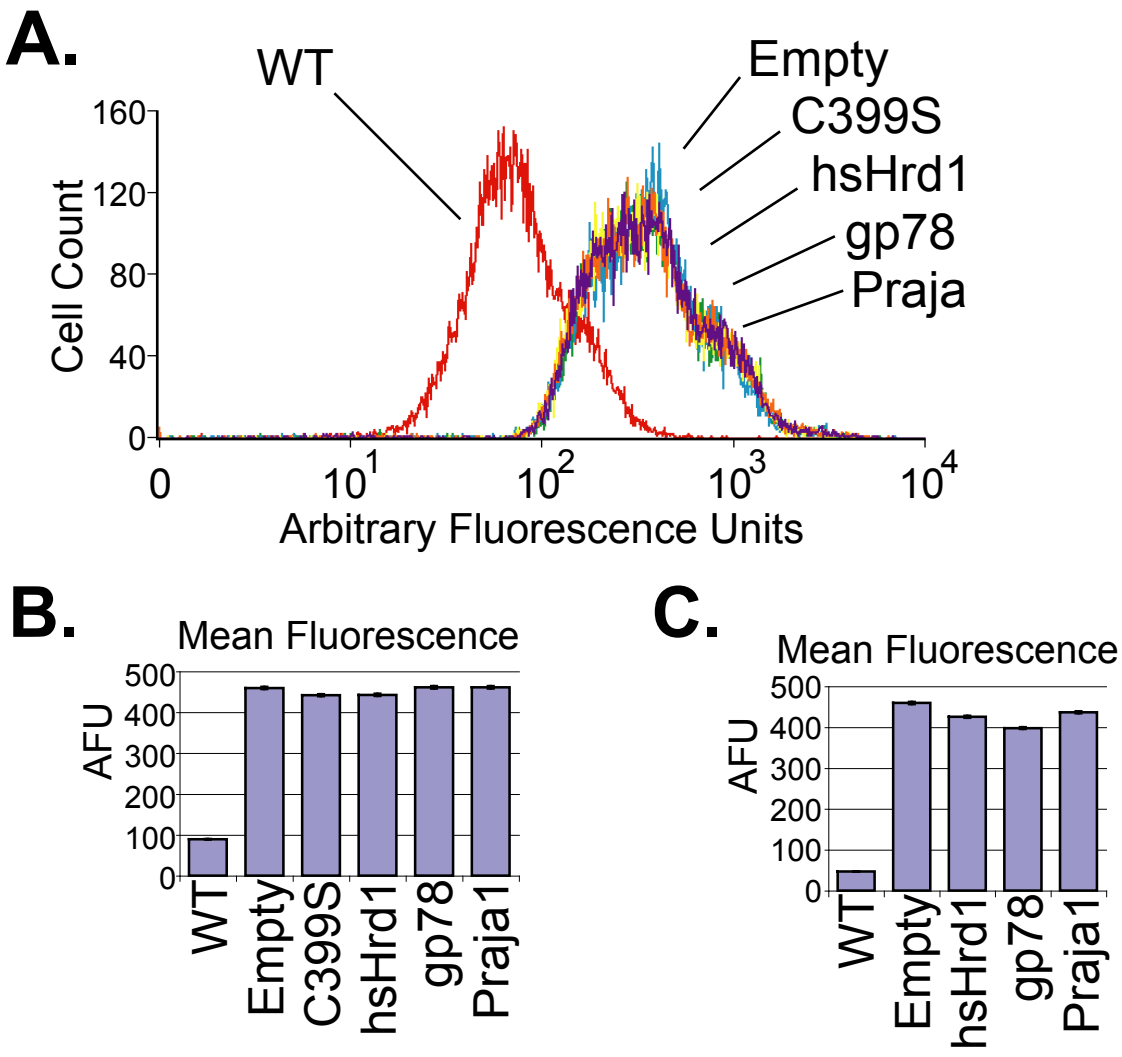


**Figure 3-2: Hrd1p with replaced RINGs does not function.**

**A.** WT and the described RING-replaced versions of Hrd1p were expressed from the *HRD1* promoter in strains with a *hrd1*Δ null allele and expressing Hmg2p-GFP. For each strain, fluorescence of 20,000 cells was measured by flow cytometry. Histograms were generated by plotting the number of cells (y-axis) having a given arbitrary fluorescence (x-axis). Degradation of Hmg2p-GFP was restored only by the WT RING version of Hrd1p, and none of the others tested. **B.** Mean fluorescence from histograms in A. Error bars depict the standard error of the mean.
**C.** Overexpression of replaced-RING-Hrd1p does not support ERAD. WT and replaced-RING versions of Hrd1p were expressed from the strong *TDH3* promoter in strains with a *hrd1*Δ null allele and Hmg2p-GFP. For each strain, fluorescence of 20,000 cells was measured by flow cytometry, and the mean fluorescence was plotted as in B.

Hmg2p-GFP, lowering steady-state Hmg2p-GFP levels, and shifting the fluorescence histogram to the left (Figure 3-2*A*, "WT"). Expression of the inactivated C399S mutant of Hrd1p is unable to support Hmg2p-GFP degradation, as shown by overlap of the C399S strain's histogram with the *hrd1*Δ null mutant (Figure 3-2*A*, "C399S"). Using this assay we tested the activity of the RING-replaced Hrd1p variants. The chimeric Hrd1p constructs with RING motifs from gp78, hsHRD1 and Praja1 were all unable to function when expressed at native levels *in vivo*, as indicated by the overlapping of histograms with those of *hrd1*Δ null or C399S strains (Figure 3-2*A*), or the mean fluorescence of the cell populations (Figure 3-2*B*). Overexpressing each chimeric Hrd1p from the strong TDH3 promoter (about 30 fold higher expression by immunoblotting) caused slight but reproducible suppression of the ERAD defect (Figure 3-2*C*), with gp78-RING-substituted Hrd1p having the most activity, about 1/8 that of authentic Hrd1p in lowering Hmg2p-GFP steady-state levels. As expected, native *HRD1* expressed at these levels caused further degradation of the reporter, since it is rate-limiting for degradation of Hmg2p (Bays, et al., 2001; Gardner, et al., 2000). In each experiment, all Hrd1p proteins were expressed at similar levels as discerned by immunoblotting (Figure 3-3A, and data not shown). Thus, even functionally related RING-H2 motifs from mammalian ERAD ubiquitin ligases were not sufficient to restore normal Hrd1p-dependent ERAD *in vivo*.

RING E3s mediate transfer of ubiquitin from E2 to substrate. The lack of *in vivo* function in the chimeric Hrd1p proteins could either be due to an inability to engage the Ubc7p E2, or an inability to catalyze transfer of ubiquitin to the Hmg2p-

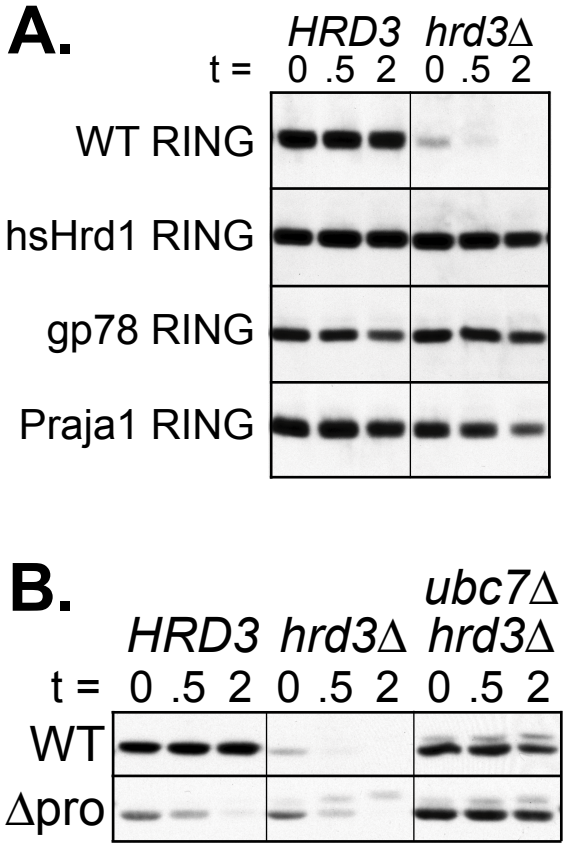


**Figure 3-3 Replaced RINGs in Hrd1p do not engage E2.**

**A.** WT and replaced-RING versions of Hrd1p-3HA were expressed from the *HRD1* promoter in strains with either a *hrd1*Δ null allele (HRD3) or *hrd1*Δ and *hrd3*Δ null alleles (*hrd3*Δ). Cycloheximide-chase assays were performed for the indicated number of hours to elucidate the stability of each Hrd1p variant. In the absence of Hrd3p, HA-tagged Hrd1p undergoes degradation (top panel), but the replaced-RING Hrd1p proteins did not. **B.** The Loop1 portion of Hrd1p is not required for E2 engagement. A portion of the Hrd1p RING in Loop1 not shared with other RINGs tested (see Figure 3-1, asterisk), was removed and this Δpro version of Hrd1p-3HA (Δpro) and WT RING-Hrd1p-3HA (WT) were tested as in D, with the addition of a hrd1Δ, hrd3Δ, and ubc7Δ null allele strain (*hrd3*Δ*, ubc7*Δ), revealing the Ubc7p-dependence of Hrd1p and Hrd1p-Δpro degradation.

GFP substrate. These two possibilities could be distinguished experimentally, because native Hrd1p undergoes Ubc7p-dependent, RING-H2-dependent self-ubiquitination when Hrd3p is absent (Gardner, et al., 2000). In a *hrd3Δ* null mutant, the normal Hrd1p protein undergoes rapid, Ubc7p-dependent degradation with a half-life of 5-10 minutes, resulting in a drastic drop in Hrd1p steady-state level. In the same conditions, the C399S-RING mutant is completely stable. By removing Hrd3p, we could evaluate the stability of each chimeric Hrd1p protein, and thus the ability of each variant to engage Ubc7p *in vivo*. Strains with either native *HRD3* or a *hrd3*Δ null allele expressing the 3HA-tagged Hrd1p chimeras were subjected to a cycloheximide-chase assay. Hrd1p chimeras with RINGs from gp78, hsHrd1, or Praja1 were stable, while the native-RING Hrd1p protein underwent the expected, rapid self-degradation when Hrd3p was absent (Figure 3-3A). Each chimeric Hrd1p was as stable as native-RING Hrd1p in the presence of Hrd3p (Figure 3-3A), or Hrd1p with a C399S mutation that abrogates RING activity (data not shown). Thus, these heterologous RINGs did not engage Ubc7p *in vivo*, despite containing a similar RING-H2 motif.

Examination of the sequence alignment in Figure 3-1 reveals an expansion in the Loop1 region of the Hrd1p RING motif not present in the other RINGs tested. We wondered if this unique sequence insertion in Hrd1p might determine Hrd1p's unique ability to engage Ubc7p. To test this idea, we made a version of Hrd1p whose RING was missing the 13 proline-flanked residues (Hrd1p-Δpro) in the Loop 1 region as indicated in Figure 3-1, and examined its stability in the presence and absence of Hrd3p. Surprisingly, Hrd1p-Δpro was able to engage Ubc7p, as revealed by its

Ubc7p-dependent degradation (Figure 3-3B). However, this construct lost regulation by Hrd3p: in the presence or absence of Hrd3p, the Hrd1p-Δpro construct was degraded at the same rate, although not as rapidly as Hrd1p with a normal RING. Hrd1p-Δpro was stabilized by the removal of Ubc7p, or the addition of the inactivating C399S RING mutation (data not shown), indicating it engaged Ubc7p and underwent self-degradation. Also, overexpression of Hrd1p-Δpro partially restored Hmg2p-GFP degradation (data not shown). Thus, the Loop1 sequence in Hrd1p is not the source of Ubc7p-specificity. This is consistent with structural analyses of E2-RING ligase complexes that suggest the Loop2 residues are involved in making E2 contacts, while Loop1 residues predominantly contact other residues in the E3 (Zheng, et al., 2002; Zheng, et al., 2000).

Despite the apparent similarity of the RINGs, particularly in the Loop2 region, we next evaluated if the failure of the substituted RINGs to engage Ubc7p was due to their intrinsic inability to recognize Ubc7p, by testing the biochemical activity of each isolated RING motif. Many RING-containing proteins will catalyze self-ubiquitination *in vitro* when combined with E1, E2, ubiquitin, and ATP (Bays, et al., 2001; Joazeiro, et al., 1999; Katoh, et al., 2003; Lorick, et al., 1999; Swanson, et al., 2001). We adapted this approach to study the isolated RING motifs in otherwise identical fusions with GST, using both authentic Ubc7p or the widely used and highly promiscuous HUBC4. Recombinant Ubc7p was expressed from the pTYB2 vector as an intein-cleavable fusion to a chitin-binding domain (CBD/intein), and purified using chitin affinity beads (Chong, et al., 1997). Addition of reducing-agent stimulates the

intein protein-cleavage reaction, liberating free Ubc7p from the resin. *In vitro* reactions were run by combining ATP, ubiquitin, E1, E2, and affinity-purified GST-RING fusions. The formation of polyubiquitin chains in the reaction mixes was evaluated directly by SDS-PAGE and anti-ubiquitin immunoblotting. The isolated RINGs from Hrd1p, hsHrd1, gp78, or Praja1 all catalyzed polyubiquitin formation with the HUBC4 enzyme (Figure 3-4, left panel) showing that the RING motifs possessed autonomous ubiquitin ligase activity. This activity required the presence of an active RING motif, as revealed by the inactivity of the GST alone and C399S RING controls. In the same assay using Ubc7p as E2, the mammalian gp78 RING showed significant ubiquitination activity with yeast Ubc7p, the hsHrd1 RING showed slight activity, and the Praja1 RING showed no ubiquitination, as did negative controls (Figure 3-4, right panel). Surprisingly, the Hrd1p RING showed no formation of polyubiquitin with Ubc7p despite its natural role using Ubc7p *in vivo*. Thus, the gp78 RING could engage Ubc7p *in vitro*, but could not do so when part of full-length Hrd1p *in vivo*. Conversely, the Hrd1p RING could not engage Ubc7p *in vitro*, while it was the only RING tested that could function in full-length Hrd1p *in vivo*. Thus, lone RING motifs can indeed exhibit E3 activity as well as E2 selectivity. However, in these examples the specificity was entirely different from that of the full length ligase functioning *in vivo*. We have used this "molecular irony" as a staring point to discern the conditions and requirements for biochemical study of Hrd1p. The correct *in vitro* conditions for accurate, biologically relevant E2 engagement by Hrd1p

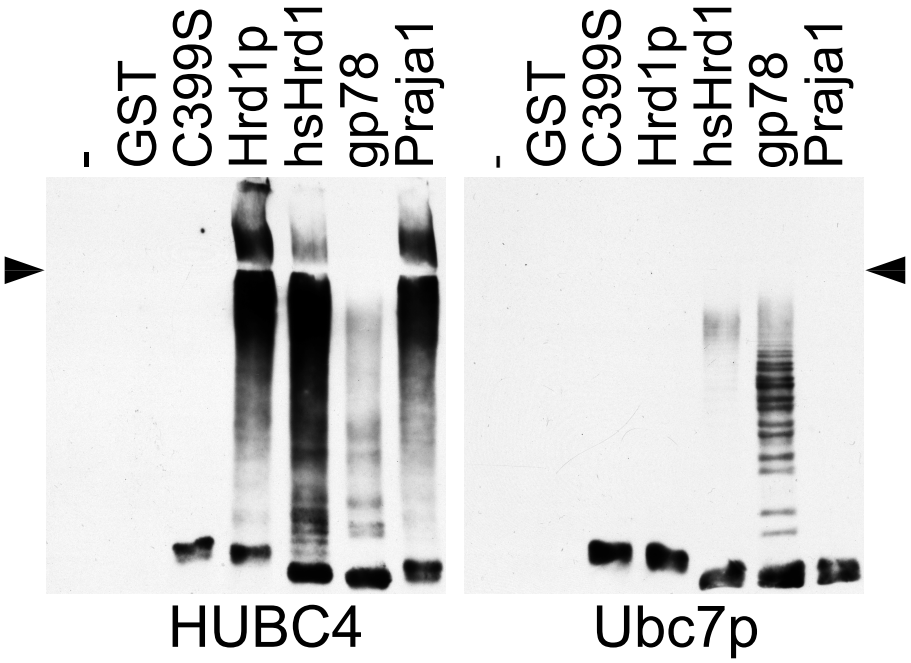


**Figure 3-4: Lone RING motifs used to replace Hrd1p RING function *in vitro.***

Ubiquitination reactions were run with GST fusions to each RING motif indicated, and either HUBC4 or Ubc7p as the E2. Immunoblotting of total reaction mix samples with anti-ubiquitin antibody revealed which RING motifs could form high molecular weight polyubiquitin chains. Arrowheads indicate the boundary between 8% running gel and 4% stacking gel.

will not merely allow the Hrd1p RING to function with Ubc7p, but also prohibit the gp78 RING from engaging Ubc7p as observed *in vivo*.

The Hrd1p RING motif was necessary for Ubc7p engagement *in vivo*, but the lone Hrd1p RING could not use this E2 *in vitro*. We next asked if other portions of the Hrd1p cytoplasmic domain were required for RING-dependent engagement of Ubc7p. We produced Hrd1p-GST fusions with Hrd1p cytoplasmic domain regions flanking either or both sides of the RING motif (Figure 3-5A). These included a construct in which the Hrd1p RING was flanked by 62 N-terminal residues and 132 C-terminal residues (GST-N-R-C), a construct with only the N-flanking portion and RING (GST-N-R), and a construct with only the C-flanking portion and RING (GST-R-C). *In vitro* ubiquitination reactions were run with equal concentrations of these GST fusions, using HUBC4 as positive control to confirm activity of the test proteins (Figure 3-5B, left panel). GST-R, GST-N-R-C, and GST-N-R showed strong ubiquitination with HUBC4 (Figure 3-5B, left panel). Although GST-R-C showed little ubiquitination with HUBC4, it was active with HUBC4 when tested at higher concentrations (data not shown), as used previously (Bays, et al., 2001). We then tested the fusions for function with Ubc7p. As expected, the lone Hrd1p RING (GST-R) was inactive, as was the GST-R-C extension, while GST-N-R had some capacity to employ Ubc7p. However, the presence of both flanks in GST-N-R-C allowed strong activity with Ubc7p (Figure 3-5B, right panel), and this activity was entirely lost by mutation of the RING to the inactive form (GST-N-C399S-C). Thus, the presence of

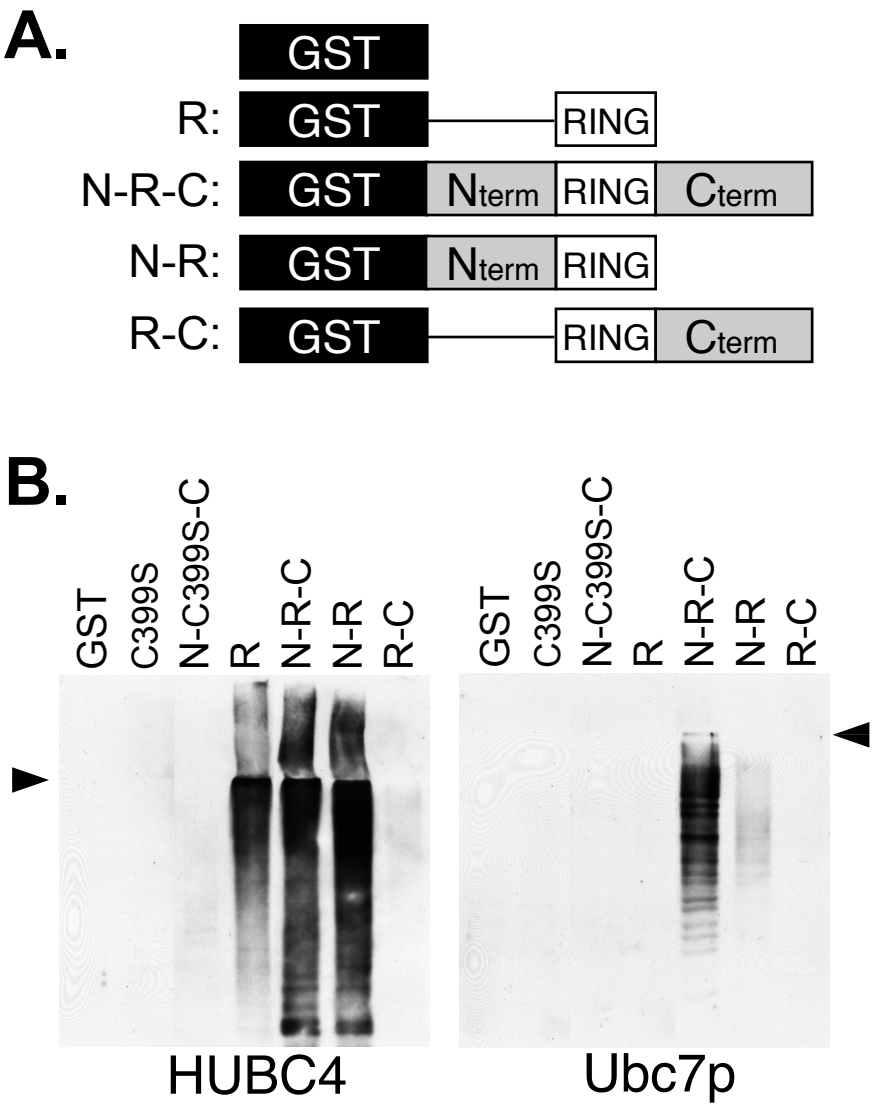


**Figure 3-5: Effect of Hrd1p N or C RING-flanking regions on activity of GST-RING fusions.**

**A.** GST fusions to the RING of Hrd1p were made that also contained portions of cytoplasmic Hrd1p N-terminal and/ or C-terminal to RING. N signifies 62 residues from the cytoplasmic domain N-terminal to RING. C signifies the 132 residues C-terminal to RING. **B.** GST fusions were tested in ubiquitination assays *in vitro* using HUBC4 or Ubc7p as the E2. Immunoblotting of total reaction mixes with anti-ubiquitin revealed which portions of Hrd1p could form high molecular weight polyubiquitin chains. Arrowheads indicate boundary between 8% running gel and 4% stacking gel.

both the Hrd1p N and C flanks were needed for engagement of Ubc7p by the Hrd1p RING.

The lone gp78 RING recognized Ubc7p *in vitro*, but failed to function in the context of full length Hrd1p. We wondered if the longer sequences of Hrd1p that allow the natural RING to engage Ubc7p *in vitro* would also preclude use of this E2 by the gp78-RING. We made and tested the analogous, extended N- and C- fusions with the gp78 RING (GST-N-gp78-C). As shown in direct comparison, the GST-N-gp78-C fusion was much *less* active with Ubc7p than the GST-N-R-C with native Hrd1p RING (Figure 3-6, top panel), while this fusion could still employ HUBC4 (data not shown). Thus, the sequences flanking the Hrd1p RING play a critical role both in allowing productive engagement of Ubc7p by the natural RING, and inhibiting this ability in the similar gp78 RING. Taken alone, these results would indicate that the RING-flanking sequences are sufficient to impose the stringent requirement for the native Hrd1p RING *in vivo*. However, the analyses below reveal critical roles for trans factors and membrane context in addition to these *in cis* determinants.

Ubc7p is presented to membrane-bound Hrd1p by the ER-localized anchoring protein Cue1p (Biederer, et al., 1997). This protein has a single N-terminal membrane span and a cytoplasmic C-terminal region that strongly binds Ubc7p, imparting surface ER localization to this otherwise soluble E2. The Cue1p protein is absolutely required for *HRD*-dependent ERAD (Gardner, et al., 2001). There are at least two ways that Cue1p can affect Hrd1p-dependent ubiquitination. Cue1p increases the effective concentration of Ubc7p accessible to Hrd1p by anchoring it to the ER

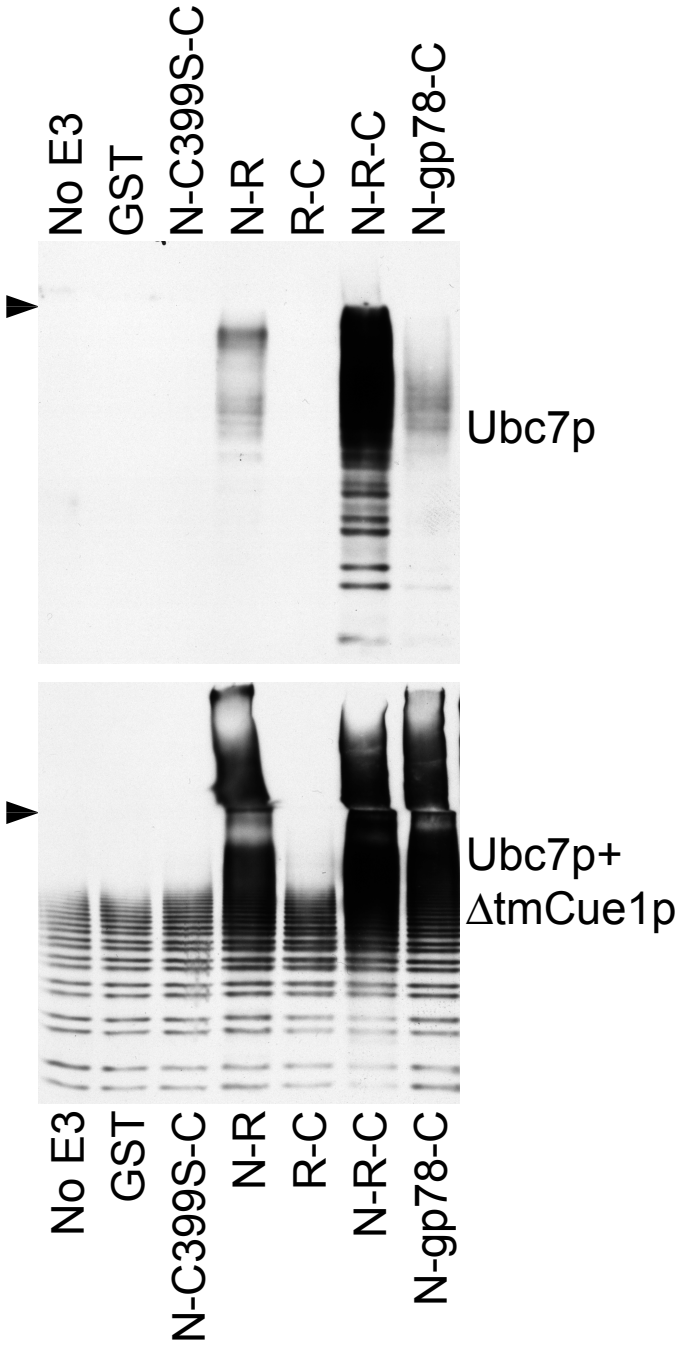


**Figure 3-6: Cue1p affects the activity of Ubc7p.**

GST-fusions were tested in ubiquitination assays *in vitro* using soluble Ubc7p (top panel), or soluble ΔtmCue1p-Ubc7p (bottom panel) as the E2 source. Assays were run as in Figure 3-4, using the indicated GST-fusion as the E3. Arrows indicate discontinuity between 8% running gel and 4% stacking gel.

membrane; in a *cue1Δ* null strain, Ubc7p is soluble and not ER-bound. However, we wondered if Cue1p binding might also increase the activity of Ubc7p. To address this, we purified a soluble Cue1p-Ubc7p heterodimer to test in our *in vitro* ubiquitination assay. We co-expressed Cue1p lacking its transmembrane-spanning anchor (ΔtmCue1p), and the Ubc7p-CBD/intein-fusion from a single bacterial expression plasmid. Chitin affinity purification of Ubc7p from the bacterial lysates resulted in co-purification of Ubc7p and the bound ΔtmCue1p. Coomassie staining of the eluted intein-cleaved product indicated that the two proteins bound with 1:1 stoichiometry (data not shown). Ubc7p or Ubc7p+ΔtmCue1p were then tested in the *in vitro* ubiquitination assays by SDS-PAGE and ubiquitin immunoblotting of reaction mixtures, using identical concentrations of Ubc7p in both reactions. In contrast to Ubc7p alone (Figure 3-6, top panel), ΔtmCue1p-Ubc7p strongly enhanced the ubiquitination activity of GST-N-R and GST-N-R-C causing the appearance of very large polyubiquitin chains (Figure 3-6, bottom panel). Strikingly, ΔtmCue1p-Ubc7p also allowed the GST-N-gp78-C construct to form polyubiquitin chains as effectively as the same protein with native Hrd1p RING (GST-N-R-C). Thus, the use of the ΔtmCue1p-Ubc7p heteromer completely removed the inhibitory effect of the N and C terminal flanks on the gp78 RING, making it as reactive as the analogous protein with the native Hrd1p RING.

Unlike free Ubc7p, Ubc7p with ΔtmCue1p was able to form intermediate size ubiquitin chains in the absence of any E3, as seen in the No E3, GST, and inactive RING controls. Immunoblotting of the E3-independent reactions suggested these

products were polymers of ubiquitin and not the result of either Cue1p or Ubc7p multiubiquitination (data not shown). However, additional formation of large polyubiquitin chains caused by the presence of active RING proteins was easily distinguishable from this E3-independent activity.

Clearly, the presence of ΔtmCue1p increased both the basal and RING-stimulated formation of polyubiquitin chains by Ubc7p. Furthermore, this heteromer drastically increased the ability of the nearly inactive GST-N-R and GST-N-gp78-C proteins to form ubiquitin polymers. Thus, we wondered if the presence of the Cue1p binding partner somehow lessened the selectivity of Ubc7p for particular rings, making it more like HUBC4, or whether Cue1p simply increased the RING-specific activity of Ubc7p. To test this idea further, we evaluated the effect of ΔtmCue1p-Ubc7p on the ubiquitination activity of the isolated RING motifs fused to GST used earlier in Figure 3-4. These experiments showed that ΔtmCue1p enhanced ubiquitination activity only in the presence of RINGs that could recognize free Ubc7p *in vitro* (Figure 3-7). Only gp78-RING, and to a lesser extent hsHrd1-RING functioned with free Ubc7p, and only those RINGs produced very large ubiquitin chains with ΔtmCue1p-Ubc7p. The free Hrd1p-RING showed only very slight activity, and the Praja1-RING show no reaction with either Ubc7p or ΔtmCue1p-Ubc7p. It is also noteworthy that, in Figure 3-6, the RING constructs that were strongly activated by ΔtmCue1p were not completely inactive with Ubc7p alone. We conclude from these results that Cue1p did not participate in or modify the selection of E3, but that it stimulated Ubc7p to be more active with those E3s it could engage.

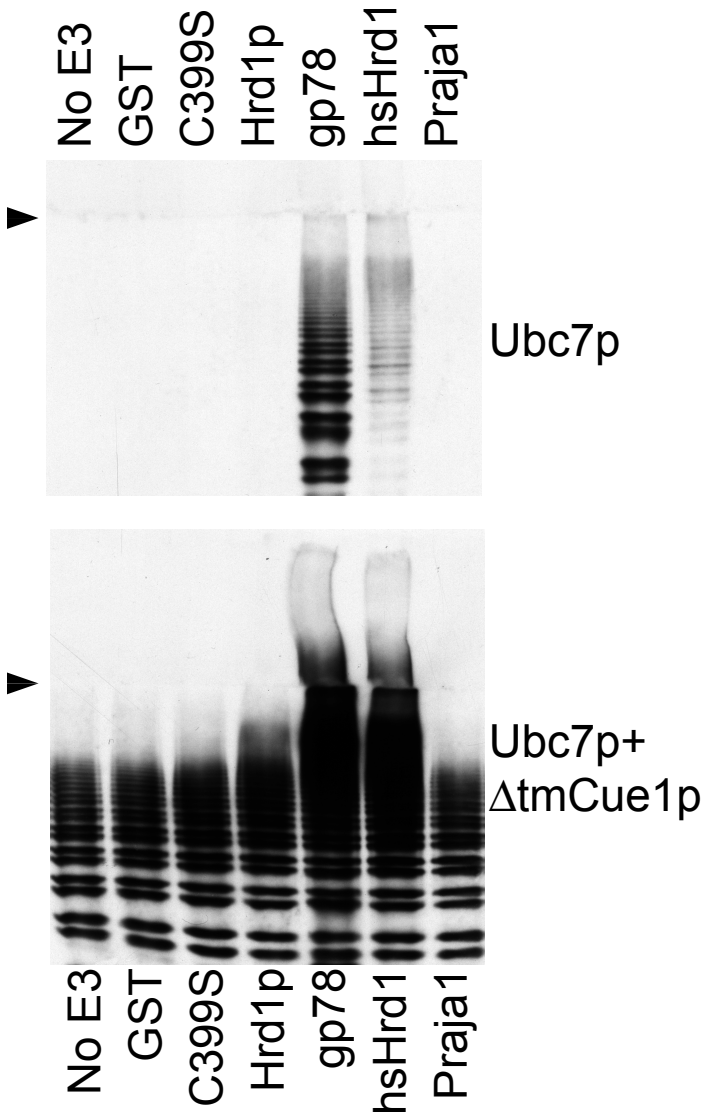


**Figure 3-7: Cue1p enhances activity of Ubc7p without reducing specificity for lone RINGs.**

GST-Fusions used in Figure 3-3 were tested with Ubc7p and Ubc7p+ΔtmCue1p. Cue1p substantially enhanced polyubiquitin chain formation only with RINGs that were also active with Ubc7p alone. Arrows indicate discontinuity between 8% running gel and 4% stacking gel.

Comparing the results using the authentic Hrd1p RING or the related gp78 RING raises an interesting dilemma. *In vivo*, the gp78 RING did not substitute for the Hrd1p RING when part of the full-length protein. This selectivity for the authentic RING was recapitulated *in vitro* with soluble proteins, using free Ubc7p and sufficient portions of the Hrd1p cytoplasmic domain: GST-N-R-C was reactive with Ubc7p, while GST-N-gp78-C was not. Taken alone, this result would indicate that high specificity for the authentic Hrd1p RING *in vivo* is imposed by the *in cis* context of the soluble cytoplasmic domain. That is, the Hrd1p RING functioned with Ubc7p in the large soluble N-R-C fusion, while the N-gp78-C fusion did not. However, when the Ubc7p was presented as part of the Cue1p-Ubc7p heterodimer, the N-gp78-C fusion functioned as well as the same construct with the native RING. *In vivo*, Cue1p is absolutely required for Hrd1p engagement of Ubc7p, but with inclusion of Cue1p, the RING specificity seen *in vivo* is no longer recapitulated in the soluble *in vitro* experiment. This implies that when Hrd1p is anchored in the ER membrane, other features impose the high selectivity for the Hrd1p RING and the observed intolerance *in vivo* for replacement with the RING from gp78, even when Cue1p is presenting Ubc7p. To explore this idea, we used a microsome assay we have recently developed for examination of full-length, ER-localized Hrd1p *in vitro* (Flury, et al., 2005).

The various HA-tagged Hrd1p proteins studied in Figure 3-3 were expressed from the strong *TDH3* promoter in *ubc7*Δ null strains. Microsomes prepared from these strains provided membrane-localized, full-length Hrd1p with native or substituted RING. These microsomes were added to *in vitro* ubiquitination reactions

with either HUBC4 or Ubc7p, and after incubation, the mix was solubilized in detergent buffer. Then full-length Hrd1p was immunoprecipitated and immunoblotted to evaluate Hrd1p self-ubiquitination.

The microsomal ubiquitination assay accurately reflected the *in vivo* engagement of Ubc7p by the Hrd1p variants. Wild-type Hrd1p showed abundant ubiquitination activity, the gp78-Hrd1p chimera was much less active and the hsHrd1p or Praja1 chimeras were nearly as low as the inactive-RING or *hrd1*Δ null controls (Figure 3-8, right panel). In microsome ubiquitination reactions with HUBC4, wild-type Hrd1p and the Hrd1p chimeras with hsHrd1 or Praja1 RING all showed substantial ubiquitination activity. However, the gp78-RING-Hrd1p was also inactive with this normally very promiscuous E2 (Figure 3-8, right panels). Thus, when the gp78 RING chimera was evaluated in the context of ER-bound Hrd1p, it lacked activity for both Ubc7p and HUBC4. This result would not be predicted from the soluble *in vitro* studies above and indicated the importance of correct cell biological context in the study of these proteins.

The relative Ubc7p-dependent activities of full-length, membrane-localized, native Hrd1p and gp78-Hrd1p resembled the *in vitro* results observed with these soluble RINGs in Figure 3-6 when Cue1p was absent. However, Cue1p was not absent from the microsomes. As expected for this integral membrane protein (Biederer, et al., 1997), nearly all of cellular Cue1p partitioned to the microsome fractions. Immunoblotting with anti-Cue1p antibodies (a gift from Thomas Sommer) confirmed that Cue1p in the microsome fractions was as abundant as Cue1p in whole-

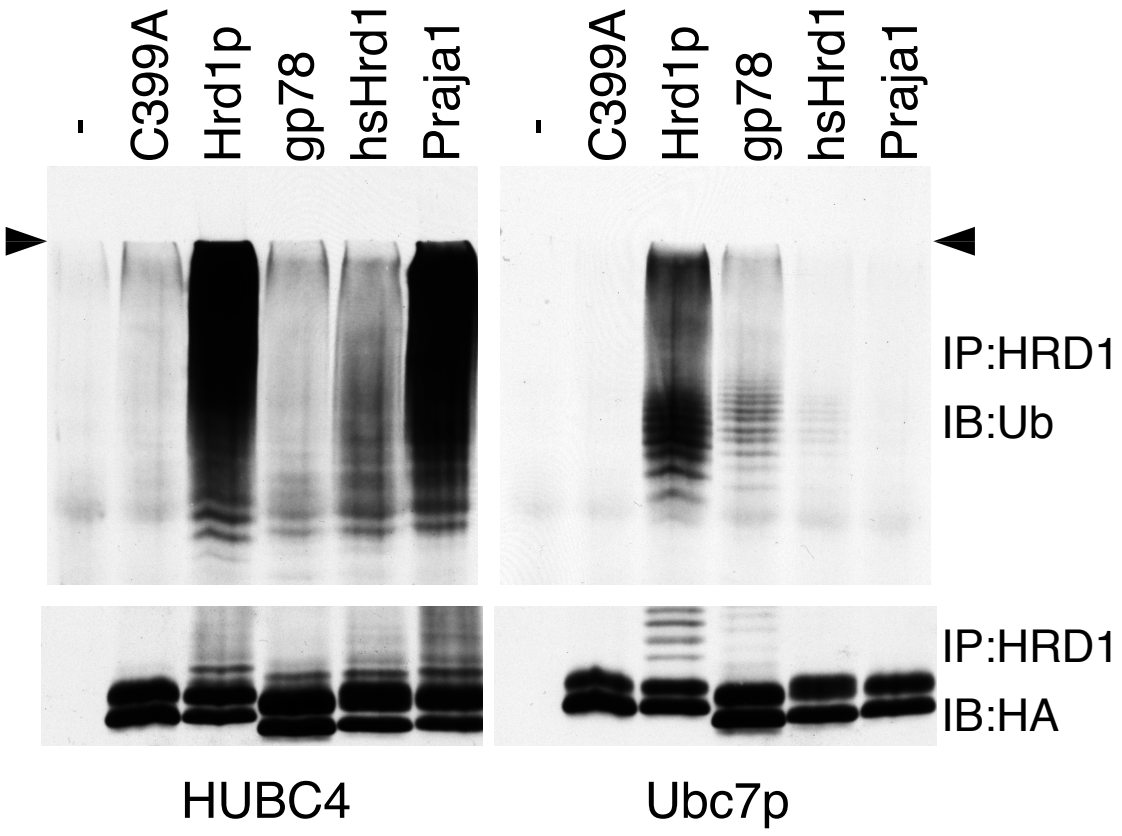


**Figure 3-8:** ***In vitro*** **self-ubiquitination of RING-replaced Hrd1p in microsomes.**

Microsomes were prepared from strains expressing the indicated chimeric-RING-Hrd1p with 3HA epitope tag. *In vitro* ubiquitination reactions were prepared and run as described with either HUBC4 (Left) or Ubc7p (Right). Each Hrd1p was immunoprecipitated, and immunoblotted with either anti-ubiquitin antibody (upper panels) or anti-HA antibody (lower panels). Arrows indicate discontinuity between 8% running gel and 4% stacking gel.

cell lysates (data not shown). Thus, we wondered if Cue1p was required for Hrd1p-Ubc7p engagement in our microsome ubiquitination assay. We compared reactions using microsomes from strains with normal *CUE1* gene or a *cue1Δ* null allele, using a range of Ubc7p concentrations. In contrast to the absolute *in vivo* requirement of Cue1p for Ubc7p-dependent activity, Cue1p moderately improved presentation of Ubc7p to Hrd1p in this microsome ubiquitination assay, shifting the concentration dependence by about 3 fold by visual inspection (Figure 3-9, bottom, compare lanes 3-5 with lanes 7-9). This enhancement by Cue1p was specific for Ubc7p, as Cue1p showed no effect on the concentration curve for HUBC4-dependent ubiquitination of Hrd1p (Figure 3-9, top). Although Cue1p showed Ubc7p-specific enhancement of Hrd1p microsome ubiquitination, the activity was not completely Cue1p-dependent as is observed *in vivo* (Gardner, et al., 2001).

To more closely approximate the *in vivo* conditions where strong Cue1p-dependence is manifest, we performed the assay using cytosolic extracts that provided endogenous Ubc7p for the assay. Cytosolic extracts were prepared from strains over-expressing epitope-tagged Ubc7p. Microsomes were prepared as before from strains with normal *CUE1* gene or a *cue1Δ* null allele, incubated with the Ubc7p-containing cytosol, after which Hrd1p was immunoprecipitated and immunoblotted for ubiquitin. As was the case *in vivo*, *in vitro* Hrd1p ubiquitination was now strongly dependent on the presence of Cue1p in the microsomes (Figure 3-10A, lanes 11, 12). This Cue1p-dependence was also observed by adding recombinant Ubc7p to cytosol prepared from *ubc7*Δ null strains. By using identically-tagged Ubc7p for the endogenous or

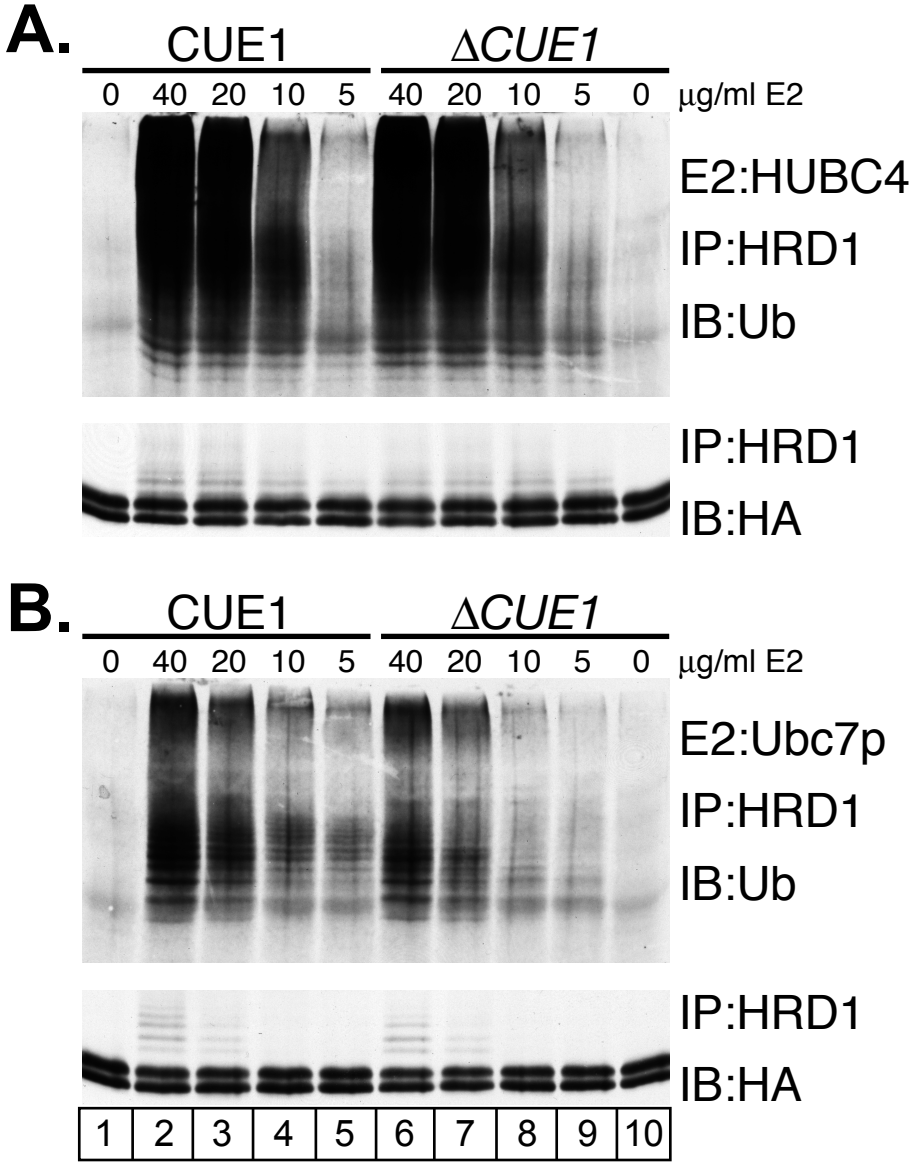


**Figure 3-9: Participation of Cue1p in *in vitro* microsomal ubiquitination.**

**A.** Cue1p has no effect on the concentration of HUBC4 required for ubiquitination of Hrd1p. Microsomes were prepared from strains expressing Hrd1p-3HA. *In vitro* ubiquitination reactions were prepared with indicated serially diluted concentrations of HUBC4 (µg/ml). Hrd1p was immunoprecipitated, and immunoblotted with either anti-ubiquitin antibody (upper panel) or anti-HA antibody (lower panel). **B.** Cue1p reduces the concentration of Ubc7p required for ubiquitination of Hrd1p. Same as in A, but with serially diluted Ubc7p.

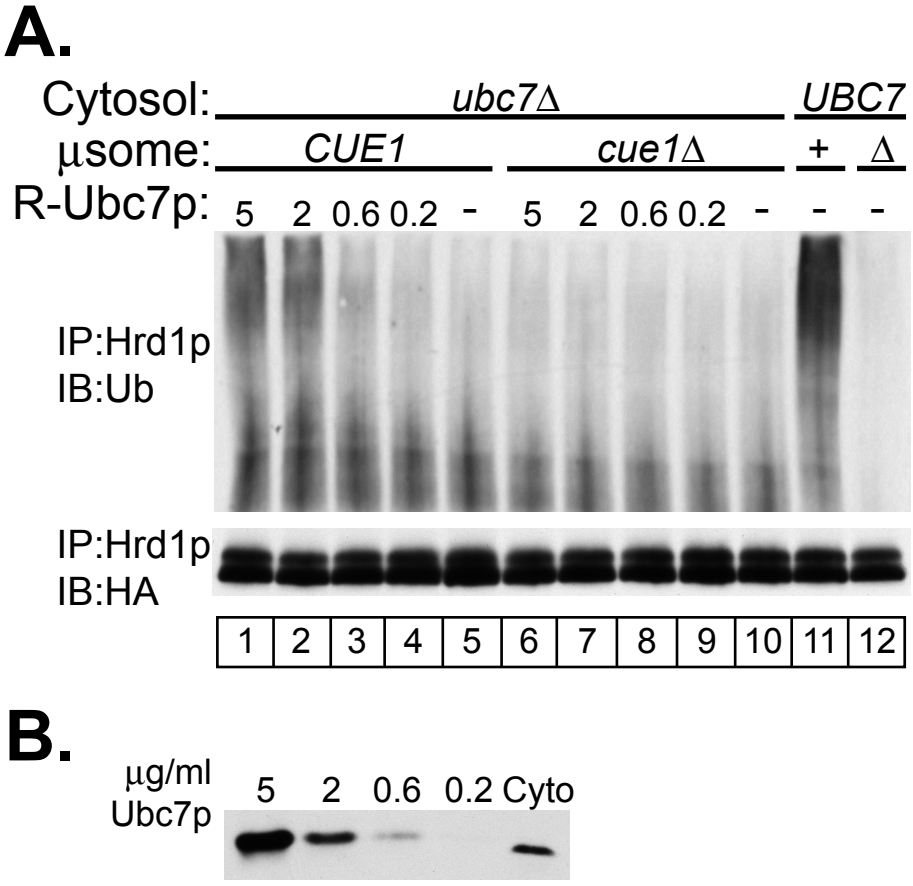


**Figure 3-10:** ***In vitro*** **microsomal Hrd1p self-ubiquitination is strongly Cue1p-dependent in the presence of cytosol.**

**A.** Ubiquitination of Hrd1p in cytosol is strongly Cue1p-dependent. Cytosol was prepared from strains with either a *ubc7*Δ null allele (*ubc7*Δ) or from strains overexpressing Ubc7p-2HA (*UBC7*). Microsomes were prepared from strains expressing Hrd1p-3HA and with either native Cue1p (*CUE1*, +) or a *cue1*Δ null allele (*cue1*Δ, Δ). Reactions with *ubc7*Δ cytosol were supplemented with the indicated concentrations (μg/ml) of recombinant Ubc7p-2HA (R-Ubc7p). The resultant ubiquitination reactions were immunoprecipitated with anti-Hrd1p antibodies and immunoblotted with either anti-ubiquitin antibody (upper panel) or anti-HA antibody (lower panel). **B.** Determination of Ubc7p concentration in cytosolic extract. Cytosol prepared from strains over-expressing Ubc7p-2HA was compared to known concentrations of recombinant Ubc7p-2HA by immunoblotting with anti-HA antibody. Ubc7p-2HA at indicated concentrations (μg/ml) was loaded next to cytosolic extract (Cyto).

recombinant Ubc7p, we determined the amount of E2 provided by the Ubc7p cytosol (Figure 3-10B), and ran *in vitro* reactions using this range of added, recombinant Ubc7p. Again, we observed strong Cue1p-dependence of Hrd1p ubiquitination (Figure 3-10A, compare lanes 1-5 and 6-10). Curiously, the Ubc7p expressed in yeast cytosol appeared more active than the recombinant E2 (Figure 3-10A, compare lane 11 with lanes 1-3), but in both cases Cue1p was absolutely required for Ubc7p-dependent polyubiquitination of membrane-bound Hrd1p.

The addition of cytosol to the *in vitro* reactions faithfully recapitulated the strong dependence on Cue1p for ubiquitination of Hrd1p. Thus, we tested if these conditions would allow the gp78-RING substituted Hrd1p to function, as was the case with the soluble chimeric protein. Despite the fact that the Ubc7p in this assay was presented by the Cue1p anchor, the gp78 chimera was still significantly less active than the native Hrd1p protein, (Figure 3-11A). Importantly, the small amount of gp78-chimeric ubiquitination was fully Cue1p-dependent, confirming that the Ubc7p was presented to the chimera as part of the Cue1p-Ubc7p heteromer. Nevertheless, the activity of the gp78 chimera was much lower than the native protein, indicating again that the cell biological context imposed restrictions on the Hrd1p ligase not observed with the analogous soluble proteins.

The above biochemical studies used self-ubiquitination to examine the requirements for observing physiologically relevant Hrd1p action. With this approach, we have shown that the high specificity for the Hrd1p RING depended on *cis* flanking sequences and the presence of Hrd1p in the correct context of the ER

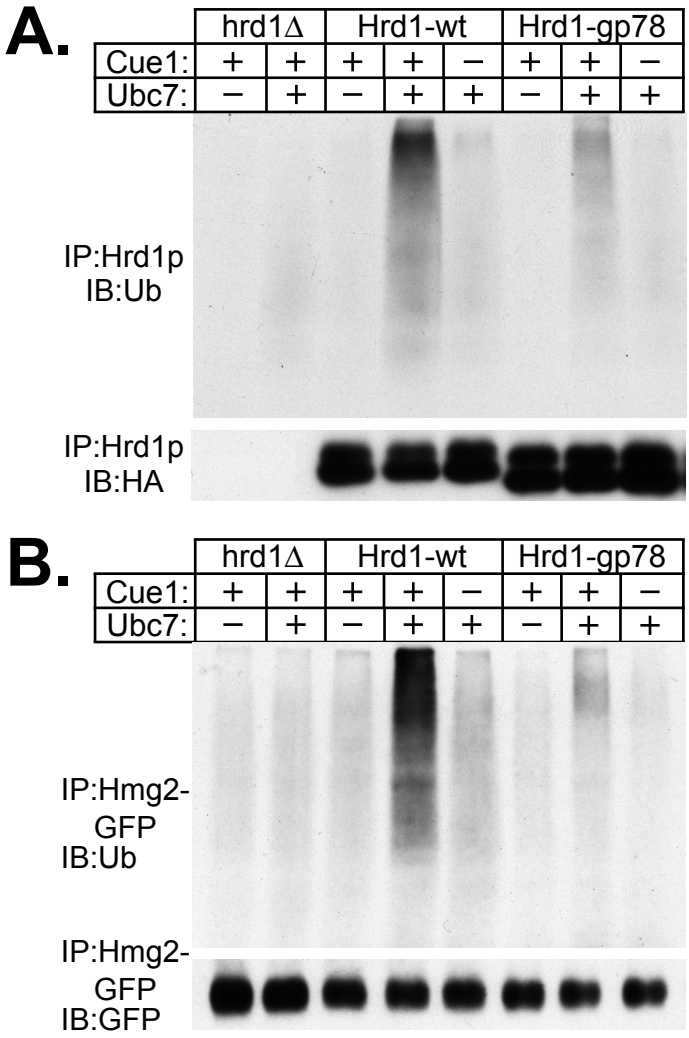


**Figure 3-11: Comparison of *in vitro* Hrd1p self-ubiquitination or Hrd1p-catalysed ubiquitination of Hmg2p-GFP.**

**A.** Ubiquitination of Hrd1p with WT or gp78 RING is highly Cue1p-dependent. Microsomes were prepared from strains with either no Cue1p or native Cue1p and expressing no Hrd1p (hrd1Δ), epitope-tagged Hrd1p with WT RING (Hrd1-WT), or epitope-tagged Hrd1p with gp78-RING (Hrd1-gp78) as indicated. Cytosol was prepared from strains without Ubc7p, or overexpressing Ubc7p, as indicated. Ubiquitination reactions were immunoprecipitated with anti-Hrd1p antibody, and immunoblotted with either anti-ubiquitin antibody (upper panel) or anti-HA antibody (lower panel). **B.** Ubiquitination of Hmg2p-GFP correlates with ubiquitination of the Hrd1p construct expressed. Hmg2p-GFP was expressed in the microsome strains used in A. Portions of the same reactions used in A were immunoprecipitated with anti-GFP antibody, and immunoblotted with either anti-ubiquitin antibody (upper panel) or anti-GFP antibody (lower panel).

membrane. Self-ubiquitination is a straightforward way to study the action of ubiquitin ligases: however, their ultimate function is to catalyze ubiquitination of substrates, such as Hmg2p in the case of Hrd1p. Thus, we extended our *in vitro* analysis with a direct test of substrate ubiquitination (Flury, et al., 2005), using Hmg2p, a natural substrate of Hrd1p-dependent ERAD (Hampton, et al., 1996). Hmg2p-GFP was expressed in the microsome strain along with Hrd1p, with a *ubc7Δ* null allele to preclude ubiquitination until the E2 is introduced in the *in vitro* reaction. These microsomes were prepared as above and incubated in cytosol prepared from Ubc7p-expressing or *ubc7*Δ null strains. Hmg2p-GFP ubiquitination was examined by immunoprecipitation with anti-GFP antibodies followed by immunoblotting for ubiquitin. Ubiquitin transfer to Hmg2p in this assay is entirely dependent on Hrd1p and Ubc7p (Flury, et al., 2005). As expected, Hmg2p-GFP ubiquitination was entirely dependent on the presence of both Ubc7p and Cue1p (Figure 3-11B). Moreover, ubiquitination of Hmg2p-GFP was proportional to the ubiquitination of Hrd1p in identical conditions (compare Figure 3-11A and Figure 3-11B). Native-RING Hrd1p was able to support transfer of ubiquitin to Hmg2p-GFP as well as the previously observed self-ubiquitination. By contrast, gp78-RING Hrd1p showed little transfer of ubiquitin to Hmg2p-GFP, in accord with its weak self-ubiquitination. Ubiquitination of Hmg2p-GFP by native Hrd1p was approximately 9 fold more than that seen with the gp78-Hrd1p. This correlates well with the results seen by flow-cytometry in Figure 3-2*C* where at similar levels of ligase expression, the gp78-Hrd1p showed about 8 fold less effect on Hmg2p-GFP levels *in vivo* than authentic Hrd1p. The

similarity of self-ubiquitination and transfer function validates the use of self-ubiquitination as a readout of authentic Hrd1p ubiquitin ligase activity.

## Discussion:

In these studies we have systematically examined the requirements of the Hrd1p ubiquitin ligase for selective function with its preferred E2, Ubc7p. Our purpose was twofold: to better understand the conditions and requirements for study of Hrd1p *in vitro*, and to delineate conditions or principles that may be operating in other ligases.

The RING motif is necessary for engagement of E2s in many ubiquitin ligases, and makes contacts with the E2 molecule in the few structures that have been resolved (Brzovic, et al., 2003; Zheng, et al., 2002; Zheng, et al., 2000). Thus, we began by testing *in vivo* the importance of the native Hrd1p RING motif. Since RING removal or inactivation was already known to eliminate Hrd1p function, RING sequences from other ubiquitin ligases were used to precisely replace the native RING sequence (Figure 3-1) in full-length native-level Hrd1p. The RING sequences were chosen from known ubiquitin ligase proteins, including homologs of Hrd1p. Despite this, the native RING motif was essential for functional engagement of Ubc7p by the Hrd1p protein; the gp78 RING provided very little activity in the Hrd1p protein, and hsHrd1 and Praja1 RINGs were completely non-functional in this context. This was somewhat surprising, because gp78 and hsHrd1 are the most closely related mammalian proteins to Hrd1p. Like Hrd1p, they participate in ERAD, and gp78 performs ERAD with Ube2g2, an E2 homologous to Ubc7p (Chen, et al., 2006; Kikkert, et al., 2004). To test if the Hrd1p RING was sufficient to specify Ubc7p

engagement, we studied the previously tested RING motifs in isolation by expressing recombinant GST-RING fusions, with either the widely-used E2 HUBC4, or authentic Ubc7p purified using an intein-fusion approach. Surprisingly, the gp78 RING engaged Ubc7p while the Hrd1p RING did not, although both could function with the promiscuous HUBC4. This inversion of the expected specificity revealed that conditions *in vivo* imparted constraints on E2-E3 pairing that the simplest direct assay of the soluble RING motifs did not.

An *in vitro* assay with the correct specificity as observed *in vivo* should permit Ubc7p to function with the Hrd1p RING, and restrict Ubc7p function with the gp78 RING. We used the gp78 and Hrd1p RING pair to evaluate the features and factors that bring about preference for the Hrd1p RING *in vivo*. The experiments revealed that a combination of conditions operate to this end. The *cis* sequence context of the cytoplasmic RING domain played a critical role, in that both the N and C terminal regions were required for robust use of Ubc7p by the Hrd1p RING. This was not simply due to the *cis* elements making the RING active, since the isolated Hrd1p RING was quite efficient at engaging another E2. Furthermore, these same flanking sequences made the gp78 RING less efficient at engaging Ubc7p, while retaining engagement of HUBC4. Taken alone, these data might be thought to completely explain the *in vivo* results, in which the authentic RING functions with Ubc7p but the gp78 RING does not. However, inclusion of Cue1p in our *in vitro* reactions showed that the actual case is more complex. When co-purified ΔtmCue1p-Ubc7p protein was used as the E2, the N-gp78-C fusion was equally active as the N-R-C fusion with the

Hrd1p RING. This restoration of activity to the N-gp78-C fusion by the presence of ΔtmCue1p indicated that the N and C *cis* flanking regions were not the sole cause of gp78 RING inactivity in full-length Hrd1p *in vivo*. Moreover, inclusion of Cue1p is biologically relevant since Cue1p is absolutely required for Ubc7p function *in vivo* (Biederer, et al., 1997; Gardner, et al., 2001).

In the context of the full-length, membrane-anchored protein, the gp78 RING was significantly less active with Ubc7p, and was similarly inactive with HUBC4. Even in the presence of cytosol, where the *in vitro* activity of Ubc7p was completely dependent on Cue1p, the gp78 RING-substituted Hrd1p was similarly less active than the native Hrd1p protein. This was distinct from the behavior of the large soluble Hrd1p fusion with gp78 RING, which reacted efficiently with ΔtmCue1p-Ubc7p, and with HUBC4. Thus, the correct analysis of E2 selection by the Hrd1p-RING domain required being in the membrane-bound context, since that was the condition where the *in vivo* behavior of the native and chimeric Hrd1p proteins was recapitulated *in vitro*.

Many studies of E3 ligases, including our earlier work, are performed with partial, RING-containing portions of the E3 proteins, and/or convenient heterologous E2s (Bays, et al., 2001; Kikkert, et al., 2004; Lorick, et al., 1999; Swanson, et al., 2001). In this systematic analysis of the requirements for highly specific RING and E2 function of Hrd1p *in vivo*, it is clear that a variety of conditions strongly determine these features of Hrd1p that were not included in our earlier assays. In fact, each alteration from "lone RING" to full-length membrane-bound Hrd1p caused a change in the use of RING and E2. The requirements for Ubc7p to both engage Hrd1p RING

and exclude gp78 RING are more readily evaluated in table form (Figure 3-12). This table summarizes Hrd1p RING or gp78 RING activity and Cue1p-dependence in each of the *in vitro* assays and *in vivo*. As can be seen, each new condition allowed a different result, and the only condition that faithfully recapitulated the *in vivo* RING and E2 selectivity was examination of the full-length protein in its membrane of origin. At present, we do not know if this restrictive behavior that results in E2 and RING selectivity is due to *cis* elements in the membrane anchor of Hrd1p, or to the proximity of the ER surface. An examination of the activity of full-length Hrd1p in micelles, or other circumstances that can separate these contributions may reveal the underpinning of this "context effect". Additionally, the strong Cue1p-dependence observed *in vivo* was seen *in vitro* only with full-length Hrd1p in microsomes incubated with cytosol. It may be that the lower Ubc7p concentrations in cytosol amplified the need for the enhancing and concentrating effects of Cue1p. Alternatively, unknown factors in the cytosol may contribute to the need for Cue1p *in vivo*. In either case, it is noteworthy that the Ubc7p derived from cytosol was more potent than similar concentrations of recombinant Ubc7p. Collectively, these results justify the use of the microsome assay in studying Hrd1p, and show that caution must be applied when examining an E3 in conditions distinct from its native circumstances.

In these *in vitro* conditions we have examined the ubiquitination of Hrd1p itself to report on Hrd1p ubiquitin ligase function. Although Hrd1p is itself an ERAD substrate when out of stoichiometric balance with Hrd3p (Gardner, et al., 2000), we also wanted to observe Hrd1p transfer ubiquitin to a substrate other than itself.

| | | Ubiquitination | |
|---|---|---|---|
| | | **Hrd1p RING** | **gp78 RING** |
| **GST-RING** | − Cue1p | − | + |
| | + Cue1p | − | + |
| **GST-N-RING-C** | − Cue1p | + | − |
| | + Cue1p | + | + |
| **Hrd1p in microsomes** | − Cue1p | + | − |
| | + Cue1p | + | − |
| **Hrd1p in microsomes +cytosol** | − Cue1p | − | − |
| | + Cue1p | + | − |
| ***in vivo*** | − Cue1p | − | − |
| | + Cue1p | + | − |

**Figure 3-12: Summary of *in vitro* function of Hrd1p and gp78 RINGs.**

Under the various conditions tested the Hrd1p and gp78 RINGs only recapitulated both the RING specificity and Cue1p-dependence observed *in vivo* (bottom) when examined in the context of full-length microsomal Hrd1p in the presence of cytosol.

Indeed, full-length Hrd1p in microsomes with cytosol was able to transfer ubiquitin to Hmg2p-GFP. Like the Hrd1p ubiquitination assay, *in vitro* ubiquitin transfer to Hmg2-GFP observed *in vivo* RING selectivity. This activity was also Cue1p-dependent, validating the self-ubiquitination assay of Hrd1p function as a genuine readout of ubiquitin ligase activity. These results also suggest a quantitative correlation with *in vivo* degradation. *In vitro* Hmg2-GFP ubiquitination with native-RING-Hrd1p was eight to ten times better than with gp78-RING-Hrd1p. Interestingly, *in vivo* reduction of Hmg2p-GFP with overexpressed RING-Hrd1p (Figure 3-2*C*) also showed an eight-fold greater effect by native-RING-Hrd1p than gp78-RING-Hrd1p, emphasizing that these more complex *in vitro* assay conditions correctly predict *in vivo* function.

In the studies above, we examined Hrd1p function alone, without its accessory protein Hrd3p, either due to isolation *in vitro* or because in the microsomal assay overexpressed Hrd1p is in excess of Hrd3p. When both Hrd1p and Hrd3p are present at normal levels, the Hrd1p protein is very stable and able to perform ERAD (Gardner, et al., 2000). It may be that one of the functions of Hrd3p is to bias the ligase activity away from Hrd1p self-ubiquitination and channel it towards substrate ubiquitination. Although we have not compared self-ubiquitination to substrate transfer when Hrd1p and Hrd3p are at normal levels, we are currently working on this more challenging assay. It is possible that other E3s have evolved regulatory factors like Hrd3p that could preferentially influence transfer of ubiquitin to either ligase or substrate, allowing regulation of E3 level through degradation.

The Hrd1p RING is distinguished from the other RINGs tested by an insert on the N-terminal half (Loop1) of the RING motif (Figure 3-1). The C-terminal half (Loop2) of all the RINGs are quite similar. We surmised that the highly selective utilization of the Hrd1p RING *in vivo* would be due to this unique additional sequence in Loop1. We removed this sequence from Hrd1p and the resulting Hrd1p-Δpro was still able to engage Ubc7p. Instead, Hrd1p regulation by Hrd3p was lost, suggesting involvement of a portion of the RING in the kind of "self vs. substrate" regulation described above. The Loop1 expansion in Hrd1p is prevalent among the Hrd1p homologs in fungi, and is absent in the mammalian homologs. It is consistent that a portion of the Hrd1p RING which mediates regulation by Hrd3p resides in a region of the RING thought to be involved in E3-specific residue interactions, and not in interactions with E2s. Many RINGs have a large insertion in the Loop1 region, including San1p and Hrt1/Roc1/Rbx1. It is as yet unclear how often these Loop1 regions are simply involved in maintaining the structure of the E3, or whether they facilitate an undiscovered means for regulation of those ligases. In addition to the structural predictions that Loop2 is involved in E2 interactions, domain swap experiments indicate that Loop2 in the Hrd1p RING is responsible for the ability to engage Ubc7p *in vivo* (data not shown). Thus the large insert in the Hrd1p RING was not the determining factor of Ubc7p engagement. Rather, subtle features of the RING domain determine the function of Hrd1p when present in the ER membrane.

Because Cue1p has been studied only in its ER-anchored state (Biederer, et al., 1997; Gardner, et al., 2001; Ravid, et al., 2006), it has not been clear whether Cue1p

only plays a concentrating role by localizing Ubc7p to the ER surface, or if Cue1p additionally affects intrinsic Ubc7p activity. Our studies with soluble ΔtmCue1p-Ubc7p complex clearly showed that this protein strongly affects the biochemistry of the E2, independent of any membrane-concentrating effects. Thus, Cue1p is an integral component of the Ubc7p E2. It will be interesting to see if the specificity or action of Ubc7p is altered by Cue1p. Despite its higher activity, the ΔtmCue1p-Ubc7p still failed to react with Praja1 RING, and only poorly reacted with Hrd1p RING, implying that the E2 activity of Ubc7p was enhanced without changing specificity, but this must be examined in more detail. The effect of Cue1p allows the possibility that other E2 binding or interacting factors may similarly activate their cognate E2s.

gp78 and Hrd1p are related ubiquitin ligases with N-terminal transmembrane domains and soluble C-terminal RING-containing domains involved in ERAD, but there are several noteworthy differences. It is clear that Cue1p binds to Ubc7p (Biederer, et al., 1997), but how this Cue1p-Ubc7p complex is recruited to Hrd1p, if it is at all specifically recruited, is not understood. gp78 contains a CUE domain in its soluble cytoplasmic region, but it is not involved in recruiting the E2 Ube2g2 to gp78 (Chen, et al., 2006). That is accomplished by a distinct Ube2g2-binding region, also in the soluble cytoplasmic region of gp78. Like other CUE proteins, the CUE domain of gp78 promotes binding to polyubiquitin, but yeast Cue1p is notable for its lack of polyubiquitin binding (Shih, et al., 2003). Our work suggests that, like Ubc7p and Cue1p interaction in yeast, Ube2g2-binding by the cytosolic portion of gp78 in mammals may result in activation of Ube2g2.

Taken together, these studies indicate that the specific E3-E2 function of Hrd1p and Ubc7p is complex and involves multiple necessary conditions. These include the presence of *cis*-acting portions of the Hrd1p soluble cytoplasmic domain, the presence of the Ubc7p-activating Cue1p, and the placement of the Hrd1p ubiquitin ligase in the ER membrane, which all must be included in biochemical analyses to ensure that successful reconstitutions are physiologically meaningful ones as well.

## Methods:

*Recombinant DNA* - Detailed plasmid information is available in supplemental data. PCR primer information will be provided upon request. All DNA segments synthesized by PCR were verified by sequencing. The production of coding regions was described for Hrd1p-3HA and C399S Hrd1p-3HA (Gardner, et al., 2000), as well as Ubc7p-2HA (Gardner, et al., 2001). gp78, hsHrd1, and Praja1 RING motifs were amplified by PCR from published template plasmids (Kikkert, et al., 2004; Lorick, et al., 1999), and joined to Hrd1p sequences by a PCR SOEing method to precisely replaced the native Hrd1p RING motif, (Ho, et al., 1989; Horton, et al., 1989) and subcloned into plasmids containing Hrd1p-3HA to yield gp78, hsHrd1, or Praja1 RING chimera in otherwise full-length Hrd1p-3HA. These were then subcloned into yeast expression plasmids with either the native *HRD1* promoter or the strong *TDH3* promoter. Hrd1p-Δpro was made using PCR SOEing to join the sequences of Hrd1p on either side of the proline-flanked deletion (see Figure 3-1), and subcloned into appropriate Hrd1p-3HA plasmids. C399A-Hrd1p was made by PCR SOEing, and was found to be as potent a RING mutant as C399S-Hrd1p (data not shown).

All GST fusions were expressed from the pET42b(+) bacterial expression plasmid (Novagen). The isolated RING motifs were amplified by PCR from plasmids above and subcloned into pET42b(+). pRH1466, the plasmid expressing GST-R-C, was previously described (Bays, et al., 2001). GST-N-R and GST-N-R-C were made by PCR of the appropriate sequence from Hrd1p plasmid and subcloning into pRH1466. GST-N-c399s-C and GST-N-gp78-C were made by PCR of sequence

encoding the mutant C399S-Hrd1p or chimeric gp78-RING-Hrd1p. The c399 refers to the last cysteine of the Hrd1p RING that normally occupies position 399 of full-length Hrd1p.

The Ubc7p or Ubc7p-2HA coding region was amplified by PCR and subcloned into pTYB2 (New England Biolabs) to produce the Ubc7p-Chitin Binding Domain/ Intein fusion vector pRH1946. ΔtmCue1p, which lacks amino acids 2-22 of Cue1p (and thus the included transmembrane span) was amplified by PCR from pTX129 (Biederer, et al., 1997) and cloned into a pET bacterial expression vector. Then, the ribosomal binding site and ΔtmCue1p were amplified by PCR and cloned behind Ubc7p-CBD/Intein in pRH1946 to produce pRH2061, with a polycistronic message encoding both Ubc7p-CBD/Intein and ΔtmCue1p proteins in one inducible operon.

6His-tagged mouse UBA1 (E1) and HUBC4 were purified from bacterial lysates as described previously (Bays, et al., 2001; Joazeiro, et al., 1999; Mori, et al., 1997).

*Strains and Media* - Yeast were cultured at 30˚C as described (Hampton, et al., 1996; Hampton and Rine, 1994), in minimal media with 2% glucose and amino acid supplements. Detailed strain information is presented in supplemental data. All yeast strains were derived from the same genetic background used in our previous work (Hampton, et al., 1996; Hampton and Rine, 1994). Strains for evaluating the *in vivo* degradation of Hmg2p-GFP were derived from the previously described RHY853 (Gardner, et al., 2000), expressing Hmg2-GFP and the independently-expressed catalytic domain of Hmg2p as its sole source of HMG-CoA reductase. *HRD1* was

replaced in RHY853 with the G418-resistance marker kanMX (Guldener, et al., 1996) to produce RHY2814. The various HA-epitope-tagged Hrd1p chimeric-RING plasmids or controls were integrated into this *hrd1*Δ strain at the *TRP1* locus. To evaluate Hrd1p degradation, *HRD3* was deleted in RHY2814 with the selectable *LEU2* marker to produce RHY3005. Into this *hrd1*Δ *hrd3*Δ strain, the various Hrd1p RING replaced plasmids or controls were also integrated at the *TRP1* locus. To evaluate Ubc7p-dependence of Hrd1p degradation, *UBC7* was deleted in RHY3005 with the nourseothricin- (ClonNat) resistance marker natMX (Goldstein and McCusker, 1999) to produce RHY3559, into which Hrd1p RING replaced plasmids or controls were integrated at the *TRP1* locus.

Strains used to produce microsomal membranes for the *in vitro* assay were *pep4*Δ *ubc7*Δ *hrd1*Δ, and expressed Hmg2p-GFP. The full-length Hrd1p-3HA chimeras tested in microsomes were expressed in these strains from the strong *TDH3* promoter by integration of the appropriate plasmid at the *TRP1* locus. *cue1*Δ nulls were generated from these strains by deletion of *CUE1* with the nourseothricin- (ClonNat) resistance marker natMX (Goldstein and McCusker, 1999). Strains for the production of cytosol were also *pep4*Δ *hrd1*Δ *ubc7*Δ and included either empty vector or Ubc7p-2HA expressed from the *TDH3* promoter.

*Flow Cytometry* - Log phase cultures ($O.D._{600} < 0.5$) grown in minimal medium at 30˚C were transferred to flow cytometer sample tubes and measured with a Becton Dickinson FACScalibur instrument. Flow microfluorimetric data were

analyzed and histograms were generated using CellQuest flow cytometry software. In all cases, histograms represented 20,000 individual cells.

*Cycloheximide-Chase Degradation Assay* - This assay was performed as described (Hampton and Rine, 1994). Briefly, log phase cultures of cells expressing HA-epitope tagged Hrd1p or RING variants were treated with 50µg/ml cycloheximide to arrest protein synthesis. At indicated times, 1 O.D. of log phase cells were harvested, lysed, and 0.1 O.D. equivalents were resolved by SDS-PAGE and immunoblotted for epitope-tagged protein.

*Protein Purification* - All recombinant proteins were expressed in Rosetta(DE3) *E. coli* (Novagen) grown in LB with appropriate antibiotics. 0.6 O.D./ml cultures were induced with 0.5mM IPTG for 12-16 hours at 15˚C. Bacterial pellets were harvested, washed in normal saline (0.9M NaCl), and frozen at –80˚C. Pellets were thawed and resuspended in extraction buffers as described below, and lysed using a Branson Sonifier 450 (VWR) with six rounds of 30s sonication/30s ice incubation. After affinity column purification, elution, and concentration, proteins were dialyzed into buffer HDB containing 10% glycerol. Single-use aliquots were flash-frozen with liquid nitrogen and stored at –80˚C. Recombinant protein concentrations were determined by Coomassie staining of SDS-PAGE resolved samples and comparison to BSA.

His-Tag purification of E1 and HUBC4: - Bacterial pellets from 2L cultures expressing 6-His mouse UBA1 or HUBC4 were resuspended in 40mls of His-Extraction Buffer (50mM sodium phosphate pH 7.4, 300mM NaCl, 0.5% Nonidet NP-

40, 5% glycerol) with protease inhibitors (13 μM AEBSF, 3.6 μM TPCK, 2.6μM leupeptin, 1.8 μM pepstatin, 0.56mM 6-aminohexanoic acid, 0.56mM benzamidine, and 2.5mM 2-mercaptoethanol), sonicated as above, and centrifuged at 12,000 x g for 20minutes in an SS34 rotor. The supernatant was transferred to a new tube with 0.5ml Talon Cell-Thru resin (BD Biosciences) equilibrated in His-Extraction Buffer, and gently nutated for 20min at room temperature. The resin was centrifuged (3000g) and washed with 10mls His-Extraction Buffer and protease inhibitors above for 10min at RT two times. The washed resin was then transferred to a 1cm diameter column and washed with 30mls His-Wash Buffer (50mM sodium phosphate pH 7.8, 300mM NaCl, 5mM imidazole, 5% glycerol, 10mM 2-mercaptoethanol). His-tagged E1 was eluted from the resin with 3mls of His Wash Buffer + 150mM imidazole, and was collected in 500ul fractions and analyzed by Bradford assay with BSA standard. Fractions with more than 0.1 mg/ml protein were pooled and concentrated with Amicon Ultra-15 5,000 MWCO filters (Millipore). Concentrated protein was dialyzed over 24 hours with 3x 1L HDBG (25mM HEPES, 0.7mM sodium phosphate, 137mM NaCl, 5mM KCl, pH 7.4, 10% glycerol) in a 3ml 10,000 MWCO Slide-A-Lyzer cassette (Pierce).

GST Protein Purification: - Each bacterial pellet from 1L of culture expressing a GST protein was resuspended in 25mls of buffer HDB (25mM HEPES, 0.7mM sodium phosphate, 137mM NaCl, 5mM KCl, pH 7.4) + protease inhibitors (7.5 mM EDTA, 1.5 mM PMSF, 10μM leupeptin, 7μM pepstatin, 28 μM TPCK, 130 μM AEBSF, 2.5 mM 6-aminohexanoic acid, 2.5mM benzamidine, and 7.5mM DTT), and

sonicated as above. To this was added 6ml 2.5M NaCl, 600ul 1M sodium phosphate pH7.4, and 1.2ml 25% Triton X-100. This was nutated 20min at 4˚C, and centrifuged for 1hr at 40,000g in SS34 rotor. Buffer HDBW (HDB + 20mM sodium phosphate pH7.4, 10 mM 2-mercaptoethanol, 1% Triton X-100.) was added to the supernatant to a final volume of 50ml along with 1ml of glutathione-Sepharose-4B resin (Pharmacia) and nutated for 1hr at 4˚C. The resin was transferred to a 1cm diameter column and washed with 10 mls of each of the following buffers: HDBW with 1.25mM PMSF and 5mM EDTA; HDBW with 1.25mM PMSF, 5mM EDTA, and 0.5M NaCl; HDBW with 0.5% deoxycholate; HDBW with 0.5M NaCl; and Final Wash (40mM Tris pH8.0, 50mM NaCl, 10mM 2-mercaptoethanol). Protein was eluted in 1ml fractions of Elution Buffer (20mM Tris pH 8, 10mM 2-mercaptoethanol, 20mM reduced glutathione) incubated with column resin for 10min before recovery. Fractions were analyzed by Bradford assay with BSA standard. Fractions with more than 0.1 mg/ml protein were pooled and concentrated with Amicon Ultra-15 5,000 MWCO filters (Millipore). Concentrated protein was dialyzed for 24 hours with 3x 1L HDBG (25mM HEPES, 0.7mM sodium phosphate, 137mM NaCl, 5mM KCl, pH 7.4, 10% glycerol) in a 0.5ml 3,000 MWCO Slide-A-Lyzer cassette (Pierce).

Intein / Chitin Binding Domain Fusion Purification: - Each bacterial pellet from 1L of culture expressing an Intein/CBD fusion was resuspended in 25mls of Intein Lysis Buffer (ILB: 50mM Tris pH8.0, 500mM NaCl, 1mM EDTA, 0.1% Triton X-100) with protease inhibitors (260µM AEBSF, 105µM leupeptin, 73µM pepstatin, 142µM TPCK), and sonicated as above. Lysate was centrifuged at 20,000g for 30min

in an SS34 rotor. Supernatant was filtered through 0.45µm and 0.2µm filters, and added to 15mls of chitin beads (New England Biolabs) equilibrated in ILB, and nutated for 90min at 4˚C. The adsorbed resin was placed in a 2.5cm column and washed with 350-400 mls of ILB. Next, the resin was nutated in 10mls of ILB + 50mM DTT for 20 hrs at 4˚C to promote intein cleavage, and chitin beads were washed with ILB to collect intein-cleaved proteins. 40 mls of fluid were collected and concentrated using Amicon Ultra-15 5,000 MWCO filters (Millipore). Concentrated protein was dialyzed against 3x 1L HDBG (25mM HEPES, 0.7mM sodium phosphate, 137mM NaCl, 5mM KCl, pH 7.4, 10% glycerol) for 24 hours in a 0.5ml 3,000 MWCO Slide-a-Lyzer cassette (Pierce). Proteins were ultra-centrifuged at 100,000g to remove any aggregates, and supernatant was aliquoted as above.

*In Vitro Ubiquitination* - Ubiquitin was resuspended from lyophilized powder in Ubiquitin Storage Buffer (50mM Tris pH7.5, 50mM NaCl, 10% glycerol) and frozen. Reactions were performed in 1x Ubiquitination Buffer (50mM Tris pH7.5, 2.5mM $MgCl_2$, 0.5mM DTT) with 3mM ATP, 80µg/ml ubiquitin, 6µg/ml E1, 20µg/ml E2, in a total volume of 15µl. Reactions mixtures were prepared on ice, then incubated at 30˚C for 2 hours, and stopped with an equal volume of 2x sample buffer (4% SDS (w/v), 8M urea, 75mM MOPS pH6.8, 200mM DTT, 0.2 mg/ml bromphenol blue) and analyzed by SDS-PAGE and anti-ubiquitin immunoblotting.

*Microsome Preparation and Ubiquitination* - 20 $O.D._{600}$ units of log phase cells grown in minimal media were harvested and resuspended in 400µl of ice cold Membrane Fractionation Buffer (MFB: 20mM Tris pH7.5, 0.1M NaCl, 0.3M sorbitol)

with protease inhibitors (260µM AEBSF, 105µM leupeptin, 73µM pepstatin, 142µM TPCK). Glass beads were added to just below the liquid level. Lysis was performed at 4˚C with six cycles of 1min vortexing (max speed) and 1min incubation on ice. Lysate was harvested by removing supernatant from beads, and washing beads twice with 400µl MFB, pooling the washes and lysate. The resulting pooled lysate was cleared by repeated 10s microcentrifuge pulses to remove unlysed cells and large debris. The cleared supernatant contains microsome membranes, which were harvested by centrifugation at 21,000g for 30 min. Microsome pellets were resuspended in 60µl Ubiquitination Buffer, and the yield from 5 O.D. of cells (15µl) was added to each reaction. Reactions were performed in Ubiquitination Buffer with 6µg/ml E1, 40µg/ml E2 (except as noted in E2 dilution experiments), 160µg/ml ubiquitin and 3mM ATP in 60µl reactions. Reaction mixes were prepared on ice, then incubated at 30˚C for 2 hours. Reactions were stopped with 200 ml of SUME (1%w/v SDS, 8M urea, 10mM MOPS pH6.8, 10mM EDTA) with protease inhibitors above and 5mM N-ethylmaleimide, followed by addition of 600 ml of IP buffer (15mM sodium phosphate, 150mM NaCl, 10mM EDTA, 2% Triton X-100, 0.1% SDS, 0.5% deoxycholate,), immunoprecipitation of Hrd1p as described (Bays, et al., 2001), and immunoblotting the SDS-PAGE resolved immunoprecipitate for ubiquitin with anti-ubiquitin antibodies (Zymed Laboratories, South San Francisco, CA) or for Hrd1p with anti-HA ascites fluid (Jackson ImmunoResearch).

*Microsome Ubiquitination Assay with Cytosol* - Cytosol and microsomes were prepared as previously described (Flury, et al., 2005). Briefly, microsomes were

prepared as above and resuspended in 60µl B88 buffer (20 mM HEPES pH 6.8, 250 mM sorbitol 150 mM KOAc, 5mM MgOAc, 1mM DTT) with protease inhibitors (1mM PMSF, 260mM AEBSF, 100µM leupeptin, 76µM pepstatin A, 5mM 6-aminohexanoic acid, 5mM benzamidine, and 142µM TPCK). At the same time, cytosol from a Ubc7p overexpressing, *hrd1D* null strain was prepared in the manner of Spang and Schekman (Spang and Schekman, 1998). Control cytosol was prepared in parallel from an otherwise identical *ubc7D* null strain. Briefly, 500 O.D. equivalents of cells were pelleted, rinsed once with water, once with B88 buffer, and resuspended in 500 ml of B88 buffer. The resulting suspension was poured into a liquid nitrogen-containing mortar, and the resulting fast-frozen pellet was ground with a pestle until a fine powder. The frozen powder was next transferred to a microfuge tube, raised to 1mM ATP with a 500 mM stock solution in water (pH 7.5), and allowed to thaw on ice. The thawed cytosol lysate was centrifuged at 3000 x g for 5 min, to remove debris, and the resulting supernatant was removed and centrifuged at 20,000g for 15 minutes. Finally, the resulting 20,000g supernatant was removed and ultracentrifuged (100,000g) for 1 hr. The resulting supernatant was collected, and diluted to 25 mg/ml protein for use in the ubiquitination assay. The *in vitro* ubiquitination assay was initiated by addition of 20 ml of microsomes (with Hrd1p and Hmg2p), 12 ml of cytosol, and sufficient concentrated stock of ATP to yield 30 mM ATP, followed by incubation at 30°C, typically for an hour. The assay was terminated by solubilization with 200 ml of SUME with protease inhibitors above and 5mM N-ethylmaleimide, followed by addition of 600 ml of IP buffer, and subsequent immunoprecipitation of

either Hrd1p or Hmg2p. Immunoprecipitated samples were SDS-PAGE resolved and immunoblotted for ubiquitin and for either Hrd1p or Hmg2p-GFP using anti-HA ascites fluid, or anti-GFP monoclonal antibodies as described (Bays, et al., 2001).

**Table 3-1. Strains used in chapter three.**

| Strain | *Genotype* | Figure |
|---|---|---|
| RHY2940 | *MATα ade2-101 met2 lys2-801 his3Δ200 leu2Δ hmg1Δ::LYS2 hmg2Δ::HIS3 ura3-52::URA3::pTDH3-HMGcd::pTDH3-HMG2-GFP hrd1Δ::KanMX trp1::hisG::TRP1(pRH311)* | 3-2A, 3-2B, 3-3A |
| RHY2941 | *MATα ade2-101 met2 lys2-801 his3Δ200 leu2Δ hmg1Δ::LYS2 hmg2Δ::HIS3 ura3-52::URA3::pTDH3-HMGcd::pTDH3-HMG2-GFP hrd1Δ::KanMX trp1::hisG::TRP1::HRD1-3HA(pRH642)* | 3-2A, 3-2B, 3-3A, 3-3B |
| RHY2943 | *MATα ade2-101 met2 lys2-801 his3Δ200 leu2Δ hmg1Δ::LYS2 hmg2Δ::HIS3 ura3-52::URA3::pTDH3-HMGcd::pTDH3-HMG2-GFP hrd1Δ::KanMX trp1::hisG::TRP1::C399S-HRD1-3HA(pRH1245)* | 3-2A, 3-2B, 3-3A |
| RHY2944 | *MATα ade2-101 met2 lys2-801 his3Δ200 leu2Δ hmg1Δ::LYS2 hmg2Δ::HIS3 ura3-52::URA3::pTDH3-HMGcd::pTDH3-HMG2-GFP hrd1Δ::KanMX trp1::hisG::TRP1::PRAJA1-RING-HRD1-3HA(pRH1644)* | 3-2A, 3-2B, 3-3A |
| RHY2946 | *MATα ade2-101 met2 lys2-801 his3Δ200 leu2Δ hmg1Δ::LYS2 hmg2Δ::HIS3 ura3-52::URA3::pTDH3-HMGcd::pTDH3-HMG2-GFP hrd1Δ::KanMX trp1::hisG::TRP1::gp78-RING-HRD1-3HA(pRH1646)* | 3-2A, 3-2B, 3-3A |
| RHY3508 | *MATα ade2-101 met2 lys2-801 his3Δ200 leu2Δ hmg1Δ::LYS2 hmg2Δ::HIS3 ura3-52::URA3::pTDH3-HMGcd::pTDH3-HMG2-GFP hrd1Δ::KanMX trp1::hisG::TRP1:hsHrd1-RING-HRD1-3HA(pRH1883)* | 3-2A, 3-2B, 3-3A |
| RHY2942 | *MATα ade2-101 met2 lys2-801 his3Δ200 leu2Δ hmg1Δ::LYS2 hmg2Δ::HIS3 ura3-52::URA3::pTDH3-HMGcd::pTDH3-HMG2-GFP hrd1Δ::KanMX trp1::hisG::TRP1::pTDH3-HRD1-3HA(pRH730)* | 3-2C |
| RHY2945 | *MATα ade2-101 met2 lys2-801 his3Δ200 leu2Δ hmg1Δ::LYS2 hmg2Δ::HIS3 ura3-52::URA3::pTDH3-HMGcd::pTDH3-HMG2-GFP hrd1Δ::KanMX trp1::hisG::TRP1::pTDH3-PRAJA1-RING-HRD1-3HA(pRH1645)* | 3-2C |

**Table 3-1 continued. Strains used in chapter three.**

| Strain | *Genotype* | Figure |
|---|---|---|
| RHY2947 | *MATα ade2-101 met2 lys2-801 his3Δ200 leu2Δ hmg1Δ::LYS2 hmg2Δ::HIS3 ura3-52::URA3::pTDH3-HMGcd::pTDH3-HMG2-GFP hrd1Δ::KanMX trp1::hisG::TRP1::pTDH3-gp78-RING-HRD1-3HA(pRH1647)* | 3-2C |
| RHY3710 | *MATα ade2-101 met2 lys2-801 his3Δ200 leu2Δ hmg1Δ::LYS2 hmg2Δ::HIS3 ura3-52::URA3::pTDH3-HMGcd::pTDH3-HMG2-GFP hrd1Δ::KanMX trp1::hisG::TRP1::pTDH3-hsHrd1-RING-HRD1-3HA(pRH1912)* | 3-2C |
| RHY2995 | *MATα ade2-101 met2 lys2-801 his3Δ200 leu2Δ hmg1Δ::LYS2 hmg2Δ::HIS3 ura3-52::URA3::pTDH3-HMGcd::pTDH3-HMG2-GFP hrd1Δ::KanMX hrd3Δ::LEU2 trp1::hisG::TRP1::HRD1-3HA(pRH642)* | 3-3A, 3-3B |
| RHY2998 | *MATα ade2-101 met2 lys2-801 his3Δ200 leu2Δ hmg1Δ::LYS2 hmg2Δ::HIS3 ura3-52::URA3::pTDH3-HMGcd::pTDH3-HMG2-GFP hrd1Δ::KanMX hrd3Δ::LEU2 trp1::hisG::TRP1::PRAJA1-RING-HRD1-3HA(pRH1644)* | 3-3A |
| RHY3001 | *MATα ade2-101 met2 lys2-801 his3Δ200 leu2Δ hmg1Δ::LYS2 hmg2Δ::HIS3 ura3-52::URA3::pTDH3-HMGcd::pTDH3-HMG2-GFP hrd1Δ::KanMX hrd3Δ::LEU2 trp1::hisG::TRP1::gp78-RING-HRD1-3HA(pRH1646)* | 3-3A |
| RHY3532 | *MATα ade2-101 met2 lys2-801 his3Δ200 leu2Δ hmg1Δ::LYS2 hmg2Δ::HIS3 ura3-52::URA3::pTDH3-HMGcd::pTDH3-HMG2-GFP hrd1Δ::KanMX hrd3Δ::LEU2 trp1::hisG::TRP1:hsHrd1-RING-HRD1-3HA(pRH1883)* | 3-3A |
| RHY3574 | *MATα ade2-101 met2 lys2-801 his3Δ200 leu2Δ hmg1Δ::LYS2 hmg2Δ::HIS3 ura3-52::URA3::pTDH3-HMGcd::pTDH3-HMG2-GFP hrd1Δ::KanMX hrd3Δ::LEU2 ubc7Δ::NatR trp1::hisG::TRP1::HRD1-3HA(pRH642)* | 3-3B |

**Table 3-1 continued. Strains used in chapter three.**

| Strain | *Genotype* | Figure |
|---|---|---|
| RHY4083 | *MATα ade2-101 met2 lys2-801 his3Δ200 leu2Δ hmg1Δ::LYS2 hmg2Δ::HIS3 ura3-52::URA3::pTDH3-HMGcd::pTDH3-HMG2-GFP hrd1Δ::KanMX trp1::hisG::TRP1::Hrd1pΔpro-RING-HRD1-3HA(pRH1949)* | 3-3B |
| RHY4090 | *MATα ade2-101 met2 lys2-801 his3Δ200 leu2Δ hmg1Δ::LYS2 hmg2Δ::HIS3 ura3-52::URA3::pTDH3-HMGcd::pTDH3-HMG2-GFP hrd1Δ::KanMX hrd3Δ::LEU2 trp1::hisG::TRP1:hsHrd1-RING-HRD1-3HA(pRH1949)* | 3-3B |
| RHY4097 | *MATα ade2-101 met2 lys2-801 his3Δ200 leu2Δ hmg1Δ::LYS2 hmg2Δ::HIS3 ura3-52::URA3::pTDH3-HMGcd::pTDH3-HMG2-GFP hrd1Δ::KanMX hrd3Δ::LEU2 ubc7Δ::NatR trp1::hisG::TRP1:hsHrd1-RING-HRD1-3HA(pRH1949)* | 3-3B |
| RHY3751 | *MATα ade2-101 met2 lys2-801 his3Δ200 leu2Δ hmg2Δ::1MYC-HMG2 ura3-52::URA3::pTDH3-HMG2-GFP pep4Δ::HIS3 hrd1Δ::KanMX ubc7Δ::LEU2 trp1::hisG::TRP1::empty vector(pRH311)* | 3-8, 3-11 |
| RHY3752 | *MATα ade2-101 met2 lys2-801 his3Δ200 leu2Δ hmg2Δ::1MYC-HMG2 ura3-52::URA3::pTDH3-HMG2-GFP pep4Δ::HIS3 hrd1Δ::KanMX ubc7Δ::LEU2 trp1::hisG::TRP1::pTDH3-HRD1-3HA(pRH730)* | 3-8, 3-9, 3-10A, 3-11 |
| RHY3753 | *MATα ade2-101 met2 lys2-801 his3Δ200 leu2Δ hmg2Δ::1MYC-HMG2 ura3-52::URA3::pTDH3-HMG2-GFP pep4Δ::HIS3 hrd1Δ::KanMX ubc7Δ::LEU2 trp1::hisG::TRP1::pTDH3-PRAJA1-RING-HRD1-3HA(pRH1645)* | 3-8 |
| RHY3754 | *MATα ade2-101 met2 lys2-801 his3Δ200 leu2Δ hmg2Δ::1MYC-HMG2 ura3-52::URA3::pTDH3-HMG2-GFP pep4Δ::HIS3 hrd1Δ::KanMX ubc7Δ::LEU2 trp1::hisG::TRP1::pTDH3-gp78-RING-HRD1-3HA(pRH1647)* | 3-8 |

**Table 3-1 continued. Strains used in chapter three.**

| Strain | *Genotype* | Figure |
|---|---|---|
| RHY3762 | *MATα ade2-101 met2 lys2-801 his3Δ200 leu2Δ hmg2Δ::1MYC-HMG2 ura3-52::URA3::pTDH3-HMG2-GFP pep4Δ::HIS3 hrd1Δ::KanMX ubc7Δ::LEU2 trp1::hisG::TRP1::pTDH3-hsHrd1-RING-HRD1-3HA(pRH1912)* | 3-8 |
| RHY3764 | *MATα ade2-101 met2 lys2-801 his3Δ200 leu2Δ hmg2Δ::1MYC-HMG2 ura3-52::URA3::pTDH3-HMG2-GFP pep4Δ::HIS3 hrd1Δ::KanMX ubc7Δ::LEU2 trp1::hisG::TRP1::pTDH3-C399A-HRD1-3HA(pRH1900)* | 3-8 |
| RHY3929 | *MATα ade2-101 met2 lys2-801 his3Δ200 leu2Δ hmg2Δ::1MYC-HMG2 ura3-52::URA3::pTDH3-HMG2-GFP pep4Δ::HIS3 hrd1Δ::KanMX ubc7Δ::LEU2 cue1Δ::NatR trp1::hisG::TRP1::pTDH3-HRD1-3HA(pRH730)* | 3-9, 3-10A, 3-11 |
| RHY3930 | *MATα ade2-101 met2 lys2-801 his3Δ200 leu2Δ hmg2Δ::1MYC-HMG2 ura3-52::URA3::pTDH3-HMG2-GFP pep4Δ::HIS3 hrd1Δ::KanMX ubc7Δ::LEU2 cue1Δ::NatR trp1::hisG::TRP1::pTDH3-gp78-RING-HRD1-3HA(pRH1647)* | 3-11 |
| RHY4295 | *MATα ade2-101 met2 lys2-801 his3Δ200 leu2Δ hmg2Δ::1MYC-HMG2 ura3-52 pep4Δ::HIS3 hrd1Δ::KanMX ubc7Δ::LEU2 trp1::hisG::TRP1::pTDH3-Ubc7p-2HA(pRH373)* | 3-10, 3-11 |
| RHY4288 | *MATα ade2-101 met2 lys2-801 his3Δ200 leu2Δ hmg2Δ::1MYC-HMG2 ura3-52 pep4Δ::HIS3 hrd1Δ::KanMX ubc7Δ::LEU2 trp1::hisG* | 3-10, 3-11 |

**Table 3-2. Plasmids used in chapter three.**

| Plasmid | Construction |
|---|---|
| pRH311 | pRS404, *TRP1*/YIp<br>Genetics 122: 19-27 (May, 1989) |
| pRH642 | $P_{HRD1}$-Hrd1p-3HA. *TRP1*/YIp<br>Native promoter Hrd1p from *hrd1-1* complementing plasmid was subcloned into pRS404 to make pRH507. PCR SOEing was used to add the 3HA tag. |
| pRH1245 | $P_{HRD1}$-C399S-Hrd1p-3HA. *TRP1*/YIp<br>Gardner, R.G. et al. (2000) JCB 151(1)69-82 |
| pRH1644 | $P_{HRD1}$-Praja1-RING-Hrd1p-3HA. *TRP1*/YIp<br>The Praja1 RING was amplified by PCR from p2641 (Lorick, K. L. et al. 1999) and PCR SOEing was used to join it to the *HRD1* sequences adjacent to the Hrd1p RING. This was then subcloned into pRH642 to make pRH1644. |
| pRH1646 | $P_{HRD1}$-gp78-RING-Hrd1p-3HA. *TRP1*/YIp<br>The gp78 RING was amplified by PCR from GST-gp78C2 (Fang weissman AM 2001) and PCR SOEing was used to join it to the *HRD1* sequences adjacent to the Hrd1p RING. This was then subcloned into pRH642 to make pRH1646, |
| pRH1883 | $P_{HRD1}$-hsHrd1-RING-Hrd1p-3HA. *TRP1*/YIp<br>The Hrd1p RING was amplified by PCR from a human Hrd1-containing plasmid (Marjolein Kikkert / Wiertz Lab) and PCR SOEing was used to join it to the *HRD1* sequences adjacent to the Hrd1p RING. This was then subcloned into pRH642 to make pRH1883. |
| pRH1949 | $P_{HRD1}$-ΔproRING-Hrd1p-3HA. *TRP1*/YIp<br>PCR SOEing was used to make a Hrd1p RING without the sequence encoding the Δpro residues in the Loop1 region of the Hrd1p RING. This was then subcloned into pRH642 to make pRH1949. |
| pRH730 | $P_{TDH3}$-Hrd1p-3HA. *TRP1*/YIp<br>Gardner, R.G. et al. (2000) JCB 151(1)69-82 |
| pRH1645 | $P_{TDH3}$-Praja1-RING-Hrd1p-3HA. *TRP1*/YIp<br>The Praja1 RING was amplified by PCR from p2641 (Lorick, K. L. et al. 1999) and PCR SOEing was used to join it to the *HRD1* sequences adjacent to the Hrd1p RING. This was then subcloned into pRH730 to make pRH1645. |

**Table 3-2 continued. Plasmids used in chapter three.**

| Plasmid | Construction |
|---|---|
| pRH1647 | $P_{TDH3}$-gp78-RING-Hrd1p-3HA. *TRP1*/YIp<br>The gp78 RING was amplified by PCR from GST-gp78C2 (Fang weissman AM 2001) and PCR SOEing was used to join it to the *HRD1* sequences adjacent to the Hrd1p RING. This was then subcloned into pRH730 to make pRH1647. |
| pRH1912 | $P_{TDH3}$-hsHrd1-RING-Hrd1p-3HA. *TRP1*/YIp<br>A human Hrd1 RING-containing BglII/NcoI fragment from pRH1883 was subcloned into pRH730. |
| pRH1900 | $P_{TDH3}$-C399S-Hrd1p-3HA. *TRP1*/YIp<br>PCR SOEing was used to generate the C399S point mutation, and this fragment was subcloned into pRH730 with BglII/NsiI. |
| pRH1749 | GST-Hrd1p-RING<br>The Hrd1p RING was amplified by PCR from pRH642 and subcloned into pET42b(+) with NcoI and EcoRI. |
| pRH1750 | GST-C399S-Hrd1p-RING<br>The C399S-Hrd1p RING was amplified by PCR from pRH1245 and subcloned into pET42b(+) with NcoI and EcoRI. |
| pRH1751 | GST-Praja1-RING<br>The Praja1 RING was amplified by PCR from pRH1644 and subcloned into pET42b(+) with NcoI and EcoRI. |
| pRH1752 | GST-gp78-RING<br>The gp78 RING was amplified by PCR from pRH1646 and subcloned into pET42b(+) with NcoI and EcoRI. |
| pRH1915 | GST-hsHrd1-RING<br>The hsHrd1 RING was amplified by PCR from pRH1883 and subcloned into pET42b(+) with NcoI and EcoRI. |
| pRH1466 | GST-R-C, GST fused to the C-terminal 203 amino acids of Hrd1p in pET42b(+)<br>Bays, NCB |
| pRH1726 | GST-N-R-C, GST fused to amino acids 262 to 551 of Hrd1p. Hrd1p sequence cut from pRH730 with BsrFI and SpeI, was subcloned into pRH1466 using AgeI and AvrII. |
| pRH1728 | GST-N-gp78-C, GST fused to amino acids 262 to 551 of Hrd1p with gp78-replacement in RING. gp78-RING-Hrd1p sequence was cut from pRH1647 with BsrFI and SpeI, was subcloned into pRH1466 using AgeI and AvrII. |

**Table 3-2 continued. Plasmids used in chapter three.**

| Plasmid | Construction |
|---|---|
| pRH1729 | GST-N-R, GST fused to amino acids 262 to 419 of Hrd1p. Hrd1p sequence from a c-terminal truncation was cut from pRH1654 with BsrFI and SpeI, and subcloned into pRH1466 using AgeI and AvrII. |
| pRH1946 | Ubc7p-Intein/Chitin Binding Domain. Ubc7p was amplified by PCR from pRH373( Gardner et al (2001) MCB) and subcloned into NEB IMPACT vector TYB2. |
| pRH1289 | 6HIS-E1 (mouse UBA1) Mori, S. et al. *European Journal of Biochemistry* **247**, 1190-6 (1997). |
| pRH1290 | 6HIS-HUBC4 Mori, S. et al. *European Journal of Biochemistry* **247**, 1190-6 (1997). |
| pRH2061 | ΔTMCue1p and Ubc7p-Intein/Chitin Binding Domain. Cue1p was amplified by PCR and subcloned into a pET vector with NdeI and XhoI to place ΔTMCue1p sequence near a ribosomal binding site. Then, the pET vector's ribosomal binding site and, ΔTM Cue1p were amplified by PCR and subcloned into pRH1946 using PstI, and screened for correct orientation. |
| pRH1947 | Ubc7p-2HA-Intein/Chitin Binding Domain. Ubc7p was amplified by PCR from pRH373( Gardner et al (2001) MCB) and subcloned into NEB IMPACT vector TYB2. |
| pRH1600 | pRH730 was digested with AflIII followed by partial digestion with NcoI and reclosure. promoterless Hrd1p-3HA. *TRP1*/YIp. |

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## References:


1. Allen, J. R., Nguyen, L. X., Sargent, K. E., Lipson, K. L., Hackett, A. and Urano, F. (2004). High ER stress in beta-cells stimulates intracellular degradation of misfolded insulin. *Biochem Biophys Res Commun* **324** (1) 166-170.

2. Amano, T., Yamasaki, S., Yagishita, N., Tsuchimochi, K., Shin, H., Kawahara, K., Aratani, S., Fujita, H., Zhang, L., Ikeda, R., Fujii, R., Miura, N., Komiya, S., Nishioka, K., Maruyama, I., Fukamizu, A. and Nakajima, T. (2003). Synoviolin/Hrd1, an E3 ubiquitin ligase, as a novel pathogenic factor for arthropathy. *Genes Dev* **17** (19) 2436-2449.

3. 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. *Nat Cell Biol* **3** (1) 24-29.

4. Biederer, T., Volkwein, C. and Sommer, T. (1997). Role of Cue1p in ubiquitination and degradation at the ER surface. *Science* **278** (5344) 1806-1809.

5. Bordallo, J., Plemper, R. K., Finger, A. and Wolf, D. H. (1998). Der3p/Hrd1p is required for endoplasmic reticulum-associated degradation of misfolded lumenal and integral membrane proteins. *Mol Biol Cell* **9** (1) 209-222.

6. Brodsky, J. L. and McCracken, A. A. (1999). ER protein quality control and proteasome-mediated protein degradation. *Semin Cell Dev Biol* **10** (5) 507-513.

7. Brzovic, P. S., Keeffe, J. R., Nishikawa, H., Miyamoto, K., Fox, D., 3rd, Fukuda, M., Ohta, T. and Klevit, R. (2003). Binding and recognition in the assembly of an active BRCA1/BARD1 ubiquitin-ligase complex. *Proc Natl Acad Sci U S A* **100** (10) 5646-5651.

8. Chen, B., Mariano, J., Tsai, Y. C., Chan, A. H., Cohen, M. and Weissman, A. M. (2006). The activity of a human endoplasmic reticulum-associated degradation E3, gp78, requires its Cue domain, RING finger, and an E2-binding site. *Proc Natl Acad Sci U S A* **103** (2) 341-346.

9. Chong, S., Mersha, F. B., Comb, D. G., Scott, M. E., Landry, D., Vence, L. M., Perler, F. B., Benner, J., Kucera, R. B., Hirvonen, C. A., Pelletier, J. J., Paulus, H. and Xu, M. Q. (1997). Single-column purification of free recombinant proteins using a self-cleavable affinity tag derived from a protein splicing element. *Gene* **192** (2) 271-281.

10. Cronin, S. R., Khoury, A., Ferry, D. K. and Hampton, R. Y. (2000). Regulation of HMG-CoA reductase degradation requires the P-type ATPase Cod1p/Spf1p. *J Cell Biol* **148** (5) 915-924.

11. Fang, S., Ferrone, M., Yang, C., Jensen, J. P., Tiwari, S. and Weissman, A. M. (2001). The tumor autocrine motility factor receptor, gp78, is a ubiquitin protein ligase implicated in degradation from the endoplasmic reticulum. *Proc Natl Acad Sci U S A* **98** (25) 14422-14427.

12. Fang, S., Lorick, K. L., Jensen, J. P. and Weissman, A. M. (2003). RING finger ubiquitin protein ligases: implications for tumorigenesis, metastasis and for molecular targets in cancer. *Semin Cancer Biol* **13** (1) 5-14.

13. Flury, I., Garza, R., Shearer, A., Rosen, J., Cronin, S. and Hampton, R. Y. (2005). INSIG: a broadly conserved transmembrane chaperone for sterol-sensing domain proteins. *Embo J* **24** (22) 3917-3926.

14. Gardner, R., Cronin, S., Leader, B., Rine, J. and Hampton, R. (1998). Sequence determinants for regulated degradation of yeast 3-hydroxy-3-methylglutaryl-CoA reductase, an integral endoplasmic reticulum membrane protein. *Mol Biol Cell* **9** (9) 2611-2626.

15. Gardner, R. G., Nelson, Z. W. and Gottschling, D. E. (2005). Degradation-mediated protein quality control in the nucleus. *Cell* **120** (6) 803-815.

16. 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. *Mol Cell Biol* **21** (13) 4276-4291.

17. Gardner, R. G., Swarbrick, G. M., Bays, N. W., Cronin, S. R., Wilhovsky, S., Seelig, L., Kim, C. and Hampton, R. Y. (2000). Endoplasmic reticulum degradation requires lumen to cytosol signaling. Transmembrane control of Hrd1p by Hrd3p. *J Cell Biol* **151** (1) 69-82.

18. Goldstein, A. L. and McCusker, J. H. (1999). Three new dominant drug resistance cassettes for gene disruption in Saccharomyces cerevisiae. *Yeast* **15** (14) 1541-1553.

19. Guldener, U., Heck, S., Fielder, T., Beinhauer, J. and Hegemann, J. H. (1996). A new efficient gene disruption cassette for repeated use in budding yeast. *Nucleic Acids Res* **24** (13) 2519-2524.

20. Hampton, R. Y., Gardner, R. G. and Rine, J. (1996). Role of 26S proteasome and HRD genes in the degradation of 3-hydroxy-3-methylglutaryl-CoA reductase,

an integral endoplasmic reticulum membrane protein. *Mol Biol Cell* **7** (12) 2029-2044.

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

22. Hatakeyama, S., Yada, M., Matsumoto, M., Ishida, N. and Nakayama, K. I. (2001). U box proteins as a new family of ubiquitin-protein ligases. *J Biol Chem* **276** (35) 33111-33120.

23. Haynes, C. M., Caldwell, S. and Cooper, A. A. (2002). An HRD/DER-independent ER quality control mechanism involves Rsp5p-dependent ubiquitination and ER-Golgi transport. *J Cell Biol* **158** (1) 91-101.

24. Hershko, A. and Ciechanover, A. (1998). The ubiquitin system. *Annu Rev Biochem* **67** 425-479.

25. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K. and Pease, L. R. (1989). Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* **77** (1) 51-59.

26. Hochstrasser, M. (1996). Ubiquitin-dependent protein degradation. *Annu Rev Genet* **30** 405-439.

27. 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.

28. Huang, T. T. and D'Andrea, A. D. (2006). Regulation of DNA repair by ubiquitylation. *Nat Rev Mol Cell Biol* **7** (5) 323-334.

29. Joazeiro, C. A., Wing, S. S., Huang, H., Leverson, J. D., Hunter, T. and Liu, Y. C. (1999). The tyrosine kinase negative regulator c-Cbl as a RING-type, E2-dependent ubiquitin-protein ligase. *Science* **286** (5438) 309-312.

30. Kaneko, M., Ishiguro, M., Niinuma, Y., Uesugi, M. and Nomura, Y. (2002). Human HRD1 protects against ER stress-induced apoptosis through ER-associated degradation. *FEBS Lett* **532** (1-2) 147-152.

31. Katoh, S., Hong, C., Tsunoda, Y., Murata, K., Takai, R., Minami, E., Yamazaki, T. and Katoh, E. (2003). High precision NMR structure and function of the RING-H2 finger domain of EL5, a rice protein whose expression is increased

upon exposure to pathogen-derived oligosaccharides. *J Biol Chem* **278** (17) 15341-15348.

32. Kikkert, M., Doolman, R., Dai, M., Avner, R., Hassink, G., van Voorden, S., Thanedar, S., Roitelman, J., Chau, V. and Wiertz, E. (2004). Human HRD1 is an E3 ubiquitin ligase involved in degradation of proteins from the endoplasmic reticulum. *J Biol Chem* **279** (5) 3525-3534.

33. Lorick, K. L., Jensen, J. P., Fang, S., Ong, A. M., Hatakeyama, S. and Weissman, A. M. (1999). RING fingers mediate ubiquitin-conjugating enzyme (E2)-dependent ubiquitination. *Proc Natl Acad Sci U S A* **96** (20) 11364-11369.

34. Mori, S., Tanaka, K., Kanaki, H., Nakao, M., Anan, T., Yokote, K., Tamura, K. and Saito, Y. (1997). Identification of an ubiquitin-ligation system for the epidermal-growth-factor receptor--herbimycin A induces in vitro ubiquitination in rabbit-reticulocyte lysate. *Eur J Biochem* **247** (3) 1190-1196.

35. Nakayama, K. I. and Nakayama, K. (2006). Ubiquitin ligases: cell-cycle control and cancer. *Nat Rev Cancer* **6** (5) 369-381.

36. Petroski, M. D. and Deshaies, R. J. (2005). Function and regulation of cullin-RING ubiquitin ligases. *Nat Rev Mol Cell Biol* **6** (1) 9-20.

37. Pickart, C. M. (2001). Mechanisms underlying ubiquitination. *Annu Rev Biochem* **70** 503-533.

38. Pickart, C. M. and Eddins, M. J. (2004). Ubiquitin: structures, functions, mechanisms. *Biochim Biophys Acta* **1695** (1-3) 55-72.

39. 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. *J Biol Chem* **273** (49) 32848-32856.

40. Ravid, T., Kreft, S. G. and Hochstrasser, M. (2006). Membrane and soluble substrates of the Doa10 ubiquitin ligase are degraded by distinct pathways. *Embo J* **25** (3) 533-543.

41. Saha, T., Vardhini, D., Tang, Y., Katuri, V., Jogunoori, W., Volpe, E. A., Haines, D., Sidawy, A., Zhou, X., Gallicano, I., Schlegel, R., Mishra, B. and Mishra, L. (2006). RING finger-dependent ubiquitination by PRAJA is dependent on TGF-beta and potentially defines the functional status of the tumor suppressor ELF. *Oncogene* **25** (5) 693-705.

42. Sasaki, A., Masuda, Y., Iwai, K., Ikeda, K. and Watanabe, K. (2002). A RING finger protein Praja1 regulates Dlx5-dependent transcription through its ubiquitin ligase activity for the Dlx/Msx-interacting MAGE/Necdin family protein, Dlxin-1. *J Biol Chem* **277** (25) 22541-22546.

43. Seol, J. H., Feldman, R. M., Zachariae, W., Shevchenko, A., Correll, C. C., Lyapina, S., Chi, Y., Galova, M., Claypool, J., Sandmeyer, S., Nasmyth, K. and Deshaies, R. J. (1999). Cdc53/cullin and the essential Hrt1 RING-H2 subunit of SCF define a ubiquitin ligase module that activates the E2 enzyme Cdc34. *Genes Dev* **13** (12) 1614-1626.

44. Shih, S. C., Prag, G., Francis, S. A., Sutanto, M. A., Hurley, J. H. and Hicke, L. (2003). A ubiquitin-binding motif required for intramolecular monoubiquitylation, the CUE domain. *Embo J* **22** (6) 1273-1281.

45. Song, B. L., Sever, N. and DeBose-Boyd, R. A. (2005). Gp78, a membrane-anchored ubiquitin ligase, associates with Insig-1 and couples sterol-regulated ubiquitination to degradation of HMG CoA reductase. *Mol Cell* **19** (6) 829-840.

46. Spang, A. and Schekman, R. (1998). Reconstitution of retrograde transport from the Golgi to the ER in vitro. *J Cell Biol* **143** (3) 589-599.

47. Staub, O. and Rotin, D. (2006). Role of ubiquitylation in cellular membrane transport. *Physiol Rev* **86** (2) 669-707.

48. 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 Dev* **15** (20) 2660-2674.

49. Weissman, A. M. (2001). Themes and variations on ubiquitylation. *Nat Rev Mol Cell Biol* **2** (3) 169-178.

50. Wilhovsky, S., Gardner, R. and Hampton, R. (2000). HRD gene dependence of endoplasmic reticulum-associated degradation. *Mol Biol Cell* **11** (5) 1697-1708.

51. Zhang, M., Windheim, M., Roe, S. M., Peggie, M., Cohen, P., Prodromou, C. and Pearl, L. H. (2005). Chaperoned ubiquitylation--crystal structures of the CHIP U box E3 ubiquitin ligase and a CHIP-Ubc13-Uev1a complex. *Mol Cell* **20** (4) 525-538.

52. Zheng, N., Schulman, B. A., Song, L., Miller, J. J., Jeffrey, P. D., Wang, P., Chu, C., Koepp, D. M., Elledge, S. J., Pagano, M., Conaway, R. C., Conaway, J. W., Harper, J. W. and Pavletich, N. P. (2002). Structure of the Cul1-Rbx1-Skp1-F boxSkp2 SCF ubiquitin ligase complex. *Nature* **416** (6882) 703-709.

53. Zheng, N., Wang, P., Jeffrey, P. D. and Pavletich, N. P. (2000). Structure of a c-Cbl-UbcH7 complex: RING domain function in ubiquitin-protein ligases. *Cell* **102** (4) 533-539.

54. Zhong, X., Shen, Y., Ballar, P., Apostolou, A., Agami, R. and Fang, S. (2004). AAA ATPase p97/valosin-containing protein interacts with gp78, a ubiquitin ligase for endoplasmic reticulum-associated degradation. *J Biol Chem* **279** (44) 45676-45684.

# Chapter 4:

# Cue1p is an activator of Ubc7p E2 activity

## Introduction:

In Chapter 3, we examined the conditions required *in vitro* for the ubiquitin ligase Hrd1p to recapitulate its *in vivo* specificity for Ubc7p. In doing so, we performed *in vitro* ubiquitination assays comparing the E2 activity of Ubc7p to that of Ubc7p co-expressed with Cue1p (Figure 3-6 and Figure 3-7). To our surprise, in these soluble, membrane-free, *in vitro* ubiquitination assay conditions, Cue1p strongly enhanced the ubiquitination activity of Ubc7p with RING motif-containing E3s. Additionally, we observed E3-independent formation of large polyubiquitin chains in the presence of Ubc7p and Cue1p, but not with Ubc7p alone.

Cue1p is required for the Ubc7p-dependent ubiquitination and degradation of substrates in the ER (Biederer, et al., 1997; Gardner, et al., 2001; Ravid, et al., 2006; Walter, et al., 2001). In cell lysates, Ubc7p is tightly associated with the membrane fraction and this peripheral membrane-association requires Cue1p (Biederer, et al., 1997; Gardner, et al., 2001). Because proximity and interaction between E2 and E3 are critical for ubiquitination function (VanDemark and Hill, 2002), it was concluded that Cue1p's concentrating of Ubc7p at the ER membrane surface was necessary for Ubc7p to engage ER localized E3s. However, studies of Cue1p have not addressed the possibility highlighted by our *in vitro* experiments, that Cue1p might play a role stimulating the catalytic activity of Ubc7p.

Aware that *in vitro* ubiquitination reactions do not always predict *in vivo* functions, we explored in greater detail our initial observation that Cue1p could stimulate E2 activity *in vitro*. We examined the nature of the polyubiquitin chains

formed in these E3-independent *in vitro* reactions. We discovered that *in vitro*, Ubc7p is autonomously able to direct polyubiquitination through lysine-48 linkages, and that the soluble portion of Cue1p stimulated the activity of Ubc7p to form lysine-48 linked polyubiquitin chains in the presence or absence of E3. We then designed chimeric proteins that, when expressed *in vivo*, separated the established anchoring function of Cue1p from its putative activation function. We tested which of these roles of Cue1p were important for the *in vivo* function of Ubc7p in ERAD. We found that both functions were important: Cue1p not only anchors Ubc7p to the ER membrane, but also stimulates the ubiquitination activity of Ubc7p. Moreover, both are required for efficient Hrd1p-dependent ERAD. Taken together, these results reveal a previously unknown role for Cue1p as an activator of Ubc7p E2 activity, and suggest that other E2s may have similar stimulating cofactors.

## Results:

In the course of our studies of Hrd1p *in vitro*, we found that Cue1p had the ability to catalyze Ubc7p-dependent ubiquitination both in the presence or absence of E3. The recombinant E3 (referred to in Chapter 3 as GST-N-R-C) was expressed as a GST fusion and affinity-purified using glutathione-sepharose beads. Recombinant E2 (Ubc7p or Ubc7p+ΔtmCue1p) was expressed as an intein-cleavable fusion to a chitin-binding domain and purified using chitin affinity beads. *In vitro* reactions were run by combining ATP, ubiquitin, E1, E2, and E3. The formation of polyubiquitin chains in the reactions was evaluated directly by SDS-PAGE and ubiquitin immunoblotting. Reactions with Ubc7p as E2 formed polyubiquitin chains only with the Hrd1p-containing GST-N-R-C fusion, and reactions without E3 showed no ubiquitin immunoreactivity (Figure 4-1, left panels). In contrast, reactions using Ubc7p+ΔtmCue1p as E2 showed more ubiquitin immunoreactivity than observed with Ubc7p alone (Figure 4-1, right panels). Ubc7p+ΔtmCue1p catalyzed more polyubiquitination than lone-Ubc7p, both in the presence or absence of E3. Moreover, ubiquitination by Ubc7p+ΔtmCue1p without E3 was comparable to that of Ubc7p with E3, highlighting the strong enhancement of Ubc7p ubiquitination activity by Cue1p. Individual rungs of the polyubiquitin ladder were observed in each of the Ubc7p+ΔtmCue1p reactions. However, it was puzzling how these ubiquitin-containing chains could have formed without E3 in the reactions. Thus, we wanted to evaluate in more detail the *in vitro* ubiquitination catalyzed by co-purified Ubc7p and ΔtmCue1p.

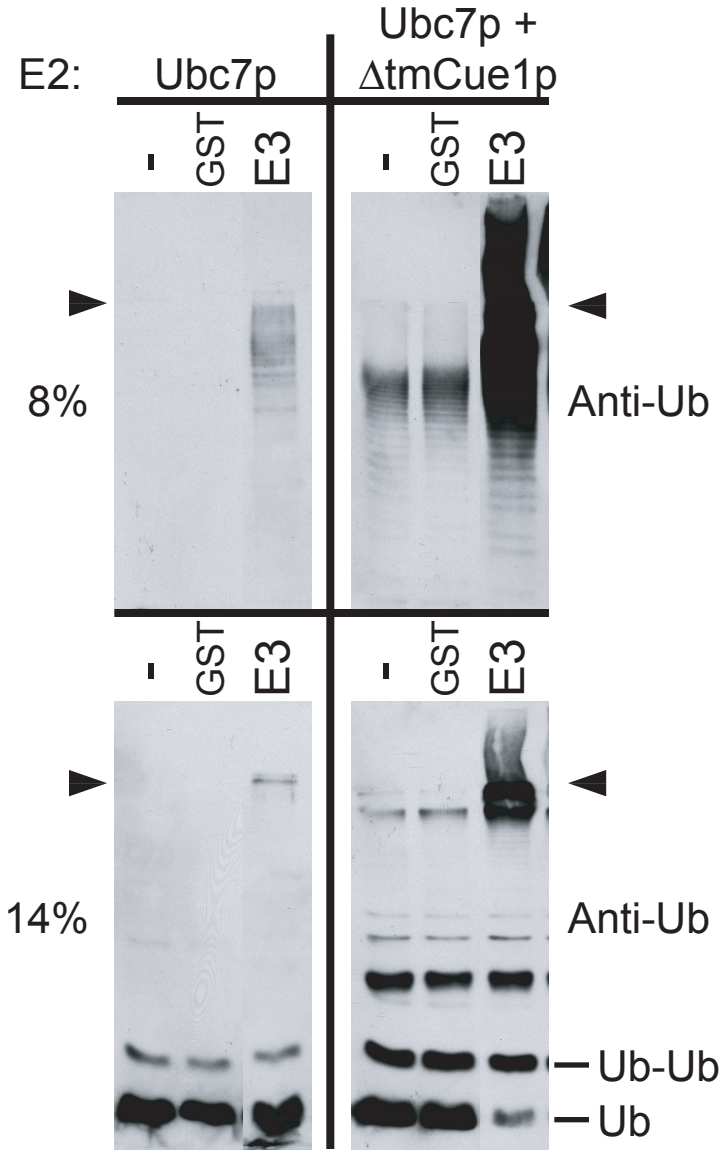


**Figure 4-1: ΔtmCue1p enhanced production of both large and small ubiquitin-immunoreactive bands by Ubc7p.**

*In vitro* ubiquitination reactions with no E3 (-), GST alone (GST), or GST-N-RING-C (E3), were prepared with either Ubc7p (left panels) or Ubc7p+ΔtmCue1p (right panels). Ubiquitination was E3 dependent with Ubc7p, while Ubc7p+ΔtmCue1p enhanced ubiquitination both in the presence and absence of E3 (compare left and right panels). Identical samples were resolved by SDS-PAGE using 8% gels (upper panels) or 14% gels (lower panels), revealing the production of both large and small ubiquitin-immunoreactive bands. Arrowheads indicate the discontinuity between 4% stacking gels and running gels. The molecular weights of mono-ubiquitin (Ub) and di-ubiquitin (Ub-Ub) are indicated.

Cdc34p, an E2 associated with the SCF ubiquitin ligase complex, is the most closely related E2 to Ubc7p in *Saccharomyces cerevisiae* (Jones, et al., 2002; Kraft, et al., 2005; Ptak, et al., 2001). In the absence of E3, Cdc34p was observed to link two ubiquitin molecules into ubiquitin dimers *in vitro,* and this activity was strongly enhanced by addition of purified SCF ubiquitin ligase complex (Petroski and Deshaies, 2005). Given the homology between Ubc7p and Cdc34p, and that Cue1p is required for Hrd1p-dependent ERAD, it seemed possible that the E3-independent, intermediate-sized polyubiquitin chains observed with Ubc7p *in vitro* might be produced by a mechanism similar to that observed with Cdc34p. To examine this possibility, an identical portion of each *in vitro* ubiquitination reaction above was resolved with high-percentage SDS-PAGE gels, then immunoblotted for ubiquitin. In the presence of Ubc7p+ΔtmCue1p, the *in vitro* reactions produced multiple low molecular weight bands not observed with Ubc7p alone, including a 16kD band consistent with a ubiquitin dimer (Figure 4-1, lower panels). Formation of the ubiquitin dimer band with Ubc7p alone was weak compared to Ubc7p+ΔtmCue1p. Also, none of the lower molecular weight bands was impacted by the presence or absence of E3.

Because these low molecular weight bands were detected by high-sensitivity immunoblotting, they could have been low in abundance, representing only a small pool of the protein in the reaction. To examine the extent to which the ubiquitin dimer and other low molecular weight bands were produced, we analyzed the reaction mixes with bulk protein staining. We prepared E3-independent ubiquitination reactions as

above, using Ubc7p, Ubc7p+ΔtmCue1p, or HUBC4 as E2. These reactions were run as before, then resolved by high-percentage SDS-PAGE and stained with Coomassie Brilliant Blue. E2s alone were loaded alongside the *in vitro* reactions to help differentiate the E2 bands from bands produced during the ubiquitination reaction. In the reaction with Ubc7p+ΔtmCue1p as E2, Coomassie staining easily detected the ubiquitin dimer, as well as other reaction products absent from the "no E2" control reaction (Figure 4-2). In the reaction with lone Ubc7p as E2, a ubiquitin dimer was faintly detectable, but not the higher molecular weight bands seen with Ubc7p+ΔtmCue1p. No bands were produced in the HUBC4 or No E2 control reactions. The E1 in these reactions was not observed because the high percentage SDS-PAGE gels do not resolve the 115 kD protein. The bands produced in the Ubc7p+ΔtmCue1p reaction were abundant, staining as strongly as the E2 bands. Though immunoblotting indicated these bands contained ubiquitin, these results did not unambiguously identify the composition of these new bands. The calculated molecular weights of the E2 proteins tested and of Cue1p are all similar: HUBC4 is 20.8 kD, Ubc7p is 19.5 kD, and Cue1p is 20.3 kD. Ubiquitin immunoreactivity and the commensurate Coomassie staining band at 16 kD strongly suggested a dimer of the 8 kD ubiquitin protein. However, it was unclear whether the higher molecular weight bands produced in only the Ubc7p+ΔtmCue1p reactions were ubiquitinated Ubc7p or Cue1p, or ubiquitin multimers. The addition of a third ubiquitin to the 16kD dimer could have electrophoretic mobility similar to mono-ubiquitinated Ubc7p or Cue1p.

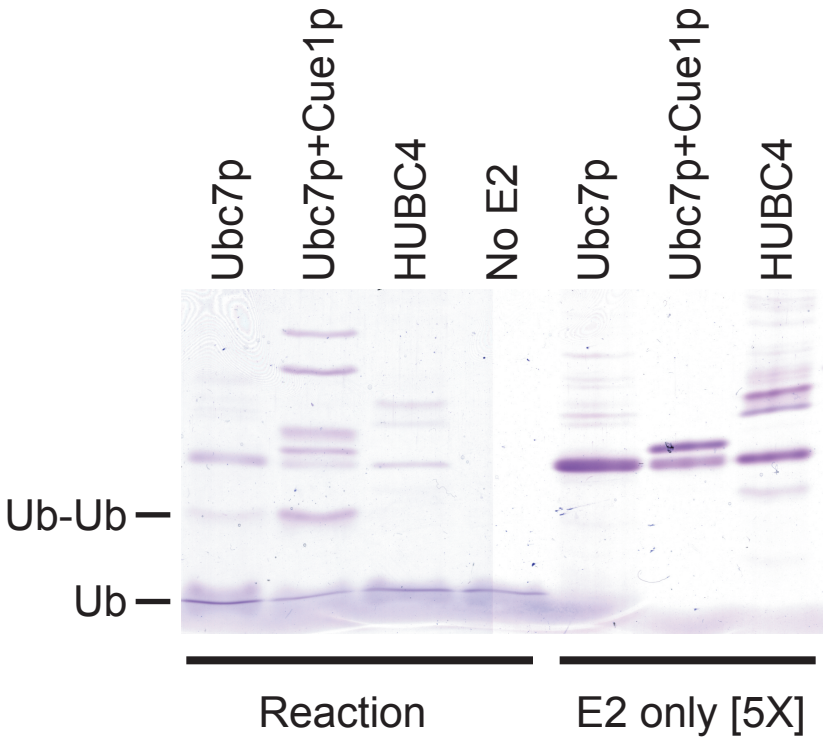


**Figure 4-2: Coomassie stained E3-independent *in vitro* ubiquitination reactions, and E2s.**

Ubiquitination reactions without E3 and with either Ubc7p, Ubc7p+ΔtmCue1p, HUBC4 or no E2, were resolved with 14% SDS-PAGE and Coomassie stained to observe the size and quantity of small protein products produced by the *in vitro* ubiquitination reactions (Reaction). Purified E2 preparations were loaded on the same gel at five times higher concentration than in the ubiquitination reactions (E2 only [5X]) to identify all bands contributed by the E2 preparations.

Likewise, a ubiquitin dimer on Ubc7p or Cue1p would also be difficult to distinguish from a tetramer of ubiquitin.

We wanted to discern among these possibilities and identify the composition of the products formed by the E3-independent ubiquitination reactions. Cue1p antibodies were obtained (T. Sommer, Max Delbrück Center, Berlin) and HA-tagged versions of recombinant Ubc7p and Ubc7p+ΔtmCue1p were purified, allowing immunoblotting of each protein in these reactions. We ran E3-independent *in vitro* ubiquitination reactions as before, using Ubc7p-2HA+ΔtmCue1p or Ubc7p-2HA as E2, and resolved the reaction mixes directly by SDS-PAGE. Equal portions of each reaction were loaded, and either Coomassie stained or immunoblotted for ubiquitin, HA-epitope, or Cue1p. With Ubc7p-2HA+ΔtmCue1p, Coomassie staining revealed the ubiquitin dimer and higher bands as expected, as well as commensurate ubiquitin immunoreactivity (Figure 4-3, lane 1). However, we observed no mobility shift of Ubc7p-2HA or Cue1p, suggesting that the higher molecular weight bands produced in the Ubc7p-2HA+ΔtmCue1p reactions were comprised exclusively of ubiquitin.

Polyubiquitin chains join the c-terminus of one ubiquitin protein with the lysine residue of another ubiquitin. There are seven lysines on the ubiquitin protein, and each has been observed to receive ubiquitin (Peng, et al., 2003), though the predominant linkage targeting proteasomal degradation is through lysine 48 (Thrower, et al., 2000). Mutants of ubiquitin that modify these lysine residues disrupt polyubiquitination while allowing mono-ubiquitination on substrate lysines (Chau, et al., 1989; Finley, et al., 1994). We wanted to characterize the type of lysine linkages

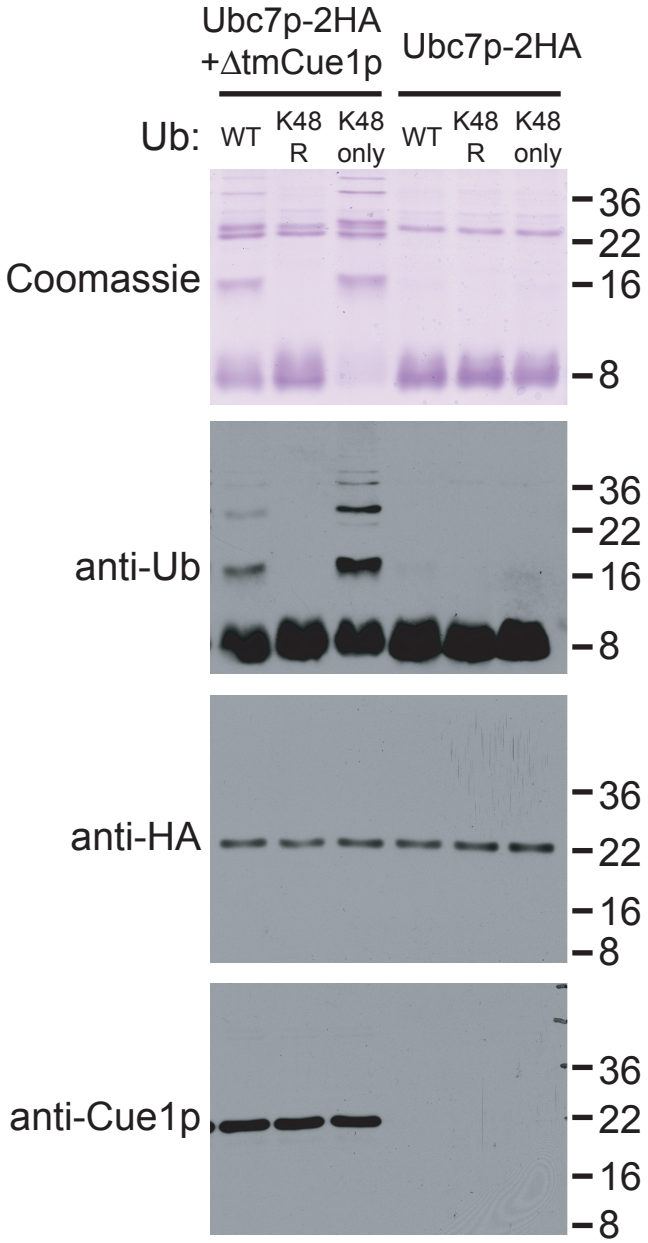


**Figure 4-3: E3-indpendent ubiquitination produces only multimers of ubiquitin linked through lysine-48.**

*In vitro* reactions with either Ubc7p-2HA+ΔtmCue1p or Ubc7p-2HA, were prepared with one of three versions of ubiquitin: wild-type ubiquitin (WT), ubiquitin with lysine-48 changed to arginine (K48R), or ubiquitin with all lysines other than lysine-48 changed to arginine (K48Only). Reactions were split into 4 identical portions and resolved by 14% SDS-PAGE. Coomassie staining revealed that E3-independent reaction products required lysine-48 of ubiquitin. Immunoblotting revealed that these higher molecular weight products were comprised only of ubiquitin protein (anti-Ub), and not of ubiquitinated Ubc7p (anti-HA) or Cue1p (anti-Cue1p).

allowed by Ubc7p with Cue1p in this E3-independent process. We prepared reactions using K48R-ubiquitin, which replaces lysine-48 with arginine, or K48only-ubiquitin, which replaces all lysines with arginine except lysine-48. The reactions were run, resolved by SDS-PAGE, and immunoblotted as before, containing wild type or mutant ubiquitin as indicated. The bands generated in the E3-independent Ubc7p+ΔtmCue1p reactions were completely inhibited by K48R-ubiquitin, and completely restored by K48only-ubiquitin (Figure 4-3, compare lanes 1-3). The lysine-48 requirement for Ubc7p+ΔtmCue1p ubiquitin chains is a strong indicator that the ubiquitin immunoreactivity observed in these E3-independent reactions was comprised exclusively of polyubiquitin. Lysine-48 linkages also imply that the activation of Ubc7p ubiquitination by Cue1p may be physiologically relevant, because polyubiquitin chains formed on ERAD substrates are also linked through lysine 48 (Flierman, et al., 2003).

It is well established that Ubc7p (and all E2s) must interact with an E1 to be charged with thioester-linked ubiquitin (Glickman and Ciechanover, 2002; Hershko and Ciechanover, 1998). We considered that Cue1p might enhance Ubc7p activity by promoting the charging of Ubc7p by E1. To examine this we adapted a previously described method to assay the ubiquitin-charging of an E2 by E1 (Gosink and Vierstra, 1995). *In vitro* reactions were run by combining E1, ubiquitin, ATP, and either Ubc7p-2HA or Ubc7p-2HA+ΔtmCue1p. Reaction mixes were resolved by non-reducing SDS-PAGE and immunoblotted for either HA-epitope or ubiquitin. Multiple concentrations of each E2 were tested as indicated. Ubiquitin immunoblotting

revealed a band at the molecular weight of a ubiquitin-Ubc7p thioester adduct (Figure 4-4, upper panel). This band was absent from "no ubiquitin" controls and its intensity diminished with E2 concentration, suggesting involvement of both ubiquitin and E2 as expected for an E2-ubiqutin adduct. There was little difference in the intensity of this band between Ubc7p-2HA and Ubc7p-2HA+ΔtmCue1p, while a two-fold reduction in E2 concentration strongly reduced its intensity. HA-eptiope immunoblotting also revealed a ubiquitin-dependent band of similar size that diminished with E2 concentration (Figure 4-4, lower panel). Again, the presence or absence of Cue1p had little effect on this band. Together, these results strongly suggest that Cue1p does not enhance E1-charging of Ubc7p with ubiquitin. It is important to emphasize that the Ubc7p-ubiquitin adduct was only detectable in these experiments when analyzed with non-reducing SDS-PAGE, while the earlier *in vitro* experiments used reducing SDS-PAGE which cleaves the thioester bond linking ubiquitin to E2. The ubiquitin dimer was not observed in these experiments because these E1 activity assays were incubated for less time than the previous reactions. The non-reducing conditions caused the appearance of HA-epitope immunoreactivity in the lone-Ubc7p experiment (Figure 4-4, asterisk). Given these results, it seemed that the mechanism by which Cue1p stimulated Ubc7p activity was not at the level of ubiquitin transfer from E1 to Ubc7p. Although we have not yet determined a clear mechanism for the activation of Ubc7p ubiquitination activity by Cue1p, we have established that it involves the production of very small ubiquitin chains linked through lysine 48.

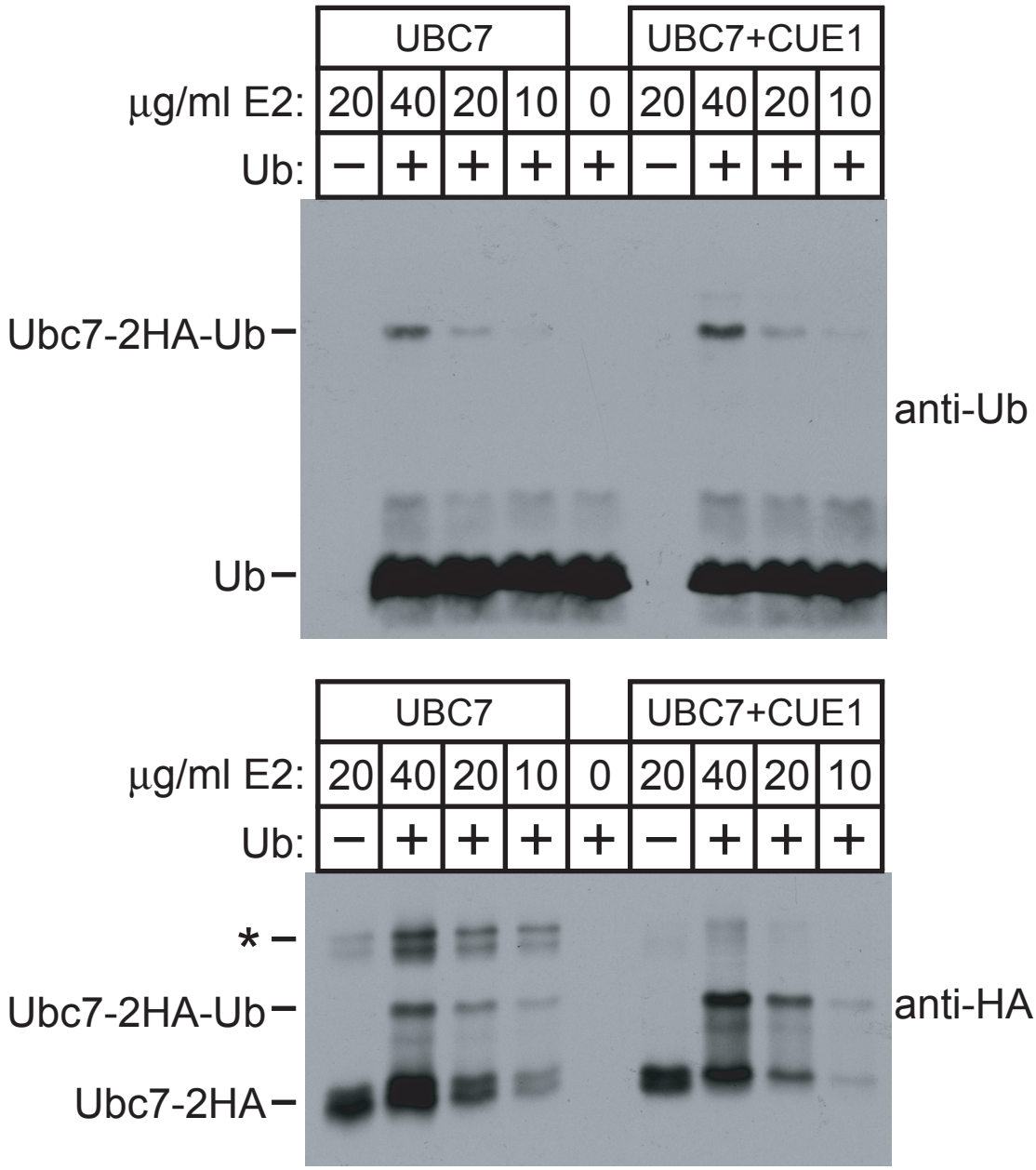


**Figure 4-4: Cue1p did not enhance the ubiquitin-charging of Ubc7p by E1.**

*In vitro* assays of ubiquitin-Ubc7p thioester formation were performed with the indicated concentrations of Ubc7p-2HA (left lanes) or Ubc7p-2HA+ΔtmCue1p (right lanes), and resolved with non-reducing SDS-PAGE. Ubiquitin immunoblotting (top panel) and HA-epitope immunoblotting (bottom panel) both revealed a band the size of ubiquitin-Ubc7p thioester (Ubc7p-2HA-Ub) that diminished with lower E2 concentration. Ubc7p-2HA and Ubc7p-2HA+ΔtmCue1p produced ubiquitin-Ubc7p thioester with similar efficiency. Asterisk (*) indicates a band of HA-epitope immunoreactivity whose appearance is prevalent in non-reducing conditions.

While the E3 independent ubiquitination observed with Ubc7p and Cue1p is surprising and interesting, we want to know whether this activity is relevant to the *in vivo* degradation of ERAD substrates by Ubc7p. It is known that the integral membrane protein Cue1p is required for Ubc7p-dependent ERAD (Biederer, et al., 1997; Denic, et al., 2006; Gardner, et al., 2001; Ravid, et al., 2006; Walter, et al., 2001). Cue1p binds to Ubc7p, localizing this E2 to the ER membrane (Biederer, et al., 1997; Gardner, et al., 2001), and overexpression of Ubc7p does not restore ERAD in strains lacking Cue1p (Biederer, et al., 1997). Also, deletion of *CUE1* reduces crosslinking between Ubc7p and its ERAD substrate Hmg2p (Gardner, et al., 2001). Thus, the membrane-tethering function of Cue1p was thought to increase the local concentration of Ubc7p at the ER membrane above a threshold required for ERAD. However, the *in vitro* stimulation of Ubc7p by Cue1p, observed in conditions with no membranes, suggested a role for Cue1p in activating Ubc7p in addition to tethering Ubc7p to the ER surface. To examine *in vivo* the contributions of both anchoring Ubc7p to the ER and activation of Ubc7p by Cue1p, we designed constructs to separate these two functions, and assayed their *in vivo* function.

Our *in vivo* measure of Ubc7p function was the degradation of the Hrd1p-dependent ERAD substrate Hmg2p-GFP, which can be assayed both biochemically and by flow cytometry (Bays, et al., 2001; Cronin and Hampton, 1999; Cronin, et al., 2000; Gardner, et al., 1998). Low Hmg2p-GFP levels in a wild-type strain reflect the short half-life of this rapidly degraded protein (Gardner, et al., 1998; Hampton, et al., 1996). Hmg2p-GFP cannot be degraded in a *ubc7*Δ null allele strain, resulting in

increased fluorescence compared to a wild-type strain (Wilhovsky, et al., 2000). In Figure 4-5 panel A, histograms plotting the GFP fluorescence of wild-type and *ubc7*Δ strains are superimposed for comparison. Expression of Ubc7p-2HA in the *ubc7*Δ null strain restored degradation of Hmg2p-GFP when expressed from either the native promoter (Figure 4-5, panel B) or the strong *TDH3* promoter (Figure 4-5 Panel C), as indicated by the overlap of these histograms with that of the wild-type strain. In contrast, expression of Cue1p from the strong *TDH3* promoter did not restore degradation of Hmg2p-GFP (Figure 4-5, panel D), as the fluorescence of this strain was equal to that of the *ubc7*Δ null strain.

To separate the anchoring function of Cue1p from its cytosolic portion that caused E2 activation *in vitro*, we fused the transmembrane span of Cue1p (amino acids 1-22) to full-length Ubc7p-2HA, producing a membrane-anchored Ubc7p with HA-epitope tag (TM-Ubc7p). We tested this construct for complementation of the *ubc7*Δ null allele by expressing it from the strong *TDH3* promoter and assaying Hmg2p-GFP levels as above. The fluorescence histogram of the strain expressing TM-Ubc7p was indistinguishable from that of the *ubc7*Δ null control (Figure 4-5, panel E), indicating that TM-Ubc7p could not promote the degradation of Hmg2p-GFP. Despite expression of TM-Ubc7p beyond levels sufficient for unanchored Ubc7p to complement *ubc7*Δ, (data not shown), this membrane-anchored version of Ubc7p showed no measurable ERAD of Hmg2p-GFP.

We considered that a transmembrane span immediately adjacent to the n-terminus of Ubc7p could constrain this E2 in a manner that inhibits proper function.

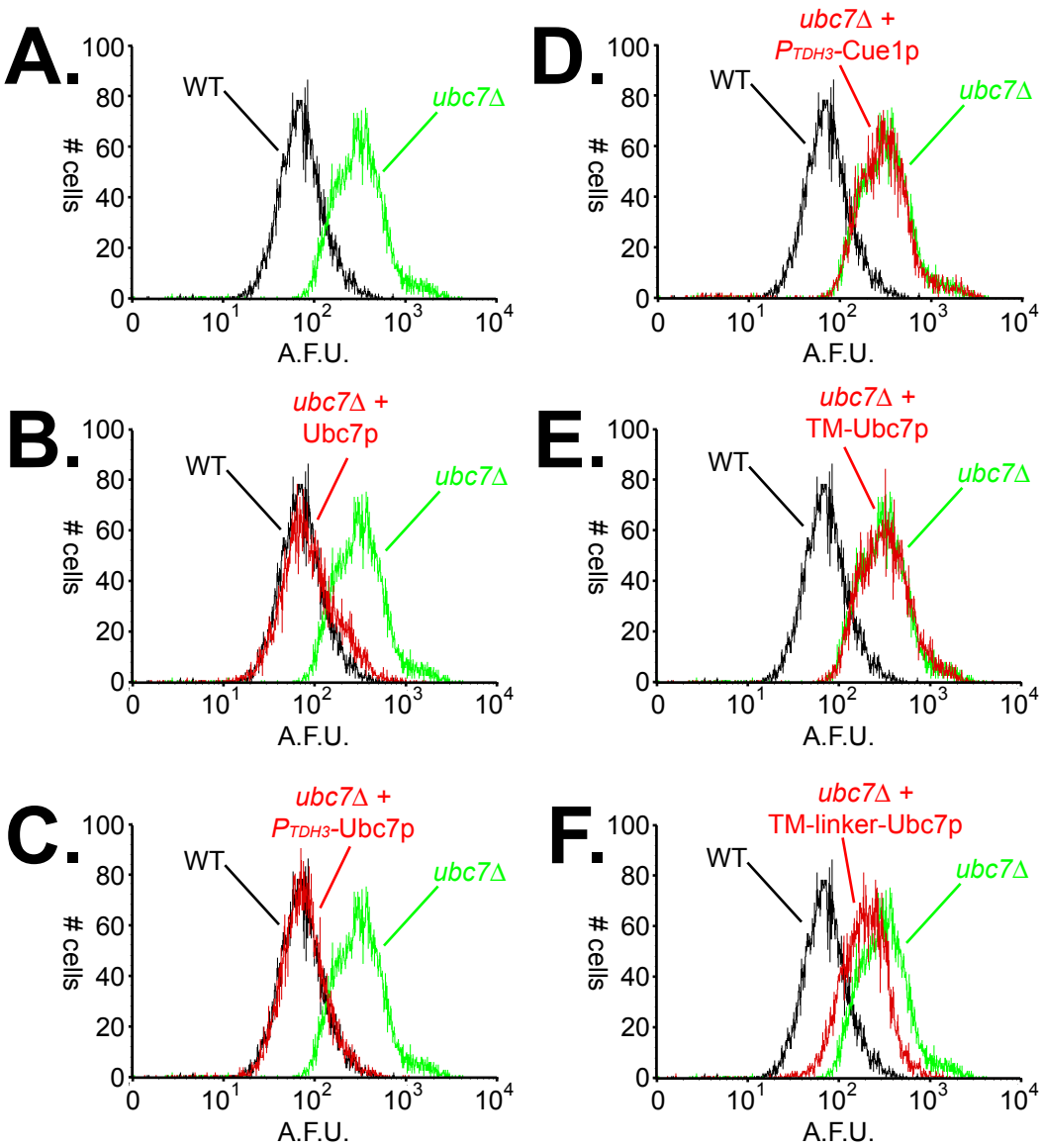


**Figure 4-5: ER-anchored Ubc7p partially restores ERAD in *ubc7*Δ strains.**

Hmg2p-GFP levels were measured in *ubc7*Δ strains expressing either empty vector or the indicated ERAD proteins. Histograms plot the number of cells (y-axis) having a given arbitrary fluorescence (x-axis). **A.** Hmg2p is degraded in a wild-type strain (WT) and stabilized in a *ubc7*Δ null strain (*ubc7*Δ). **B-F.** The WT and *ubc7*Δ histograms (black and green) are superimposed on the histogram of a *ubc7*Δ strain expressing an ERAD protein (red), to test complementation of *ubc7*Δ. **B.** Ubc7p-2HA from the native promoter complemented *ubc7*Δ. **C.** Ubc7p-2HA from the strong *TDH3* promoter complemented *ubc7*Δ. **D.** Cue1p-2HA from the strong *TDH3* promoter did not complement *ubc7*Δ. **E.** Membrane-anchored Ubc7p-2HA (TM-Ubc7p) did not complement *ubc7*Δ. **F.** Membrane-anchored Ubc7p-2HA with linker (TM-linker-Ubc7p) partially complemented *ubc7*Δ.

To address this concern, we modified the non-functional TM-Ubc7p construct to include a linker of 88 amino acids between the transmembrane span and the n-terminus of Ubc7p-2HA. This TM-linker-Ubc7p construct was expressed from the strong *TDH3* promoter in the *ubc7*Δ null allele strain, and GFP fluorescence was measured by flow cytometry as above. Hmg2p-GFP levels were lower in the presence of TM-linker-Ubc7p than in the *ubc7*Δ control. This long-tethered version of membrane-anchored Ubc7p partially complemented the ERAD defect in the *ubc7*Δ null allele strain (Figure 4-5 panel F). To confirm that proteolysis of the long-tether on Ubc7p did not generate a pool of soluble Ubc7p *in vivo* that restored Ubc7p function, we prepared whole-cell lysates and immunoblotted for HA-epitope, confirming the presence of only full-length TM-linker-Ubc7p (data not shown). Thus, we generated a functioning version of Ubc7p with its own ER anchor. With this construct, we could examine *in vivo* if there was an anchoring-independent requirement for Cue1p in ERAD.

If the only required function of Cue1p in ERAD was anchoring Ubc7p to the ER membrane, then the TM-linker-Ubc7p construct tested above ought to allow ERAD in the absence of Cue1p. TM-linker-Ubc7p was expressed from the strong *TDH3* promoter in a *ubc7*Δ *cue1*Δ strain expressing Hgm2p-GFP, and GFP fluorescence was measured as before. Compared to the empty vector control, TM-linker-Ubc7p supported little degradation of Hmg2p-GFP in the *ubc7*Δ *cue1*Δ strain (Figure 4-6, panel A). Thus, localization of Ubc7p to the ER membrane was not sufficient to allow ERAD. The same TM-linker-Ubc7p construct allowed degradation

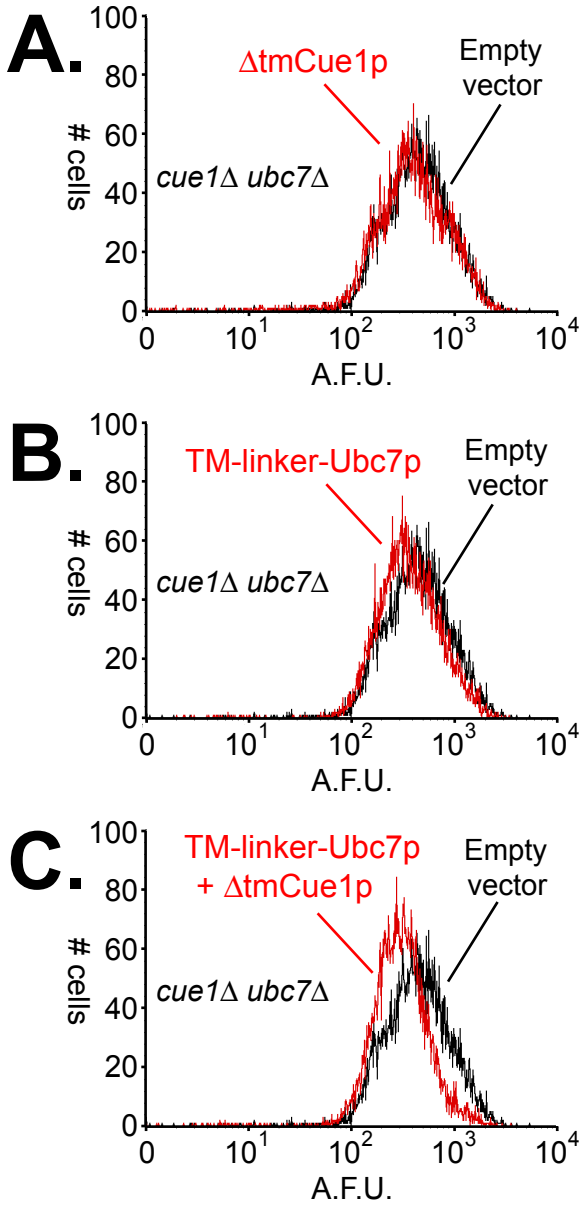


**Figure 4-6: Soluble Cue1p enhanced ERAD by anchored Ubc7p *in vivo*.**

Hmg2p-GFP levels were measured as above, in *cue1*Δ *ubc7*Δ strains with either empty vector or the indicated ERAD proteins. **A.** ΔtmCue1p showed no reduction in Hmg2p-GFP levels. **B.** TM-linker-Ubc7p showed a very small reduction in Hmg2p-GFP. **C.** When expressed together, TM-linker Ubc7p and ΔtmCue1p synergistically reduced Hmg2p-GFP levels, partially complementing the *cue1*Δ *ubc7*Δ double-null.

of Hmg2p-GFP when tested in the strain with native *CUE1* allele (Figure 4-5 panel F). This suggested that degradation of Hmg2p-GFP *in vivo* required Cue1p for a function independent of Ubc7p membrane-localization.

This result implied that Cue1p had a separable activating function *in vivo* akin to Cue1p enhanced Ubc7p ubiquitination *in vitro*. However, the Cue1p that allowed ERAD with TM-linker-Ubc7p was native, membrane-anchored Cue1p. Complete separation of the putative Ubc7p-activating function from the established Ubc7p-localizing function would require removal of the transmembrane span from Cue1p. ΔtmCue1p, a version of Cue1p with no transmembrane span, was expressed from the strong *TDH3* promoter and tested for ERAD function *in vivo*. As before, Hmg2p-GFP was assayed by flow cytometry. ΔtmCue1p did not stimulate any Hmg2p-GFP degradation in a *cue1*Δ null strain (data not shown), indicating that membrane-anchoring is necessary for Cue1p function. A similar lack of ERAD function was seen when expressing ΔtmCue1p in the *ubc7*Δ *cue1*Δ strain used above (Figure 4-6 panel B). However, when ΔtmCue1p and TM-linker-Ubc7p were expressed together in a *ubc7*Δ *cue1*Δ strain, Hmg2p-GFP levels were lowered (Figure 4-6 panel C). This change in GFP fluorescence exceeded that caused by either protein expressed alone (Figure 4-6 panels A and B), and was comparable to the shift observed in Figure 4-5 panel F, where TM-linker-Ubc7p was shown to partially complement a *ubc7*Δ allele. Therefore, ΔtmCue1p stimulated TM-linker-Ubc7p similarly to native Cue1p protein.

These results are consistent with a model whereby both ER localization of Ubc7p and stimulation of Ubc7p activity are required *in vivo* for ERAD function.

These results were obtained by comparing Hmg2p-GFP levels between strains that, except for the empty vector or expression-plasmid, were isogenic. To confirm that the changes in steady-state GFP levels observed above resulted from degradation of Hmg2p-GFP, cycloheximide-chase assays were conducted. In log-phase cultures, the addition of cycloheximide causes arrest in translation, while degradation of the already-expressed proteins can proceed. We conducted cycloheximide-chase assays on the *ubc7*Δ *cue1*Δ strains used in Figure 4-6 to confirm that ΔtmCue1p and TM-linker-Ubc7p were allowing degradation of Hmg2p-GFP. Log-phase cultures of each strain were split, and incubated for 2 hours with either cycloheximide or no drug, after which GFP fluorescence was measured as before. The histograms compared in each overlay are from identical strain populations, only differing in cycloheximide treatment. There was no change in GFP fluorescence in the empty vector strain (Figure 4-7 panel A) or the strain expressing ΔtmCue1p alone (Figure 4-7 Panel B). The TM-linker-Ubc7p construct revealed a small but reproducible reduction in GFP fluorescence upon cycloheximide treatment, suggesting that membrane localized Ubc7p could support some Hmg2p-GFP degradation even without the cytosolic portion of Cue1p (Figure 4-7 panel C). The presence of ΔtmCue1p and TM-linker-Ubc7p together caused the most reduction in Hmg2p-GFP levels, indicating the strongest stimulation of ERAD (Figure 4-7 panel D). Although it has been reported that levels of cytosolic Ubc7p diminish in the absence of Cue1p (Biederer, et al., 1997; Gardner, et al., 2001), we determined by immunoblotting of whole-cell lysates that the addition of ΔtmCue1p had no effect on TM-linker-Ubc7p levels in these strains (data

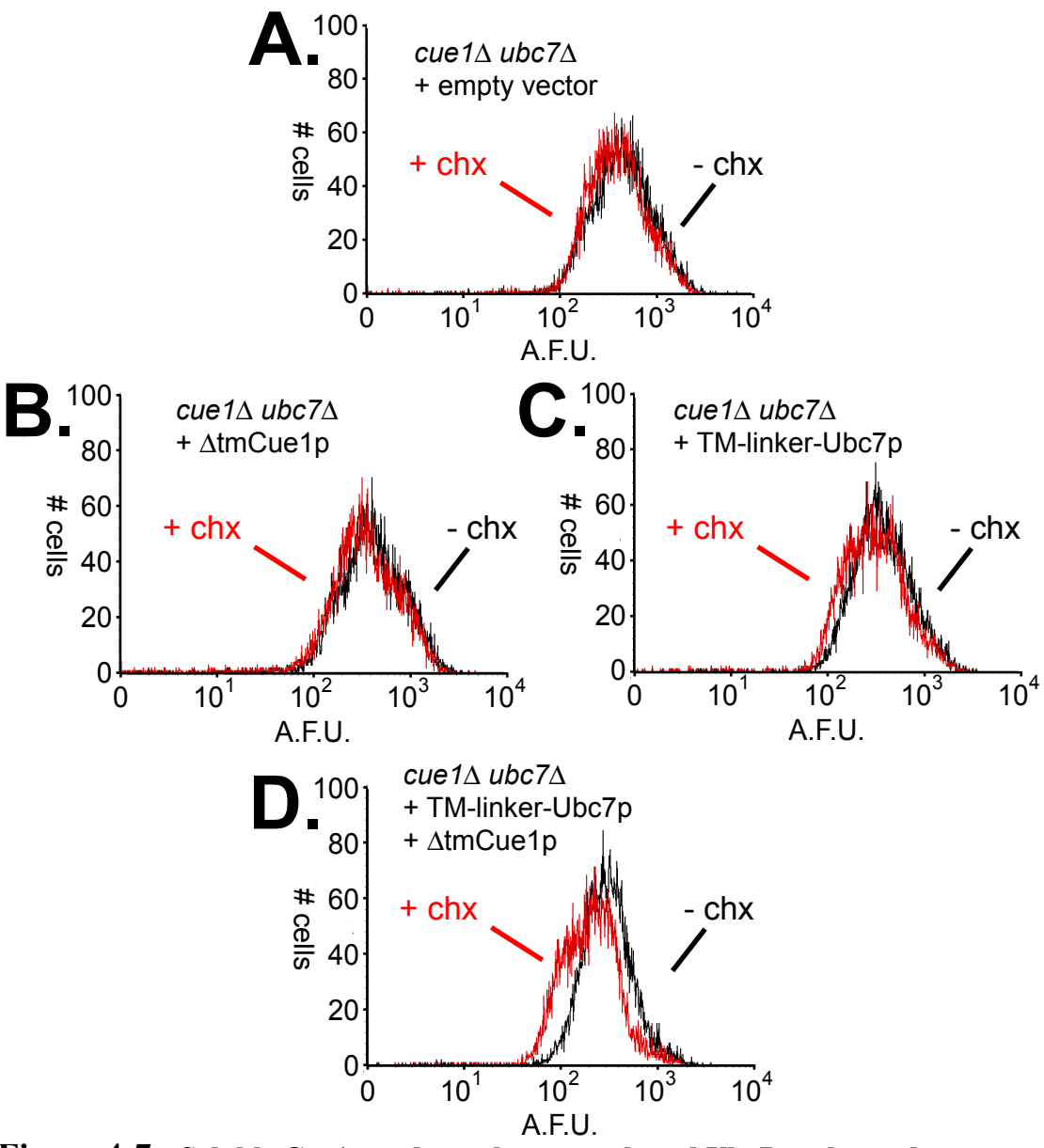


**Figure 4-7: Soluble Cue1p and membrane-anchored Ubc7p enhanced degradation of Hmg2p-GFP *in vivo*.**

Cycloheximide chase degradation assays were performed to confirm Hmg2p-GFP degradation in *cue1*Δ *ubc7*Δ strains expressing either empty vector or the indicated ERAD proteins. For each strain, histograms for no drug (black) or 50µg/ml cycloheximide treatment for 2 hrs (red) are superimposed. **A.** *cue1*Δ *ubc7*Δ strains expressing empty vector showed no degradation of Hmg2p-GFP. **B.** Expression of only ΔtmCue1p also showed no degradation of Hmg2p-GFP. **C.** TM-linker-Ubc7p stimulated Hmg2p-GFP degradation to a small degree, even without Cue1p. **D.** Co-expression of ΔtmCue1p with TM-linker-Ubc7p enhanced Hmg2p-GFP degradation beyond that allowed by TM-linker-Ubc7p alone.

not shown). The data in Figure 4-7 recapitulated the trends observed in Figure 4-6, confirming that membrane-anchored Ubc7p and soluble Cue1p improved degradation of Hmg2p-GFP *in vivo*. These results are also consistent with the behavior of Ubc7p in our *in vitro* ubiquitination assays: ubiquitination activity was present with Ubc7p alone, but strongly enhanced by addition of Cue1p.

The *in vivo* experiments above separated the two roles of Cue1p as activator and localizer of Ubc7p, indicating that each of these roles was necessary for Ubc7p to perform Hrd1p-dependent degradation in the ER. It remains possible that the requirement for Cue1p in ERAD is not to activate Ubc7p in a manner analogous to Ubc7p activation *in vitro*. The Cue1p soluble domain could instead play a necessary role in assembling a functional ERAD complex. Cue1p has recently been identified as a component of the Doa10p ERAD ligase complex, and as an indirect interactor with Hrd1p (Carvalho, et al., 2006), and it is possible that Cue1p has similar association with the Hrd1p ERAD complex. This alternative hypothesis is consistent with the *in vivo* data, as is the idea that Cue1p stimulates Ubc7p *in vivo*. Also, TM-linker-Ubc7p was not as active as Ubc7p (compare Figure 4-5F with 4-5B), and this reduced efficacy may have been due to spatial or stearic problems imposed by anchoring to the membrane surface.

To address these issues, we wanted to examine the Cue1p enhancement of Ubc7p function *in vivo*, but in a context removed from the ER and ERAD machinery. Previous studies suggested an approach to do this using Cdc34p. Cdc34p is the most closely related E2 to Ubc7p in *Saccharomyces cerevisiae* (Figure 4-8A). *CDC34* is an

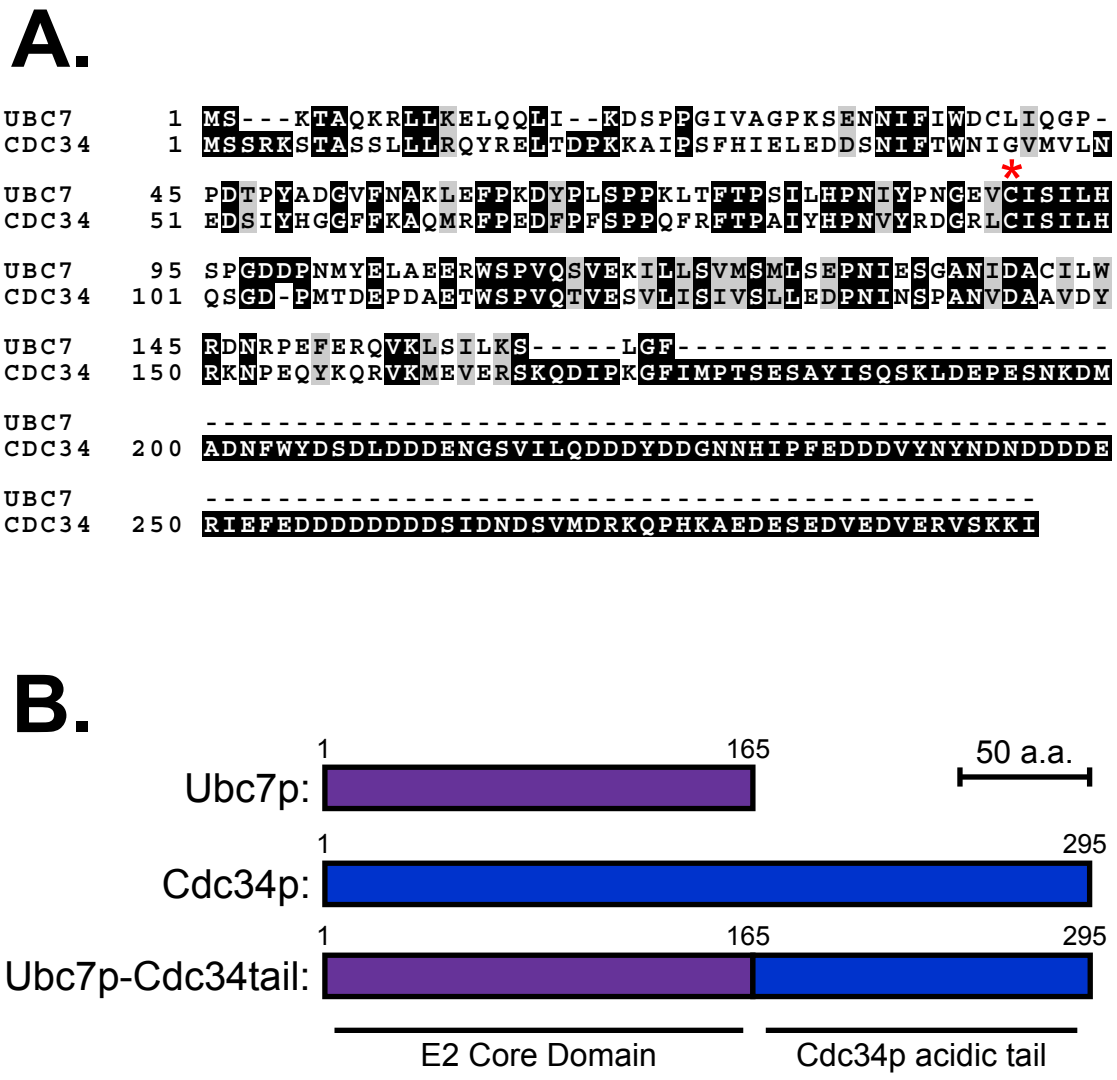


**Figure 4-8: A strategy to test Ubc7p function independent of ERAD.**

**A.** Primary sequence alignment of Ubc7p and Cdc34p. Red asterisk indicates the conserved cysteine residue (C89 in Ubc7p, C95 in Cdc34p) where the ubiquitin thioester forms. Cdc34p has an acidic c-terminal tail required for localization to the SCF ubiquitin ligase complex. **B.** Schematic of Ubc7p, Cdc34p, and a construct fusing the E2 domain of Ubc7p with the c-terminus of Cdc34p (Ubc7p-Cdc34tail) to target Ubc7p to the SCF ubiquitin ligase complex.

essential gene with well-characterized conditional alleles. Cdc34p associates with the cytosolic SCF ubiquitin ligase complex, which regulates cell cycle progression by targeting key regulatory proteins for ubiquitination and degradation (Seol, et al., 1999). Cdc34p is localized to the SCF ubiquitin ligase complex through an acidic region c-terminal to the conserved E2 domain. It was reported that a yeast E2 (Rad6p) fused to this c-terminal tail domain of Cdc34p could partially complement a temperature-sensitive (ts) allele of *CDC34* (Kolman, et al., 1992; Silver, et al., 1992).

We made a similar construct fusing Ubc7p to the c-terminal tail of Cdc34p (Figure 4-8B), and expressed the Ubc7p-Cdc34tail protein in strains whose only copy of *CDC34* was the recessive *cdc34-2* ts allele. By evaluating rescue of the *cdc34-2* ts phenotype caused by Ubc7p-Cdc34tail, we could assay Ubc7p function independent of Hrd1p, ERAD substrates, or the ER membrane. Ubc7p-Cdc34tail expressed from the strong *TDH3* promoter fully complemented the *cdc34-2* ts phenotype, but Ubc7p-Cdc34tail expressed from the weaker *CDC34* promoter resulted in much weaker complementation of the *cdc34-2* allele (data not shown). This complementation by Ubc7p-Cdc34tail also required the conserved catalytic cysteine residue essential for E2 function, suggesting that *cdc34-2* was rescued by the E2 activity of Ubc7p-Cdc34tail.

Using this *in vivo*, ER-free assay of Ubc7p activity, we could test whether Cue1p enhanced this Ubc7p activity. If Cue1p were an enhancer of Ubc7p activity *in vivo*, then expression of ΔtmCue1p in these *cdc34-2* strains should stimulate the ubiquitination activity of Ubc7p-Cdc34tail, improving complementation of the *cdc34-*

*2* ts phenotype. In a strain with *cdc34-2* as the only copy of *CDC34*, we added empty vector or vectors expressing one of Cdc34p, Ubc7-Cdc34tail, or Ubc7p, all from the *CDC34* promoter (Figure 4-9). At the permissive temperature (30˚C) all the strains grew equally well. At the non-permissive temperatures (33˚C, 35˚C), the native Cdc34p gene strongly improved growth relative to the empty vector control, indicating complementation of the *cdc34-2* ts phenotype. As mentioned above, neither Ubc7p-Cdc34tail nor Ubc7p could complement the ts phenotype to the same extent (Figure 4-9). However, expression of ΔtmCue1p markedly improved complementation of co-expressed Ubc7-Cdc34tail construct, but not the strains expressing Cdc34p or normal Ubc7p without the c-terminus from Cdc34p. This suggested that, even removed from the context of the ER and ERAD, Ubc7p *in vivo* required tethering to its ubiquitin ligase complex, and its activity was enhanced by Cue1p.

Cue1p enhanced the production of lysine-48 linked ubiquitin chains by Ubc7p *in vitro*. Hrd1p-dependent ERAD *in vivo* required Ubc7p to be tethered to the ER membrane, and also required the presence of the soluble cytosolic portion of Cue1p. Ubc7p activity was measured *in vivo* by two distinct criteria: degradation of the ERAD substrate Hmg2p-GFP, and complementation of the *cdc34-2* allele. In both cases, Cue1p improved the activity of Ubc7p. Collectively, the evidence presented here implicates Cue1p as an *in vivo* activator of Ubc7p, revealing a previously uncharacterized mode of action for this protein. In addition, these findings confirm the importance of Ubc7p membrane-localization by Cue1p for efficient degradation of ERAD substrates.

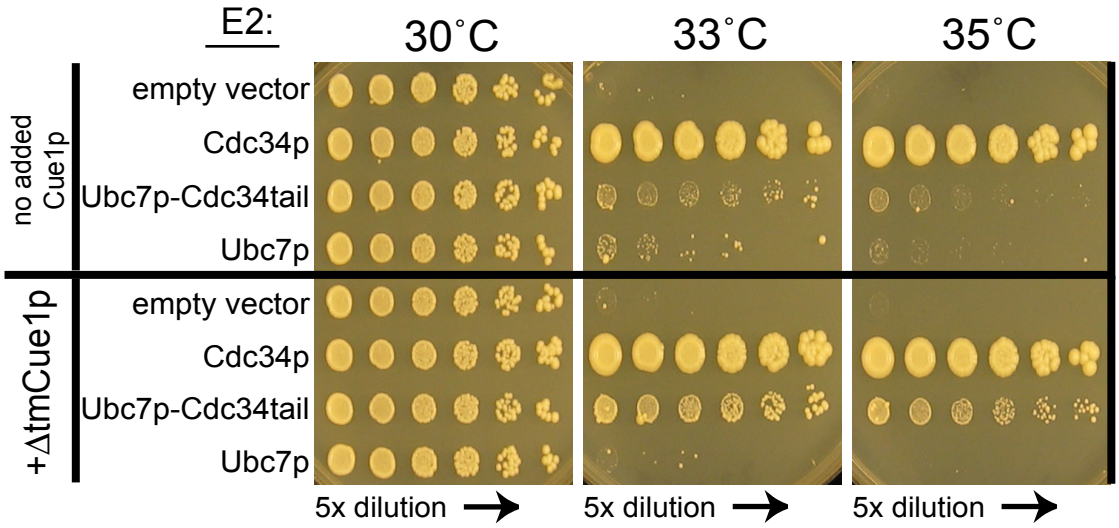


**Figure 4-9: Cue1p enhanced Ubc7p activity in an ERAD-independent context.**

Restoration of Cdc34p function complements the *cdc34-2* temperature-sensitive (ts) growth phenotype. Cdc34p, chimeric Ubc7p-Cdc34tail, and Ubc7p were each expressed in the *cdc34-2* strain. These strains also contained either empty vector ("no added Cue1p", top 4 rows of each panel) or a vector expressing ΔtmCue1p ("+ΔtmCue1p", bottom 4 rows of each panel). These strains were grown at permissive (30˚C) or non-permissive (33˚C, 35˚C) temperatures. Ubc7p-Cdc34tail slightly improved complementation of the ts-phenotype, and the addition of ΔtmCue1p enhanced *cdc34-2* complementation of the ts-phenotype by Ubc7p-Cdc34tail, but not the other E2 constructs.

## Discussion:

Our previous studies in Chapter 3 first identified E3-independent ubiquitination activity of Ubc7p in the presence of Cue1p (Bazirgan, et al., 2006). We were surprised to find this ubiquitination activity produced very small ubiquitin chains, including dimers of ubiquitin (Figure 4-1). These polyubiquitin chains formed unconnected to any other proteins in these *in vitro* ubiquitination reactions. Also surprising was the strong preference of these chains to form lysine 48 linkages, suggesting that lysine linkages were specified independent of the E3 Hrd1p (Figure 4-3). We also observed that formation of the ubiquitin-Ubc7p thioester adduct was equal in the presence and absence of Cue1p (Figure 4-4). Thus, rather than improving transfer of ubiquitin from E1 to E2, the mechanism by which Cue1p stimulated Ubc7p activity was downstream of ubiquitin-E2 thioester formation. Our studies *in vivo* suggested separable functions of Cue1p for both anchoring Ubc7p to the membrane, and for activating Ubc7p, which were each required for maximum Ubc7p function. Most convincingly, only the co-expression of anchored Ubc7p with unanchored Cue1p improved Ubc7p activity *in vivo*, as measured by the degradation of the ERAD protein Hmg2p-GFP. Additionally, ectopic localization of Ubc7p to the SCF E3 complex confirmed that Cue1p enhanced Ubc7p activity in a soluble, membrane-free, ERAD-independent context *in vivo*.

It is interesting that the self-anchored TM-linker-Ubc7p did not support ERAD to the same extent as normal, unanchored Ubc7p, even in the presence of Cue1p (Figure 4-5, compare panel F with B or C). Though TM-linker-Ubc7p was expressed

at higher levels than Ubc7p, the degradation of Hmg2p-GFP it stimulated was relatively weak. E2-E3 structural studies suggest that RING motifs interact with E2s on a surface near the E2 n-terminus (Zheng, et al., 2000). It is possible that adding the transmembrane span and linker to the n-terminus of Ubc7p impaired E2 activity. C-terminal modification of Ubc7p with the 2HA epitope-tag had little effect on Ubc7p function (data not shown). If c-terminal-anchored Ubc7p were to function well in a *ubc7*Δ strain, it could provide a more potent demonstration of the activating power of ΔtmCue1p in a *ubc7*Δ *cue1*Δ strain than the TM-linker-Ubc7p construct tested (Figures 4-6 and 4-7).

Alternatively, if Ubc7p with a c-terminal transmembrane anchor were also impaired in Hmg2p-GFP degradation, like the n-terminally anchored Ubc7p, it would suggest that Ubc7p needs the freedom to dissociate from the membrane for optimal ERAD function. We have often wondered why Ubc7p is localized to the ER membrane through a non-covalent interaction, rather than through a membrane anchor *in cis* like that of Ubc6p (Sommer and Jentsch, 1993). A ubiquitin ligase like Hrd1p that promotes processive assembly of polyubiquitin chains might require a steady supply of ubiquitin-charged E2 in proximity to it and its substrates. It is possible that Ubc7p is more efficiently charged by E1 if it can escape from the plane of the ER into the cytosol. It would be both functional and elegant if ubiquitin-charged Ubc7p had higher affinity for Cue1p than Ubc7p alone. If this were the case, discharged Ubc7p would tend to cycle off of Cue1p into the cytosol for recharging by the cytosolic E1, and charged ubiquitin-Ubc7p would tend to engage Cue1p at the membrane surface

for another round of ubiquitination. Although Ubc7p and Cue1p have been observed to interact tightly *in vitro*, this interaction has not been studied at physiological ionic strength, and Ubc7p might transiently dissociate from Cue1p *in vivo*. The relationship of Ubc7p and Cue1p is analogous to that of Pex4p/Ubc10p and Pex22p. Pex4p is an E2 required for peroxisome protein import that peripherally associates with peroxisome membranes through Pex22p, which is required for Pex4p function (Koller, et al., 1999). Pex22p and Cue1p have 16% amino acid identity, and roughly 30% amino acid similarity, suggesting that Pex22p and Pex4p could function like Cue1p and Ubc7p.

There are numerous parallels between Ubc7p and Cdc34p that make their cross-complementation a reasonable prediction. Both have an insertion of acidic residues within the core E2 homology domain that is absent from all other E2s in *Saccharomyces cerevisiae*. Both E2s use RING-motif containing E3s that target substrates for proteasomal degradation by building lysine-48 linked polyubiquitin chains. The RING motif in each ubiquitin ligase has an insertion in the Loop1 region that interacts with other members of the ligase complex. Both are localized to a multi-subunit ubiquitin ligase complex: Ubc7p is recruited in proximity to Hrd1p, Hrd3p and other ERAD machinery by Cue1p, while Cdc34p has a c-terminal tail with affinity to a subunit of the SCF ubiquitin ligase complex. Both E2-E3 complexes degrade a wide variety of substrates. The SCF complex recruits diverse substrates using numerous interchangeable F-box modules that each recruit a substrate to the SCF complex, positioning the substrate in proximity to Cdc34p. The quality-control Hrd1p

ubiquitin ligase complex also recruits many different misfolded protein substrates for ubiquitination, all of which are in the ER and in proximity to ER-anchored Ubc7p.

The possibility of relocating an E2 other than Cdc34p to the SCF complex to rescue *cdc34* mutants was suggested by early studies of yeast E2s. A sequence fusing the E2 homology portion of *RAD6/UBC2* and the c-terminal extension of *CDC34/UBC3* was able to complement mutations in the essential *CDC34* gene (Kolman, et al., 1992; Silver, et al., 1992). Complementation was strong when the chimeric Rad6p-Cdc34tail E2 was expressed from strong promoters or high-copy vectors, and weak under low-copy or native-promoter expression. The c-terminal portion of Cdc34p was later appreciated to interact with the SCF ubiquitin ligase complex (Mathias, et al., 1998). Because we wanted an ER-independent assay of Ubc7p function, and Ubc7p is more closely related to Cdc34p than Rad6p, it seemed reasonable to explore complementation of *cdc34* mutant alleles with a chimeric Ubc7p-Cdc34tail E2.

We chose to test complementation of the recessive *cdc34-2* temperature-sensitive (ts) allele, which has an easily observable growth defect at non-permissive temperatures (Prendergast, et al., 1995). The protein encoded by *cdc34-2* is degraded at non-permissive temperatures (Richard G. Gardner, personal communication). The growth defect of the *cdc34-2* strains worsened with increased temperature, and complementation of this allele by the chimeric E2s was dosage dependent. Thus, examination of complementing constructs at several temperatures allowed for a very sensitive assay of E2 function measured by rescue of the *cdc34-2* growth defect. The

*cdc34-2* strain grew well at 30˚C, and poorly at 34˚C, and barely at all at 37˚C. We determined that Ubc7p-Cdc34tail could complement the *cdc34-2* ts-phenotype when expressed from the strong *TDH3* promoter. The *cdc34-2* strain overexpressing Ubc7p-Cdc34tail grew as well as the native *CDC34* strain at 34˚C, but at 37˚C rescue was incomplete (data not shown). When expressed from the weaker *CDC34* promoter, Ubc7p-Cdc34tail showed poor complementation of the ts-phenotype at 34˚C. This was consistent with the previous studies using Rad6p-Cdc34tail chimeric E2s: strong expression led to complementation of *cdc34* mutants, but weak expression did not complement.

This lack of complementation by weaker expression of Ubc7p-Cdc34tail made it possible to detect the improvement of Ubc7p activity caused by unanchored Cue1p in a cellular setting removed from the ER. The addition of ΔtmCue1p improved the ts-phenotype complementation by Ubc7p-Cdc34tail, and not by Ubc7p alone or Cdc34p alone (Figure 4-9). Our results suggested that the ubiquitin ligase activity of Ubc7p-Cdc34tail was enhanced *in vivo* by Cue1p in an ER-independent assay of Ubc7p activity. Though we have not directly examined the degradation of Cdc34p-dependent and SCF-dependent substrates, doing so would confirm that restored E2 activity from Ubc7p-Cdc34tail and ΔtmCue1p caused rescue of the *cdc34-2* ts-phenotype.

Because of the many parallels between Cdc34p and Ubc7p, we thought that a recent study of the mechanism by which Cdc34p synthesizes lysine 48-linked ubiquitin chains by Petroski and Deshaies might be particularly relevant to our

understanding of Ubc7p (Petroski and Deshaies, 2005). *In vitro*, Cdc34p with a ubiquitin thioester conjugate was found to release its ubiquitin to unbound ubiquitin in solution only on lysine 48, and this activity was strongly enhanced by the addition of purified SCF complex. They did not examine which specific component of the SCF complex was responsible for this stimulation, but they did determine that the SCF-enhanced reaction was due to stimulation of the Cdc34p-ubiquitin adduct to release ubiquitin (increased Vmax), and not due to improved affinity for ubiquitin in solution (Km did not change). If we imagine Cue1p to be a part of the Hrd1p complex, then perhaps the stimulation of Ubc7p-activity by Cue1p to make lysine 48 chains occurs by a similar mechanism as Cdc34p stimulation by the SCF complex. Cue1p could be a sort of "ubiquitin exchange factor" that promotes the release of ubiquitin from Ubc7p, perhaps by stabilizing a transition intermediate.

The acidic residues in the loop that Cdc34p shares with Ubc7p were critical for Cdc34p to guide lysine specificity of chain formation, but irrelevant to the ubiquitination of substrate lysines. Consistent with this, we have observed Ubc7p *in vitro* to form only lysine-48 polyubiquitin chains. This chain linkage seemed unaffected by the presence of Cue1p or which RING E3 was present (data not shown), suggesting that the lysine linkage of polyubiquitin chains was completely dictated by the E2. The model proposed for Cdc34 function is biphasic. First, a slow rate-limiting ubiquitination of one lysine on a substrate occurs. This first reaction does not require the acidic loop residues that guide lysine-48 linkage. Then, the newly-attached ubiquitin is rapidly ubiquitinated on lysine-48 in a manner dependent on the acidic

loop. If this model for Cdc34p function is also the mechanism by which Ubc7p functions, we can test Ubc7p to see if it fulfills this model's predictions.

One prediction is that mutants in the acidic loop of Ubc7p will produce shorter ubiquitin chains on targets of Hrd1p ubiquitination like Hmg2p-GFP. Since we are able to lyse cells and immunoprecipitate specific substrates to examine their ubiquitination (Gardner, et al., 2001), we can introduce mutants of Ubc7p *in vivo* and see if ubiquitin chains on known degradation substrates are shorter. These shorter chains are also predicted to have disorderly lysine linkages, not lysine-48 chains. Another prediction is that the acidic loop modifications to Ubc7p would slow the rate of ubiquitination, and possibly Hmg2p-GFP degradation. Because the degradation of Hgm2p-GFP is subject to feedback regulation by the sterol pathway and pharmacological manipulation of the pathway signals, we could treat cells to cause accumulation of Hmg2p-GFP, then remove the block to degradation and rapidly quantify Hmg2p-GFP levels at different time points by flow cytometry. Analysis of the proposed acidic loop mutants in Ubc7p should reveal an impairment in the rate at which substrate is degraded *in vivo*. In addition to examining degradation substrates ubiquitinated *in vivo*, we can use the *in vitro* assay defined in the previous chapter to examine the ubiquitination of ER proteins using recombinantly expressed mutants of Ubc7p to better understand the mechanism by which polyubiquitin chains are constructed.

Alternatively, Ubc7p might not function by the same mechanism as Cdc34p, and there are other models to explain the means by which Cue1p stimulates Ubc7p

activity. The strong interaction between Ubc7p and Cue1p observed *in vitro* during purification, as well as the cellular localization of Ubc7p at the ER (Biederer, et al., 1997; Gardner, et al., 2001; Huh, et al., 2003), suggest that these two proteins may exist as a dimer in cytosol. CUE domains with homology to Cue1p are able to bind polyubiquitin but Cue1p has been observed to lack this function (Shih, et al., 2003). Cue1p might bind individual ubiquitin molecules, or enhance a non-covalent interaction between charged Ubc7p and an additional ubiquitin in solution, as has been suggested for another E2 (Brzovic, et al., 2006). Cue1p could also multimerize, recruiting a multi-Ubc7p complex that could arrange the E2s for rapid and processive polyubiquitination.

Another surprising result observed with Ubc7p and Cue1p *in vitro* is the extent to which the E3-dependent ubiquitination was enhanced by addition of Cue1p. It is not clear whether the improved ubiquitination observed with E3 and Ubc7p+ΔtmCue1p was due completely to the activation of Ubc7p (Figure 4-1, compare left and right panels). Cue1p may have affinity for a portion of the Hrd1p soluble domain used as E3 in the experiment (GST-N-R-C), in addition to its stimulation of Ubc7p. However, in pulldown experiments using solution conditions that approximate the *in vitro* reactions, we have not been able to detect an interaction between Ubc7p and Hrd1p or Ubc7p+ΔtmCue1p and Hrd1p (data not shown). The interactions of E2 and RING ubiquitin ligases are thought to have very low affinity, and a requirement for transience in this interaction has been suggested by a point mutation in Cdc34p whose increased affinity for the SCF E3 resulted in reduced

ubiquitination of substrate (Deffenbaugh, et al., 2003). A requirement for weak affinity between E2 and E3 might strike a balance between two competing needs that both promote continued ubiquitination by the E3: recruiting the E2 with charged ubiquitin, and exchanging the discharged E2 with a new ubiquitin-charged E2.

We first identified the enhancing effect of Cue1p on Ubc7p-dependent ubiquitination *in vitro*, but it appears that this is also critical for the function of Ubc7p *in vivo*. Although we have not determined the mechanism by which Cue1p enhances Ubc7p-dependent polyubiquitin chain formation, we have determined some details of the reaction, and they are consistent with the mechanism proposed for Cdc34p function with the SCF E3 complex. It is possible that the mechanism of polyubiquitin chain construction, and the requirement of an activating factor, are shared by Ubc7p, Cdc34p, and other E2s that promote polyubiquitination.

## Methods:

*Recombinant DNA* - All DNA segments synthesized by PCR were verified by sequencing. GST and the GST fusion protein were expressed from the pET42b(+) bacterial expression plasmid (Novagen). GST-N-R-C was made by PCR of the appropriate sequence from Hrd1p plasmid and subcloning into pRH1466, which was derived from pET42b(+). The Ubc7p coding region was amplified by PCR and subcloned into pTYB2 (New England Biolabs) to produce the Ubc7p-Chitin Binding Domain/ Intein fusion vector pRH1946. Ubc7p-2HA coding region was amplified by PCR and subcloned into pTYB2 to produce the Ubc7p-2HA-Chitin Binding Domain/ Intein fusion vector pRH1947. ΔtmCue1p, which lacks amino acids 2-22 of Cue1p (and thus the included transmembrane span) was amplified by PCR from pTX129 (Biederer, et al., 1997) and cloned into a pET bacterial expression vector. Then, the ribosomal binding site and ΔtmCue1p were amplified by PCR and subcloned behind Ubc7p-CBD/Intein in pRH1946 to produce pRH2061, whose polycistronic message encoded both Ubc7p-CBD/Intein and ΔtmCue1p proteins in one inducible operon. The ribosomal binding site and ΔtmCue1p were also subcloned behind Ubc7p-2HA-CBD/Intein in pRH1947 to produce pRH2064, whose polycistronic message encoded both Ubc7p-CBD/Intein and ΔtmCue1p proteins in one inducible operon. 6His-tagged mouse UBA1 (E1) and HUBC4 were purified from bacterial lysates as described previously (Bays, et al., 2001; Bazirgan, et al., 2006; Joazeiro, et al., 1999; Mori, et al., 1997). Ubc7p with two HA-epitope tags was expressed in yeast from the strong *TDH3* promoter using the previously described vector pRH373 (Gardner, et al., 2001).

The identical coding sequence for Ubc7p-2HA was amplified by PCR and subcloned into a yeast expression vector containing the native *UBC7* promoter for expression of Ubc7p-2HA (pRH2193). For expression of Cue1p in yeast, sequence encoding full-length Cue1p was amplified by PCR and subcloned into an existing yeast expression vector between its *TDH3* promoter and three HA-epitope tags (pRH1334). Membrane anchored versions of Ubc7p were made by a PCR SOEing method (Ho, et al., 1989; Horton, et al., 1989). Sequences encoding the n-terminal 22 amino acid transmembrane region of Cue1p and the entire coding region of Ubc7p-2HA were amplified by PCR, joined by PCR SOEing, and this chimeric PCR product was subcloned into a vector allowing expression of TM-Ubc7p from the strong *TDH3* promoter (pRH2190). To produce TM-linker-Ubc7p, the Cue1p transmembrane span and Ubc7p-2HA were amplified exactly as above by PCR. The linker sequence encodes amino acids 531-618 of Hmg2p, a portion of the cytosolic linker region between the multispanning transmembrane portion of Hmg2p and its conserved cytosolic catalytic domain. This 88 amino acid linker was amplified from pRH469 by PCR and joined to the PCR products above by PCR SOEing to produce TM-linker-Ubc7p. This chimeric PCR product was subcloned into a vector allowing expression of TM-linker-Ubc7p from the strong *TDH3* promoter (pRH2191). To express ΔtmCue1p in yeast, the sequence encoding amino acids 23-203 and the adjacent three HA-epitope tags was amplified by PCR from pRH1334 and subcloned behind the strong *TDH3* promoter in a yeast expression vector (pRH2198). Sequence encoding Cdc34p was amplified from genomic DNA and subcloned into the previously

described p416-GPD vector (Mumberg, et al., 1995) between the *TDH3* promoter and *CYC1* terminator to produce pRH1939. The native *CDC34* promoter was amplified from pRG721 (from Richard G. Gardner, University of Washington) and subcloned into pRH1939 to make pRH1971, expressing Cdc34p from the *CDC34* promoter. Ubc7p and the tail domain of Cdc34p were fused by a PCR SOEing method. Sequence encoding amino acids 171-294 of Cdc34p were amplified by PCR and fused to the sequence encoding Ubc7p. DNA encoding this chimeric Ubc7p-Cdc34tail was subcloned into pRH1939 to produce pRH1968. pRH1969, expressing the function-blocking C89S mutant version of Ubc7p-Cdc34tail, was made as above except Ubc7p sequence was amplified from a template with the C89S point mutation. Ubc7p was expressed from the same vector as these *CDC34* constructs by PCR amplifying Ubc7p encoding sequence and subcloning it into pRH1939. These constructs were then subcloned into pRH1971 to express them from the *CDC34* promoter: pRH1983 expressed Ubc7p-Cdc34tail, pRH1985 expressed Ubc7p-Cdc34tail with C89S, and pRH1987 expressed Ubc7p.

*Strains and Media* - Yeast were cultured as described (Hampton, et al., 1996; Hampton and Rine, 1994), in minimal media with 2% glucose and amino acid supplements, at 30˚C unless otherwise indicated. All yeast strains were derived from the same genetic background used in our previous work (Hampton, et al., 1996; Hampton and Rine, 1994). Strains for evaluating the *in vivo* degradation of Hmg2p-GFP were derived from RHY853 (Gardner, et al., 2000), expressing the catalytic domain of Hmg2p as its sole source of HMG-CoA reductase, and Hmg2-GFP. To test

complementation of Ubc7p and Ubc7p-containing constructs, *UBC7* was replaced with the selectable *HIS3* marker, producing the *ubc7*Δ strain RHY1848. Constructs expressing Ubc7p, Cue1p, and membrane-anchored versions of Ubc7p were introduced into this strain to test their restoration of Ubc7p function. To examine restoration of both Ubc7p and Cue1p function, the *CUE1* gene was replaced in RHY1848 with the nourseothricin- (ClonNat) resistance marker natMX (Goldstein and McCusker, 1999) to produce the *ubc7*Δ *cue1*Δ strain RHY5917. In this strain ΔtmCue1p and TM-linker-Ubc7p were expressed (individually and together) to test restoration of ERAD function. To convert the native *CDC34* locus to the *cdc34-2* allele encoding the G58R mutation, the pRG721 plasmid encoding *cdc34-2* was integrated into RHY2863 at the *CDC34* locus, placing the selectable *URA3* gene between two copies of *CDC34* (one wt, one mutant). Spontaneous recombination between the *CDC34* loci left some cells with one copy of *CDC34* gene, causing spontaneous loss of *URA3*, which was selected by growth on 5'FOA. RHY3802 was screened for temperature sensitivity and loss of *URA3*, confirming the presence of only the *cdc34-2* allele. This strain was transformed with expression plasmids for Cdc34p, Ubc7p-Cdc34tail, or Ubc7p, and in turn these were transformed with either empty vector, or ΔtmCue1p expression plasmid to assess the activation of Ubc7p by Cue1p.

*Protein Purification* - All recombinant proteins were expressed in Rosetta(DE3) *E. coli* (Novagen) grown in LB with appropriate antibiotics. 0.6 OD/ml cultures were induced with 0.5mM IPTG for 12-16 hours at 15˚C. Bacterial pellets

were harvested, washed in normal saline (0.9M NaCl), and frozen at –80˚C. Pellets were thawed and resuspended in extraction buffers as described below, and lysed using a Branson Sonifier 450 (VWR) with six rounds of 30s sonication/30s ice incubation. After affinity column purification, elution, and concentration, proteins were dialyzed into buffer HDB containing 10% glycerol. Single-use aliquots were flash-frozen with liquid nitrogen and stored at –80˚C. Recombinant protein concentrations were determined by Coomassie staining of SDS-PAGE resolved samples and comparison to BSA.

His-Tag purification of E1 and HUBC4: - Bacterial pellets from 2L cultures expressing 6-His mouse UBA1 or HUBC4 were resuspended in 40mls of His-Extraction Buffer (50mM sodium phosphate pH 7.4, 300mM NaCl, 0.5% Nonidet NP-40, 5% glycerol) with protease inhibitors (13 μM AEBSF, 3.6 μM TPCK, 2.6μM leupeptin, 1.8 μM pepstatin, 0.56 mM 6-aminohexanoic acid, 0.56mM benzamidine, and 2.5mM 2-mercaptoethanol), sonicated as above, and centrifuged at 12,000 x g for 20minutes in an SS34 Rotor. The supernatant was transferred to a new tube with 0.5ml Talon Cell-Thru resin (BD Biosciences) equilibrated in His-Extraction Buffer, and gently nutated for 20min at room temperature. The resin was centrifuged (3000g) and washed with 10mls His-Extraction Buffer and protease inhibitors above for 10min at RT two times. The washed resin was then transferred to a 1cm diameter column and washed with 30mls His-Wash Buffer (50mM sodium phosphate pH 7.8, 300mM NaCl, 5mM imidazole, 5% glycerol, 10mM 2-mercaptoethanol). His-tagged E1 was eluted from the resin with 3 mls of His Wash Buffer + 150mM imidazole, and was

collected in 500ul fractions and analyzed by Bradford assay with BSA standard. Fractions with more than 0.1 mg/ml protein were pooled and concentrated with Amicon Ultra-15 5,000 MWCO filters (Millipore). Concentrated protein was dialyzed over 24 hours with 3x 1L HDBG (25mM HEPES, 0.7mM sodium phosphate, 137mM NaCl, 5mM KCl, pH 7.4, 10% glycerol) in a 3ml 10,000 MWCO Slide-A-Lyzer cassette (Pierce).

GST Protein Purification: - Each bacterial pellet from 1L of culture expressing a GST protein was resuspended in 25mls of buffer HDB (25mM HEPES, 0.7mM sodium phosphate, 137mM NaCl, 5mM KCl, pH 7.4) + protease inhibitors (7.5 mM EDTA, 1.5 mM PMSF, 10μM leupeptin, 7μM pepstatin, 28 μM TPCK, 130 μM AEBSF, 2.5 mM 6-aminohexanoic acid, 2.5mM benzamidine, and 7.5mM DTT), and sonicated as above. To this was added 6ml 2.5M NaCl, 600ul 1M sodium phosphate pH7.4, and 1.2ml 25% Triton X-100. This was nutated 20min at 4˚C, and centrifuged for 1hr at 40,000g in SS34 rotor. Buffer HDBW (HDB + 20mM sodium phosphate pH7.4, 10 mM 2-mercaptoethanol, 1% Triton X-100.) was added to the supernatant to a final volume of 50ml along with 1ml of glutathione-Sepharose-4B resin (Pharmacia) and nutated for 1hr at 4˚C. The resin was transferred to a 1cm diameter column and washed with 10 mls of each of the following buffers: HDBW with1.25mM PMSF and 5mM EDTA; HDBW with 1.25mM PMSF, 5mM EDTA, and 0.5M NaCl; HDBW with 0.5% deoxycholate; HDBW with 0.5M NaCl; and Final Wash (40mM Tris pH8.0, 50mM NaCl, 10mM 2-mercaptoethanol). Protein was eluted in 1ml fractions of Elution Buffer (20mM Tris pH 8, 10mM 2-mercaptoethanol, 20mM reduced

glutathione) incubated with column resin for 10min before recovery. Fractions were analyzed by Bradford assay with BSA standard. Fractions with more than 0.1 mg/ml protein were pooled and concentrated with Amicon Ultra-15 5,000 MWCO filters (Millipore). Concentrated protein was dialyzed for 24 hours with 3x 1L HDBG (25mM HEPES, 0.7mM sodium phosphate, 137mM NaCl, 5mM KCl, pH 7.4, 10% glycerol) in a 0.5ml 3,000 MWCO Slide-A-Lyzer cassette (Pierce).

Intein / Chitin Binding Domain Fusion Purification: - Each bacterial pellet from 1L of culture expressing an Intein/CBD fusion was resuspended in 25mls of Intein Lysis Buffer (ILB: 50mM Tris pH8.0, 500mM NaCl, 1mM EDTA, 0.1% Triton X-100) with protease inhibitors (260µM AEBSF, 105µM leupeptin, 73µM pepstatin, 142µM TPCK), and sonicated as above. Lysate was centrifuged at 20,000g for 30min in an SS34 rotor. Supernatant was filtered through 0.45µm and 0.2µm filters, and added to 15mls of chitin beads (New England Biolabs) equilibrated in ILB, and nutated for 90min at 4˚C. The adsorbed resin was placed in a 2.5cm column and washed with 350-400 mls of ILB. Next, the resin was nutated in 10mls of ILB + 50mM DTT for 20 hrs at 4˚C to promote intein cleavage, and chitin beads were washed with ILB to collect intein-cleaved proteins. 40 mls of fluid were collected and concentrated using Amicon Ultra-15 5,000 MWCO filters (Millipore). Concentrated protein was dialyzed against 3x 1L HDBG (25mM HEPES, 0.7mM sodium phosphate, 137mM NaCl, 5mM KCl, pH 7.4, 10% glycerol) for 24 hours in a 0.5ml 3,000 MWCO Slide-a-Lyzer cassette (Pierce). Proteins were ultra-centrifuged at 100,000g to remove any aggregates, and supernatant was aliquoted as above.

*In Vitro Ubiquitination* - Ubiquitin was resuspended from lyophilized powder in Ubiquitin Storage Buffer (50mM Tris pH7.5, 50mM NaCl, 10% glycerol) and frozen. Mutant varieties of ubiquitin were purchased from Boston Biochem, Inc. (Cambridge, MA). Reactions were performed in 1x Ubiquitination Buffer (50mM Tris pH7.5, 2.5mM $MgCl_2$, 0.5mM DTT) with 3mM ATP, 80µg/ml ubiquitin, 6µg/ml E1, 20µg/ml E2, in a total volume of 15µl. Reactions mixtures were prepared on ice, then incubated at 30˚C for 2 hours, and stopped with an equal volume of 2x sample buffer (4% SDS (w/v), 8M urea, 75mM MOPS pH6.8, 200mM DTT, 0.2 mg/ml bromphenol blue) and analyzed by SDS-PAGE and immunoblotting or Coomassie staining as indicated.

*Ubiquitin-Ubc7p thioester formation* - Ubiquitin was resuspended from lyophilized powder in Ubiquitin Storage Buffer (50mM Tris pH7.5, 50mM NaCl, 10% glycerol) and frozen. Reactions were performed in 1x Ubiquitination Buffer (50mM Tris pH7.5, 2.5mM $MgCl_2$, 0.5mM DTT) with 3mM ATP, 80µg/ml ubiquitin, 6µg/ml E1, 40µg/ml E2, in a total volume of 15µl. Reactions mixtures were prepared on ice, then incubated at room temperature for 5 minutes, and stopped with an equal volume of non-reducing 2x sample buffer (4% SDS (w/v), 8M urea, 75mM MOPS pH6.8, 0.2 mg/ml bromphenol blue) and analyzed by SDS-PAGE and immunoblotting for ubiquitin or HA-epitope.

*Flow Cytometry* - Log phase cultures ($O.D._{600} < 0.5$) grown in minimal medium at 30˚C were transferred to flow cytometer sample tubes and measured with a Becton Dickinson FACScalibur instrument. Flow microfluorimetric data were

analyzed and histograms were generated using CellQuest flow cytometry software. In all cases, histograms represented 10,000 individual cells.

*Cycloheximide-Chase Assay* – Log phase cultures ($O.D._{600} < 0.1$) grown in minimal medium at 30˚C were split into two tubes. One was treated with 50µg/ml cycloheximide, the other with no drug. These were incubated for 2 hours at 30˚C, then transferred to flow cytometer sample tubes and measured as above.

*Growth assay for temperature-sensitive phenotype* – Log phase cultures ($O.D._{600} < 0.5$) grown in minimal medium at 30˚C for each strain tested were normalized to equal $O.D._{600}$ with additional medium. 5-fold serial dilutions were performed in a 96 well plate, and cells were replica plated with a 48-pin replicator onto Synthetic Complete medium without uracil and leucine, and incubated at the indicated temperatures for 3 days. Images are representative of 3 experiments with duplicate plates for each temperature.

**Table 4-1. Strains used in chapter four.**

| Strain | *Genotype* | Figure |
|---|---|---|
| RHY5930 | *MATα ade2-101 met2 lys2-801 his3Δ200 leu2Δ hmg1Δ::LYS2 hmg2Δ::HIS3 ura3-52::URA3::pTDH3-HMGcd::pTDH3-HMG2-GFP trp1::hisG::TRP1(pRH311)* | 4-5 |
| RHY5931 | *MATα ade2-101 met2 lys2-801 his3Δ200 leu2Δ hmg1Δ::LYS2 hmg2Δ-4 ura3-52::URA3::pTDH3-HMGcd::pTDH3-HMG2-GFP ubc7Δ::HIS3 trp1::hisG::TRP1(pRH311)* | 4-5 |
| RHY5947 | *MATα ade2-101 met2 lys2-801 his3Δ200 leu2Δ hmg1Δ::LYS2 hmg2Δ-4 ura3-52::URA3::pTDH3-HMGcd::pTDH3-HMG2-GFP ubc7Δ::HIS3 trp1::hisG TRP1-Ubc7p-2HA(pRH2193)* | 4-5B |
| RHY5933 | *MATα ade2-101 met2 lys2-801 his3Δ200 leu2Δ hmg1Δ::LYS2 hmg2Δ-4 ura3-52::URA3::pTDH3-HMGcd::pTDH3-HMG2-GFP ubc7Δ::HIS3 trp1::hisG::TRP1::pTDH3-Ubc7p-2HA(pRH373)* | 4-5C |
| RHY5935 | *MATα ade2-101 met2 lys2-801 his3Δ200 leu2Δ hmg1Δ::LYS2 hmg2Δ-4 ura3-52::URA3::pTDH3-HMGcd::pTDH3-HMG2-GFP ubc7Δ::HIS3 trp1::hisG::TRP1::pTDH3-Cue1p-3HA (pRH1334)* | 4-5D |
| RHY5941 | *MATα ade2-101 met2 lys2-801 his3Δ200 leu2Δ hmg1Δ::LYS2 hmg2Δ-4 ura3-52::URA3::pTDH3-HMGcd::pTDH3-HMG2-GFP ubc7Δ::HIS3 trp1::hisG::TRP1::pTDH3-Cue1TM-Ubc7p-2HA(pRH2190)* | 4-5E |
| RHY5943 | *MATα ade2-101 met2 lys2-801 his3Δ200 leu2Δ hmg1Δ::LYS2 hmg2Δ-4 ura3-52::URA3::pTDH3-HMGcd::pTDH3-HMG2-GFP ubc7Δ::HIS3 trp1::hisG:: TRP1::pTDH3-Cue1TM-Hmg2pLinker-Ubc7p-2HA(pRH2191)* | 4-5F |
| RHY6026 | *MATα ade2-101 met2 lys2-801 his3Δ200 hmg1Δ::LYS2 hmg2Δ-4 ura3-52::URA3::pTDH3-HMGcd::pTDH3-HMG2-GFP ubc7Δ::HIS3 cue1Δ::NatR trp1::hisG leu2Δ::LEU2 (pRH312)* | 4-6, 4-7 |
| RHY6025 | *MATα ade2-101 met2 lys2-801 his3Δ200 hmg1Δ::LYS2 hmg2Δ-4 ura3-52::URA3::pTDH3-HMGcd::pTDH3-HMG2-GFP ubc7Δ::HIS3 cue1Δ::NatR trp1::hisG leu2Δ::LEU2::ΔtmCue1p-3HA (pRH2198)* | 4-6, 4-7 |

**Table 4-1 continued. Strains used in chapter four.**

| Strain | *Genotype* | Figure |
|---|---|---|
| RHY6052 | *MATα ade2-101 met2 lys2-801 his3Δ200 hmg1Δ::LYS2 hmg2Δ-4 ura3-52::URA3::pTDH3-HMGcd::pTDH3-HMG2-GFP ubc7Δ::HIS3 cue1Δ::NatR trp1::hisG::TRP1::pTDH3-Cue1pTM-Hmg2pLinker-Ubc7p-2HA leu2Δ::LEU2 (pRH312)* | 4-6, 4-7 |
| RHY6053 | *MATα ade2-101 met2 lys2-801 his3Δ200 hmg1Δ::LYS2 hmg2Δ-4 ura3-52::URA3::pTDH3-HMGcd::pTDH3-HMG2-GFP ubc7Δ::HIS3 cue1Δ::NatR trp1::hisG::TRP1::pTDH3-Cue1pTM-Hmg2pLinker-Ubc7p-2HA leu2Δ::LEU2::ΔtmCue1p-3HA (pRH2198)* | 4-6, 4-7 |
| RHY5984 | *MATa ade2-101 met2 lys2-801 his3Δ200 trp1::hisG cdc34-2 leu2Δ::LEU2(pRH312) ura3-52 URA3/YCp (pRH317)* | 4-9 |
| RHY5987 | *MATa ade2-101 met2 lys2-801 his3Δ200 trp1::hisG cdc34-2 leu2Δ::LEU2(pRH312) ura3-52 URA3/YCp-CDC34 (pRH1972)* | 4-9 |
| RHY5990 | *MATa ade2-101 met2 lys2-801 his3Δ200 trp1::hisG cdc34-2 leu2Δ::LEU2(pRH312) ura3-52 URA3/YCp-Ubc7p-CDC34tail (pRH1984)* | 4-9 |
| RHY5993 | *MATa ade2-101 met2 lys2-801 his3Δ200 trp1::hisG cdc34-2 leu2Δ::LEU2(pRH312) ura3-52 URA3/YCp-Ubc7p (pRH1988)* | 4-9 |
| RHY5985 | *MATa ade2-101 met2 lys2-801 his3Δ200 trp1::hisG cdc34-2 leu2Δ::LEU2::pTDH3-ΔtmCue1p-3HA ura3-52 URA3/YCp (pRH317)* | 4-9 |
| RHY5988 | *MATa ade2-101 met2 lys2-801 his3Δ200 trp1::hisG cdc34-2 leu2Δ::LEU2::pTDH3-ΔtmCue1p-3HA ura3-52 URA3/YCp-CDC34 (pRH1972)* | 4-9 |
| RHY5991 | *MATa ade2-101 met2 lys2-801 his3Δ200 trp1::hisG cdc34-2 leu2Δ::LEU2::pTDH3-ΔtmCue1p-3HA ura3-52 URA3/YCp-Ubc7p-CDC34tail (pRH1984)* | 4-9 |
| RHY5994 | *MATa ade2-101 met2 lys2-801 his3Δ200 trp1::hisG cdc34-2 leu2Δ::LEU2::pTDH3-ΔtmCue1p-3HA ura3-52 URA3/YCp-Ubc7p (pRH1988)* | 4-9 |

**Table 4-2. Plasmids used in chapter four.**

| Plasmid | Construction |
|---|---|
| pRH1726 | GST-N-R-C, GST fused to amino acids 262 to 551 of Hrd1p. Hrd1p sequence cut from pRH730 with BsrFI and SpeI, was subcloned into pRH1466 using AgeI and AvrII. |
| pRH1946 | Ubc7p-Intein/Chitin Binding Domain. Ubc7p was amplified by PCR from pRH373( Gardner et al (2001) MCB) and subcloned into NEB IMPACT vector TYB2. |
| pRH2061 | ΔTMCue1p and Ubc7p-Intein/Chitin Binding Domain. Cue1p was amplified by PCR and subcloned into a pET vector with NdeI and XhoI to place ΔTMCue1p sequence near a ribosomal binding site. Then, the pET vector's ribosomal binding site and, ΔTM Cue1p were amplified by PCR and subcloned into pRH1946 using PstI, and screened for correct orientation. |
| pRH1289 | 6HIS-E1 (mouse UBA1) Mori, S. et al. *European Journal of Biochemistry* **247**, 1190-6 (1997). |
| pRH1290 | 6HIS-HUBC4 Mori, S. et al. *European Journal of Biochemistry* **247**, 1190-6 (1997). |
| pRH1947 | Ubc7p-2HA-Intein/Chitin Binding Domain. Ubc7p was amplified by PCR from pRH373( Gardner et al (2001) MCB) and subcloned into NEB IMPACT vector TYB2. |
| pRH2064 | ΔTMCue1p and Ubc7p-2HA-Intein/Chitin Binding Domain. Cue1p was amplified by PCR and subcloned into a pET vector with NdeI and XhoI to place ΔTMCue1p sequence near a ribosomal binding site. Then, the pET vector's ribosomal binding site and, ΔTM Cue1p were amplified by PCR and subcloned into pRH1947 using PstI, and screened for correct orientation. |
| pRH311 | pRS404, *TRP1*/YIp Genetics 122: 19-27 (May, 1989) |
| pRH312 | pRS405, *LEU2*/YIp Genetics 122: 19-27 (May, 1989) |
| pRH317 | pRS416, *URA31*/YCp Genetics 122: 19-27 (May, 1989) |
| pRH373 | *TDH3* driven Ubc7p-2HA. *TRP1/YIp* Gardner et. al. 2001, MBC |
| pRH2193 | Native promoter Ubc7p-2HA. *TRP1/YCp* pRH373 digested with PacI/SphI and subcloned into pRH549, YCplac22 with *UBC7* promoter |

**Table 4-2 continued. Plasmids used in chapter four.**

| Plasmid | Construction |
|---|---|
| pRH1334 | *TDH3* driven Cue1p-3HA. *TRP1/YIp* |
| pRH2190 | *TDH3* driven Cue1pTM-Ubc7p-2HA. *TRP1/YIp*<br>PCR SOEing to fuse Cue1pTM to Ubc7p-2HA |
| pRH2191 | *TDH3* driven Cue1pTM-linker-Ubc7p-2HA. *TRP1/YIp*<br>PCR SOEing to fuse Cue1pTM and Ubc7p-2HA to linker from Hmg2p. |
| pRH2198 | *TDH3* driven ΔtmCue1p. LEU2*1/YIp*<br>PCR amplification of pRH1334 without TM digested with NcoI/SphI and subcloned into LEU2*1/YIp* vector. |
| pRH1972 | *CDC34* promoter driven Cdc34p. URA3/*YCp* |
| pRH1984 | *CDC34* promoter driven Ubc7p-Cdc34tail. URA3/*YCp* |
| pRH1988 | *CDC34* promoter driven Ubc7p. URA3/*YCp* |

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## References:


1. 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. *Nat Cell Biol* **3** (1) 24-29.

2. Bazirgan, O. A., Garza, R. M. and Hampton, R. Y. (2006). Determinants of RING-E2 Fidelity for Hrd1p, a Membrane-anchored Ubiquitin Ligase. *J Biol Chem* **281** (51) 38989-39001.

3. Biederer, T., Volkwein, C. and Sommer, T. (1997). Role of Cue1p in ubiquitination and degradation at the ER surface. *Science* **278** (5344) 1806-1809.

4. Brzovic, P. S., Lissounov, A., Christensen, D. E., Hoyt, D. W. and Klevit, R. E. (2006). A UbcH5/ubiquitin noncovalent complex is required for processive BRCA1-directed ubiquitination. *Mol Cell* **21** (6) 873-880.

5. 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.

6. Chau, V., Tobias, J. W., Bachmair, A., Marriott, D., Ecker, D. J., Gonda, D. K. and Varshavsky, A. (1989). A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein. *Science* **243** (4898) 1576-1583.

7. Cronin, S. R. and Hampton, R. Y. (1999). Measuring protein degradation with green fluorescent protein. *Methods Enzymol* **302** 58-73.

8. Cronin, S. R., Khoury, A., Ferry, D. K. and Hampton, R. Y. (2000). Regulation of HMG-CoA reductase degradation requires the P-type ATPase Cod1p/Spf1p. *J Cell Biol* **148** (5) 915-924.

9. Deffenbaugh, A. E., Scaglione, K. M., Zhang, L., Moore, J. M., Buranda, T., Sklar, L. A. and Skowyra, D. (2003). Release of ubiquitin-charged Cdc34-S - Ub from the RING domain is essential for ubiquitination of the SCF(Cdc4)-bound substrate Sic1. *Cell* **114** (5) 611-622.

10. 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.

11. Finley, D., Sadis, S., Monia, B. P., Boucher, P., Ecker, D. J., Crooke, S. T. and Chau, V. (1994). Inhibition of proteolysis and cell cycle progression in a multiubiquitination-deficient yeast mutant. *Mol Cell Biol* **14** (8) 5501-5509.

12. Flierman, D., Ye, Y., Dai, M., Chau, V. and Rapoport, T. A. (2003). Polyubiquitin serves as a recognition signal, rather than a ratcheting molecule, during retrotranslocation of proteins across the endoplasmic reticulum membrane. *J Biol Chem* **278** (37) 34774-34782.

13. Gardner, R., Cronin, S., Leader, B., Rine, J. and Hampton, R. (1998). Sequence determinants for regulated degradation of yeast 3-hydroxy-3-methylglutaryl-CoA reductase, an integral endoplasmic reticulum membrane protein. *Mol Biol Cell* **9** (9) 2611-2626.

14. 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. *Mol Cell Biol* **21** (13) 4276-4291.

15. Gardner, R. G., Swarbrick, G. M., Bays, N. W., Cronin, S. R., Wilhovsky, S., Seelig, L., Kim, C. and Hampton, R. Y. (2000). Endoplasmic reticulum degradation requires lumen to cytosol signaling. Transmembrane control of Hrd1p by Hrd3p. *J Cell Biol* **151** (1) 69-82.

16. Glickman, M. H. and Ciechanover, A. (2002). The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. *Physiol Rev* **82** (2) 373-428.

17. Goldstein, A. L. and McCusker, J. H. (1999). Three new dominant drug resistance cassettes for gene disruption in Saccharomyces cerevisiae. *Yeast* **15** (14) 1541-1553.

18. Gosink, M. M. and Vierstra, R. D. (1995). Redirecting the specificity of ubiquitination by modifying ubiquitin-conjugating enzymes. *Proc Natl Acad Sci U S A* **92** (20) 9117-9121.

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

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

21. Hershko, A. and Ciechanover, A. (1998). The ubiquitin system. *Annu Rev Biochem* **67** 425-479.

22. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K. and Pease, L. R. (1989). Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* **77** (1) 51-59.

23. 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.

24. Huh, W. K., Falvo, J. V., Gerke, L. C., Carroll, A. S., Howson, R. W., Weissman, J. S. and O'Shea, E. K. (2003). Global analysis of protein localization in budding yeast. *Nature* **425** (6959) 686-691.

25. Joazeiro, C. A., Wing, S. S., Huang, H., Leverson, J. D., Hunter, T. and Liu, Y. C. (1999). The tyrosine kinase negative regulator c-Cbl as a RING-type, E2-dependent ubiquitin-protein ligase. *Science* **286** (5438) 309-312.

26. Jones, D., Crowe, E., Stevens, T. A. and Candido, E. P. (2002). Functional and phylogenetic analysis of the ubiquitylation system in Caenorhabditis elegans: ubiquitin-conjugating enzymes, ubiquitin-activating enzymes, and ubiquitin-like proteins. *Genome Biol* **3** (1) RESEARCH0002.

27. Koller, A., Snyder, W. B., Faber, K. N., Wenzel, T. J., Rangell, L., Keller, G. A. and Subramani, S. (1999). Pex22p of Pichia pastoris, essential for peroxisomal matrix protein import, anchors the ubiquitin-conjugating enzyme, Pex4p, on the peroxisomal membrane. *J Cell Biol* **146** (1) 99-112.

28. Kolman, C. J., Toth, J. and Gonda, D. K. (1992). Identification of a portable determinant of cell cycle function within the carboxyl-terminal domain of the yeast CDC34 (UBC3) ubiquitin conjugating (E2) enzyme. *Embo J* **11** (8) 3081-3090.

29. Kraft, E., Stone, S. L., Ma, L., Su, N., Gao, Y., Lau, O. S., Deng, X. W. and Callis, J. (2005). Genome analysis and functional characterization of the E2 and RING-type E3 ligase ubiquitination enzymes of Arabidopsis. *Plant Physiol* **139** (4) 1597-1611.

30. Mathias, N., Steussy, C. N. and Goebl, M. G. (1998). An essential domain within Cdc34p is required for binding to a complex containing Cdc4p and Cdc53p in Saccharomyces cerevisiae. *J Biol Chem* **273** (7) 4040-4045.

31. Mori, S., Tanaka, K., Kanaki, H., Nakao, M., Anan, T., Yokote, K., Tamura, K. and Saito, Y. (1997). Identification of an ubiquitin-ligation system for the epidermal-growth-factor receptor--herbimycin A induces in vitro ubiquitination in rabbit-reticulocyte lysate. *Eur J Biochem* **247** (3) 1190-1196.

32. Mumberg, D., Muller, R. and Funk, M. (1995). Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. *Gene* **156** (1) 119-122.

33. Peng, J., Schwartz, D., Elias, J. E., Thoreen, C. C., Cheng, D., Marsischky, G., Roelofs, J., Finley, D. and Gygi, S. P. (2003). A proteomics approach to understanding protein ubiquitination. *Nat Biotechnol* **21** (8) 921-926.

34. Petroski, M. D. and Deshaies, R. J. (2005). Mechanism of lysine 48-linked ubiquitin-chain synthesis by the cullin-RING ubiquitin-ligase complex SCF-Cdc34. *Cell* **123** (6) 1107-1120.

35. Prendergast, J. A., Ptak, C., Arnason, T. G. and Ellison, M. J. (1995). Increased ubiquitin expression suppresses the cell cycle defect associated with the yeast ubiquitin conjugating enzyme, CDC34 (UBC3). Evidence for a noncovalent interaction between CDC34 and ubiquitin. *J Biol Chem* **270** (16) 9347-9352.

36. Ptak, C., Gwozd, C., Huzil, J. T., Gwozd, T. J., Garen, G. and Ellison, M. J. (2001). Creation of a pluripotent ubiquitin-conjugating enzyme. *Mol Cell Biol* **21** (19) 6537-6548.

37. Ravid, T., Kreft, S. G. and Hochstrasser, M. (2006). Membrane and soluble substrates of the Doa10 ubiquitin ligase are degraded by distinct pathways. *Embo J* **25** (3) 533-543.

38. Seol, J. H., Feldman, R. M., Zachariae, W., Shevchenko, A., Correll, C. C., Lyapina, S., Chi, Y., Galova, M., Claypool, J., Sandmeyer, S., Nasmyth, K. and Deshaies, R. J. (1999). Cdc53/cullin and the essential Hrt1 RING-H2 subunit of SCF define a ubiquitin ligase module that activates the E2 enzyme Cdc34. *Genes Dev* **13** (12) 1614-1626.

39. Shih, S. C., Prag, G., Francis, S. A., Sutanto, M. A., Hurley, J. H. and Hicke, L. (2003). A ubiquitin-binding motif required for intramolecular monoubiquitylation, the CUE domain. *Embo J* **22** (6) 1273-1281.

40. Silver, E. T., Gwozd, T. J., Ptak, C., Goebl, M. and Ellison, M. J. (1992). A chimeric ubiquitin conjugating enzyme that combines the cell cycle properties of CDC34 (UBC3) and the DNA repair properties of RAD6 (UBC2):

implications for the structure, function and evolution of the E2s. *Embo J* **11** (8) 3091-3098.

41. Sommer, T. and Jentsch, S. (1993). A protein translocation defect linked to ubiquitin conjugation at the endoplasmic reticulum. *Nature* **365** (6442) 176-179.

42. Thrower, J. S., Hoffman, L., Rechsteiner, M. and Pickart, C. M. (2000). Recognition of the polyubiquitin proteolytic signal. *Embo J* **19** (1) 94-102.

43. VanDemark, A. P. and Hill, C. P. (2002). Structural basis of ubiquitylation. *Curr Opin Struct Biol* **12** (6) 822-830.

44. Walter, J., Urban, J., Volkwein, C. and Sommer, T. (2001). Sec61p-independent degradation of the tail-anchored ER membrane protein Ubc6p. *Embo J* **20** (12) 3124-3131.

45. Wilhovsky, S., Gardner, R. and Hampton, R. (2000). HRD gene dependence of endoplasmic reticulum-associated degradation. *Mol Biol Cell* **11** (5) 1697-1708.

46. Zheng, N., Wang, P., Jeffrey, P. D. and Pavletich, N. P. (2000). Structure of a c-Cbl-UbcH7 complex: RING domain function in ubiquitin-protein ligases. *Cell* **102** (4) 533-539.

# Chapter 5:

# Toward the future

## Summary:

In total, these studies have substantially improved and expanded our understanding of how Hrd1p functions to degrade proteins. In Chapter 2 we discovered that the isolated RING motif was a sufficient fragment of ubiquitin ligase to transfer ubiquitin to another protein *in vitro*, but that the *in vivo* action of Hrd1p is not recapitulated by study of the isolated RING motif, or a larger portion of Hrd1p, as is often done with *in vitro* studies. In Chapter 3 we found that the RING motif specified E2 recognition *in vivo*, and also effected regulation of Hrd1p by Hrd3p. While the RING motif alone was sufficient to function with an E2, this was not sufficient for Hrd1p to work with its native E2, Ubc7p. The bulk of the Hrd1p soluble domain was important for efficient ubiquitination with Ubc7p *in vitro*. The sequences in addition to the RING motif made requirement for the native RING more stringent. In the membrane, Hrd1p showed the strictest preference for the native RING to function with Ubc7p. *In vitro* activity of Hrd1p in the membrane was improved in the presence of Cue1p, and at cellular Ubc7p concentrations, Cue1p was completely required for Hrd1p activity. Finally, we saw Hrd1p self-ubiquitination in microsomes was proportional to Hmg2p ubiquitination, validating self-ubiquitination as an assay of E3 activity. We also noticed soluble Cue1p enhanced ubiquitination *in vitro*, with or without an E3, and in Chapter Four we examined this function of Cue1p in detail. Soluble Cue1p seemed to promote the transfer of ubiquitin from Ubc7p to other ubiquitin molecules in solution. We also established that this stimulation by Cue1p was essential for Ubc7p function *in vivo*. These properties of Ubc7p and Cue1p also

bore sufficient similarities to another well-studied E2 to suggest that they may share the mechanism by which they form polyubiquitin chains on a substrate lysine. We hope that future studies can elucidate the detailed mechanisms underlying these processes.

**Weak interactions between E2 and E3**

In general, the interactions between E2 and RING-motif E3s are weak, which makes analysis of this interaction challenging (VanDemark and Hill, 2002). It is thought that the transience of RING-E3 interaction with E2 is important to balance the competing needs for an E3 to bind charged E2, and then release the E2 after ubiquitin discharge to seek out another charged E2 molecule. For example, a mutant of Cdc34p with improved affinity for the SCF complex was found to have impaired SCF ligase activity (Deffenbaugh, et al., 2003). Due to their transience, E2-E3 interactions are largely studied by structural methods (Brzovic, et al., 2003; Brzovic, et al., 2006; Katoh, et al., 2003; Zhang, et al., 2005; Zheng, et al., 2000), inferred from functional assays *in vivo* (Bays, et al., 2001; Gardner, et al., 2000; Hampton and Bhakta, 1997), or captured using crosslinking reagents (Bays, et al., 2001; Gardner, et al., 2001). We were not able to detect co-precipitation of Ubc7p or HUBC4 with any fragment of Hrd1p used in the *in vitro* ubiquitination reactions (data not shown). Cue1p might improve the function of Ubc7p by enhancing its interaction with Hrd1p, but the presence of Cue1p did not improve Ubc7p co-precipitation with Hrd1p (data not shown). Although Hrd1p and Ubc7p interaction was not directly observable with

soluble recombinant proteins, the consequences of this interaction were, as they catalyze the formation of polyubiquitin chains. The interactions of Ubc7p with Hmg2p, and of Ubc7p with Hrd1p, can be observed *in vivo* using crosslinking reagents (Bays, et al., 2001; Gardner, et al., 2001). Using a crosslinking approach *in vitro* with recombinant proteins, we might be able to detect interaction of Ubc7p with soluble Hrd1p. This could be developed into an interaction assay to test whether Cue1p, other ERAD proteins, or certain solvent conditions, promote the interaction of Ubc7p with Hrd1p, or fragments of Hrd1p, to understand how these proteins engage one another *in vivo*. It is also possible that Ubc7p and Hrd1p interact only indirectly through other proteins, as implied in a recent interaction-mapping study of ERAD proteins where Ubc7p was observed to interact with Doa10, but not Hrd1p (Carvalho, et al., 2006).

The challenge of identifying these weak E2-E3 interactions is an imposing barrier toward mapping all the E2-E3 interactions in a given organism. In *Saccharomyces cerevisiae* there are 10 E2s, at least 50 RING motif-containing proteins, and 6 known HECT-domain ligases; and other ligase classes such as the U-box and PHD E3s are emerging (Hatakeyama and Nakayama, 2003; Mansouri, et al., 2003; Patterson, 2002). In humans there are more of every category. Despite this diversity of subunits, structural studies of E2 and RING E3 proteins suggest that they interact mostly at a small surface on the E2 and at the second loop of the RING motif (Brzovic, et al., 2003; Katoh, et al., 2003; Zheng, et al., 2000). Our *in vivo* analyses of this RING loop confirm the importance of this structurally predicted interaction for *in vivo* function of Hrd1p (Bazirgan, et al., 2006). It would be highly informative if this

interaction surface could be sufficiently understood to predict putative E2-E3 pairings. Some systematic analyses of E2-E3 ubiquitination activity have been performed using *in vitro* assays with recombinant proteins (Kraft, et al., 2005; Stone, et al., 2005), but the preceding chapters highlight that *in vitro* assays can give both false-negative and false-positive predictions of E2-E3 function. Thus, such systematic evaluations of E2-E3 pairings still require confirmation with *in vivo* experiments, which in turn demand sufficient knowledge of the protein's biological function for an *in vivo* assay.

Alternatively, we could leverage our knowledge of Hrd1p function to test the entire scope of RING motif variability that will allow function with Ubc7p. A Hrd1p plasmid can be engineered to allow facile mutagenesis of the 15 amino acids that comprise the second loop of the RING motif that is responsible for Ubc7p engagement. Oligoprimers with Hrd1p sequence could be synthesized with random nucleotide bases in the positions encoding the RING motif second loop. These could be annealed, then reintroduced into a Hrd1p-encoding plasmid by ligation or other means, essentially forming a library of *HRD1* sequence with 45 random bases encoding the 15 amino acids in loop 2 of the RING motif. The functional Hrd1p variants could be screened for successful restoration of Hmg2p-GFP degradation in a *hrd1*Δ strain. If Ubc7p-dependence of this degradation is confirmed, retrieval of the Hrd1p mutant plasmid and sequencing would identify a RING motif capable of engaging Ubc7p. In aggregate, the consensus sequence of these mutants would allow prediction of which RING motifs in the genome function with Ubc7p. Since Ubc1p is also known to play a minor role in Hrd1p function, this approach might also identify

RING motifs that perform Hrd1p function using Ubc1p. Other E2s might be amenable to similar analysis using a suitable E3 and fluorescently tagged substrate. If technically feasible, this sort of analysis has the potential to unlock the code of physical interactions underlying E2-E3 specificity.

**The Hrd1p transmembrane span**

The Hrd1p transmembrane span traverses the ER membrane six times (Deak and Wolf, 2001) and is necessary for Hrd1p function (Gardner, et al., 2000). Hrd3p in the ER lumen rescues Hrd1p from self-degradation by affecting the cytosolic activity of Hrd1p through the ER membrane (Gardner, et al., 2000). Though we have not identified mutations in the Hrd1p transmembrane span that fail to transmit this signal across the membrane, there are mutants in the cytosolic portion of Hrd1p that fail to respond to it. A small deletion of Hrd1p in the cytosolic RING motif is unresponsive to this signal, and is equally unstable in either the presence or absence of Hrd3p (Figure 3-3B, Δpro). C-terminal truncations of Hrd1p *in vivo* were also unresponsive to the presence of Hrd3p, as indicated by their degradation despite the presence of Hrd3p (Figure 2-7). Although we do not know the route by which information about Hrd3p in the lumen is delivered to the cytosolic portion of Hrd1p, the Hrd1p transmembrane domain is well positioned to do so. It is possible that Hrd3p regulates self-ubiquitination of Hrd1p through a mechanism that modulates the dimerization or multimerization of Hrd1p, as we have not established if Hrd1p self-ubiquitination occurs *in cis* or *in trans*. Hrd1p could also recruit other factors that influence the

preference of Hrd1p for self-ubiquitination vs. substrate ubiquitination. This can occur both through the loops of Hrd1p that emerge from the membrane, as well as laterally with other proteins embedded in the membrane.

The Hrd1p transmembrane spans could also act as an intramembrane scaffold to recruit other ER proteins, like the Ubc7p membrane anchor Cue1p. The Cue1p transmembrane span could have specific affinity for the Hrd1p transmembrane domain, bringing Ubc7p and Hrd1p together in the cytosol, and explaining the lack of interaction observed when testing the cytosolic domains alone. To test this possibility, we could replace the transmembrane span of Cue1p with that from another ER-localized protein. If the anchor-replaced Cue1p has reduced ERAD function compared to native Cue1p, it might indicate specific affinity between the Hrd1p and Cue1p transmembrane spans. The transmembrane span of Hrd1p may recruit other proteins as well. Recent studies of the Hrd1p ubiquitin ligase complex identified several membrane-spanning proteins, including Ubx2p, Usa1p, and Der1p, as components of a wider HRD complex (Carvalho, et al., 2006; Denic, et al., 2006).

Analogous to the protein translocating channel that allows secretory pathway and ER-resident proteins into the ER, ERAD substrates must be retro-translocated to exit the ER for proteasomal degradation (Tsai, et al., 2002). Luminal ERAD substrates are topologically excluded from the cytosolic ubiquitination machinery until they emerge from the ER. Hrd1p is required for ERAD of both multispanning transmembrane proteins and completely luminal proteins (Bordallo, et al., 1998; Hampton, et al., 1996; Plemper, et al., 1998; Wilhovsky, et al., 2000). Hrd3p is

normally required for Hrd1p-dependent ERAD, but overexpression of Hrd1p can rescue ERAD function in the absence of Hrd3p (Denic, et al., 2006; Gardner, et al., 2000; Plemper, et al., 1999). Interestingly, the ERAD restoration is complete for the membrane anchored Hmg2p, and partial for the luminal CPY*. It is possible that Hrd1p associates with this predicted but unidentified retro-translocation complex to ubiquitinate these proteins soon after they emerge from the ER. Hrd1p may even form the retrotranslocation channel as a multimer of Hrd1p proteins, or as a mixed multimer with other proteins in a complex. Others in the Hampton lab are currently exploring this exciting possibility.

**The Role of Hrd3p**

Hrd3p is required for ERAD, and with a large luminal domain it is well positioned to coordinate substrate detection in the ER lumen with ubiquitination at the cytosolic ER surface. Hrd3p interacts with Hrd1p, and by a mechanism that is not understood, signals through the ER membrane to stop Hrd1p self-degradation (Gardner, et al., 2000). When Hrd3p is absent, Hrd1p is rapidly degraded, resulting in too little Hrd1p to perform ERAD (Gardner, et al., 2000). This keeps Hrd1p and Hrd3p levels equal, and destroys any Hrd1p unassociated with Hrd3p. Interestingly, a Hrd3p truncation capable of stabilizing Hrd1p is still defective in ERAD (Gardner, et al., 2000). Despite stabilizing Hrd1p, this Hrd3p truncation shows diminished crosslinking between Ubc7p and Hmg2p (Gardner, et al., 2001). Hrd3p might be interacting with intra-membrane proteins, in addition to Hrd1p, to influence the

recruitment of Ubc7p or otherwise promote substrate ubiquitination. This could prime the cytosolic side of the HRD complex in preparation to receive luminal substrates. Consistent with this, recent studies have identified Yos9p and the prototypical ERAD substrate CPY* as interactors with Hrd3p (Carvalho, et al., 2006; Denic, et al., 2006; Gauss, et al., 2006). Yos9p is an ER luminal lectin that is necessary for both interaction with and degradation of luminal ERAD substrates (Bhamidipati, et al., 2005; Kim, et al., 2005; Szathmary, et al., 2005). Hrd3p was also found to physically interact specifically with CPY* and not native carboxypeptidase Y, independent of Yos9p (Denic, et al., 2006; Gauss, et al., 2006). Thus, Hrd3p can sense ERAD substrates, recruit other ERAD substrate interactors, and modify the ligase activity of Hrd1p. Although there is no evidence that these are coordinated or synchronized actions of Hrd3p, it is an ideal candidate to signal the cytosolic side of the membrane that substrates in the lumen await exit form the ER.

Though Hrd3p is positioned to be the critical element of luminal substrate recognition, Hrd3p can be completely overruled by Hrd1p. Overexpression of Hrd1p suppresses the ERAD defect of a *hrd3*Δ null strain, allowing degradation of Hmg2p, CPY*, and the unglycosylated CPY*0000 without Hrd3p (Denic, et al., 2006; Gardner, et al., 2000; Plemper, et al., 1999). When overexpressed, Hrd1p seems to be the key mediator of misfolded protein recognition and Hrd3p appears dispensable. We have imagined that Hrd3p causes Hrd1p to prefer substrate over self-ubiquitination. Hrd3p might accomplish this by providing constant inhibition of Hrd1p that is only lifted when substrates are present. We hope that further understanding of the HRD

complex, ERAD, and the mechanism of substrate recognition will help us resolve the biological underpinnings of this paradox. The techniques developed for the study of Hrd1p function in membranes, as described in Chapter Three, with be beneficial to exploring the coordination of action amongst HRD-complex members.

**Cue1p**

Cue1p concentrates Ubc7p at the ER membrane and is necessary for ERAD *in vivo* (Biederer, et al., 1997; Gardner, et al., 2001). *In vitro*, membrane preparations from strains with Cue1p require a lower concentration of Ubc7p to achieve ubiquitination (Figure 3-9) (Bazirgan, et al., 2006). In Chapter Four, a soluble form of Cue1p showed a surprising stimulation of Ubc7p E2 activity both *in vitro* and *in vivo*. Cue1p improved E3-stimulated ubiquitination and also allowed E3-independent ubiquitination (Figure 4-1). The E3-independent polyubiquitin chains were lysine 48-linked, and were built unattached to other substrates (Figure 4-3). Importantly, lysine-48 linked ubiquitin chains are those recognized by the proteasome, suggesting this E3-free *in vitro* activity is relevant to the cellular function of Ubc7p and Cue1p. We have not yet discerned how these polyubiquitin chains were formed, but they were not caused by enhanced E1 function (Figure 4-4). We have not tested whether Cue1p can stimulate other E2s, but we predict this Cue1p function is Ubc7p-specific because Cue1p interacts tightly with Ubc7p in yeast extracts and during purification from bacterial extracts, and *cue1*Δ strains phenocopy *ubc7*Δ strains.

The strong E3-independent activity of Ubc7p with Cue1p suggests that Cue1p has potent effects on Ubc7p not explainable by improved E3 affinity. Cue1p might promote processivity of Ubc7p by bring soluble ubiquitin near the Ubc7p-ubiquitin adduct, so that the E2 can more readily ubiquitinate it and make polyubiquitin. Structural analyses of UbcH5 and BRCA-1 identified such an additional ubiquitin affinity site on UbcH5 that is required for processive ubiquitination by BRCA-1 (Brzovic, et al., 2006). Cue1p binding to Ubc7p might allow a multimer of Ubc7p molecules to form, positioning multiple charged ubiquitin-E2s for rapid polyubiquitination. We can study these potential mechanisms of interaction-mediated Ubc7p enhancement using epitope-tagged Ubc7p, and affinity purification to determine if Cue1p improves co-interaction among Ubc7p molecules, or improved interaction with ubiquitin. Gradient centrifugation or gel filtration techniques, can reveal whether the tightly bound Ubc7p and Cue1p form a heterodimer or larger complex.

Cue1p could also be modifying the enzymatic activity of Ubc7p by changing the kinetics of Ubc7p. Ubc7p stimulation could result from Cue1p stabilizing a transition intermediate in the ubiquitination reaction. Careful analysis of Ubc7p enzyme kinetics, and a determination of Vmax and Km in the presence and absence of Cue1p will help discern whether Cue1p is improving identification of ubiquitin and substrate lysines or enhancing the enzyme's rate of reaction, or both. Additionally, a structural analysis of Ubc7p and Cue1p could be very informative. Little is known about what residues on Ubc7p and Cue1p are required for their interaction. Mutants

that abrogate this interaction might suggest the relative orientation of the two proteins, which could further refine our ideas about how this activation works. These and other mutants of Ubc7p and Cue1p could also be useful tools in the study of other aspects of ERAD, and might reveal unexpected functions for these proteins, or insights into their mechanism of action.

Of course, a structure for the Ubc7p/Cue1p complex would be extremely informative. The X-ray crystal structure of Ubc7p has been determined to 2.9Å (Cook, et al., 1997). If the Ubc7p-Cue1p complex can be crystallized, and it's structure determined, this can be readily compared to the solved Ubc7p structure to determine what, if any, changes occur in Ubc7p when interacting with Cue1p. Alternatively, because Ubc7p and Cue1p are 18.5kD and 22.7kD respectively, they are individually within the limits of protein NMR spectroscopy, and the 41kD dimer is also within the limits of newly developed NMR techniques (Foster, et al., 2007; Xu, et al., 2006). If Ubc7p or Cue1p can be individually expressed and isotopically labeled with C13 and N15 to determine their structures, then the other unlabeled protein could be added to determine which residues are involved in the interaction. Additionally, NMR is ideally suited to determine the residues involved in transient interactions, like those of ubiquitin or E3 with the Ubc7p/Cue1p complex. With such structural information, we could determine with more certainty a likely mechanism for Ubc7p activation by Cue1p, and extrapolate this to other E2 homologs of Ubc7p.

**Lessons from homologous proteins**

By primary sequence, Ubc7p and Cdc34p are the most closely related E2s in *Saccharomyces cerevisiae* (Figure 4-8A). The numerous parallels between Ubc7p and Cdc34p suggest functional commonalities might also exist. Both have an insertion of acidic residues within the core E2 homology domain that is absent from all other E2s in *S. cerevisiae*. Both are localized to a multi-subunit ubiquitin ligase complex: Ubc7p is recruited in proximity to the HRD ligase complex by Cue1p, and Cdc34p has a c-terminal tail region that binds it to the SCF ubiquitin ligase complex (Figure4-8B) (Biederer, et al., 1997; Mathias, et al., 1998). Both E2s use RING-motif containing E3s that target substrates for degradation by building lysine-48 linked polyubiquitin chains. The RING motif in each ubiquitin ligase has an insertion in the Loop1 region that interacts with other members of the complex (Bazirgan, et al., 2006; Zheng, et al., 2002). Both E3 complexes recruit a wide variety of substrates: the SCF complex uses numerous F-box modules to recruit diverse substrates positioned in proximity to Cdc34, while the quality control nature of the HRD complex demands that it also ubiquitinate many different ER proteins when they are placed in proximity to Ubc7p.

Because of these many parallels between Cd34p and Ubc7p, we considered that the mechanism by which Cdc34p synthesizes lysine 48-linked ubiquitin chains might be particularly informative toward our understanding of Ubc7p (Petroski and Deshaies, 2005; Petroski, et al., 2006). Cdc34p-ubiquitin was found to release its ubiquitin thioester onto another ubiquitin using only lysine 48, and this was enhanced in the presence of SCF complex through increased reaction rate and not improved

substrate binding. Cue1p and its Ubc7p stimulating activity could be an analogous subunit of the HRD complex to the unidentified portion of SCF complex that promotes Cdc34p activity. It is noteworthy that the free chains built by Ubc7p-Cue1p complex were also lysine-48 linked (Figure 4-3). It is also interesting that a sequence insertion into the E2 core homology domain occurring only with Ubc7p and Cdc34p has residues that, for Cdc34p, were required for specific lysine-48 polyubiquitin chain formation (Petroski and Deshaies, 2005). The model proposed for Cdc34p ubiquitination is biphasic, involving a slow rate-limiting ubiquitination of one substrate lysine, followed by rapid cycles of lysine 48-linked ubiquitination on the most distal ubiquitin of the chain. We expect from all these similarities and correlations that HRD complex ubiquitination will proceed by a similar mechanism to SCF complex ubiquitination, and we hope to verify this experimentally.

## References:


1. 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. *Nat Cell Biol* **3** (1) 24-29.

2. Bazirgan, O. A., Garza, R. M. and Hampton, R. Y. (2006). Determinants of RING-E2 Fidelity for Hrd1p, a Membrane-anchored Ubiquitin Ligase. *J Biol Chem* **281** (51) 38989-39001.

3. Bhamidipati, A., Denic, V., Quan, E. M. and Weissman, J. S. (2005). Exploration of the topological requirements of ERAD identifies Yos9p as a lectin sensor of misfolded glycoproteins in the ER lumen. *Mol Cell* **19** (6) 741-751.

4. Biederer, T., Volkwein, C. and Sommer, T. (1997). Role of Cue1p in ubiquitination and degradation at the ER surface. *Science* **278** (5344) 1806-1809.

5. Bordallo, J., Plemper, R. K., Finger, A. and Wolf, D. H. (1998). Der3p/Hrd1p is required for endoplasmic reticulum-associated degradation of misfolded lumenal and integral membrane proteins. *Mol Biol Cell* **9** (1) 209-222.

6. Brzovic, P. S., Keeffe, J. R., Nishikawa, H., Miyamoto, K., Fox, D., 3rd, Fukuda, M., Ohta, T. and Klevit, R. (2003). Binding and recognition in the assembly of an active BRCA1/BARD1 ubiquitin-ligase complex. *Proc Natl Acad Sci U S A* **100** (10) 5646-5651.

7. Brzovic, P. S., Lissounov, A., Christensen, D. E., Hoyt, D. W. and Klevit, R. E. (2006). A UbcH5/ubiquitin noncovalent complex is required for processive BRCA1-directed ubiquitination. *Mol Cell* **21** (6) 873-880.

8. 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.

9. Cook, W. J., Martin, P. D., Edwards, B. F., Yamazaki, R. K. and Chau, V. (1997). Crystal structure of a class I ubiquitin conjugating enzyme (Ubc7) from Saccharomyces cerevisiae at 2.9 angstroms resolution. *Biochemistry* **36** (7) 1621-1627.

10. 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. *J Biol Chem* **276** (14) 10663-10669.

11. Deffenbaugh, A. E., Scaglione, K. M., Zhang, L., Moore, J. M., Buranda, T., Sklar, L. A. and Skowyra, D. (2003). Release of ubiquitin-charged Cdc34-S - Ub from the RING domain is essential for ubiquitination of the SCF(Cdc4)-bound substrate Sic1. *Cell* **114** (5) 611-622.

12. 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.

13. Foster, M. P., McElroy, C. A. and Amero, C. D. (2007). Solution NMR of large molecules and assemblies. *Biochemistry* **46** (2) 331-340.

14. 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. *Mol Cell Biol* **21** (13) 4276-4291.

15. Gardner, R. G., Swarbrick, G. M., Bays, N. W., Cronin, S. R., Wilhovsky, S., Seelig, L., Kim, C. and Hampton, R. Y. (2000). Endoplasmic reticulum degradation requires lumen to cytosol signaling. Transmembrane control of Hrd1p by Hrd3p. *J Cell Biol* **151** (1) 69-82.

16. Gauss, R., Jarosch, E., Sommer, T. and Hirsch, C. (2006). A complex of Yos9p and the HRD ligase integrates endoplasmic reticulum quality control into the degradation machinery. *Nat Cell Biol* **8** (8) 849-854.

17. Hampton, R. Y. and Bhakta, H. (1997). Ubiquitin-mediated regulation of 3-hydroxy-3-methylglutaryl-CoA reductase. *Proc Natl Acad Sci U S A* **94** (24) 12944-12948.

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

19. Hatakeyama, S. and Nakayama, K. I. (2003). U-box proteins as a new family of ubiquitin ligases. *Biochem Biophys Res Commun* **302** (4) 635-645.

20. Katoh, S., Hong, C., Tsunoda, Y., Murata, K., Takai, R., Minami, E., Yamazaki, T. and Katoh, E. (2003). High precision NMR structure and function of the RING-H2 finger domain of EL5, a rice protein whose expression is increased upon exposure to pathogen-derived oligosaccharides. *J Biol Chem* **278** (17) 15341-15348.

21. Kim, W., Spear, E. D. and Ng, D. T. (2005). Yos9p detects and targets misfolded glycoproteins for ER-associated degradation. *Mol Cell* **19** (6) 753-764.

22. Kraft, E., Stone, S. L., Ma, L., Su, N., Gao, Y., Lau, O. S., Deng, X. W. and Callis, J. (2005). Genome analysis and functional characterization of the E2 and RING-type E3 ligase ubiquitination enzymes of Arabidopsis. *Plant Physiol* **139** (4) 1597-1611.

23. Mansouri, M., Bartee, E., Gouveia, K., Hovey Nerenberg, B. T., Barrett, J., Thomas, L., Thomas, G., McFadden, G. and Fruh, K. (2003). The PHD/LAP-domain protein M153R of myxomavirus is a ubiquitin ligase that induces the rapid internalization and lysosomal destruction of CD4. *J Virol* **77** (2) 1427-1440.

24. Mathias, N., Steussy, C. N. and Goebl, M. G. (1998). An essential domain within Cdc34p is required for binding to a complex containing Cdc4p and Cdc53p in Saccharomyces cerevisiae. *J Biol Chem* **273** (7) 4040-4045.

25. Patterson, C. (2002). A new gun in town: the U box is a ubiquitin ligase domain. *Sci STKE* **2002** (116) PE4.

26. Petroski, M. D. and Deshaies, R. J. (2005). Mechanism of lysine 48-linked ubiquitin-chain synthesis by the cullin-RING ubiquitin-ligase complex SCF-Cdc34. *Cell* **123** (6) 1107-1120.

27. Petroski, M. D., Kleiger, G. and Deshaies, R. J. (2006). Evaluation of a diffusion-driven mechanism for substrate ubiquitination by the SCF-Cdc34 ubiquitin ligase complex. *Mol Cell* **24** (4) 523-534.

28. Plemper, R. K., Bordallo, J., Deak, P. M., Taxis, C., Hitt, R. and Wolf, D. H. (1999). Genetic interactions of Hrd3p and Der3p/Hrd1p with Sec61p suggest a retro-translocation complex mediating protein transport for ER degradation. *J Cell Sci* **112 ( Pt 22)** 4123-4134.

29. 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. *J Biol Chem* **273** (49) 32848-32856.

30. Stone, S. L., Hauksdottir, H., Troy, A., Herschelb, J., Kraft, E., Callis, J. (2005). Functional analysis of the RING-type ubiquitin ligase family of Arabidopsis.. *Plant Physiol* **137** (1) 13-30.

31. Szathmary, R., Bielmann, R., Nita-Lazar, M., Burda, P. and Jakob, C. A. (2005). Yos9 protein is essential for degradation of misfolded glycoproteins and may function as lectin in ERAD. *Mol Cell* **19** (6) 765-775.

32. Tsai, B., Ye, Y. and Rapoport, T. A. (2002). Retro-translocation of proteins from the endoplasmic reticulum into the cytosol. *Nat Rev Mol Cell Biol* **3** (4) 246-255.

33. VanDemark, A. P. and Hill, C. P. (2002). Structural basis of ubiquitylation. *Curr Opin Struct Biol* **12** (6) 822-830.

34. Wilhovsky, S., Gardner, R. and Hampton, R. (2000). HRD gene dependence of endoplasmic reticulum-associated degradation. *Mol Biol Cell* **11** (5) 1697-1708.

35. Xu, Y., Zheng, Y., Fan, J. S. and Yang, D. (2006). A new strategy for structure determination of large proteins in solution without deuteration. *Nat Methods* **3** (11) 931-937.

36. Zhang, M., Windheim, M., Roe, S. M., Peggie, M., Cohen, P., Prodromou, C. and Pearl, L. H. (2005). Chaperoned ubiquitylation--crystal structures of the CHIP U box E3 ubiquitin ligase and a CHIP-Ubc13-Uev1a complex. *Mol Cell* **20** (4) 525-538.

37. Zheng, N., Schulman, B. A., Song, L., Miller, J. J., Jeffrey, P. D., Wang, P., Chu, C., Koepp, D. M., Elledge, S. J., Pagano, M., Conaway, R. C., Conaway, J. W., Harper, J. W. and Pavletich, N. P. (2002). Structure of the Cul1-Rbx1-Skp1-F boxSkp2 SCF ubiquitin ligase complex. *Nature* **416** (6882) 703-709.

38. Zheng, N., Wang, P., Jeffrey, P. D. and Pavletich, N. P. (2000). Structure of a c-Cbl-UbcH7 complex: RING domain function in ubiquitin-protein ligases. *Cell* **102** (4) 533-539.