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In vitro analysis of ER-associated protein degradation

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In vitro analysis of ER-associated protein degradation

A dissertation submitted in partial satisfaction of the requirements for the degree
Doctor of Philosophy

in

Biology

by

Renee Marie Garza

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2008
The Dissertation of Renee Marie Garza is approved, and it is acceptable in quality and form for publication on microfilm:

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Chair

University of California, San Diego

2008
Dedication

I dedicate this thesis to the supportive and loving individuals I have been very fortunate to have in my life.

My devoted family, John Shimashita and our son James, has steadfastly believed in me, and my abilities.

My wonderful parents, Margaret and Larry Garza, stressed education more than they realized. Their unconditional love has never wavered.

My sister, Gloria Weingarten, is an amazing person, who has had a significant influence on the person I am today.

Ralph Marquez and Brian Weingarten have given me never-ending encouragement.

Annie Ventura and Alyssa Jimenez have given me friendships that are genuine and unfaltering.

John Chappell and Dr. Rodrigo Lois, for whom I was privileged to know during my time spent at CSUF, positively influenced my view on life.
Epigraph

Advances are made by answering questions.
Discoveries are made by questioning answers.

_Bernard Haisch_

Science knows only one commandment -- contribute to science.

_Bertolt Brecht_
## Table of Contents

Signature Page ........................................................................................................ iii  
Dedication ................................................................................................................. iv  
Epigraph ..................................................................................................................... v  
Table of Contents ........................................................................................................ vi  
List of Figures ............................................................................................................. vii  
List of Tables .............................................................................................................. xi  
Acknowledgements .................................................................................................. xii  
Vita .............................................................................................................................. xiv  
Abstract .................................................................................................................... xv  
Chapter 1: ER-associated degradation ..................................................................... 1  
Chapter 2: In vitro Hmg2p ubiquitination assay development ................................. 13  
Chapter 3: HRD-mediated retrotranslocation of Hmg2p ........................................ 47  
Chapter 4: Geranylgeranyl pyrophosphate accelerates Hmg2p degradation ......... 94  
Chapter 5: Future Directions .................................................................................... 127  
Appendix 1: Materials and Methods ....................................................................... 136  
References ................................................................................................................ 149
List of Figures

Figure 1-1:  
The Ubiquitin-Proteasome System................................................................. 4

Figure 2-1:  
In vitro ubiquitination of Hmg2p-GFP requires Ubc7p, Hrd1p, and Hrd1p's intact RING domain................................. 21

Figure 2-2:  
Cue1p is required for in vitro Hmg2-GFP ubiquitination......................... 22

Figure 2-3:  
Lysine-6 is required for in vitro Hmg2-GFP ubiquitination............ 24

Figure 2-4:  
ATP is required for in vitro Hmg2p-GFP ubiquitination................... 26

Figure 2-5:  
In vitro Hmg2p-GFP ubiquitination requires hydrolyzable \( \beta-\gamma \) bond of ATP................................................................. 27

Figure 2-6:  
In vitro ubiquitination of unregulated variants of Hmg2p-GFP........ 29

Figure 2-7:  
In vitro Hmg2-GFP ubiquitination levels decrease with lovastatin pre-treatment of microsome donor strain.............. 32

Figure 2-8:  
In vitro ubiquitination of lovastatin-pretreated microsome donor strains expressing Hmg2p-GFP, NR1-Hmg2-GFP, or 6myc-Hmg2p-GFP................................................................. 34

Figure 2-9:  
Lovastatin pre-treatment of both microsome and cytosol donor strains decreases in vitro Hmg2p-GFP ubiquitination............... 36

Figure 2-10:  
In vitro ubiquitination of Hmg2p-GFP and its unregulated variants using cytosol prepared from strains grown in synthetic complete media with or without lovastatin...................................................... 37
Figure 2-11: Native Hrd1p levels support in vitro ubiquitination of unregulated variant NR1-Hmg2p-GFP and to a lesser extent WT Hmg2p-GFP. .............................................................. 39

Figure 2-12: Chemical chaperone glycerol decreases in vitro ubiquitination of Hmg2p-GFP with no effect on 6myc-Hmg2p-GFP. .......................................................... 41

Figure 2-13: Chemical chaperone glycerol decreases in vitro ubiquitination of Hmg2p-GFP with little effect on ubiquitin ligase Hrd1p. ............................................. 42

Figure 3-1: In vitro ubiquitination assay conditions support retrotranslocation of ubiquitinated Hmg2p-GFP into supernatant fraction. ............................................ 54

Figure 3-2: Proteasomal inhibition enhances in vitro Hmg2-GFP ubiquitination and retrotranslocation. ............................................................... 56

Figure 3-3: Hrd1p overexpression bypasses the requirement for Hrd3p in Hmg2p-GFP ubiquitination and retrotranslocation. ............................................. 58

Figure 3-4: Retrotranslocated Hmg2p-GFP can be immunoprecipitated with antisera that recognize cytosolic and transmembrane determinants. ............................................................... 60

Figure 3-5: Retrotranslocated Hmg2p-GFP is an intact molecule. ............................................ 62

Figure 3-6: Retrotranslocated Hmg2p-GFP is full-length. ............................................. 64

Figure 3-7: Hmg2p-GFP retrotranslocation is dependent on Cdc48p. ............................................. 67

Figure 3-8: In vitro Hmg2p-GFP ubiquitination with GST-ubiquitin does not support Hmg2p-GFP retrotranslocation. ............................................. 69

Figure 3-9: AMP-PNP does not support Hmg2p-GFP retrotranslocation. ............................................. 72
Figure 3-10: Cdc48p receptor Ubx2p is necessary for in vitro Hmg2p-GFP retrotranslocation....................................................... 74

Figure 3-11: Ubiquitin-proteasome shuttling factors Rad23p and Dsk2p are required for in vitro Hmg2p-GFP retrotranslocation............... 76

Figure 3-12: Putative retrotranslocons, Der1p, Dfm1p, and Sec61p, are not required for in vitro Hmg2p-GFP retrotranslocation............... 79

Figure 3-13: Full-length Hmg1-Hrd1p fusion is retrotranslocated in vitro........ 81

Figure 4-1: The Mevalonate Pathway.......................................................... 96

Figure 4-2: The multidrug transporter Pdr5p does not affect GGPP-induced Hmg2p-GFP degradation.............................................. 100

Figure 4-3: GGPP dose response and time course........................................ 102

Figure 4-4: GGPP did not induce Hmg1p-GFP degradation.......................... 104

Figure 4-5: Ubiquitin ligase Hrd1p is required for GGPP-induced Hmg2p-GFP degradation............................................................ 106

Figure 4-6: GGPP induces Hmg2p-GFP ubiquitination.................................. 107

Figure 4-7: GGPP specifically induces wild-type Hmg2p-GFP degradation, and not degradation of its variants................................. 109

Figure 4-8: Overexpression of GGPP synthase gene, BTS1, lowers Hmg2p-GFP steady-state levels. ...................................................... 111
Figure 4-9: Overexpression of squalene synthase did not shift the GGPP dose response. ................................................................. 113

Figure 4-10: Hmg2p-GFP is degraded in a bts1Δ null........................................... 114

Figure 4-11: GGPP does not induce Hmg2p-GFP degradation in a hmg1Δ HMG2 strain........................................................................ 116

Figure 4-12: GGPP, and not ZA, stimulates Hmg2p-GFP degradation in cells treated with lovastatin.................................................. 118

Figure 4-13: GGPP induces full-length 1-myc-Hmg2p degradation................. 120

Figure 5-1: A proposed model for Hmg2p ERAD......................................... 134
List of Tables

Table A-1:  
Strains used in Chapter 2............................................................. 146

Table A-2:  
Strains used in Chapter 3............................................................. 147

Table A-3:  
Strains used in Chapter 4............................................................. 148
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ABSTRACT OF THE DISSERTATION

In vitro analysis of ER-associated protein degradation

by

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Doctor of Philosophy in Biology

University of California, San Diego, 2008

Professor Randolph Hampton, Chair

Endoplasmic reticulum-associated degradation (ERAD) is part of the cellular protein quality control system. Substrates of protein quality control are usually ubiquitinated proteins that are degraded by the proteasome. Hrd1p, a principal ER ubiquitin ligase, mediates ERAD of proteins residing in the ER to those that are prevented from passing through the ER to final destinations in
every other part of the cell. Protein quality control pathways not only degrade misfolded or mutant proteins. They are also involved in the regulated destruction of otherwise normal proteins. The ER-localized Hmg2p is an example of a natural substrate recognized and degraded by the protein quality control pathway ERAD and specifically by Hrd1p. HMG2 is one of two isozymes in yeast encoding the rate-limiting enzyme for the production of isoprenoids and sterols. At the intersection of sterol synthesis and ERAD, Hmg2p regulated degradation is unique for the study of two important pathways. We have developed an in vitro assay that allows testing both Hmg2p regulation-specific and ERAD hypotheses. In vitro Hmg2p ubiquitination was in agreement with established in vivo criteria. In vitro analysis was broadened to the study how Hmg2p, a multi-spanning transmembrane protein, is removed from the ER membrane (retrotranslocation). Studies revealed full-length Hmg2p is liberated into the cytosol after retrotranslocation and Rad23p and Dsk2p play an unexpected role in this step. Furthermore, reputed retrotranslocation channels were not found to have a role in Hmg2p retrotranslocation. In vivo examination of Hmg2p degradation uncovered geranylgeranyl pyrophosphate (GGPP) stimulates Hmg2p degradation. Future GGPP analysis includes its addition to in vitro assays described in this thesis.
Chapter 1:

ER-associated degradation
Protein quality control pathways are in place to eliminate mutated, misfolded, and unassembled proteins. Misfolded and aggregated proteins can lead to cellular dysfunction, as they are hallmarks of serious disorders such as Alzheimer's, Parkinson's, and polyglutamine disease (Berke and Paulson, 2003). Oftentimes, part of the pathology is that these diseases affect other aspects of the protein quality control system. In a normal state, cells employ molecular chaperones to recognize common characteristics found in misfolded and damaged proteins that are otherwise varied in composition. Whether promoting folding or degradation, preventing aggregation is key.

The proteasome is the primary way proteins are degraded for protein quality control. The proteasome is a multi-subunit complex of approximately 2.4 MD that can be divided into two major components, the 20S core particle (CP) and the 19S regulatory particle (RP). Two RPs typically flank the CP. The CP's protein subunits form four stacks of rings, resembling the shape of a barrel. The two inner rings contain three different proteolytic activities and the two outer rings prevent access to those proteolytic subunits. Therefore, the CP is inert until it is activated. The RP recognizes substrates, regulates the CP, and guides the substrates into the chamber (Liu et al., 2006). The ring of RP subunits that contact the CP contain AAA ATPase subunits (Bochtler et al., 1999). For recognition of the substrates by the RP, the substrate is usually covalently tagged with the 8 kD protein ubiquitin.

A series of enzymes are required to tag proteins with ubiquitin. The ubiquitin activating enzyme, or E1, activates the C-terminus of ubiquitin with
ATP and then forms a high-energy thiolester bond with ubiquitin. The E1 then passes the ubiquitin onto an active site cysteine of a ubiquitin conjugating enzyme (E2). E3s, or ubiquitin ligases, specifically bind substrate and mediate the interaction required for the transfer of the ubiquitin from the E2 to a specific lysine of the substrate (Herschko and Ciechanover, 1998). Typically the ubiquitin is transferred from the E2, however there is a small group of E3s that directly ubiquitinates substrates (Kostova et al., 2007). After successive rounds of ubiquitin transfer to the lysine of a previously attached ubiquitin, a multiubiquitin chain is formed. The minimal chain length recognized by the proteasome is at least four ubiquitins long, however most substrates have longer chains in vivo. The chain linkages hold information about whether a protein should be degraded, with lysine-48 ubiquitin linkages signaling degradation (Thrower et al., 2000). Substrate modification by a single ubiquitin or a chain of lysine-63 linkages are involved in other cellular processes such as vesicular trafficking and membrane fusion.

Once a substrate is multiubiquitinated, the RP recognizes it and prepares it for entry into the CP where the substrate will be degraded. The RP has integrated and associated activities that are involved in unfolding and deubiquitinating the substrate, in order for the substrate to gain access into the chamber and to minimize ubiquitin loss. The formation and actions of the 26S proteasome are ATP-dependent. Figure 1-1 shows a cartoon of the ubiquitin proteasome system.
**Figure 1-1: The Ubiquitin-Proteasome System.** The E1 is charged with ubiquitin. The E2 then accepts the ubiquitin. The E2 recognizes a specific E3. The E3 binds substrate and typically orients substrate and E2, for ubiquitin transfer to the substrate. The multiubiquitin chain is recognized by RP of proteasome and allows movement of substrate into the CP where the substrate is degraded.
ER-associated degradation

Protein quality control is important for the management of misfolded and unassembled proteins in every part of the cell, including the endoplasmic reticulum (ER). Having roles that affect most aspects of normal cell function, protein quality control at the ER (ER-associated degradation or ERAD) is intensely studied. When the ER is overloaded with misfolded proteins, the Unfolded Protein Response (UPR) is activated, resulting in the increased transcriptional expression of chaperones and degradation machinery. Genetic studies in yeast have exposed a vital need for ERAD in resistance to ER stress (Friedlander et al., 2000). ERAD factors are found residing inside, outside, and through the ER membrane, for the recognition of substrates, movement of the substrate to the cytoplasm (or retrotranslocation), and degradation by the proteasome.

Ubiquitination

Ubiquitin ligases (E3) provide specificity to the ubiquitin pathway. There are two major E3s in the ER, Hrd1p and Doa10p, each with sizable transmembrane spans (six for Hrd1p and ten for Doa10p). In general, Hrd1p mostly degrades ER lumenal and membrane proteins whereas Doa10p degrades cytoplasmic and nuclear soluble substrates as well as some membrane proteins. Attempts have been made to classify ERAD substrates based on where the substrate defect lies, in the cytoplasm, lumen, or membrane (Vashist and Ng, 2004). However, especially true for membrane
proteins, a mutation on one side of the membrane may influence the rest of the protein found on the opposite side of the membrane (Kostova et al., 2007). Thus, further study of ERAD will reveal how E3s recognize substrates.

The HRD screen (for Hmg CoA reductase degradation) was carried out to identify genes involved in the degradation of Hmg2p, one of two Hmg-CoA reductase isozymes in yeast. Hmg2p is an ER-resident transmembrane protein that catalyzes the rate-limiting step for the production of sterols. Genes identified in the screen encoded HRD1, the ER ubiquitin ligase; HRD2, a proteasome RP subunit; HRD3, a membrane-anchored ER protein (Hampton et al., 1996a); and HRD4, part of the Cdc48p complex (Bays et al., 2001b). This HRD pathway was discovered to be responsible for the recognition and ubiquitination of many misfolded proteins, such as the mutated soluble vacuolar protein carboxypeptidase yscY* or CPY*, a mutant form of the major anterograde ER translocon sec61-2, a mutated form of the ATP-binding cassette multidrug transporter Pdr5*p, and others. Hrd1p was also identified in a screen to identify DER proteins, involved in degradation in the ER of the substrate CPY* (Bordallo et al., 1998). The natural substrate Hmg2p was degraded by a protein quality pathway that typically recognizes mutant proteins.

The HRD complex consists of members that localize to both sides of the ER membrane. The HRD1 E3 ubiquitin ligase belongs to the RING family of ubiquitin ligases. It has six transmembrane spans and a cytosolic RING domain. The core of the HRD complex is made up of Hrd1p and Hrd3p.
Hrd3p stabilizes Hrd1p and is involved in substrate recognition (Gardner et. al., 2000). Hrd3p has a large luminal domain and a single transmembrane anchor. Hrd1p employs the E2 Ubc7p and its partner, single transmembrane spanning Cue1p, to ubiquitinate substrates. Another E2, Ubc1p, plays a minor role in HRD mediated ubiquitination (Bays et. al., 2001a). Other complex members are involved in the recognition of luminal substrates. Yos9p is a lectin, which resides in the ER lumen, binding both Hrd3p and the ER chaperone Kar2p, and is involved in glycoprotein recognition (Gauss et al., 2006a). Another protein found in complex with Hrd1p is Der1p. Long known to be involved in CPY* degradation, its function has not been made clear (Knop et al., 1996). Usa1p is another complex member and effects luminal substrates and Hmg2p degradation (Carvahlo et al., 2006; Carroll and Hampton, manuscript submitted).

Unlike Hrd1p, the other major ER ligase, Doa10p, does not have many binding partners. It does utilize the E2 Ubc7p and Cue1p for substrate ubiquitination, and the ER membrane bound E2 Ubc6p. Some of the substrates Doa10p degrades are the E2 Ubc6p, a truncated version of the mating receptor STE6, Ste6-166, and the transcription factor MAT alpha 2 (Swanson et al., 2001; Huyer et al., 2004; Ravid et al. 2006). Cytosolic chaperones are often involved in Doa10p dependent degradation, however how substrates are recognized requires more study.

The two E3 complexes share common ground once substrates are recognized and ubiquitinated. The Cdc48p complex and its ER receptor
Protein Ubx2p have been purified in both the HRD and DOA complexes (Carvahlo et al., 2006; Denic et al., 2006).

**Retrotranslocation**

Movement of an ERAD substrate from the ER to the cytoplasm is usually termed retrotranslocation or dislocation. A clear channel or retrotranslocon of ERAD substrates has not been identified. However, integral to this process is the Cdc48 complex, which is formed by a homo-hexamer of the AAA-ATPase Cdc48p and its co-factors Npl4p and Ufd1p. Both Ufd1p and Cdc48p bind ubiquitin (Ye et al., 2003). Ubx2p is an ER-membrane protein receptor of the Cdc48p complex (Schuberth et al., 2004). Interaction studies show that co-immunoprecipitation between either Hrd1p or Doa10p ubiquitin ligases with the Cdc48p complex are detectable only when Ubx2p is present.

The Cdc48p complex also binds other factors. One such protein is Ufd2p. Ufd2p has E3 ubiquitin ligase activity, however instead of initiating a ubiquitin chain like characterized E3s, Ufd2p elongates ubiquitin chains formed by other E3s. Ufd2p competes with the proteasome for Rad23p binding. The current model of ERAD is that the ubiquitinated substrate is passed from factor to factor until the substrate reaches the proteasome (Richly et al., 2005).

In mammalian cells, derlins, which are homologues to yeast Der1p, have been shown to be involved in ERAD. They also demonstrate binding to both p97/Cdc48p (Lilley and Ploegh, 2004, and Ye et al., 2004), and ER ubiquitin ligases (Lilley and Ploegh, 2005, and Ye et al., 2005). Presently, the
derlins are favored retrotranslocon candidates (Sun et al., 2006). However, the Sec61p anterograde translocon and even ubiquitin ligases are potential candidates as well (Plemper and Wolf, 1999; Plemper et al., 1998; Plemper et al., 1999).

**Degradation**

The final event in ERAD is substrate degradation by cytoplasmic proteasomes. Proteasome mutants stabilize ubiquitinated proteins in general. The *hrd2-1* mutant uncovered in the Hmg2p screen stabilizes ERAD substrates as well as other proteasome substrates (Hampton et al., 1996a). A subunit of the RP base, Rpt4p has AAA ATPase activity. Rpt4p mutants have defective ERAD and an activated UPR. Interestingly, high levels of Cdc48p partially complemented the *RPT4* mutant (Lipson et al., 2008). The RP and Cdc48p complex ATPase activities may serve similar functions in ERAD but possibly with different substrate specificities.

The proteasome has other associated proteins. Rpn1p (*HRD2*) and Rpn2p are large subunits in the RP base subcomplex that serve as scaffolds for the associated proteins. They bind proteins involved in deubiquitination, ubiquitination, and delivery of substrates to the proteasome (Leggett et al., 2002). The proteasome contains an in-house deubiquitinating enzyme in the base subunit Rpn11p that removes the entire ubiquitin chain from a substrate. Ubp6p is one of the proteasome-associated proteins that bind Rpn1p. Ubp6p deubiquitinating activity involves removal of individual ubiquitins from a chain, and plays a role in regulating the proteasome for the maintenance of ubiquitin
pools (Hanna et al., 2007). Rpn2p has been shown to bind the Ubl domains of the shuttling or delivery factors, Rad23p and Dsk2p (Medicherla et al., 2004). The ubiquitin ligase Hul5p also binds Rpn2p. Hul5p extends multiubiquitin chains.

An emerging view of the proteasome is that its composition changes in response to different cellular contexts (Hanna and Finley, 2007). In yeast, proteasomes are found associated with ER membranes (Enenkel et al., 1998). Whether there are specific ERAD proteasomes remains to be determined.

**HMG-CoA reductase regulation**

Yeast Hmg2p and mammalian HMG-CoA reductase (HMGR) share general sensing and modes of degradation. Each is made up of eight membrane spans and a cytosolic domain. Within their transmembrane domain resides the sterol sensing domain, or SSD. For mammalian HMGR, the SSD is important for sensing sterols. Its degradation is dependent on the interaction with other ER proteins, Insigs. The ubiquitin ligase gp78, a Hrd1p homologue, binds Insig in low and high sterol conditions. However, HMGR is recruited to the Insig-gp78 complex in the presence of high sterols (Sever et al., 2003a, 2003b; Song and DeBose-Boyd, 2004). Thus, HMGR is ubiquitinated by gp78 and degraded by the proteasome (Ravid et al., 2000).

Hmg2p, likely senses with its SSD like HMGR. Mutational analysis of conserved residues in the SSD result in misregulation (Gardner et al., 1998; Gardner and Hampton, 1999; Davis and Hampton, unpublished observations). Instead of sterols signaling degradation, Hmg2p degradation relies on a non-
sterol isoprenoid derived from FPP (Gardner and Hampton, 1999). Yeast Insig homologues, Nsg1p and Nsg2p, are not required for Hmg2p degradation. They bind Hmg2p but promote stabilization, not degradation. Unlike HMGR, which requires another protein to broker interaction of it with the ubiquitin ligase, Hmg2p interacts with Hrd1p, in both low and high degradation signal conditions (Gardner et al., 2001b). However, in the presence of high degradation signal, Hmg2p is ubiquitinated by Hrd1p and degraded by the proteasome. Therefore, general mechanisms of signal recognition and ubiquitination/degradation are similar in mammalian cells and yeast HMGR regulation.

**In vitro ERAD assays**

There is a need to study ERAD in vitro. The identities of many ERAD factors have been revealed using genetic screens as well as mass spectrometry of purified protein complexes. Both genetic and physical interaction studies have been useful for building models. However, the functional significance and ordering of these interactions cannot be assumed. The HRD pathway is responsible for the ERAD of a growing list of substrates. Unlike most HRD substrates, Hmg2p is a physiological substrate resembling a misfolded protein when exposed to degradation signals. The Hampton laboratory uncovered the HRD pathway in our efforts to understand the regulated degradation of Hmg2p. In vitro analyses can be used to address mechanistic and biochemical questions that are specific to Hmg2p regulated ubiquitination and degradation, and may be applied to general ERAD.
In the following chapters, I will describe an in vitro assay for the study of Hmg2p degradation. I accomplished reconstitution of Hmg2p ubiquitination and retrotranslocation in vitro. In vivo Hmg2p degradation studies resulted in the discovery of GGPP functioning as an Hmg2p degradation signal.
Chapter 2:

In vitro Hmg2p ubiquitination assay development
Introduction

Endoplasmic reticulum-associated degradation (ERAD) is important for the clearance of misfolded and unpartnered proteins that either transit through the secretory pathway or reside in the endoplasmic reticulum (ER). ERAD is also responsible for the degradation of numerous normal proteins, including Hmg2p, one of two HMGR (3-hydroxymethylglutaryl-coenzyme A reductase) isozymes in *S. cerevisiae*. HMGR is a rate-limiting enzyme of the sterol pathway (Goldstein and Brown, 1980). Unlike most ERAD substrates, Hmg2p is degraded in a regulated manner, so that levels of sterol pathway intermediates influence its stability (Hampton and Bhakta, 1997; Hampton and Rine, 1994; Gardner and Hampton, 1999a). When sterol pathway signals are high, degradation is fast. Conversely when sterol synthesis is slow, Hmg2p is stabilized, partially alleviating the paucity of sterol synthesis.

ERAD substrates are degraded through a series of steps by the ubiquitin proteasome pathway. The ubiquitin-activating enzyme or E1 activates ubiquitin in an ATP-dependent manner. The activated ubiquitin is then transferred to the active site cysteine of the ubiquitin conjugating enzyme (UBC) or E2. E2s interact with specific E3s, or ubiquitin ligases. E3s recognize substrate, bringing the E2 in close proximity to the substrate for the covalent attachment of ubiquitin to the substrate (Hershko and Ciechanover, 1998). More ubiquitin-charged E2, then transfers additional ubiquitins until a multiubiquitin chain is formed on the substrate. Although a chain consisting of
four or more ubiquitins serves as a proteasomal degradation signal, substrates often have many more ubiquitins added in the course of in vivo degradation (Thrower et al., 2000).

Hrd1p is an E3 ubiquitin ligase that plays a central role in ERAD. Hrd1p has an N-terminal, multi-spanning, ER transmembrane anchor followed by a cytosolic RING-H2 domain (Gardner et al., 2000; Bordallo and Wolf, 1999). Hrd1p's intact RING-H2 domain is essential for the ubiquitination and degradation of many ER proteins in vivo, including Hmg2p (Hampton and Gardner, 1996; Bays et al., 2001a; Bordallo and Wolf, 1999). Hrd1p has a binding partner, Hrd3p, which has one ER transmembrane span attached to a large ER-lumenal domain (Gardner et al., 2001). Hrd3p participates in the stabilization of Hrd1p, the recognition of substrate, and the recruitment of other ERAD lumenal factors (Gardner et al., 2000, Denic et al., 2006; Carvalho et al., 2006; Gauss et al., 2006a). Hrd1p E3 action primarily depends on the E2 Ubc7p and to a lesser extent Ubc1p (Bays et al., 2000). Ubc7p is a cytosolic protein that localizes to the ER membrane through interaction with the single span ER membrane protein Cue1p (Biederer et al., 1997). How or if Ubc1p localizes to the ER has not been determined. Hmg2p ubiquitination involves each of these factors.

The Hampton laboratory has demonstrated that Hmg2p ubiquitination and degradation is regulated by a signal derived from farnesyl pyrophosphate (FPP), and to a small extent by an oxysterol-derived signal (Hampton and Bhakta, 1997; Gardner and Hampton, 1999a; Gardner et al., 2001a).
current model for Hmg2p degradation is that Hmg2p becomes an ERAD substrate because of a change in its transmembrane domain structure, induced by an FPP-derived molecule, making it acquire features of a misfolded protein and thus be recognized by the HRD pathway. An in vitro limited proteolysis assay showed that Hmg2p was more sensitive to protease digestion when Hmg2p was incubated with farnesol, the alcohol derivative of FPP (Shearer and Hampton, 2005). Direct addition of farnesol to cultures kills yeast cells precluding direct analysis and highlighting the importance of in vitro assays for studying how farnesol or other molecules not conducive to in vivo analysis influence Hmg2p regulated degradation (Fairn et al., 2007).

Once an ERAD substrate is ubiquitinated other proteins are necessary for its retrotranslocation or removal from the ER lumen or membrane into the cytosol for delivery to the proteasome. Multiple laboratories have demonstrated the critical involvement of the Cdc48p/Ufd1p/Npl4p complex after the substrate is ubiquitinated and in some instances in the recognition of ERAD substrates even before ubiquitination (Bays et al., 2001b and Ye et al., 2003). The involvement of Cdc48p complex in ERAD is indisputable but how and when it functions is not well understood. Reconstitution of the post ubiquitin steps in vitro would greatly facilitate our understanding and study of these poorly understood parts of the pathway.

To explore the mechanism of regulated ERAD, I developed an in vitro assay for HRD-dependent Hmg2p ubiquitination. In this chapter the
ubiquitination assay is developed and characterized. In the next chapter, it is harnessed to study biologically relevant retrotranslocation.

To begin to resolve the requirements for Hmg2p in vitro regulated ubiquitination, we determined the contributions of known factors in the yeast cytosol and in ER-derived microsomes during the ERAD of Hmg2p. I first determined that Hrd1p was active in this assay and its presence and activity were essential for Hmg2p ubiquitination. The presence of the E2 Ubc7p and its membrane-bound binding partner Cue1p were critical as well. I then tested if Hmg2p's key lysines, shown to be important for in vivo degradation, were required for in vitro ubiquitination. My results revealed a strong dependence on lysine-6 of Hmg2p. I have made some progress in the observation of Hmg2p regulated ubiquitination, however this aspect has been challenging, exposing the need for other approaches or more sensitive detection methods. Interestingly, the chemical chaperone glycerol decreased in vitro Hmg2p ubiquitination, consistent with its ability to slow Hmg2p in vivo degradation. In vivo, Hrd1p has the capacity to distinguish the difference between a less folded and more folded Hmg2p, and it appears that this discrimination is occurring in vitro.

Results

In vitro ubiquitination assay design

Previous work from the Hampton laboratory has shown that Hmg2p ubiquitination depends on factors in the ER membrane and in the cytosol. I
took advantage of this natural division to control the in vitro ubiquitination assay. The assay involves mixing microsomes and cytosol from separate strains to bring together all proteins required for Hmg2p ubiquitination. The ER-anchored substrate Hmg2p was expressed in a microsome donor strain in the absence of the E2 Ubc7p, whereas a donor strain expressing Ubc7p but no Hmg2p served as a source of cytosol. The ER-enriched microsomes also contained the membrane bound factors, the E3 Hrd1p and the Ubc7p-binding protein Cue1p, and in addition to Ubc7p, the yeast cytosol contained native E1 and ubiquitin. The necessity of specific factors could be easily tested by genetic modification of either the cytosol or microsome donor strains. The use of simply prepared microsomes and cytosol allows for participation of any ERAD proteins not yet identified by our previous studies.

To improve signal in vitro, I used a microsome strain that overexpressed Hrd1p. Previous Hampton studies have shown that Hrd1p expressed from the strong \( \text{TDH3} \) promoter is rate-limiting for Hmg2p ubiquitination, and operates independently of several other ERAD factors. Importantly, even in the presence of high levels of Hrd1p, Hmg2p degradation can be regulated to a certain degree, however it is biased toward degradation (Bays et al., 2001a).

We used the ERAD substrate Hmg2p-GFP in the in vitro ubiquitination assay. The soluble, catalytic domain of Hmg2p was replaced with the GFP reporter for facile detection and optical quantification (Hampton et al., 1996b). In vivo Hmg2p-GFP ubiquitination and degradation is regulated in the same
manner as full-length Hmg2p and is dependent on the E3 Hrd1p and the E2 Ubc7p (Hampton and Bhakta, 1997; Bays et al., 2001a; Cronin and Hampton, 1999; Gardner and Hampton, 1999a), not surprisingly since the ER transmembrane domain of Hmg2p is sufficient for regulated degradation (Hampton and Rine, 1996; Gardner et al., 1998; Gardner and Hampton, 1999).

Hmg2p ubiquitination did not proceed in the microsome strain due to the presence of the \textit{ubc7}\textsuperscript{Δ} null mutant. The HRD pathway reaction thus would start only when the E2 Ubc7p was delivered with other soluble factors upon cytosol addition. In this way the reaction is initiated upon addition of the Ubc7p-containing cytosol. Expressed at native levels, Ubc7p is stably bound to the ER membrane protein Cue1p, but when overexpressed, unbound Ubc7p localizes to the cytosol (Biederer et al., 1997).

We used a cytosol preparation modified from the Schekman laboratory. The cytosol donor cells were lysed under liquid nitrogen with a mortar and pestle. The cytosol fraction was then separated from the membrane fraction by ultracentrifugation (Spang and Schekman, 1998). ER microsomes were prepared from the microsome donor strain by membrane fractionation. Cells resuspended in a hypo-osmotic buffer were lysed by bead-beating and membranes were collected by centrifugation. This protocol produces greater than 95% of the microsomes in the correct orientation (Cronin et al., 2002). To start the reaction the cytosol and the microsomes are mixed in the presence of added ATP, and incubated for typically one hour at 30°C. Hmg2p-GFP was then immunoprecipitated with polyclonal anti-GFP antiserum, and detected
with either anti-GFP or anti-ubiquitin. This method for Hmg2p in vitro ubiquitination was successful, yielding a distribution of ubiquitin-reactive bands from immunoprecipitated Hmg2p-GFP (Figure 2-1).

To confirm the biological relevance of in vitro Hmg2p-GFP ubiquitination, we tested the known ERAD factors required for in vivo Hmg2p-GFP ubiquitination in the in vitro assay. This test entailed generating microsome and cytosol donor strains deleted of the genes that encode Hrd1p, Ubc7p, and Cue1p. Hmg2p-GFP was not ubiquitinated when any one of these proteins was missing from the reactions. Hmg2p-GFP ubiquitination was dependent on the Hrd1p RING-H2 domain, as the C399S mutation in the RING-H2 domain greatly decreased Hmg2p-GFP ubiquitination (Bays et al., 2001a). The cytosol-introduced Ubc7p was active and presumably able to interact with its membrane binding protein Cue1p, supporting Hmg2p-GFP ubiquitination. In the absence of Cue1p, Hmg2p-GFP was not ubiquitinated. Thus, the in vitro Hmg2p-GFP ubiquitination assay adhered to all relevant criteria tested also important for in vivo degradation (Figures 2-1 and 2-2).

The degree to which Hmg2p-GFP in vitro ubiquitination was specific was next tested. In vivo, Hmg2p degradation is dependent on two key lysines at positions 6 and 357. When lysine-6 or lysine-357 are mutated to arginine, Hmg2p is no longer ubiquitinated or degraded, even in response to high degradation signal (Gardner and Hampton, 1999b). We made microsome donor strains expressing the K6R, K357R, and double mutant Hmg2p-GFP variants to test whether these two lysines are required for Hmg2p-GFP in vitro
Figure 2-1: In vitro ubiquitination of Hmg2p-GFP requires Ubc7p, Hrd1p, and Hrd1p's intact RING domain. Following one hour reactions consisting of WT, hrd1Δ, or RING mutant microsomes with ubc7Δ or UBC7 cytosol, the reaction was solubilized and Hmg2p-GFP was immunoprecipitated with anti-GFP antiserum. Immunoprecipitated Hmg2p-GFP was detected by western blot with anti-ubiquitin for detection of ubiquitinated Hmg2p-GFP and anti-GFP for levels of Hmg2p-GFP in the sample.
Figure 2-2: Cue1p is required for in vitro Hmg2-GFP ubiquitination. In vitro ubiquitination reactions consisted of CUE1 or cue1Δ microsomes incubated with ubc7Δ or UBC7 cytosol. Hmg2p-GFP was immunoprecipitated with anti-GFP antiserum and detected by immunoblot with anti-ubiquitin and anti-GFP.
ubiquitination. K6R-Hmg2p-GFP ubiquitination was much lower than wild-type Hmg2p-GFP, whereas K357R-Hmg2p-GFP ubiquitination rivaled that of wild-type Hmg2p-GFP. The double mutant was not ubiquitinated, similar to K6R-Hmg2-GFP (Figure 2-3).

This was not the expected result, since in vivo, the K357R mutation would be expected to prevent Hmg2p-GFP ubiquitination (Gardner and Hampton, 1999b). However, these in vivo studies were done with Hrd1p at its native levels, whereas the in vitro assay employs elevated levels of Hrd1p. Thus, I hypothesized that high Hrd1p levels destabilized K357R-Hmg2p-GFP in vivo as well. To test this, the wildtype and variant microsome donor strains were modified to express \textit{UBC7}. Both the K6R-Hmg2p-GFP and double mutant were stable in the presence and absence of Ubc7p, whereas steady state levels of wildtype and K357R-Hmg2p-GFP were low, only in the presence of Ubc7p (data not shown). Thus, the in vitro assay accurately predicted a hitherto unknown feature of the in vivo pathway: that at high levels of Hrd1p, degradation of K357R-Hmg2p-GFP proceeded.

\textbf{ATP dependence}

The ubiquitin system is dependent on ATP. Specifically the E1, or ubiquitin-activating enzyme, hydrolyzes ATP to activate the C-terminus of ubiquitin with AMP. The activated ubiquitin is then transferred to the E2, or ubiquitin-conjugating enzyme. I tested if exogenous ATP was required for Hmg2-GFP ubiquitination and, if so, the optimal concentration. No addition of
Figure 2-3: Lysine-6 was required for in vitro Hmg2-GFP ubiquitination. Microsomes were prepared from strains expressing WT and Hmg2p-GFP variants, K6R-Hmg2p-GFP, K357R-Hmg2p-GFP, and K6R,K357R-Hmg2p-GFP. Reactions contained indicated microsomes with *ubc7Δ* (-) or *UBC7* (+) cytosol. Hmg2p-GFP was processed as described in previous figures.
ATP resulted in undetectable levels of in vitro Hmg2p-GFP ubiquitination and the greatest level of ubiquitination was typically supported by 30 mM ATP (Figure 2-4).

Ubiquitin activation requires hydrolysis of the α-β bond of ATP to yield AMP and PPi. To test if the α-β hydrolysis is sufficient for in vitro Hmg2p ubiquitination the ATP analogue, AMP-PNP, can be utilized. AMP-PNP is a β-γ-nonhydrolyzable analogue of ATP that can support ubiquitin activation by the E1 (Johnston and Cohen, 1991). AMP-PNP was added to the reactions at the same concentration as ATP, 30 mM. In vitro Hmg2p-GFP ubiquitination was significantly weaker with AMP-PNP than with ATP (data not shown). This result was surprising because in vitro ubiquitination of mammalian HMGR is similar between reactions containing either ATP or AMP-PNP (Song et al., 2005).

I next tested if AMP-PNP affected in vitro ubiquitination in general or Hmg2p specifically. To address this, general in vitro ubiquitination was detected from reactions. The microsomes and cytosol were incubated in the presence of varying concentrations of AMP-PNP or ATP. Instead of immunoprecipitating and detecting ubiquitinated Hmg2p-GFP, the appearance of all ubiquitinated proteins, after initiating the reaction, was detected. At most concentrations tested, AMP-PNP was not as effective as ATP for ubiquitin conjugate formation, but at 15 and 30 mM AMP-PNP, the levels of ubiquitin conjugates were similar to 10 mM ATP (Figure 2-5 A). In vitro Hmg2p-GFP ubiquitination was re-examined using the concentrations of ATP that were
Figure 2-4: ATP was required for in vitro Hmg2p-GFP ubiquitination. Various concentrations of ATP, as indicated, were added to reactions containing WT microsomes and UBC7 cytosol. Reactions were solubilized and Hmg2p-GFP was immunoprecipitated with anti-GFP antiserum. Hmg2p-GFP was detected by immunoblot with anti-ubiquitin and anti-GFP.
Figure 2-5: In vitro Hmg2p-GFP ubiquitination requires the hydrolyzable $\beta-\gamma$ bond of ATP. A. Various concentrations of ATP and AMP-PNP were added to in vitro ubiquitination reactions, as indicated. One hour reactions were solubilized and detected by immunoblot with anti-ubiquitin, to detect whole reaction ubiquitination. B. In vitro Hmg2p-GFP ubiquitination reactions were carried out with indicated concentrations of AMP-PNP that supported the highest level of whole reaction ubiquitination and ATP concentrations that supported whole reaction ubiquitination levels similar in extent. Hmg2p-GFP was immunoprecipitated and detected as described in previous figures.
found to equal the greatest level of general in vitro ubiquitination supported by AMP-PNP. Reactions with "optimal" concentrations of AMP-PNP were unable to promote Hmg2p-GFP ubiquitination to the extent of every concentration of ATP tested (Figure 2-5 B). Using a better way to directly compare ATP and AMP-PNP, results indicated that the $\alpha$-$\beta$ bond of nucleotide hydrolysis is clearly not sufficient for in vitro Hmg2p-GFP ubiquitination. Instead, these results imply that an action of a protein requiring $\beta$-$\gamma$ ATP hydrolysis is involved in Hmg2p-GFP ubiquitination. The prime candidate is Cdc48p, which binds Hrd1p in a RING-dependent manner (Gauss et al., 2006b). In fact the human homologue of Cdc48p, p97, is known to bind MHC Class I molecules prior to their ubiquitination when induced by US11, a human cytomegalovirus protein that promotes its degradation (Ye et al., 2003).

Furthermore, lower levels of in vitro Hmg2p-GFP ubiquitination are detected from reactions composed of mutant $\text{cdc48-2}$ cytosol and microsomes (Chapter 3). Perhaps, Cdc48p is performing a similar task in Hmg2p degradation as p97.

Regulation of Hmg2p ubiquitination in vitro

Hmg2p does not contain one specific sequence, or degron, targeting it for degradation, but important structural information embedded throughout its transmembrane domain (Gardner and Hampton, 1999a). The working model is that Hmg2p undergoes a structural transition in the presence of degradation signal, giving Hmg2p characteristics of a quality control substrate recognized by the HRD pathway.
Figure 2-6: In vitro ubiquitination of unregulated variants of Hmg2p-GFP. A. 6myc-Hmg2p-GFP in vitro ubiquitination is Hrd1p dependent and Ubc7p dependent. B. NR1-Hmg2p-GFP in vitro ubiquitination is Ubc7p dependent. WT Hmg2p-GFP and variants were immunoprecipitated with anti-GFP antiserum and detected by immunoblotting with anti-ubiquitin and anti-GFP.
Incorporation of Hmg2p-GFP variants into the in vitro assay was necessary for evaluating if Hmg2p-GFP ubiquitination can be regulated in vitro. As a control, a Hrd1p substrate was included that was not influenced by levels of the degradation signal, 6myc-Hmg2p-GFP. The six tandem myc epitope tags disrupt folding of the Hmg2p-GFP variant, removing regulation but allowing HRD-dependent degradation (Hampton et al., 1996a). I tested if in vitro ubiquitination of 6myc-Hmg2p-GFP was dependent on Hrd1p and Ubc7p. This in vitro assay met these qualifications for in vitro 6myc-Hmg2p-GFP, yielding a useful system for asking questions about Hmg2p-GFP regulated degradation (Figure 2-6A).

In addition, NR1-Hmg2p-GFP was also used as an unregulated Hmg2p variant. The NR1-Hmg2p-GFP variant is only altered in five residues, at the end of the sixth transmembrane span of Hmg2p (Gardner et al., 1998; Shearer and Hampton, 2005). This mutant is, like 6-myc-Hmg2p-GFP, constitutively degraded by the HRD pathway but it is unaffected by altering degradation signals. NR1-Hmg2p-GFP reactions were carried out in the presence and absence of Ubc7p. NR1-Hmg2p-GFP ubiquitination was mostly dependent on Ubc7p. However, even in the absence of Ubc7p, NR1-Hmg2p-GFP was ubiquitinated to a small extent. Whether this ubiquitination is attributed to in vivo pre-ubiquitination or native Ubc1p acting in the reaction remains to be determined (Figure 2-6 B). With the addition of two unregulated Hmg2p-GFP variants for in vitro study, regulation of in vitro Hmg2p-GFP ubiquitination
could be explored. Relevant perturbations to the signaling pathway in vitro should only affect regulated Hmg2p-GFP and not these unregulated variants.

Lovastatin treatment of intact cells lowers the FPP degradation signal, stabilizing Hmg2p or Hmg2p-GFP (Hampton and Rine, 1994). Thus, in vitro Hmg2p-GFP ubiquitination levels were evaluated when the degradation signal was decreased by the pre-treatment of the microsome donor strain with lovastatin. The microsome donor strain was treated first because the degradation signal is hydrophobic and would likely partition into the membrane fraction. The microsome donor strain was incubated with lovastatin for increasing time, after which microsomes were prepared, and used in the in vitro assay with cytosol not treated with lovastatin. With longer lovastatin pre-incubation, less Hmg2-GFP was ubiquitinated in vitro (Figure 2-7). In direct comparison with the control variants, wild-type Hmg2p-GFP was ubiquitinated to a lesser extent with lovastatin pre-treatment whereas NR1-Hmg2p-GFP and 6myc-Hmg2p-GFP were ubiquitinated to the same extent with or without lovastatin pre-treatment (Figure 2-8). In vivo, similar lovastatin treatment abolishes Hmg2p-GFP ubiquitination and degradation, whereas in vitro pre-treatment only diminished Hmg2p-GFP ubiquitination, albeit with the appropriate specificity.

Multiple attempts have been made to stimulate in vitro Hmg2p-GFP ubiquitination with the putative degradation signaling molecules, FPP or farnesol. However, high levels of Hrd1p in the reactions may have prevented detection of small increases of Hmg2p-GFP ubiquitination when FPP and
Figure 2-7: In vitro Hmg2p-GFP ubiquitination levels decrease with lovastatin pre-treatment of microsome donor strain. Microsomes were prepared from microsome donor strains pre-treated with 25 μg/mL lovastatin for indicated times. In vitro Hmg2p-GFP ubiquitination reactions were carried out with cytosol prepared from untreated donor strains.
farnesol were directly added to the reactions (data not shown). By decreasing the degradation signal endogenously, by lovastatin pre-treatment, we reasoned that addition of FPP or farnesol to the reactions would stimulate ubiquitination. No detectable increase of Hmg2p-GFP ubiquitination was observed when either FPP or farnesol was added to reactions in which microsomes were prepared from lovastatin-treated donor strains (data not shown). The mechanism required for degradation signal presentation may be missing in the in vitro ubiquitination assay or it may be that FPP and farnesol are not Hmg2p degradation signals.

I next directly tested if cytosol contained degradation signal. Unfortunately, the use of lovastatin in cultures grown in YPD is not optimal, because of non-specific sequestration of lovastatin, which decreases the drug's effectiveness. YPD is a rich medium made from yeast extract, peptone, and dextrose. Pre-treating the cytosol donor strain with ten-fold higher concentrations of lovastatin gave variable results, sometimes preventing Hmg2p-GFP ubiquitination and other times having no effect. When both microsome and cytosol donor strains were treated with lovastatin, we detected less Hmg2p-GFP ubiquitination (Figure 2-9). Although these results were promising they have been difficult to reproduce. For this reason, the use of synthetic complete (SC) medium in growing the cytosol donor strain was explored. Lovastatin was predictably effective in cultures grown in SC medium. Steady state levels of Hmg2p-GFP, measured by flow cytometry, increased when cells grown in SC medium were treated with lovastatin (data
Figure 2-8: In vitro ubiquitination of lovastatin-pretreated microsome donor strains expressing Hmg2p-GFP, NR1-Hmg2-GFP, or 6myc-Hmg2p-GFP. Microsome donor strains were either untreated (-) or treated with 25 μg/mL lovastatin (+) for 12 hours. Cytosol donor strains ubc7Δ (Δ) and UBC7 were not treated with lovastatin. Following the in vitro ubiquitination reactions, Hmg2p-GFP, unregulated variants NR1-Hmg2p-GFP and 6myc-Hmg2p-GFP were immunoprecipitated with anti-GFP antiserum and detected by immunoblot with anti-ubiquitin and anti-GFP.
not shown). Cytosol was then prepared from donor strains grown in SC medium to ascertain if, when added to reactions, would provide all of the necessary factors and active Ubc7p necessary to support Hmg2p-GFP in vitro ubiquitination. The cytosol was able to support in vitro Hmg2p-GFP ubiquitination (Figure 2-10). Next tested in the in vitro assay was cytosol prepared from the cytosol donor strain pre-treated with lovastatin to determine if in vitro Hmg2p-GFP ubiquitination is affected by cytosolic signal levels. The cytosol donor strain was grown in SC media in the presence or absence of lovastatin. Cytosol was prepared from each culture and incubated with microsomes not treated with lovastatin. The level of in vitro Hmg2p-GFP ubiquitination in reactions with cytosol prepared from pre-treated cells was much lower than the control reactions. However, in vitro ubiquitination of either unregulated variant, NR1-Hmg2p-GFP or 6myc-Hmg2p-GFP, was also reduced in reactions containing cytosol prepared from lovastatin-treated cultures but not to the extent of wild-type Hmg2p-GFP (Figure 2-10). Lovastatin pre-treatment of the cytosol donor strain may have been too long, possibly affecting ubiquitination in general. Growing cytosol donor strains in SC media instead of YPD appears to be a feasible way to manipulate signal levels in the cytosol but needs further exploration.

In contrast to using high levels of Hrd1p, in vitro Hmg2p-GFP ubiquitination by native levels of Hrd1p has been more challenging. However, to further understand Hmg2p regulation, I began characterizing in vitro Hmg2p-GFP ubiquitination in microsome donor strains with native levels of
Figure 2-9: Lovastatin pre-treatment of both microsome and cytosol donor strains decreases in vitro Hmg2p-GFP ubiquitination. Microsomes and cytosol were prepared from donor strains not treated or pre-treated with lovastatin. In vitro ubiquitination reactions were carried out with combinations of no drug and lovastatin microsomes and cytosol. M denotes the Hrd1p RING mutant.
Figure 2-10: In vitro ubiquitination of Hmg2p-GFP and its unregulated variants using cytosol prepared from strains grown in synthetic complete media with or without lovastatin. Cytosol was prepared from UBC7 donor strains grown in SC media in the absence or presence of lovastatin. ubc7Δ (∆) cytosol donor strain was not treated with lovastatin. Microsomes were prepared from donor strains expressing WT Hmg2p-GFP, NR1-Hmg2p-GFP, or 6myc-Hmg2p-GFP.
Hrd1p. The levels of in vitro ubiquitination of Hmg2p-GFP from strains either expressing Hrd1p from the \textit{TDH3} promoter or Hrd1p's native promoter, in an otherwise isogenic background, were directly compared. There was a stark difference in the levels of ubiquitinated Hmg2p-GFP between the two strains, with native levels of Hrd1p supporting little Hmg2p-GFP ubiquitination whereas overexpressed Hrd1p driven Hmg2p-GFP ubiquitination was strong (Figure 2-11). The low level of Hmg2p-GFP ubiquitination may facilitate detection of even minor increases in the level of in vitro Hmg2p-GFP ubiquitination induced by putative degradation molecules.

An intriguing observation emerged when this experiment was used to compare Hmg2p-GFP to its unregulated variant NR1-Hmg2p-GFP. A microsome donor strain expressing NR1-Hmg2p-GFP and native levels of Hrd1p was made and used in the in vitro assay. In contrast to the regulated wild-type construct, the constitutively degraded NR1-Hmg2p-GFP was strongly ubiquitinated in native Hrd1p microsomes (Figure 2-11). These results showed that native levels of Hrd1p support ubiquitination of the constitutively degraded Hmg2p-GFP variant in vitro. Furthermore, the resistance of the Hmg2p-GFP to ubiquitination may be due to a paucity of signals that are not required in the unregulated NR1-Hmg2p-GFP reaction. Thus, incubation of wild-type Hmg2p-GFP with putative degradation molecules introduced directly or by pharmacological and genetic manipulation, may allow its recognition as a misfolded protein, and thus increase its in vitro ubiquitination. If the cytosol strain grown in SC medium supports in vitro ubiquitination in reactions with
Figure 2-11: Native Hrd1p levels support in vitro ubiquitination of unregulated variant NR1-Hmg2p-GFP and to a lesser extent WT Hmg2p-GFP. WT Hmg2p-GFP and NR1-Hmg2p-GFP were expressed in strains with either PTDH3-driven or PHRD1-driven Hrd1p. In vitro ubiquitination reactions and detection were carried out as previously described.
microsome strains expressing Hrd1p at lower levels, pharmacological manipulation of signal levels in the cytosol donor strain will be tested as well.

Previous work from the Hampton laboratory has shown that Hmg2p behaves like a misfolded protein when it is in conditions of rapid degradation. For instance, chemical chaperones such as glycerol will stabilize Hmg2p in vivo, and will improve the folding of Hmg2p in vitro (Gardner et al., 2001b; Shearer and Hampton, 2004). The model is that a fraction of Hmg2p appears misfolded, and these molecules are susceptible to Hrd1p dependent degradation. High degradation signal increases this fraction, whereas chemical chaperones lower it. If chemical chaperones similarly stabilize Hmg2p-GFP in the in vitro ubiquitination assay, this would indicate that the Hrd1p ligase is sensitive to the folding state of Hmg2p-GFP. Indeed, Hmg2p-GFP ubiquitination levels dropped with increasing glycerol concentration, suggesting glycerol promoted a more structured Hmg2-GFP. The misfolded variant 6myc-Hmg2p-GFP is not responsive to the stabilizing effect of glycerol in vivo or in vitro (Shearer and Hampton, 2004). In contrast to wild-type Hmg2p-GFP, 6myc-Hmg2p-GFP ubiquitination was not affected by glycerol addition (Figure 2-12). In a separate experiment, the effect of glycerol on Hrd1p in vitro ubiquitination was tested. Within the same in vitro ubiquitination reactions both Hrd1p and Hmg2p-GFP can be assayed. Hrd1p ubiquitination levels were not affected by glycerol treatment whereas Hmg2p-GFP ubiquitination levels were lower with increasing glycerol concentration (Figure 2-13). This shows that the effect of glycerol was not due to wholesale
Figure 2-12: Chemical chaperone glycerol decreases in vitro ubiquitination of Hmg2p-GFP with no effect on 6myc-Hmg2p-GFP. Microsomes were prepared from strains expressing Hmg2p-GFP or 6myc-Hmg2p-GFP and cytosol was prepared from a *UBC7* strain. Glycerol was added to reactions to indicated final concentrations.
Figure 2-13: Chemical chaperone glycerol decreases in vitro ubiquitination of Hmg2p-GFP with little effect on ubiquitin ligase Hrd1p. Double reactions were carried out with the WT microsome donor strain that expresses TDH3-driven Hrd1p and Hmg2p-GFP and UBC7 cytosol. Glycerol was added to reactions to indicated final concentrations. Half of the reaction was immunoprecipitated with anti-GFP antiserum and other half was immuno-precipitated with anti-Hrd1p antiserum. Hmg2p-GFP was detected by immunoblot with anti-ubiquitin and anti-GFP and Hrd1p was detected by immunoblot with anti-ubiquitin and anti-HA (Hrd1p is tagged with 3 HA-epitopes).
inhibition of the Hrd1p ligase, but rather specific inhibition of Hrd1p-mediated transfer to the glycerol-stabilized substrate.

Even though Hrd1p was present at high levels, the in vitro ubiquitination assay provided an environment in which Hrd1p could distinguish a less structured Hmg2p from a more structured Hmg2p.

**Discussion**

I have developed an in vitro ubiquitination assay for studying HRD-dependent Hmg2p-GFP ubiquitination. The assay met numerous biological criteria for in vivo relevance. Hrd1p and its RING-H2 domain were required for Hmg2p-GFP ubiquitination as were the E2 Ubc7p and its binding partner Cue1p. The key lysine-6 of Hmg2p-GFP was necessary for in vitro ubiquitination as it is in vivo. Pre-treatment of the microsome and cytosol donor strains with the signal-lowering lovastatin decreased the level of Hmg2p-GFP in vitro ubiquitination. Also observed was less Hmg2p-GFP in vitro ubiquitination with the structure stabilizing chemical chaperone glycerol.

Induction of in vitro Hmg2p-GFP ubiquitination in response to putative degradation signals has been difficult to observe. In overexpressed Hrd1p microsomes, we did not see any increase in Hmg2p-GFP ubiquitination when reactions included either FPP or farnesol. Data suggesting that FPP and farnesol were not Hmg2p degradation signals recently led to the discovery that geranylgeranyl pyrophosphate (GGPP) induces in vivo Hmg2p ubiquitination and degradation (See Chapter 4). Future applications of the assay should be
carried out with native Hrd1p level microsome donor strains. High levels of Hrd1p make detection of small changes in ubiquitination levels difficult to see. In contrast, in vitro Hmg2p-GFP ubiquitination by native levels of Hrd1p are barely detectable. Of particular interest is if Hmg2p ubiquitination is higher in reactions in which GGPP was added. However, there is the possibility that Hmg2p in vitro ubiquitination is similar to that of mammalian HMGR. In mammalian HMGR degradation, a non-sterol is required for HMGR degradation as well, and identified to be geranylgeraniol (GGOH). Addition of GGOH to in vitro mammalian HMGR ubiquitination reactions does not enhance HMGR ubiquitination (Sever et al., 2003a). The DeBose-Boyd laboratory favors the idea that the non-sterol molecule is involved in the removal of HMGR from the membrane (retrotranslocation) because sterols alone can induce HMGR ubiquitination but in the absence of GGOH or upstream molecule mevalonate, HMGR is not degraded (Sever et al., 2003a; Goldstein and Brown, 1990). Hmg2p in vitro ubiquitination and retrotranslocation will be evaluated in the presence of GGPP.

In vitro ubiquitination experiments with mammalian HMGR are similar to Hmg2p in vitro reactions in that only a small fraction of HMGR is ubiquitinated in vitro (Song et al., 2005; Song and DeBose-Boyd, 2004; Sever et al., 2003a). Recent work by another graduate student from the Hampton laboratory suggests a model, based on the cell biology of the ER, that would explain this low throughput, and perhaps the lack of regulation seen in vitro. In vivo, structures made up of Hmg2p-GFP are formed when there are high levels of
Hmg2p-GFP (Parrish et al., 1995). Unpublished work has shown that Hrd1p does not immunolocalize to these Hmg2p-GFP rich structures. In contrast, an unregulated variant of Hmg2p-GFP does co-localize with Hrd1p (Federovitch and Hampton, manuscript submitted). It may be that the regulatory signal, through causing misfolding, allows Hmg2p-GFP access to Hrd1p in a regulated manner. This would explain why only a small amount of Hmg2p-GFP is ubiquitinated, since microsome preparations may disrupt further transfer to the Hrd1p site. This model would also explain the apparent lack of regulation seen in vitro. Disrupting the structure of the ER would similarly prohibit enhanced access of Hmg2p-GFP to Hrd1p caused by signal added in vitro. Future work will entail testing if an intact cell is required for regulated in vitro Hmg2 ubiquitination.

Hmg2p-GFP in vitro ubiquitination has been the focus of this stage of research but application of this assay to learn more about ERAD in general is intended. The in vitro assay will be applied to study more in-depth how factors identified in genetic screens and in vivo co-immunoprecipitation experiments function in the ubiquitination of Hmg2p and other substrates. Also, integrating recombinant proteins and their mutant versions to the assay should provide more mechanistic insight into ERAD. The development of this in vitro assay was the first phase in the pursuit of understanding the complex events of ERAD.
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Chapter 3:

HRD-mediated retrotranslocation of Hmg2p
**Introduction**

The ER-associated degradation (ERAD) pathway mediates the destruction of numerous integral membrane or lumenal ER-localized proteins. ERAD functions mainly in the disposal of misfolded or unassembled proteins, but also participates in the physiological regulation of some normal residents of the organelle. This ER-localized degradation pathway has been implicated in a wide variety of normal and pathophysiological processes, including sterol synthesis (Hampton, 2002; Song et al., 2005), rheumatoid arthritis (Amano et al., 2003), fungal differentiation (Swanson et al., 2001), cystic fibrosis (Cheng et al., 1990; Kopito et al., 1999), and several neurodegenerative diseases (Berke and Paulson, 2003). Accordingly, there is great impetus to understand the molecular mechanisms that mediate this broadly important route of protein degradation.

ERAD proceeds by the ubiquitin-proteasome pathway, by which an ER-localized substrate is covalently modified by addition of multiple copies of 7.6 kD ubiquitin to form a multiubiquitin chain that is recognized by the cytosolic 26S proteasome (Thrower et al., 2000; Hershko and Ciechanover, 1998). Ubiquitin is added to the substrate by the successive action of three enzymes. The E1 ubiquitin activating enzyme uses ATP to covalently add ubiquitin to an E2 ubiquitin conjugating (UBC) enzyme. Ubiquitin is then transferred from the charged E2 to the substrate or the growing ubiquitin chain by the action of an E3 ubiquitin ligase, resulting in a substrate-attached multiubiquitin chain that is
recognized by the proteasome, leading to degradation of the ubiquitinated substrate. This is a skeletal picture; in most cases ancillary factors participate in substrate recognition and transfer of the ubiquitinated substrate to the proteasome (Bays et al., 2001b; Medicherla et al., 2004; Ye et al., 2001; Meyer et al., 2000; Jarosch et al., 2002).

The ERAD pathway presents the cell with a topological conundrum. Substrates are either sequestered in the lumen or embedded in the ER membrane with lumenal portions, yet the proteasome, E2s and E3 active sites are all cytosolic. Thus, it was realized early on that a critical step in the ERAD pathway involves transfer of the ERAD substrate to the cytosol for proteasomal degradation by a process referred to as retrotranslocation or dislocation. Retrotranslocation is energy-requiring, and the hexameric AAA-ATPase called Cdc48p in yeast and p97 in mammals has been proposed to drive transfer of ubiquitinated ERAD substrates across the ER membrane. By analogy with the delivery of proteins to the ER membrane and lumen, it is thought that a protein channel mediates the passing of ER substrates across the membrane. Several candidate channels have been proposed, with the most prominent being the derlins (Ye et al., 2004). The derlins are found in ERAD E3 ligase complexes (Lilley et al., 2005; Ye et al., 2005), and are required in a reconstituted system to be required for the movement of a model soluble ERAD substrate across the mammalian ER membrane (Wahlman et al., 2007). Other candidates include the Sec61p channel used in anterograde
transfer of proteins, and the multi-spanning domains of the ER ligases themselves (Plemper et al., 1997; Weirtz et al., 1996).

The yeast HRD pathway mediates ERAD of numerous misfolded ER proteins and the physiologically regulated degradation of normal HMG-CoA reductase (HMGR), a key enzyme in sterol synthesis (Hampton, 2002). Specifically, the yeast HMGR isozyme Hmg2p-GFP undergoes HRD pathway-mediated degradation that is regulated in response to signals from the sterol pathway (Gardner et al., 2001b). The integral membrane ER ligase Hrd1p, in conjunction with Hrd3p, mediates the recognition and ubiquitination of Hmg2p-GFP, which is an 8-spanning integral membrane ER protein. Ubiquitinated Hmg2p-GFP requires the Cdc48p/Ufd1p/Npl4p complex for efficient delivery to the proteasome, presumably by promoting retrotranslocation of ER-embedded Hmg2p-GFP, although this process has not been directly observed. Subsequent recognition of ubiquitinated Hmg2p-GFP and delivery to the proteasome has been proposed to be mediated by ubiquitin-binding adaptors Dsk2p and Rad23p. This model is the result of numerous laboratories using both in vivo degradation and assays of interaction to posit the role and order of action of these factors. The reconstitution of this process will allow detailed testing of the model, and mechanistic analysis of the known and novel steps that lead an ERAD substrate to destruction.

We have been studying the HRD-dependent degradation of Hmg2p-GFP in order to understand both regulation of the sterol pathway and the molecular mechanisms of ERAD. As part of this effort we have reconstituted
Hrd1p-mediated ubiquitination and retrotranslocation of Hmg2p-GFP in vitro (Flury et al., 2005; Bazirgan et al., 2006; Chapter 2). We demonstrated that the entire 8-spanning Hmg2p-GFP protein is removed from the membrane by this process, remaining intact yet soluble after retrotranslocation. Using this assay we have discerned a core set of proteins that can mediate the recognition and removal of Hmg2p-GFP from the ER, and addressed a number of questions pertinent to current models of ERAD.

Results

Hrd1p-mediated ubiquitination of Hmg2p-GFP involves both membrane-bound and soluble proteins. The E2 Ubc7p is soluble, as are ubiquitin, the proteasome, the Cdc48p complex and other coupling factors such as Rad23p. Conversely, the E3 ligase, the substrate, and Cue1p, the required anchor for Ubc7p, are integral membrane proteins. Our assay uses two distinct strains as sources of ER membranes and cytosol that are mixed to initiate the in vitro reaction (Chapter 2). The microsome strain expresses epitope-tagged ligase 3HA-Hrd1p, substrate Hmg2p-GFP, Cue1p, and any other membrane-bound proteins required for the process. The microsome strain harbors a null mutation in **UBC7**, which encodes the principal E2 for Hrd1p-mediated ERAD. The **ubc7Δ** null prevents nearly all ERAD of Hmg2p-GFP while the microsome strain is intact (Bays et al., 2001a). The cytosol strain is devoid of Hmg2p-GFP, and overexpresses Ubc7p from a strong
The TDH3 promoter to provide a pool of soluble E2 in the cytosol fraction that is available for the ERAD reaction when mixed with the microsomes.

The reaction is started by addition of Ubc7p-containing cytosol to separately prepared microsomes, followed by incubation at 30°C. Ubiquitin transfer is measured by solubilization of the entire reaction mix, immunoprecipitation of Hmg2p-GFP (or any other substrate being tested), and SDS-PAGE immunoblotting of the precipitates for Hmg2p-GFP itself or for ubiquitin to detect the conjugates. The reaction as used in this work follows a number of biological criteria of specificity, including strict dependence on Hrd1p, the lysine-6 of Hmg2p-GFP, dependence on Ubc7p and Cue1p. A typical ubiquitination reaction is shown in Chapter 2, demonstrating the dependence on Hrd1p.

In the assay, HRD1 is expressed from the TDH3 promoter, producing levels sufficient to drive Hmg2p-GFP ubiquitination and retrotranslocation, independent of a number of ERAD factors including Hrd3p (see below), Usa1p (Carroll and Hampton, manuscript submitted), and Yos9p (Carroll and Hampton, unpublished observation). The assay thus defines the minimal components sufficient for successful retrotranslocation, providing the best avenue for complete reconstitution. In addition, Hrd1p at this level of expression drives Hmg2p-GFP ubiquitination independently of the level of sterol pathway signals required for Hmg2p-GFP degradation at lower levels of the ligase, thus obviating the need to preserve or supply the signal after lysis. Finally, this level of Hrd1p allows for the direct recovery and detection of the
retrotranslocated substrate (see below), eventually allowing study of the
dislocated Hmg2p-GFP species and analysis the partner molecules needed
for solubilization of a multi-spanning membrane protein (see below).

To directly evaluate Hmg2p-GFP retrotranslocation, we fractionated the
in vitro reaction mix to assess the amount of ubiquitinated Hmg2p-GFP
present in the soluble phase. The reaction mix was divided into two equal
volumes. One was processed without fractionation to evaluate total
ubiquitination and the amount of Hmg2p-GFP present. The remaining half
was centrifuged at 25,000 xg. The supernatant was removed and the resulting
membrane pellet was resuspended in reaction buffer to the same volume as
the removed supernatant. All three equal volume samples were then analyzed
for ubiquitinated Hmg2p-GFP. The results of this retrotranslocation assay are
shown in Figure 3-1, as three immunoblotting lanes labeled T (total reaction),
S (supernatant) and P (pellet). Each lane shows both the ubiquitin
immunoblot (for ubiquitinated Hmg2p-GFP) and the anti-GFP blot for
unmodified Hmg2p-GFP. As can be seen, a significant portion of the
ubiquitinated Hmg2p-GFP is in the supernatant, consistent with
retrotranslocation. The extent of the fractionation is indicated by the lack of
native mobility Hmg2p-GFP in the S lane. Furthermore, essentially the same
results are obtained with 100,000 xg fractionation (Figure 3-1).

Immunoprecipitation of the supernatant with pre-immune serum (see below)
resulted in no anti-ubiquitin immunoreactivity, indicating that the ubiquitin
Figure 3-1: In vitro ubiquitination assay conditions support retrotranslocation of ubiquitinated Hmg2p-GFP into supernatant fraction. Microsomes with overexpressed Hrd1p and Hmg2p-GFP were incubated with *ubc7Δ* (Δ) or *UBC7* cytosol and 30 mM ATP. One reaction equivalent was centrifuged at 25,000 xg and another was centrifuged at 100,000 xg, each for one hour. Hmg2p-GFP was immunoprecipitated with anti-GFP antiserum from a total (T) reaction with *ubc7Δ*, a total (T) reaction with *UBC7* cytosol, the supernatant (S) fractions after centrifugations, and the resuspended pellet (P) fractions after centrifugations. Hmg2p-GFP was detected by immunoblot with anti-ubiquitin and anti-GFP.
immunoreactivity was from Hrd1p-modified Hmg2p-GFP generated in the reaction.

Retrotranslocation is thought to precede proteasomal degradation. Since there is an abundant pool of proteasomes present in the cytosol fraction we tested if proteasomal degradation was occurring in the course of the assay. We repeated the retrotranslocation experiment in the presence of proteasome inhibitor MG132. The intensity of the signal for retrotranslocated protein was more intense (compare S lanes) indicating the 20S core particle protease activity was contributing to decreased signal, presumably due to in vitro degradation of a fraction of the substrate (Figure 3-2). Thus, we used proteasome inhibitors in subsequent experiments.

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

It has been suggested that membrane-embedded substrates and lumenal substrates can be divided into two sub-pathways of ERAD referred to as ERAD-M and ERAD-L, respectively. ERAD-L requires more components, presumably because recognition, recruitment and exposure of the substrate to the cytosolic ubiquitination machinery requires separate processes that are not needed to find integral membrane substrates. Some of these components
**Figure 3-2: Proteasomal inhibition enhances in vitro Hmg2-GFP ubiquitination and retrotranslocation.**

DMSO or proteasome inhibitor MG132 was added to reactions. Following fractionation, Hmg2p-GFP was immunoprecipitated with anti-GFP antiserum and detected by immunoblot with anti-ubiquitin or anti-GFP.
include lectin/chaperones such as Yos9p (Gauss et al., 2006a), and traditional chaperones like Kar2p (Denic et al., 2006; Nishikawa et al., 2001). These lumenal proteins are not required for regulated degradation of Hmg2p-GFP. Furthermore, sufficient levels of Hrd1p will allow ERAD of both lumenal and membrane-bound substrates in the absence of Hrd3p (Denic et al., 2006; Gardner et al., 2000). Because Hrd3p serves as a protein link between the cytosol and the lumen (Gardner et al., 2000), we examined if Hrd3p is required for the in vitro reactions described herein. Our suspicion was that Hrd3p would be dispensable for this reaction, since increasing Hrd1p above its genomic levels without increasing Hrd3p increases the proportion of Hmg2p-GFP that is both ubiquitinated and retrotranslocated. Testing an otherwise isogenic \textit{hrd3}\textsuperscript{Δ} null strain confirmed this; the ubiquitination and retrotranslocation of Hmg2p-GFP were indistinguishable when compared to the normal \textit{HRD3} strain (Figure 3-3). Thus, Hrd1p appears to play a central role in recognition and retrotranslocation of its natural substrate Hmg2p-GFP, as anticipated from its central role defined by earlier genetic studies.

ER degradation of Hmg2p-GFP appears to be processive. A variety of tags have never revealed any fragments of this substrate produced by ERAD in our hands. This processivity is thought to be a common feature of ERAD and is consistent with wholesale removal of protein substrates by retrotranslocation machinery. If this is the case then full-length Hmg2p-GFP would be predicted to be transported as a ubiquitinated molecule into the S cytosolic fraction in the assay. Alternatively, the ubiquitin immunoreactivity
Figure 3-3: Hrd1p overexpression bypasses the requirement for Hrd3p in Hmg2p-GFP ubiquitination and retrotranslocation. Hmg2p-GFP retrotranslocation was evaluated in WT and hrd3Δ microsome strains incubated with either ubc7Δ (Δ) or UBC7 cytosol. Hmg2p-GFP was immunoprecipitated from each fraction with anti-GFP antiserum and detected by immunoblot with anti-ubiquitin or anti-GFP.
that was precipitated with anti-GFP antiserum could be GFP-containing products cleaved from the transmembrane region in the microsomal membrane. We employed an antiserum raised against the last lumenal loop (normally within the ER) to test if normally lumenal transmembrane domain determinants are present in the retrotranslocated ubiquitinated material. The results obtained with the anti-lumenal antiserum were identical to those obtained with anti-GFP antiserum, indicating that both transmembrane domain and GFP epitopes are present in the soluble ubiquitinated substrate (Figure 3-4).

Fully processive retrotranslocation would be predicted to result in the movement of intact Hmg2p-GFP to the soluble fraction in our assay. We next used serial immunoprecipitations with the anti-GFP and anti-lumenal loop antisera to discern if the ubiquitinated GFP and transmembrane determinants were on the same or different molecules, that is, if the ubiquitinated Hmg2p-GFP in the soluble fraction was intact. If full-length ubiquitinated Hmg2p-GFP was moved to the cytosolic fraction in the assay, then either antiserum should completely remove the ubiquitinated material from the soluble fraction. Three pairs (six samples total) of reaction supernatants were immunoprecipitated with either anti-GFP (2 samples), anti-LOOP (2 samples), or pre-immune serum (2 samples). The two identical supernatants from each pair were next re-precipitated with either anti-GFP or anti-LOOP antisera. The precipitated material from all reactions was then subjected to ubiquitin immunoblotting, with
Figure 3-4: Retrotranslocated Hmg2p-GFP can be immunoprecipitated with antisera recognizing cytosolic and transmembrane determinants. Hmg2p-GFP was immunoprecipitated from each fraction with anti-GFP antiserum, which recognizes the cytosolic domain of the molecule, or anti-LOOP antiserum, which recognizes the first loop of the transmembrane domain of Hmg2p-GFP. Immunoprecipitated Hmg2p-GFP was detected by immunoblot with either anti-ubiquitin or anti-GFP.
the first round results in the top panel and the second round results in the bottom panel (Figure 3-5).

The serial IP experiment indicated that the retrotranslocated Hmg2p-GFP was intact. Immunoprecipitation of the soluble, ubiquitinated Hmg2-GFP with either GFP or lumenal antisera completely cleared all the ubiquitin immunoreactivity, resulting in no further pull-down of ubiquitinated Hmg2p-GFP in the second IP. Conversely, re-immunoprecipitation of the supernatants from the pre-immune pair with either antiserum resulted in a signal that was identical to either initial IP. This pre-immune control demonstrates that Hmg2p-GFP must be pulled down to observe a ubiquitin signal. Second, it shows that the supernatant samples from the first round of IP are competent for a second round, and that the absence of signal in the second IPs (two right pairs) was due to a lack of any more ubiquitinated Hmg2p-GFP being present after either first round precipitations. These studies strongly indicate that intact molecules of Hmg2p-GFP are present in the supernatant, despite the large transmembrane region.

The Hmg2p-GFP N-terminal ER anchor has 8 transmembrane spans. Normally Hmg2-GFP is only found in membrane fractions. Accordingly, the presence of this intact molecule in the soluble fraction of the retrotranslocation assay was surprising, but consistent with current models of ERAD. We did another experiment to confirm that the intact, ubiquitinated Hmg2p-GFP had been solubilized, by running the immunoprecipitation of the cytosolic "S" fraction without detergent, using an immunoprecipitation buffer that is typically
**Figure 3-5: Retrotranslocated Hmg2p-GFP is an intact molecule.** Six supernatant fractions from six retrotranslocation reactions were subjected to serial immunoprecipitations. Two supernatant fractions were first individually immunoprecipitated with either pre-immune, anti-GFP, or anti-LOOP antisera (top panel). The unbound material from the first immunoprecipitation was subjected to a second immunoprecipitation by either anti-GFP or anti-LOOP antisera (bottom panel). Immunoprecipitates were detected by anti-ubiquitin immunoblots.
only used for soluble proteins. Parallel samples of the supernatant fraction were subjected to detergent-free immunoprecipitation with either pre-immune serum or anti-GFP antiserum. Although there is a higher background of non-specific ubiquitin immunoreactivity than seen with the normal IP procedure with detergent, there is a clear increase of ubiquitinated protein brought down by the anti-GFP serum (Figure 3-6 A), indicating that this ubiquitinated, polytopic membrane protein is present in the cytoplasmic fraction as a soluble protein, presumably in a complex with factors that mediate retrotranslocation.

The above experiments all indicate that ubiquitinated Hmg2p-GFP is moved to the soluble fraction intact. To directly test if full-length Hmg2p-GFP was retrotranslocated, we used recombinant Ubiquitin-specific protease 2 catalytic domain (Usp2-cc), which efficiently strips ubiquitin from multiubiquitin chains on substrates (Ryu et al., 2006). Removal of ubiquitin from the immunoprecipitated protein allowed us to directly examine the molecular weight of the retrotranslocated GFP by immunoblotting. Before immunoprecipitating the retrotranslocated protein, we divided the sample into two aliquots and treated one with buffer and the other with Usp2-cc (Figure 3-6 B). The upper panel shows an anti-ubiquitin blot of the sample, and demonstrates the effect of the Usp2-cc "ubiquitin strippase". The lower panel shows the same sample immunoblotted with anti-GFP antibodies. Without ubiquitin stripping there is little detectable GFP, whereas in the stripped sample, the GFP immunoreactivity increases as a discrete band with the molecular weight of Hmg2p-GFP. Importantly, no other sizes of GFP
Figure 3-6: Retrotranslocated Hmg2p-GFP is full-length.
A. Retrotranslocated Hmg2p-GFP was immunoprecipitated in non-detergent conditions with pre-immune or anti-GFP antiserum. B. Supernatant fractions were either treated with buffer or the ubiquitin-specific protease Usp2-cc. Hmg2p-GFP was immunoprecipitated with anti-GFP antiserum and detected by immunoblot with anti-ubiquitin and anti-GFP.
immunoreactivity were liberated by Usp2-cc; only the mobility of full-length Hmg2p-GFP was detected. This experiment directly demonstrates that intact Hmg2p-GFP is being moved to the cytosol as a multiubiquitinated protein, as indicated by the serial IP and as predicted by processive retrotranslocation. In this ubiquitin-stripping procedure, the immunoprecipitated protein from the “S” fraction is immunoblotted directly for GFP. We noticed that a band of Hmg2p-GFP immunoreactivity can be seen in the “unstripped” control, at varying intensities between experiments. This background immunoreactivity is not dependent on ubiquitination or the genetic factors shown below as needed for retrotranslocation (see below), and thus represents a non-specific background. Perhaps it is caused by some fragmentation of the ER into small vesicles, although that remains to be tested. This background signal is not present when ubiquitin is used to detect the retrotranslocated material. Thus, the use of ubiquitin immunoblotting as an assay of retrotranslocated Hmg2p-GFP provides a way to examine this process without this background signal. Nevertheless, it was important to demonstrate that Hmg2p-GFP is moved in its entirety to the soluble fraction.

A unifying feature of many ERAD pathways is the proposed role of the hexameric AAA-ATPase Cdc48p (p97 in mammals) in retrotranslocation (Bays et al., 2001b; Ye et al., 2003). This essential complex, in conjunction with its binding partners Ufd1p and Npl4p, has been implicated in a post ubiquitination step and has been directly shown in a permeabilized cell assays to mediate retrotranslocation of MHC-I molecules in the viral ERAD pathway promoted by
the US2 and US11 proteins (Ye et al., 2003; Flierman et al., 2003). Hmg2p-GFP is strongly stabilized by mutations in the Cdc48p complex, with a block that occurs after ubiquitination. We tested the role of Cdc48p in the in vitro retrotranslocation assay. We incorporated the cdc48-2 allele into assay strains, and simultaneously confirmed that this allele shows a strong in vivo block in Hmg2p-GFP degradation, even at the permissive temperature of 30°C (data not shown). The cdc48-2 allele was first evaluated in vitro when present in both the microsome and cytoplasm strains. Although Ubc7p-dependent ubiquitination occurred, there was a nearly complete block in release of ubiquitinated Hmg2p-GFP into the cytoplasmic "S" fraction (Figure 3-7: cdc48-2 microsomes and cdc48-2 cytosol). The block to ERAD occurs at the permissive growth temperature of 30°C, as did the block to retrotranslocation. It is interesting to note that the in vitro assay with cdc48-2 shows a small but consistent decrease in Hmg2p-GFP ubiquitination.

The Cdc48p complex has binding sites for ubiquitin, located both on Ufd1p and Cdc48p itself (Flierman et al., 2003; Richly et al., 2005). The importance of these sites have been demonstrated for the case of virally-mediated MHC-I retrotranslocation by mammalian p97, and we hypothesized that the multiubiquitin chains served a similar "guiding" role in directing retrotranslocation with polytopic Hmg2p-GFP in yeast. p97-mediated recognition of multiubiquitin chains does not occur when bulky GST-ubiquitin fusion is used instead of native ubiquitin. Similarly, Hmg2p-GFP retrotranslocation was dependent on native ubiquitin. Use of the N-terminal
Figure 3-7: Hmg2p-GFP retrotranslocation is dependent on Cdc48p. In vitro retrotranslocation assay reactions were carried out with either WT (CDC48) or cdc48-2 microsomes incubated with ubc7Δ (Δ), CDC48 UBC7, or cdc48-2 UBC7 cytosol.
GST-ubiquitin fusion allowed in vitro ubiquitination to proceed producing large conjugates in the reaction mix (Figure 3-8). However there was little or no movement of the GST-ubiquitin-derivatized Hmg2p-GFP to the cytosol, underscoring the importance of the ubiquitin molecule in the retrotranslocation process. Because Hmg2p-GFP was effectively ubiquitinated, this also ruled out simple "ratcheting models" by which ubiquitin addition caused movement of the Hmg2p-GFP out of the ER due solely to that exergonic modification.

The Cdc48p complex is found both in the microsome fraction and in the cytoplasmic pool. In fact, recent work has shown that Cdc48p is bound to the HRD complex (Gauss et al., 2006b). Thus we tested whether membrane-bound or soluble Cdc48p provided the activity for retrotranslocation. Because our assay divides cytoplasmic and membrane-bound components, we could examine this question by judicious use of cdc48-2 or wild-type assay strains. We prepared microsomes and cytosol with either normal CDC48 or the cdc48-2 allele and ran the retrotranslocation assay with the various combinations of mutant or wild-type material, as indicated (Figure 3-7: CDC48 microsomes with cdc48-2 cytosol and cdc48-2 microsomes with CDC48 cytosol). Although the strongest effect on retrotranslocation was seen when both membrane and cytosol were cdc48-2, it was clear that the cytosol alone contributes the majority of the needed Cdc48p activity. This implies that soluble Cdc48p complexes are recruited from the aqueous medium for their role in retrotranslocation.
Figure 3-8: In vitro Hmg2p-GFP ubiquitination with GST-ubiquitin does not support Hmg2p-GFP retro-translocation. Exogenous ubiquitin or GST-ubiquitin was added to the in vitro assay.
Cdc48p is a member of the large AAA-ATPase family. The hexameric structure allows concerted harnessing of substantial chemical energy to drive polytopic membrane protein retrotranslocation. To examine the role of ATP in retrotranslocation, we needed to separate the use of ATP in ubiquitination from that in retrotranslocation, since ubiquitination is also required for this step (see above). We capitalized on the differing use of ATP by the ubiquitin E1 or the AAA-ATPase domains of Cdc48p. Ubiquitin addition to the E1 is driven by hydrolysis of the \( \alpha \) phosphodiester bond, to produce an AMP-ubiquitin intermediate. In contrast, the AAA-ATPases hydrolyze the \( \gamma \)-phosphodiester bond. In numerous cases, the immido analog of ATP, called AMP-PNP, with an intact \( \alpha \) bond and a non-hydrolyzable phosphodiimido linkage at the \( \gamma \) position, will drive ubiquitination (Johnston and Cohen, 1991), but not reactions that depend on the \( \gamma \) bond. We employed AMP-PNP to test for a role of ATP in retrotranslocation separate from its known involvement in ubiquitination. The direct approach of simply adding AMP-PNP to the retrotranslocation assay rather than normal ATP was not an effective way to ask this question. In our in vitro assay, while AMP-PNP did support Hmg2p-GFP ubiquitination, the reaction did not proceed to nearly the same extent as those run with ATP, prohibiting direct comparison between the two nucleotides (Chapter 2). Thus we first ran a ubiquitination phase of the assay with ATP to ensure sufficient Hmg2p-GFP ubiquitination, and then a retrotranslocation phase with either ATP or AMP-PNP to test for ATP dependence of retrotranslocation of the ubiquitinated Hmg2p-GFP. The major contribution of
soluble Cdc48p to Hmg2p-GFP retrotranslocation allowed us to accomplish this separation. We first allowed ubiquitination to proceed with little or no retrotranslocation, and then retrotranslocation to occur with minimal ubiquitination. This was done by first running the “ubiquitination phase” with \textit{cdc48-2} mutant microsomes, \textit{cdc48-2} mutant Ubc7p cytosol, and ATP. After a reaction period, we recovered the microsomes from the reaction, and initiated the second “retrotranslocation phase” by adding \textit{CDC48} cytosol from a \textit{ubc7Δ} null. This \textit{CDC48 ubc7Δ} cytosol was supplemented with either ATP or AMP-PNP, and a control in which buffer was added to the microsomes was run in addition. The reaction mixes were then fractionated to evaluate soluble and membrane-bound ubiquitinated Hmg2p-GFP (Figure 3-9). The reaction with AMP-PNP did not support retrotranslocation, but did allow continued ubiquitination when compared to the buffer control. In contrast, the cytosol with ATP, retrotranslocated Hmg2p-GFP, and also supported further ubiquitination. Clearly a $\gamma$ ATP bond is needed for Cdc48p-dependent retrotranslocation.

A significant portion of the Cdc48p complex is found in the cytosol, and this pool can function in retrotranslocation from the experiments above. This leads naturally to the question of how it engages the HRD complex at the ER surface. It has been proposed from co-immunoprecipitation and in vivo experiments that the ER-localized integral membrane protein Ubx2p serves as an ER-localizing "receptor" or docking site for the Cdc48p complex (Schuberth and Buchberger, 2005; Wilson et al., 2006; Neuber et al., 2005). We used the
Figure 3-9: AMP-PNP does not support Hmg2p-GFP retrotranslocation. Five in vitro ubiquitination reactions were carried out with *cdc48-2* microsomes and *cdc48-2 UBC7* cytosol for one hour to minimize retrotranslocation. The reacted microsomes were pelleted and resuspended in *CDC48 ubc7Δ* cytosol and incubated for an additional hour in the presence of buffer, ATP, or AMP-PNP. The supernatant and pellet fractions were separated by centrifugation and Hmg2p-GFP was immunoprecipitated from each fraction with anti-GFP antiserum. Hmg2p-GFP was detected by immunoblot with anti-ubiquitin and anti-GFP. M denotes the starting ubiquitination level in the membrane fraction before the retrotranslocation phase of the experiment.
retrotranslocation assay to directly test this idea. The \textit{ubx2Δ} null allele was introduced into the microsome strain, since Ubx2p is an integral membrane protein. When the assay was run with \textit{ubx2Δ} null membranes, it was clear that retrotranslocation was completely abrogated, as shown by the complete lack of immunoreactivity in the soluble fraction (Figure 3-10). However, the loss of microsomal Ubx2p also showed unexpected ubiquitination of Hmg2p-GFP in the absence of added Ubc7p. This ubiquitination of Hmg2p-GFP is present in the microsome fraction before the reaction is initiated. This "pre-ubiquitination" is highly reproducible and implies that Ubx2p may have roles at other positions in the ERAD pathway, at least of Hmg2p-GFP. At present we do not know how this process occurs.

We confirmed the generality of Ubx2p's requirement in retrotranslocation by examining Hrd1p itself as a substrate. Normally Hrd1p is present in stoichiometric balance with Hrd3p and is quite stable. In this assay, Hrd1p levels are sufficiently elevated to allow Hrd1p self-degradation, mediated by Hrd1p's own RING domain (Bazirgan et al., 2006). Hrd1p undergoes Ubc7p-dependent self-ubiquitination in vitro. As with Hmg2p-GFP, a portion of the ubiquitinated Hrd1p is retrotranslocated but this does not occur in the \textit{ubx2Δ} microsomes (data not shown). Curiously, Hrd1p does not show the "preubiquitination" in the \textit{ubx2Δ} microsomes that was seen with Hmg2p-GFP.

The block to retrotranslocation caused by \textit{ubx2Δ} is strong with both Hmg2p-GFP and Hrd1p. However, it is important to note that in vivo, loss of
Figure 3-10: Cdc48p receptor Ubx2p is necessary for in vitro Hmg2p-GFP retrotranslocation. Hmg2p-GFP retrotranslocation was evaluated in WT (UBX2) and ubx2Δ microsomes incubated with ubc7Δ (Δ) or UBC7 cytosol. Hmg2p-GFP was immunoprecipitated with anti-GFP antiserum and detected by immunoblot with anti-ubiquitin or anti-GFP.
Ubx2p only partially blocks ERAD (data not shown). Clearly, there must be other ways for Cdc48p to get to the ERAD process, since a complete null of the *UBX2* gene has a less strong effect on Hmg2p-GFP ERAD than a partially functioning hypomorph of *cdc48-2*. Nevertheless, the combination of the in vitro assay and in vivo analysis allows us to examine detailed mechanisms of known ERAD components and place their function in the broader context of multiple pathways and mechanisms of ERAD.

ERAD involves the transfer of ubiquitinated substrates to the proteasome from the ER surface. A number of genetic and biochemical studies have implicated a set of adaptor proteins that facilitate this transfer. These adaptors have both ubiquitin-binding Uba motifs and proteasome-binding Ubl motifs, and thus appear well-suited for this job (Wilkinson et al., 2001; Rao and Sastry, 2002). Yeast has two of these factors, Dsk2p and Rad23p, which have been previously implicated in ERAD in yeast (Medicherla et al., 2004). They are sufficiently similar that the double null is required to observe an ERAD defective phenotype in vivo. By the simplest model, we would expect the *rad23Δ dsk2Δ* double null to have no effect on retrotranslocation in our assay, since we use proteasome inhibitors due to the enhancement of signal described above. We first tested reactions in which both the cytosol and the microsomes were derived from double null strains. Surprisingly, the absence of these two adaptors had a strong effect on Hmg2p-GFP retrotranslocation (Figure 3-11).
**Figure 3-11:** Ubiquitin-proteasome shuttling factors Rad23p and Dsk2p are required for in vitro Hmg2p-GFP retrotranslocation. Hmg2p-GFP in vitro retrotranslocation was evaluated in reactions containing either WT or rad23Δ dsk2Δ microsomes mixed with either WT or rad23 dsk2Δ microsomes.
Both Rad23p and Dsk2p are soluble proteins; they can be purified and studied in vitro in solution (Kang et al., 2007). Not surprisingly, the use of the rad23Δ dsk2Δ double null cytosol had significant effect on Hmg2p-GFP retrotransloction. However, the combined use of both microsomes and cytosol from double null strains had the largest effect (Figure 3-11), implying that there might be a role for these adaptors at the microsome that is not the same as that provided by the soluble pool. Consistent with this, removal of Rad23p and Dsk2p in only the microsome fraction did not appear wild type, and showed retrotranslocation of only very high molecular weight Hmg2p-GFP species. It is also clear from the figure that the extent of Hmg2p-GFP ubiquitination is lessened when these two proteins are missing from the cytosolic fraction. This is seen by comparing the total from the wild-type reaction with the other reactions carried out with rad23Δ dsk2Δ double null cytosol. In all cases, the loss of Dsk2p and Rad23p caused a reproducible decrement of extent of Hmg2p-GFP ubiquitination, with the cytosolic pool having the larger role. Taken together, the results indicate that these ubiquitin-binding adaptors could be involved in retrotranslocation as well as delivery to the proteasome.

Along with an energy source such as the γ-bond of ATP, it is widely and reasonably thought that a pore or channel is required for the removal of ERAD substrates. In the case of Hmg2p-GFP, the intact molecule is removed to the cytosol, requiring the dislocation of 8 transmembrane spans and four luminal loops of up to 20 kD which would present a substantial energy barrier for movement across the ER bilayer. The best candidates for such a pore include
members of the derlin family (Lilley et al., 2005; Ye et al., 2005), or the anterograde pore Sec61p, both of which have been suggested to be broadly involved in dislocation with the strongest evidence favoring the derlins. Yeast has two members of this superfamily, the original Der1p protein, and a homologous protein Dfm1p that has demonstrable Cdc48p-binding activity (Sato and Hampton, 2006). We tested membranes from a der1Δ dfm1Δ double null strain in the retrotranslocation assay (Figure 3-12 A). We also tested membranes from the sec61-2 strain, which has reproducible effects on the in vivo ERAD of Hmg2p-GFP (Figure 3-12 B). In no case was there any effect of loss of these channel candidates on directly observed retrotranslocation of Hmg2p-GFP.

The centrality of Hrd1p in ERAD, and the lack of evidence for a role of known channels in the retrotranslocation of Hmg2p-GFP led us to examine if Hrd1p itself was providing channel function in addition to its role as the E3 ligase. This idea is appealing from a structural perspective, since Hrd1p has a multi-spanning membrane domain N-terminal to the cytosolic RING ligase domain. Furthermore, the Hrd1p N-terminal anchor has a substantial number of hydrophilic residues, which might be expected in a structure that forms an aqueous channel through which retrotranslocation substrates travel. However, the simple experiment of removing Hrd1p was not feasible because it also is necessary for Hmg2p ubiquitination, which is a prerequisite for subsequent removal by Cdc48p.
Figure 3-12: Putative retrotranslocons, Der1p, Dfm1p, and Sec61p are not required for Hmg2p-GFP retrotranslocation. Hmg2p-GFP in vitro retrotranslocation was evaluated in der1Δ dfm1Δ in (A) and sec61-2 in (B).
To separate the Hrd1p ligase function from other possible activities, we devised a “self-destructive” substrate that employs the Hrd1p RING domain in the absence of its transmembrane region. We did this by fusing the Hrd1p cytosolic RING domain to the transmembrane domain of the normally highly stable Hmg1p HMG-CoA reductase isozyme, called Hmg1-Hrd1p. In vivo, Hmg1-Hrd1p behaves in all ways like a HRD pathway substrate, but does not require Hrd1p’s presence due to the in-cis RING domain. Its degradation is very rapid, and strongly dependent on both Ubc7p and the intact RING domain. Hmg1-Hrd1p degradation is also slowed by the cdc48-2 mutant or the RPN1 hypomorph hrd2-1 (data not shown). The degradation of Hmg1-Hrd1p occurs in a hrd1Δ null, in a doa10Δ null, and in the hrd1Δ doa10Δ double null with very similar kinetics and extent as in a wild-type strain (data not shown). The rapid proteasomal degradation of Hmg1-Hrd1p implied that the autonomously degrading fusion protein undergoes retrotranslocation. We tested this directly in vitro, and indeed, ubiquitinated Hmg1-Hrd1p showed the expected movement to the soluble supernatant fraction (Figure 3-13 A). Preliminary work shows in vitro retrotranslocation of the fusion was partially blocked in an assay run with both cytosol and supernatant from the cdc48-2 mutant (data not shown). Finally, we test if intact Hmg1-Hrd1p retrotranslocation had occurred by again using the Usp2-cc “strippase” to demonstrate recovery of the full-length fusion protein from the retrotranslocated S fraction (Figure 3-13 B). Thus, retrotranslocation of this
Figure 3-13: Full-length Hmg1-Hrd1p fusion is retrotranslocated in vitro. A. In vitro retrotranslocation of Hmg1-Hrd1p (HA epitope-tagged) fusion. Hmg1-Hrd1p was immunoprecipitated with anti-Hrd1p antiserum and immunoblotted with anti-ubiquitin and anti-HA. B. Supernatant fractions were either treated with buffer or ubiquitin-specific protease Usp2-cc. After one hour incubation, fusion was processed as described in (A).
HRD pathway substrate, can occur in the complete absence of the Hrd1p transmembrane domain.

**Discussion**

In this study we have successfully reconstituted Hrd1p-dependent retrotranslocation of a natural multi-spanning membrane substrate of the HRD pathway, Hmg2p-GFP. Once ubiquitinated, full-length Hmg2p-GFP is moved to the supernatant fraction, despite having 8 transmembrane spans and several lumenal loops. We have shown this by several criteria, including: immunoprecipitation studies with both GFP and lumenal-domain antisera, the ability to capture ubiquitinated Hmg2p-GFP in the absence of detergent, and the direct recovery of full-length Hmg2p-GFP in the retrotranslocated fraction after removal of ubiquitin with USP2. Thus, complete retrotranslocation occurs in the in vitro assay, opening the door to analysis and detailed mechanistic understanding of this thermodynamically heroic event that seems to occur in all ERAD pathways. An important question that remains is whether completely retrotranslocated Hmg2p-GFP has a transient existence during its ERAD in vivo. Alternatively, proteasomal delivery could be sufficiently rapid to not allow buildup of this retrotranslocated intermediate. It appears that different substrates show different degrees of cytoplasmic buildup of fully retrotranslocated species during in vivo ERAD (Elkabetz et al., 2004; Medicherla et al., 2004). Nevertheless, the ability to observe this process with
Hmg2p-GFP both defines the capacity of this transfer mechanism and allows its intimate mechanistic study in ways that are not possible using intact cells.

In our approach, we have used sufficient Hrd1p levels to drive ubiquitination of Hmg2p-GFP without need for Hrd3p or Usa1p. Thus, we are examining the minimal requirements for in vitro ubiquitination and retrotranslocation. Importantly, in vitro ERAD of Hmg2p-GFP by this method has all the features of in vivo Hmg2p-GFP degradation. Ubc7p and its anchor Cue1p are required, as is the critical lysine-6 of Hmg2p-GFP (Gardner et al., 2001b; Gardner and Hampton, 1999b). Furthermore, Hrd1p-dependent ubiquitination of Hmg2p-GFP is specifically blocked by a chemical chaperone, which also prevents in vivo degradation of the substrate (Gardner et al., 2001b). Thus, information derived from our assay will be informative about the mechanism and energetics of retrotranslocation in the functioning in vivo HRD pathway. These results also reinforce the idea that Hrd1p is a central organizer of ERAD, consistent with multiple observations that Hrd1p alone can drive ERAD of membrane-anchored substrates.

In both mammals and yeast, the AAA-ATPase Cdc48p/p97 is a key component of the post-ubiquitination step in ERAD of multiple substrates. Elegant in vitro analyses of the virally-mediated degradation of MHC-I molecules by the US11 protein of cytomegalovirus clearly demonstrated the importance of this energy-consuming protein in the dislocation of that single-spanning substrate. Our studies above show that Cdc48p is similarly required for the complete removal of Hmg2p-GFP from the ER membrane. The
requirement for ubiquitin is reasonable considering the ubiquitin-binding capacity of the Cdc48p/Npl4p/Ufd1p multimer. The assay uses microsomes and cytosol prepared from separate yeast strains. This allowed us to evaluate the relative importance of cytosolic or membrane-bound Cdc48p in Hrd1p-dependent retrotranslocation, by using either mutant or wild-type strains for each component. It was clear that the cytosolic pool of Cdc48p was the most important source of this activity: cdc48-2 microsomes supported retrotranslocation only slightly lower that that in the all-wild-type experiment, whereas cdc48-2 cytosol with wild-type microsomes resulted in almost no retrotranslocation of Hmg2p-GFP. There is a pool of Cdc48p (as well as p97 in mammals) that is tightly bound to microsomal membranes, and that Cdc48p associates with Hrd1p, which taken alone might indicate that the bound pool to be important in ERAD. Nevertheless, the observed importance of soluble Cdc48p implies that movement from the cytosol to the surface of the ER occurs during the ERAD process. Perhaps Cdc48p plays a role in stabilizing the retrotranslocated Hmg2p-GFP, and so it must be replenished from the soluble pool as the reaction proceeds, as has been proposed from structural studies of the mammalian p97 protein’s role in ERAD.

Cdc48p/p97 is a member of the large AAA-ATPase family. This activity is thought to power the movement of retrotranslocation substrates across the ER membrane. By capitalizing on the differing requirements for ATP by E1 (which hydrolyzes the ATP α bond) and Cdc48p (which hydrolyzes the ATP γ bond) we were able to demonstrate an ATP requirement for Hmg2p-GFP
retrotranslocation distinct from ubiquitination. This separation depended on using AMP-PNP during the retrotranslocation phase of an experiment to show the specific need for a $\gamma$ bond in this step. Interestingly, the simple method of separating ubiquitination from retrotranslocation by use of AMP-PNP instead of ATP in the complete reaction was not feasible, because AMP-PNP only poorly supports ubiquitination in the reconstituted HRD reactions. We ran a separate ubiquitination phase with both membranes and cytosol from $cdc48$-2 mutants in the presence of ATP, and then a separate retrotranslocation phase using $CDC48$ wild-type cytosol with AMP-PNP present. This technical separation was possible because of our observed main role of cytosolic $CDC48$ in Hmg2p-GFP retrotranslocation. This variation clearly demonstrated a requirement for the $\gamma$-bond in Hmg2p-GFP retrotranslocation.

The added requirement for a $\gamma$-bond ATP for optimal ubiquitination of Hmg2p-GFP may be due to a requirement for Cdc48p in Hmg2p-GFP ubiquitination. Consistent with this idea, we observed reproducibly decreased in vitro Hmg2p-GFP ubiquitination when the reactions were run with $cdc48$-2 mutants. It has been posited that Cdc48p may play a role in MHC-I recognition as well as retrotranslocation in the US2-dependent pathway (Ye et al., 2003), as have other AAA-ATPases been implicated in the recognition of misfolded proteins for destruction (Ye, 2006; Ito and Akiyama, 2005). This additional role for Cdc48p will be investigated in the future. The in vitro approach makes the separation of such intertwined functions feasible.
The participation of soluble Cdc48p in Hmg2p-GFP retrotranslocation strengthens the idea that there must be ways for Cdc48p to fruitfully interact with the ER membrane in order to effect its ERAD functions. A number of studies have implicated the ER-localized Ubx2p as an ER-receptor for Cdc48p. We directly tested this idea and found that Ubx2p is required for retrotranslocation of both Hmg2p-GFP and Hrd1p itself. While the effects of Ubx2p are quite clear in vitro, in intact cells Hmg2p-GFP degradation is more strongly effected by the partial loss of function \textit{cdc48-2} mutant than it is by a complete \textit{ubx2Δ} null in otherwise isogenic strains. This genetic observation implies that there must be other ways that Cdc48p can engage the ERAD machinery in the absence of Ubx2p. This is consistent with the observation that loss of Ubx2p does not completely remove Cdc48p association from the ER membrane (Wilson et al., 2006). Nevertheless, our results are consistent with the role for Ubx2p as a receptor for Cdc48p. Perhaps our assay is more sensitive due to the inevitable dilution of the cytosol fraction that occurs during its preparation, rendering the system more dependent on the ability of the Ubx2p microsomes to attract Cdc48p. It is important to note that the functions of Ubx2p are likely to be broader than only providing efficient delivery of Cdc48p to the ERAD pathways, since we have observed that a \textit{ubx2Δ} null has a profound upregulation of the UPR that is much greater than that caused by strong ERAD-inhibiting mutants of Cdc48p (unpublished observation), again indicating roles that transcend this receptor function. The in vitro assay is particularly useful when this is considered, since in intact cells, a \textit{ubx2Δ} null
mutant is causing ongoing strong regulatory responses that may cloud observation of its direct ERAD functions.

It appears that the entire ERAD pathway for Hmg2p can occur in our in vitro assay. Addition of proteasome inhibitors to our assay caused an increase in ubiquitinated Hmg2p-GFP, indicating that proteasomal destruction was occurring. Thus, we examined the involvement of the ubiquitin-binding, proteasome delivery adaptors Rad23p and Dsk2p in vitro. These proteins bind multibubiquitin chains and the proteasome by virtue of their Uba and Ubl domains, respectively. Current models predict that these soluble adaptors would have a role after retrotranslocation, and thus would be expected to have no effect on the removal of ubiquitinated Hmg2p-GFP from the membrane. We observed that loss of these redundant factors in fact caused a strong block to retrotranslocation, implicating them in a step more upstream in the pathway. It could be that their ability to bind a ubiquitinated substrate contributes to the stabilization of the retrotranslocated substrate. It is also possible that they participate in formation of a complex that functions in several post-ubiquitination steps, including removal from the membrane and delivery to the proteasome. The Rad23p and Dsk2p adaptors are soluble proteins, and would be expected to function in the cytosolic fraction, and we saw the strongest effect when the cytosol fraction alone was prepared from the rad23Δ dsk2Δ double null. In addition, we noted that in experiments using cytosol devoid of Rad23p and Dsk2p resulted in a lessened extent of ubiquitination. This could be due to a role in recruiting the “E4” Ufd2p, which has been
posited to enhance ubiquitination of ERAD substrates including Hmg2p-GFP (Richly et al., 2005). We do not know if the effects on retrotranslocation are due to this lessening of ubiquitination extent. However we think this is unlikely because in many experiments, we have observed that Hmg2p-GFP with a similar extent of ubiquitination in wild-type experiments does show robust retrotranslocation. Another intriguing possibility is that these ubiquitin-binding/proteasome-binding adaptors might recruit the proteasome to the newly ubiquitinated Hmg2p-GFP, and so provide additional impetus for retrotranslocation that works in conjunction with Cdc48p. Whatever the mechanism, it is clear that these adaptors may function at multiple points in the ERAD pathway.

One of the challenging open questions concerning ERAD is the mechanism of exit from the ER membrane. Both lumenal and membrane-bound substrates are expected to require a channel or pore for successful exit from the ER. In the case of Hmg2p-GFP, there are significant lumenal regions of polypeptide. However, the identity of this channel remains unclear. There is evidence for members of the polytopic derlin family having a role in retrotranslocation of some proteins, and the derlins have a compelling association with the p97 in mammalian cells which both indicate a possible role in mediating dislocation of ERAD substrates. Furthermore, the anterograde pore Sec61p has not been ruled out, and can be found to have some role in lumen-to-cytosol movement of some test molecules (Plemper et al., 1999). We directly examined both the pair of derlins, Der1p and Dfm1p,
and Sec61p in our assay, and confirmed that neither the double mutant $der1\Delta dfm1\Delta$ nor the temperature-sensitive $sec61-2$ mutant had any detectable effects on retrotranslocation of Hmg2p-GFP. This is consistent with our in vivo studies, which similarly showed a lack of effect of the $der1\Delta dfm1\Delta$ double mutant, or even the $der1\Delta dfm1\Delta sec61-2$ triple mutant on in vivo degradation of Hmg2p-GFP (Sato and Hampton, 2006).

An appealing idea consistent with the lack of an obvious, separate channel is that the ligases themselves function as channels, in addition to their roles in ubiquitin transfer. Both Hrd1p and Doa10p, the two principle ERAD ligases in yeast, have large transmembrane regions (Swanson et al., 2001; Gardner et al., 2000; Bordallo and Wolf, 1999). The transmembrane spans of these ligases have a substantial proportion of relatively hydrophilic amino acids, as might be predicted for a membrane protein that forms an aqueous channel for transmission of peptide strands out of the ER. By this model the ligases would mediate movement of substrates across the ER membrane and ubiquitinate them as they emerged. We tested this idea for Hrd1p by formation of a “self-destructive” fusion protein that had Hrd1p’s ubiquitination activity but lacked the Hrd1p transmembrane domain. When fused to the Hrd1p cytoplasmic domain, the normally stable Hmg1p underwent dramatically rapid degradation that required Ubc7p, the proteasome and Cdc48p. Appending the Hrd1p ligase domain to Hmg1p was sufficient for rapid ERAD that had many of the features of a typical HRD substrate. The resulting “self-destructive” Hmg1-Hrd1p fusion underwent in vivo degradation in both a $hrd1\Delta$
null and a \textit{doa10} ∆ \textit{hrd1} ∆ double null, indicating that delivery to the proteasome could occur without either of the transmembrane regions being present in the cell. The retrotranslocation assay directly confirmed this: Hmg1-Hrd1p underwent Cdc48p-dependent retrotranslocation, and like the Hmg2p-GFP substrate, was recovered intact upon removal of the ubiquitin with Usp2-cc. The retrotranslocation of this fusion occurred in the \textit{hrd1} ∆ null, indicating that the Hrd1p transmembrane domain was not needed for retrotranslocation of this substrate from the ER membrane.

The result with the Hmg1-Hrd1p fusion indicated that the Hrd1p transmembrane domain is not required for removal of a HRD substrate from the ER membrane. There are three possibilities suggested from these and other results on ERAD retrotranslocation. Perhaps there is a yet-undiscovered protein required for retrotranslocation of ERAD substrates that has evaded numerous screens and direct protein analyses that have been performed. This factor could be essential to cells, or a poor genetic target, making its isolation by screening difficult. Alternatively, it may be that the bone fide ERAD channel is a composite of several proteins that can each function when a given component is removed. That is, there may an optimal channel of several proteins that can still function upon the removal of any one member. Finally, it is possible that retrotranslocation of Hmg2p occurs in a way that does not require a channel, but instead involves the recruitment of lipids to form a soluble intermediate. One version of this idea has been suggested in a recent essay (Ploegh, 2007). While we find this possibility unlikely, it cannot
be ruled out until the lack of a channel is understood by its discovery or the complete reconstitution of the process in a pure system devoid of channel candidates.

A striking result of this study is the observed movement of full-length Hmg2p-GFP into the cytosolic fraction by the combined action of Hrd1p and Cdc48p. Recent work on the Ste6-166p transmembrane substrate implied that this multi-spanning membrane protein was similarly moved to the soluble fraction, using arguments of molecular weight of the ubiquitinated intermediates (Nakatsukasa et al., 2008). The greater mass of Hmg2p-GFP moved by this immunoblotting assay allowed us to directly detect the retrotranslocated substrate. Combined, these results indicate that movement of entire transmembrane substrates is commonly occurring in ERAD, as has been suggested from in vivo studies with CFTR-Δ508 in mammalian cells. It is not clear how the 8-spanning integral membrane protein Hmg2p-GFP remains soluble in the cytosol. It will be important to analyze the physical state and binding partners of this molecular species to gain insight into what processes and proteins are responsible for this heroic thermodynamic event.

A perplexing feature of our and other in vitro assays is the low extent of substrate conversion that occurs. While only a small fraction of Hmg2p-GFP is ubiquitinated, modification of a given Hmg2p-GFP molecule appears to be very efficient, such that the small fraction of Hmg2p-GFP subjected to in vitro ubiquitination is modified with many copies of ubiquitin, as we have also seen in vivo. The lack of continued substrate processing appears to be a general
feature of the several reported in vitro ER ubiquitination assays (Song et al., 2005; Nakatsukasa et al., 2008). Conversely, the retrotranslocation of Hmg2p-GFP, once ubiquitinated, is quite efficient. Our estimates, using the Usp2-cc stripping procedure of the total reaction mix vs. the supernatant fraction, is that about 30%-50% of the ubiquitinated Hmg2p-GFP is moved to the soluble fraction by retrotranslocation. Although we do not yet understand why such a small percentage of the Hmg2p-GFP is recruited for ubiquitination, it appears that once that occurs, the subsequent movement of it out of the ER is very efficient.

This and other in vitro approaches will allow detailed mechanistic analysis of the ERAD pathway. Our future directions will include the analysis of the nature and composition of the retrotranslocated Hmg2p-GFP, and the role of the sterol regulatory signals that control Hmg2p-GFP degradation in coordinating this key step with the earlier events that are required for movement of substrates from the ER to their proteasomal destruction.
Acknowledgements

Chapter 3, in part, is a manuscript in preparation as "In vitro analysis of Hrd1p-mediated retrotranslocation of its natural substrate HMG-CoA reductase," Garza, Renee M.; Sato, Brian K.; Hampton, Randolph Y. The dissertation author was the primary investigator and author of this work.
Chapter 4:

Geranylgeranyl pyrophosphate accelerates Hmg2p degradation
Introduction

There are two major classes of molecules produced by the mevalonate pathway: isoprenoids and sterols. Both isoprenoids and sterols are critical for cell survival. Sterols are important for cell membranes and isoprenoids are involved in many diverse cellular functions such as mannosylation, mitochondrial respiration, and protein prenylation (Brown and Goldstein, 1990). The enzyme HMG CoA reductase (HMGR) is the first committed step in the mevalonate pathway. HMGR activity is rate limiting, and undergoes regulation, both transcriptionally and post-translationally (Brown and Goldstein, 1980).

*S. cerevisiae* has two isozymes of HMGR, Hmg1p and Hmg2p. Unlike Hmg1p, Hmg2p undergoes regulated degradation that is controlled by the activity of the mevalonate pathway. Pharmacological treatments reveal that the non-sterol degradation signal is derived from farnesyl pyrophosphate (FPP). FPP levels are lowered in cells treated with lovastatin, an inhibitor of HMGR action, thus stabilizing Hmg2p (Hampton and Bhakta, 1997; Hampton and Rine, 1994). Zaragozic acid (ZA) treatment results in a build-up of FPP that causes Hmg2p degradation (Hampton and Bhakta, 1997; Hampton and Rine, 1994). Genetic analyses substantiate that FPP or one of its derivatives functions to induce Hmg2p degradation (Gardner and Hampton, 1999). FPP holds a unique place in the mevalonate pathway, serving as precursor to a variety of molecules, including sterols, dolichol, heme, ubiquinone, and
Figure 4-1: The Mevalonate Pathway.
Figure 4-1 Continued: The Mevalonate Pathway.
geranylgeranyl pyrophosphate (GGPP) (See Figure 4-1). Therefore, the degradation signal may be FPP or one of its derivatives. Identification of the degradation signal would allow better understanding of Hmg2p regulation.

Our and others’ work has implicated farnesol, the alcohol derivative of FPP, to be the Hmg2p degradation signal. High levels of farnesol are detected when pharmacological and genetic means are used to increase FPP levels (Chambon et al., 1990; Gardner et al., 2001a). An in vitro limited proteolysis assay was used to test if farnesol and other isoprenoids induce a detectable change in Hmg2p structure (Shearer and Hampton, 2004; Shearer and Hampton, 2005). Hmg2p is believed to acquire features of a misfolded protein in the presence of its degradation signal. Farnesol was found to specifically hasten the proteolytic susceptibility of Hmg2p and fulfilled other biologically relevant criteria (Shearer and Hampton, 2005). As the degradation signal, we would expect farnesol to promote recognition of Hmg2p by the degradation machinery.

The HRD pathway degrades Hmg2p. Ubiquitination, and thus, degradation of Hmg2p by the HRD pathway depends on the levels of FPP, or a molecule derived from FPP. Farnesol, the strongest candidate, would be expected to increase Hmg2p ubiquitination and degradation if it were the degradation signal. We have employed both in vivo and in vitro methods to test if FPP or farnesol affects Hmg2p ubiquitination and degradation. Exogenous FPP was added to growing yeast cells as a way to increase farnesol levels within the cells, since farnesol added directly kills yeast cells
(Fairn et al., 2007). FPP did not have any detectable effect on Hmg2p degradation, however FPP entry into yeast cells could not be confirmed and has not been reported. We also added FPP and farnesol to the in vitro Hmg2p ubiquitination assay (described in Chapter 2). We found that FPP and farnesol had no detectable effect on in vitro Hmg2p-GFP ubiquitination. These results prompted us to re-evaluate different mevalonate pathway intermediates as possible degradation signals, thus shifting our analysis back into the live, intact cell.

Our analysis revealed that direct addition of geranylgeranyl pyrophosphate (GGPP) to growing cells promoted Hmg2p degradation. After further evaluation, we now propose that GGPP is a key physiological regulator of Hmg2p.

**Results**

We decided to survey pathway molecules distinct from FPP to evaluate their ability to drive Hmg2p degradation. pdr5Δ null cells were used in our initial survey. This null mutant allows a variety of drugs that are otherwise ineffective to enter yeast cells (Emter et al., 2002). We reasoned that the pdr5Δ null might similarly increase isoprenoid entry into cells. The reporter Hmg2p-GFP has been extensively shown to have the same regulation as full-length wild-type Hmg2p-GFP (Cronin and Hampton, 1999). Therefore, we utilized strains expressing Hmg2p-GFP to facilitate optical detection of Hmg2p-GFP steady state levels by flow cytometry. A shift of the fluorescence
Figure 4-2: The multidrug transporter Pdr5p does not affect GGPP-induced Hmg2p-GFP degradation. Strains expressing Hmg2p-GFP reporter were incubated with no drug, vehicle, 11 µM GGPP, or ZA for one hour. 10,000 cells were measured by flow cytometry resulting in histograms plotting fluorescence vs cell count.
histogram plot to the left indicates Hmg2p degradation, and to the right indicates Hmg2p-GFP stabilization. We tested the following isoprenoid pyrophosphates: isopentenyl pyrophosphate (IPP), dimethyl allyl pyrophosphate (DMAPP), geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP), and geranylgeranyl pyrophosphate (GGPP) (See Figure 4-1). The histograms were superimposable for each isoprenoid tested except for one (data not shown). GGPP, and not FPP, was the only isoprenoid to decrease Hmg2p-GFP steady state levels. Unexpectedly, the pdr5Δ null was unnecessary for Hmg2p-GFP degradation by GGPP (Figure 4-2). We further investigated this surprisingly specific effect. Since GGPP is synthesized from FPP, we hypothesized that it might be the molecule that mediates FPP-dependent regulation of Hmg2p.

We tested a range of GGPP concentrations on Hmg2p-GFP degradation. Concentrations less than 3 µM (EC₅₀=2.75 µM) were sufficient to induce Hmg2p-GFP degradation however we decided to continue studies with 11 µM GGPP (Figure 4-3 A), which shows close to maximal degradation in one hour incubations (Figure 4-3 C). Western blot analysis of Hmg2-GFP expressing strains corroborated flow cytometry results. 11 µM GGPP was effective at decreasing Hmg2p-GFP levels (Figure 4-3 B). Based on the structure of GGPP, we would not have predicted that GGPP could gain access into yeast cells.

Even though the two isozymes of HMGR have transmembrane domains with 50% identity, Hmg1p-GFP is stable and Hmg2p-GFP is degraded
Figure 4-3: GGPP dose response and time course.
A, B. Cells expressing Hmg2p-GFP reporter were incubated for one hour with various concentrations of GGPP, as indicated. A. Hmg2p-GFP was measured by flow cytometry and, B. by immunoblot of whole cell lysates with anti-GFP.
C. Time course of Hmg2p-GFP degradation in cells treated with ZA, control, and 11 μM GGPP.
(Gardner et al., 1998). Raising levels of mevalonate pathway molecules stimulates Hmg2p-GFP degradation, whereas Hmg1p-GFP is unaffected. Thus, we would not expect Hmg1p-GFP to undergo degradation in response to GGPP if it is increasing the physiological degradation signal of Hmg2p-GFP (Hampton and Rine, 1994). As predicted, Hmg1p-GFP was not degraded with GGPP treatment (Figure 4-4). Degradation induced by GGPP was specific for Hmg2p-GFP.

Interestingly, geranylgeraniol (GGOH), like farnesol, increased the rate of proteolytic digestion of Hmg2p-GFP in the limited proteolysis assay (Shearer and Hampton, 2005). Moreover, mammalian cells exposed to GGOH have enhanced HMGR degradation (Sever et al., 2003a). Thus, we next directly tested GGOH addition to cells for any effects on Hmg2p-GFP degradation. At every concentration tested (34 µM - 3.4 mM), GGOH did not have any detectable effect on Hmg2p-GFP steady state levels (data not shown). As the alcohol derivative of GGPP, it was surprising that GGOH did not have an effect on Hmg2p-GFP degradation.

Our past studies have conclusively showed Hmg2p degradation is dependent on the HRD pathway and thus mediated by the Hrd1p ubiquitin ligase. To determine if GGPP is promoting Hmg2p degradation through the biologically relevant pathway, we tested the effect of GGPP in *HRD1* or *hrd1Δ* null strains. GGPP was only able to lower steady state levels of Hmg2p-GFP in the *HRD1* strain; it had no effect on Hmg2p-GFP steady state levels in the
Figure 4-4: GGPP did not induce Hmg1p-GFP degradation. Strains expressing Hmg2p-GFP or Hmg1p-GFP were treated with no drug, ZA, vehicle, or GGPP for one hour. GFP fusions were detected by flow cytometry.
$hrd1\Delta$ null (Figure 4-5). GGPP-induced degradation of Hmg2p-GFP was dependent on the HRD pathway.

We next tested if GGPP, like ZA (Bays et al., 2001a), would cause increased ubiquitination of Hmg2p-GFP. Incubation with ZA for a little as ten minutes induces Hmg2p-GFP ubiquitination in intact cells. We tested if GGPP also induced Hmg2-GFP ubiquitination. After we incubated cultures for ten minutes with ZA, GGPP, or vehicle controls, we evaluated Hmg2p-GFP ubiquitination by immunoprecipitation with ubiquitin immunoblotting. Cells were lysed, Hmg2p-GFP was immunoprecipitated with polyclonal anti-GFP antiserum, and the precipitated protein was immunoblotted with anti-ubiquitin or anti-GFP to evaluate Hmg2p-GFP ubiquitination or Hmg2p-GFP levels, respectively. A burst of Hmg2p-GFP ubiquitination was detected in cells exposed to either ZA or GGPP (Figure 4-6). Hmg2p-GFP ubiquitination was similar between ZA and GGPP, implying a similar mechanism for this induction.

Our considerable knowledge of Hmg2p-GFP degradation facilitated the analysis of GGPP and its function as a potential biologically relevant signal for Hmg2p-GFP degradation. We have a collection of Hmg2p variants that do not respond to altered levels of mevalonate pathway intermediates. The ubiquitination and regulated degradation of Hmg2p-GFP is dependent on two critical lysines. Arginine substitution of lysines 6 or 357, result in Hmg2p-GFP variants that are stable even in the presence of high degradation signals (Gardner and Hampton, 1999b). We tested if these lysines were required for
Ubiquitin ligase Hrd1p is required for GGPP-induced Hmg2p-GFP degradation. HRD1 and hrd1Δ cells were incubated with no drug, ZA, vehicle, or GGPP for one hour and Hmg2p-GFP fluorescence was measured by flow cytometry.
**Figure 4-6: GGPP induces Hmg2p-GFP ubiquitination.**
Hmg2p-GFP from cells treated with no drug, ZA, or GGPP were immunoprecipitated with anti-GFP antiserum. Western blots of ubiquitinated Hmg2p-GFP were detected with anti-ubiquitin and levels of Hmg2p-GFP were detected with anti-GFP.
Hmg2p-GFP degradation induced by GGPP. Strains expressing wild-type Hmg2p-GFP or the Hmg2p-GFP mutant strains were incubated with GGPP, and evaluated by flow cytometry. GGPP lowered only wild-type Hmg2p-GFP steady state levels and did not have any detectable effect on the stable mutants of Hmg2p-GFP (Figure 4-7). Two other Hmg2p-GFP variants are degraded by the HRD pathway but are unresponsive to changes in degradation signal. One is misfolded, 6myc-Hmg2p-GFP, and the other, NR1-Hmg2p-GFP, has a more subtle, five amino acid substitution in its sterol-sensing domain (Hampton et al., 1996a; Gardner et al., 1998; Shearer and Hampton, 2005). Both 6myc-Hmg2p-GFP and NR1-GFP are constitutively degraded by the HRD pathway. That is, altering the degradation signal with drugs has no effect on their rates of degradation. GGPP was added to strains expressing these variants to test if GGPP action was specific to wild-type Hmg2p-GFP. Steady state levels of NR1-Hmg2p-GFP and 6myc-Hmg2p-GFP were unchanged when exposed to GGPP in conditions that levels of wild-type dropped (6myc-Hmg2p-GFP not shown; NR1-Hmg2p-GFP Figure 4-7). Taken together, these results show that GGPP causes Hmg2p-GFP degradation in the same manner as the physiological signal derived from FPP.

Raising levels of GGPP outside of the cell by exogenous addition drives Hmg2p-GFP degradation. Our model is that GGPP enters the cell, and then either serves as, or is converted to, the signal for Hmg2p degradation. Thus, we predicted that increasing GGPP levels intracellularly would similarly drive Hmg2p-GFP degradation. There is only one known gene in S. cerevisiae that
Figure 4-7: GGPP specifically induces wild-type Hmg2p-GFP degradation, and not degradation of its variants. Strains expressing wild-type Hmg2p-GFP, NR1-Hmg2p-GFP, K6R-Hmg2p-GFP, or K357R-Hmg2p-GFP were treated with no drug, ZA, vehicle, or 11 μM GGPP for one hour. Flow cytometry was used to measure fluorescence.
synthesizes GGPP, called \textit{BTS1}, which encodes geranylgeranyl synthase (Jiang et al., 1995). Bts1p catalyzes the condensation of FPP with IPP to make GGPP (Figure 4-1). We introduced a galactose-inducible promoter-driven \textit{BTS1}, to cells expressing Hmg2p-GFP. Placement of cells with this plasmid in galactose-containing medium causes activation of the strong \textit{GAL} promoter, resulting in elevation of the Bts1p enzyme. Yeast cells were either grown in glucose or galactose, and levels of Hmg2p-GFP fluorescence were analyzed by flow cytometry. Cultures grown in galactose had lower steady state levels of Hmg2p-GFP. The degradation effect was reversible with lovastatin treatment, indicating that inhibition of HMGR activity inhibited downstream GGPP production by Bts1p (Figure 4-8). Galactose did not have any effect on Hmg2p degradation in the empty vector control strain (data not shown). Thus, both internal and external sources of GGPP induce Hmg2p-GFP degradation.

The structure of squalene synthase inhibitor ZA is similar to the squalene synthase (\textit{ERG9}) substrate FPP. GGPP is similar to FPP as well, only different by the addition of the 5-carbon IPP to the FPP structure. We hypothesized that GGPP affected Hmg2p-GFP stability in the same manner as ZA, by inhibiting squalene synthase, and thus accumulating FPP and driving Hmg2p degradation (Hampton and Bhakta, 1996; Gardner and Hampton, 1999). Work from our laboratory had previously demonstrated that potency of ZA on Hmg2p stability was strongly diminished by elevating the expression of its target Erg9p. \textit{ERG9} expression from the strong \textit{TDH3} promoter leads to
Figure 4-8: Overexpression of GGPP synthase gene, \textit{BTS1}, lowers Hmg2p-GFP steady-state levels. Strains expressing GAL-driven Bts1p were grown in either glucose (Non-induced) or galactose (GAL). A portion of the induced strain (GAL) was incubated for the final four hours with lovastatin. Hmg2p-GFP fluorescence was measured by flow cytometry.
higher levels of Erg9p that require a higher concentration of ZA to stimulate Hmg2p-GFP degradation (Gardner and Hampton, 1999b). We thus tested if increased squalene synthase would similarly raise the effective concentration of GGPP. In contrast to ZA, elevation of Erg9p did not shift the dose-response curve of GGPP in both wild type and overexpressing ERG9 strains whereas it did shift that of ZA in the high ERG9 strain (Figure 4-9). GGPP was acting through a separate mechanism from ZA.

GGPP is synthesized from the condensation of FPP with IPP. The only protein known to catalyze this reaction in S. cerevisiae is geranylgeranyl pyrophosphate synthase, or Bts1p. If GGPP is the signal for Hmg2p-GFP degradation, eliminating BTS1 is predicted to stabilize Hmg2p-GFP. BTS1 is a non-essential gene therefore we generated a bts1Δ null strain. As previously reported, the bts1Δ null was found to show cold-sensitive growth (17°C) (Jiang et al., 1995). We tested the dependence of Bts1p activity in Hmg2p-GFP degradation by incubating BTS1 and bts1Δ null cells with cycloheximide, which inhibits protein synthesis. Examination by flow cytometry showed both BTS1 and bts1Δ strains degraded Hmg2p-GFP (Figure 4-10). However, Hmg2p-GFP degradation was approximately two-fold slower in the bts1Δ null strain. We also tested Hmg2p-GFP accelerated degradation by ZA treatment in both the BTS1 and the bts1Δ null strains. Hmg2p-GFP degradation was similar between the two strains (Figure 4-10). We interpret Hmg2p-GFP degradation in the bts1Δ null as GGPP production by an enzyme distinct from Bts1p.
Figure 4-9: Overexpression of squalene synthase did not shift the GGPP dose response. Cells with either native (ERG9) or TDH3-driven squalene synthase (PTDH3-ERG9) were treated with various concentrations of ZA or GGPP, as indicated, for one hour. Hmg2p-GFP was detected by flow cytometry.
Figure 4-10: Hmg2p-GFP is degraded in a bts1Δ null. BTS1 and bts1Δ null strains expressing Hmg2p-GFP were treated with ZA or cycloheximide (CX) for one hour. Flow cytometry was used to measure Hmg2p-GFP fluorescence.
We would expect exogenous application of GGPP to have a strong degradation effect on Hmg2p-GFP in cells with low levels of degradation signal. Hmg2p stability is determined the level of degradation signal set by the genetic background. As mentioned, *S. cerevisiae* has two HMGR isozymes, Hmg1p and Hmg2p. Native Hmg1p is stable and is expressed at higher levels than native Hmg2p. When cells express only native Hmg2p as the essential HMG-CoA reductase source, Hmg2p-GFP steady state levels are very high, due to the low levels of degradation signal (Cronin and Hampton, unpublished observation). Cells expressing Hmg2p-GFP in a strain with only *HMG2* were incubated with either ZA or GGPP, and analyzed by flow cytometry. Unexpectedly, GGPP did not have any effect on Hmg2p-GFP steady state levels whereas ZA did trigger Hmg2p-GFP degradation (Figure 4-11). Even GGPP concentrations 10-fold higher did not have an effect on Hmg2p-GFP degradation (data not shown). There are many plausible reasons GGPP did not have an effect on Hmg2p-GFP degradation in a strain expressing *HMG2* only. The simplest is that GGPP did not gain entrance into cells of this background. There are numerous examples of perturbations of the sterol pathway causing altered and lower permeability of the yeast (Umebayashi and Nakano, 2003).

We devised a distinct, acute way of lowering the mevalonate pathway flux to test for direct action of added GGPP, by use of the HMGR inhibitorLovastatin. Strains were grown in the presence of Lovastatin for either four hours or two hours. They were divided, with half incubated with ZA and the
Figure 4-11: GGPP does not induce Hmg2p-GFP degradation in a hmg1Δ HMG2 strain. Cells expressing Hmg2p-GFP were incubated for one hour with ZA, control, or GGPP.
other half with GGPP during the final hour of incubation prior to flow cytometry analysis. Control strains not exposed to lovastatin were responsive to both ZA and GGPP for both time incubations (Figure 4-12, top histogram). After four hours of lovastatin treatment, both ZA and GGPP incubation could not induce Hmg2p-GFP degradation (data not shown). However, after two hours of lovastatin incubation, ZA did not stimulate Hmg2p-GFP degradation whereas GGPP did (Figure 4-12). We were not surprised that ZA could not exert an effect in strains treated with the upstream HMGR inhibitor lovastatin because ZA action is dependent on the build-up of the mevalonate pathway product FPP. The fact that GGPP did induce Hmg2p-GFP degradation in cells treated with two hours of lovastatin treatment, supports the idea that GGPP can directly mediate degradation. In contrast, lowering the degradation signal chronically, incubating with lovastatin for four hours or solely expressing HMG2 as the reductase source, prevents GGPP action. How higher concentrations and longer incubations of GGPP affect chronic signal inhibition remains to be determined. These results indicate ZA and GGPP act through different mechanisms to drive Hmg2p-GFP degradation.

We have extensively analyzed how Hmg2p-GFP is degraded specifically by GGPP in the work presented above. Previously, our lab has demonstrated thoroughly that the regulated degradation of Hmg2p-GFP is comparable to that of full-length, wild-type Hmg2p. To test if this was the case in response to GGPP as well, we exposed strains expressing wild-type Hmg2p, tagged with a single myc epitope, and its stable K6R version, to
Figure 4-12: GGPP, and not ZA, stimulates Hmg2p-GFP in cells treated with lovastatin. Cells expressing Hmg2p-GFP were treated for a total of two hours with lovastatin and during the last hour with no drug (Control), ZA, vehicle, or GGPP.
GGPP for one or three hours. Wild-type Hmg2p levels declined and the stable mutant levels remained the same in response to GGPP incubation (Figure 4-13). Thus, GGPP stimulates the degradation of authentic Hmg2p.

**Discussion**

We have made a strong case that GGPP’s effect on Hmg2p degradation operates through the same mechanisms of physiological regulation. Hmg2p-GFP was ubiquitinated in response to exogenously added GGPP, and its degradation was *HRD* dependent. Furthermore, variants of Hmg2p-GFP not affected by degradation signal levels were also not affected by GGPP. Increasing expression of the enzyme that synthesizes GGPP, Bts1p, also drove Hmg2p-GFP degradation. GGPP action could be distinguished from that of ZA. GGPP did not exert its effect by inhibiting squalene synthase as did ZA. Moreover, acute inhibition of the mevalonate pathway prevented ZA action, but not GGPP action. Chronic inhibition of the mevalonate pathway blocks the effect of exogenous GGPP, perhaps through altered plasma membrane permeability. Surprisingly, Bts1p was not required for ZA-stimulated degradation. However, the rate of Hmg2p-GFP degradation is slower in a *bts1Δ* null. Our studies have uncovered GGPP or a derivative of GGPP as the degradation signal of Hmg2p-GFP and full-length Hmg2p.

Our discovery that GGPP is a positive degradation signal for Hmg2p is similar to findings with mammalian HMGR. Mammalian HMGR degradation is enhanced by the addition of GGOH to cells. In vitro, GGOH did not have an
Figure 4-13: GGPP induces full-length 1-myc-Hmg2p degradation. Cells expressing either myc-tagged, full-length wild-type Hmg2p or its K6R variant were treated with 0, 1, or 3 hours of 11 μM GGPP. Whole cell lysates were prepared at each time point and Hmg2p was detected by immunoblot with anti-myc.
effect on HMGR ubiquitination (Sever et al., 2003a). It has been proposed that GGOH undergoes processing by an in vivo isoprenyl alcohol salvage pathway to generate GGPP (Crick et al., 1997). GGPP action is speculated to enhance the retrotranslocation of HMGR, by the action of a geranylgeranylated protein (Goldstein et al., 2006). As far as we know, we are the first to report GGPP introduction into yeast cells whereas GGPP uptake by mammalian cells has been documented (Cheng et al., 2002; Danesi et al., 1995). It would be interesting to know if attempts were made to use GGPP to stimulate HMGR degradation in the mammalian cells. The experimental design in mammalian HMGR degradation experiments typically consists of pre-incubation with compactin, an HMGR inhibitor like lovastatin (Sever et al., 2003a). When mevalonate, or GGOH in its place, and sterols are added to the cells, HMGR is strongly ubiquitinated and degraded (Sever et al., 2003b). Mammalian cells pre-treated with compactin may be similar to yeast cells treated with four hours of lovastatin. GGPP could not effect Hmg2p degradation in severe signal-lowering conditions. On the other hand, if there is a conserved mechanism shared between the GGPP induced-degradation of yeast Hmg2p and GGOH-induced degradation of mammalian HMGR, we should add GGOH to yeast cells pre-treated with four hours of lovastatin. The GGOH salvage pathway may be conserved in yeast.

Our model of Hmg2p degradation is that an FPP-derived molecule, possibly GGPP or GGPP metabolite, interacts with Hmg2p in a way to promote its ubiquitination by Hrd1p. Hrd1p is continuously scanning proteins
for potential targeting for degradation, including Hmg2p. When the structure of Hmg2p changes in response to the degradation signal, Hrd1p facilitates E2 Ubc7p binding to Hmg2p and thus Hmg2p ubiquitination and degradation (Gardner et al., 2001b). We have not ruled out the possibility that GGPP also influences Hmg2p retrotranslocation. The in vitro assays described in the preceding chapters will be employed for testing GGPP action.

GGPP is an important molecule involved in cell signaling through its post-translational role in geranylgeranylation. However, we do not believe Hmg2p degradation is dependent on geranylgeranylation. GGPP, or one of its derivatives appears to have a direct effect on the structure of Hmg2p. Previous research from our laboratory has examined Hmg2p structure with the limited proteolysis assay in high and low degradation signal conditions (Shearer and Hampton, 2005). Hmg2p from cells treated with the signal-lowering drug lovastatin was less accessible to proteolytic digestion than Hmg2p from cells treated with the signal-raising drug ZA. The limited proteolysis assay will be used to analyze the structure of Hmg2p after cells are treated with GGPP. Further analysis would be to test direct interaction between Hmg2p and GGPP. Fluorescently labeled GGPP and FPP molecules (Dursina et al., 2006), may facilitate testing if GGPP, and not FPP, specifically interacts with Hmg2p.

We were surprised to see that the bts1Δ null strain supported ZA-stimulated degradation. If GGPP or a derivative of GGPP is signaling degradation, then we expected Bts1p, the only enzyme in S. cerevisiae
directly shown to have geranylgeranyl synthase activity, would be required for Hmg2p degradation. However, it is possible that other enzymes can synthesize GGPP. Recently, studies in *S. cerevisiae*, demonstrated that the cold-sensitive growth of a *bts1*Δ null was complemented by FPP synthase (*ERG20*) overexpression (Ye et al., 2007). If overexpressing *ERG20* complemented *bts1*Δ null cold sensitivity, then it is not unimaginable that ZA treatment still works in the *bts1*Δ null because GGPP can also be produced by FPP synthase. In fission yeast, the FPP synthase fps1 and an FPS synthase-like protein spo9, form a heteromer with GGPP synthase activity (Ye et al., 2007). Although spo9 provided the bulk GGPP synthase activity to the heteromer, a low level of GGPP was still detected in the absence of spo9. spo9− cells were defective in sporulation and exhibited cold-sensitive growth. Reminiscent of *ERG20* overexpression complementing *bts1*Δ null cold-sensitivity, overexpressed fps1 complemented spo9− phenotypes. Cells have managed a way to maintain GGPP synthesis for, likely, its role in geranylgeranylation, which is critical for cell viability.

We are considering two isoprenyl synthases that have homology to Bts1p as potential sources of GGPP synthase activity. Protein BLAST results show that Coq1p has the greatest similarity to Bts1p. Coq1p is the first enzyme in the production of ubiquinone, an important component of the electron transport system (Ashby and Edwards, 1990). The hexaprenyl pyrophosphosphate synthase Coq1p is believed to bind either GGPP or FPP, and add IPP until hexaprenyl pyrophosphate is formed, and does not use GPP
or DMAPP as substrates (See Figure 4-1). Not surprisingly, Erg20p is the other protein similar to Bts1p by protein BLAST analysis. The studies mentioned above with FPP synthase overexpression in both baker's and fission yeast, bolster the idea of Erg20p having GGPP synthase activity. Furthermore, purified FPP synthase from chicken mostly catalyzed FPP from GPP and IPP, but also produced GGPP to a small extent (Reed and Rilling, 1976). **ERG20** is an essential gene but its transcription can be lowered when driven by the **MET** promoter, and grown in high levels of methionine (Gardner and Hampton, 1999b). We will generate **bts1Δ** and **coq1Δ** single nulls and double null mutants in the **MET**-driven **ERG20** background. When cells are incubated with methionine, we will test if ZA-stimulated degradation is inhibited or stunted, and Hmg2p degradation by GGPP is intact. Separating the effects of FPP from GGPP is complicated given GGPP synthesis is dependent on FPP synthesis.

Chronic lowering of the mevalonate pathway in the four hour lovastatin treatment and in the **hmg1Δ HMG2** strain may inhibit GGPP action through a common mechanism. In conditions with low levels of isoprenoids and sterols, the yeast cell may have altered plasma membrane permeability. This effect of sterol pathway inhibition has been observed in transport of other small molecules. For instance, plasma membrane-localized tryptophan transport activity is lower when the sterol pathway is down modulated (Umebayashi and Nakano, 2003). Preliminary results show Hmg2p-GFP is degraded in **hmg1Δ HMG2** cells incubated with GGPP for two hours, instead of one hour, however,
a time course is still needed. We will be testing if the lovastatin block can also be overcome with longer time exposure to GGPP as well. If GGPP and ZA were distinguishable in longer incubations, our results would strongly suggest GGPP or something derived from it is in fact Hmg2p's degradation signal.

Understanding how GGPP gets in the cell and how it mediates Hmg2p-GFP degradation will be important to further study. If GGPP is stable in agar plate media, a genetic screen may be useful in finding the putative transporter(s) and other genes involved in GGPP action. Hmg2p-GFP has been a useful reporter for screens carried out in our lab. Using a filter-modified projector, we can see GFP fluorescence on plated colonies (Cronin et al., 2000). We would be able to screen the null library or mutagenized cells, looking for colonies that were not responsive to GGPP, and therefore bright. Bright colonies with normal mevalonate pathway activity would hold promising candidate genes.

It is an exciting time for further research into determining what GGPP is doing, how GGPP is made, and how GGPP gets into yeast cells.
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Chapter 4, in part, is a manuscript in preparation as "Degradation of HMG-CoA reductase is stimulated by geranylgeranyl pyrophosphate," Garza, Renee M.; Tran, Peter N.; Hampton, Randolph Y. The dissertation author was the primary investigator and author of this work.
Chapter 5:

Future Directions
We described in vitro assays in Chapter 2 and 3 that have fulfilled numerous biological criteria and unveiled interesting aspects of Hmg2p ubiquitination and retrotranslocation.

Our approach was to control Hmg2p ubiquitination by generating strains that separate the expression of known factors necessary for in vivo Hmg2p ubiquitination. Some of the components are membrane-bound and others are found in the cytosol. When cytosol was combined with microsomes and incubated in the presence of ATP, we observed in vitro Hmg2p ubiquitination.

Our analyses suggest certain aspects of Hmg2p regulated ubiquitination and retrotranslocation were lost when Hrd1p was overexpressed. Native levels of Hrd1p do not support detectable levels of retrotranslocation and ubiquitination of wild-type Hmg2p. In future studies it will be necessary to employ more sensitive methods of detection. The in vitro retrotranslocation of another ERAD substrate, ste6-166, has been successfully detected in strains with native levels of its E3 Doa10p, using radioactive iodinated ubiquitin (Nakatsukasa et al., 2008). Radioactive ubiquitin will also allow a greater level of sensitivity for definitively testing if direct addition of putative degradation signals to in vitro reactions with native Hrd1p levels stimulate Hmg2p in vitro ubiquitination.

In pursuit of in vitro regulated degradation, our attention turned to in vivo analysis in Chapter 4. We discovered GGPP stimulates Hmg2p degradation and this will be an active area of research in the future.
As in every scientific endeavor, the understanding of Hmg2p ERAD is a work in progress. However, the work presented in this thesis has led to many ways in which Hmg2p degradation and ERAD in general can be studied.

**Mass Spectrometry of Retrotranslocated Hmg2-GFP**

We described the retrotranslocation of the multiubiquitinated, full-length Hmg2p-GFP. Mass spectrometry of the retrotranslocated Hmg2p-GFP should help identify factors involved in maintaining this multi-spanning transmembrane protein in the soluble, supernatant fraction, before proteasomal degradation. Potential candidates include the shuttling factors Rad23p and Dsk2p. Each contains Uba domains that bind ubiquitin (Kang et al., 2006) and Ubl domains that bind the proteasome (Elsasser et al., 2002; Saeki et al., 2002). Our in vitro results demonstrated that Rad23p and Dsk2p were required for Hmg2p-GFP retrotranslocation. This was surprising and different than what was seen with lumenal ERAD substrate, CPY*, which accumulates in the cytosol in the absence of Rad23p and Dsk2p (Medicherla et al., 2004). To distinguish ubiquitin-binding proteins from Hmg2p-binding proteins, the supernatant fraction should be either untreated or pre-treated with the non-specific deubiquitinating enzyme before mass spectrometry analysis. It will be interesting to see if there are two classes of associated proteins, ones that bind multiubiquitin chains and others that are specific to Hmg2p-GFP, or other HRD pathway substrates. Many factors have been identified in Hmg2p ERAD and their functions will be revealed with continued in vitro study.
The Identification of the Retrotranslocon

Clearly, a retrotranslocon is necessary for lumenal ERAD substrates but it remains to be determined if multi-spanning proteins such as Hmg2p, require a channel for their retrotranslocation. Using the in vitro retrotranslocation assay, we tested candidate retrotranslocons.

Sec61p is vital for anterograde translocation into the ER and for many years, was the prime candidate for mediating retrotranslocation. However, direct evidence of its involvement has been lacking. In our analysis, we used the sec61-2 mutant to test if Sec61p was necessary for in vitro retrotranslocation. In vivo Hmg2p-GFP degradation is approximately two-fold slower in the sec61-2 (Sato and Hampton, 2006), however Sec61p does not seem to be involved in Hmg2p-GFP retrotranslocation using this same mutant. There is evidence that ribosomes compete with proteasomes for binding to Sec61p (Ng et al., 2007; Kalies et al., 2005). The Romish lab generated mutants of Sec61p that cannot bind proteasomes. These mutants may be more suitable for testing Sec61p involvement in the retrotranslocation assay.

The derlins appear to play a key role in the retrotranslocation of many ERAD substrates in mammalian cells (Lilley and Ploegh, 2005; Ye et al., 2005). However, derlin homologues, Der1p and Dfm1p, were not required for Hmg2p-GFP retrotranslocation. Our lab had previously tested the role of these proteins in vivo and found that Dfm1p was not required for the
degradation of many model ERAD substrates, including Hmg2p-GFP (Sato and Hampton, 2006). Der1p has long been known to be involved in the in vivo degradation of the lumenal substrate CPY* (Knop et al., 1996), but not Hmg2p.

More recently, it has been proposed that E3s mediate retrotranslocation. The self-degrading fusion protein described in Chapter 3, consisting of the catalytic domain of Hrd1p attached to the stable Hmg1p transmembrane domain did not require Hrd1p for its retrotranslocation. In vivo, Doa10p and Hrd1p were also not necessary for its degradation. Our results did not support the idea that E3s mediate retrotranslocation.

A combination of in vivo and in vitro assays will be necessary for identifying the retrotranslocon(s). Having this in vitro retrotranslocation assay in our arsenal will be useful in testing more potential transmembrane protein candidates.

**Hmg2p degradation signal**

In Chapter 4, we presented evidence that GGPP or a derivative of GGPP is the signal for Hmg2p degradation. We were surprised that direct addition of GGPP to cells led to the ubiquitination and degradation of Hmg2p. This observation has led to many potential areas of investigation such as how GGPP enters the cells, how GGPP promotes Hmg2p degradation, and how the cell synthesizes GGPP.

A fundamental concern is whether there are multiple enzymes in *S. cerevisiae* that synthesize GGPP. Hmg2p was degraded in response to ZA,
even in a strain lacking the only known GGPP synthase Bts1p. If there is
another source of GGPP synthase activity then our results would be explained
and if no other source is detected then it would imply that more than one FPP
derived molecule is able to accelerate Hmg2p degradation. The
complementation of bts1Δ null phenotypes by FPP synthase (Erg20p)
suggests Erg20p may be the source of GGPP in our studies (Ye et al., 2007).

The direct way in which GGPP can be added to cells was a
breakthrough for our Hmg2p regulation studies. Up until now, only genetic
and pharmacological means were used to manipulate the degradation signal.
This more direct effect that GGPP seems to have on Hmg2p degradation
makes GGPP an obvious molecule to add to in vitro Hmg2p ubiquitination
reactions. The lack of in vitro regulated ubiquitination may be attributed to
overexpressed levels of Hrd1p and/or conditions that prohibit its detection.
However, with future studies involving native levels of Hrd1p, examination of
Hmg2p in vitro ubiquitination with GGPP will be imperative.

High levels of microsomal Hrd1p were necessary to observe Hmg2p-GFP in vitro ubiquitination. Even so, the percentage of Hmg2p-GFP that is
ubiquitinated in vitro is estimated to be approximately 1-2% of the total
Hmg2p-GFP in the microsomes. The reason why Hrd1p cannot recruit more
Hmg2p-GFP for ubiquitination may be due to the lack of a cell biological
context. This "cell biological" model was suggested by the in vivo immuno-
fluorescence of Hmg2p with Hrd1p (Federovitch and Hampton, manuscript
submitted). Misfolded Hmg2p variants co-localized with Hrd1p whereas wild-
type Hmg2p excluded Hrd1p. Therefore, a limited amount of Hmg2p-GFP may be accessible to Hrd1p in the in vitro assay. Furthermore, breaking up the ER into microsomes may inhibit Hmg2p movement into regions that Hrd1p localize, even in response to degradation signal. Permeabilized cells may need to be explored for use in the in vitro assay.

**Closing Remarks**

Understanding the regulation of this natural ERAD substrate may uncover mechanisms that the degradation machinery utilizes for the recognition of substrates and thus, may be valuable for therapeutic usage in the treatment of protein quality control maladies.

Figure 5-1 proposes a model of Hmg2p degradation that incorporates contributions made by the work in this thesis and present ERAD models.
Figure 5-1: A proposed model for Hmg2p ERAD. A. Elevated levels of GGPP induce a structural change in Hmg2p. E3 Hrd1p recognizes Hmg2p and bridges the interaction between E2 Ubc7p and Hmg2p, thus promoting ubiquitination of Hmg2p. Other ATP-dependent factor(s) may be involved in Hmg2p recognition (not shown). B. Many factors are recruited to ubiquitinated Hmg2p and Hrd1p (not shown). Rad23p/Dsk2p (dark green) bind the ubiquitin chain with their Uba domains. Ubx2p is the docking site for the Cdc48p complex. Cdc48p and co-factors Ufd1p and Npl4p bind the ubiquitin chain. Ufd2p binds to Cdc48p and elongates the ubiquitin chain. Rad23p/Dsk2p bind Ufd2p with their Ubl domains.
Figure 5-1 Continued: A proposed model for Hmg2p ERAD.

C. Factor binding and AAA ATPase action of Cdc48p are necessary to extract ubiquitinated Hmg2p from the ER membrane. A channel may be involved in this process. Unknown chaperones keep Hmg2p soluble in the cytoplasm. Rad23p/Dsk2p help target Hmg2p to 26S proteasome. The proteasome degrades Hmg2p.
Appendix 1:

Materials and Methods
Materials

Lovastatin and zaragozic acid were gifts from Merck. GGPP, AMP-PNP, and MG132 was purchased from Sigma Aldrich. ATP was acquired from Pharmacia Biotech. Cycloheximide was obtained from Fisher Scientific. Rabbit polyclonal anti-GFP, anti-LOOP, and anti-Hrd1p antisera were prepared in collaboration with Scantibodies, Inc. Monoclonal mouse anti-GFP was obtained from BD Biosciences. Mouse anti-HA ascites fluid was purchased from Covance. Monoclonal mouse anti-ubiquitin was obtained from Invitrogen. 9E10 cell culture supernatant was prepared in our lab from hybridoma cells (ATCC). Goat anti-mouse conjugated with HRP was purchased from Jackson ImmunoResearch. Protein A Sepharose CL-4B was acquired from Pharmacia Biotech. Western Lightning chemiluminescence immunodetection reagents were purchased from Perkin Elmer. Ubiquitin-specific protease Usp2-cc was a gift from the Kopito lab. GAL-driven BTS1 was a gift from the Matsuda lab. GST-ubiquitin and ubiquitin were obtained from Sigma Aldrich. Protran BA 85 nitrocellulose (0.45 µM) was made by Schleisicher and Schuell.

Strains and Media

All strains described were derived from the same genetic background used in our previous work and grown at 30°C with aeration by shaking or rotation (Hampton and Rine, 1994). For in vitro ubiquitination and retrotranslocation, microsome donor strains were grown to log phase (OD600<0.35) in minimum medium with 2% glucose and appropriate amino
acid supplements. Cytosol donor strains were typically grown in YPD medium (OD600<1.2), and as indicated, in synthetic complete (SC) medium (OD600<0.6). Lovastatin pre-treatment of microsome donor strains and cytosol donor strain grown in SC was up to 12 hours with 25 µg/mL lovastatin and for cytosol grown in YPD was 250 µg/mL lovastatin for 12 hours. Cultures used for flow cytometry or whole cell lysate preparation were grown to log phase in minimum medium with 2% glucose and appropriate amino acid supplements. Glucose was replaced with galactose to induce expression of GAL-driven Bts1p. For in vivo analysis, lovastatin concentration was 25 µg/mL for indicated times and zaragozic acid was 10 µg/mL for one hour in flow cytometry experiments, 10 minutes in in vivo ubiquitination, and as indicated for time course. Geranylgeranyl pyrophosphate was typically used at 11 µM except where indicated, for one hour. Vehicle control was prepared with 7 parts methanol and 3 parts 10 mM NH₄OH.

Standard yeast techniques were used to integrate plasmids. All GFP fusion plasmids were linearized with Stul and integrated at the ura3-52 locus. HRD1 constructs PTDH3-HRD1, PHRD1-HRD1, and PTDH3-HRD1-C399S (RING mutant) were linearized with BsgI and integrated at the trp locus. For in vitro ubiquitination and retrotranslocation strains deletions of PEP4, HRD1, and UBC7 were each plasmid mediated. The remaining gene deletions were PCR product-mediated. Primers were designed to incorporate sequences specific to the 5' and 3' genomic regions of gene to be deleted into sequences that flank either the geneticin resistance marker or the nourseothricin
resistance marker, which are on plasmids, pRH728 and pRH1838, respectively. PCR was used to confirm deletion and when possible, restrictive temperature sensitivity. Mutants $cdc48-2$ and $sec61-2$ were introduced into strains using two PCR products. One PCR product is an amplification of the mutant allele synthesized with a primer that carries sequence that overlaps with the nourseothricin resistance marker PCR product. The second PCR product is an amplification of the nourseothricin resistance marker with a primer that carries sequence found downstream of the mutant allele. Both PCR products are introduced to the cell by standard transformation techniques. Confirmation of mutant allele strains was by temperature sensitivity at 35°C and PCR.

**In vitro ubiquitination**

Microsomes from a $ubc7\Delta$ null yeast strain expressing 3HA-Hrd1p from the $TDH3$ promoter or $HRD1$ promoter and Hmg2p-GFP or variants from the $TDH3$ promoter were prepared by glass bead lysis. For each 10 OD$_{600}$ units of log-phase cells, 200 µl of MF buffer (20 mM Tris pH 7.5, 100 mM NaCl, 300 mM sorbitol, with the following protease inhibitors (PIs): 1 mM PMSF, 260 mM AEBSF, 100 mM leupeptin hemisulfate, 76 mM pepstatin A, 5 mM e-aminocaproic acid, 5 mM benzamidine, and 142 mM TPCK) was added. At 4°C, cells were lysed using hand vortexing for 6 x 1 minute intervals with 1 minute intervals on ice between each vortexing. The lysate was collected and pooled with two bead rinses with MF to give the crude microsomal lysate. The crude lysate was centrifuged for 5 seconds at room temperature at full speed
(16,000 xg). The resulting supernatant was transferred to a fresh tube. This was repeated until no cellular debris could be seen. Next the microsomes were pelleted at 14,000 xg for 45 minutes at 4 °C. The pellets are resuspended in B88 buffer (20 mM Hepes pH 6.8, 250 mM sorbitol, 150 mM KOAc, 5 mM MgOAc, 1 mM DTT, and the same cocktail of PIs used in the lysis buffer), to a final concentration of 0.3 OD equivalent units/µL of B88.

Cytosol is prepared from strains overexpressing Ubc7p in a hrd1Δ ubc7Δ double null strain in a similar manner as the Schekman lab (Spang and Schekman, 1998). Control cytosol was prepared in parallel from an otherwise identical ubc7Δ null strain. 500 OD equivalents of cells were pelleted, rinsed twice with water, once with B88 buffer, and resuspended in 500 µL of B88 buffer with PIs. The resuspended cells were transferred to a mortar previously chilled and containing liquid nitrogen. The frozen cells were ground with a pestle until a fine powder resulted, which involves repeated addition of liquid nitrogen and pestle grinding. The frozen powder was transferred to microfuge tube and thawed on ice. Typically, 1 mM ATP from a 500 mM stock solution in H2O pH 7.5 is added, however ATP was not added into AMP-PNP experiments. Once thawed, the crude cytosol lysate was centrifuged at 3000 xg for 5 min, to remove large debris, and then supernatant was centrifuged at 20,000 xg for 15 minutes. The supernatant was then ultracentrifuged at 100,000 xg for one hour. Protein concentration was measured using Bradford reagent. Cytosol concentrations were adjusted with B88 to 25 mg/mL for
ubiquitination and retrotranslocation assays. MG132 was added to a final concentration of 300 µM to microsomes and cytosol separately.

One in vitro ubiquitination reaction typically consisted of 10 µL of microsome resuspension and 12 µL cytosol. ATP (500 mM stock) was added to each reaction to a final concentration of 30 mM. The reactions were incubated for one hour at 30°C. The assay was stopped by solubilization with 200 µl of SUME (1% SDS, 8 M urea, 10 mM MOPs pH 6.8, 10 mM EDTA) with PIs and 5 mM N-ethylmaleimide (NEM). 600 µL immunoprecipitation buffer (IPB: 15 mM Na₂HPO₄, 150 mM NaCl, 2% Triton X-100, 0.1% SDS, 0.5% DOC, 10 mM EDTA, pH 7.5) with protease inhibitors and NEM is added and followed by addition of 15 µL-25 µL rabbit polyclonal antisera (anti-GFP, anti-LOOP, or anti-Hrd1p antisera). Precipitated material was collected by centrifugation and supernatant was then transferred to a fresh tube. Immunoprecipitation (IP) incubation was carried out at 4°C for 12-16 hours with nutation. Protein A sepharose was added to IP and incubated for 2-4 hours at 4°C with nutating. Protein A sepharose with bound protein was washed once with IPB and once with IPW (50 mM NaCl, 10 mM Tris, pH 7.5). Beads were aspirated to dryness and 55 µL 2X urea sample buffer (2XUSB: 8 M urea, 4% SDS, 10% β-mercaptoethanol, 125 mM Tris, pH 6.8). The slurry was incubated at 50°C for 7 minutes. IPs were resolved on 8% SDS-PAGE Tris-glycine gels and transferred to nitrocellulose. For anti-ubiquitin immunoblotting, nitrocellulose membranes were rinsed with water and autoclaved for 30 minutes at liquid setting and 15 minutes at gravity setting.
Blocking and antibody incubations were carried out with Tris-buffered saline containing 0.45% Tween-20 and 20% heat-inactivated bovine serum (Swerdlow et al., 1986). Blots were washed with TBS-HT. For anti-GFP or anti-HA blots, nitrocellulose membranes were blocked with 5% nonfat dried milk in Tris-buffered saline (TBS-T: 10 mM Tris, pH 8.0, 140 mM NaCl, 0.05% Tween 20) and antibody incubations were in 2% non-fat dried milk in TBS-T.

**In vitro Retrotranslocation**

In vitro ubiquitination reactions were scaled up or individual reactions were pooled, for retrotranslocation analysis. The reactions were terminated by 5 mM NEM, not SUME. One reaction equivalent was transferred to one tube designated as total (T) and another reaction equivalent was transferred to another tube that was centrifuged for one hour at 25,000 xg in 4°C cold room. The resulting supernatant (S) was carefully removed and dispensed into a fresh tube containing 200 µL SUME with PIs and NEM and the resulting pellet (P) was resuspended in the same volume as the supernatant with B88 containing PIs and NEM and then solubilized with 200 µL SUME with PIs and NEM. The total reaction was also solubilized with 200 µL SUME with PIs and NEM. Each fraction is immunoprecipitated and detected as described above.

**Non-detergent Immunoprecipitation**

In vitro ubiquitination was carried out and fractions were separated by centrifugation as described above. Instead of adding SUME to supernatant fractions, 800 µL of non-detergent IP buffer (15 mM Na₂HPO₄, 150mM NaCl, 10 mM EDTA) was added. Either pre-immune or anti-GFP antisera was
added and incubated. Protein A sepharose suspended in IPB was washed with non-detergent IP buffer before addition to non-detergent IPs. After addition and incubation with Protein A sepharose, protein-bound beads were washed with non-detergent IP buffer. Immunoprecipitated Hmg2p-GFP was removed from beads by addition of 2XUSB, resolved by 8% SDS-PAGE gels, and detected as described above.

**Ubiquitin-specific protease (Usp2-cc) Experiments**

In vitro ubiquitination and retrotranslocation was carried out as described above, except that leupeptin was not added to any of the buffers used in the preparation of cytosol and microsomes. In vitro ubiquitination reactions were carried out and not terminated by SUME or NEM. The reactions were centrifuged at 25,000 xg and the supernatant fraction was removed and pooled with the other supernatant fractions. The pooled fractions were divided into two tubes. One tube containing 2 reaction equivalents received 5 µL of Usp2-cc (0.8 - 1.6 mg/mL) and the other buffer. The Usp2-cc reactions were incubated for one hour in a 37°C incubator. The reactions were stopped with 200 µL SUME, containing leupeptin, other PIs, and NEM, and processed the same way as described above. Half of each IP was used for detection of anti-ubiquitin and other half was used to detect Hmg2p-GFP with anti-GFP or Hmg1-Hrd1p with anti-HA antibodies.

**GST-ubiquitin experiment**

The microsomes and cytosol were prepared for in vitro ubiquitination as described. Reactions were modified by the addition of either GST-ubiquitin or
ubiquitin to a final concentration of 20 µM. Retrotranslocation and detection was carried out as shown above.

**Whole Cell Lysates**

Preparation of whole cell lysates was previously described (Gardner et al., 1998). Pelleted cells (1 OD equivalent) were resuspended in 100 µL SUME and PIs. 100 µL of acid-washed glass beads were added to suspension and cells were broken by vortexing for 3 x 1 minute intervals with 1 minute intervals on ice between each vortexing. Following addition of 100 µL of 2XUSB, the slurry was incubated at 50°C for 10 minutes. Lysates were clarified by centrifugation for 5 minutes at 16,000 xg. Proteins were resolved by 8% SDS-PAGE and transferred to nitrocellulose blots. 5% nonfat-dried milk in TBS-T was used to block blots and 2% non-fat dried milk in TBS-T was used in antibody incubations. Hmg2p-GFP was detected with anti-GFP and 1myc-Hmg2p was detected with anti-myc hybridoma cell supernatant 9E10.

**In vivo Hmg2p-GFP Ubiquitination**

Whole cell lysates were prepared by cell lysis with glass beads and SUME with PIs and NEM. After vortexing, the crude lysate was removed from beads. The beads were then washed twice with 500 µL IPB with PIs and NEM. The washes were combined with crude lysate. This mixture was centrifuged for 5 minutes at 16,000 xg and the cleared supernatant was transferred to a fresh tube. Anti-GFP was added to the supernatant and precipitates were cleared by centrifugation. The IP was incubated at 4°C with nutating for 12 hours. Protein A sepharose was added to IPs and incubated
for 2-4 hours. Beads were washed once with 1 mL IPB and once with 1 mL IPW. After aspirating beads to dryness, bound proteins were removed by addition of 55 µL 2XUSB and incubation at 50°C for 7 minutes. SDS-PAGE and anti-ubiquitin and anti-GFP immunoblotting were carried out as described above.

**Flow Cytometry**

Flow cytometry was carried out as previously described (Cronin et al., 2000). Yeast were grown in minimum medium with 2% glucose and appropriate amino acids into log phase (OD600 < 0.2). Cultures were incubated with drugs for times indicated. The BD Biosciences FACS Calibur flow cytometer (David laboratory) measured the individual fluorescence of 10,000 cells. The CellQuest software was used to analyze the data and plotted fluorescence vs. cell count histograms.
Table A-1: Strains used in Chapter 2. Background genotype for cytosol and microsome strains in bold.

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Table A-2: Strains used in Chapter 3. Background genotype for cytosol and microsome strains in bold.

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