UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Isolation and Characterization of Genes Required for Endoplasmic Reticulum-Associated Protein Degradation

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

by

Nathan Wesley Bays

Committee in charge:

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Professor Joanne Chory
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2002

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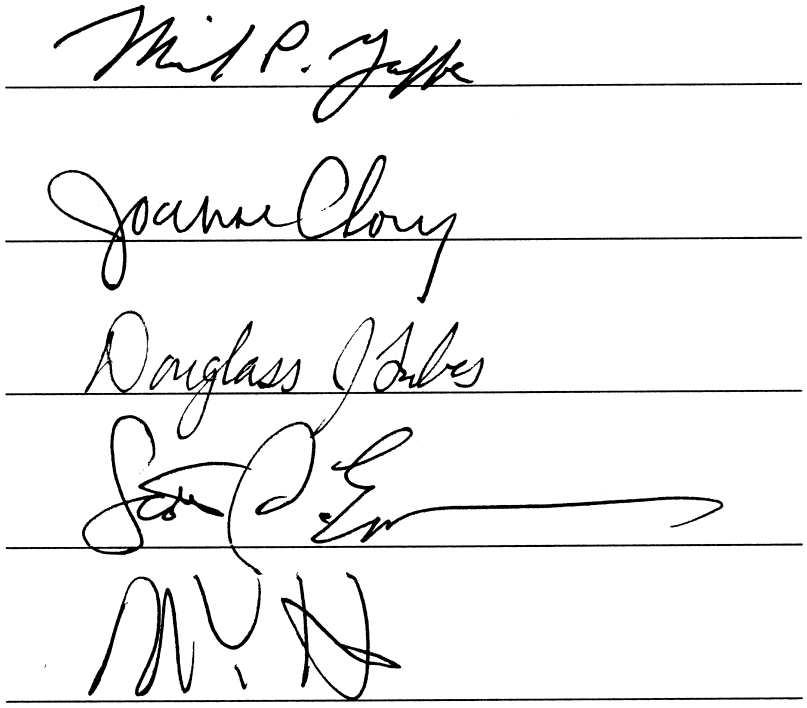

Chair

University of California, San Diego

2002

This work is dedicated

to the memory

of my mother

Marie Elizabeth Bays

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Randy Hampton deserves primary acknowledgement for his devotion to training independent scientists. His most invaluable instruction is by example: Randy has high standards for how science should be performed. Randy also has extensive knowledge of biology and is able to impart this knowledge accurately to others. Randy is exactly what an advisor should be. Even more importantly, Randy is a fantastic person rich with character, experience and compassion. I have confidence Randy will one day have a fitting and more eloquent acknowledgement (hopefully in Sweden), but perhaps this will suffice for now.

The *hrd* selection required a formidable amount of work, especially in the definition of complementation groups. I received invaluable assistance in this effort from Linda Seelig, Ami Goradia, Kelley Hodgkiss-Harlow, and Ngoc Luu. Not only were these individuals scientifically talented, but each unique personality was a joy to work with. This is true of several individuals in the Hampton lab. Stephen Cronin is a prime example, despite his tendency to study "Eeyore Degradation" rather than the ER variety.

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Nathan W. Bays

Professional Experience

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Genetics: Initiated a large-scale genetic screen and developed novel techniques to maximize discovery of new genes in yeast.

Biochemistry: Developed new assays to investigate functions of ubiquitin ligases in vitro. Significantly improved detection methods and assays for a diverse array of applications.

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National Science Foundation Undergraduate Researcher
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Publications

Bays, N., L. Seelig, A. Goradia, K. Hodgkiss-Harlow and R. Hampton "*HRD4/NPL4* is required for proteasomal processing of ubiquitinated ER proteins." 2001. *Molecular Biology of the Cell* **12:**4114-4128

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Gardner, R., G. Swarbrick, N. Bays, S. Cronin, S. Wilhovsky, L. Seelig, C. Kim and R. Hampton "Endoplasmic reticulum degradation requires lumen to cytosol signaling: transmembrane control of Hrd1p by Hrd3p." 2000. *Journal of Cell Biology* **151:**69-82

Mehdy, M., Y. Sharma, K. Sathasivan and N. Bays "The role of activated oxygen species in plant disease resistance." 1996. *Physiologia Plantarum* **98**:365-374

ABSTRACT OF THE DISSERTATION

The Isolation and Characterization of Genes Required
for Endoplasmic Reticulum-Associated Protein Degradation

by

Nathan Wesley Bays
Doctor of Philosophy in Biology
University of California, San Diego, 2002
Professor Randolph Hampton, Chair

The endoplasmic reticulum is a major site for protein degradation, and the substrates for ER-associated degradation (ERAD) include a diverse array of misfolded and regulated proteins. In the degradation of misfolded proteins, ERAD maintains "quality control" over the secretory pathway. For regulated proteins, ERAD can be used to permanently remove proteins like HMG-CoA reductase (HMGR) from the cell (and thus permanently remove their activity). HMGR is a rate-limiting enzyme in sterol synthesis, and its degradation is regulated by a classic feedback loop where an abundance of pathway products signals HMGR degradation. This work describes a large-scale genetic study to identify

genes required for the degradation of HMG-CoA reductase and other ERAD substrates, including high-throughput assays and genetic strategies that greatly enhance the discovery of *HRD* (HMG-CoA Reductase Degradation) genes. So far, 21 *HRD* genes have been identified and are represented by mutants with a diverse array of phenotypes.

The study of *HRD* genes indicates that ERAD proceeds by the ubiquitin-proteasome pathway where the successive addition of ubiquitin to a protein targets it for degradation by the 26S proteasome. The addition of ubiquitin to proteins (ubiquitination) requires a cascade of enzymes, but the E3, or ubiquitin-protein ligase, serves as the key specificity-determining factor. This work describes the identification of Hrd1p as the first E3 known to participate in ERAD. Hrd1p is rate-limiting for ubiquitination and degradation of ERAD substrates *in vivo,* and Hrd1p uses only Ubc7p or Ubc1p to promote *in vivo* ubiquitination. Purified, recombinant Hrd1 protein catalyzes the polyubiquitination of proteins *in vitro,* and Hrd1p shows an apparent preference for misfolded proteins in this *in vitro* action.

Whereas Hrd1p promotes the actual ubiquitination of proteins, other Hrd proteins act downstream of ubiquitination. This work describes the action of Hrd4p at a previously undescribed step in ERAD after ubiquitination but before proteasomal degradation. Hrd4p exists in a complex with Cdc48p and Ufd1p in the cell, and these proteins are also required for ERAD. These data and others suggest that the Cdc48p-Ufd1p-Hrd4p complex acts in the recognition of ubiquitinated proteins and their subsequent presentation to the 26S proteasome.

1

Proteolytic regulation of the proteome

Proteins are not eternal. They are passively and actively degraded into smaller peptides by a variety of processes ranging from spontaneous breakage of the peptide bond to processive degradation catalyzed by large multiprotein complexes (Daniel et al., 1996; Zwickl et al., 1999). In the living cell, protein degradation is a carefully orchestrated process used to tame the proteome through modulation of protein activities and the elimination of misfolded or otherwise damaged proteins (Hochstrasser, 1996; Wickner et al., 1999). Several different pathways degrade proteins in the cell, including the lysosome/vacuole and the 26S proteasome. The lysosome is membrane-bounded organelle readily identified by its acidic pH and abundance of proteolytic enzymes (Holtzman, 1989). The lysosome is a site for the degradation of cellular constituents ranging from plasma membrane receptors to entire organelles, (Klionsky and Emr, 2000; Mullins and Bonifacino, 2001). The importance of the lysosome to cell function is illustrated by several disorders caused by defects in the lysosome function (Watts and Gibbs, 1986; Winchester et al., 2000).

The bulk of non-lysosomal protein degradation is carried out by the 26S proteasome, a large multisubunit protease in the cell (DeMartino and Slaughter, 1999; Hilt and Wolf, 2000; Walz et al., 1998). The 26S proteasome consists of a 20S proteolytic core (Coux et al., 1996; Groll et al., 1997, See Figure 1-3 on page 5) and a 19S "cap" or "regulatory particle" that functions in the presentation of protein substrates to the 20S core (Braun et al., 1999; Glickman et al., 1998a; Glickman et al., 1998b). The 20S pro-

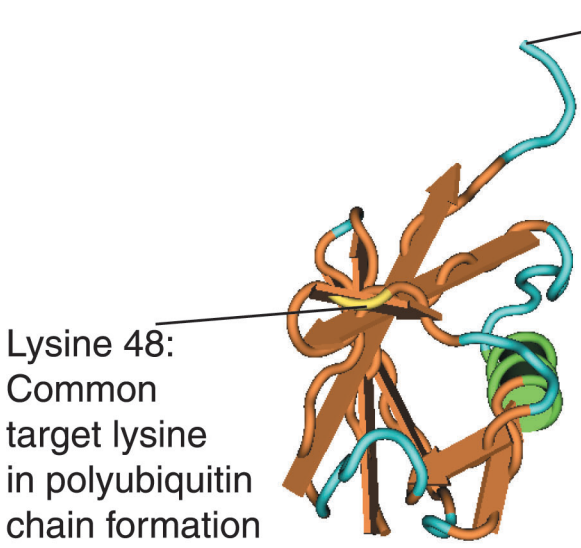

Figure 1-1

The ubiquitin protein

Image rendered using previously published crystallographic data (Vijay-Kumar et al., 1987b) and the Cn3D 3.0 software package (National Institutes of Health, Bethesda, Maryland)

teasome consists of four heptameric rings assembled from proteins closely related in sequence and structure (Groll et al., 1997; Kopp et al., 1997). The four rings of the 20S proteasome create a central cylindrical core where proteolysis occurs (Groll et al., 2000; Groll et al., 1997, See Figure 1-4 on page 6). Most proteins are targeted for proteasomal degradation by a polyubiquitin tag (Thrower et al., 2000). Ubiquitin is an 8 kDa protein that can be covalently attached to proteins and then attached to itself to form polyubiquitin chains that target proteins to the 26S proteasome for destruction (Pickart, 2001; Vijay-Kumar et al., 1987a; Vijay-Kumar et al., 1987b, See Figure 1-1 on page 3 for ubiquitin structure and Figure 1-2 on page 4 for ubiquitin enzymology). A cascade of

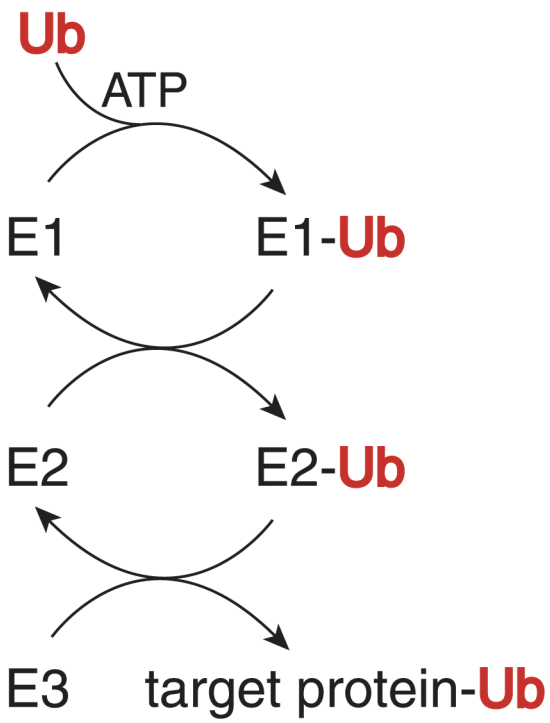

Figure 1-2

Ubiquitin enzymology

Ubiquitin is first covalently attached to a **ubiquitin-activating enzyme (E1)** via a thioester bond. Ubiquitin can then be passed to a cysteine residue in the **ubiquitin-conjugating enzyme (E2)**. The actual ubiquitination of a target protein is catalyzed by a **ubiquitin-protein ligase (E3)** which specifies both the target protein to be ubiquitinated and the E2 that will transfer ubiquitin to a lysine of the target protein.

enzymes is required for the polyubiquitination of target proteins, with a ubiquitin-protein ligase (or E3) serving as the key specificity-determining factor (Figure 1-2 on page 4 summarizes ubiquitin enzymology and Chapter 3 discusses ubiquitin enzymology in more detail beginning with the section "The enzymology of ubiquitination" on page 140. See also Hershko et al., 1983; Pickart, 2001; Weissman, 2001).

It has long been assumed that the proteasome itself can recognize polyubiquitin chains, despite the fact that the only proteasome subunit implicated in polyubiquitin chain recognition is dispensable for proteasome function (Fu et al., 1998; van Nocker et al., 1996). New data included in this work suggest that, at least for protein degradation

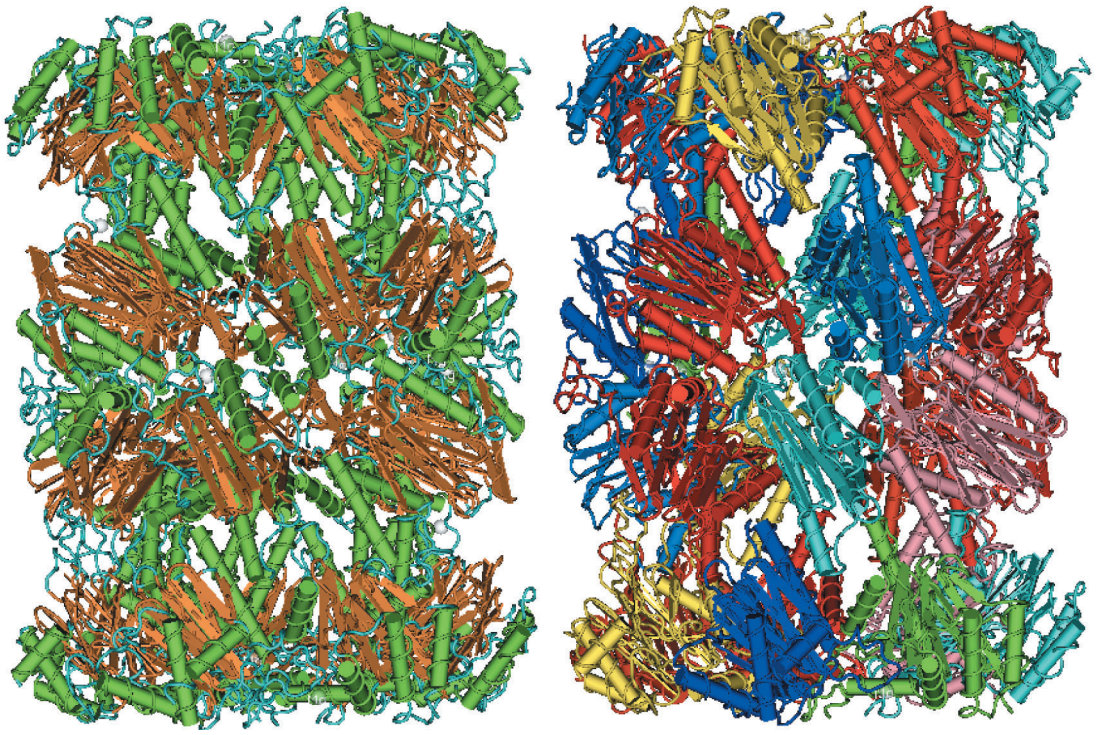

Figure 1-3

The 20S proteasome

Image rendered using previously published crystallographic data (Groll et al., 2000) and the Cn3D 3.0 software package (National Institutes of Health, Bethesda, Maryland). The left image is colored by secondary structure with alpha-helices green, beta-sheets brown, and random coils blue. The right image is colored by individual protein subunit.

at the endoplasmic reticulum, the 26S proteasome is not capable of recognizing ubiquitinated proteins by itself and requires other factors (see Chapter 4). Discerning how proteins are targeted for the degradation by the 26S proteasome is no doubt complicated by the impressive array of proteins that manage to be degraded by the proteasome. In gen-

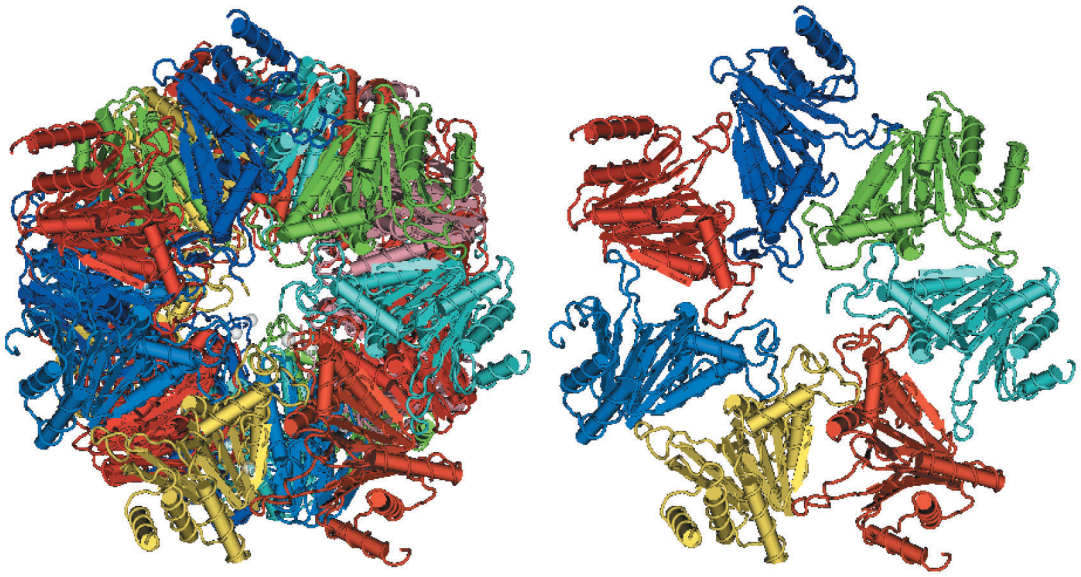

Figure 1-4

The central proteolytic core of the 20S proteasome

The same crystallographic data was used as in Figure 1-2, but a top down view of the proteasome is rendered (left) along with a top down view of only one ring (alpha subunits, right).

eral, these proteins fall into two broad categories: misfolded protains and regulated proteins.

Misfolded proteins are degraded as a means of quality control

Cells contain a large number of proteins devoted to detecting and removing misfolded proteins. Recent studies have revealed that the protein folding and degradation machineries do not even wait for proteins to be fully translated before making the decision to eliminate proteins from the cell via ubiquitination and processive degradation by the 26S proteasome (Schubert et al., 2000; Turner and Varshavsky, 2000). Since many,

if not most, nascent polypeptides are associated with chaperones (Hardesty and Kramer, 2001; Hardesty et al., 1995; Kolb et al., 1995), this cotranslational monitoring of protein folding is likely dependent on the nature of chaperone interaction with nascent polypeptide chains. However, the criteria required to either spare or doom nascent polypeptide chains are not clear. In fact, it is not even clear whether quality control is the sole reason for this cotranslational ubiquitination and degradation. Because the products of proteasomal degradation are presented by MHC proteins to immune cells as antigen, it might be argued that cotranslational ubiquitination and degradation exist to produce a somewhat random sample of selfantigen (Yewdell et al., 2001). This argument is complicated, however, by observations of very active cotranslational ubiquitination and degradation in single cell organisms (Turner and Varshavsky, 2000). It appears more likely that cotranslational ubiquitination and degradation of proteins occurs because of errors in protein synthesis and that the immune system has conveniently taken advantage of this inherent error rate to gain a supply of antigen. Unfortunately, investigation into the compelling questions raised by the degradation of nascent polypeptides is complicated by the rapid nature of cotranslational degradation as well as the daunting number and heterogeneity of ribosomal-polypeptide products in the cell at any one time.

Fully-translated but misfolded proteins are also subject to degradation (Table 1-1 on page 9). Such proteins include mutant proteins that fail to fold properly or even proteins that fail to fold at a sufficiently rapid rate (Ellgaard et al., 1999; Wickner et al., 1999). Like cotranslational degradation, proper folding of fully translated proteins is facilitated,

monitored, and even timed by chaperone-target protein interaction (Ellgaard et al., 1999; Hebert et al., 1996; Lehrman, 2001; Ritter and Helenius, 2000; Wickner et al., 1999). However, the role for chaperones in the degradation of fully folded proteins becomes less clear for proteins that have passed their initial "folding stage" and are no longer regularly associated with chaperones. Although several full-length misfolded proteins are found associated with chaperones, there is conflicting data about whether chaperones are actually required to target proteins for degradation by the 26S proteasome (Bennett et al., 1998; Knop et al., 1996b; Mancini et al., 2000; McCracken and Brodsky, 1996; Parlati et al., 1995). This leaves open the critical question of how misfolded proteins are recognized as misfolded and consequently targeted for degradation. Chapter 3 of this work raises the intriguing possibility that the Hrd1 ubiquitin ligase may actually possess the ability to recognize misfolded proteins and target them for proteasomal degradation – raising the idea that ubiquitin protein ligases and their associated proteins may play a major role in scanning the proteome for misfolded proteins and targeting them for degradation by the 26S proteasome.

Protein	Normal Location	Site of degradation	References
Carboxypeptidase Y* (CPY*, Prc1-1p) ** indicates mutant protein*	Vacuole/Lysosome	ER	Bordallo et al., 1998; Finger et al., 1993; Hiller et al., 1996
Uracil Permease* (UP*, Fur4-430p)	Plasma Membrane	ER	Galan et al., 1998; Wilhovsky et al., 2000
Ste6p* (Ste6-166p)	Plasma Membrane	ER	Loayza et al., 1998
Dipeptidyl-peptidase IV* (DPP-IV*)	Plasma Membrane	ER	Tsuji et al., 1992
α1-antitrypsin* (mutant forms)	Secreted	ER	Qu et al., 1996; Teckman et al., 2000
Vph1p (when not assembled into ATPase)	Mitochondria	ER	Hill and Cooper, 2000
T-Cell Receptor (unassembled α,β chains)	Plasma Membrane	ER	Huppa and Ploegh, 1997; Lee, 1998; Yang et al., 1998; Yu and Kopito, 1999
MHC, Class I protein (misfolded)	Plasma Membrane	ER	Hughes et al., 1997
MHC, Class I protein (upon CMV infection)	Plasma Membrane	ER	Shamu et al., 1999; Wiertz et al., 1996a; Wiertz et al., 1996b
CD4 protein (upon HIV infection)	Plasma Membrane	ER	Fujita et al., 1997; Schubert et al., 1998
Opioid Receptor (δ; misfolded forms)	Plasma Membrane	ER	Petaja-Repo et al., 2001
Sec61-2p*	ER	ER	Biederer et al., 1996; Sommer and Jentsch, 1993

Table 1-1

Examples of quality-control substrates for the ubiquitin-proteasome pathway

Regulation by destruction

The activity of a protein can be modulated by a variety of mechanisms including reversible modifications like phosphorylation and acetylation (Berger, 2001; Hunter, 1998; Nakatani, 2001). The specific degradation of proteins is also a very effective means of regulation as it ensures a rapid and irreversible removal of a protein (and its activity) from the cell. Indeed, a rapidly increasing body of data details the use of ubiquitin-mediated proteasomal degradation in a broad array of regulatory axes including cell cycle progression and checkpoint control, transcriptional regulation and the feedback regulation of metabolic pathways. Table 1-2 on page 11 summarizes several examples of protein degadation in the regulation of diverse proteins. In each of these examples, protein degradation is a highly specific means of regulation where specific cues signal the ubiquitination of specific proteins, targeting them for degradation by the 26S proteasome.

Protein	Cell Process	Cues for Degradation	E3 Involved	References
Cyclin E	Cell Cycle (G1/S transition)	Autophosphorylation of cyclin E by cyclinE-CDK2. Phosphorylated cyclin E is then recognized by the SCF^{CDC4} ubiquitin-protein ligase.	SCF Complex with CDC4 as F box protein (SCF = Skp1-Cullin-F Box)	Dealy et al., 1999; Glotzer et al., 1991; Hershko et al., 1991; Moberg et al., 2001; Skowyra et al., 1999; Strohmaier et al., 2001
Cyclin B	Cell Cycle (G2/M transition)	A complex regulatory network including modulation of Cdc20p and Cdh1p expression as well as regulation of activity by the metaphase spindle checkpoint. Activation of the APC also regulated by cyclinB-CDC2/CDK1 phosphorylation	APC with Cdh1p/ Hct1p as substrate receptor. (APC = Anaphase-Promoting Complex)	Aristarkhov et al., 1996; Kotani et al., 1999; Listovsky et al., 2000; Schwab et al., 2001; Sudakin et al., 1995; Visintin et al., 1997; Yudkovsky et al., 2000; Zachariae et al., 1998
Pds1p/ Securin	Cell Cycle (Anaphase Inhibitor)	As above, regulation of the APC substrate receptors/activators Cdc20p and Cdh1p.	APC with Cdc20p as substrate receptor.	Gmachl et al., 2000; Hilioti et al., 2001; Salah and Nasmyth, 2000; Schwab et al., 2001
Sic1	Cell Cycle (CDK Inhibitor)	Phosphorylation by cyclinE-CDC25 at the G1/S transtition	SCF complex with CDC4 as the F Box protein	Feldman et al., 1997; Lyapina et al., 1998; Seol et al., 1999; Skowyra et al., 1997; Verma et al., 1997

Table 1-2

Proteins regulated by degradation via the ubiquitin-proteasome pathway

MyoD	Transcription Factor (myoblast)	Phosphorylation by cyclinE-CDK2 at G1/S transition	Large nuclear-localized E3 of unknown identity.	Abu Hatoum et al., 1998; Breitschopf et al., 1998; Floyd et al., 2001; Tintignac et al., 2000
p53	Cell Cycle Checkpoint	Many factors can regulate MDM2-p53 association including phosphorylation of p53 (abrogates binding), cellular localization of MDM2, and degradation of MDM2 itself. MDM2 activity is also regulated by association with other proteins and its own phosphorylation.	MDM2	Craig et al., 1999; Fang et al., 2000; Fuchs et al., 1998; Honda and Yasuda, 1999; Jackson and Berberich, 2000; Mayo et al., 1997; Stad et al., 2001; Strachan et al., 2001; Tanimura et al., 1999; Tao and Levine, 1999; Unger et al., 1999
E2F-1	Cell Cycle Checkpoint	Dissociation with the retinoblastoma gene product (pRB) caused by phosphorylation of pRB	Not known, but some hint for SCF complex	Campanero and Flemington, 1997; Hateboer et al., 1996; Hofmann et al., 1996
HMG-CoA reductase	Metabolism; Sterol Synthesis	Accumulation of mevalonate pathway intermediates	Hrd1p	Bays et al., 2001; Gardner and Hampton, 1999; Hampton and Rine, 1994; Nakanishi et al., 1988; Ravid et al., 2000; Roitelman and Simoni, 1992
MATα repressor	Transcription factor (repressor protein)	Rapidly degraded in yeast cells with α mating type (MATα cells). Dimerization with Mata repressor protects α repressor from degradation in diploid cells.	Doa10p	Hochstrasser et al., 1991; Hochstrasser and Varshavsky, 1990; Johnson et al., 1998; Swanson et al., 2001

Table 1-2 (continued)

Proteins regulated by degradation via the ubiquitin-proteasome pathway

Protein degradation at the endoplasmic reticulum

The secretory pathway begins at the endoplasmic reticulum (Rothblatt et al., 1994). As such, the ER is a major site of both protein synthesis and protein degradation (Bonifacino and Weissman, 1998; Brodsky and McCracken, 1999; Enenkel et al., 1998; Palmer et al., 1996; Schubert et al., 2000). Along with components of the unfolded protein response (UPR)[1], ER-associated degradation (ERAD) plays an essential role in quality control over the secretory pathway (Friedlander et al., 2000; McCracken and Brodsky, 2000; Travers et al., 2000). There are now multiple examples of mutant, misfolded proteins that are retained in the ER and degraded rather than passing through the rest of the secretory pathway and reaching the cellular destination of their normally folded counterparts (Table 1-1 on page 9). ERAD, however, is not solely devoted to eliminating misfolded proteins. ERAD is also used to regulate the activities of proteins like the metabolic enzyme HMG-CoA reductase (HMGR).

HMG-CoA reductase is an integral ER-resident membrane protein whose sequence and function are conserved from yeast to humans (Basson et al., 1988; Basson et al., 1986; Brown and Simoni, 1984). HMGR catalyzes a rate-limiting step in sterol

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1. The Unfolded Protein Response (UPR) is a coordinated transcriptional regulation of genes coding for proteins involved in protein folding and organelle maintenance (see page 208 in Chapter 4 for further discussion of the UPR).

biosynthesis (see Figure 1-5 on page 15), and as such, is subject to several different modes of regulation, including degradation of the actual HMGR protein (Ericsson and Dallner, 1993; Hampton and Rine, 1994; Nakanishi et al., 1988; Stermer et al., 1994). The regulated degradation of HMG-CoA reductase is a classic feedback loop where the abundance of pathway intermediates[2] regulates HMGR stability (Hampton and Rine, 1994; Nakanishi et al., 1988). When pathway intermediates are abundant, HMGR is rapidly degraded by the ubiquitin-proteasome pathway in both yeast and mammals (Hampton and Bhakta, 1997; Ravid et al., 2000). When these same intermediates are scarce, HMGR is stable. This regulated degradation can be demonstrated by drugs that inhibit different enzymes in the mevalonate pathway. The drug lovastatin, for instance, inhibits the enzymatic activity of HMG-CoA reductase itself, blocking the production of pathway intermediates (See Figure 1-5 on page 15; Alberts, 1988). As a result,

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2. In both yeast and mammals, this pathway intermediate is derived from farnesyl pyrophosphate but the exact identity is not known (Gardner and Hampton, 1999; Meigs and Simoni, 1997). In addition, there is evidence that an oxysterol signal for degradation is also conserved from yeast to mammals (Edwards and Ericsson, 1999; Gardner et al., 2001; Mark et al., 1996; Schroepfer, 2000). Finally, in mammals, cholesterol levels regulate HMGR stability (Chin et al., 1985; Chun and Simoni, 1992; Jingami et al., 1987; Roitelman and Simoni, 1992). A role for sterol regulation of HMGR degradation in yeast has not been found but remains under investigation.

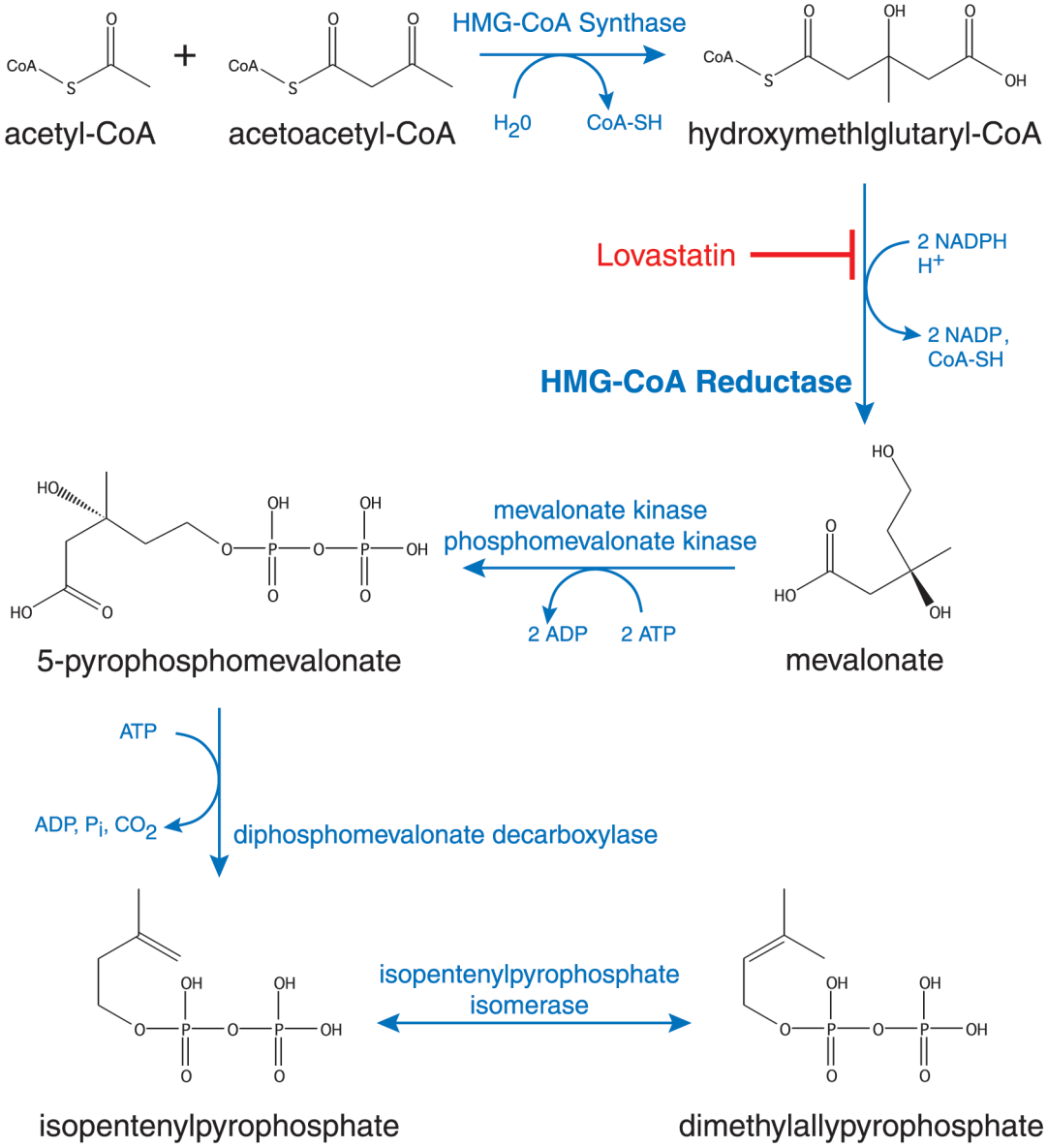

Figure 1-5

The mevalonate pathway

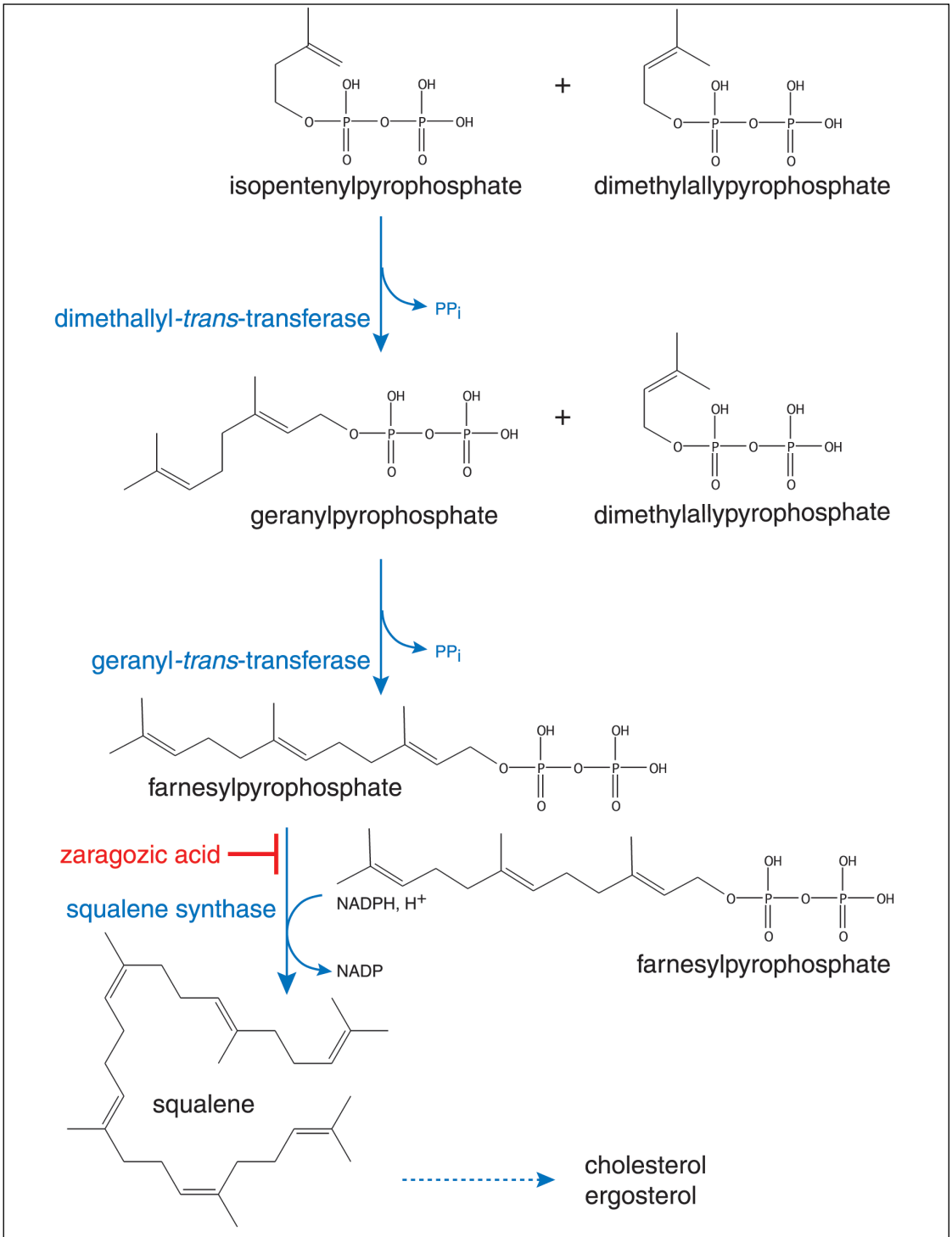

Figure 1-5 (continued)

The mevalonate pathway

HMG-CoA reductase is stable in lovastatin-treated cells (Hampton and Rine, 1994; Nakanishi et al., 1988). Another drug, zaragozic acid, is an inhibitor of squalene synthase (Bergstrom et al., 1993). Pathways signals dramatically increase in zaragozic acid-treated cells, and HMGR is rapidly degraded (Hampton and Bhakta, 1997).

The mechanism of ER-associated degradation

Although ERAD is central to quality control in the secretory pathway and to regulatory axes like the feedback regulation of HMG-CoA reductase, significant progress in the elucidation of ERAD mechanism has only recently been made. Much of this progress has been made by study of *HRD* (HMG-CoA Reductase Degradation) genes required for HMGR degradation. This work details the discovery of *HRD* genes and their action at all stages in endoplasmic reticulum-associated protein degradation. Chapter 2 describes several versions of the *hrd* selection and the diverse array of *HRD* genes identified using this genetic approach. Chapter 3 examines the role of Hrd1p as a ubiquitin-protein ligase (or E3), a critical specificity-determining factor in ubiquitin-mediated degradation. Chapter 4 details the role of Hrd4p/Npl4p, Cdc48p and Ufd1p at a previously uncharacterized post-ubiquitination but pre-proteasomal step in ERAD. In all, the data in this work describe the role of the ubiquitin-proteasome system in ER-associated degradation, offering a mechanism for how ERAD substrates are targeted for ubiquitination and delivered to the proteasome for processive degradation. Perhaps just

as importantly, this work also provides numerous tools for further study of ERAD and ubiquitin-mediated degradation in general.

Protein	Molecular Function	Comments	References
Ubc1p	Ubiquitin-Conjugating Enzyme (E2)	Used by the Hrd1p E3 along with Ubc7p. Although required for ERAD, Ubc1p is a soluble, cytosolic protein. Recruitment to the ER may be mediated by a Cue1p-like protein yet to be characterized.	This work, Friedlander et al., 2000
Ubc6p	E2	Although Ubc6p is the only E2 that is an integral ER membrane protein, Ubc6p is not the major E2 in ERAD. Exhibits physical interaction with Ubc7p and also affected by loss of Cue1p.	This work, Biederer et al., 1996; Biederer et al., 1997; Chen et al., 1993; Wilhovsky et al., 2000
Ubc7p	E2	The major E2 involved in ERAD. Most ERAD substrates tested require Ubc7p for their degradation. Requires Cue1p to function at the ER.	This work, Biederer et al., 1997; Hampton and Bhakta, 1997; Hiller et al., 1996; Wilhovsky et al., 2000
Cue1p	Anchor for Ubc7p	Cue1p is an ER membrane protein that recruits Ubc7p to the ER.	Biederer et al., 1997
Hrd1p	Ubiquitin-Protein Ligase (E3)	An integral ER membrane protein, Hrd1p promotes ubiquitination of both misfolded and regulated proteins at the ER (via Ubc1p or Ubc7p *in vivo*).	This work, Bordallo et al., 1998; Hampton et al., 1996; Wilhovsky et al., 2000
Doa10p	E3	Also an integral ER membrane protein, Doa10p is required for degradation of MATα2p, but data also indicate that Doa10p functions with Hrd1p in eliminating misfolded proteins from the ER.	Swanson et al., 2001

Table 1-3

Proteins required for Endoplasmic Reticulum-Associated Degradation

Hrd3p	Regulator of Hrd1p E3 activity	Hrd3p forms a complex with Hrd1p *in vivo* and regulates Hrd1p activity across the ER membrane. Deletion studies of Hrd3p suggest that Hrd3p is required for another function in ERAD other than Hrd1p regulation.	Gardner et al., 2000; Hampton et al., 1996; Plemper et al., 1999; Wilhovsky et al., 2000
Der1p	Unknown	Der1p appears to be required for the degradation of lumenal ER proteins, but not membrane proteins.	Knop et al., 1996a
Kar2p, Sec61p	Retrotranslocation of ERAD substrates from the ER lumen to cytosol.	Retrotranslocation is suggested by several genetic studies, but direct demonstration of a Sec61p and Kar2p function in retrotranslocation remains elusive.	Pilon et al., 1997; Plemper et al., 1997[a]
Cdc48p, Hrd4p/Npl4p, Ufd1p	Recognition of ubiquitinated ER proteins	Cdc48p, Ufd1p, and Hrd4p/Npl4p form a complex that appears to act in the recognition of ubiquitinated proteins andserves to present these ubiquitinated proteins for proteasomal processing	This work, Meyer et al., 2000
26S proteasome	Multisubunit protease	Processive degradation of ERAD substrates requires the 26S proteasome. The exact path of a ubiquitinated protein from the ER to the proteasome is an area of active investigation.	This work, Biederer et al., 1996; Hampton et al., 1996; Hiller et al., 1996

Table 1-3 (continued)

Proteins required for Endoplasmic Reticulum-Associated Degradation

a. References regarding some *SEC61* alleles have been omitted intentionally.

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2

The *hrd* selection

Introduction

The *hrd* selection was originally devised to identify genes required for HMG-CoA reductase degradation — making an understanding of HMG-CoA reductase biology a primary goal in the study of the resulting *hrd* complementation groups. However, the very fact that HMG-CoA reductase is an integral ER membrane protein meant that the *hrd* selection could also illuminate another poorly understood question in biology, namely how proteins at the endoplasmic reticulum are degraded. Even the union of the these two questions could be answered by the *hrd* selection, for it was not clear whether there was a separate degradation machinery in the ER specific for HMG-CoA reductase or whether HMGR degradation employed a common ER degradation pathway used in the degradation of many other ER proteins. Fortunately, the *hrd* selection has lived up to its potential in all of these areas. In this chapter, the theoretical basis of the *hrd* selection is described and the results of the first *hrd* selection are discussed. A modified *hrd* selection is then detailed where modifications to the original *hrd* selection make possible the ultimate goal of identifying all genes required for HMG-CoA reductase degradation. The success of this strategy is then revealed with a discussion of the genetic richness emerging from the modified *hrd* selection.

Theoretical basis of the *hrd* selection

HMG-CoA reductase activity is essential for the life of organisms from yeast to humans (Brown and Goldstein, 1980; Stermer et al., 1994). In the yeast *Saccharomyces*

cerevisiae, for example, deletion of HMG-CoA reductase genes results in yeast strains that cannot grow on normal laboratory media[3] (Basson et al., 1986). HMG-CoA reductase activity can also be eliminated from cells through the use of drugs like lovastatin that inhibit the enzymatic activity of HMG-CoA reductase (See Figure 1-5 on page 15, Alberts, 1988; MacDonald et al., 1988). Indeed, lovastatin is toxic to cells at high concentrations (see Figure 2-1 on page 42 for chemical structure of lovastatin and Figure 2-2 on page 43 for lovastatin toxicity in a strain of the yeast *Saccharomyces cerevisiae*). Cells can generate resistance to lovastatin in a variety of ways. For instance, amplification of genes coding for HMG-CoA reductase has been found in several lovastatin-resistant cell lines (Luskey et al., 1983; Skalnik et al., 1985). This increase in mRNA coding for HMG-CoA reductase

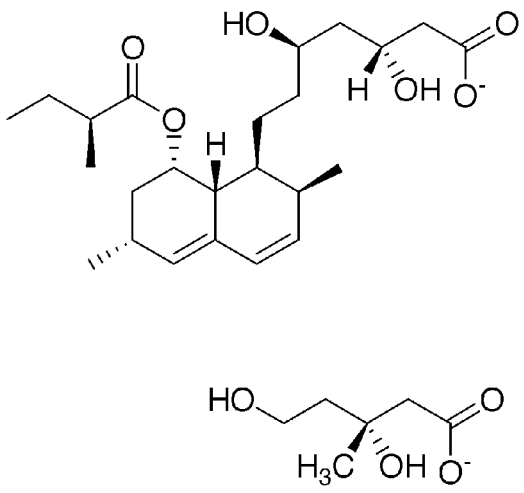

Figure 2-1

Chemical structure of lovastatin (top) and mevalonate (bottom)

3. Strains lacking HMG-CoA reductase activity are mevalonate auxotrophs, and their growth can be restored by the addition of mevalonate to the growth medium.

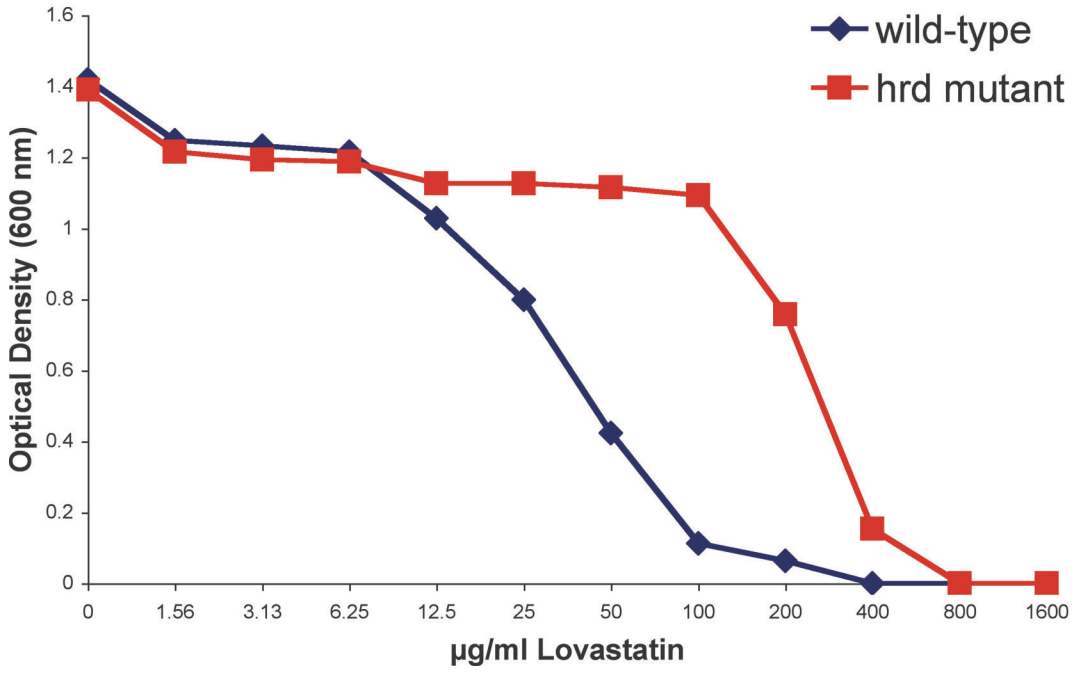

Figure 2-2

***hrd* mutants are resistant to lovastatin**

Cultures of the indicated strains were inoculated at low cell density (OD_{600} < 0.001) in yeast minimal media and added to serial dilutions of lovastatin also made in the same media. Cells were allowed to grow until cultures without lovastatin grew to an OD_{600} of 1.4.

can lead to a 1000-fold increase in the amount of HMG-CoA reductase protein in the cell and consequently, a massive expansion of the endoplasmic reticulum itself. (Luskey et al., 1983). Since HMG-CoA reductase is degraded (Chapter 1), one might also expect cells deficient in degradation to also accumulate higher levels of HMG-CoA reductase protein and develop resistance to lovastatin. Indeed, Randolph Hampton successfully used lovastatin resistance to isolate mutants deficient in the degradation of

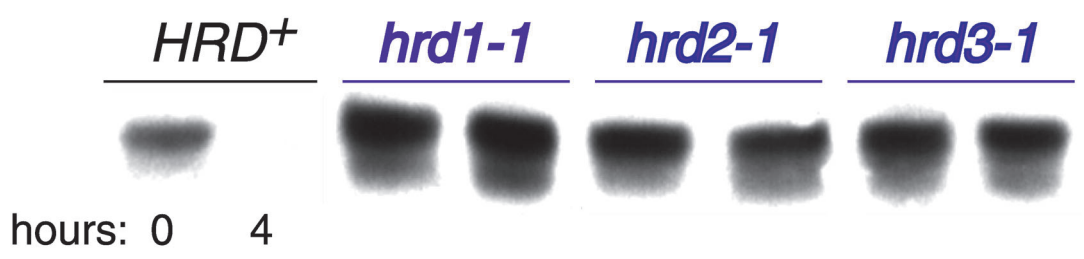

Figure 2-3

***hrd* mutants are defective in the degradation of HMG-CoA reductase**

Cycloheximide chase of the indicated strains was performed by adding cycloheximide to log-phase cells, harvesting cells at the indicated times, and mechanochemical lysis. Lysates were subjected to SDS-PAGE and loss of Hmg2p due to degradation was followed by immunoblotting for the myc epitope tag in 1myc-Hmg2p (an epitope-tagged isozyme of *S. cerevisiae* HMG-CoA reductase).

HMG-CoA reductase: developing a genetic selection now known as the *hrd* selection (Hampton et al., 1996a).

The *hrd* selection is performed in a strain of *Saccharomyces cerevisiae* that expresses a variant of HMG-CoA reductase that is constitutively degraded. This "6myc-Hmg2p" is degraded like native yeast Hmg2p, but its degradation is no longer regulated by the mevalonate pathway (Hampton et al., 1996a). 6myc-Hmg2p is used in the *hrd* selection to reduce the background of lovastatin resistance that could be generated by *regulation* of Hmg2p degradation (Hampton et al., 1996a). Using 6myc-Hmg2p strains, Hampton was able to isolate lovastatin resistant colonies which proved to be strains deficient in the degradation of 6myc-Hmg2p (Hampton et al., 1996a). Importantly, these strains were

also unable to degrade normally regulated Hmg2p (Hampton et al., 1996a and Figure 2-3 on page 44). The use of lovastatin and 6myc-Hmg2p proved to be a successful strategy to isolate *hrd* mutants.

With *hrd* mutants isolated, Hampton was able to identify three different genes required for the degradation of Hmg2p: *HRD1, HRD2,* and *HRD3* (Hampton et al., 1996a). These three genes alone have provided a wealth of information about the biology of HMG-CoA reductase degradation as well as ER protein degradation in general (see Chapter 1, Chapter 3 and references therein). My goal at the beginning of my graduate work was to reveal the identify of as many *HRD* genes as possible. Because the original *HRD* selection was so successful, I simply repeated the procedure to see which new *HRD* genes would be revealed. The results of this repetition made clear several problems that would have to be overcome if more genes were to be identified.[4] Although these problems ranged from nuisances to formidable obstacles, they were each overcome to

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4. Here, "problem" does not mean that the original *hrd* selection was somehow flawed. In fact, it is my opinion that the original *hrd* selection was an innovative and successful approach. "Problems" only arose upon the attempt to expand the selection to its maximum capacity – limitations that were not an issue for the success of the original *hrd* selection and identification of the first *HRD* genes.

Statistics for a second repetition of the *hrd* selection.

Genotype of selection strain (RHY400): *MATa ade2-101 lys2-801 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 met2 ura3-52::P_{TDH3}-6MYC-HMG2*

Source of cells: 20 independent cultures were grown in YPD to a density of 2 x 10^7 cells/ml. 2 x 10^6 unmutagenized cells from each culture were plated onto solid yeast minimal medium containing 250 μg/ml lovastatin.

Mean Lov^r colonies emerging per plate: 16

Standard Deviation: 16

Median: 13; Maximum: 79; Minimum: 6

Frequency of Lov^r: 1 in 125,000 cells

% breeding true for Lov^r: 65%

Frequency of Lov^r (adjusted) 1 in 200,000

% of Lov^r cells that stabilize Hmg2p: 80%

Frequency of *hrd* mutants: 1 in 250,000

Table 2-1

Second Repetition of the *hrd* selection

generate a new generation of the *hrd* selection capable of identifying a multitude of new *HRD* genes.

Results

Defects in mitochondrial function unrelated to the Hrd$^-$ phenotype.

No temperature-sensitive *hrd* mutants were isolated in the original *hrd* selection (R. Hampton, unpublished results). This absence of temperature-sensitive mutants was no surprise, however, as the original *hrd* selection was performed at 34˚C[5] in order to reduce the recovery of temperature-sensitive mutants (Hampton et al., 1996a). In subsequent repetitions of the *hrd* selection, the growth temperature was lowered to 30˚C to allow the recovery of temperature-sensitive *hrd* mutants – mutants that might well lead to the identification of *HRD* genes essential for life.

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5. Most laboratory strains of *Saccharomyces cerevisiae* are grown at 30˚C (Guthrie and Fink, 1991). The strains discussed in this chapter arose from JRY527, an S288C derivative (Hampton and Rine, 1994). These strains grow best at 30˚C and cannot tolerate temperatures higher than 37˚C (data not shown). Many temperature-sensitive strains isolated in this background are inviable at 35˚C (see this Chapter, Chapter 4, and Appendix A)

#	+ HRD1 (pRH433)		+ HRD2 (pRH482)		+ HRD3 (pRH485)	
	Lov(r)?	Ts(-)?	Lov(r)?	Ts(-)?	Lov(r)?	Ts(-)?
1	yes	yes	yes	yes	NO	YES
2	yes	yes	yes	yes	NO	YES
3	yes	yes	yes	yes	NO	YES
4	yes	yes	yes	yes	NO	YES
5	NO	YES	yes	yes	yes	yes
6	yes	yes	yes	yes	NO	YES
7	NO	YES	yes	yes	yes	yes
8	NO	YES	yes	yes	yes	yes
9	NO	YES	yes	yes	yes	yes
10	NO	YES	yes	yes	yes	yes

Table 2-2

Phenotypes of *hrd* mutants after transformation with *HRD* plasmids

Lovastatin resistance but not temperature sensitivity is reversed by *HRD1* or *HRD3* plasmids.

In the second repetition of the *hrd* selection, a number of lovastatin-resistant yeast strains were isolated that could grow at 30°C, but not at 37°C. In fact, almost 70% of the *hrd* mutants isolated in the second repetition were temperature-sensitive (data not shown). It appeared, therefore, that simply lowering the selection temperature had allowed a bountiful harvest of new temperature-sensitive *hrd* mutants. It was initially

assumed that none of these newly-isolated Ts^{-} [6] mutants expressed mutant alleles of previously identified *HRD* genes *HRD1* and *HRD3* since even null alleles of *HRD1* and *HRD3* fail to display temperature sensitivity (Hampton et al., 1996a). To test this assumption, a group of 10 temperature-sensitive mutants were transformed with plasmid DNAs coding for the previously isolated *HRD* genes: *HRD1, HRD2* and *HRD3* (Table 2-2 on page 48). The results of this transformation were surprising. The lovastatin resistance of the mutants could be reversed by transformation with plasmids bearing either wild-type *HRD1* or *HRD3* alleles. However, these same plasmids did nothing to reverse the temperature-sensitivity of the 10 *hrd* mutant strains (Table 2-2 on page 48).

To begin addressing the genetic questions raised by this observation, the 10 mutants were backcrossed to the parent strain to determine if the temperature-sensitivity and lovastatin-resistance phenotypes mapped to the same or different loci. The diploids resulting from the backcross were all lovastatin-sensitive and showed no temperature-sensitivity – indicating that recessive allele(s) were conferring both phenotypes (data not shown). The diploids were sporulated, tetrads were dissected and segregants[7] screened for relevant phenotypes. It was quickly apparent that lovastatin-resistance and temperature-sensitivity were not segregating together. In fact, the occurrence of phenotypes in

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6. In this work, Ts^{-} indicates a temperature-sensitive strain while Ts^{+} indicates the wild-type phenotype (able to grow at the assay temperature).

Mutant 1 x wild-type parent strain

Mutant 1 phenotypes: Ts^- Lov^r MATa

parent strain phenotypes: Ts^+ Lov^s MATα

Segregant #	Phenotypes
1a	Ts^- Lov^s MATα
1b	Ts^- Lov^s MATa
1c	Ts^+ Lov^r MATa
1d	Ts^+ Lov^r MATα
2a	Ts^+ Lov^r MATa
2b	Ts^- Lov^s MATa
2c	Ts^- Lov^s MATα
2d	Ts^+ Lov^r MATα
3a	Ts^- Lov^s MATα
3b	Ts^+ Lov^r MATa
3c	Ts^+ Lov^r MATa
3d	Ts^- Lov^s MATα
4a	Ts^+ Lov^r MATα
4b	Ts^- Lov^s MATa
4c	Ts^- Lov^s MATa
4d	Ts^+ Lov^r MATα

Table 2-3

Two different alleles are responsible for temperature-sensitivity and lovastatin-resistance in a *hrd* mutant.

The haploid “mutant 1” was crossed to its wild-type “parent” strain. The resulting diploid was sporulated and tetrads dissected. Only the first four tetrads are shown.

Segregation of temperature-sensitivity and lovastatin-resistance indicate that these phenotypes are caused by mutations residing at two unlinked loci.

Mutant 7 x wild-type parent strain

Mutant 7 phenotypes: Ts^- Lov^r MATa

parent strain phenotypes: Ts^+ Lov^s MATα

Segregant #	Phenotypes
1a	Ts^+ Lov^s MATa
1b	Ts^+ Lov^r MATα
1c	Ts^+ Lov^s MATa
1d	Ts^+ Lov^r MATα
2a	Ts^+ Lov^r MATa
2b	Ts^+ Lov^s MATa
2c	Ts^+ Lov^s MATα
2d	Ts^+ Lov^r MATα
3a	Ts^+ Lov^s MATα
3b	Ts^+ Lov^r MATa
3c	Ts^+ Lov^r MATa
3d	Ts^+ Lov^s MATα
4a	Ts^+ Lov^r MATα
4b	Ts^+ Lov^s MATa
4c	Ts^+ Lov^s MATa
4d	Ts^+ Lov^r MATα

Table 2-4

The Ts^- phenotype is lost after crossing mutant 7 to a wild-type strain.

The haploid “mutant 7” was crossed to its wild-type “parent” strain. The resulting diploid was sporulated and tetrads dissected. Only the first four tetrads are shown.

No temperature-sensitive segregant was recovered.

Mutant 1 x wild-type parent strain (expanded)

Mutant 1: Ts^-, Lov^r, white, slow-growing, unable to grow on glycerol, MATa

parent strain: Ts^+, Lov^s, pink, normal growth on glycerol, MATα

Segregant	Phenotypes
1a	Ts^-, Lov^s, white, slow-growing, unable to grow on glycerol,MATα
1b	Ts^-, Lov^s, white, slow-growing, unable to grow on glycerol,MATa
1c	Ts^+, Lov^r, pink, normal growth on glycerol, MATa
1d	Ts^+, Lov^r, pink, normal growth on glycerol, MATα
2a	Ts^+, Lov^r, pink, normal growth on glycerol, MATa
2b	Ts^-, Lov^s, white, slow-growing, unable to grow on glycerol,MATa
2c	Ts^-, Lov^s, white, slow-growing, unable to grow on glycerol,MATα
2d	Ts^+, Lov^r, pink, normal growth on glycerol, MATα

Table 2-5

Additional phenotypes for mutant 1 backcross

“Petite” phenotypes show 2:2 segregation. “Petite” mutation is not linked to *hrd* mutation.

3a	Ts^{-}, Lov^{s}, white, slow-growing, unable to grow on glycerol,MATα
3b	Ts^{+}, Lov^{r}, pink, normal growth on glycerol, MATa
3c	Ts^{+}, Lov^{r}, pink, normal growth on glycerol, MATa
3d	Ts^{-}, Lov^{s}, white, slow-growing, unable to grow on glycerol,MATα
4a	Ts^{+}, Lov^{r}, pink, normal growth on glycerol, MATα
4b	Ts^{-}, Lov^{s}, white, slow-growing, unable to grow on glycerol,MATa
4c	Ts^{-}, Lov^{s}, white, slow-growing, unable to grow on glycerol,MATa
4d	Ts^{+}, Lov^{r}, pink, normal growth on glycerol, MATα

Table 2-5 (continued)

Additional phenotypes for mutant 1 backcross

"Petite" phenotypes show 2:2 segregation. "Petite" mutation is not linked to *hrd* mutation.

each of the segregants indicated that the Ts^- and Lov^r phenotypes were being caused by mutations in two unlinked genes (One instance is given in Table 2-3 on page 50). Evidence for two unlinked alleles was obtained from the genetic analysis of all diploids studied except for one very notable exception.

One diploid formed by the mating of mutant "#7" to its wild-type parent strain displayed Lov^s and Ts^+ phenotypes just like all the other diploids formed by a mutant-parent backcross (data not shown). In the resulting tetrads, 2 segregants were Lov^r and 2 segregants were Lov^s, as expected (on page 51). However, all 4 segregants in each tetrad were *not* temperature sensitive (all 4 segregants were wild-type [Ts^+], see on page 51). There are several possible explanations for such an observation. 4:0 segregation from a heterozygous diploid can be caused by conversion of one allele into the other through a homologous recombination event in the diploid. If such "gene conversion" had occurred in the "7 x wt" diploid, it would have to have occurred soon after formation of the diploid as *all* tetrads obtained from this diploid showed 4:0 segregation. Addition-

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7. Here, segregant refers to clonal cells arising from one individual spore produced from meiosis in the diploid parent. In this case, the segregant strain should have a phenotype identical to that of the original spore which was the original product of meiosis in the diploid parent.

ally, subsequent analysis of separate "7 x wt" matings revealed the same pattern of 4:0 segregation (data not shown).

4:0 segregation can also occur for traits that are cytoplasmically inherited: traits transmitted by non-nuclear, and thus non-Mendelian, elements. There are several examples of cytoplasmic inheritance from many different organisms, including *S. cerevisiae* (Aufderheide and Johnson, 1976; Austin and Hall, 1992; Cox, 1994; Michaelis, 1976; Stuart, 1970; Uchida and Suda, 1976; Wickner, 1994; Wilkie, 1975). Probably the most well-characterized examples of cytoplasmic inheritance, however, are found in the study of mitochondria. In order to conduct oxidative metabolism, mitochondria require proteins coded by nuclear genes, and these genes are inherited in the same manner as any other nuclear gene (Dietrich et al., 1992; Grossman and Lomax, 1997). However, mitochondria also possess their own genomes containing genes required for mitochondrial function (Berger and Yaffe, 2000; Hermann and Shaw, 1998). The inheritance of the mitochondrial genome follows the inheritance of the mitochondria itself. There is no chromosome pairing, no independent segregation of alleles. These mitochondrially-encoded traits thus follow a "cytoplasmic inheritance" rather than a "nuclear inheritance" (Aufderheide and Johnson, 1976; Carnevali et al., 1969; Wilkie, 1975). For the yeast *Saccharomyces cerevisiae*, the loss of mitochondrial genome function leads to a cytoplasmic[8] "petite" phenomenon characterized by slower growing "petite" colonies appearing among larger "grande" colonies (Chen and Clark-Walker, 2000; Ephrussi et al., 1949a; Ephrussi et al., 1949b; Whittaker, 1979). Cells from these "petite" colonies

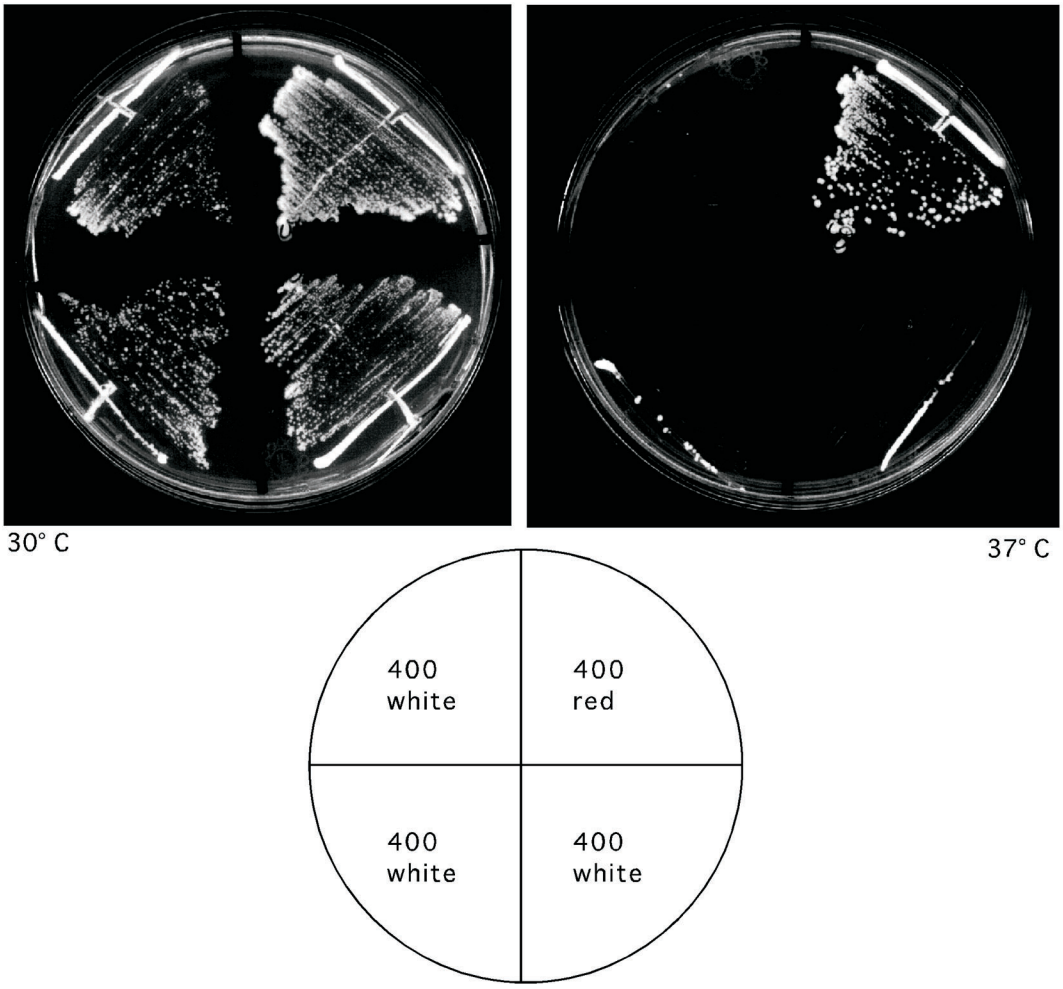

Figure 2-4

White RHY400 colonies are also temperature-sensitive

Indicated strains were plated on yeast minimal media and grown at either 30°C (permissive temperature) or 37°C (restrictive temperature).

are unable to grow on nonfermentable carbon sources such as glycerol, consistent with their loss of mitochondrial function (Ephrussi et al., 1949a; Kraepelin, 1967; Kraepelin, 1972; Whittaker, 1979). There is a striking visual indication of the "petite" phenotype in *Saccharomyces cerevisiae* cells bearing mutations in the *ADE2* gene: "petite" colonies composed of *ade2* mutant cells appear white whereas "grande" *ade2* mutant colonies appear pink (Lai-Zhang et al., 1999)[9].

Several observations indicated that *hrd* mutant #7 carried a cytoplasmic petite mutation. Mutant #7 cells produced slow-growing, white colonies and apparently transmitted these traits in a cytoplasmic manner (on page 51). Furthermore, mutant 7 cells were unable to grow on media containing glycerol as the only carbon source although its parent strain grew normally on the same media (data not shown and Figure 2-5 on page 58). Mutant 7 was not alone in its respiratory deficiency: Every temperature-sen-

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8. Mutant alleles of nuclear genes required for mitochondrial function also cause a "petite" phenotype. The resulting petites are sometimes referred to as *nuclear* petites.

9. The relationship between the *ade2* red phenotype and the petite mutation is well known, but its first documentation is not clear. For instance, the Lie-Zhang reference clearly shows that petite colonies are white for a variety of mutants and even use the white/red difference as a way to detect petites, but they do not provide a reference for the red/white grande/petite phenomenon.

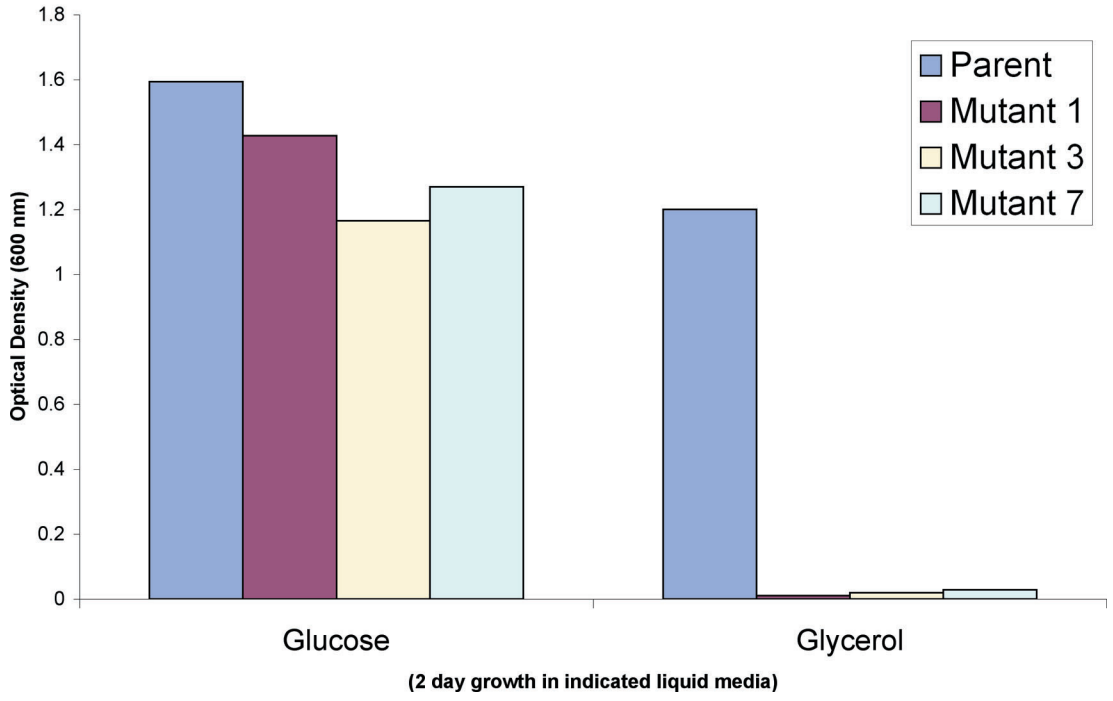

Figure 2-5

Petite mutant growth in media containing glycerol as the carbon source

Indicated strains were inoculated at low cell densities (OD_{600} < 0.001) in yeast minimal media with either glucose or glycerol as a carbon source. Strains were allowed to grow for 2 days to allow for the possibility that mutant strains were simply slow growing in glycerol. However, there was no indication that mutants 1,3 or 7 could utilize glycerol as a carbon source even after extended periods of incubation in glycerol-containing media.

sitive *hrd* mutant isolated in the second repetition of the *hrd* selection was also unable to grow on media containing solely glycerol as a carbon source (data not shown and Figure 2-5 on page 58). Genetic analysis of the *hrd* mutants also indicated that phenotypes of slow growth, white colony color, temperature sensitivity, and inability to grow on glycerol all segregated together after a backcross to the wild-type parent strain (data not shown and Table 2-5 (continued) on page 53). Therefore, a single locus was responsible for the petite phenotype in each the *hrd* mutants isolated. With the exception of mutant 7, these loci (or locus) displayed normal nuclear, Mendelian inheritance. Finally, "petite" colonies were isolated from the parent-strain that had never been subjected to selection on lovastatin. These colonies were also white, slow-growing, and unable to grow at all on glycerol (data not shown). Importantly, these "petite" colonies were also temperature-sensitive (Figure 2-4 on page 56).

Wild-type cells from the parent strain in the *hrd* selection were subjected to a "mock *hrd* selection" where independent cultures were prepared in the same manner as in the second repetition of the *hrd* selection, but the cells were plated onto media *without* lovastatin. After two days, the proportion of "petite" Ts^- and "grande" Ts^+ cells were determined. The results of this experiment indicated that the method of preparing cells for the *hrd* selection generated a large percentage of petites (63%) and that selection on lovastatin did *not* appear to increase the number of petites appearing in the population as about 65% of the *hrd* mutants isolated in the second repetition were petite and temperature sensitive – a proportion very similar to cells plated on media lacking lovastatin

(data not shown). The occurrence of petites was apparently a feature of the parent strain and the manner in which cells were prepared for plating on lovastatin (the normal rate of petite occurrence for the parent strain on solid media is about 5-10% [data not shown]).

Once it was discovered that petite mutations and temperature-sensitivity were unrelated to the Hrd phenotype, a protocol were devised to rid the *hrd* selection of this phenotypic noise. Fortunately, the problem was much easier to solve than it was to detect. Once cells were grown with glycerol as the sole carbon source before plating onto lovastatin, petites rarely emerged among Lov^r mutants. Because mitochondrial function was never intended to be the subject of this thesis, the petite question was not dealt with after it ceased to be a problem in the *hrd* selection. Several questions, therefore, remain unanswered. For instance, it is not clear why petites in this strain background are temperature-sensitive. Although specific temperature-sensitive petite mutations have been characterized in other strain backgrounds (Guerrini et al., 1975; Yang and Trumpower, 1994), the majority are not reported as temperature-sensitive (Chen and Clark-Walker, 2000; Whittaker, 1979) – raising a potentially interesting question about the sensitivity of this strain to loss of mitochondrial function.

90% of *hrd* mutants isolated are *hrd1* or *hrd3* mutants

After clearing the mitochondrial smoke from the *hrd* selection, another obstacle for the *hrd* selection was made apparent. In two repetitions of the *hrd* selection, it was found

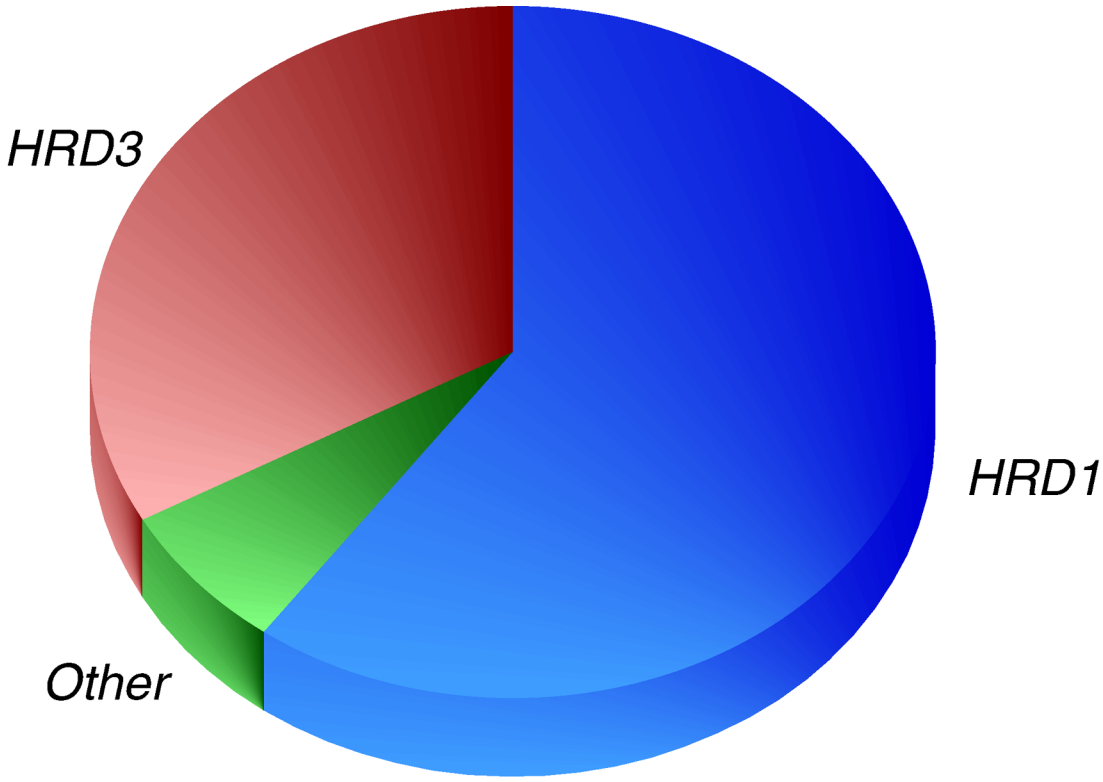

Figure 2-6

Over 90% of *hrd* mutants isolated are *hrd1* or *hrd3* mutants

Percentages for each *HRD* complementation group were obtained in two seperate repetitions of the *hrd* selection.

that over 90% of the *hrd* mutants isolated contained mutant alleles of either *HRD1* or *HRD3* (Table 2-2 on page 48 and data not shown; illustrated in Figure 2-6 on page 61). This hampered the isolation of mutants other than *hrd1* or *hrd3* mutants. For instance, in the second repetition of the *hrd* selection, mutations were isolated in only one other gene, *HRD4* (Chapter 4). Also, mutant alleles of *HRD2* were not recovered in the second repetition of the *hrd* selection (data not shown). This was a discomforting observation as it indicated that the *hrd* selection, as performed in the second repetition, was not supplying mutant alleles of known *HRD* genes – much less *HRD* genes yet to be identified. It became clear that the *HRD1/HRD3* bias would have to be eliminated if the *hrd* selection were going to be used to identify all *HRD* genes required for the degradation of yeast HMG-CoA reductase.

The *HRD1/HRD3* bias was an unfortunate roll of the genetic dice: the probability of *hrd1/hrd3* mutant alleles appearing in the *hrd* selection was much higher than for mutant alleles of other *hrd* genes (Table 2-2 on page 48 and data not shown). Although the *hrd* dice were loaded, they did not necessarily have to remain that way – especially in a genetically tractable organism like *Saccharomyces cerevisiae*. One solution to the *HRD1/HRD3* bias, therefore, might arise from changing the probability of *hrd1/hrd3* mutant alleles appearing in the selection. One strategy to alter the bias was based on the rationale that if additional copies of the *HRD1* and *HRD3* genes were added to the parent yeast strain, then it might become multiplicatively harder to obtain mutant alleles of *HRD1* or *HRD3* [10]. To implement this strategy, *HRD1::TRP1* and *HRD3::LEU2* plas-

New *hrd* selection strain

MATa ade2-101 lys2-801 his3Δ200 HRD1 HRD3 hmg1Δ::LYS2 hmg2Δ::HIS3 leu2Δ::HRD3::LEU2 met2 trp1::hisG::HRD1::TRP1 ura3-52::P_{TDH3}-6MYC-HMG2

Figure 2-7

Genotype of new *hrd* selection strain

HRD1 and *HRD3* genes are duplicated.

mids were cloned. These *HRD1* and *HRD3* plasmids were then integrated into the selection strain at the *TRP1* and *LEU2* loci, respectively. The resulting strain now contained two copies each of the *HRD1* and *HRD3* genes located both at the *HRD1* and *HRD3* loci as well as the separate *TRP1* and *LEU2* loci. This new parent strain would then be used in the third repetition of the *hrd* selection with the intent of reducing the occurrence of *hrd1* and *hrd3* mutants.

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10. In these repetitions of the *hrd* selection, only recessive alleles were considered for use in identifying genes required for Hmg2p degradation. Dominant alleles of *HRD1/HRD3* would therefore not be an issue even if they occurred at a high frequency.

High-throughput modifications to the *hrd* selection

While constructing the new *hrd* selection strain, several modifications to the selection were designed to increase the number of *hrd* mutants that could be analyzed genetically and biochemically – as well as greatly decreasing the processing time for each step of the analysis. These modifications included the construction of wild-type backcross strains with reciprocal auxotrophies and mating types to the selection strains so that mating could be done by selection on the appropriate media instead of isolating zygotes by microscopy and micromanipulation. Other technical modifications were also made to the way mutant strains were organized in the genetic selection. The most beneficial modification, however, was made in the way that the actual degradation defect was measured in candidate *hrd* mutants. This screening for a true degradation defect is critical because lovastatin resistance does not always arise from a defect in degradation of HMG-CoA reductase (Nathan Bays and Randolph Hampton, unpublished results). More fundamentally, the Hrd^- phenotype is a defect in the degradation of HMG-CoA reductase and a degradation defect is the phenotype that a strain must have in order to be a *hrd* mutant. The degradation defect is measured biochemically, and traditionally, strains were subjected to a "chase" protocol: either a pulse-chase, cycloheximide chase or stationary chase (Hampton and Rine, 1994).[11] Each of these protocols requires growth of cells to a particular density (which is often complicated by differing growth rates for mutants), a chase period, mechanochemical lysis, SDS-PAGE, and for the latter two, immunoblotting (Hampton and Rine, 1994). Since an expanded *hrd* selection would

require the analysis of several hundred samples daily, a more efficient, "high-throughput" method of measuring protein degradation was devised.

The new degradation assay was termed a "plate-chase." In a plate-chase, colonies are grown on solid synthetic medium containing casamino acids. Cells are grown for 2.5 days to allow cells to enter a stationary phase of growth. Although growth slows as cells enter stationary phase, degradation of Hmg2p continues (Hampton et al., 1996a; Hampton and Rine, 1994). As a result, wild-type (Hrd^+) cells display lower levels of Hmg2p immunoreactivity in stationary phase. *hrd* mutant cells, however, fail to degrade Hmg2p and thereby retain high levels of Hmg2p immunoreactivity in stationary phase. When performed with liquid cultures and SDS-PAGE, this assay is termed a "stationary chase" (Hampton et al., 1996a; Hampton and Rine, 1994). In the plate chase, however, immunoblotting is performed directly on the colonies themselves without the need for mechanical lysis, SDS-PAGE, and transblotting of proteins. The elimination of these

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11. These protocols to measure protein degradation are used extensively in the following chapters where they are described in more detail. The "cycloheximide chase" is described in Hampton and Rine, 1996 and is an assay where cycloheximide is added to stop protein synthesis. Degradation continues after addition of cycloheximide, and loss of protein by degradation is determined by immunoblotting (or flow cytometry for Hmg2-GFP).

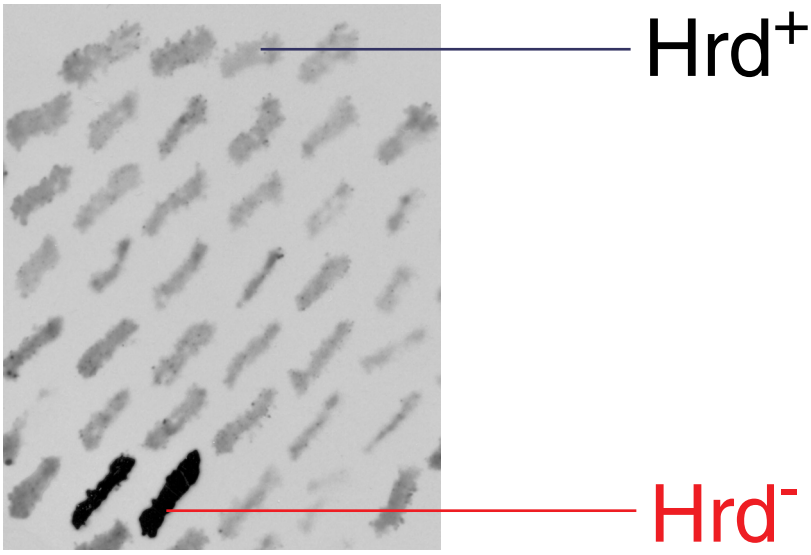

Figure 2-8

Example of "plate-chase" assay

Individual colonies were streaked onto a casamino acid media plate to form each stripe pictured. Degradation of 6myc-Hmg2p in these cells was assayed by allowing cells to grow 2.5 days and thus approach stationary phase. At this stage of growth, synthesis of 6myc-Hmg2p slows, but degradation continues. *hrd* mutants, however, are unable to degrade 6myc-Hmg2p like wild-type cells. As a result, *hrd* mutants retain a high level of 6myc-Hmg2p in stationary phase while wild-type cells show reduced levels of 6myc-Hmg2p in stationary phase.

Levels of 6myc-Hmg2p were determined by transferring cells onto nitrocellulose and chemical lysis directly on the membrane. After inactivation of endogenous peroxidases by acetic acid, membranes are subjected to immunoblotting using anti-myc antibody to detect 6myc-Hmg2 protein.

Pictured is a portion of a plate containing diploids created by crossing a set of *hrd* mutants to a *hrd15* mutant strain. Most of these diploids show normal degradation. Two diploids show noncomplementation of the *hrd15* allele.

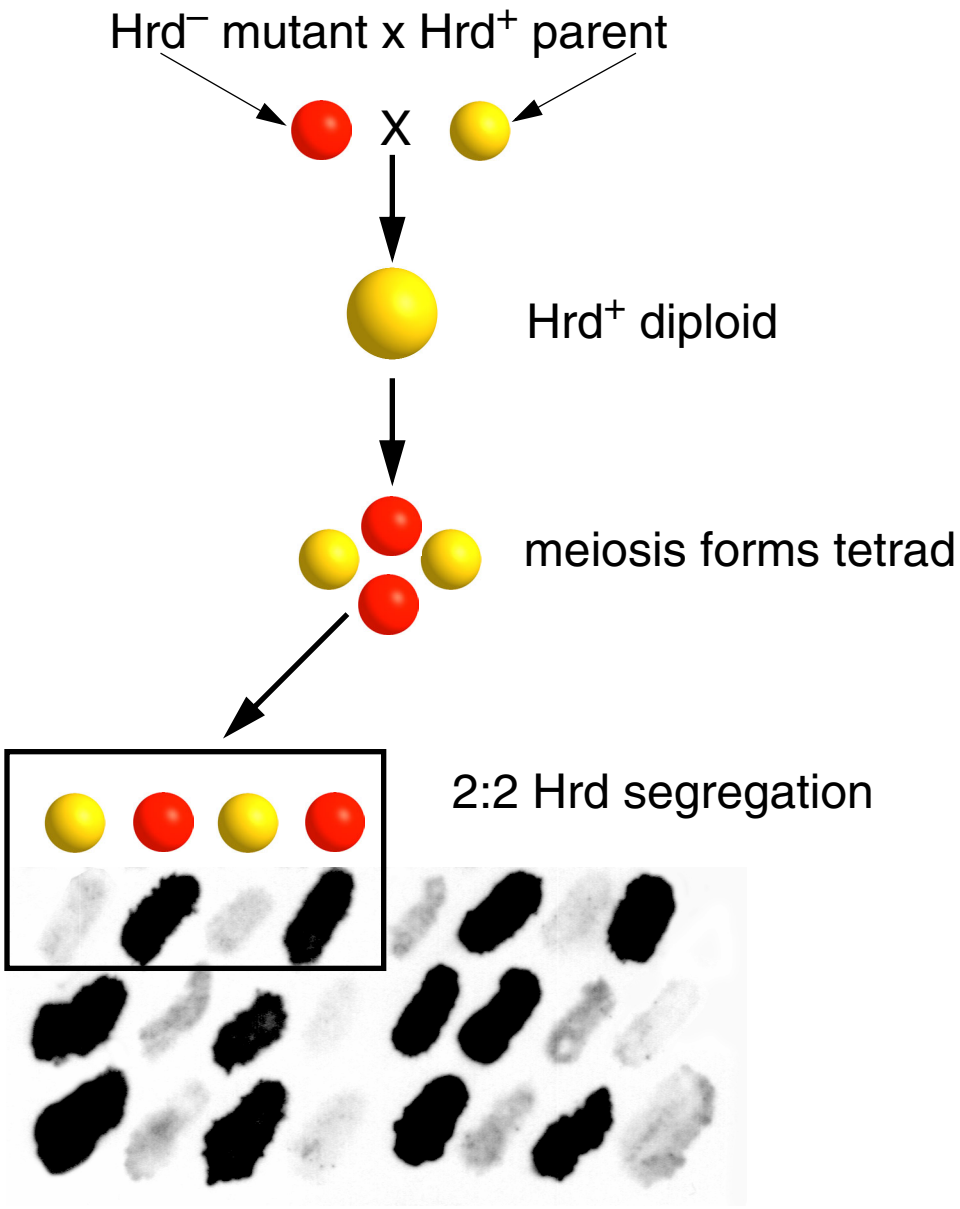

Figure 2-9

Use of "plate-chase" assay in genetic analysis of a *hrd* mutant

A *hrd* mutant strain was crossed to a Hrd$^+$ strain. The resulting diploid was sporulated and tetrads dissected. Degradation of 6myc-Hmg2p was assayed in each segregant by "plate-chase."

steps and the ability to grow 50-100 colonies on a single plate greatly increases the number of *hrd* mutants that can be analyzed in a single experiment, and the time required to determine the Hrd phenotype for an individual strain is drastically reduced. An example of a plate-chase is given in Figure 2-8 on page 66 and in Figure 2-9 on page 67.

Modifications to the *hrd* selection permit the identification of new *HRD* genes.

A third repetition of the *hrd* selection was performed to test new modifications created to overcome the limitations of the first two *hrd* selections. These modifications were designed to eliminate the large background of Ts^- mitochondrial petites, reduce the overwhelming bias of *hrd1* and *hrd3* mutants, and to increase the scale of *hrd* selection using high-throughput techniques – with the ultimate goal of identifying more *HRD* genes. As analysis of mutants progressed, the success of these modifications became apparent. For instance, mitochondrial petites no longer clouded the results of the *hrd* selection. Although the petite phenotype was observed in almost 65% of *hrd* candidates in the second selection, it was only observed in less than 13% of the lovastatin-resistant colonies recovered in the third repetition (data not shown and Appendix B).[12] Furthermore, several mutants were isolated where temperature-sensitivity and lovastatin resistance were conferred by mutation of a single locus (data not shown and Appendix B).

Several such mutants have led to the identification of the first essential *HRD* genes (discussed below).

Genetic analysis of mutants was greatly facilitated by use of the plate-chase technique. The plate-chase technique was first used to test lovastatin-resistant colonies for stabilization of 6myc-Hmg2p (i.e. for the true Hrd^- phenotype). The plate-chase assay was then used in the first backcross of candidate mutants to the wild-type parent strain in order to identify well-behaved segregants where stabilization of 6myc-Hmg2p segregated 2:2 after sporulation of the heterozygous "mutant x parent" diploid (Figure 2-9 on page 67). Finally, plate-chase was used to help assign complementation groups to the newly isolated *hrd* mutants by crossing the panel of new *hrd* mutants to both known *hrd* mutants and to mutants isolated in the third repetition (Data for complementation group assignment is given in Appendix B).

The assignment of complementation groups revealed the ultimate success of the modifications to the *hrd* selection: Although the third repetition was initially intended as a moderate trial run of the new *hrd* selection, 21 different *HRD* complementation

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12. Colonies unable to grow at all on glycerol were excluded from further genetic analysis. Some mutants exhibited poor growth on glycerol. These strains were not excluded from consideration, and often the glycerol growth phenotype was linked to the same allele conferring lovastatin resistance.

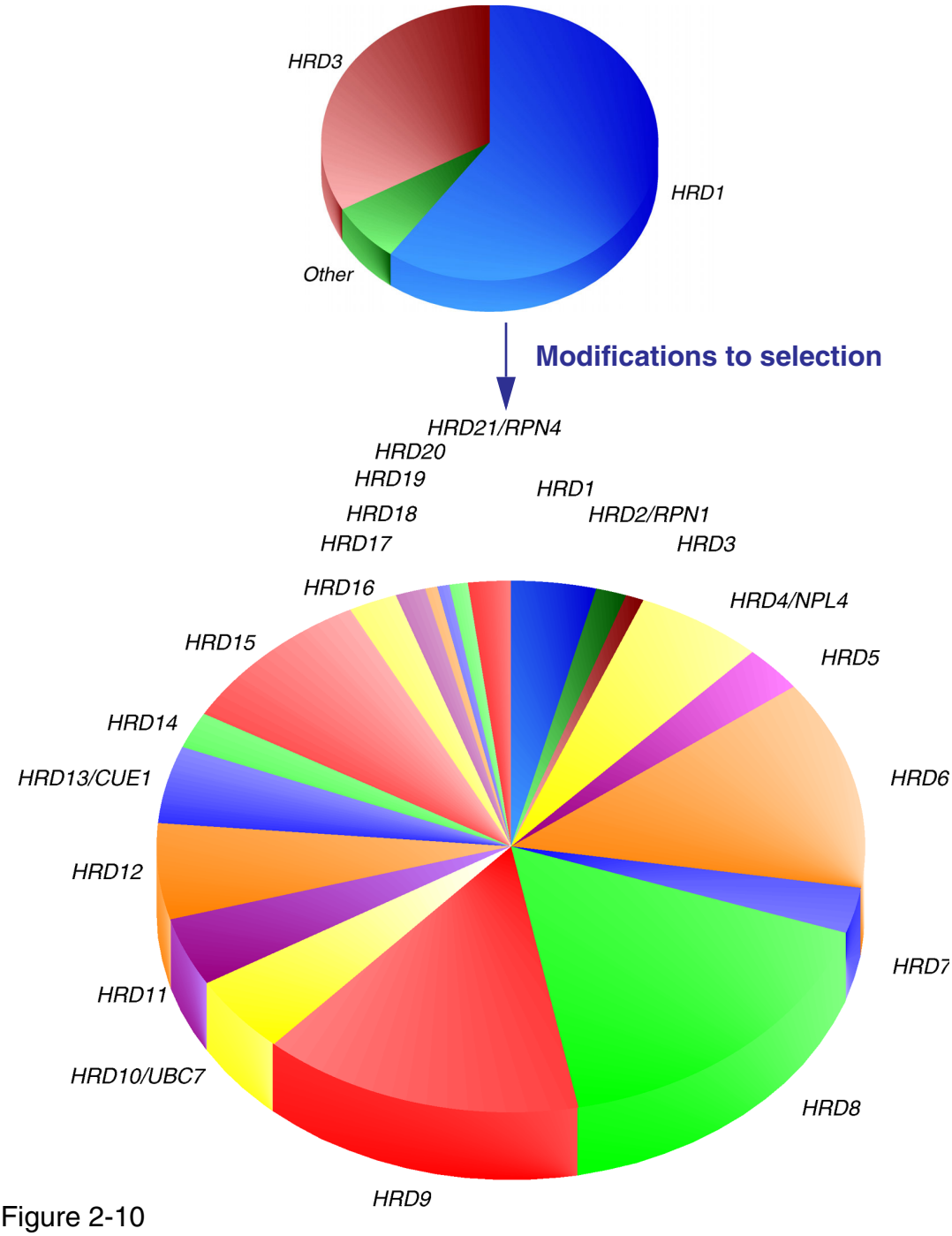

Figure 2-10

A new *hrd* selection allows the discovery of new *HRD* genes

Relative abundance of each complementation group isolated in new selection.

groups were identified – a dramatic improvement from the previous 3 (Figure 2-10 on page 70, data not shown, and Appendix B). These complementation groups included all previously known *HRD* genes: *HRD1-3* and *UBC7* (Hampton and Bhakta, 1997; Hampton et al., 1996a). The assignment of complementation groups also indicated that duplication of the *HRD1* and *HRD3* genes in the selection strain was very effective at reducing the number of *hrd1* and *hrd3* mutants isolated in the new *hrd* selection. Whereas *hrd1* and *hrd3* mutants accounted for over 90% of the mutants isolated in the previous repetitions of the *hrd* selection, less than 10% of the mutants isolated in the third repetition were *hrd1/hrd3* mutants (Figure 2-10 on page 70, data not shown, and Appendix B).

The function of two *HRD* genes, *HRD1* and *HRD4,* will be discussed in some detail in the following chapters along with *HRD2*, *HRD3* and *HRD10/UBC7*. Other *HRD* genes, however, will not enjoy as much discussion. The current knowledge about mutants defining new *HRD* complementation groups is given in the following section. Some of these mutants hint at new *HRD* genes with quite interesting and informative features. All testify to the genetic richness of the *hrd* selection, beginning with *hrd5* mutants.

HRD5 is tightly linked to the *URA3::6MYC-HMG2* locus

hrd5 strains fully complement every other recessive *hrd* mutant upon formation of a heterozygous diploid, and the three *hrd5* strains fail to complement each other when

$$\text{centiMorgans} = \frac{\text{numberoftetratype} + 6(\text{numberofnonparentalditype})}{\text{totalnumberoftetrads}}$$

Equation 1

mated to form a *hrd5/hrd5* diploid (data not shown). Additionally, segregants obtained from these diploids are all lovastatin resistant (data not shown). These results indicate that each *hrd5* allele is located at the same locus and define a single complementation group. The *hrd5* locus is also very tightly linked to the *URA3* locus. In one experiment a Ura^- Lov^r Hrd^- *hrd5* strain was mated to a Ura^+ Lov^s Hrd^+ $HRD5^+$ strain. The resulting diploid was Ura^+ Lov^s Hrd^+ indicating that the *hrd5* allele was recessive (data not shown). Sporulation of the diploid and dissection of the resulting tetrads produced segregants where every Lov^r Hrd^- segregant was also Ura^-, with only one Lov^r Hrd^- Ura^+ segregant as an exception (data not shown). All phenotypes segregated 2:2 (Hrd, Ura, Leu, Trp and mating type), and there was no linkage seen between the *hrd5* and *LEU2, TRP1* or *MAT* loci. Using Equation 1, the map distance between the *HRD5* and *URA3* loci was estimated to be about 4 cM.[13] For this region of the yeast genome, this map

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13. The data for the equation was as follows: number of tetratype=1, number of non-parental ditype=0, total tetrads=13.

distance roughly equals 5 kilobases of DNA (Cherry et al., 1997).

The linkage between the *HRD5* and *URA3* loci was especially interesting since *URA3* is the site of integration for the gene coding 6myc-Hmg2p, the constitutively degraded HMG-CoA reductase used in the *hrd* selection (Hampton et al., 1996a). Linkage between the *hrd5* and *URA3::6MYC-HMG2* loci raised the interesting possibility that *hrd5* alleles may actually be mutant alleles of the gene coding 6myc-Hmg2p. To help test this idea, another Hmg2p fusion was employed: Hmg2-GFP (Hampton et al., 1996b). In Hmg2-GFP, the catalytic domain of Hmg2p is replaced with GFP. Because only the transmembrane domain of Hmg2p is required for regulated degradation (Chun and Simoni, 1992; Gardner et al., 1998; Gardner and Hampton, 1999; Jingami et al., 1987; Sekler and Simoni, 1995), Hmg2-GFP is degraded and regulated just like native Hmg2p (Cronin and Hampton, 1999; Hampton et al., 1996b). The presence of GFP allows detection of Hmg2-GFP levels in the cell by measuring cell fluorescence (with flow cytometry, for instance). Hmg2-GFP also does not possess a HMGR catalytic domain, so the fusion confers no lovastatin resistance when added to 6myc-Hmg2p stains (Stephen Cronin and Nathan Bays, unpublished results). In one experiment, a *URA3::6MYC-HMG2* ***hrd5-1*** *leu2*Δ strain was crossed to a *ura3-52::6MYC-HMG2* ***HRD5***$^{+}$ *leu2*Δ*::LEU2::HMG2-GFP* strain. In the resulting diploid, both Hmg2-GFP and 6myc-Hmg2p were degraded normally (data not shown). In the segregants, however, Ura^{+} cells were *always* lovastatin resistant and 6myc-Hmg2p was stabilized regardless of whether the strains were expressing *LEU2::HMG2-GFP* or not. Additionally,

Hmg2-GFP was *always* degraded even when the cells expressed the *hrd5-1* allele (as detected by resistance to lovastatin and plate-chase, data not shown and Figure 2-11 on page 75).

If *HRD5* is *not* identical to the *6MYC-HMG2* gene, this result implies that *HRD5* would be required for 6myc-Hmg2p degradation and *not* Hmg2-GFP degradation. This would be surprising because every other *hrd* mutant isolated and tested has stabilized Hmg2-GFP (This work, Bays et al., 2001; Cronin and Hampton, 1999; Gardner et al., 2000), and there is no data to suggest that 6myc-Hmg2p and Hmg2p degradation should have different *HRD* gene requirements. To the contrary, several data suggest that 6myc-Hmg2p is simply always misfolded whereas Hmg2p is misfolded in a regulated manner (Gardner et al., 2001). In light of these data and their resulting model, it is important to know whether *HRD5* is an allele of *6MYC-HMG2* or not.

If *HRD5* is indeed identical to *6MYC-HMG2*, then the Hmg2-GFP result suggests the possibility that the transmembrane domain of Hmg2-GFP is not sufficient to complement the *hrd5-1* allele and that a catalytic domain is required to complement the *in cis hrd5/6myc-hmg2* mutation. (Recall that *6MYC-HMG2*$^{+}$ can complement the *hrd5* allele). This implication would also be surprising since it has long been observed that only the transmembrane domain of Hmg2p is required for the regulated degradation of Hmg2p (Chun and Simoni, 1992; Gardner et al., 1998; Gardner and Hampton, 1999; Jingami et al., 1987; Sekler and Simoni, 1995). To help clarify the nature of *HRD5*, a *6MYC-HMG2-GFP* fusion is currently being cloned. In this fusion, GFP would replace

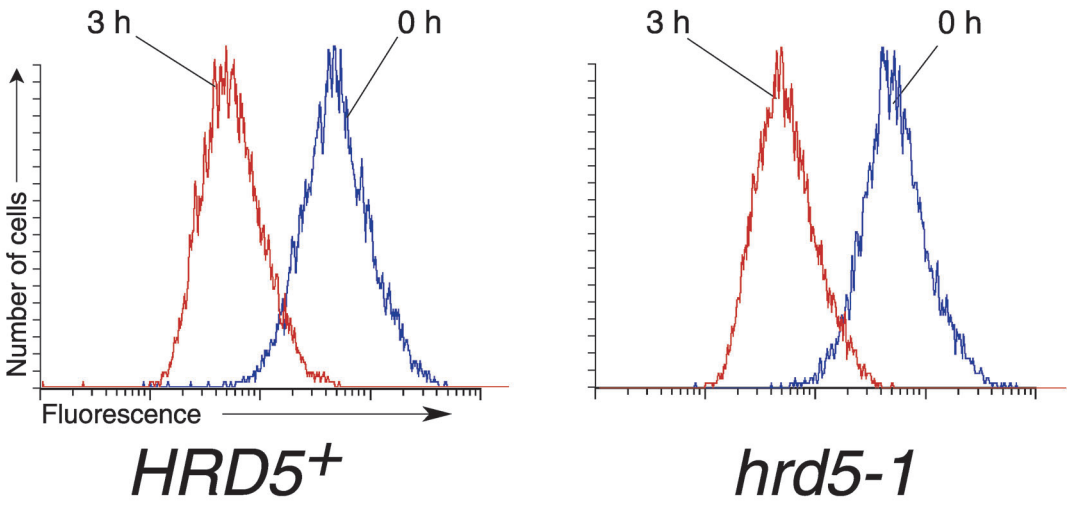

Figure 2-11

Hmg2-GFP is degraded normally in *hrd5-1* cells

Degradation of Hmg2-GFP was assayed by cycloheximide chase. *HRD5* and *hrd5-1* cells expressing Hmg2-GFP were grown into log phase (OD_{600}=0.1). Cycloheximide was added to stop protein synthesis three hours before measuring cell fluorescence by flow cytometry. Each histogram is constructed from the measurement of 20,000 cells.

Both strains also expressed 6myc-Hmg2p, but only *hrd5-1* cells were lovastatin-resistant.

the catalytic domain of 6myc-Hmg2p as it does in the Hmg2-GFP fusion. The degradation of both 6myc-Hmg2p and 6myc-Hmg2-GFP can then be determined in a *hrd5* mutant strain. The possible results from this experiment are summarized inTable 2-6 on page 76 with their most likely interpretation. Two likely results could be quite informative: If *6MYC-HMG2-GFP* is added to *hrd5* cells and both 6myc-Hmg2p and 6myc-

Nature of *hrd5* mutation	6myc-Hmg2p stability	6myc-Hmg2-GFP stability	Interpretation
in cis (mutation in *6MYC-HMG2* gene itself)	stable	stable	Not consistent with phenotype of heterozygous *hrd5/HRD5* diploid
in cis	stable	degraded	**Complementation of *hrd5* requires *6MYC-HMG2* gene coding for catalytic domain**
in cis	degraded	stable	Not consistent with 6myc-Hmg2p stabilization in *hrd5* cells.
in cis	degraded	degraded	6myc-Hmg2-GFP can complement *hrd5* mutation but Hmg2-GFP cannot.
in trans (mutation in a gene other than *6MYC-HMG2*)	stable	stable	**Consistent with *hrd5* as a mutation in a gene other than *6MYC-HMG2*, but also suggests *HRD5* is only required for 6myc-Hmg2p degradation and not native Hmg2p.**
in trans	stable	degraded	Not consistent unless catalytic domain is required for stabilization by *hrd5* which is not likely
in trans	degraded	stable	Not consistent with *in trans* mutation
in trans	degraded	degraded	Not consistent with *in trans* mutation

Table 2-6

Possible outcomes from 6myc-Hmg2-GFP expression in *hrd5* cells

Hmg2-GFP are stable, then future analysis would likely focus on *HRD5* as a gene separate from *6MYC-HMG2* (Recall that 6myc-Hmg2p is degraded normally in a *hrd5/HRD5*$^{+}$ heterozygous diploid.) However, if 6myc-Hmg2p and 6myc-Hmg2-GFP are *both* degraded, then *hrd5* is likely to be an *in cis* mutation in 6myc-Hmg2p itself.

Because of the *in cis* potential of *hrd5*, an important experiment is the rescue of the *6MYC-HMG2::ura3-52* allele from the *hrd5* mutant strain. This allele was introduced into the parent strain by homologous recombination of a linearized plasmid into the genome (Hampton et al., 1996a). This process can be reversed to recover the *6MYC-HMG2::ura3-52* allele from the *hrd5* strain as follows: Genomic DNA isolated from *hrd5* strains is digested with the linearizing restriction endonuclease (StuI), incubated with T4 DNA ligase, and ligation reaction transformed into *E. coli* followed by selection for Amp^{r} bacterial colonies. The recovered *6MYC-HMG2::ura3-52* allele can then be reintegrated into a strain lacking any HMG-CoA reductase (a mevalonate auxotroph). Transformants are recovered simply by plating on YPD. (Only those cells gaining *6MYC-HMG2::ura3-52* [gaining an HMG-CoA reductase] would be viable on media lacking mevalonate) 6myc-Hmg2p degradation can then be assayed in this strain: if 6myc-Hmg2p is stable, then it has finally been demonstrated that *hrd5* cells contain a mutation in *6MYC-HMG2*. If 6myc-Hmg2p is normally degraded, then *HRD5* is simply a gene closely linked to *URA3*. This result can then be combined with the previous genetic data and data gained from Hmg2-GFP and 6myc-Hmg2-GFP experiments to provide a model of Hrd5p in the degradation of Hmg2p.

HRD6

Representative mutants from different *HRD* complementation groups were tested for several global phenotypes including canavanine sensitivity (Figure 2-13 on page 81), cadmium sensitivity (Figure 2-14 on page 82) and temperature sensitivity (Figure 2-12 on page 80). Canavanine is an amino acid structurally similar to arginine (Figure 2-15 on page 79). In fact, canavanine is transported into cells using an arginine transporter (Can1p in *S. cerevisiae*), assembled into normally arginyl tRNAs and finally incorporated into proteins in place of arginine (Grenson et al., 1966; Opekarova et al., 1998; Opekarova et al., 1993; Opekarova and Kubin, 1997). Canavanine is highly toxic to cells, and causes gross protein misfolding as a result of its substitution for arginine (Attias et al., 1969; Knowles and Ballard, 1978; Knowles et al., 1975; Prouty, 1976; Rosenthal et al., 1976). Not surprisingly, mutations in genes required for protein folding and protein degradation render cells more sensitive to canavanine. These genes include chaperones and many subunits of the 26S proteasome (Heinemeyer et al., 1991; Hilt et al., 1993; Jubete et al., 1996; Lambertson et al., 1999; Rubin et al., 1998; Yokota et al., 1996). Therefore, canavanine sensitivity can provide some information about how mutations in a particular *hrd* gene affect the ability of cells to handle the stress of misfolded proteins.

The heavy metal cadmium is also toxic to cells (Rikans and Yamano, 2000; Trevors et al., 1986). Although the exact mechanisms of cadmium toxicity remain a subject of debate, several models argue that cadmium increases the production of active oxygen species (such as hydroxyl radicals) which then lead to an increase in oxidative protein and

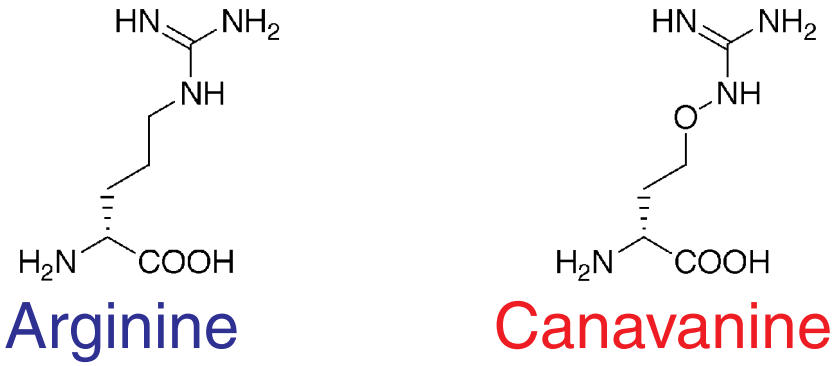

Figure 2-15

Chemical structures of arginine and canavanine

Incorporation of canvanine in proteins instead of arginine leads to gross protein misfolding and cell death.

DNA damage (Brennan and Schiestl, 1996; Hussain et al., 1987; Martins et al., 1991; Ochi and Ohsawa, 1985; Snyder, 1988; Theocharis et al., 1991). Cadmium sensitivity is of particular interest to the study of *HRD* genes because mutations in several genes required for ER-associated protein degradation lead to cadmium sensitivity, including mutation in genes required for the actual ubiquitination of proteins at the ER: *UBC7, UBC1, HRD1,* and *DOA10* (Bays et al., 2001; Jungmann et al., 1993; Swanson et al., 2001).

hrd6 mutants are temperature-sensitive, show no more canavanine sensitivity than *HRD6*$^{+}$ strains, and are only slightly sensitive to cadmium (Figure 2-12 on page 80,

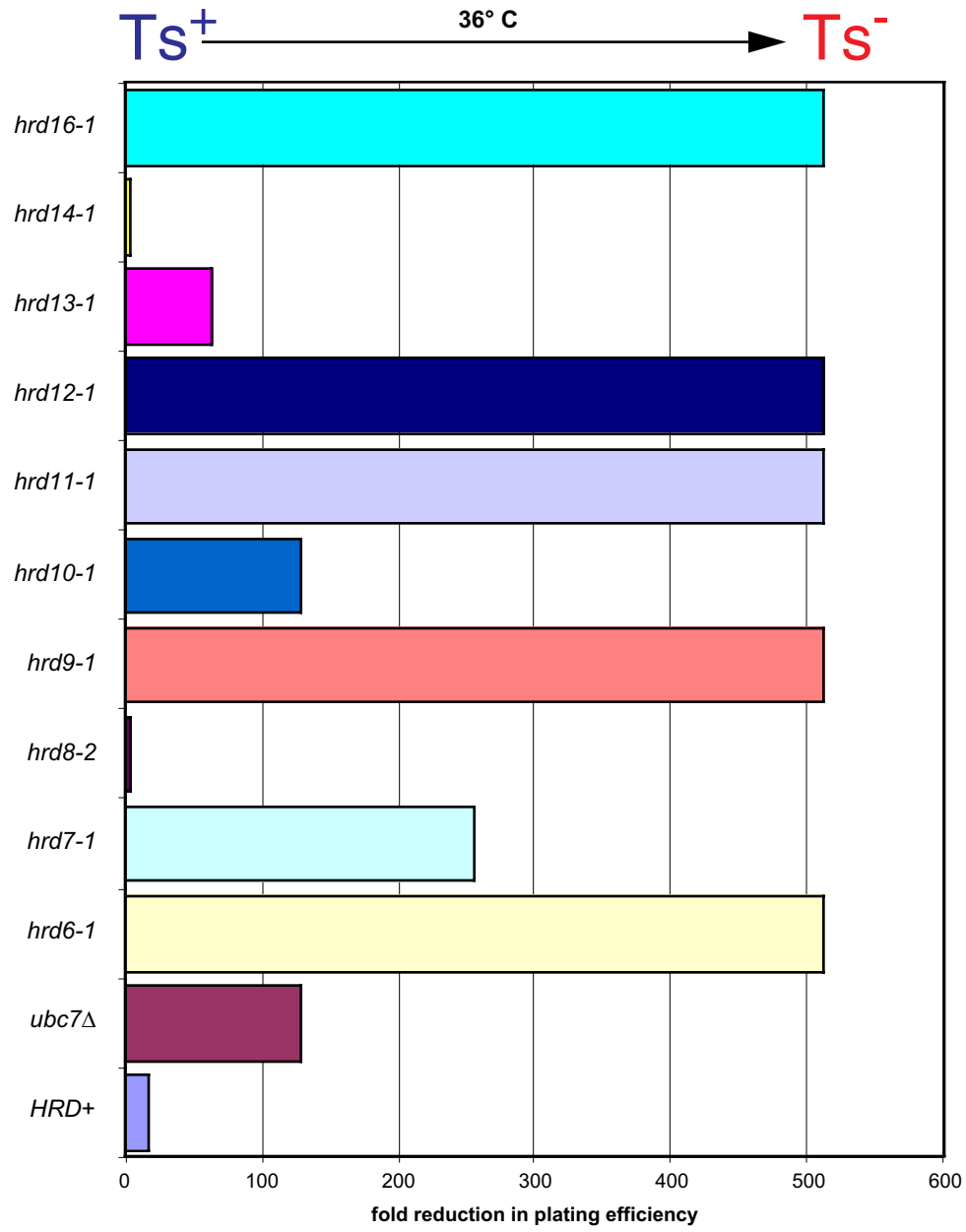

Figure 2-12

Temperature sensitivity of *hrd* mutants

Cells were incubated on yeast minimal media at 36°C. Temperature sensitivity was indicated by loss of plating efficiency compared to 30°C.

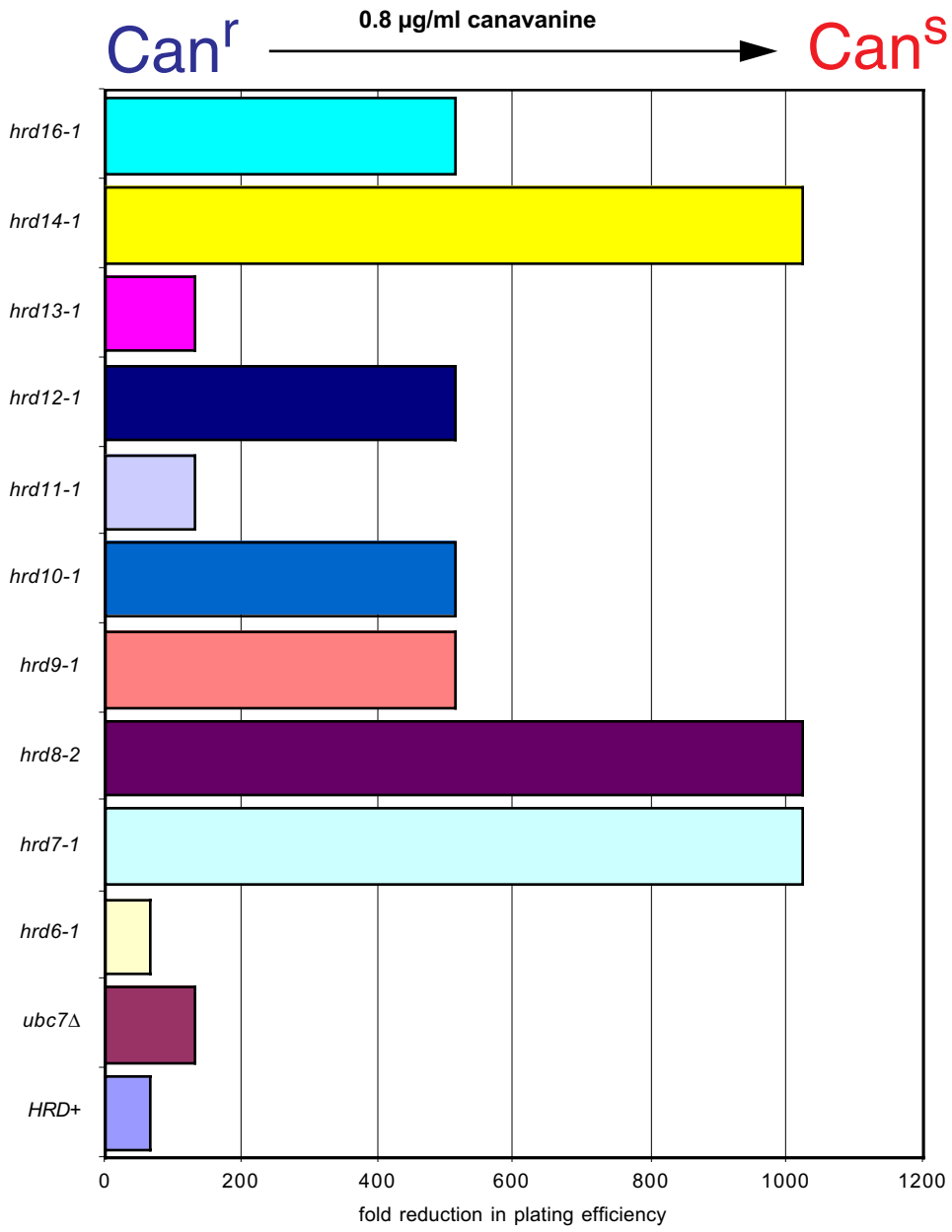

Figure 2-13

Canavanine sensitivity of *hrd* mutants

Cells were incubated on yeast minimal media containing canvanine. Sensitivity was indicated by loss of plating efficiency compared to no drug.

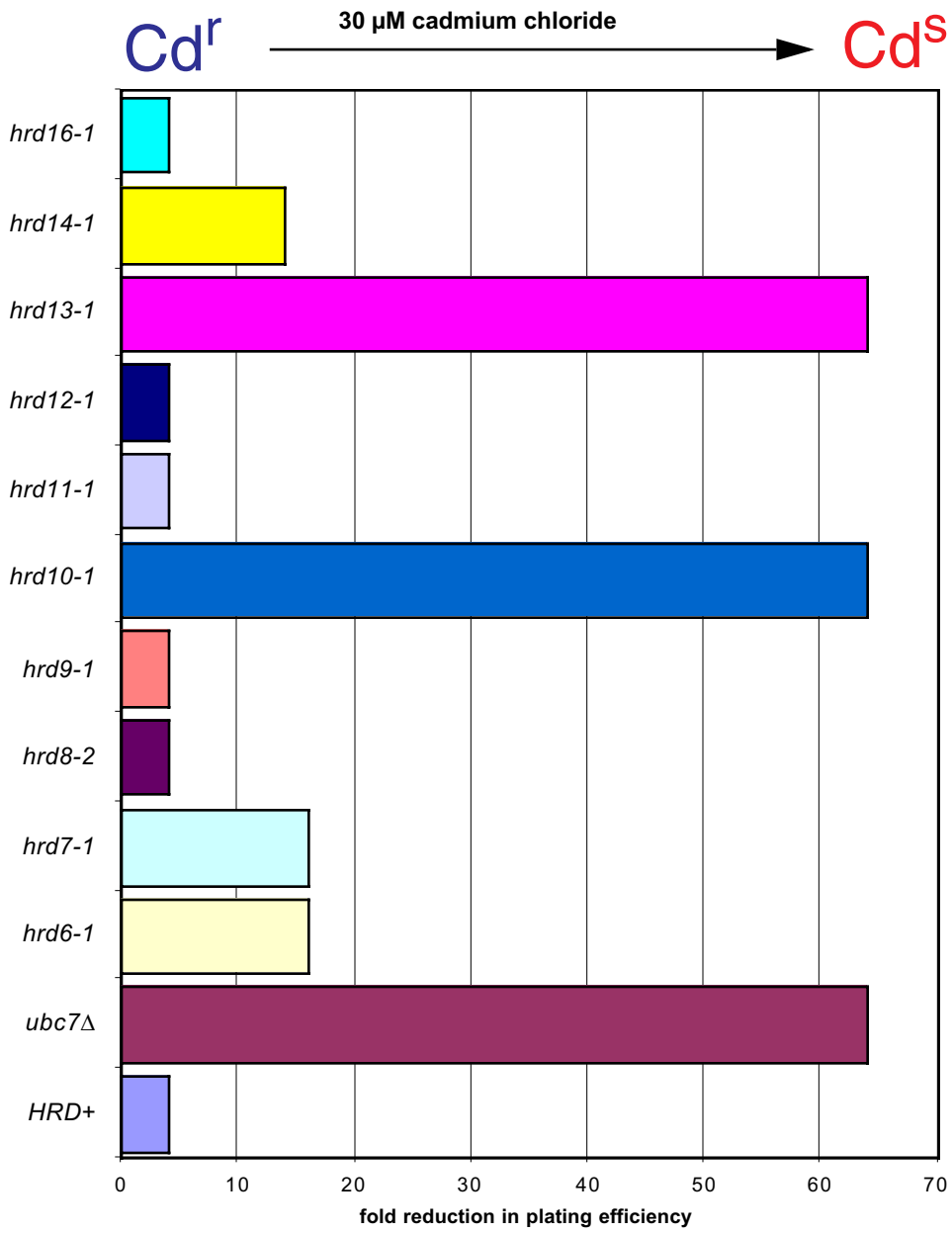

Figure 2-14

Cadmium sensitivity of *hrd* mutants

Cells were incubated on yeast minimal media containing $CdCl_2$. Sensitivity was indicated by loss of plating efficiency compared to plates with no $CdCl_2$.

Figure 2-13 on page 81, and Figure 2-14 on page 82). Furthermore, *hrd6/HRD6* diploids show a higher than normal rate for the emergence of Lov^r colonies (higher "escaper" rate, data not shown). 19 different mutant strains have been assigned to the *HRD6* complementation group. The gene corresponding to *HRD6* has not yet been cloned.

HRD7 may code for a subunit of the 26S proteasome

hrd7 mutants are both temperature sensitive and canavanine sensitive (Figure 2-12 on page 80 and Figure 2-13 on page 81). Four *hrd7* strains were recovered in the *hrd* selection and all these strains fail to grow at 36°C (Appendix B). A strategy to clone the gene corresponding to the *hrd7* mutation was based on the temperature sensitivity of *hrd7* strains: a *hrd7* mutant strain was transformed with a yeast genomic library. After growth into individual colonies at 30°C, transformants were replica plated onto fresh media and incubated at 36°C to select for cells transformed with a plasmid capable of reversing the temperature sensitivity phenotype. Five different colonies were recovered that showed complementation of both the Ts^- and Lov^r phenotypes (Figure 2-16 on page 84 and data not shown). Plasmid DNA was recovered from these colonies, amplified in *E. coli* and used to retransform the original *hrd7* mutant strain. Since the pure plasmid DNA was capable of reversing both phenotypes (Figure 2-16 on page 84 and data not shown), two different recovered plasmids were sequenced. Both plasmids contained overlapping fragments of chromosome XV (Dujon et al., 1997). Five open read-

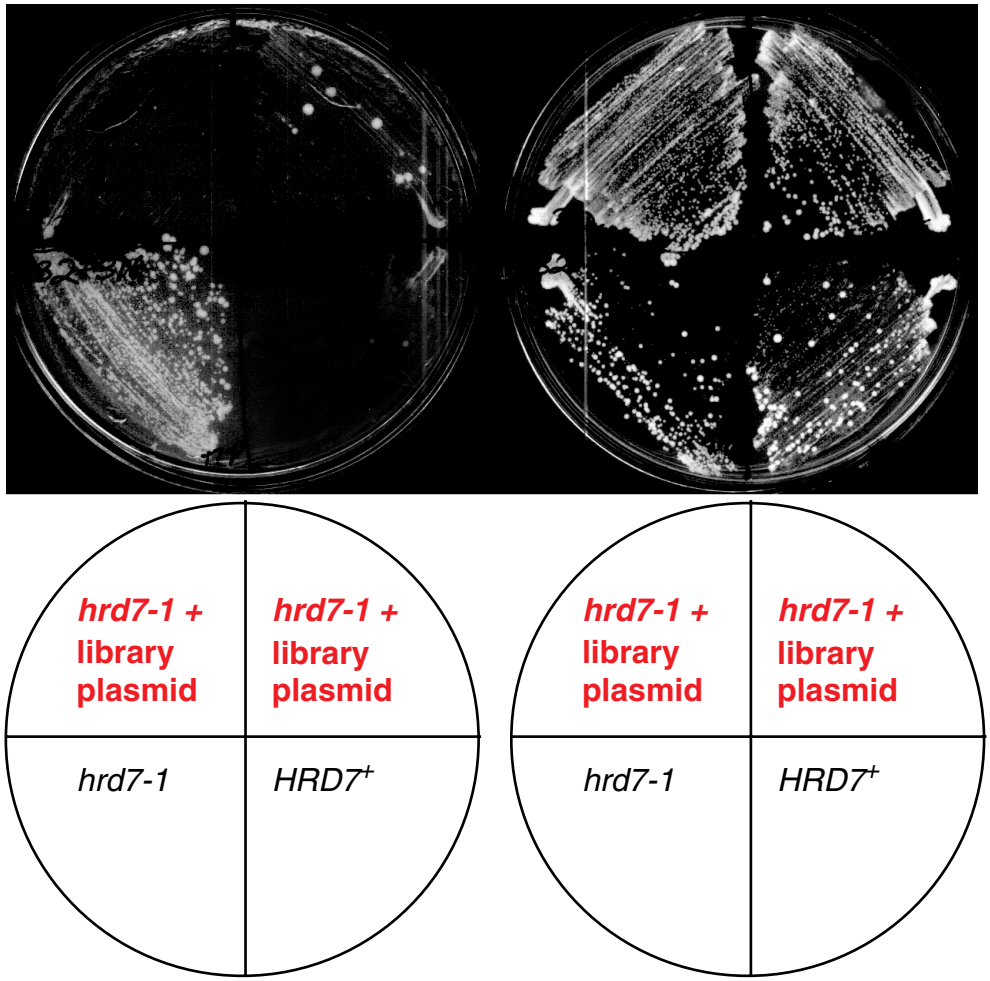

Figure 2-16

Complementation of the *hrd7-1* mutation by a genomic library plasmid

hrd7-1 cells were transformed with a *LEU2* library plasmid isolated by virtue of its ability to confer growth to *hrd7-1* cells at 36°C. The indicated transformants were also plated on yeast minimal media containing lovastatin to test the ability of the genomic library plasmid to reverse the lovastatin resistance phenotype of *hrd7-1* cells.

ing frames (orf)s are contained in this region (Cherry et al., 2001). One of these is the gene coding for Rpn8p, a subunit of the 26S proteasome (Dubiel et al., 1995; Glickman et al., 1998; Tsurumi et al., 1995). Rpn8p is highly homologous to the product of the mouse MOV-34 gene as well as the gene coding for human p40. (Dubiel et al., 1995; Gridley et al., 1990; Gridley et al., 1991; Soriano et al., 1987; Tsurumi et al., 1995). Rpn8p/Mov-34/p40 was identified as a subunit of the 26S proteasome by virtue of biochemical evidence of its assembly into the 19S regulatory subunit of proteasome (Dubiel et al., 1995; Glickman et al., 1998; Tsurumi et al., 1995). Consistent with its role in the 26S proteasome, *RPN8* transcripts are elevated during several stress conditions, along with transcripts for other proteasome subunits (Jelinsky et al., 2000).

RPN8 is a strong candidate for the gene mutated in *hrd7* strains as the 26S proteasome is known to be required for the degradation of 6myc-Hmg2p (Hampton et al., 1996a). Unfortunately because of its biochemical assignment to the proteasome, no phenotypic data is reported for *rpn8* mutants other than the observation that *rpn8*Δ cells are not viable (Winzeler et al., 1999). However, *rpn8* mutants may well join other proteasome subunits in displaying temperature sensitivity and canavanine sensitivity like *hrd7*mutants. Currently, a plasmid containing only the *RPN8* orf and the *URA3* gene is being cloned. This plasmid will allow the full genetic analysis required to determine whether *HRD7* is identical to *RPN8*.[14]

HRD1 is a high-copy suppressor of *hrd8*

During analysis of mutants recovered in the third repetition of the *hrd* selection, *hrd8* mutants displayed an abundance of different phenotypes. *hrd8* mutants stood out early in the selection because they consistently gave a very strong signal in the plate-chase assay. *hrd8* cells also displayed unique growth phenotypes. *hrd8* cells grew very slowly in liquid media until cultures reached an OD_{600} of ~0.8 when cells suddenly grew at wild-type rates (data not shown). On solid media containing glucose, *hrd8* cells grew more slowly than wild-type cells, but this difference was greatly exacerbated when *hrd8* cells were plated on glycerol (data not shown). Despite these growth phenotypes, *hrd8* cells show no detectable temperature sensitivity (Figure 2-12 on page 80). *hrd8* cells, however, are canavanine sensitive (Figure 2-13 on page 81). When *hrd8-1*[15] cells were plated on solid media, fast-growing colonies appeared at a rate of about 1 in 1,000. These large colonies appeared at a rate of about 1 in 3,500 in *hrd8-2* cells (data not shown). In about

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14. An example of such genetic analysis is given in Chapter 4 in the cloning of *HRD4*.

15. Two different alleles are presumed here because in two independently-generated *hrd8* isolates (i.e. isolates unlikely to be siblings), two different growth and "escaper" rates are consistently seen. Because growth phenotypes can be variable, one must leave open the possibility that these growth differences are due to physiological and not genetic reasons.

80% of these large colonies, fast growth and lovastatin-sensitivity were observed. However, fast-growing but lovastatin-resistant colonies were also isolated from plating *hrd8* cells. It is not known whether this reversion is due to conversion of a mutant *hrd8* allele to *HRD8*$^{+}$ or whether several different cases of extragenic suppression are occurring. The following data, however, indicate that at least one mechanism of extragenic suppression for *hrd8* is possible.

Once complementation groups were assigned to *hrd* mutants isolated in the third repetition, representative mutants were transformed with plasmids containing either *HRD1, HRD2,* or *HRD3* genes to test whether these plasmids were capable of reversing lovastatin resistance in newly isolated *hrd* mutants – just in case the earlier genetic analysis had failed to detect that a "new" complementation group was actually a previously identified *HRD* gene. When *hrd8-1* cells were transformed with these plasmids, a low-copy plasmid bearing the *HRD1* gene was able to confer lovastatin sensitivity. This was not expected for the simple fact that *hrd1* mutants do not exhibit any of the growth phenotypes associated with *hrd8* mutants (Hampton et al., 1996). To ask whether *hrd8* was an allele of *HRD1*, *hrd8-1* and *hrd8-2* cells were crossed to a *hrd1*Δ strain. The resulting diploids were lovastatin sensitive (Figure 2-18 on page 89), indicating neither *hrd8-1* or *hrd8-2* was not an allele of *HRD1.* Further analysis indicated that the suppression by the low-copy *HRD1* plasmid was not complete: *hrd8* cells were made more lovastatin sensitive but were still more lovastatin resistant than wild-type cells (Figure 2-17 on page 88). Complete suppression was obtained, however, when *hrd8-1* cells were transformed with

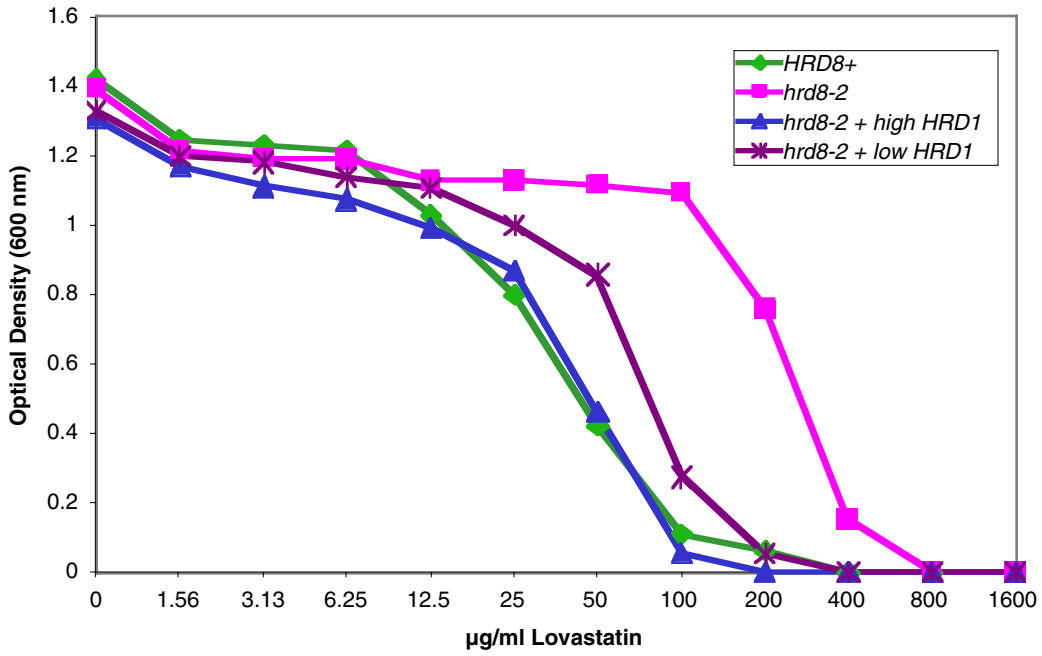

Figure 2-17

Suppression of lovastatin resistance in *hrd8-2* cells by *HRD1*

Cultures of the indicated strains were inoculated at low cell density (OD_{600} < 0.001) in yeast minimal media and added to serial dilutions of lovastatin also made in the same media. Cells were allowed to grow until cultures without lovastatin grew to an OD_{600} of 1.4

either a high-copy 2µ plasmid or an integrating plasmid expressing *HRD1* from the strong, constitutive *TDH3* promoter. The same suppression was also seen when *hrd8-2* cells were transformed with *HRD1* plasmids (Figure 2-17 on page 88). Interestingly, *HRD1* only suppressed the lovastatin resistance phenotype of *hrd8* cells. *HRD1* did not suppress the growth phenotypes of *hrd8* cells (data not shown).

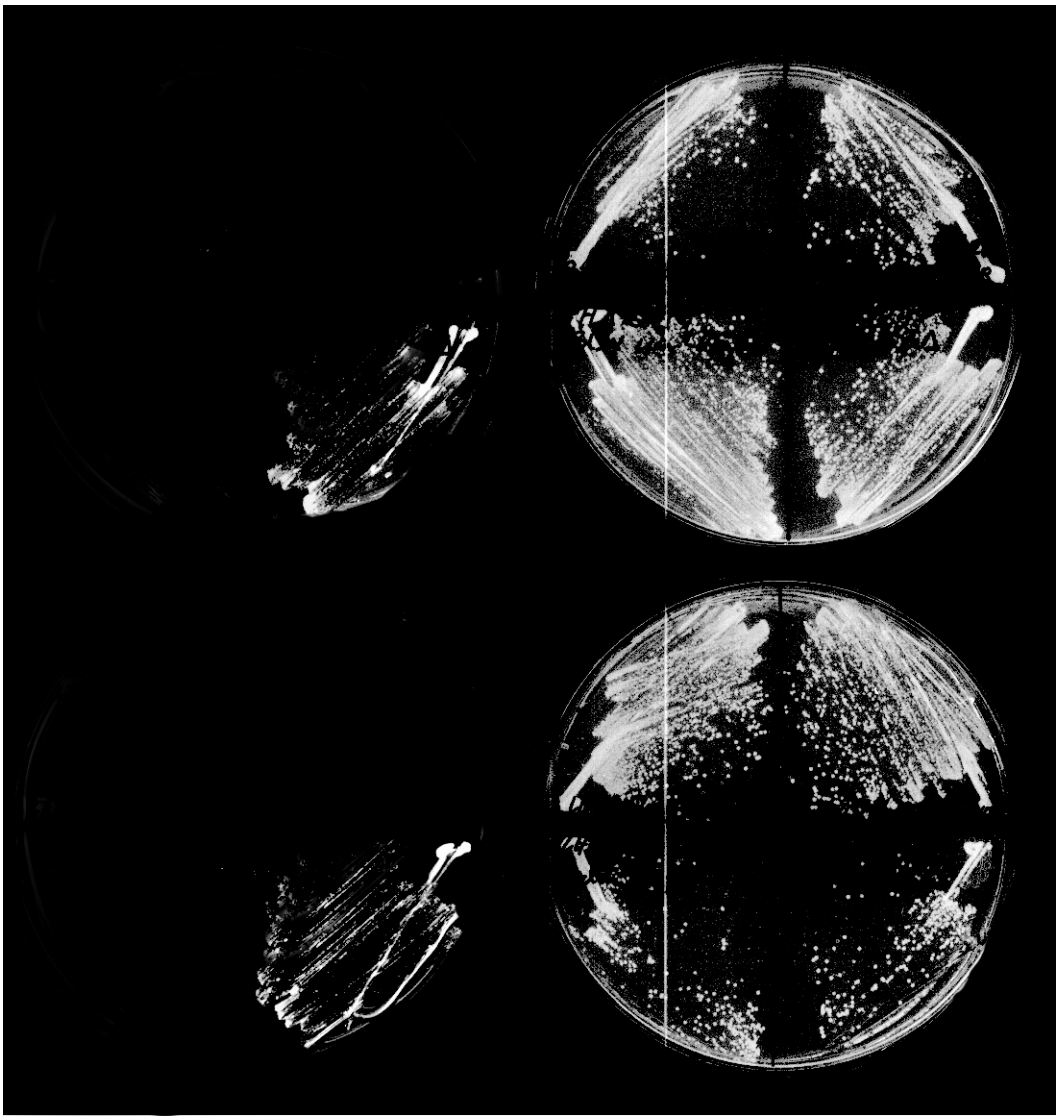

Figure 2-18

hrd8* alleles are not alleles of *HRD1

hrd8-1 and *hrd8-2* strains were crossed to *hrd1Δ* and *HRD*$^{+}$ strains. The resulting diploids were then assayed for lovastatin resistance.

A legend can be found on the next page.

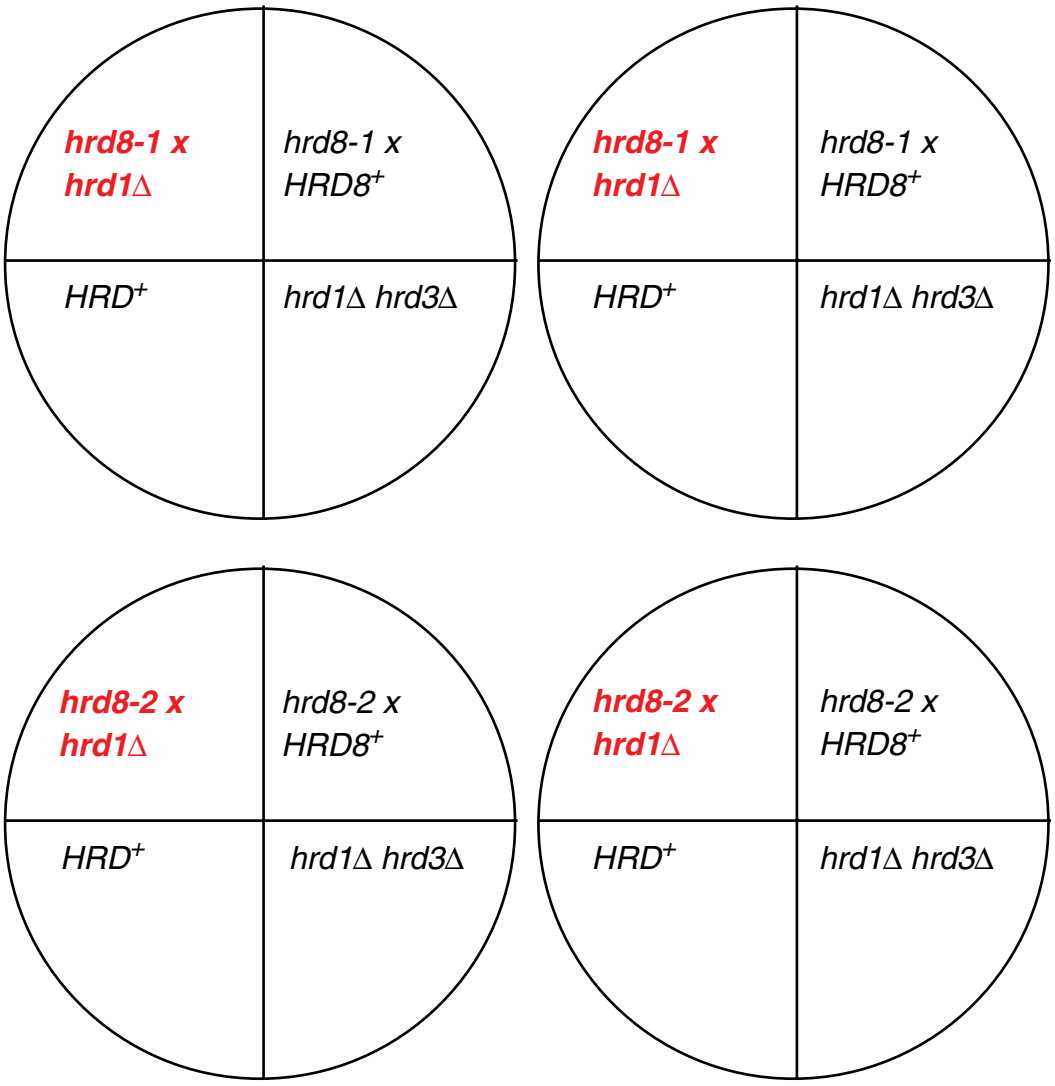

Figure 2-18 (continued)

hrd8* alleles are not alleles of *HRD1

Legend for plates on the previous page.

It would be interesting enough that *HRD8* shows a genetic interaction with another *HRD* gene, but *HRD1* is not just any *HRD* gene. As detailed in the following chapter, the Hrd1 protein is a ubiquitin-protein ligase and serves as a critical specificity-determining factor in ER protein degradation. Furthermore, *hrd8* mutants are not the only mutants suppressed by increased *HRD1* expression: *HRD1* can completely suppress *ubc7Δ, ubc1Δ* and *hrd3Δ* mutants as well (Chapter 3). All three of these genes are required for the actual ubiquitination of ER proteins and their suppression by *HRD1* reveals important features of ERAD itself. Therefore, suppression of *hrd8* by *HRD1* may well provide additional information about the mechanism of ERAD.

Reaping the full harvest of information from *hrd8* mutants requires the identification of the gene corresponding to the *hrd8* mutation. Unfortunately, attempts at cloning *HRD8* have not been successful. Four attempts have been made with two different genomic libraries (F. Spencer and P. Hieter, unpublished results; Rose et al., 1987) and two different *hrd8* alleles. No plasmid capable of fully complementing *hrd8* phenotypes has been found. The reason for this recalcitrance is not clear. It is possible, for instance, that the *HRD8* DNA is toxic to *E. coli* as are some other regions of *S. cerevisiae* DNA (Brown and Campbell, 1993; Estruch et al., 1989; Holzer and Hammes, 1989; Polumienko et al., 1986; Webster and Dickson, 1983; Wei and Friedberg, 1998). In light of this possibility, a cloning strategy is being devised that relies on inactivating the *HRD8* coding region in a heterozygous *hrd8-2/HRD8*$^{+}$ diploid. As *hrd8-2* is a recessive allele of *HRD8,* a *hrd8-2/HRD8*$^{+}$ diploid is sensitive to lovastatin. However, inactivation of

the wild-type allele should create a lovastatin-resistant *hrd8-2/hrd8*Δ diploid. Transposon mutagenesis would be ideal for this inactivation of the wild-type allele because the insertion of a transposable element (marked, for instance, with the *KanMX* gene[16]) could be recovered from the genome by restriction digestion and ligation to generate plasmids bearing the transposable element flanked by yeast genomic DNA in the *HRD8* region. In the worst-case scenario, the inactivated *hrd8* would still be toxic to bacteria and the yeast genomic DNA could be sequenced using primers identical to the ends of the transposable element. Unfortunately, the current transposon-tagged libraries of yeast genes that could be used for this approach were constructed in *E. coli*,[17] and thus run the same risk of losing clones to DNA toxicity as untagged genomic libraries (Burns et al., 1994; Huisman et al., 1987; Seifert et al., 1986). One approach to circumvent DNA toxicity would be to conduct transposon mutagenesis *in zyme*.[18] Transposable elements native to *S. cerevisiae* would likely not be used in these experiments because the site of integration for these elements is far from random and many genes would simply never be mutagenized (Jacq et al., 1997; Kunz et al., 1994; Roeder and Fink, 1982; Roeder et al., 1984; Warmington et al., 1986). One might begin with the Tc1/*mariner* family of

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16. *KanMX* confers resistance to kanamycin/G-418/Geneticin™ and is attractive for this use because the same *KanMX* allele confers resistance in both bacteria and yeast (Güldener et al., 1996; Jimenez and Davies, 1980; Polumienko et al., 1986; Webster and Dickson, 1983).

transposable elements that do show more promiscuous integration and function in a wide variety of species (Jarvik and Lark, 1998; Plasterk et al., 1999; Schouten et al., 1998; van Luenen and Plasterk, 1994). In this way, a transposable element + transposase like the vertebrate *Sleeping Beauty* (Ivics et al., 1997; Izsvak et al., 2000) or the fungal *impala* (Hua-Van et al., 1998; Langin et al., 1995) could be engineered with different drug resistance and/or auxotrophy markers for use in *S. cerevisiae*. The experiments to determine the feasibility of this approach are fairly straightforward because of the genetic tractability of *S. cerevisiae:* there are many different genes whose rate of mutagenesis can be quickly determined and verified. Ironically, it is probably the tractability of *S. cerevisiae* to genetics and molecular biology that has allowed the absence of an effective *in zyme* transposon mutagenesis system to continue for so long. Since such systems are critical for genetic studies in other organisms, much is known about the design and use of transposons, and it may be time to exploit that knowledge for a system in *S. cerevisiae*. Such a system will not revolutionize the use of yeast as a model organism, but it should prove useful in a variety of genetic applications.

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17. The construction of these transposon-tagged yeast libraries has involved transformation of *E. coli* with plasmids containing yeast genomic DNA and *then* transposition of a chromosome-based marked mobile element into the yeast genomic DNA (Burns et al., 1994; Huisman et al., 1987; Seifert et al., 1986).

18. "in yeast," latin base for "enzyme"

HRD9

So far, twenty-nine mutants belong to the *HRD9* complementation group. These mutants are not temperature sensitive (Figure 2-12 on page 80 and Appendix B), and are only moderately sensitive to canavanine (Figure 2-13 on page 81). *hrd9* mutants also show a slight sensitivity to cadmium (Figure 2-14 on page 82). No attempts at cloning *HRD9* have been made so far.

HRD10 is identical to *UBC7*

Ubc7p is the principal ubiquitin-conjugating enzyme involved in ER-associated protein degradation, including the degradation of Hmg2p (Bays et al., 2001; Hampton and Bhakta, 1997; Hiller et al., 1996; Swanson et al., 2001; Wilhovsky et al., 2000). *ubc7* mutants were not isolated, however, in the first two repetitions of the *hrd* selection, and the involvement of Ubc7p in Hmg2p degradation was first tested based on its previously identified role in ER-associated degradation (Hampton and Bhakta, 1997). The absence of *ubc7* mutants from the *hrd* selection was one glaring indication of the limitations in the previous *hrd* selection: The previous selection was not producing mutants in genes known to be required for Hmg2p degradation. The modified *hrd* selection, however, was able produce mutant alleles of *UBC7*. A low-copy ARS/CEN plasmid bearing the *UBC7* gene was able to reverse the lovastatin resistance of *hrd10* mutants (data not shown). When a *hrd10-1* strain was crossed to a *ubc7*Δ strain, a lovastatin-resistant diploid was formed (data not shown). Dissection of this diploid produced segregants that

were always lovastatin-resistant, indicating that the *hrd10-1* and *ubc7*Δ alleles reside at the same locus (data not shown). These data indicated that *hrd10-1* and *ubc7*Δ are loss-of-function alleles of the same gene, *UBC7*.

HRD11

hrd11 mutants are temperature sensitive (Figure 2-12 on page 80), but are only slightly canavanine sensitive (Figure 2-13 on page 81). Furthermore, *hrd8* mutants show a weaker Hrd$^-$ phenotype than other *hrd* mutants in that *hrd8* mutants are less lovastatin resistant and do not show full stabilization of 6myc-Hmg2p as measured in a plate-chase assay (data not shown).

HRD12 and non-complementation with *HRD1*

hrd12 mutants are also temperature sensitive (Figure 2-12 on page 80), but display a moderate sensitivity to canavanine (Figure 2-13 on page 81). *hrd12* mutants also somewhat defective in mating. Specifically, *hrd12* mutants appear to mate at a low efficiency with haploids of either the opposite or the same mating type (a or α). Such cells are sometimes called "bimaters." *hrd12* cells of both mating types display this low-efficiency bimating (data not shown). The source of this phenotype is unknown, but will likely be illuminated by the actual identity of the gene mutated in *hrd12* strains. When *hrd12* strains are crossed to a *hrd1* mutant strain, some non-complementation is seen in the resulting diploid: 6myc-Hmg2 is slightly stabilized as measured in the plate-chase assay (data not shown). This slight non-complementation is also seen when *hrd3* or *hrd14* cells

are mated to *hrd1* strains. Although the genetic analysis of *hrd* mutants in this selection generated thousands of diploids, non-complementation was only seen in the following matings: *hrd3* x *hrd1, hrd12* x *hrd1,* and *hrd14* x *hrd1.* Since each of these complementation groups contained at least 9 members, there were multiple instances of non-complementation for each mating listed, reducing the chance that simple wobble in the plate-chase assay was responsible for the observation. Furthermore, non-complementation was also seen in lovastatin resistance for these matings (data not shown). This non-complementation was only slight and did not generate the same degradation defect as diploids homozygous for *hrd1, hrd3, hrd12,* or *hrd14.* The exact source of the non-complementation in each of these matings is not known, but the *hrd1* x *hrd3* non-complementation may well be due to a reduced amount of Hrd1p in the resulting diploid. Hrd1p is strictly rate-limiting for the degradation of Hmg2p (see Chapter 3), and Hrd3p is required for the stability of Hrd1p. Thus, in a *hrd1/HRD1*$^{+}$ *hrd3/HRD3*$^{+}$ diploid, the half-dose of Hrd1p may result in some slower degradation of 6myc-Hmg2p. A *hrd1/HRD1*$^{+}$ *HRD3*$^{+}$*/HRD3*$^{+}$ diploid may contain more Hrd1p because more Hrd3p is present. This is of course, is simply a model, and remains to be tested. Once the actual identity of *HRD12* and *HRD14* are known, it may become more apparent why their mutants show slight non-complementation with *hrd1*.

HRD13 as *CUE1*

Ubc7p is the principal ubiquitin conjugating enzyme for ER-associated degradation (Bays et al., 2001; Hampton and Bhakta, 1997; Hiller et al., 1996; Swanson et al., 2001; Wilhovsky et al., 2000). Ubc7p, however, is a soluble protein. To function at the endoplasmic reticulum, Ubc7p requires the protein Cue1p (Biederer et al., 1997). Cue1p recruits Ubc7p to the endoplasmic reticulum, and is absolutely required for Ubc7p to function as a ubiquitin-conjugating enzyme at the ER (Biederer et al., 1997). As a result, *cue1* mutants are often phenotypically identical to *ubc7* mutants for the degradation of several different ER proteins (Biederer et al., 1997; Walter et al., 2001). Therefore, it was expected that *CUE1,* like *UBC7,* would be a *HRD* gene. Once complementation groups were assigned for the new *hrd* mutants, a representative mutant from each complementation group was transformed with a low copy ARS/CEN plasmid bearing the *CUE1* gene (Biederer et al., 1997). Only a member of the *HRD13* complementation was affected by transformation with this plasmid: The *CUE1* plasmid rendered *hrd13* cells lovastatin sensitive (data not shown). This observation coupled with the similar temperature sensitivity and cadmium sensitivity of *cue1*, *ubc7,* and *hrd13* mutants (Figure 2-12 on page 80, Figure 2-14 on page 82, and data not shown) strongly suggest that *HRD13* is identical to *CUE1.* A *cue1Δ::LEU2* allele has recently been constructed in the 6myc-Hmg2p strain background and will be used to test whether *hrd13-1* is a mutant allele of *CUE1*.

HRD14: potentially proteasomal *PRE2*

Although *HRD14* strains are not temperature sensitive (Figure 2-12 on page 80), they are quite sensitive to canavanine (Figure 2-13 on page 81). It was thought that this combination might suggest a gene that was not essential (and thus not likely proteasomal) but that still coded for a protein required in some aspect of quality control, perhaps as a protein chaperone. This class of protein could be quite enlightening to the mechanism of ER-associated degradation and thus an effort was made to clone the gene corresponding to the *hrd14* mutation. Despite the previous reasoning, it appears that *HRD14* may well be another gene coding for a subunit of the 26S proteasome.

hrd14 cells were transformed with a yeast genomic library bearing *LEU2* as a marker gene. Leu^+ cells were then replica-plated onto plates with and without lovastatin. Colonies were then screened for lovastatin sensitivity. Three lovastatin-sensitive colonies were recovered from approximately 30,000 lovastatin-resistant colonies. Plasmid DNA obtained from these colonies was capable of conferring lovastatin sensitivity to the original parent strain (data not shown). The yeast genomic fragment was sequenced and found to contain a 9 kilobase region of *S. cerevisiae* chromosome XVI. This region contains the gene *PRE2/PRG1/DOA3* (a core subunit of the 26S proteasome), *FHL1* (Forkhead-like protein, a putative transcription factor), *SNT309* (complexed with the splicing factor Prp19p), and four hypothetical open reading frames (Chen et al., 1998; Chen et al., 1999; Chen and Hochstrasser, 1995; Cherry et al., 2001; Friedman et al., 1992; Heinemeyer et al., 1993; Heinemeyer et al., 1991; Hoch and Pankratz, 1996;

Weigel et al., 1989). Currently, the *PRE2* gene alone is being cloned to test whether *HRD14* is identical to *PRE2*. Since the 26S proteasome is required for the degradation of Hmg2p, *PRE2* stands as a likely candidate for *HRD14*. Despite the fact that the *PRE2* gene is essential for life (Friedman et al., 1992), several *pre2* alleles are *not* temperature sensitive although several other *pre2* alleles are compromised for growth at both 30˚C and 37˚C (Chen and Hochstrasser, 1995; Heinemeyer et al., 1997; Heinemeyer et al., 1993; Heinemeyer et al., 1991). *pre2* strains are defective in the degradation of several proteins and are reported as sensitive to canavanine (Friedman and Snyder, 1994; Heinemeyer et al., 1993; Heinemeyer et al., 1991). If *HRD14* is identical to *PRE2*, then it would mark the first reported *HRD* gene coding for a core subunit[19] of the 26S proteasome rather than a component of the 19S cap.

hrd15-1 is suppressed by *HRD2/RPN1*

As mentioned previously, representative members of each new complementation group were transformed with *HRD1, HRD2* and *HRD3* plasmids just in case genetic analysis had failed to identify a "new" complementation group as a previously isolated *HRD* gene. Although *hrd15-1* cells were transformed with a variety of different low-copy *HRD* plasmids, only a plasmid bearing the *HRD2* gene (Hampton et al., 1996a)

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19. *HRD2/RPN1* codes for a subunit of the 19S cap rather than the actual 20S proteolytic core

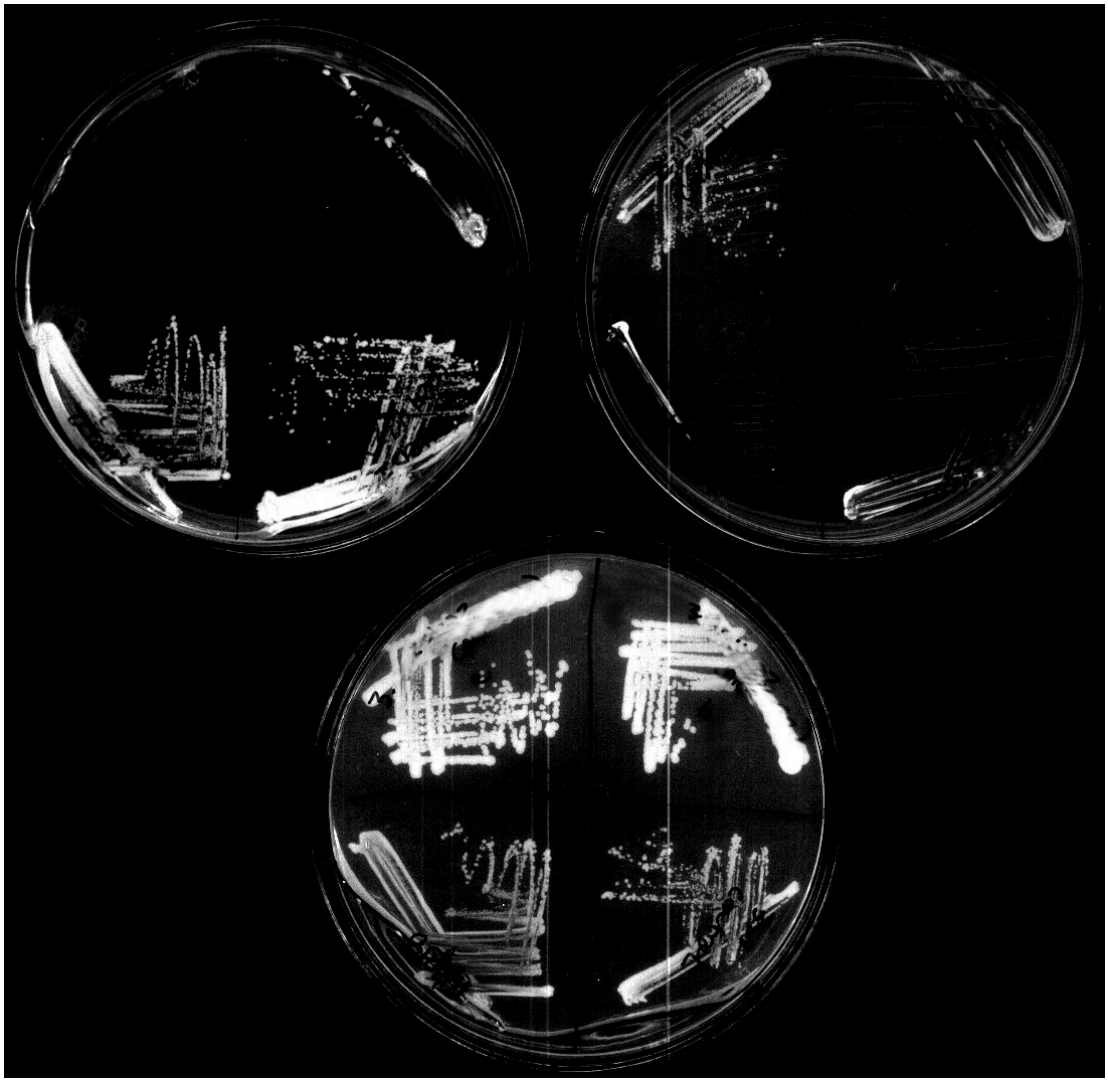

Figure 2-19

Suppression of *hrd15-1* phenotypes by *HRD2*

Indicated *hrd15-1* strains were transformed with a low copy ARS/CEN *HRD2* plasmid. Transformants were then assayed for *hrd15* phenotypes of temperature sensitivity and lovastatin resistance.

Legend for strains and media is given on the following page.

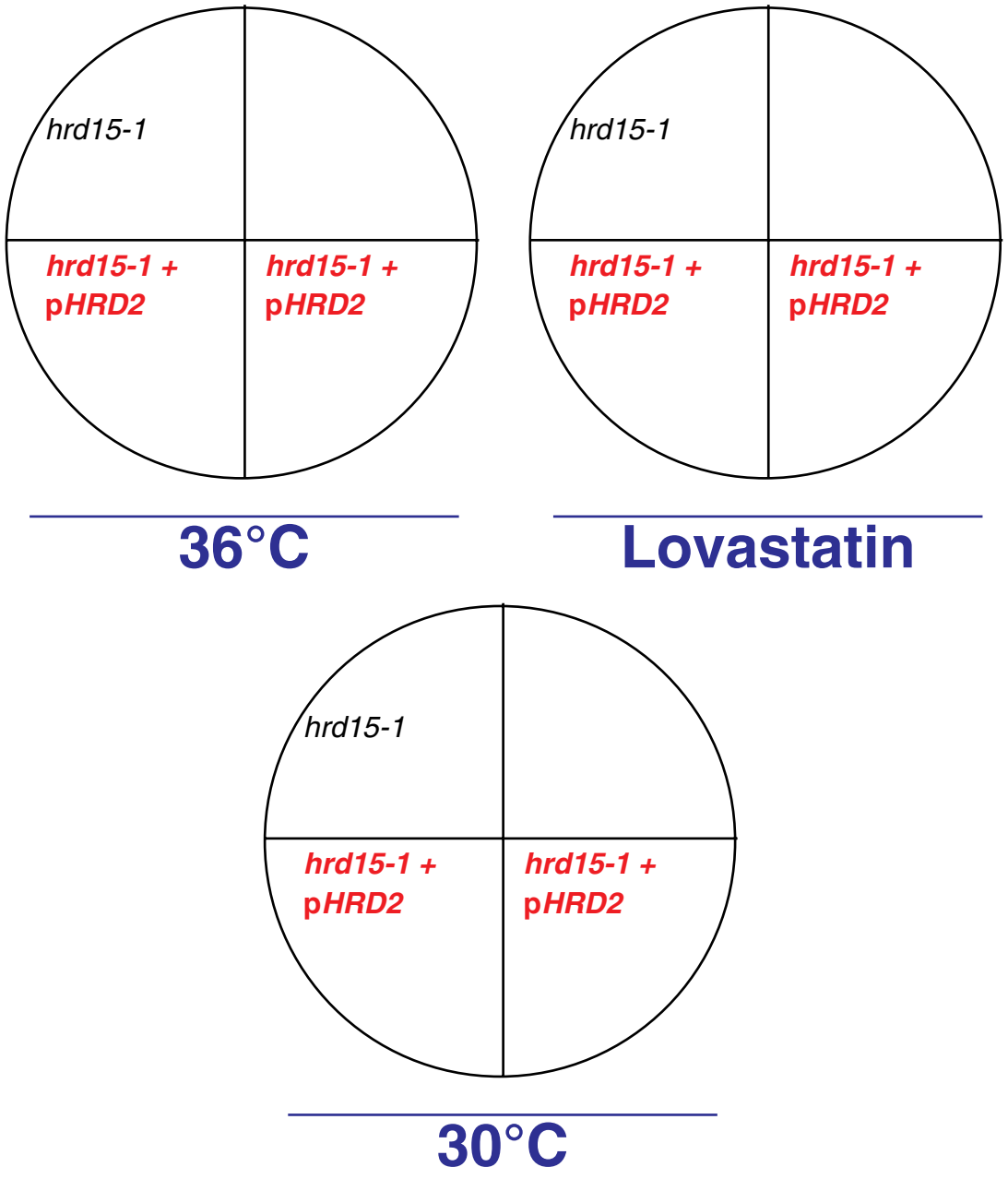

Figure 2-19 (continued)

Suppression of *hrd15-1* phenotypes by *HRD2*

Legend for plates pictured on previous page

conferred lovastatin sensitivity to *hrd15-1* cells. Unlike the suppression of *hrd8* by *HRD1,* a low copy *HRD2* plasmid was able to confer *full* lovastatin sensitivity to *hrd15-1* cells (Figure 2-19 on page 100). Furthermore, *hrd15* cells are temperature sensitive, and this phenotype was also reversed by the addition of a *HRD2* plasmid (Figure 2-19 on page 100). This full suppression of *hrd15* phenotypes strongly suggested that *hrd15* was simply an allele of *HRD2.* To test this, *hrd15-1* cells were crossed to *hrd2-1* cells to form diploids. Surprisingly, these diploids were lovastatin-sensitive and Ts^+ indicating that *hrd15-1* and *hrd2-1* were alleles of two different genes (data not shown). Diploids from the *hrd15-1* x *hrd2-1* cross were dissected, and segregation of the lovastatin-resistance phenotype indicated that *HRD15* and *HRD2* were separate, unlinked loci (data not shown).

The suppression of *hrd15-1* by a low-copy *HRD2* plasmid is quite strong (Figure 2-19 on page 100), and it raises the suspicion that *HRD15* codes for a subunit of the of the 26S proteasome. This suspicion is enhanced by previous reports of strikingly similar suppression by proteasomal genes. For instance, mutant alleles of *rpn12* can be suppressed by increased expression of *RPN10* or *RPN3* (Kominami, et al., 1997). Given that the 26S proteasome is a multisubunit complex, cases of such cross-suppression might well be expected. If Hrd15p is not a subunit of the proteasome, then *HRD2* suppression could become even more informative. *HRD2* suppression could point to a previously unknown aspect of proteasome interaction with accessory proteins, for instance. The

identity of *HRD15*, of course, is required to construct a meaningful model of Hrd15p-Hrd2p interaction.

hrd15-1 is not the only mutant allele suppressed by *HRD2*. *HRD2* is also a high-copy suppressor of the mating defect in *ste6-90* and *ste6-166* mutant cells. (Loayza et al., 1998) Ste6p is normally localized to the plasma membrane, but both Ste6-90p and Ste6-166p are retained in the endoplasmic reticulum and do not appear at the plasma membrane. The ER retention and degradation of a mutant protein is a familiar theme in the quality control of proteins passing through the secretory pathway (See Table 1-1 on page 9; Finger et al., 1993; Galan et al., 1998; Hammond and Helenius, 1994; Hill and Cooper, 2000; Kowalski et al., 1998; Qu et al., 1996; Shenkman et al., 1997). Consistent with this theme, Ste6-166p is retained and degraded at the ER (Loayza et al., 1998). Ste6-90p, however, is retained but not degraded. This suggests that Ste6-90p may actually be subject to a defect in trafficking rather than a folding defect. Unfortunately, no data have been reported to differentiate these two fates for Ste6-90p. The mechanism of *ste6-90* and *ste6-166* suppression by *HRD2* is not known, but the fact that Ste6-90p is stable and Ste6-166 is unstable complicates the construction of models related strictly to ERAD and even degradation. As a result, it should also be stated that the possibility exists that Hrd2p may exhibit some function separate from its role in the 26S proteasome. This is not simply a formal possibility. Other proteasome subunits like Rpt6p/Sug1p appear to play roles in the cell distinct from their role in the 26S protea-

some (Fraser et al., 1997; Makino et al., 1997; McDonald and Byers, 1997; Rubin et al., 1996; Weeda et al., 1997).

HRD16-HRD20

Two *hrd16* strains have been isolated so far, and both are temperature sensitive and moderately sensitive to canavanine (Figure 2-12 on page 80 and Figure 2-13 on page 81). There are also two members of the *HRD17* complementation group. *HRD18, HRD19,* and *HRD20* are each defined by one *hrd* mutant strain that complements every other *hrd* mutant strain when crossed to form a diploid. *hrd17, hrd18, hrd19,* and *hrd20* mutants are not temperature sensitive (Appendix B). The definition of *HRD16-HRD20* by two or one mutant strains has implications for the *hrd* selection that are discussed in the following section.

HRD21 is *SON1/RPN4/UFD5*

Son1p/Rpn4p/Ufd5p is a transcription factor required for the regulated transcription of genes coding for subunits of the 26S proteasome (Jelinsky et al., 2000; Mannhaupt et al., 1999). Son1p is actually physically associated with the 26S proteasome itself [20] and was named Rpn4p by virtue of that interaction (Fujimuro et al., 1998).[21]

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20. Other protocols for purifying the 19S regulatory particle do not retain Son1p as a subunit of the 19S (Glickman et al., 1998).

Interestingly, Son1p is also a short-lived protein and is degraded in a ubiquitin- and proteasome-dependent manner (Xie and Varshavsky, 2001). This suggests that Son1p may be involved in a feedback loop where decreased proteasome function allows the buildup of Son1p and hence increased transcription of proteasome subunits (Xie and Varshavsky, 2001). The requirement of Son1p for transcription of proteasomal genes is consistent with the observation that Son1p/Rpn4p/Ufd5p is required for the degradation of several short-lived proteins (Johnson et al., 1995). The requirement for Son1p in Hmg2p degradation was tested directly after it was discovered that *HRD4* was identical to *NPL4* (See Chapter 4). Since *SON1* [22] is a suppressor of mutations in another *NPL* gene, *NPL1/SEC63,* and *SON1* was already known to be required for protein degradation, *son1*Δ cells were tested for their ability to stabilize 6myc-Hmg2p (Fujimuro et al., 1998; Johnson et al., 1995; Nelson et al., 1993). When a *son1Δ::LEU2* allele was introduced into 6myc-Hmg2p strain, cells were rendered lovastatin resistant (Figure 2-20 on page 106), and the degradation of 6myc-Hmg2p was dramatically slowed (Figure 2-21 on page 108). These *son1Δ::LEU2* cells were also cold-sensitive (assayed at 15˚C, Figure

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21. *RPN* complementation groups are named Regulatory Particle Non-ATPase to denote those genes coding for subunits of the 19S cap (also called Regulatory Particle or PA700) of the proteasome that do not possess ATPase activity. (19S ATPase subunits are named *RPT).*

22. *SON* = suppressor of *npl1-1*

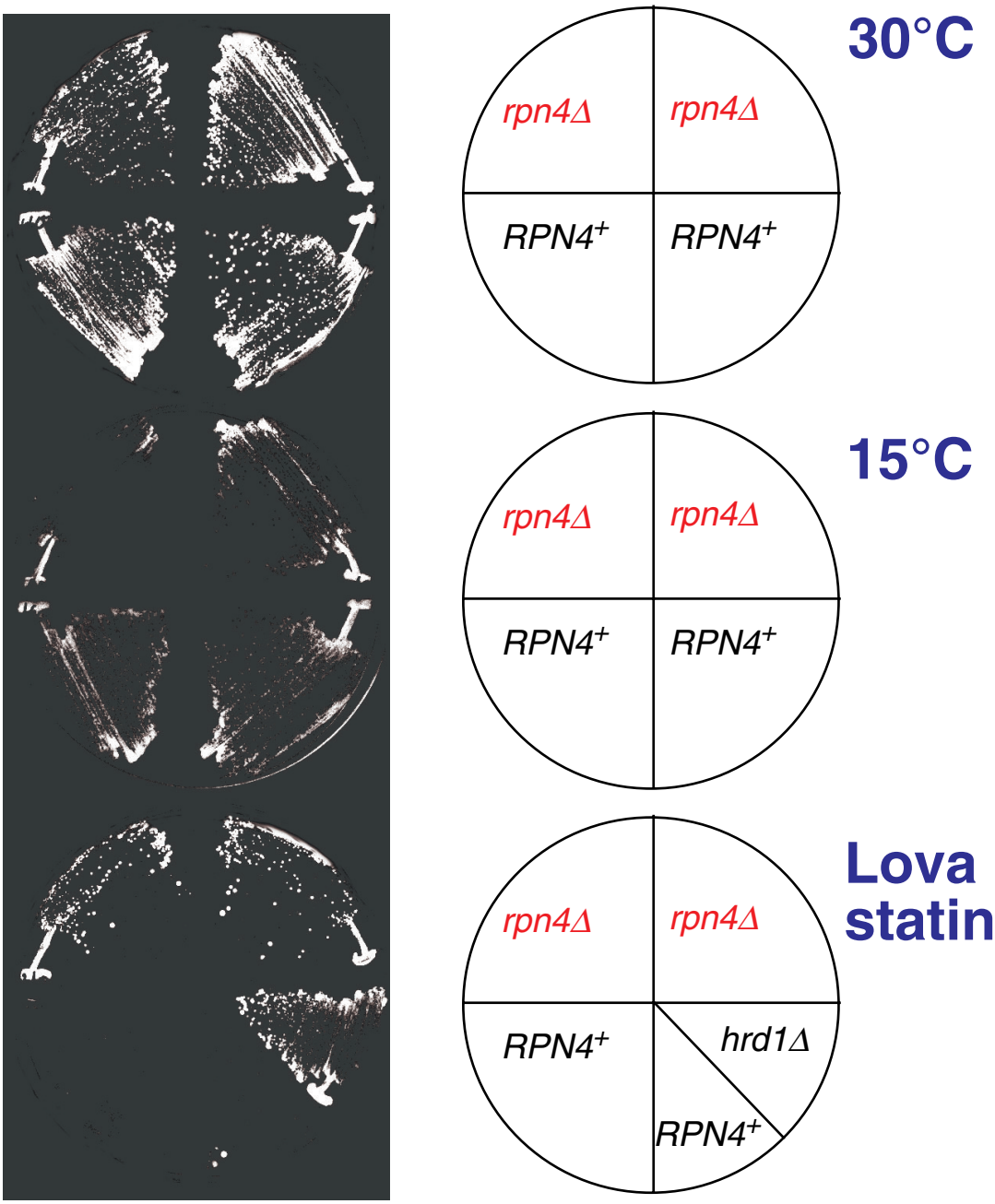

Figure 2-20

***rpn4Δ/son1Δ* cells are lovastatin-resistant and cold-sensitive**

The *rpn4Δ/son1Δ* allele was introduced into a strain expressing 6myc-Hmg2p. These transformants were then tested for lovastatin resistance and growth at low temperatures.

2-20 on page 106) as previously reported (Johnson et al., 1995; Nelson et al., 1993). These data indicated that *SON1* was required for the degradation of 6myc-Hmg2p. This is entirely consistent with the requirement for the 26S proteasome in the degradation of Hmg2p (Hampton et al., 1996a). During the third repetition of the *hrd* selection, newly isolated *hrd* mutants were crossed to *son1Δ::LEU2* cells to determine if any of the newly isolated mutants contained mutant alleles of *SON1*. 12 *hrd* mutant strains in the *HRD21* complementation group were found to produce Hrd^- diploids when mated to a *son1Δ::LEU2* strain. One of these Hrd^- diploids was sporulated and dissected. All the resulting segregants were lovastatin resistant indicating that *HRD21* was identical to *SON1* (data not shown).

Discussion

ex carcerem: freeing the *hrd* selection from its genetic prison

The *hrd* selection was designed to identify genes required for the degradation of HMG-CoA reductase in yeast (Hmg2p), and in its first use, three different genes required for the degradation of Hmg2p were identified: *HRD1, HRD2,* and *HRD3.* These genes have produced a wealth of information about how ER-associated degradation functions to eliminate both regulated and misfolded proteins from the ER. In order to expand the understanding of Hmg2p and ER-associated degradation, the *hrd* selection was used to begin the identification of genes required for the degradation of Hmg2p. Unfortunately, the initial manifestation of the *hrd* selection contained several

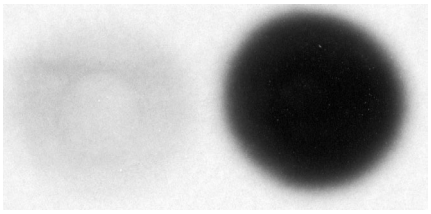

Figure 2-21

***RPN4/SON1* is required for degradation of 6myc-Hmg2p.**

Degradation of 6myc-Hmg2p in the indicated strains was assayed by stationary chase. Cells were grown for 12 h after reaching an OD_{600} of 0.2. Harvested cells were then lysed mechanochemically. Lysates were then spotted onto nitrocellulose and the membrane was subjected to immunoblotting using anti-myc antibody.

obstacles that held this goal at a distance. For instance, mitochondrial petite mutations conferring temperature sensitivity independently of the Hrd phenotype were abundant – confusing and complicating subsequent genetic analysis of new *hrd* mutants. Even more problematic was the observation that over 90% of the *hrd* mutants isolated in the *hrd* selection were *hrd1* or *hrd3*mutants: a bias that substantially hindered the isolation of new *hrd* mutants.

A series of modifications to the *hrd* selection removed the hurdles preventing its full use in identifying new *HRD* genes. The mitochondrial petite problem was discovered and eliminated by changing the growth media for the source cultures. The *HRD1/*

HRD3 bias was removed by duplicating the *HRD1* and *HRD3* genes in the haploid selection strain. The proportion of *hrd1/hrd3* mutants fell from over 90% to less than 10%. High throughput modifications like a "plate-chase" assay greatly increased the number of *hrd* mutants that could be analyzed and ultimately assigned to new *HRD* complementation groups. The ultimate judgment of these modifications lies in the ability of the modified *hrd* selection to identify new *HRD* genes. Prior to the new *hrd* selection, three *HRD* complementation groups were known with several problems blocking the discovery of additional complementation groups. Now, there are at least 21 different *HRD* complementation groups with the potential to discover many more.

Which *HRD* genes should be cloned first?

The identification of many new *HRD* genes raises a multitude of issues, scientifically and pragmatically. Principal among these is the decision to clone which genes and in what order. Fortunately, there are two important assays that should help provide answers to both the scientific and pragmatic issues arising from the abundance of new *HRD* genes. First, representative mutants from each new complementation group should be assayed for their effect on the ubiquitination of Hmg2p. Any new *hrd* mutant that blocks the ubiquitination of Hmg2p should immediately move to the top of the list of genes to be cloned. First, it would mean that such mutants are almost certainly not bearing mutant alleles of proteasomal genes. Since it is already known that the 26S proteasome is required for Hmg2p degradation and ERAD, the identification of proteasomal *HRDs*

should probably not be a top priority. Second, a new *hrd* mutant blocking ubiquitination would point to a gene that acts upstream in the process of Hmg2p degradation. As detailed in Chapter 3, such genes currently include *HRD1, HRD3, UBC7, CUE1,* and *UBC1*. For some ERAD substrates (but not Hmg2p), *UBC6, DER1,* and *DOA10* are also required. Adding any new gene to these lists could prove to be very enlightening, and would provide new information to the field of ER-associated degradation.

Another way to prioritize the cloning of new *HRD* genes would be to identify those *hrd* mutants that are defective in general proteasome activity and those *hrd* mutants that show a more specific lesion. One way to accomplish this is to transform representative mutants from each new complementation group with a short-lived *cytosolic* protein whose degradation is ubiquitin- and proteasome-dependent but does not require the ERAD ubiquitin-protein ligase Hrd1p or its associated ubiquitin-conjugating enzymes, Ubc7p and Ubc1p (Chapter 3). Such a substrate could be found in a family of GFP fusions that are short-lived and degraded by either the N-end rule or UFD pathway. These degradation pathways use different ubiquitin-protein ligases and ubiquitin-conjugating enzymes than ERAD substrates, but are very sensitive to mutations in genes coding for subunits of the 26S proteasome (Chapter 4). In fact, these GFP fusions were initially constructed exactly to ask whether general proteasome function was affected in *hrd4* mutant cells (Chapter 4). Since they are GFP fusions, protein degradation can be followed using flow cytometry in a high throughput protocol. Another short-lived GFP fusion, Deg1-GFP (see Chapter 4), could also be used. But since its degradation

requires the ER ubiquitin-conjugating enzymes Ubc6p and Ubc7p, a defect in Deg1-GFP would not place emphasis away from the ER. Again, since these assays use GFP and flow cytometry, the information can be gathered in a fairly timely and straightforward manner. With both GFP assays, it should be possible to tell which *hrd* mutants are likely to be impaired in proteasomal function rather than more upstream aspects of Hmg2p degradation. Temperature-sensitive *hrd* mutants that still allow degradation of short-lived cytosolic proteins may well be quite informative, potentially pointing to essential genes like some chaperones that are critical for protein degradation but are not part of the 26S proteasome. The use of both ubiquitination and GFP degradation assays should be able to identify the most potentially interesting mutants and place proteasomal genes in the appropriate priority for cloning. It should, of course, be said that there will certainly be exceptions to the assumptions used in prioritizing the cloning of *HRD* genes. Therefore, a long-term strategy to clone every *HRD* gene will be the only way to ensure a complete picture of Hmg2p degradation.

Are there more than 21 *HRD* genes?

The *HRD18-HRD20* complementation groups are each represented by a single *hrd* mutant. There are several statistical arguments that might be made to argue that the presence of three complementation groups with only one member indicates that the *hrd* selection has not been exhausted. But in a union of statistics and pragmatism, one would probably wish to isolate at least five mutants of each complementation group (to cover a

typical standard distribution) before arguing that the *hrd* selection has been exhausted in its ability to generate complementation groups. There is certainly no statistical argument — or more importantly any data — to suggest that there are only 21 *HRD* genes. To the contrary, the presence of four complementation groups with only one member strongly hints that there are other *HRD* complementation groups whose mutants are not represented in the current 21 groups. Only 500 lovastatin-resistant colonies were analyzed to generate the current 21 complementation groups, and modifications to the *hrd* selection discussed in the previous sections would certainly permit the analysis of a much larger numbers of mutants. Therefore, the *hrd* selection could pushed even further as a powerful tool in the understanding of HMG-CoA reductase degradation and ER-associated protein degradation in general.

Materials and Methods

Strains and Media

The parent strain for the second repetition of the *hrd* selection was RHY400 *MATa ade2-101 lys2-801 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 met2 ura3-52::*P_{TDH3}*-6MYC-HMG2* (Hampton et al., 1996a). The parent strain for the second repetition of the *hrd* selection was RHY715 *MATa ade2-101 HRD1 HRD3 leu2Δ::LEU2::HRD3 lys2-801 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 met2 trp1::hisG::TRP1::HRD1 ura3-52::*P_{TDH3}*-6MYC-HMG2*. The following alleles were described previously: *hrd1-1, hrd1Δ, hrd2-1,*

hrd3-1, hrd3Δ, ubc7Δ (Bays et al., 2001; Hampton et al., 1996a; Wilhovsky et al., 2000) and *son1Δ* (Nelson et al., 1993).

Prior to plating on lovastatin, strains were grown in YPD (Dextrose) for the second repetition and YPG (Glycerol) for the third repetition. These media were prepared as described previously (Guthrie and Fink, 1991). For the selection of lovastatin resistant colonies, cells were grown on yeast minimal (YM) media plates supplemented with 250 μg/ml lovastatin prepared as described (Hampton et al., 1996a). The plate-chase protocol used casamino acid media plates prepared as follows for 1 liter[23]: 20g Bacto-Agar™ (Difco), 6.7g yeast nitrogen base (Difco), 20g casamino acids (Difco), 20g dextrose, and water to 1L were autoclaved 30 min, 121˚C. Supplements (adenine, histidine, leucine, lysine, methionine, tryptophan and uracil) were added at levels recommended previously (Guthrie and Fink, 1991) except the concentrations of leucine and tryptophan are doubled. In the third repetition, strains were also mutagenized by EMS (ethyl methane-

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23. Note that this casamino acid media differs from other recipes in that it includes uracil. Synthetic complete media could likely also be used, but has not been tested. YPD should not be used as it is poor in several nutrients that apparently affect how degradation occurs in stationary phase. Casamino acid media is consistently rich in amino acids.

sulfonate; cells used in selection showed 30% killing under the EMS treatment) and ultraviolet radiation as described previously (Guthrie and Fink, 1991).

Plate-chase protocol

Individual colonies were streaked onto a plate containing casamino acid media and allowed to grow 2.5 to 3 days before harvest. To harvest colonies, a sheet of nitrocellulose (Butterfly™ membrane, Schleicher & Scheull) was placed directly on the colonies in the Petri dish. The top side (side not touching the colonies) was then rubbed until the entire membrane was wet (about 10 s). The nitrocellulose was then removed (cell side up) to a sheet of 3MM paper (Whatman) saturated in lysis buffer (200 mM NaOH, 0.1% SDS, 3.5 mM DTT) for a 30 min incubation. Cell debris was completely removed from the nitrocellulose by rinsing vigorously in deionized water. Filter was then submerged in 0.1 M acetic acid (pH adjusted to 2.0 with H_2SO_4) for 30 min. Filter was then rinsed twice with deionized water followed by two washes in TBST (tris-buffered saline, see below). Immunoblotting was then performed as described below.

Plasmids

To duplicate the *HRD1* gene in the selection strain, pRH507 was constructed as follows: A 4 kb BamHI-PvuII fragment from pRH433 (a genomic library plasmid containing the *HRD1* coding region, Hampton et al., 1996) was ligated into BamHI-HincII digested pRS404, a yeast integrating plasmid with *TRP1* as a marker gene (Sikorski and Hieter, 1989). pRH507 was digested with BsgI for integration at the

trp1::hisG locus in RHY609, a parent strain to RHY715. To duplicate the *HRD3* gene, the *HRD3* coding region was removed from the yeast genomic libraray plasmid pRH485 by a BstBI-HindIII fragment and cloned into HindIII-SmaI sites of pRS406 to form pRH428. A NotI-XhoI fragment of pRH428 was then cloned in to pRH405 to create pRH508, a *LEU2* yeast integrating plasmid bearing the *HRD3* gene. pRH508 was digested with EheI for integration at the *leu2*Δ locus in RHY691. The following ARS/CEN plasmids were transformed into newly isolated *hrd* mutants to test complementation by previously isolated (or suspected) *HRD* genes: pRH433 for *HRD1* (Hampton et al., 1996a), pRH482 for *HRD2* (Hampton et al., 1996a), pRH485 for *HRD3* (Hampton et al., 1996a), pRH590 for *HRD4* (Chapter 4), pRH541 for *UBC7* (Chen et al., 1993) and pRH1133 for *CUE1* (Biederer et al., 1997).

Antibodies, Immunoprecipitation and Immunoblotting

Monoclonal anti-myc antibodies were produced as cell-culture supernatant from 9e10 hybridomas obtained from ATCC. Monoclonal anti-HA antibodies (clone 12CA5) were obtained from Babco as purified antibody derived from mouse ascites fluid. Anti-GFP antisera was a gift from Charles Zuker (UCSD). Anti-Hmg2p antisera was described previously (Hampton and Rine, 1994). SDS-PAGE was performed using 8% Tris-glycine gels. Immunoblotting was performed as described in Hampton and Rine, 1994 except that tris-buffered saline contained 0.45% Tween 20, and 20% heat-inactivated bovine calf serum was used as the blocking agent.

Cloning of a *HRD7* complementing plasmid

A *leu2*Δ *hrd7* strain was transformed with a yeast genomic library constructed in a *LEU2* bearing plasmid vector (F. Spencer and P. Hieter, unpublished results). Tranformants were recovered by plating on yeast minimal (YM) media with roughly 500 colonies/plate. Colonies were then transferred to new YM plates by replica plating and incubated at 36˚C. Eight colonies emerged at 36˚C from 30 plates (~15,000 colonies). After colony purification, these transformants were again assayed to ensure that they were both Ts^+ and Lov^s. 5 colonies were clearly Ts^+ Lov^s. Plasmid DNA was recovered from these Ts^+ Lov^s cells and retransformed into *E. coli* for amplification. Two different plasmids were recovered from 10 different *E. coli* transformants (transformed in duplicate). These plasmids were sequenced with primers flanking the insertion site for the genomic fragment. Both plasmids contained overlapping fragments of chromosome XV. The smallest transforming fragment contained a region of chromosome XV from coordinates 813557 to 821242 (as plotted in the *Saccharomyces* Genome Database, http://genome-www.stanford.edu/Saccharomyces)

Cloning of a *HRD14* complementing plasmid

A *leu2*Δ *hrd14* strain was transformed with a yeast genomic library constructed in a *LEU2* vector (F. Spencer and P. Hieter, unpublished results). Tranformants were recovered by plating on yeast minimal (YM) media. 30 plates with roughly 400 colonies/plate were used as masters for subsequent analysis (roughly 120,000 colonies screened). Col-

onies were then transferred to new YM plates both with and without 250 μg/ml lovastatin by replica plating. Plates were screened for colonies that were sensitive to lovastatin. Three lovastatin-colonies were isolated and each bred true for lovastatin sensitivity. Plasmid DNA was recovered from these Lov^s cells and retransformed into *E. coli* for amplification. The same plasmid was recovered from the 6 different transformants (*E. coli* cells were transformed in duplicate). These plasmids were sequenced with primers flanking the insertion site for the genomic fragment. The plasmid contained a region of *S. cerevisiae* chromosome XVI from coordinates 725227 to 734138 (as plotted in the *Saccharomyces* Genome Database, http://genome-www.stanford.edu/Saccharomyces)

Acknowledgements

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3

Hrd1p is a membrane-anchored ubiquitin ligase required for endoplasmic reticulum-associated protein degradation.

Introduction

Ubiquitin: "Darwin's Phosphate" [26]

Ubiquitin is an 8 kDa protein whose sequence is highly conserved in eukaryotes from yeast to mammals (Doolittle, 1995; Mita et al., 1991; Ozkaynak et al., 1987). The past two decades have seen ubiquitin emerge from obscurity and specialty to a central role in cell biology, and a rapidly increasing body of data details the role of ubiquitin as a multifunctional protein tag (Figure 3-1 on page 138). Ubiquitin is directly conjugated to a vast array of proteins in the cell, and this covalent modification is reversible (D'Andrea and Pellman, 1998; Huang et al., 1995; Rotin et al., 2000; Weissman, 2001; Yan et al., 2000). In this way, ubiquitination is very much like phosphorylation, another reversible protein modification (Hunter, 1995). The two tags differ, however, in a fundamental way: ubiquitin is a protein available to evolutionary forces while phosphate is stuck in its inorganic immutability. Indeed, the ubiquitin protein itself has evolved, and numerous "ubiquitin-like" proteins are used as a reversible modification in diverse areas of biology from nuclear import/export to cell division, and ubiquitin-like proteins are even incorporated into the primary sequence of several proteins (Desterro et al., 1998;

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26. The term "Darwin's phosphate" was originally coined by Randolph Hampton, UCSD and first appeared in published form in a review article by Cecile Pickart (Pickart, 2001).

Kamitani et al., 1997; Liakopoulos et al., 1998). The conjugation of ubiquitin-like proteins like SUMO/Smt3p employs protein machinery that has apparently evolved from analogous proteins in the ubiquitin conjugation system (Hochstrasser, 2000). The system for ubiquitination itself has also evolved into a diverse functional family (Hochstrasser, 1996; Weissman, 2001). In these ways, ubiquitin might appropriately be regarded as "Darwin's phosphate" – a reversible modification fully available to evolution.

Examples of ubiquitin as a signal

Diverse pathways in the cell exploit ubiquitin as a signal. These include endocytosis (Govers et al., 1997; Hicke and Riezman, 1996; Lill et al., 2000), protein sorting in the endosome (Katzmann et al., 2001) or the trans-Golgi network (Helliwell et al., 2001; Kahana, 2001), mitochondrial inheritance (Fisk and Yaffe, 1999), viral budding (Patnaik et al., 2000; Resh, 2001), transcriptional regulation (Hoppe et al., 2000), and protein degradation (Hochstrasser, 1996). In endocytosis, ubiquitin serves as an internalization signal and is required for entry of many different plasma membrane proteins into budding endocytic vesicles destined for the lysosome/vacuole or recycling to the plasma membrane (Hicke, 1999; Rocca et al., 2001). Endocytic vesicles later join biosynthetic vesicles on their way to the lysosome, forming the late endosome (Parton et al., 1989; Prescianotto-Baschong and Riezman, 1998; Rieder et al., 1996). As the late endosome matures, proteins destined for the lumen of the lysosome are sorted away

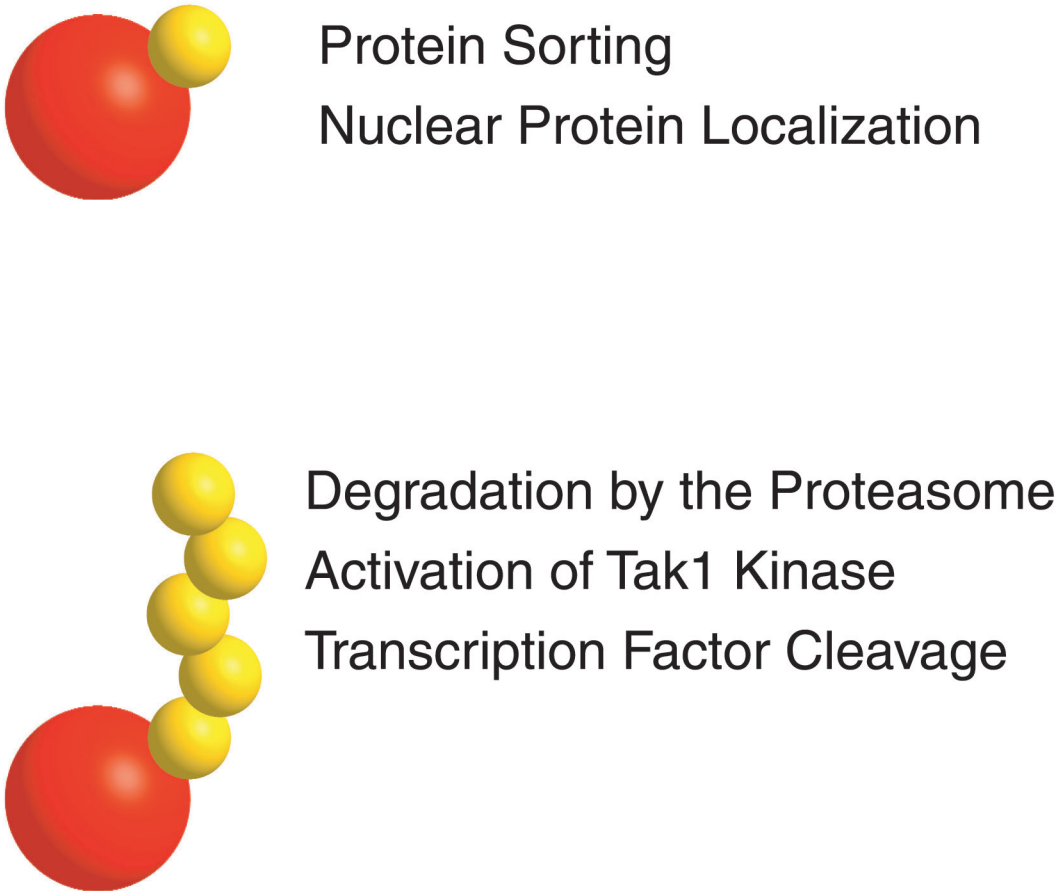

Figure 3-1

Different fates for ubiquitinated proteins

Both monoubiquitination (top) and polyubiquitination (bottom) can serve as signals in diverse cellular processes.

from proteins destined for the lysosomal membrane via structures called multivesicular bodies (Roizin et al., 1967). The pathway responsible for this protein sorting in the late endosome is called the multivesicular body (MVB) pathway (Geuze et al., 1985; Wend-

land et al., 1998). It has recently been shown that the MVB pathway recognizes monoubiquitin as a sorting signal with a protein complex called ESCRT[27]-I serving as the recognition factor (Katzmann et al., 2001). The role of ESCRT-I in protein sorting is illustrated by defects in Vps23p, an ESCRT-I component, which abolish sorting in the MVB pathway (Katzmann et al., 2001). A human homologue of Vps23p has also been implicated in the budding of viruses from mammalian cells (Garrus et al., 2001; Ver-Plank et al., 2001). Interestingly, viral budding and MVB sorting are vesicularly equivalent events (with respect to membrane topology; Simons and Garoff, 1980) and raises the possibility that a common ubiquitin-dependent process is being used for both viral budding and MVB pathways. Some data suggest that the trans-Golgi network also uses ubiquitin as a sorting signal: Changing the ubiquitination state of proteins like Gap1p can alter their trafficking to the plasma membrane, either allowing proteins to reach the plasma membrane or diverting them to the lysosome/vacuole for destruction (Helliwell et al., 2001; Kahana, 2001).

Both endocytosis and MVB protein sorting recognize monoubiquitination as a signal (Hicke, 2001; Katzmann et al., 2001; Rocca et al., 2001; Rotin et al., 2000), but other pathways recognize polyubiquitin chains (Thrower et al., 2000). An essential process utilizing such a signal is the ubiquitin-proteasome pathway of protein degradation.

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27. ESCRT=Endosomal Sorting Complex Required for Transport

In this pathway, the addition of polyubiquitin to a protein targets that protein for degradation by the 26S proteasome, a multisubunit proteolytic complex in the cell. As discussed in chapter 1, the ubiquitin-proteasome pathway is used both to eliminate misfolded proteins from the cell and to regulate the activity of proteins like cyclins, transcription factors, and metabolic enzymes like HMG-CoA reductase.

The enzymology of ubiquitination

The addition of both mono- and polyubiquitin to target proteins requires a cascade of enzymes (Figure 3-2 on page 141). The first enzyme in this cascade is called a ubiquitin-activating enzyme, or E1 (Haas and Rose, 1982; Haas et al., 1982). In the yeast *Saccharomyces cerevisiae*, there is only one E1 for ubiquitin itself (Uba1p) and one for the ubiquitin-like protein Smt3p/SUMO (Uba2p-Aos1p[28]) (Dohmen et al., 1995; Johnson et al., 1997; McGrath et al., 1991). Ubiquitin-activating enzymes use ATP to catalyze the formation of a high-energy thioester bond between the E1 and ubiquitin (Haas and Rose, 1982; Haas et al., 1982; Wee et al., 2000). This thioester-linked ubiquitin can then be transferred to a cysteine in the ubiquitin-conjugating enzyme (UBC or E2). There are 12 ubiquitin-conjugating enzymes in *S. cerevisiae* for ubiquitin and one for Smt3p/SUMO (Hochstrasser et al., 1999; Johnson and Blobel, 1997). All UBCs share a highly

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28. Uba2p and Aos1p form a heterodimer that serves as the E1 for Smt3p. Neither protein alone functions as an E3 *in vivo*.

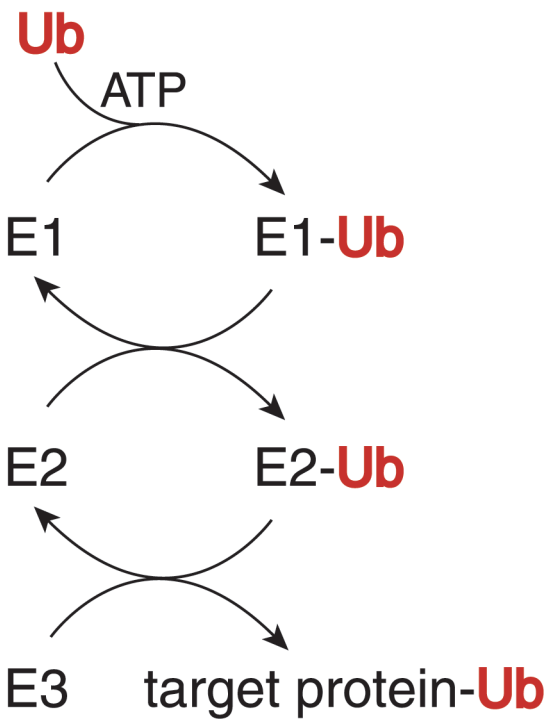

Figure 3-2

Ubiquitin enzymology

Ubiquitin is first covalently attached to a **ubiquitin-activating enzyme (E1)** via a thioester bond. Ubiquitin can then be passed to a cysteine residue in the **ubiquitin-conjugating enzyme (E2)**. The actual ubiquitination of a target protein is catalyzed by a **ubiquitin-protein ligase (E3)** which specifies both the target protein to be ubiquitinated and the E2 that will transfer ubiquitin to a lysine of the target protein.

conserved sequence motif that includes a catalytic cysteine (Hochstrasser, 1996).[29] The catalytic cysteine of a UBC is required for the acceptance of "activated" ubiquitin and its transfer to a target protein (Banerjee et al., 1995; Sung et al., 1990; Sung et al., 1991). The selection of a target protein, however, is largely not a decision for the ubiquitin-conjugating enzyme. In fact, the key specificity-determining factor in ubiquitination is the ubiquitin-protein ligase, or E3 (Ciechanover and Schwartz, 1989; Haas and Siepmann,

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29. Intriguingly, several proteins contain a UBC motif that lacks a catalytic cysteine – including Vps23p, a subunit of the ESCRT-I complex mentioned above.

1997; Hershko et al., 1983). Ubiquitin-protein ligases specify E2-mediated ubiquitination by designating both the protein(s) targeted for ubiquitination and the E2(s) that will actually transfer ubiquitin to the target protein. There are two broad classes of E3s and the exact mechanism of directing ubiquitination differs somewhat between the two, but though their mechanisms of catalysis differ, both types of E3s specifically promote the ubiquitination of target proteins by specific E2s and thus adhere to the definition of an E3.

Two classes of ubiquitin-protein ligases: HECT and RING

Members of the first class of E3s contain a sequence motif homologous to the carboxy terminus of the E6-associated protein (E6-AP) and are named HECT (Homologous to E6-AP Carboxy Terminus) E3s by virtue of this feature (Huibregtse et al., 1995). HECT E3s bind substrate and ubiquitin-conjugating enzyme via two distinct domains (Figure 3-3 on page 143), and to catalyze the transfer of ubiquitin from the ubiquitin-conjugating enzyme to substrate, HECT E3s can accept ubiquitin from the E2 via a cysteine residue in the E3 and then pass the ubiquitin to the target protein (Huang et al., 1999; Scheffner et al., 1995). It is not clear whether HECT E3s always pass ubiquitin in this "bucket-brigade" manner or if HECT E3s are commonly "pre-charged" with ubiquitin to later pass to target proteins. Regardless of the timing of E2 binding, HECT E3s can accept activated ubiquitin and pass it to a target protein. This

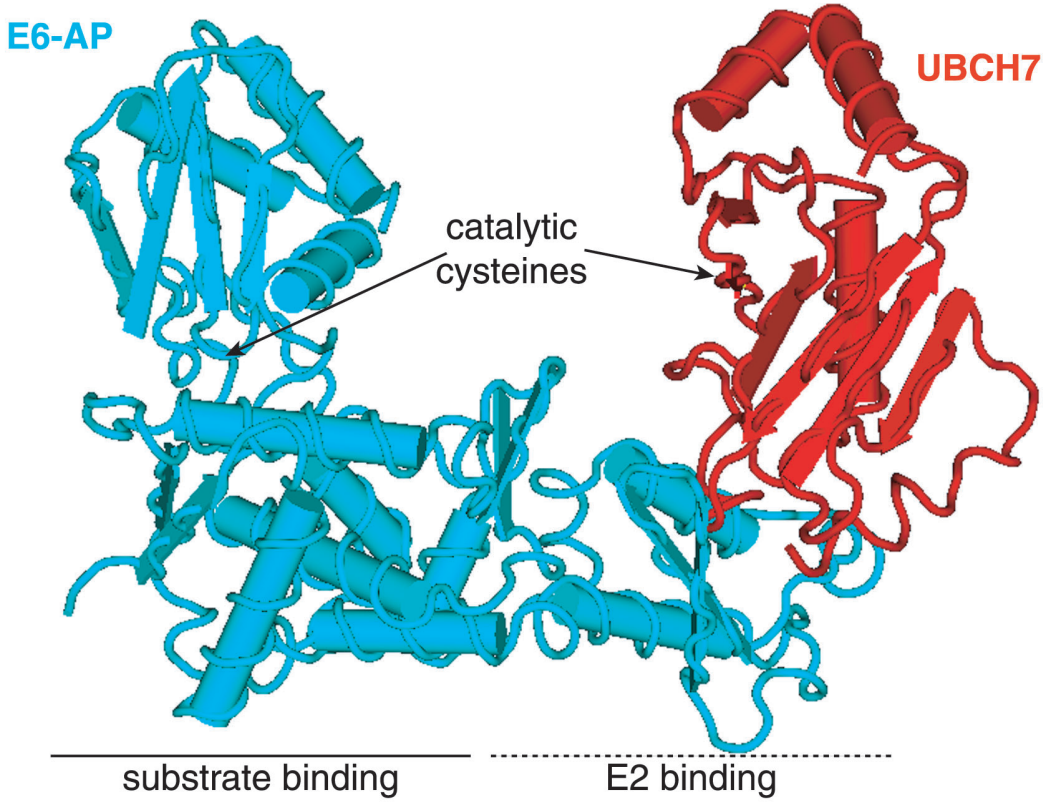

Figure 3-3

Structure of the HECT E3, E6-AP, and UBCH7

Image generated by Cn3D for MacOS (National Institutes of Health, Bethesda, MD) using published crystallographic data (Huang, et al., 1999).

mechanism is quite distinct from that used by the other family of ubiquitin-protein ligases, the "RING" E3s.

RING E3s share a conserved sequence motif characterized by eight regularly spaced cysteine and/or histidine residues (Figure 3-4 on page 144). Two sets of four cysteine

A.

C-X-X-C-$X_{(9-39)}$-C-$X_{(1-3)}$-H-$X_{(2-3)}$-H-X-X-C-$X_{(4-48)}$-C-X-X-C

B.

```
Hrd1          CIICMDELIHSPNQQTWKNKNKKPKRLPCGHILHLSCLKNWMERS-QT---CPIC
c-Cbl         CKICA-------------ENDKDVKIEPCGHLMCTSCLTSWQESEGQG---CPFC
Hrt1 (SCF)    CIECQPKAMTDT------DNECVAAWGVCNHAFHLHCINKWIKTR-DA---CPLD
Apc11 (APC)   CGICRASYNGTCPSCKFPGDQCPLVIGLCHHNFHDHCIYRWLDTP-TSKGLCPMC
Zn2+-binding  C  C                        C H  H  C              C  C
              |_____________________________|
                  H2 (or C3H2) RING
```

Figure 3-4

The RING Sequence Motif in Ubiquitin-Protein Ligases

The RING consensus sequence (A) consists of 8 regularly spaced cysteine and/or histidine residues, and is found in several E3s (B).

and/or histidine residues chelate two zinc ions (Figure 3-5 on page 145), and this chelation of zinc is required for the catalytic activity of RING E3s (Lorick et al., 1999; Zheng et al., 2000). Unlike HECT E3s, no data have been reported to suggest that RING E3s accept activated ubiquitin from an E2 for transfer to a target protein. Instead, RING E3s appear to facilitate the physical interaction between substrate and ubiquitin-conjugating enzyme, allowing the direct transfer of ubiquitin from E2 to substrate (Zheng et al., 2000, Figure 3-5 on page 145).

A recent report indicates that several proteins possessing a “U box” sequence motif also function as ubiquitin-protein ligases (Hatakeyama et al., 2001). However, it does

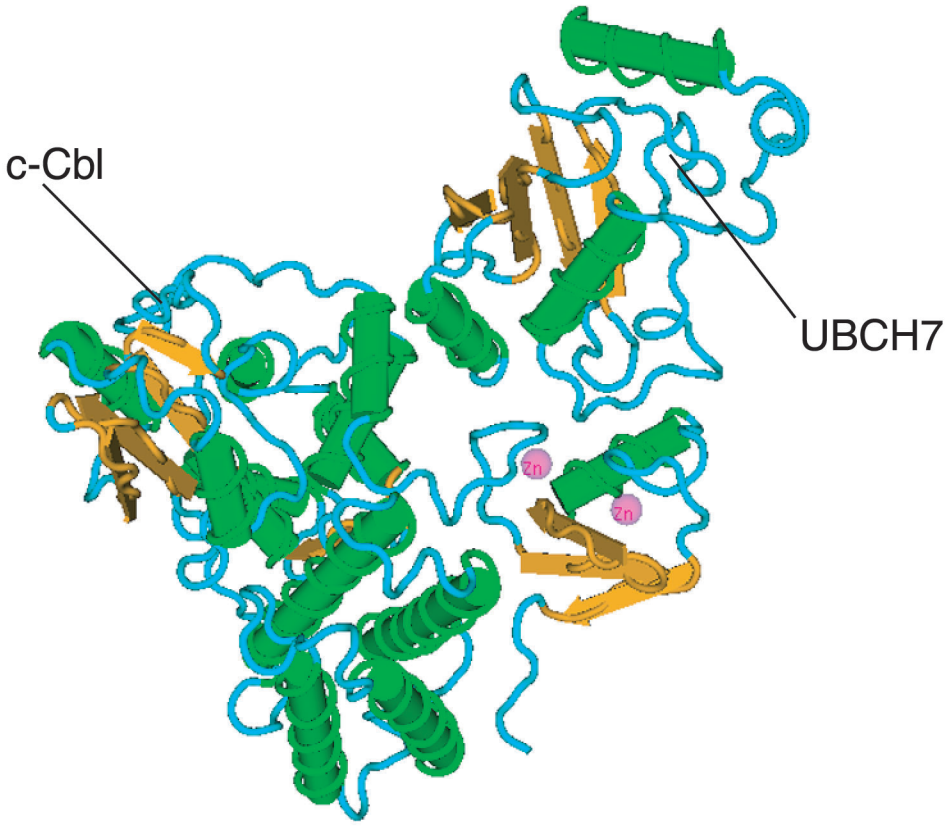

Figure 3-5

Structure of the RING E3, c-Cbl, and the E2, UBCH7

Chelated zinc ions are shown as purple spheres. Not that the RING domain is a binding site for the E2, consistent with the studies in this chapter. Image generated by Cn3D for MacOS (National Institutes of Health, Bethesda, MD) using published crystallographic data (Zheng et al., 2000).

not appear that these "U box" E3s constitute a third class of E3s, for the reason that the "U box" sequence motif is very similar to the RING motif in structure (Aravind and Koonin, 2000). Therefore, it is likely that "U box" and RING E3s follow similar catalytic mechanisms. However, much more information about both types of E3s will be required before definitive judgement can be made.

E3 Name	E3 Class	Organism	Substrates/cellular processes	References
E6-asssociated protein (E6-AP)	HECT	Human, Mouse	Tagging proteins for degradation by 26S proteasome including p53 (when E6 associated), HHR23A (human Rad23 homolog), MCM-7, and some Src protein kinases	Huibregtse et al., 1991; Kumar et al., 1999; Oda et al., 1999; Scheffner et al., 1993; Scheffner et al., 1995
Rsp5p	HECT	*S. cerevisiae*	Tagging plasma membrane proteins for endocytosis; transcription factor processing (Spt23p), protein sorting, and mitochondrial inheritance	Dunn and Hicke, 2001; Fisk and Yaffe, 1999; Galan et al., 1996; Horak and Wolf, 2001; Wang et al., 2001
SCF complex	RING (Hrt1/Rbx1/Roc1 is the core catalytic subunit of this E3 complex)	Eukaryotes	Tagging numerous proteins, including cyclins, for degradation by proteasome. Specificity conferred by F box proteins.	Deshaies, 1999; Seol et al., 1999; Skowyra et al., 1997; Skowyra et al., 1999

Table 3-1

Examples of ubiquitin-protein ligases (E3s) and their properties.

Ubr1p/ N-recognin	RING	Eukaryotes	Tagging N-end rule substrates for degradation by the proteasome	Kwon et al., 1998; Xie and Varshavsky, 1999
c-Cbl	RING	Mammals, *Drosophila, C. elegans*	Tagging receptor tyrosine kinases (EGF, PDF, CSF) for endocytosis	de Melker et al., 2001; Joazeiro et al., 1999; Lill et al., 2000; Zheng et al., 2000
Hrd1p	RING	*S. cerevisiae* Eukaryotes	Tagging both regulated (HMG-CoA reductase) and misfolded proteins (mutant forms of CPY, Sec61, Pdr5p, etc.) for destruction by the 26S proteasome	Bays et al., 2001; Bordallo et al., 1998; Hampton et al., 1996; Wilhovsky et al., 2000a

Table 3-1 (continued)

Examples of ubiquitin-protein ligases (E3s) and their properties.

ERAD proceeds by the ubiquitin-proteasome pathway

As mentioned in Chapter 1, the substrates for ER-associated degradation (ERAD) include a diverse array of both misfolded proteins and regulated ER proteins (Brodsky and McCracken, 1999). Genetic analyses indicate that ERAD substrates are degraded by the ubiquitin-proteasome pathway as components like E2s and proteasome subunits are required for ERAD. Despite their central role in specifying protein degradation, no E3 has been implicated in ERAD.

Hrd1p is an ER-resident membrane protein required for ER degradation of many substrates including HMG-CoA reductase (Hampton et al., 1996), a rate-limiting enzyme in sterol biosynthesis, and numerous misfolded proteins (Wilhovsky et al., 2000b). In fact, Hrd1p plays a critical role in removing aberrant proteins normally produced in cells (Friedlander et al., 2000; Travers et al., 2000). The Hrd1p C-terminal region contains a RING motif required for Hrd1p function (Figure 3-4 on page 144), leading to the suspicion of Hrd1p as a ubiquitin-protein ligase. By a combination of *in vitro* and *in vivo* approaches, this chapter shows that Hrd1p functions as an ER-associated E3 that specifically requires Ubc7p or Ubc1p for its action. Hrd1p is the first membrane-anchored E3 characterized *in vivo.* As such, the study of Hrd1p promises insight into the unknown mechanisms of ER substrate selection, the nature of membrane-associated ubiquitin ligases, and the growing family of RING domain proteins.

Results

Hrd1p is required for ubiquitination of an ERAD substrate, Hmg2p

The *in vivo* role of Hrd1p in ubiquitination of ER substrates was directly examined using the ERAD substrate HMG-CoA reductase (HMGR). HMGR is an essential sterol synthetic enzyme that undergoes feedback-regulated ER degradation in eukaryotes (Hampton and Rine, 1994; Nakanishi et al., 1988). ER degradation of the yeast HMGR isozyme Hmg2p proceeds by ubiquitination (Hampton and Bhakta, 1997) and requires Hrd1p (Hampton et al., 1996). Hmg2p ubiquitination was directly examined by immunoprecipitation of Hmg2p from cellular lysates followed by immunoblotting for covalently attached ubiquitin (Ub) (Figure 3-6 on page 150). In a strain with the *hrd1Δ* allele, no ubiquitination of Hmg2p was observed, even when maximally stimulated by incubation of cells with zaragozic acid (ZA), a drug that increases sterol pathway signals for degradation (Gardner and Hampton, 1999) ("+ ZA" in Figure 3-6 on page 150). Hrd1p was also required for ubiquitination of the regulated optical reporter Hmg2p-GFP (Cronin and Hampton, 1999; Gardner and Hampton, 1999) and the unregulated, misfolded 6myc-Hmg2p (Hampton et al., 1996) (data not shown). Consistent with earlier studies (Hampton and Bhakta, 1997), Hmg2p ubiquitination was strongly dependent on the ER-associated Ubc7p but unaffected by loss of Ubc6p, the other ER-associated E2 (Figure 3-6 on page 150).

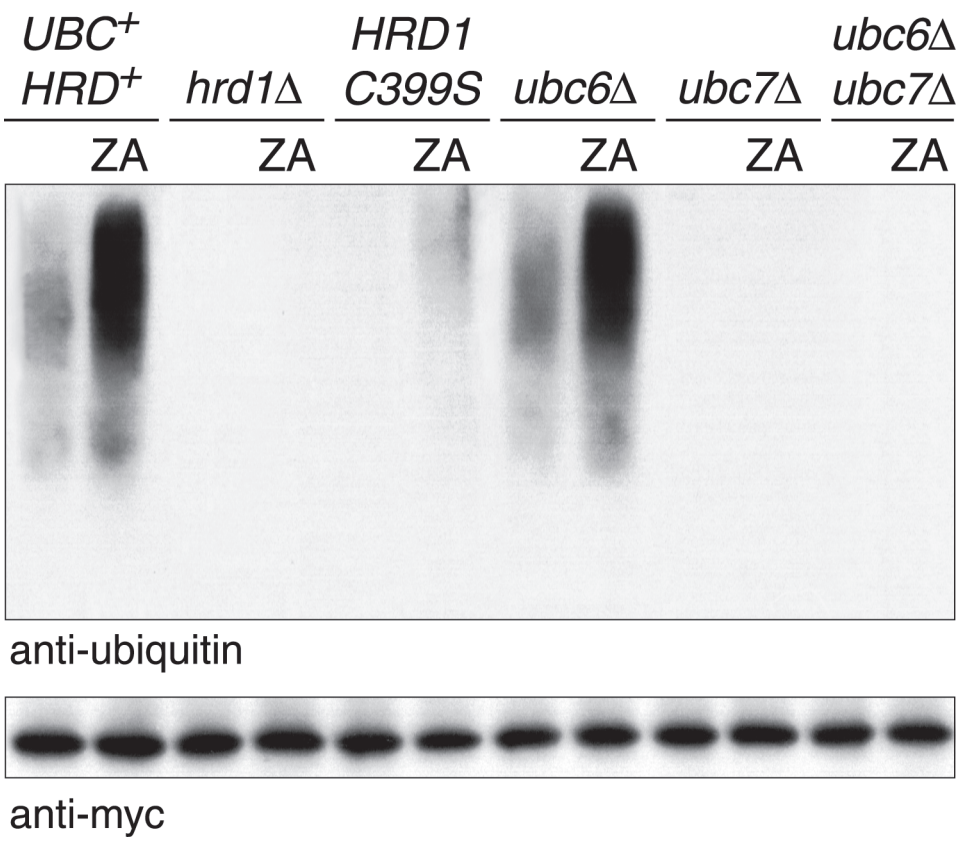

Figure 3-6

***HRD1* and *UBC7* are required for the ubiquitination of Hmg2p.**

Ubiquitination in the indicated strains was assayed by immunoprecipitation and subsequent immunoblotting for ubiquitin (top) or myc-tagged Hmg2p (bottom). The RING-H2 mutant *HRD1-C399S* was the only *HRD1* allele expressed in the indicated strain. "ZA" indicates cells treated with zaragozic acid for 10 min prior to assay to stimulate ubiquitination.

The Hrd1p C-terminal region contains a RING-H2[30] motif required for Hrd1p-dependent degradation (Figure 3-4 on page 144). Similar RING-H2 motifs are critical in the function of several soluble ubiquitin E3s, including c-Cbl, APC, and the SCF complex (Joazeiro et al., 1999; Seol et al., 1999; Zachariae et al., 1998). Experiments

were performed to test whether the Hrd1p RING-H2 motif was necessary for Hmg2p ubiquitination. Cysteine 399 of Hrd1p was chosen for mutation as C399 is a conserved RING-H2 residue in c-Cbl and related E3 proteins (Joazeiro et al., 1999; Zachariae et al., 1998). Expression of only the C399S-Hrd1p mutant as the sole source of Hrd1p severely impaired *in vivo* Hmg2p ubiquitination (Figure 3-6 on page 150) and degradation (data not shown).

Hrd1p physically associates with E2s *in vivo*

The principle E2 involved in Hrd1p-dependent degradation is Ubc7p (Hampton and Bhakta, 1997; Hiller et al., 1996; Sommer and Jentsch, 1993; Wilhovsky et al., 2000b). Physical association of Hrd1p and Ubc7p was tested using an *in vivo* crosslinking assay (Gardner et al., 2000). Intact cells expressing functional, HA epitope-tagged

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30. RING motifs can be named by the order and number of cysteine and histidine residues. Hrd1p and several other RING ligases contain an H2 ring, or more specifically a C_3H_2 ring. The C/H nomenclature lists zinc-binding RING residues in their primary sequence order. C_3H_2 or H2 specifies that the Hrd1p RING primary sequence contains 3 conserved cysteines followed by 2 conserved histidines. The 3 remaining conserved zinc-chelating residues are all cysteines in Hrd1p and not listed in the C_3H_2 designation. Another RING, the C_3H_4 ring, contains 3 cysteines followed by 4 histidines and 1 cysteine.

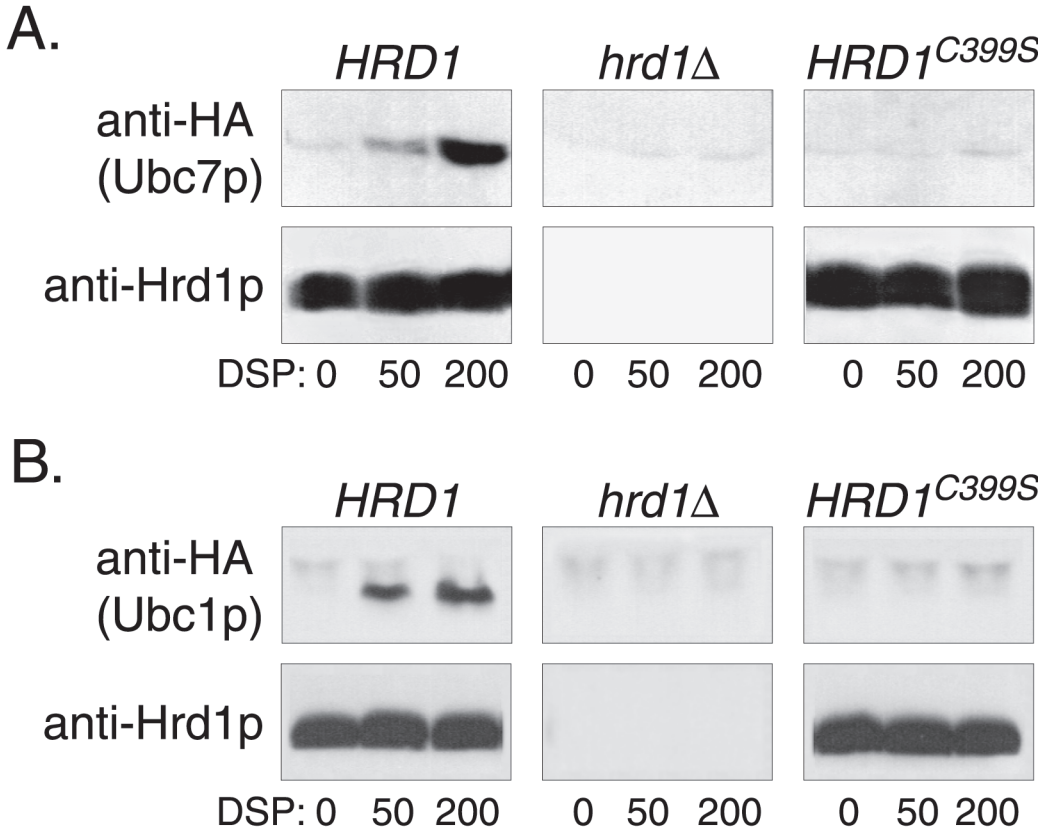

Figure 3-7

Hrd1p physically associates with relevant E2s *in vivo*

Intact cells expressing HA-Hrd1p and either HA-Ubc7p (A) or HA-Ubc1p (B) were treated with crosslinker DSP, lysed, immunoprecipitated with anti-Hrd1p antiserum, and immunoblotted for cross-linked E2 (top of A and B) or Hrd1p (bottom of A and B). DSP was added at 0, 50 and 200 µg/ml as indicated.

Ubc7p were treated with the crosslinking agent DSP (Dithiobis [Succinimidyl Propionate]), lysed, and immunoprecipitated with Hrd1p antisera. Precipitated proteins were then immunoblotted with an anti-HA antibody. HA-Ubc7p co-precipitated with Hrd1p in a crosslinker-dependent manner (Figure 3-7 on page 152). The identical

experiment performed with strains lacking Hrd1p *("hrd1Δ")* or strains expressing only the C399S point mutant of Hrd1p ("*HRD1^{C399S}*") showed no co-precipitation of Ubc7p (Figure 3-7 on page 152). Because it was determined that Ubc1p could also participate in Hrd1p E3 action (see below), the same studies were performed using tagged Ubc1p. The resulting data indicated that Hrd1p also interacted with Ubc1p in a RING-H2 dependent manner (Figure 3-7 on page 152). Thus, Hrd1p could directly associate with its two partner E2s *in vivo*, and each Hrd1p-Ubc interaction was completely dependent on the Hrd1p RING-H2 motif. In both cases, it was necessary to overexpress Hrd1p (~ 4 fold), probably because the interaction of Hrd1p with an E2 occurs with a fairly low affinity to allow effective catalytic cycling. However, the interaction was absolutely dependent on an intact RING-H2 domain, as is the case for E2 binding to other RING-H2 E3s such as c-Cbl (Joazeiro et al., 1999).

Soluble Hrd1p fusion proteins catalyze polyubiquitination *in vitro*

Purified or recombinant ubiquitin-protein ligases can catalyze *in vitro* transfer of ubiquitin from E2s to target proteins or to themselves (Joazeiro et al., 1999; Lorick et al., 1999). Direct assessment of Hrd1p E3 activity was explored using a soluble fusion (MBP-Hrd1p) between maltose binding protein and the C-terminal 203 residues of Hrd1p including the RING-H2 motif (residues 349-399, Figure 3-19 on page 175). E3-catalyzed ubiquitination requires ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (Ubc; E2), ubiquitin and ATP. Incubation of recombinant, purified

MBP-Hrd1p, E1 and E2 with ubiquitin and ATP caused the production of ubiquitin immunoreactivity at many molecular weights (Figure 3-8 on page 155). Exclusion of any reaction component completely inhibited the *in vitro* reaction (Figure 3-8 on page 155 "-E1," "-E2," etc.), as did denaturation of the complete reaction mixture before incubation (Figure 3-8 on page 155 "+SDS"). When the *in vitro* reaction was performed with a GST-Ub fusion protein (32kD) in place of native ubiquitin (8kD), the resulting products had greatly separated molecular weights, forming a ladder of discrete Ub- or GST- immunoreactive bands (Figure 3-8 on page 155 and not shown). Consistent with the *in vivo* data above, the Hrd1p RING-H2 domain was required for *in vitro* ubiquitination. MBP-C399S-Hrd1p (numbered according to full length Hrd1p) had no detectable ubiquitination activity at any concentration tested (Figure 3-8 on page 155). Furthermore, ubiquitination activity of MBP-Hrd1p was zinc-dependent, a feature of RING-H2 action (Lorick et al., 1999). MBP-Hrd1p *in vitro* activity was completely inhibited by pre-treatment with zinc chelator TPEN and completely restored by reintroduction of free zinc ion (Figure 3-8 on page 155).In the *in vitro* reactions, ubiquitin immunoreactivity was only observed at mobilities greater than MBP-Hrd1p (Figure 3-8 on page 155, arrow), implying that MBP-Hrd1p catalyzes self-ubiquitination, a feature of RING E3s (Joazeiro et al., 1999; Lorick et al., 1999). This was confirmed by purification of MBP-Hrd1p from a reaction mix with amylose resin (which binds MBP) and subsequent immunoblotting for ubiquitin (data not shown). Thus, at least a portion of the MBP-Hrd1p fusion itself was ubiquitinated, consistent

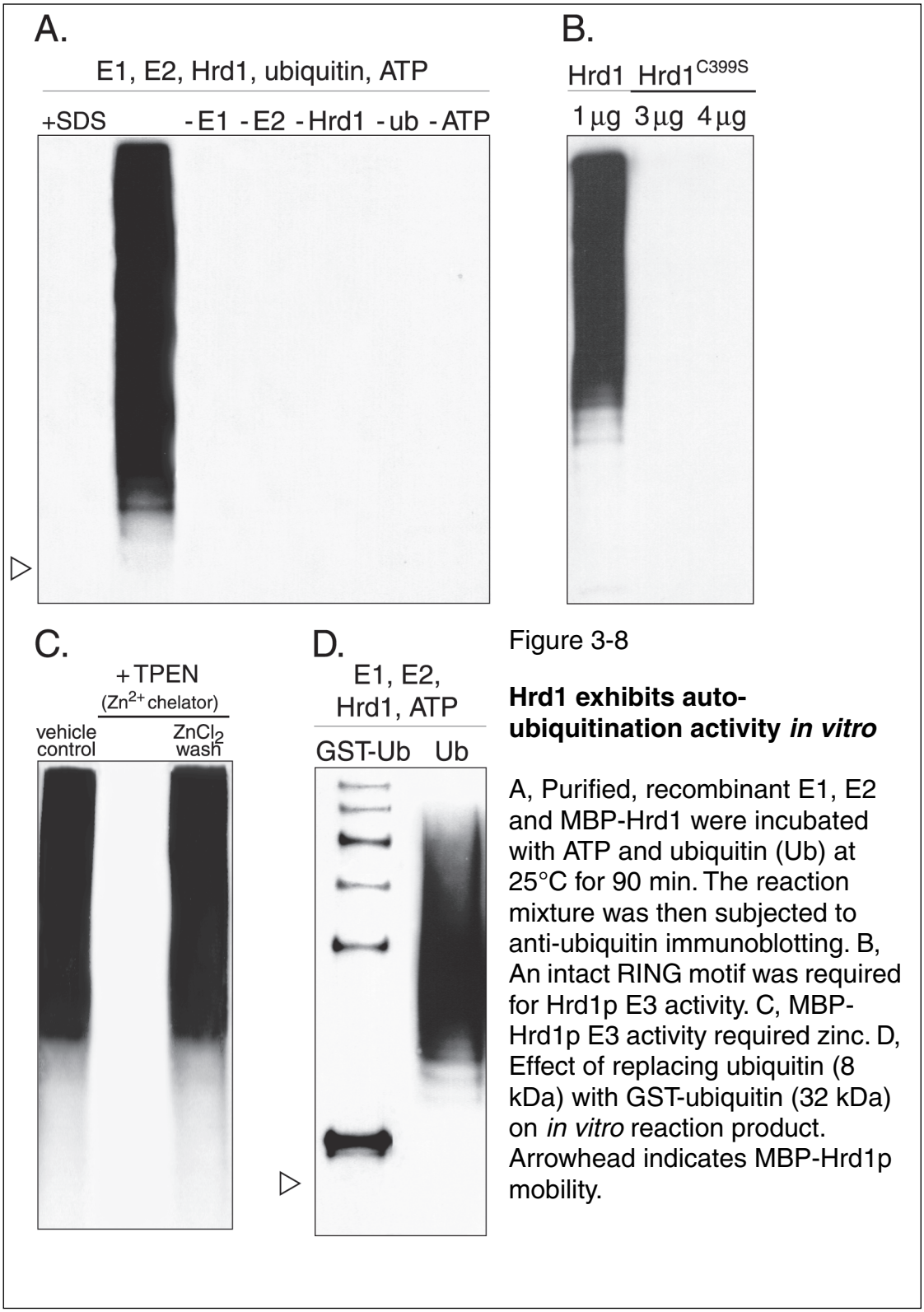

Figure 3-8

Hrd1 exhibits auto-ubiquitination activity *in vitro*

A, Purified, recombinant E1, E2 and MBP-Hrd1 were incubated with ATP and ubiquitin (Ub) at 25°C for 90 min. The reaction mixture was then subjected to anti-ubiquitin immunoblotting. B, An intact RING motif was required for Hrd1p E3 activity. C, MBP-Hrd1p E3 activity required zinc. D, Effect of replacing ubiquitin (8 kDa) with GST-ubiquitin (32 kDa) on *in vitro* reaction product. Arrowhead indicates MBP-Hrd1p mobility.

with Hrd1p's ability to autocatalyze its own degradation *in vivo* (Gardner et al., 2000). Identical behavior was observed with a GST fusion of Hrd1p (see below).

Hrd1p-mediated transfer of ubiquitin to target proteins

A variety of RING-H2 motifs, when incorporated into fusion proteins, will bind E2s and catalyze self-addition of ubiquitin (Joazeiro et al., 1999; Lorick et al., 1999). However, the principal *in vivo* function of an E3 is to catalyze processive transfer of ubiquitin from a charged E2 to a target protein. Hrd1p transfer activity was demonstrated *in vitro* using two different test substrates: a protein with its binding site engineered into a Hrd1p fusion protein and a model misfolded quality control substrate.The 10 kDa S protein binds the 15 amino acid S peptide with high affinity (Blackburn and Moore, 1982; Richards, 1958; Figure 3-9 on page 158). This interaction was exploited by cloning the Hrd1p RING-H2 domain used above (the last 203 amino acids of Hrd1p) into a GST fusion vector containing the S peptide sequence, thereby producing a Hrd1p fusion with a high affinity binding site for the S protein. The resulting GST-S peptide-Hrd1p protein catalyzed extremely efficient transfer of ubiquitin to S protein *in vitro*. This is shown by the appearance of bands of ubiquitin immunoreactivity at the M_r (relative mobility) corresponding to mono- and di- ubiquitinated S protein when the S protein substrate was included in the reaction (Figure 3-9 on page 158, lane 2). Transfer of ubiquitin to S protein was completely inhibited when S protein binding was blocked with free S peptide (lane 3). Similarly, neither of two Hrd1p fusions lacking the

S peptide sequence had any ability to transfer ubiquitin to S protein (lanes 5, 6), nor did a GST-S peptide-C399S-Hrd1p fusion (lane 4). Note that the transfer-competent Hrd1p fusions also self-ubiquitinate in all cases where they are active. This is reminiscent of Mdm2, a RING E3 that both self-ubiquitinates and transfers ubiquitin to p53 (Fang et al., 2000).

Hrd1p prefers a misfolded protein as a substrate *in vitro*

In several cases, E3 RING-H2 domains appear to participate in recognition of substrates (Fang et al., 2000; Gonen et al., 1996; Joazeiro et al., 1999). *In vivo*, Hrd1p is involved in degradation of numerous misfolded proteins (Bordallo et al., 1998; Wilhovsky et al., 2000b). Because of this role for Hrd1p in protein quality control, Hrd1p was tested for any preferential E3 activity toward misfolded proteins *in vitro*. Biotinylated BSA was used as a test substrate, comparing GST-Hrd1p-mediated ubiquitination of native BSA or protein that was briefly boiled to alter its folding state. The reaction was first performed with methylated ubiquitin, which can be charged and transferred to target proteins, but cannot participate in polyubiquitin chain formation (Hershko and Heller, 1985; Methylated ubiquitin was used to facilitate detection of new ubiquitin immunoreactivity in the presence of strong Hrd1p auto-ubiquitination.) Addition of boiled BSA to the *in vitro* reaction mix resulted in the appearance of a new ubiquitin-reactive band corresponding exactly to the M_r for BSA-ubiquitin (Figure 3-10 on page 160, lane 2). In contrast, addition of normally folded BSA showed no new product

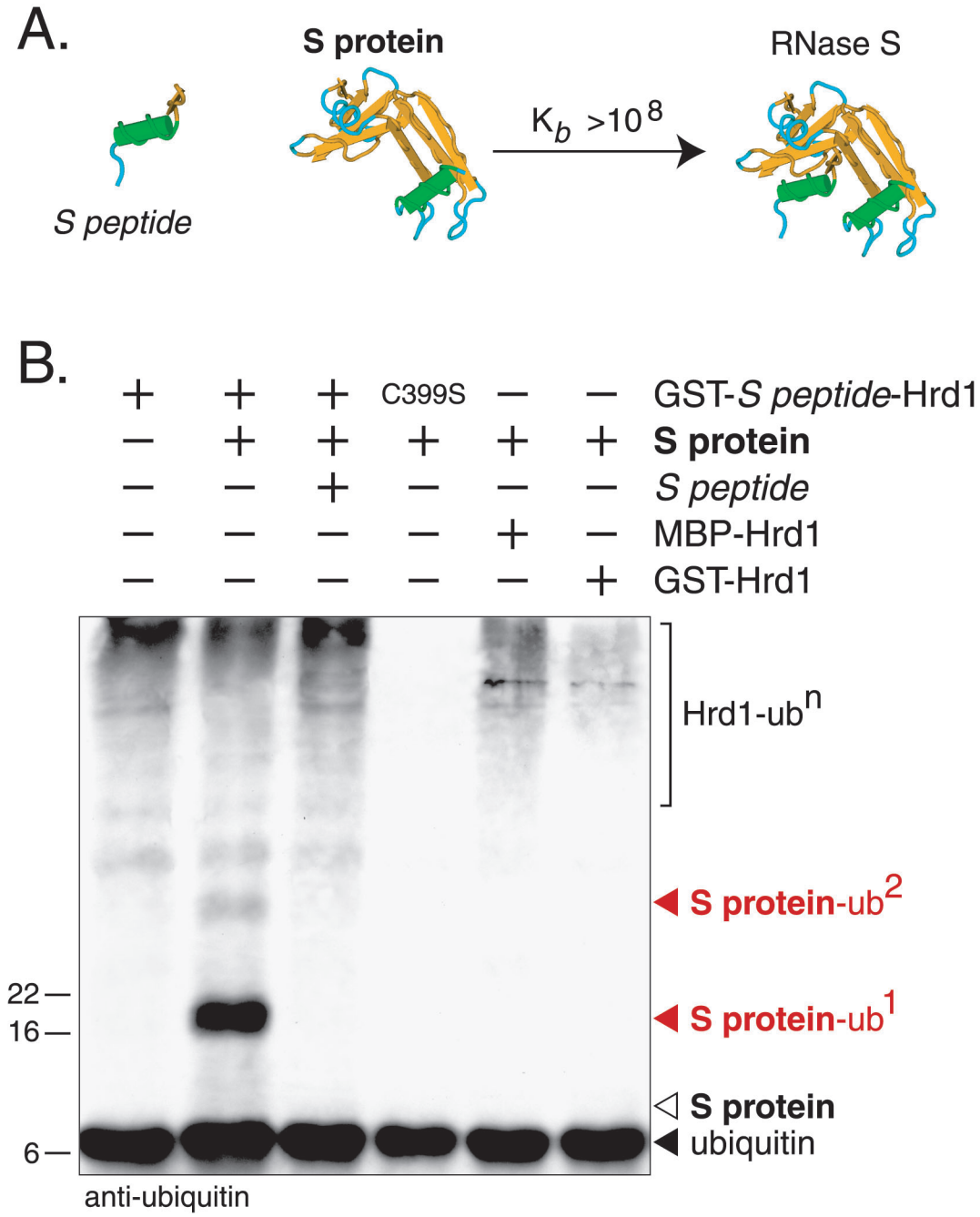

Figure 3-9

Hrd1 displays E3 activity *in vitro*

A, S protein binds S peptide (and proteins containing the S peptide sequence) with high affinity. B, Binding of S protein to Hrd1 results in the ubiquitination of S protein.

(Figure 3-10 on page 160, lane 1). GST-Hrd1p also preferentially promoted polyubiquitination of boiled BSA as shown by performing the *in vitro* reaction with native ubiquitin (Figure 3-11 on page 162). To detect multi-ubiquitinated BSA, the post-reaction mix was affinity precipitated with streptavidin beads, and the resulting precipitate was subjected to immunoblotting to detect ubiquitinated BSA (Figure 3-11 on page 162). The resulting multi-ubiquitin bands were completely absent from a reaction using boiled non-biotinylated BSA ("boiled BSA") or GST-Hrd1p inactivated by the C399S mutation (“C399S”). Blotting with streptavidin-HRP confirmed that equal amounts of BSA were precipitated from both sets of reactions (data not shown). Although equal amounts of both folded and boiled BSA were present in the indicated lanes, only misfolded BSA showed any detectable ubiquitin immunoreactivity.

Other E3s were tested for an ability to promote the ubiquitination of misfolded BSA. In one experiment, *in vitro* ubiquitination reactions were performed with GST-Hrd1 and GST fusions of the RING E3s MDM2 and Praja1 (Fang et al., 2000; Lorick et al., 1999). All three proteins were able to catalyze their own autoubiquitination, although GST-MDM2 showed much less activity than GST-Hrd1 or GST-Praja1 which both showed very high autoubiquitination activity (Figure 3-12 on page 163). However, although Hrd1 and Praja1 showed similar E3 activity as measured by autoubiquitination, *only* GST-Hrd1 promoted the ubiquitination of BSA and only when BSA was boiled (Figure 3-12 on page 163). GST-MDM2 also did not promote the ubiquitination of BSA, but its low autoubiquitination activity reduced its value as a control

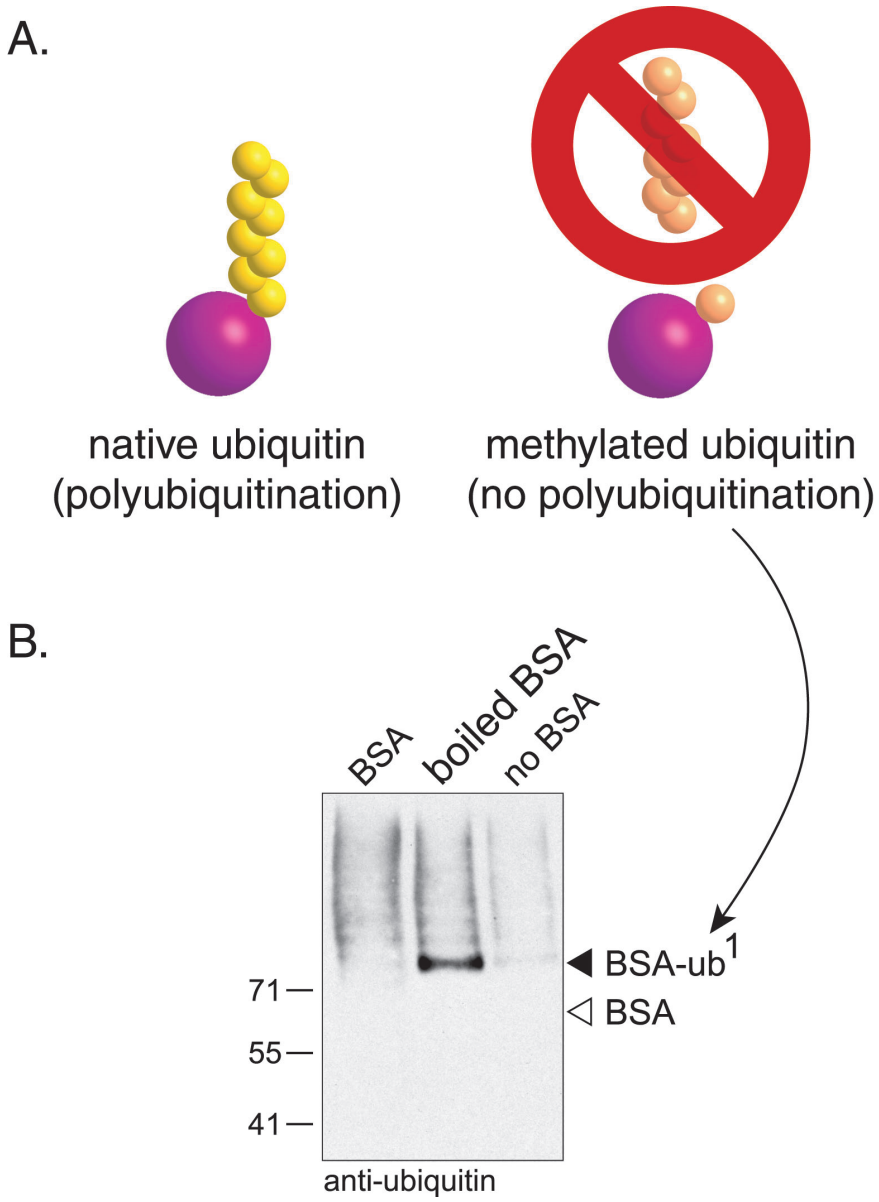

Figure 3-10

Hrd1 promotes the ubiquitination of a misfolded protein *in vitro*

A, Methylated ubiquitin cannot form polyubiquitin chains. B, *in vitro* ubiquitination reactions using Hrd1 as the E3 were performed as in the previous two figures, except that equal amounts of unboiled or boiled BSA were added to the indicated lanes and methylated ubiquitin was used in place of native ubiquitin to simplify the ubiquitination pattern.

compared to the fully active Praja1. These results indicated that BSA or even boiled BSA itself is not simply ubiquitinated by any E3 added to an *in vitro* reaction, and Hrd1 possesses a somewhat unique[31] ability to selectively promote the ubiquitination of a misfolded protein while leaving the fully folded protein untouched. Results from both S protein and BSA experiments show that the Hrd1p RING-H2 domain can catalyze processive transfer of ubiquitin between an E2 and a variety of proteins, and so functions *in vitro* as a fully *bona fide* E3.

Determining the E2 specificity of Hrd1p *in vivo*

E3s orchestrate protein degradation by facilitating transfer of ubiquitin from select E2s to specific target proteins. The crosslinking and *in vitro* studies above, as well as other work on RING-H2 E3s (Lorick et al., 1999), indicate that the RING-H2 domain can physically and functionally interact with diverse E2s. These studies taken alone leave open the question of E2 selectivity in Hrd1p action. Accordingly, experiments were conducted to determine which E2(s) participate in Hrd1p-mediated degradation *in vivo.* Like other E3s (Gray et al., 1999; Kumar et al., 1999; Visintin et al., 1997), Hrd1p

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31. "a somewhat unique ability" in that at least not every E3 possesses this Hrd1 ability to promote the ubiquitination of misfolded BSA. Which E3s do possess this activity is an open and intriguing question.

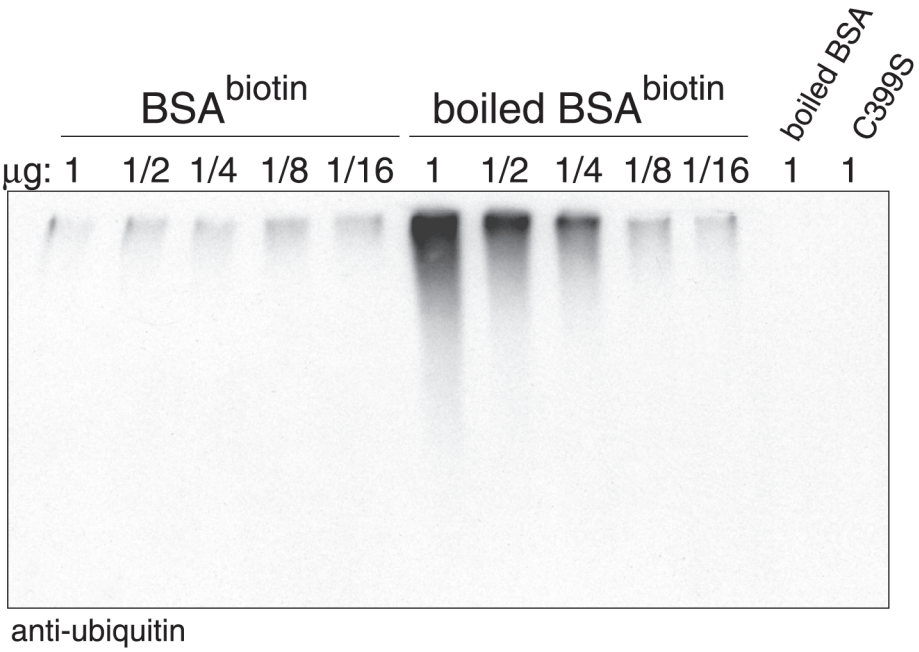

Figure 3-11

Hrd1 promotes polyubiquitination of a misfolded protein

Indicated amounts of boiled or unboiled BSA-biotin were added to ubiquitination reactions. Streptavidin-agarose was used to selectively retrieve BSA-biotin from the reaction for subsequent analysis by SDS-PAGE and immunoblotting for ubiquitin. "boiled BSA" indicates 1 µg of boiled, nonbiotinylated BSA added. "C399S" indicates 1 µg of boiled biotinylated BSA added to a reaction containing GST-Hrd1^{C399S}.

is rate-limiting for ER degradation, such that increasing levels of Hrd1p hastens degradation of its specific substrates *in vivo* (Bordallo et al., 1998), including Hmg2p-GFP (Figure 3-13 on page 165: degradation, Figure 3-14 on page 166: ubiquitination). The E2 specificity of Hrd1p was determined by examining the effects of *ubc* null mutants on Hrd1p-stimulated degradation. The optical substrate Hmg2p-GFP was used to allow analysis by both flow cytometry and biochemistry (Cronin and Hampton, 1999). Iden-

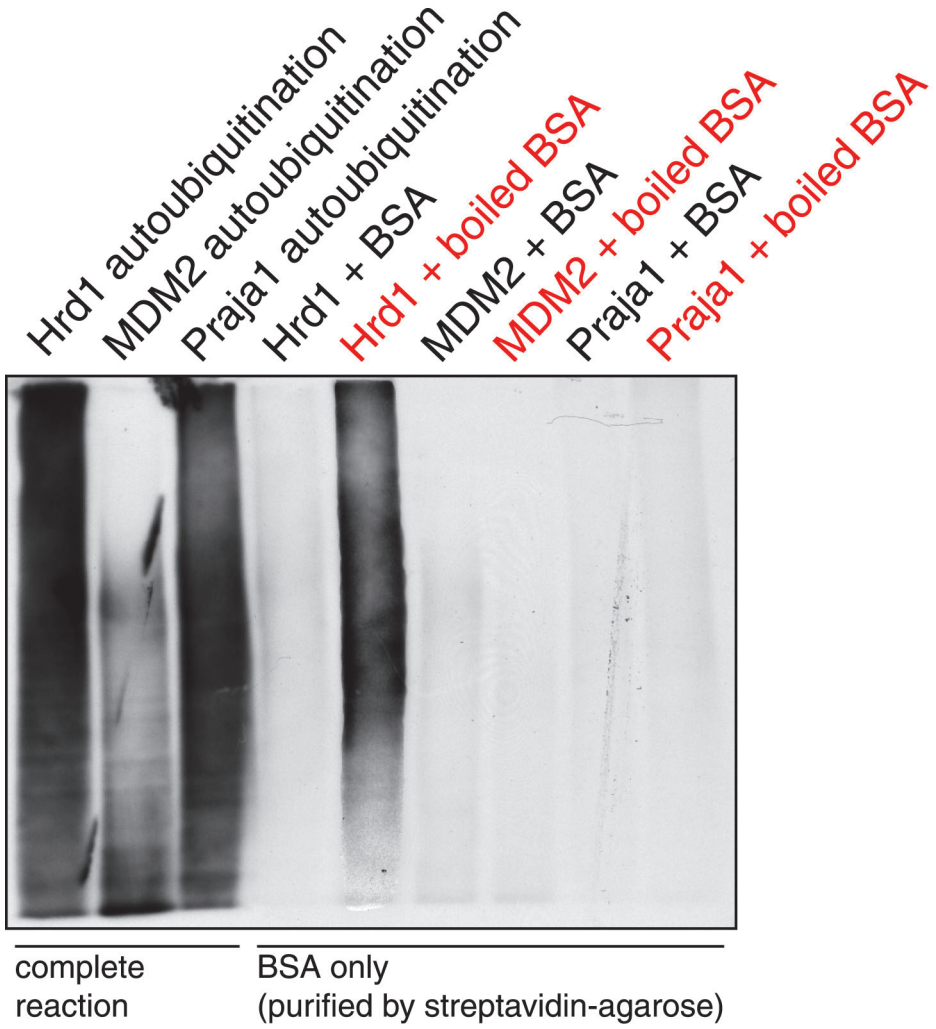

Figure 3-12

Other E3s fail to promote the ubiquitination of a misfolded protein under identical conditions *in vitro*

Reactions were performed as in Figure 3-11. Recombinant Hrd1, MDM2, and Praja1 were all GST fusion prepared by identical protocols. The first three lanes show the autoubiquitination activity of the three E3s with Hrd1 and Praja1 comparable in activity. The remaining lanes are BSA purified from reactions containing the indicated E3.

tical results were obtained using wild-type Hmg2p as the substrate (data not shown). Hrd1p could promote ubiquitination via ubiquitin-conjugating enzymes *in vivo* – thereby displaying E3 activity *in vivo* – and showed a marked specificity for Ubc7p and Ubc1p.

Two ER-associated E2s, Ubc7p and Ubc6p, were first examined for a role in Hrd1p function. These two E2s (but predominantly Ubc7p) have been implicated in Hrd1p-dependent degradation of numerous substrates (Hiller et al., 1996; Plemper et al., 1998; Sommer and Jentsch, 1993; Wilhovsky et al., 2000b). Hmg2p-GFP is strongly stabilized in both *ubc7*Δ and *ubc6Δubc7Δ* strains (Hampton and Bhakta, 1997 and Figure 3-13 on page 165). Nevertheless, modest (four-fold) overexpression of Hrd1p in the *ubc6Δubc7Δ* strain fully restored Hmg2p-GFP ubiquitination (Figure 3-14 on page 166) and degradation as measured by cycloheximide chase followed by flow cytometry (Figure 3-13 on page 165). Furthermore, the sterol pathway was able to regulate *HRD1*-restored ubiquitination and degradation in a completely normal manner. (Figure 3-14 on page 166 and data not shown). Thus, Hrd1p was still rate-limiting for ER ubiquitination and degradation in the absence of Ubc6p and Ubc7p – the two known ER-associated E2s. In fact, four-fold elevation of Hrd1p in the absence of Ubc7p and Ubc6p restored degradation of Hmg2p that was identical in magnitude and regulation to that in wild-type strains (except for ubiquitination pattern).

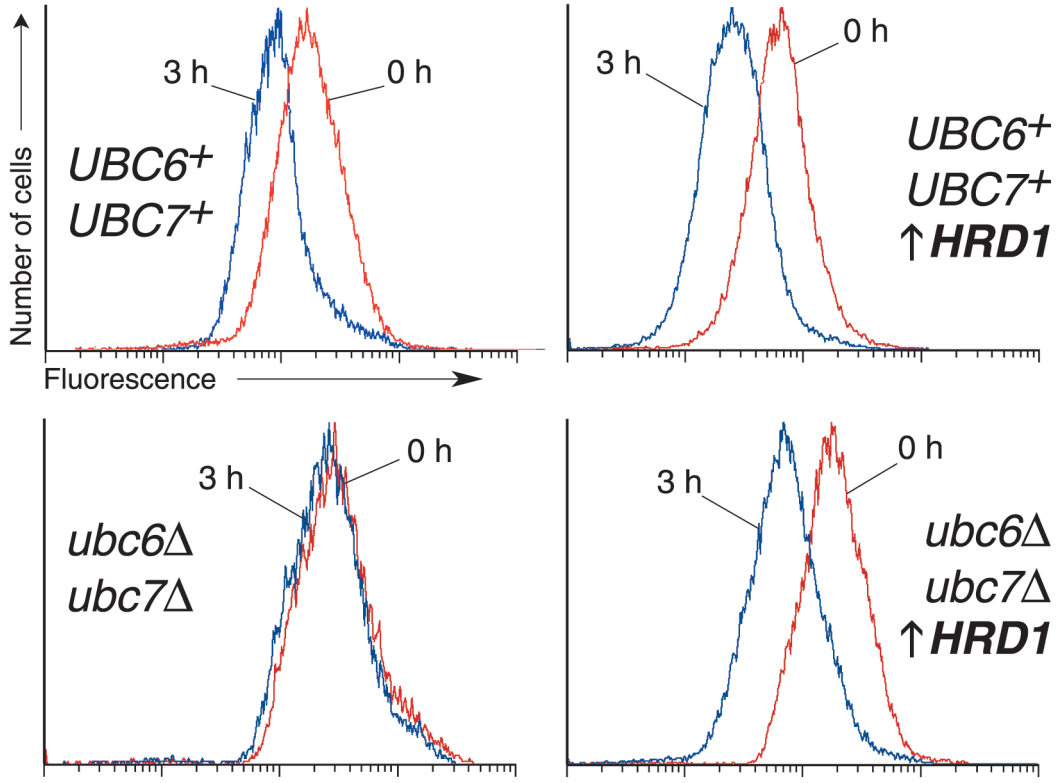

Figure 3-13

Increased *HRD1* expression suppresses the *ubc6*Δ *ubc7*Δ degradation defect.

Cycloheximide chase analysis of Hmg-GFP: each pair of histograms shows Hmg2p-GFP degradation as loss of Hmg2p-GFP fluorescence. Each histogram represents 20,000 cells.

Hrd1p uses only Ubc7p or Ubc1p *in vivo*

Because Hrd1p could function in the absence of Ubc6p and Ubc7p, other E2s were examined for their participation in Hrd1p function. Hrd1p-stimulated degradation of Hmg2p-GFP in the *ubc7Δ* strain (Figure 3-15 on page 168) or the *ubc6Δubc7Δ* strain (data not shown) was absolutely dependent on Ubc1p, an E2 not widely appreciated to

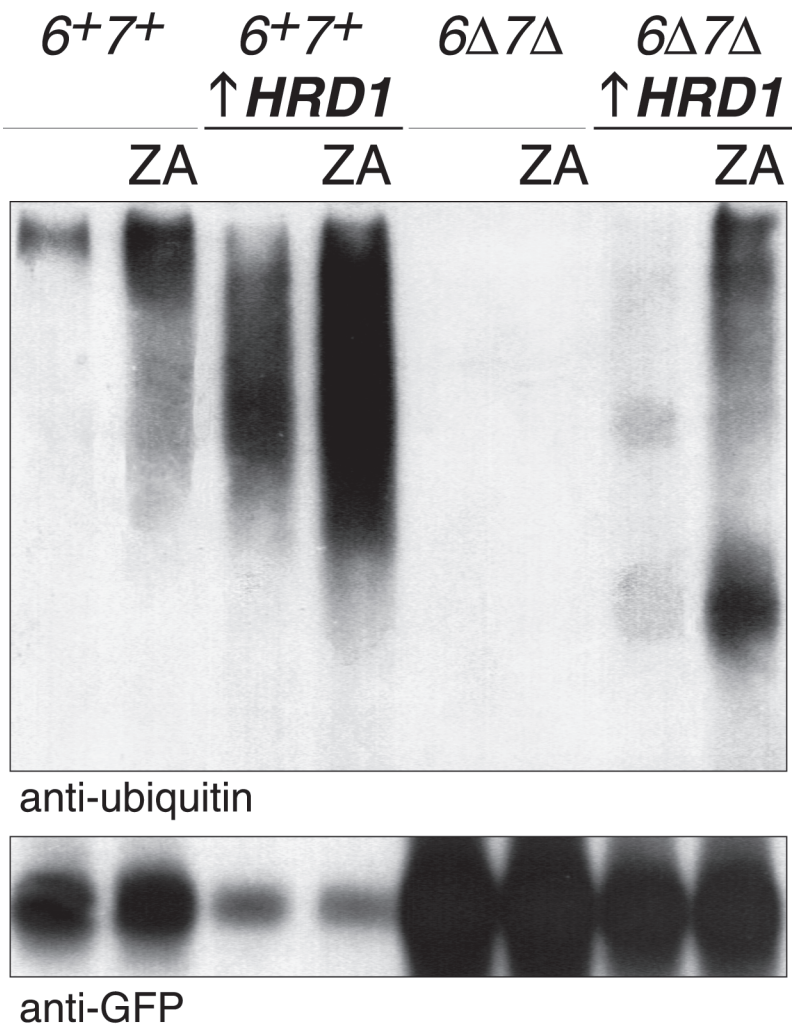

Figure 3-14

Hrd1p controls ubiquitination rate *in vivo*

Ubiquitination in the indicated strains was assayed by immunoprecipitation and subsequent immunoblotting for ubiquitin (top) or the GFP moiety of Hmg2-GFP (bottom)

participate in ER degradation. Increasing Hrd1p dosage in a *ubc1Δubc7Δ* strain had no effect on Hmg2p-GFP degradation (Figure 3-15 on page 168) or ubiquitination (Figure 3-16 on page 169), despite the presence of all other *UBC* genes including *UBC6*. In contrast, removal of Ubc5p – an E2 quite similar to Ubc1p in sequence and function (Seufert et al., 1990) – still allowed ERAD by Hrd1p overexpression in *ubc7*Δ or *ubc6*Δ*ubc7*Δ strains (data not shown). Thus, in the absence of Ubc1p and Ubc7p, Hrd1p failed to function in any detectable manner. However, Hrd1p could stimulate degradation of Hmg2p-GFP in either the *ubc1Δ* or *ubc7Δ* strains (Figure 3-15 on page 168), meaning that Hrd1p could function as an E3 *in vivo* with either Ubc7p or Ubc1p, but only with these two E2s.

Hrd1p shows identical E2 specificity in a global phenotype.

Hrd1p E2 specificity was also examined in a global phenotype associated with normal protein degradation: cellular resistance to Cd^{2+} toxicity (Jungmann et al., 1993). Wild-type yeast can grow in medium with 200 μM $CdCl_2$. Loss of *UBC7* or *UBC1* causes profound sensitivity to cadmium (Jungmann et al., 1993) (Figure 3-17 on page 170), presumably due to the increased burden of misfolded proteins degraded by ER-associated mechanisms. Elevating Hrd1p suppressed cadmium sensitivity of either *ubc6Δubc7Δ* or *ubc1Δ* strains, restoring cell growth on medium containing $CdCl_2$ (Figure 3-17 on page 170). However, the cadmium sensitivity of the *ubc1Δubc7Δ* strain was not relieved by increased expression of Hrd1p. Thus, Hrd1p displayed the same E2

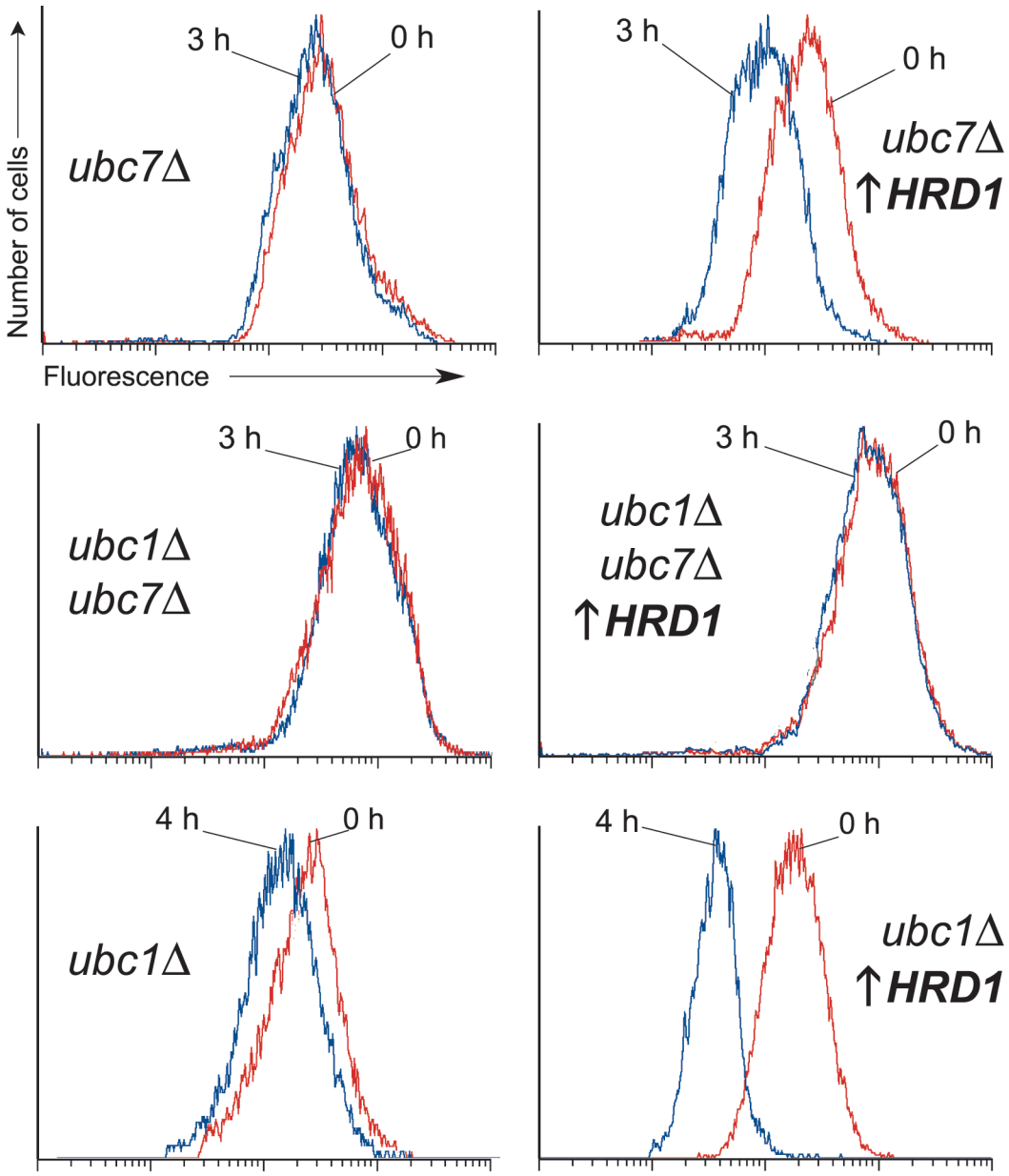

Figure 3-15

Hrd1 requires either Ubc1p or Ubc7p to function *in vivo*

Cycloheximide chase analysis of Hmg2-GFP degradation measured by flow cytometry.

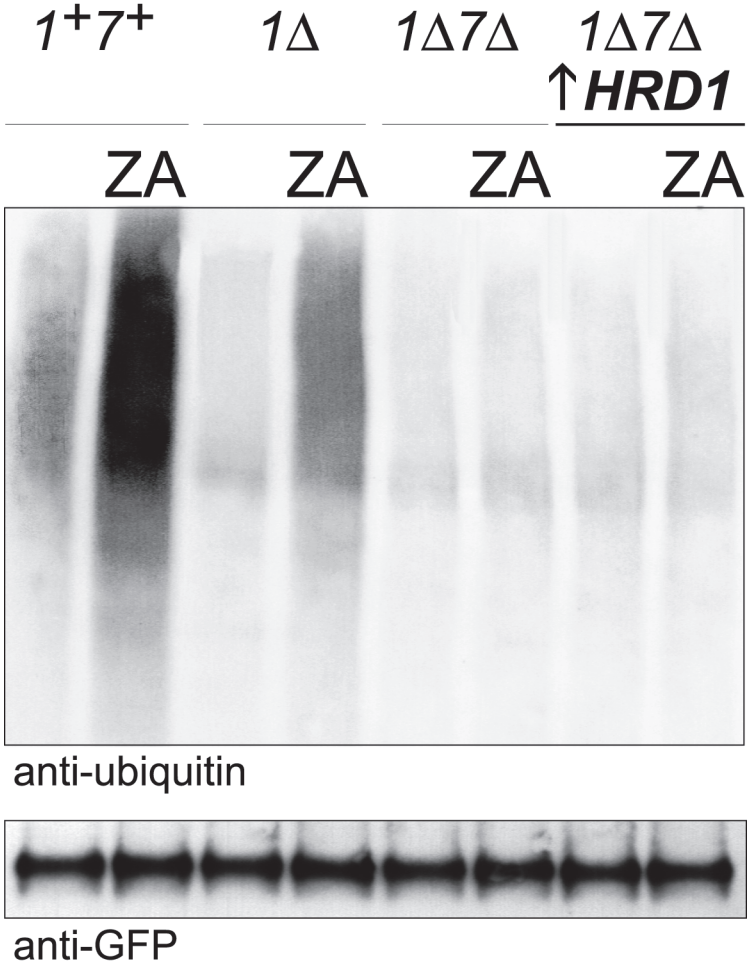

Figure 3-16

Hrd1 shows E3 activity *in vivo* by promoting ubiquitination via specific E3s: Hrd1 can promote ubiquitination by either Ubc1p or Ubc7p.

Assay of Hmg2-GFP ubiquitination as in Figure 3-14

selectivity in suppression of cadmium sensitivity as it did in Hmg2p degradation: either Ubc1p or Ubc7p allowed Hrd1p suppression of cadmium sensitivity, but only these two E2s could participate in this action of Hrd1p.

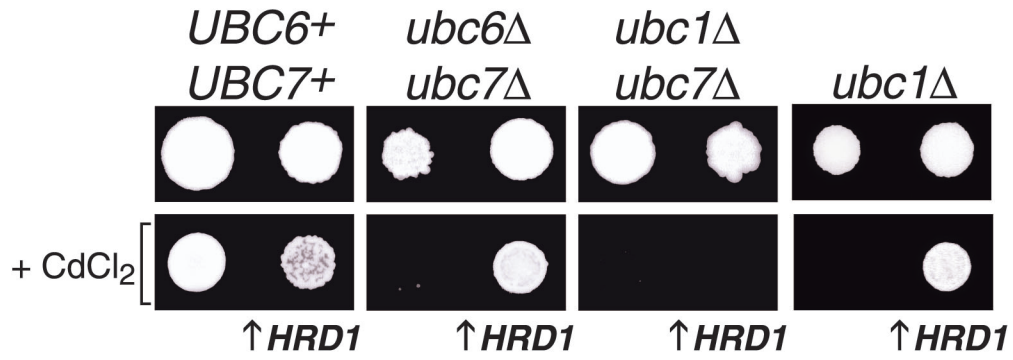

Figure 3-17

***HRD1* suppression of a global degradation phenotype**

Identical numbers of cells from indicated strains were incubated on solid medium with 200 μM $CdCl_2$ (bottom row) or no $CdCl_2$ (top row). Strains with increased expression of *HRD1* marked underneath with "↑*HRD1*."

Ubc1p normally participates in Hmg2p degradation.

These studies indicated that Ubc1p could function in Hrd1p-mediated ER-associated degradation. Direct examination revealed that Ubc1p had a demonstrable role in ER degradation even at normal levels of Hrd1p. A *ubc1Δ* strain was clearly deficient in both Hmg2p-GFP ubiquitination (Figure 3-16 on page 169) and degradation (Figure 3-15 on page 168 and Figure 3-14 on page 166: compare 3h chase for *"UBC6⁺ UBC7⁺"* and 4h chase for *"ubc1Δ"*; data not shown). Furthermore, Ubc1p has recently been implicated in the degradation of misfolded CPY*, another well-studied and Hrd1p-dependent ERAD substrate (Friedlander et al., 2000). At native levels of Hrd1p, *ubc7Δ* strains showed a greater defect than *ubc1Δ* strains in Hmg2p-GFP degradation (Hampton and Bhakta, 1997) (and Figure 3-15 on page 168). Thus, Hrd1p could function as

an E3 with two distinct E2s, Ubc1p and Ubc7p, but preferred Ubc7p. Whether Ubc1p, like Ubc7p (Biederer et al., 1997), requires localization to the ER surface to function in ERAD remains an open and clearly relevant question.

Discussion

Hrd1p as a ubiquitin-protein ligase in ERAD

The experiments in this chapter show that Hrd1p is an ER-associated ubiquitin-protein ligase for degradation of misfolded proteins and regulated HMGR. Hrd1p was required for the ubiquitination of yeast HMG-CoA reductase and physically associated with the ubiquitin-conjugating enzymes Ubc7p and Ubc1p *in vivo.* A recombinant Hrd1 protein containing the soluble RING domain showed profound autoubiquitination activity *in vitro* when added to purified, recombinant E1, E2, ubiquitin and ATP. This activity was abolished by the removal of zinc from the Hrd1 protein or by mutation of a zinc-binding residue in the RING sequence motif. Since E3s, by functional definition, must promote the transfer of ubiquitin to *other* proteins, Hrd1 was tested for its ability to promote the ubiquitination of test proteins *in vitro.* When an S protein binding site was added to the GST-Hrd1 fusion protein, GST-Hrd1 directly promoted the ubiquitination of S protein *in vitro.* The ubiquitination of S protein was not seen when Hrd1 fusions lacking the S protein binding site were used or when free S peptide (the S protein binding site) was added in excess to the reaction to compete for S protein binding to Hrd1.

In vivo, Hrd1p was rate-limiting for the ubiquitination and degradation of yeast HMG-CoA reductase, consistent with a role for Hrd1 as the key specificity-determining factor in HMGR ubiquitination. Furthermore, increased expression of *HRD1* fully suppressed a *ubc6Δ ubc7Δ* mutant, restoring full ubiquitination and degradation of yeast HMGR. However, this suppression was not seen in a *ubc1Δ ubc7Δ* strain, indicating that Hrd1 required either Ubc1p or Ubc7p in order to promote the ubiquitination of yeast HMGR. *HRD1* was also rate-limiting in a *ubc1Δ* strain, indicating that either UBC could be used to achieve full ubiquitination of HMGR. These results demonstrated that Hrd1p was capable of promoting the ubiquitination of a target protein via specific E2s *in vivo,* and thus Hrd1p displayed *bona fide* E3 activity *in vivo*. These results also indicated a previously unappreciated role for Ubc1p in the ubiquitination and degradation of HMG-CoA reductase in yeast.

It is clear that some ER substrates undergo ubiquitin-mediated degradation independent of Hrd1p (Hill and Cooper, 2000; Wilhovsky et al., 2000b), implying that other ubiquitin-protein ligases may work in conjunction with Hrd1p. Indeed, another ER-resident E3, Doa10p, has recently been identified (Swanson et al., 2001). Nevertheless, Hrd1p plays a broad and central role in ERAD of both normal and misfolded proteins (Bordallo et al., 1998; Friedlander et al., 2000; Hampton et al., 1996; Plemper et al., 1998; Travers et al., 2000). These studies delineate the molecular details of that role and extend the action of RING ubiquitin ligases to membrane surfaces and substrates –

ligases that include Hrd1p homologues found throughout eukaryotes (Figure 3-18 on page 174 and Figure 3-19 on page 175).

Hrd1p and recognition of misfolding in the quality control pathway

A brief glimpse at the substrates for Hrd1p-mediated ubiquitination and degradation immediately indicates that Hrd1p plays a major role in the destruction of mutant, misfolded proteins at the ER (Bordallo et al., 1998; Friedlander et al., 2000; Hampton et al., 1996; Plemper et al., 1998; Travers et al., 2000). Indeed, Hrd1p looks very much like an E3 for misfolded proteins and functions in protein quality control. One central question in the study of protein quality control is how misfolded proteins are recognized as misfolded. How can the quality control machinery discriminate between folded and misfolded forms of proteins with identical primary sequences and cause the rapid ubiquitination and destruction of the misfolded protein while leaving the folded protein untouched? Experiments in this chapter provide the first data to suggest that E3s can directly recognize misfolded proteins and promote their ubiquitination while leaving the folded version of that a untouched. When boiled BSA was added to an *in vitro* ubiquitination reaction, Hrd1 was capable of promoting the polyubiquitination of boiled BSA but did not promote the ubiquitination of unboiled BSA. The use of other E3s in the *in vitro* reaction with boiled BSA provided an important control for the specific nature of this Hrd1 activity. Although a RING E3 similar to Hrd1 showed autoubiquitination activity comparable to Hrd1, it was not able to promote the ubiquitination of either

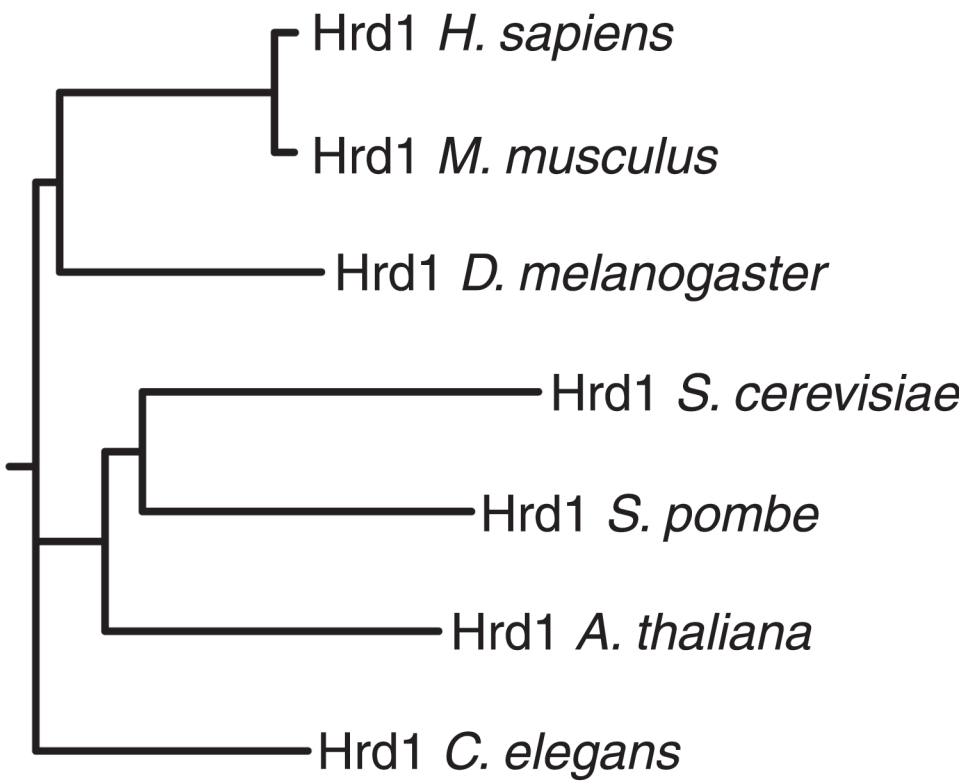

Figure 3-18

Phylogenetic tree depicting Hrd1 protein sequence divergence

The indicated proteins were aligned using the CLUSTALW algorithm (Thompson et al., 1994) and phylogentic tree based on the Hrd1 alignment was constructed using the PHYLIP package (Phylogeny Inference Package, J. Felsenstein, University of Washington, Seattle).

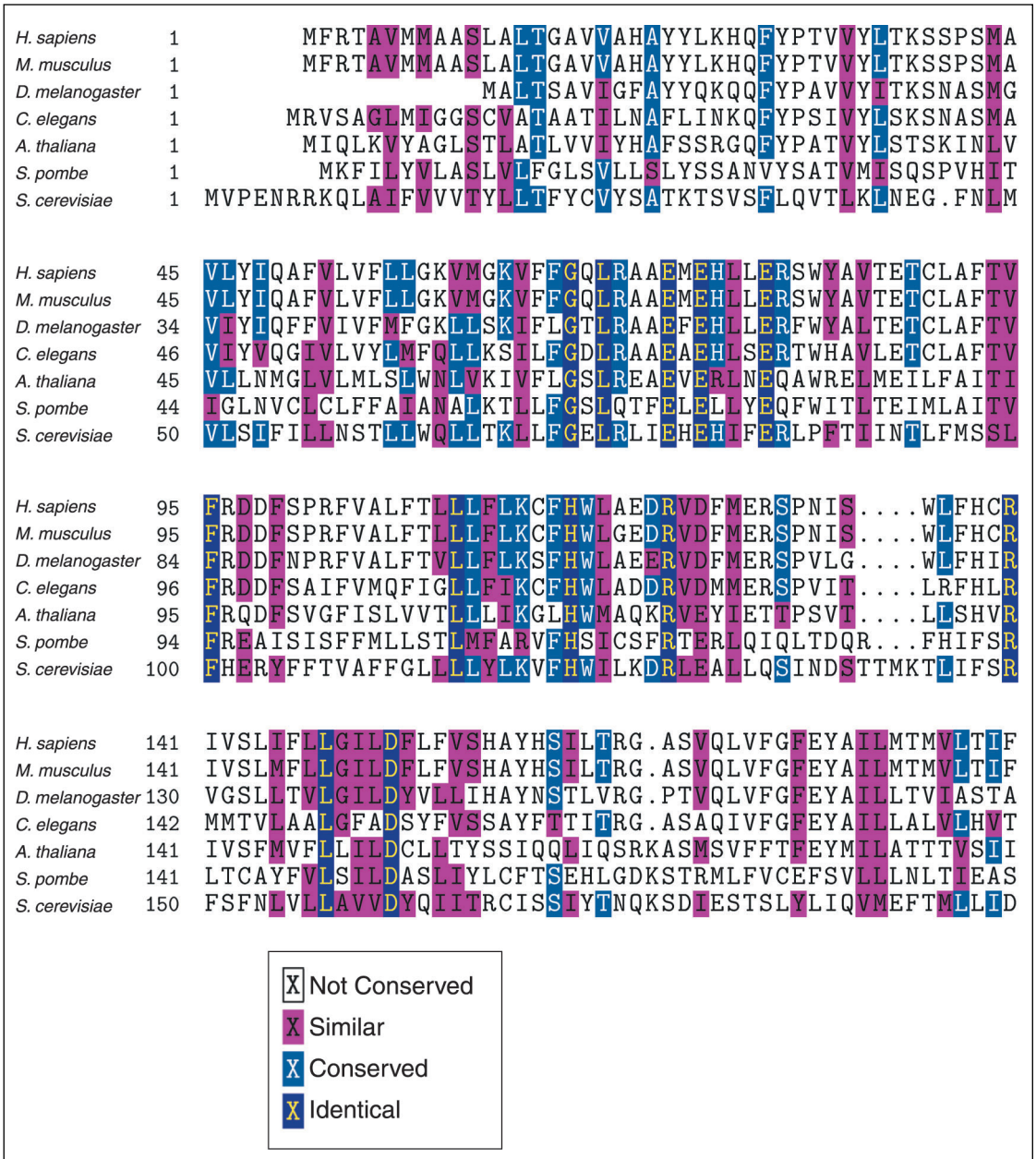

Figure 3-19

Sequence alignment of Hrd1 homologues in different species

Sequence alignment was performed using the CLUSTALW algorithm (Thompson et al., 1994). Strong sequence similarity is seen in this transmembrane region of Hrd1.

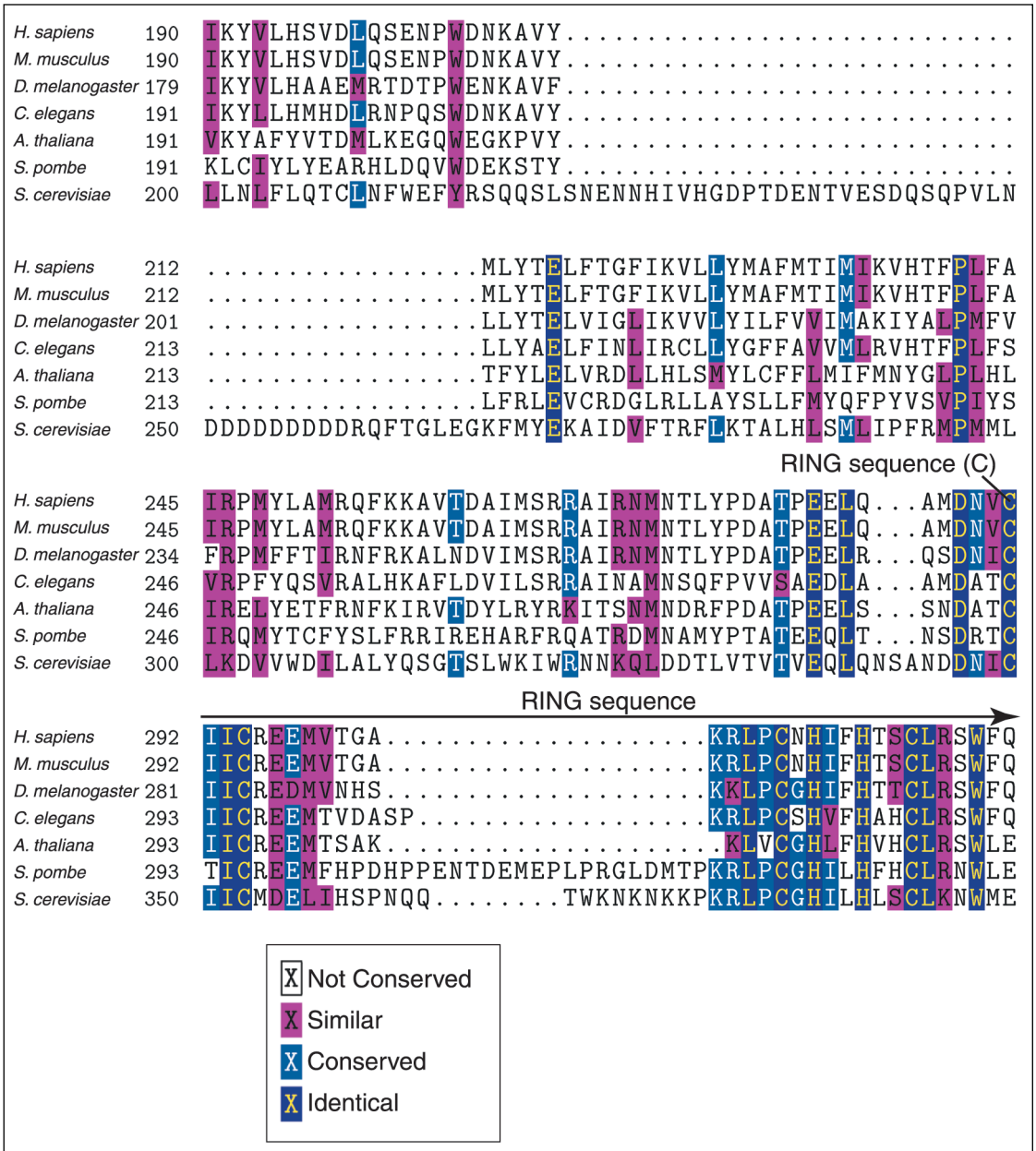

Figure 3-19 (continued)

Sequence alignment of Hrd1 homologues in different species

Sequence alignment was performed using the CLUSTALW algorithm (Thompson et al., 1994). Very strong sequence conservation is seen in the RING sequence of the Hrd1 proteins.

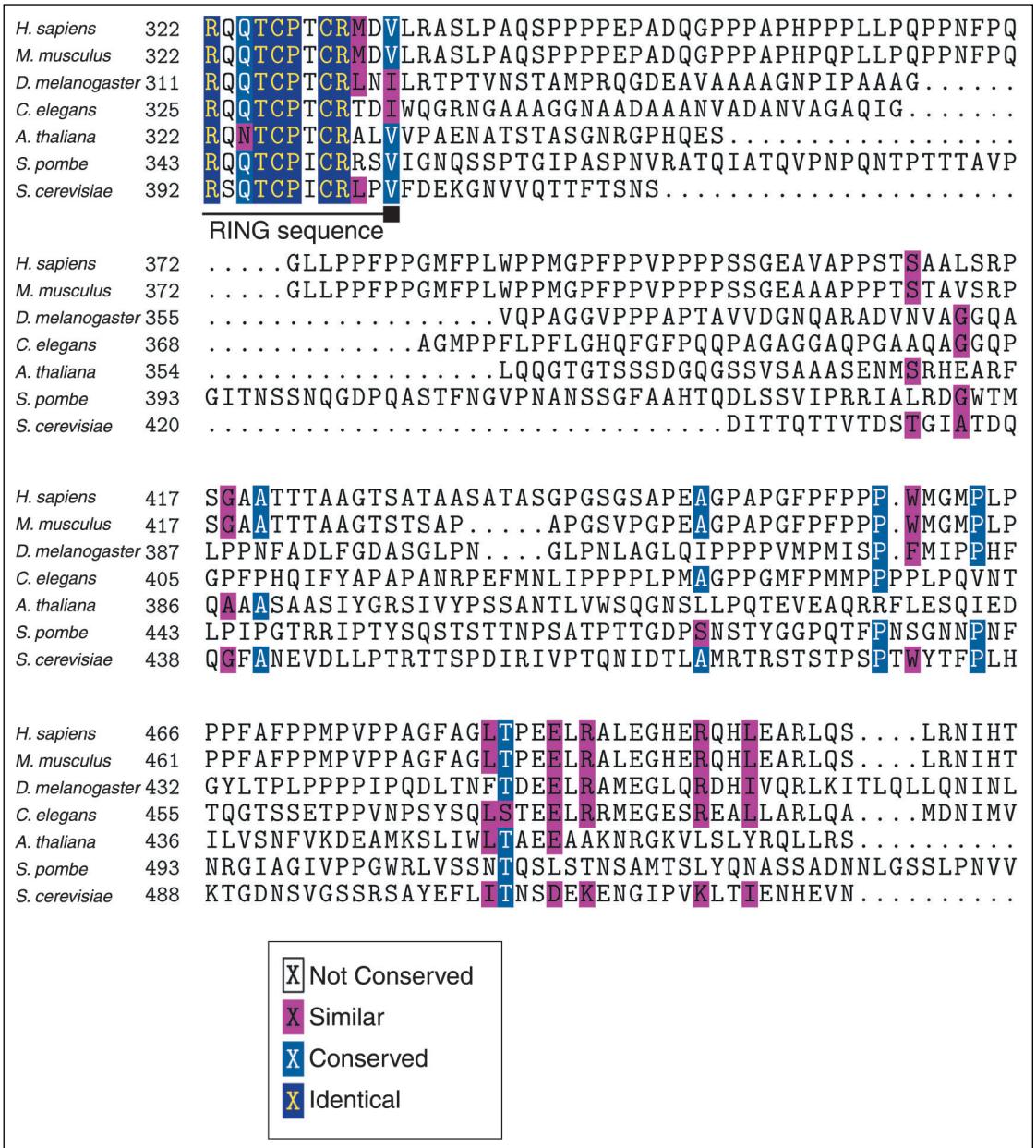

Figure 3-19 (continued)

Sequence alignment of Hrd1 homologues in different species

Sequence alignment was performed using the CLUSTALW algorithm (Thompson et al., 1994). Very strong sequence conservation is seen in the RING sequence of the Hrd1 proteins, but divergence is seen in sequence after the RING.

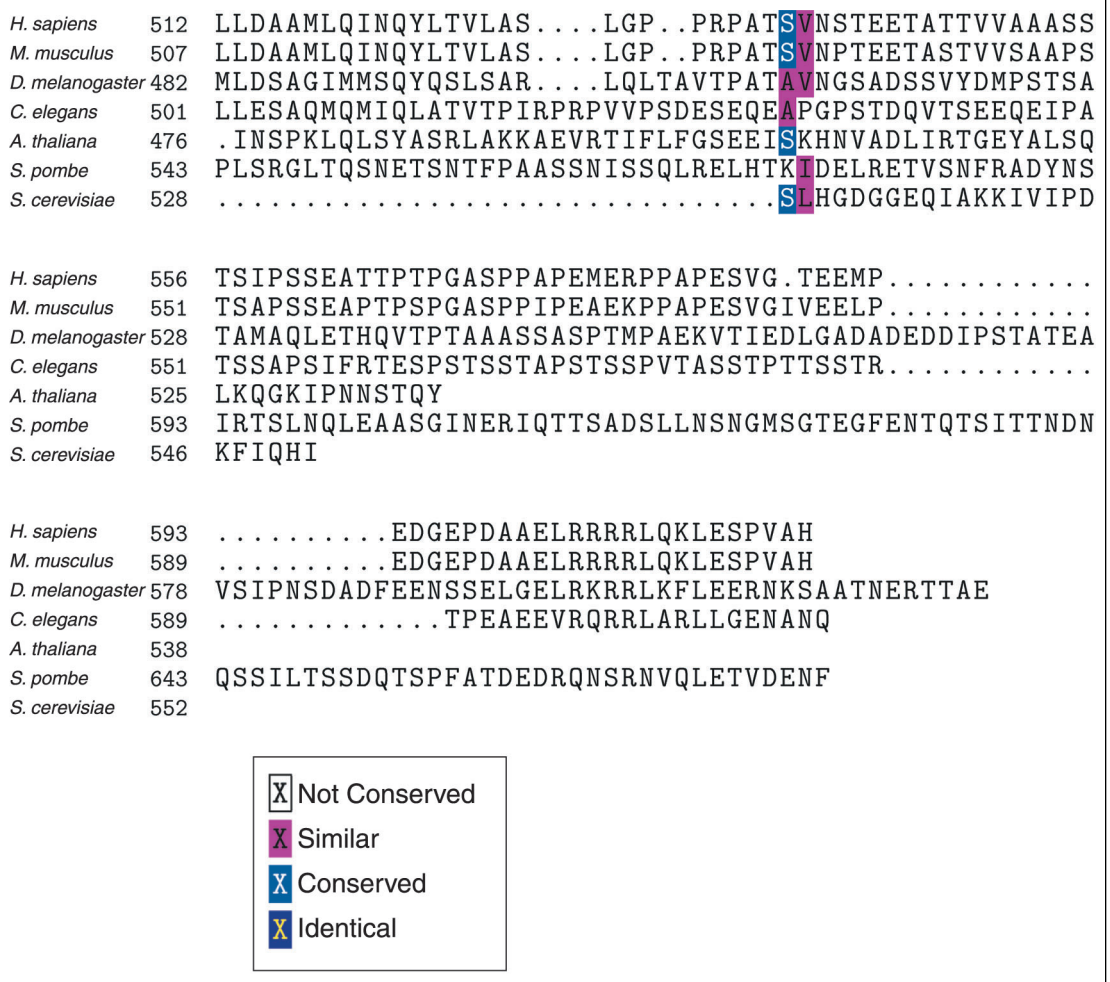

Figure 3-19 (continued)

Sequence alignment of Hrd1 homologues in different species

Sequence alignment was performed using the CLUSTALW algorithm (Thompson et al., 1994). Divergence is seen in sequence after the RING.

boiled or unboiled BSA. This indicated that boiled BSA is not simply a generic target for E3s *in vitro*, and that the Hrd1 activity is somewhat unique among E3s. The investigation of this particular Hrd1 activity is in a very early stage, but further study of Hrd1 holds promise for understanding how misfolded proteins are recognized by the quality control machinery and for the eventual modulation of Hrd1 activity as a means of targeting specific proteins for degradation or stability.

Methods

Immunoprecipitation, immunoblotting and protein crosslinking

Immunoprecipitation was performed as described but with additional protease inhibitors (*n*-ethylmaleimide, AEBSF, E-64, benzamidine and ε-amino-*n*-caproic acid). Immunoblotting was also performed as described (Hampton and Rine, 1994) except that tris-buffered saline contained 0.45% Tween 20, and 20% heat-inactivated bovine calf serum was used as the blocking agent. Ubiquitin immunoblots were processed as described. (Swerdlow et al., 1986) SDS-PAGE in Figure 3-8 on page 155, Figure 3-10 on page 160, Figure 3-12 on page 163, and Figure 3-16 on page 169 was performed using 3-8% NuPage™ tris-acetate gels (Novex/Invitrogen, Carlsbad, CA) and 14% tris-glycine gels in Figure 3-11 on page 162. All other SDS-PAGE was performed using 8% tris-glycine gels. *In vivo* protein cross-linking was performed as described (Gardner et al., 2000). Briefly, intact cells were placed in amine-free buffer, treated with crosslinker DSP (Dithiobis [Succinimidyl Propionate], Pierce), and subse-

quently lysed, immunoprecipitated with anti Hrd1p antiserum and immunoblotted for Ubc7p. For crosslinking experiments, *HRD1* and *UBC7* were expressed from the same *(TDH3)* promoter.

Antibodies

GFP was detected using a polyclonal rabbit antibody for cycloheximide chases (provided by C. Zuker, UCSD) and a mouse monoclonal antibody following immunoprecipitations (Zymed). Ubiquitin was detected with an anti bovine-Ub monoclonal antibody (Zymed). The myc epitope was detected with the 9e10 mAb (ATCC hybridoma); the HA epitope was detected with the 12CA5 mAb (Babco), Polyclonal antibodies against Hmg2p were generated previously (Hampton et al., 1996).

Protein purification and *in vitro* ubiquitination

Maltose Binding Protein (MBP) fusions were constructed and purified with amylose resin according to the manufacturer's directions (New England Biolabs) as were 6HIS fusions using TALON™ resin (Clontech) and GST fusions using glutathione-Sepharose™ (Pharmacia). Both MBP-Hrd1p and GST-S peptide-Hrd1p were fusions with the last 203 amino acids of Hrd1p (containing the RING-H2 motif). The parent vector for GST-S peptide constructs was pET42(b) (Novagen). Assays for *in vitro* ubiquitination were performed using materials and methods described previously (Joazeiro et al., 1999). Briefly, 0.1 μg of 6HIS-E1 (Anan et al., 1998, mouse), 0.2 μg of 6HIS-UBC4 (Anan et al., 1998, human), 2.5 μg of ubiquitin (bovine, Sigma) and indicated

amounts of Hrd1p fusions (1 μg unless otherwise stated) were incubated at 25˚C for 90 min in 2 mM ATP, 50 mM Tris-HCl (pH7.5), 2.5 mM $MgCl_2$ and 0.5 mM DTT. Indicated reactions contained TPEN (Fluka, 10 mM final concentration from a 100 mM stock in absolute ethanol), S protein (Sigma), or S peptide (Biozyme, San Diego). Biotinylated bovine serum albumin (Sigma) was resuspended to a stock concentration of 10 mg/ml in HDB (25 mM Hepes, 0.7 mM Na_2HPO_4, 137 mM NaCl, 5 mM KCl, pH 7.4) containing 10% glycerol. Where indicated, BSA was boiled in a microfuge tube for 5 min. To precipitate biotinylated BSA, 60 μl of a 50% Streptavidin-agarose (Fluka, HDB-equilibrated) suspension was added to each reaction along with 300 μl HDB. Reactions were incubated for 1 h at 25˚C with gentle shaking and then washed 3 times with 1 ml HDB, 3 times with 1 ml HDB containing 0.25% Triton X-100 and 0.5% deoxycholate, followed by two more washes with 1 ml HDB. The supernantant was then aspirated to dryness, 50 μl of 2X USB added, and sample boiled for 5 min before SDS-PAGE. Boiling had no effect on precipitation efficiency as determined by SDS-PAGE of precipitates and blotting using strepavidin-HRP. All proteins were resuspended and/or stored in HDB buffer.

Assays for protein degradation

Cycloheximide chase was used to measure protein stability as described (Hampton et al., 1996). Degradation was measured by immunoblotting or flow cytometry (Hmg2p-GFP strains). Flow cytometry was performed using a FACScalibur machine

(Becton Dickinson) as described (Cronin and Hampton, 1999). Statistical analysis was performed to quantitate loss of fluorescence (Young, 1977).

Plasmids

pRH808 *(P_{TDH3}-HRD1,TRP1* YIP), used to increase expression of *HRD1*, was constructed by placing the yeast *TDH3* promoter immediately before the *HRD1* coding region using PCR and Vent polymerase (New England Biolabs, Beverly, MA). pRH808 increased steady-state levels of Hrd1p by approximately four-fold in *ubc6Δubc7Δ*, *ubc7Δ* and *ubc1Δubc7Δ* strains. pRH1256 *(ubc1Δ::KanMX* disruption plasmid) was constructed by inserting the KanMX gene into PstI-BstZ17I sites of *UBC1*.

Strains and media

The parent for all yeast strains was JRY527, an S288C derivative. The parent strain for experiments with Hmg2p-GFP was RHY853 *MATalpha ade2-101 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 leu2Δ lys2-801 met2 trp1::hisG URA3::P_{TDH3}-HMG2cd::P_{TDH3}-hmg2-GFP.* The parent strain for experiments with Hmg2p was RHY1167 *MATalpha ade2-100 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 leu2Δ lys2-801 met2 trp1::hisG ura3-52:: P_{TDH3}-1MYC-HMG2*. RHY853 and RHY1167 alleles were described previously (Gardner and Hampton, 1999; Hampton et al., 1996; Hampton and Rine, 1994) as were the *hrd1Δ::TRP1, ubc6Δ::KanMX* (Wilhovsky et al., 2000b) and *ubc7Δ::HIS3* (Hampton and Bhakta, 1997) alleles. The *ubc1Δ::KanMX* allele was created by transforming RHY853 with *ubc1Δ::KanMX* fragment from pRH1256,

described above. All strains were isogenic and constructed using standard techniques in yeast genetics. Yeast strains were grown in yeast minimal media at 30°C with shaking as described (Hampton and Rine, 1994). Yeast were transformed by the lithium acetate method (Gietz et al., 1995). When strains expressed the *KanMX* gene, G-418 sulfate was added to a final concentration of 500 μg/ml.

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4

HRD4/NPL4 is required for the proteasomal processing of ubiquitinated ER proteins.

Introduction

As discussed in chapter 1, the endoplasmic reticulum is a major site for protein degradation in the cell (Arias et al., 1969; Brodsky and McCracken, 1999; Fra and Sitia, 1993). This endoplasmic reticulum-associated degradation (ERAD) serves several functions: ERAD removes aberrant, misfolded proteins from the ER as a means of "quality control" for ER proteins (Hammond and Helenius, 1995; Wickner et al., 1999). ERAD is also employed in the regulation of HMG-CoA reductase (HMGR), a rate-limiting enzyme in cholesterol biosynthesis (Hampton and Rine, 1994). Several *HRD* genes required for the degradation of Hmg-CoA Reductase Degradation have been identified (Hampton et al., 1996). The characterization of these *HRD* genes along with other studies has revealed that ERAD proceeds via the ubiquitin-proteasome pathway (Hampton and Bhakta, 1997; Hiller et al., 1996; Sommer and Jentsch, 1993). Several Hrd proteins are required for the ubiquitination of ERAD substrates including the ubiquitin-protein ligase (E3) Hrd1p (Bays et al., 2001), its associated membrane protein Hrd3p (Gardner et al., 2000), and the ubiquitin-conjugating enzymes (E2s) Ubc7p and Ubc1p (Bays et al., 2001; Hampton and Bhakta, 1997). Other Hrd proteins, like Hrd2p, are components of the 26S proteasome itself (Tsurumi et al., 1996). In order to extend the range of *HRD* genetic studies, the original *hrd* selection (Hampton et al., 1996) was modified to allow the recovery of temperature-sensitive *hrd* mutants. One mutant strain isolated expressed the *hrd* allele *hrd4-1 and* grew normally at 30°C but were inviable at

35°C. The wild-type allele corresponding to the *hrd4-1* mutation was cloned and found to be identical to the gene *NPL4*.

NPL4 is an essential gene (null alleles are not viable), and was previously identified in a selection for mutants deficient in nuclear import/export (DeHoratius and Silver, 1996). *npl4* mutants fail both to import nuclear localization signal (NLS)-bearing proteins into the nucleus as well as export poly(A)$^+$ RNA from the nucleus when shifted to the restrictive temperature. *npl4* mutants also exhibit defects in nuclear structure at the restrictive temperature. These nuclear abnormalities include herniations of the nuclear envelope, separation of the inner and outer nuclear membranes and large membrane protusions containing accumulations of poly(A)$^+$ RNA (DeHoratius and Silver, 1996).

NPL4 has also been implicated in unsaturated fatty acid (UFA) biosynthesis. Hitchcock *et al*. (2001) isolated the *OLE1* gene as a partial high copy suppressor of *npl4-1* and *npl4-2* mutant growth phenotypes. *OLE1* is an essential gene encoding the sole Δ9-fatty acid desaturase in yeast (Stukey et al., 1990). *MGA2* and *SPT23* were also isolated as partial high copy suppressors of *npl4* (Hitchcock et al., 2001). These genes were previously identified as functionally redundant transcription factors required for the production of *OLE1* transcript (Zhang et al., 1999). Recently, it has been shown that Mga2p and Spt23p reside in the endoplasmic reticulum as inactive membrane-bound transcription factors (Hoppe et al., 2000). When cells are deprived of fatty acids, the Mga2 and Spt23 proteins are cleaved from their membrane anchors in a ubiquitin- and

proteasome-dependent process, liberating the transcription factor domains to enter the nucleus and promote transcription of the *OLE1* gene. The proteasome-dependent processing of Mga2p and Spt23p requires *NPL4*: In *npl4* mutants, Mga2p and Spt23p cleavage is defective (Hitchcock et al., 2001). Furthermore, at least some of the nuclear defects in *npl4* mutants can also be suppressed by unsaturated fatty acids and increased *OLE1* expression (Hitchcock et al., 2001).

Npl4p physically associates with Cdc48p via Ufd1p to form a Cdc48p-Ufd1p-Npl4p complex (Hitchcock et al., 2001; Meyer et al., 2000). Ufd1p was previously identified in a screen for mutants that fail to degrade a fusion protein with a nonremovable ubiquitin moiety (Johnson et al., 1995). Cdc48p is a AAA ATPase required for a variety of cellular processes including cell division, protein degradation and ER membrane fusion (Ghislain et al., 1996a; Latterich et al., 1995; Moir et al., 1982). Cdc48p actually associates with two other Ufd proteins, Ufd2p and Ufd3p/Doa1p, as well as several other proteins with no known function in protein degradation (Ghislain et al., 1996a; Koegl et al., 1999). This ability to bind multiple proteins has prompted models of an "adapter" function for Cdc48p (Patel and Latterich, 1998).

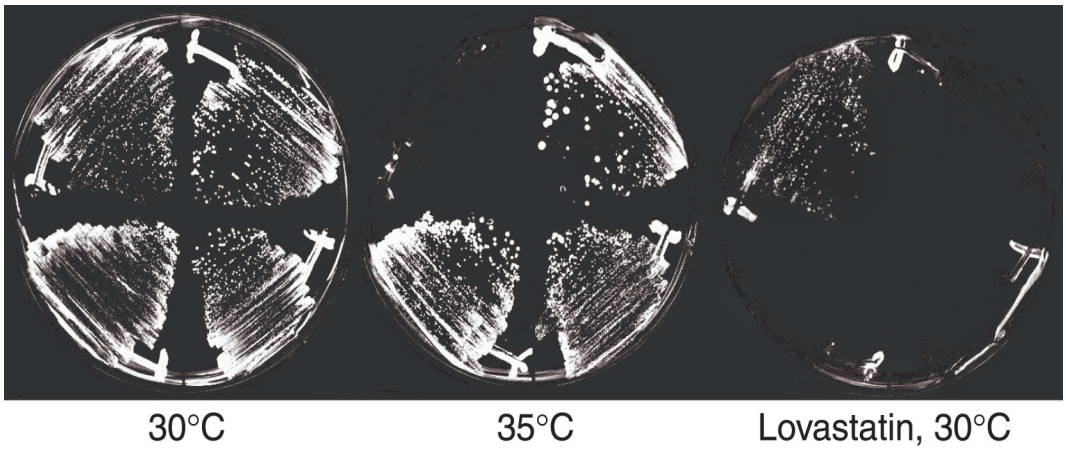
Here, these diverse phenotypes for *HRD4/NPL4* are united by the discovery of a role for Hrd4p/Npl4p in the proteasomal processing of ubiquitinated proteins at the ER. *hrd4/npl4* mutants are defective in the degradation of several ER proteins, but *HRD4/NPL4* is not required for the actual ubiquitination of ERAD substrates. Importantly, general proteasome function is not impaired in *hrd4/npl4* mutant cells. Therefore,

Hrd4p/Npl4p appears to function at a post-ubiquitination but pre-proteasomal step in ERAD. Analysis also shows that the primary defect in *hrd4/npl4* cells is a lack of proteasomal processing of ubiquitinated proteins, and not a defect in nuclear transport or fatty acid biosynthesis. These diverse phenotypes apparently arise from the loss of Hrd4p/Npl4p function in the ubiquitin-proteasome pathway.

Results

HRD4/NPL4 is required for ERAD

A selection strategy to identify *HRD* genes required for HMG-CoA reductase (HMGR) degradation was described previously (Hampton et al., 1996). This selection employed the drug lovastatin, a specific inhibitor of HMGR's essential catalytic activity, to select for mutants with elevated HMGR due to slowed degradation. The previously described *hrd* selection was modified to allow the isolation of lovastatin-resistant *hrd* mutants that were also temperature-sensitive for growth. The selection yielded mutations in several genes, including a previously unidentified *HRD* gene, *HRD4*. The gene corresponding to the *hrd4-1* mutation was cloned and found to be identical to *NPL4* an essential gene previously identified in a selection for genes involved in nuclear transport (Figure 4-1 on page 203). This work describes the characterization of the *HRD4/NPL4* gene and its role in the ubiquitin-mediated degradation of a diverse group of ER proteins.



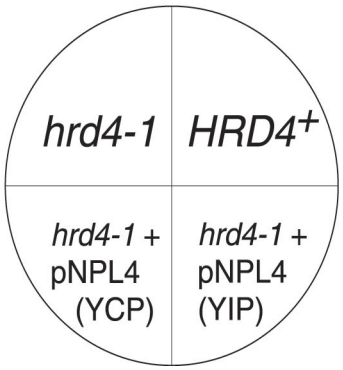


Figure 4-1

HRD4* is *NPL4

(YCP) indicates the *hrd4-1* strain was transformed with an ARS/CEN yeast genomic library plasmid isolated for its ability to complement the *hrd4-1*mutation and containing a genomic fragment bearing the *NPL4* gene. (YIP) indicates a plasmid bearing only the *NPL4* and *URA3* genes was integrated at the *ura3-52* locus in a *hrd4-1* strain. "Lovastatin" indicates the addition of 200 µg/ml lovastatin to the indicated plate.

The requirement for *HRD4/NPL4* in ERAD was first seen by testing the effect of the *hrd4-1* mutation on the degradation of 6myc-Hmg2p, the unregulated version of HMGR used in the *hrd* selection. 6myc-Hmg2p is a constitutively degraded ER membrane protein and is targeted for proteasomal degradation by the ERAD E3 complex, Hrd1p-Hrd3p (Bays et al., 2001; Gardner et al., 2000; Hampton et al., 1996). To examine the degradation of 6myc-Hmg2p, a "stationary chase" (Hampton and Rine, 1994) of

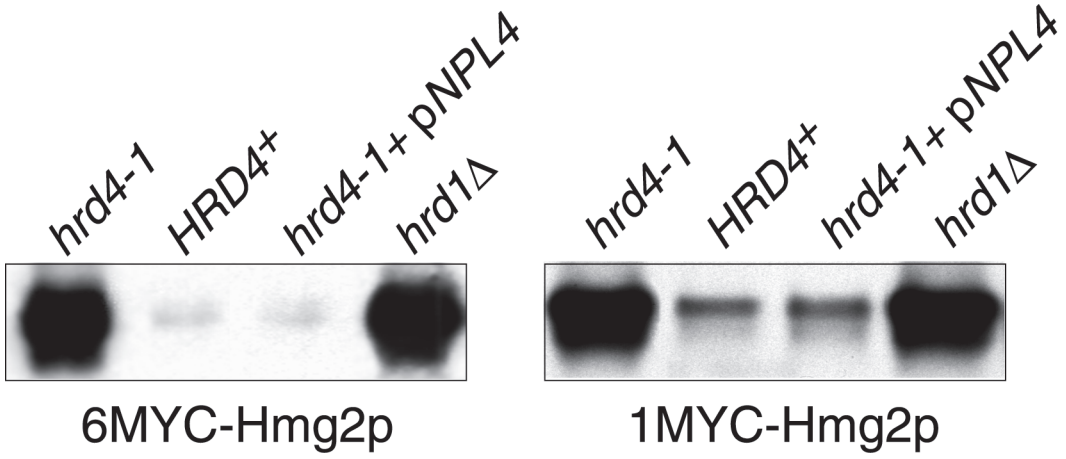


Figure 4-2

***HRD4* is required for the degradation of both constitutively-degraded and normally regulated Hmg2p**

Both panels are stationary chase assays of protein degradation. The indicated yeast strains were grown into early stationary phase (12 hours after strains reached an OD_{600} of 0.1). Equal numbers of cells were then lysed and subjected to SDS-PAGE followed by immunoblotting for the myc-epitope tag.

hrd4-1 strains was performed. In this assay, cells are grown into stationary phase where protein synthesis slows while degradation continues. As a result, *hrd* mutants display a considerably higher level of 6myc-Hmg2p in stationary phase compared to wild-type cells. *hrd4-1* cells showed significantly more 6myc-Hmg2p immunoreactivity than wild-type cells in a stationary chase, and this increase was reversed upon the addition of the *NPL4* gene to *hrd4-1* strains (Figure 4-2 on page 204). The effect of the *hrd4-1* mutation on the native Hmg2 protein was then tested. Unlike 6myc-Hmg2p, 1myc-Hmg2p undergoes mevalonate-pathway regulated degradation: When mevalonate and its derivatives are abundant, 1myc-Hmg2p degradation is fast. Conversely, when these

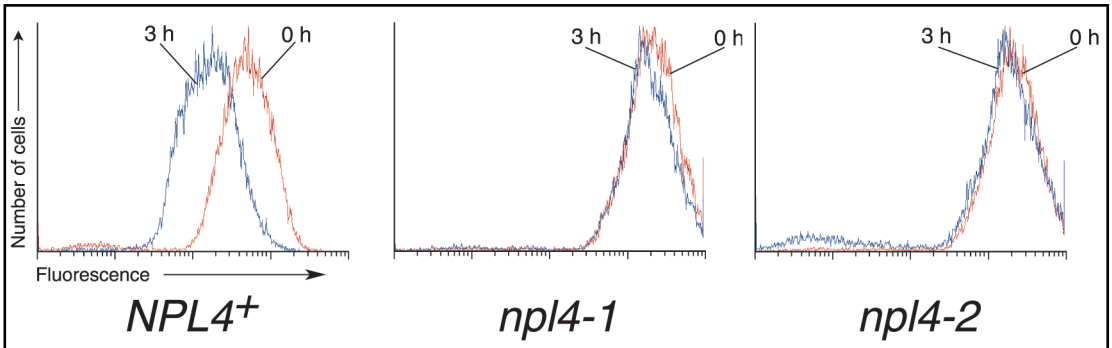


Figure 4-3

Mutant alleles of *NPL4* isolated in the *Nuclear Protein Localization* selection are also defective in protein degradation.

Degradation of Hmg2-GFP was measured by cycloheximide chase followed by flow cytometry. Cycloheximide was added to log-phase (OD600 < 0.2) cells 3 hours prior to collection and analysis by flow cytometry. Each histogram represents 20,000 cells.

same molecules are scarce, 1myc-Hmg2p degradation is slow. Regulated 1myc-Hmg2p also required *HRD4/NPL4* for degradation. When a stationary chase was performed with 1myc-Hmg2p in a *hrd4-1* strain, degradation of 1myc-Hmg2p was impaired (Figure 4-2 on page 204). 1myc-Hmg2p immunoreactivity was identical in wild-type cells and in *hrd4-1* cells transformed with a single copy of the *NPL4* gene (Figure 4-2 on page 204). Degradation of 6myc-Hmg2p and 1myc-Hmg2p was also assessed using a "cycloheximide chase" (Hampton and Rine, 1994). In this assay, cycloheximide is added to log-phase cells to stop protein synthesis. Protein degradation, however, continues and can be measured by detecting a loss of immunoreactivity (for myc-tagged Hmg2p) or a loss of fluorescence (for Hmg2-GFP). When examined by cycloheximide chase, wild-type cells showed a decrease in immunoreactivity over the chase period, while *hrd4-1*

and *hrd1-1* strains showed little loss over the same period (data not shown). Therefore, *HRD4/NPL4* was required for the degradation of normally regulated 1myc-Hmg2p and its constitutively degraded variant, 6myc-Hmg2p.

Because *NPL4* has been implicated in a phenotype quite distinct from ERAD, it was considered whether the ERAD defect seen in *hrd4* mutants was a general feature of losing *HRD4/NPL4* function or if the ERAD phenotype was unique to the *hrd4/npl4* allele isolated. *npl4-1* and *npl4-2* alleles were isolated in the original *npl* selection for mutants that fail to properly localize an NLS (nuclear localization signal)-bearing protein (DeHoratius and Silver, 1996). To determine whether *npl4-1* and *npl4-2* strains were defective in ERAD, *npl4* mutant strains were by assayed for their ability to degrade the reporter protein Hmg2-GFP. Hmg2-GFP is degraded via the Hrd pathway just like wild-type Hmg2p, and the GFP fusion allows detection of Hmg2-GFP levels by flow cytometry. When wild-type *NPL4*$^{+}$ cells expressing Hmg2-GFP were subjected to a cycloheximide chase, loss of fluorescence was observed as the Hmg2-GFP protein was degraded (Figure 4-3 on page 205). However, very little loss of fluorescence was observed during a cycloheximide chase of *npl4-1* and *npl4-2* strains (Figure 4-3 on page 205) indicating that *npl4-1* and *npl4-2* mutant strains were indeed deficient in Hmg2-GFP degradation. Therefore, several different hypomorphic alleles of *NPL4* isolated in very different genetic studies were deficient in ERAD.

The degradation of several other known ERAD substrates was tested in *hrd4-1* mutant strains. CPY* is a mutant, misfolded form of carboxypeptidase Y that is retained

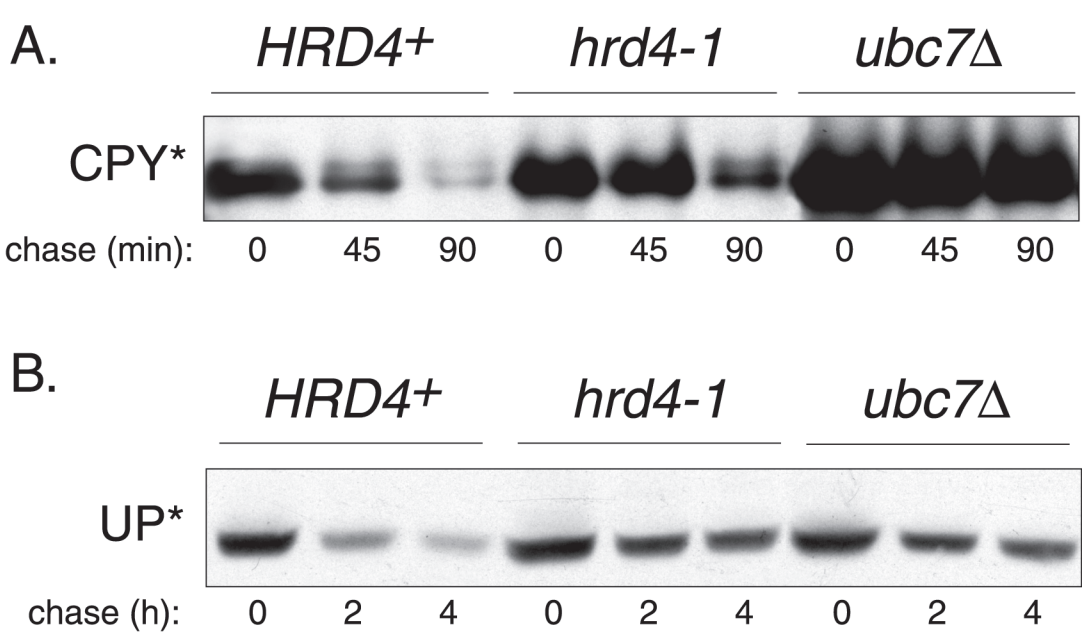


Figure 4-4

***HRD4/NPL4* is required for ER-Associated Degradation (ERAD)**

(A) CPY* degradation in the indicated strains was assayed by cycloheximide chase. Equal numbers of log-phase (OD_{600} < 0.5) cells were collected at the indicated times post cycloheximide addition, lysed and analyzed by SDS-PAGE followed by immunoblotting for the HA-epitope tag present on the CPY*-HA protein. (B) Cycloheximide chase as in C, except that immunoblotting using anti-Fur4p antibodies was performed to detect UP*

in the endoplasmic reticulum and degraded by the Hrd pathway (Bordallo et al., 1998; Finger et al., 1993). To test the effect of the *hrd4-1* mutation on the degradation of CPY*, a cycloheximide chase of CPY* was performed in *HRD4*$^{+}$ and *hrd4-1* cells. CPY* was rapidly degraded in *HRD4*$^{+}$ cells, but degradation in *hrd4-1* cells was clearly impaired (Figure 4-4 on page 207). Loss of Ubc7p, the principle ubiquitin conjugating enzyme in ERAD, also blocked the degradation of CPY* (Figure 4-4 on page 207).

The degradation of some ERAD substrates does not require the Hrd1p-Hrd3p ubiquitin ligase complex (Wilhovsky et al., 2000). For example, a mutant form of uracil permease (UP*) does not require either Hrd1p or Hrd3p for its ER-associated degradation (Wilhovsky et al., 2000). The degradation of UP* was tested in *hrd4* mutant strains to see how broadly Hrd4p/Npl4p affected ERAD. In a cycloheximide chase, UP* was degraded in wild-type cells (Figure 4-4 on page 207). However, the degradation of UP* was slowed in *hrd4-1* cells (Figure 4-4 on page 207). Loss of Ubc7p also caused a defect in UP* degradation (Figure 4-4 on page 207). Therefore, Hrd4p function was required for the degradation of a larger set of ERAD substrates than those ubiquitinated by the action of the Hrd1p-Hrd3p ubiquitin ligase complex.

As Hrd4p/Npl4p appeared to function broadly in ERAD, *hrd4* mutants were tested for an elevated unfolded protein response (UPR). The UPR is a coordinated regulation of gene expression induced upon an increase of unfolded proteins in the ER (Sidrauski et al., 1998). The genes upregulated by the UPR include those coding for protein folding machinery like the chaperone Kar2p and ERAD components like the E3 Hrd1p (Kohno et al., 1993; Travers et al., 2000). The UPR serves as sensitive indicator of conditions that increase the abundance of misfolded proteins in the ER. For instance, mutations that block ERAD often lead to an elevated UPR as cells are unable to remove misfolded proteins from the ER (Friedlander et al., 2000; Travers et al., 2000). To assess the UPR in *hrd4-1* cells, a UPR reporter construct (P_{KAR2}-*GFP*) was introduced into wild-type, *hrd4-1*, and *hrd1*Δ cells. Flow cytometry was then used to measure cell fluo-

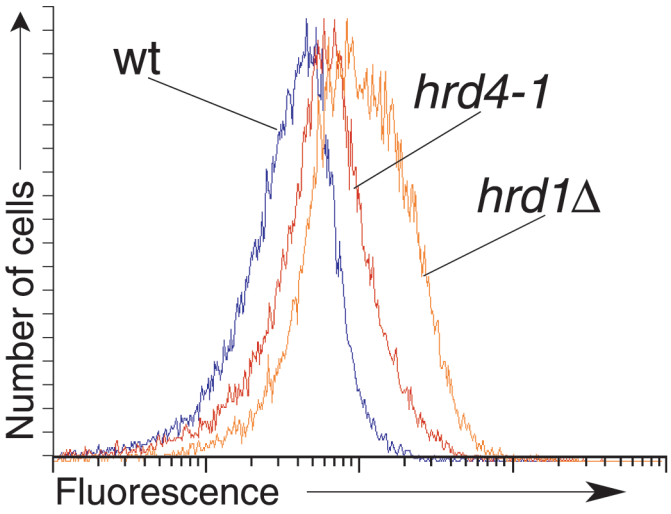


Figure 4-5

***hrd4-1* cells show an elevated Unfolded Protein Response (UPR)**

Steady-state cell fluorescence for the indicated strains expressing the UPR reporter construct, P_{KAR2}-*GFP*, was analyzed by flow cytometry during log-phase growth. All experiments were performed at the permissive temperature of 30°C.

rescence. When *hrd4-1* cells were analyzed, they showed a clear increase in cell fluorescence compared to wild-type cells – indicating that indeed *hrd4-1* cells exhibited an elevated UPR (Figure 4-5 on page 209). As previously described, *hrd1*Δ cells also displayed an increased UPR compared to wild-type cells (Friedlander et al., 2000; Travers et al., 2000). These results are consistent with a loss of Hrd4p/Npl4p that leads to an increased burden of unfolded proteins in the ER.

HRD4/NPL4 has been implicated in both nuclear transport and fatty acid biosynthesis (DeHoratius and Silver, 1996; Hitchcock et al., 2001). Therefore, it was consid-

ered whether one or both of these defects is the cause of ERAD deficiency of *hrd4/npl4* mutants. The following results indicated that neither nuclear transport nor fatty acid biosynthesis explained the defects in ERAD.

A block in nuclear import/export does not affect ERAD

npl4 mutants are defective in nuclear import/export, and were originally isolated by virtue of this deficiency. The *npl4* mutant defect in nuclear import/export, however, is seen after a shift to the restrictive temperature of 37˚C (DeHoratius and Silver, 1996). There appears to be no significant deficit in nuclear transport at 30˚C in *npl4-1, npl4-2* or *hrd4-1* cells. In contrast, the same *npl4/hrd4* cells show a substantial defect in ERAD at the *permissive* temperature of 30˚C with almost no detectable growth deficit. (Note: All degradation experiments in this paper were performed at the permissive temperature of 30˚C with the exception of those in Figure 4-6 on page 212.) Because nuclear import and export are not significantly compromised at 30˚C in *npl4/hrd4* strains, a deficiency in nuclear import/export would be insufficient to explain the ERAD defect in *npl4/hrd4* cells: The ERAD defect is present in *hrd4/npl4* cells at 30˚C while the nuclear transport defect is *not* present at 30˚C. Nonetheless, blocking nuclear import/export with a distinct mutation was performed to see if such a block had any effect on ERAD. To achieve a block in nucleocytoplasmic traffic, strains bearing the temperature-sensitive *nup116*Δ allele were used. Loss of the *bona fide* nuclear pore protein Nup116p (Ho et al., 2000; Rout et al., 2000; Strawn et al., 2000) leads to a profound block in nuclear transport after

a shift to the restrictive temperature of 37°C (Wente and Blobel, 1993). The *nup116*Δ allele was chosen for several reasons. First, a complete loss of function in a nuclear pore protein and an apparently complete block in nuclear traffic could be achieved by using *nup116*Δ cells, providing a stringent test of any requirement for nuclear transport in ERAD. Second, the block in nuclear traffic and the presence of nuclear membrane herniations in *nup116*Δ cells are strikingly similar to the phenotypes of *npl4* mutant cells at the non-permissive temperature (DeHoratius and Silver, 1996; Wente and Blobel, 1993). Therefore, *nup116*Δ offers an equivalent type of block in nuclear traffic as *npl4*. Consistent with this, *NUP116* and *NPL4* also show genetic indications of affecting similar functions as overexpression of *NPL4* can partially suppress the structural and growth defects of *nup116*Δ strains (DeHoratius and Silver, 1996). These numerous similarities make *nup116*Δ especially appropriate for testing whether a block in nuclear traffic underlies the ERAD phenotypes of *npl4* mutants.

The *nup116*Δ allele was introduced into a strain expressing 6myc-Hmg2p. The absence of Nup116 protein was verified by immunoblotting: Nup116p immunoreactivity was present in the wild-type strain, but not in the *nup116Δ* strain (Figure 4-6 on page 212). As expected from the previous characterization of the *nup116Δ* allele (Wente and Blobel, 1993), strains lacking Nup116p were temperature sensitive (data not shown). *nup116*Δ cells were not lovastatin-resistant, indicating that loss of Nup116p did not cause a significant defect in 6myc-Hmg2p degradation (data not shown). Further-

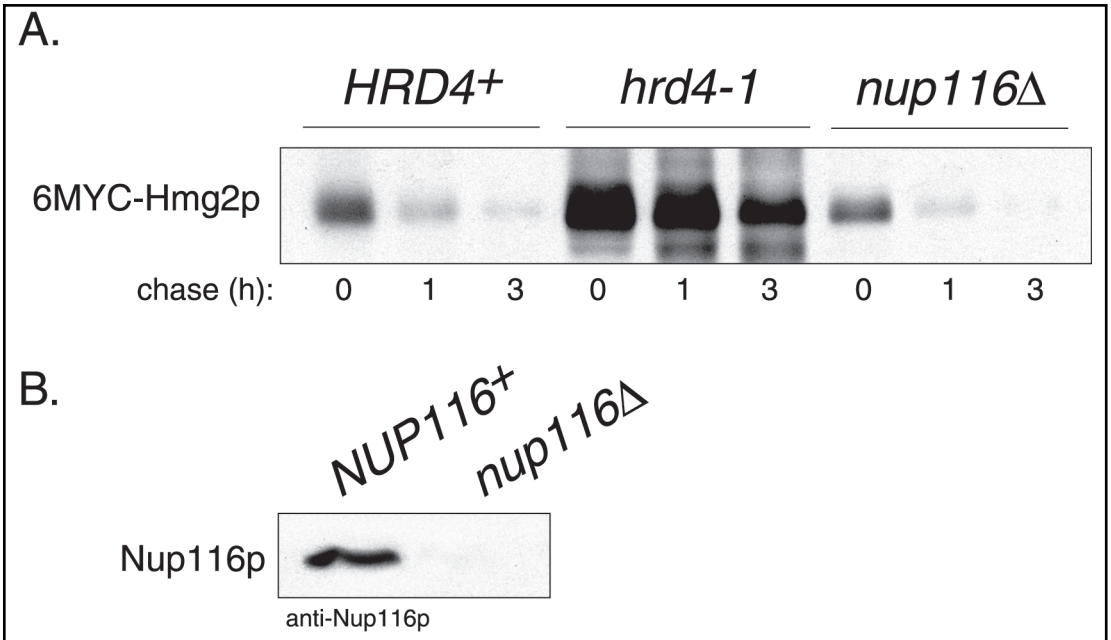


Figure 4-6

A block in nuclear transport has no effect on the degradation of an ER membrane protein.

(A) The indicated strains expressing constitutively-degraded 6MYC-Hmg2p were grown at the permissive temperature of 23°C. Log phase (OD_{600}=0.1) cells were then shifted to the restrictive temperature of 37°C for 3.5 hours before the addition of cycloheximide and subsequent chase at the indicated times. During this time wild-type cells doubled at the normal rate while the temperature-sensitive *hrd4-1* and *nup116*Δ cells failed to complete even one additional doubling following temperature shift. Equal numbers of cells were collected at the indicated times following addition of cycloheximide and lysed. These lysates were then separated by SDS-PAGE and immunoblotting for the myc epitope tag was performed. (B) Nup116 protein is absent in *nup116*Δ cells. Lysates from the indicated strains were subjected to SDS-PAGE and immunoblotting using anti-Nup116p antibodies.

more, no effect of *nup116*Δ was found using biochemical assays of 6myc-Hmg2p stability. In these assays, wild-type, *hrd4-1* and *nup116*Δ cells were grown at the permissive temperature of 23˚C. Cells were shifted to 37˚C for 3.5 hours prior to the addition of

cycloheximide and subsequent chase of 6myc-Hmg2p immunoreactivity. This shift to 37°C initiates a rapid and profound block in nuclear import/export in *nup116Δ* cells (Wente and Blobel, 1993). Despite this block in nucleocytoplasmic traffic, *nup116Δ* cells showed no detectable deficit in 6myc-Hmg2p degradation (Figure 4-6 on page 212). *hrd4-1* cells showed little degradation of the 6myc-Hmg2 protein under the same conditions (Figure 4-6 on page 212). Therefore, ERAD proceeded normally despite the block in nuclear import/export caused by the *nup116*Δ allele and despite the numerous phenotypic similarities between *nup116*Δ and *npl4/hrd4* mutants.

Unsaturated fatty acids and Npl4p

In yeast, unsaturated fatty acids (UFA) are synthesized by the action of Ole1p, a Δ9 fatty acid desaturase that catalyzes the formation of palmitoleic acid (16:1) and oleic acid (18:1) from palmitoyl-CoA (16:0) and stearolyl-CoA (18:0), respectively (Stukey et al., 1990). The *OLE1* gene is essential for life, but the addition of either palmitoleic acid or oleic acid can restore growth to *ole1*Δ cells (Zhang et al., 1999). Transcription of the *OLE1* gene requires two related and redundant transcription factors: Spt23p and Mga2p (Zhang et al., 1999). Recently, it has been shown that Spt23p and Mga2p are made as inactive proteins residing in the ER/nuclear membrane. To become active transcription factors, Spt23p and Mga2p are processed in a unsaturated fatty acid-regulated process requiring the 26S proteasome (Hoppe et al., 2000). This processing of Mga2p and Spt23p requires *HRD4/NPL4* (Hitchcock et al., 2001). Since unsaturated fatty acids are

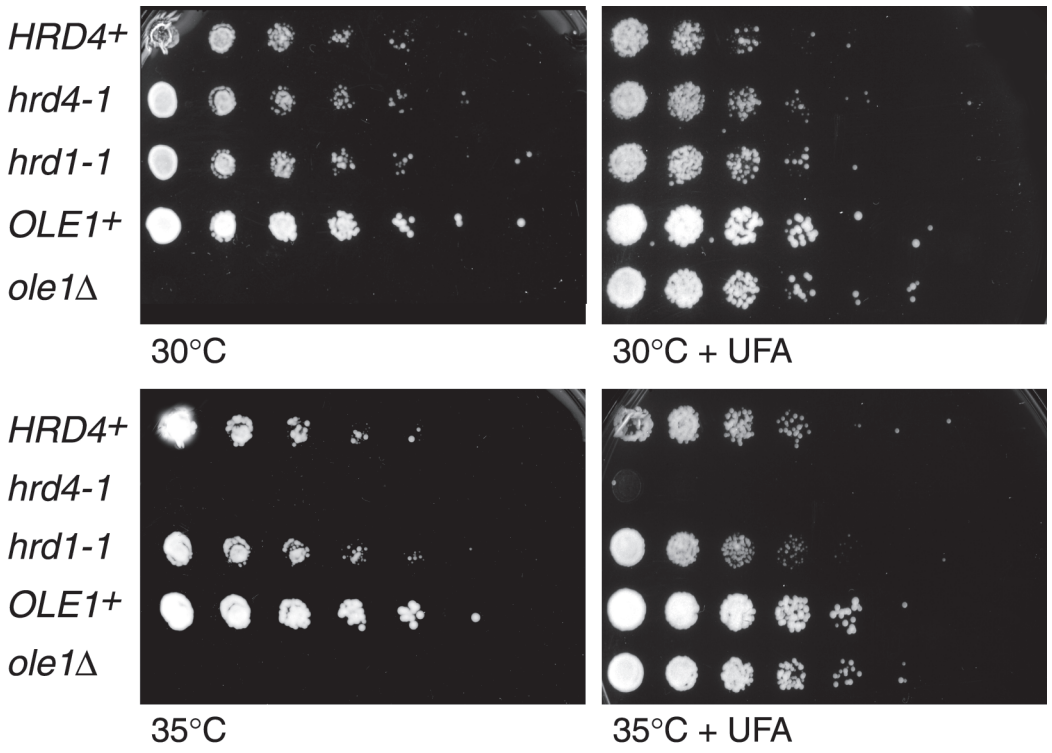


Figure 4-7

Unsaturated fatty acids do not suppress the temperature sensitivity phenotype of *hrd4-1* cells.

Serial dilutions of the indicated strains were grown at the conditions labeling each panel. "+ UFA" indicates 0.5 mM each of oleic acid and palmitoleic acid were added to the labeled plates.

critical to the function of membranes throughout the cell and ERAD occurs at the ER membrane, I was compelled to ask whether the degradation defect in *hrd4/npl4* mutant strains was due to a lack of unsaturated fatty acids (UFA). Analysis began by testing whether unsaturated fatty acids could reverse the temperature-sensitivity and lovastatin-resistance of *hrd4* mutant strains.

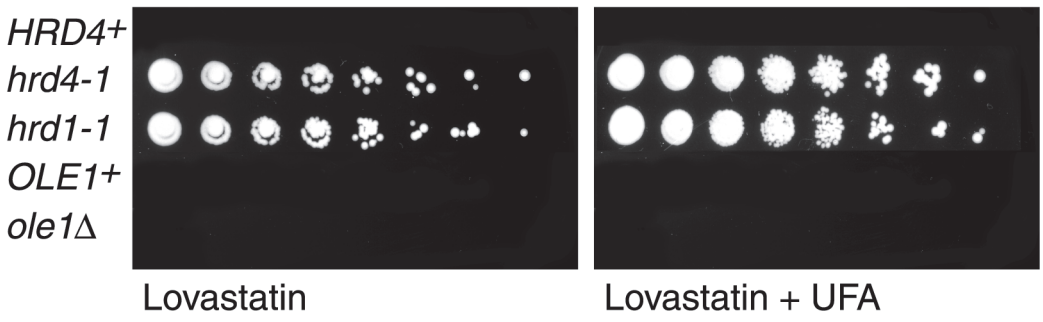


Figure 4-8

Unsaturated fatty acids do not suppress lovastatin resistance of *hrd4-1* cells.

Serial dilutions of the indicated strains were grown on the media indicated under each panel. "Lovastatin" indicates the addition of 200 µg/ml lovastatin to yeast synthetic complete media.

To test the reversal of temperature sensitivity, *HRD4*$^{+}$ and *hrd4-1* cells were plated on media with and without unsaturated fatty acids. These plates were then incubated at a series of permissive and restrictive temperatures. At 35˚C, *hrd4-1* cells failed to grow even on media containing unsaturated fatty acids (Figure 4-7 on page 214). *HRD4*$^{+}$ cells grew at 35˚C on both media (Figure 4-7 on page 214). Using a series of restrictive temperatures, colony size could slightly increase when *hrd4-1* cells were plated on unsaturated fatty acid media at 33˚C (data not shown), but at no temperature did *hrd4-1* cells grow exclusively on unsaturated fatty acid media with no growth on media lacking unsaturated fatty acids. Therefore, the addition of unsaturated fatty acids had very little, if any, effect on the Ts^{-} phenotype of *hrd4-1* cells. In the same experiments, the growth phenotype of *ole1Δ* cells was completely reversed upon the addition of unsaturated fatty

acids. *npl4-1* and *npl4-2* alleles were also tested for suppression by unsaturated fatty acids, noting that these alleles were isolated in a different parent strain than the *hrd4-1* allele. However, both *npl4-1* and *npl4-2* cells failed to grow at 37˚C even when unsaturated fatty acids were added to the media (data not shown). Although unsaturated fatty acids failed to restore wild-type growth to *npl4* mutant strains, the addition of unsaturated fatty acids did allow *npl4* mutant strains to tolerate a 1˚C increase in restrictive temperature as *npl4-1* and *npl4-2* cells could only grow at 34˚C when plated on media containing unsaturated fatty acids (data not shown). Therefore, there does appear to be a partial suppression of *npl4* temperature sensitivity by unsaturated fatty acids, but not a complete suppression. These results are consistent with those reported by Hitchcock et al. (2001) who find that unsaturated fatty acids can partially suppress the temperature sensitivity of *npl4-1* and *npl4-2* strains.

Suppression of *npl4/hrd4* temperature sensitivity was also tested using an *OLE1* 2μ (multicopy) plasmid. Suppression of *npl4/hrd4* mutants by an *OLE1* 2μ plasmid is an important test of the genetic relationship between Npl4p and Spt23p/Mga2p because the *OLE1* 2μ plasmid completely suppresses the growth and unsaturated fatty acid phenotypes of a *mga2*Δ *spt23*^{ts} double mutant strain (Zhang et al., 1999). Although *mga2*Δ *spt23*^{ts} mutant strains fail to produce *OLE1* transcript at the restrictive temperature, transformation with an *OLE1* 2μ plasmid produces an abundance of *OLE1* mRNA, allowing full complementation (Zhang et al., 1999). If the sole effect of losing Npl4p/Hrd4p function were an inability to process (and thus activate) Mga2p and Spt23p, then

npl4/hrd4 loss-of-function mutants should be phenotypically identical to *mga2 spt23* loss-of-function mutants and thus would be completely suppressed by overexpression of *OLE1*. To test suppression, *HRD4*$^{+}$ and *hrd4-1* strains were transformed with the 2μ *OLE1* plasmid isolated as a high-copy suppressor of the *mga2*Δ *spt23*^{ts} mutation. When plated at 35˚C, *hrd4-1* strains failed to survive even when bearing the *OLE1* 2μ plasmid, although a slight increase in colony size before death was seen in strains with the *OLE1* plasmid. (Figure 4-7 on page 214). For the *npl4-1* and *npl4-2* alleles, only partial suppression was seen with unsaturated fatty acid supplementation:*OLE1* overexpression allowed a 1˚C increase in the restrictive temperature but did not restore wild-type growth to *npl4-1* or *npl4-2* strains (data not shown). In contrast, transformation with the *OLE1* 2μ plasmid was able to completely restore growth to *ole1Δ* cells.

The ability of unsaturated fatty acids and *OLE1* overexpression to suppress the degradation defect in *hrd4-1* strains was also tested. *HRD4*$^{+}$ and *hrd4-1* cells were plated on media with and without lovastatin. *hrd4-1* cells showed the same plating efficiency on lovastatin even when supplemented with unsaturated fatty acids (Figure 4-8 on page 215). Likewise, *hrd1-1* cells also showed lovastatin resistance whether the plates were supplemented with UFA or not (Figure 4-8 on page 215) *HRD4*$^{+}$ cells were lovastatin-sensitive in both cases. Likewise, transformation of *hrd4-1* strains with a 2μ *OLE1* plasmid failed to restore degradation and thus reverse the lovastatin-resistance phenotype (Figure 4-9 on page 218). *HRD4*$^{+}$ cells were identically lovastatin-sensitive whether transformed with an empty or *OLE1* 2μ plasmid. These results indicated that

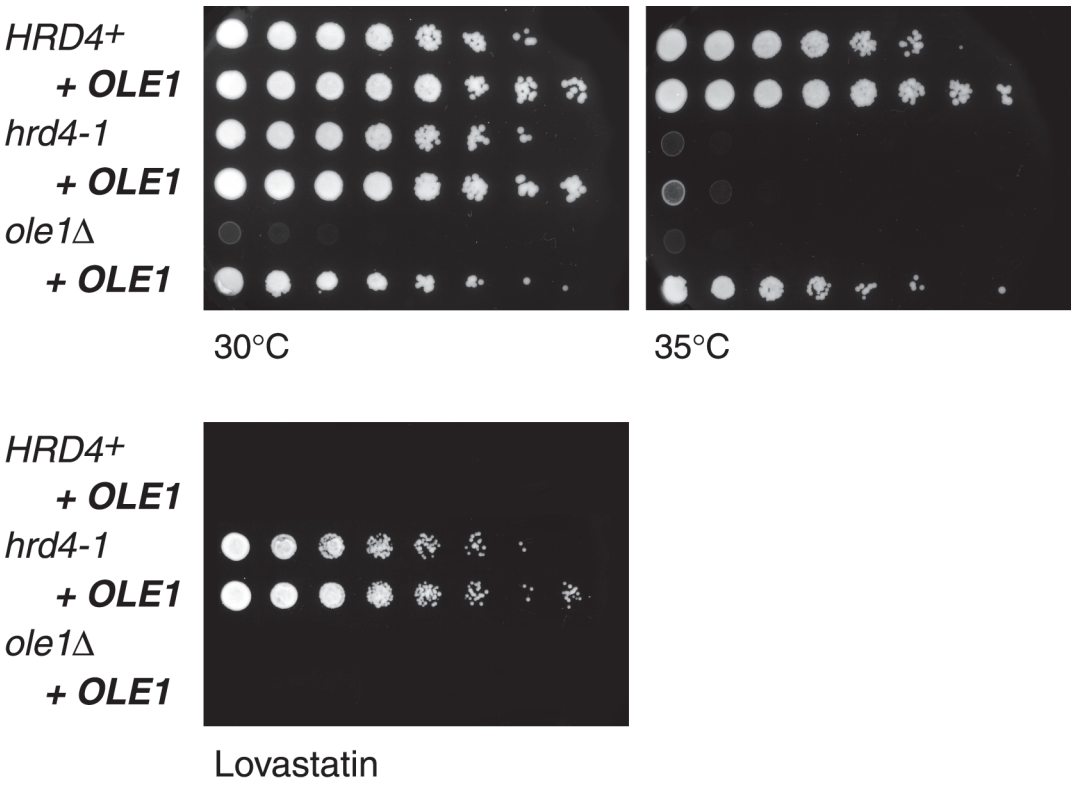


Figure 4-9

Increased *OLE1* expression does not suppress temperature sensitivity or lovastatin resistance of *hrd4-1* cells.

Serial dilutions of the indicated strains were grown at the conditions labeling each panel. "***+ OLE1***" indicates strain was stably transformed with a high copy 2μ plasmid bearing the *OLE1* gene, a plasmid originally isolated by virtue of its ability to suppress the growth phenotype of *mga2*Δ *spt23*^{ts} mutant strains. "Lovastatin" indicates the addition of 200 mg/ml lovastatin.

the *hrd4/npl4* defect in degradation was not due to an indirect effect of low unsaturated fatty acid concentrations in the cell.

Hrd4p function in the ubiquitin-proteasome pathway.

ERAD of Hmg2p and other proteins proceeds by the ubiquitin-proteasome pathway. Previously described Hrd proteins function at two distinct steps in this pathway: they are either components of the E2/E3 ubiquitination machinery (e.g. Hrd1p, Ubc7p, Ubc1p) or the 26S proteasome itself (e.g. Hrd2p/Rpn1p) (Bays et al., 2001; Hampton et al., 1996). E2 and E3 proteins are required for ubiquitination of proteins, such that their loss abrogates ubiquitination. In contrast, deficiencies in components of the 26S proteasome leave proteins fully ubiquitinated. I examined where Npl4p/Hrd4p functions in ubiquitin-mediated degradation by testing the ubiquitination of Hmg2p in *npl4/hrd4* mutant strains.

The ubiquitination of Hmg2p is subject to feedback regulation, and drugs that alter the Hmg2p (mevalonate) pathway also alter the ubiquitination of Hmg2p. Zaragozic acid, an inhibitor of the pathway enzyme squalene synthase, increases ubiquitination of Hmg2p by allowing the accumulation of a natural signal for Hmg2p degradation. (Figure 4-10 on page 220 and Gardner and Hampton, 1999; Hampton and Bhakta, 1997). When ubiquitination was assayed in a wild-type *HRD4*$^{+}$ strain, Hmg2p ubiquitination was increased dramatically by a five-minute addition of zaragozic acid (Figure 4-10 on page 220, "ZA"). This was also the case for *hrd4-1* and *hrd2-1* strains, which showed full regulated ubiquitination of Hmg2p (Figure 4-10 on page 220). In contrast, a *hrd1*Δ strain showed no ubiquitination of Hmg2p even when maximally stimulated by zaragozic acid (Figure 4-10 on page 220). Therefore, Hrd4p/Npl4p was not required for the

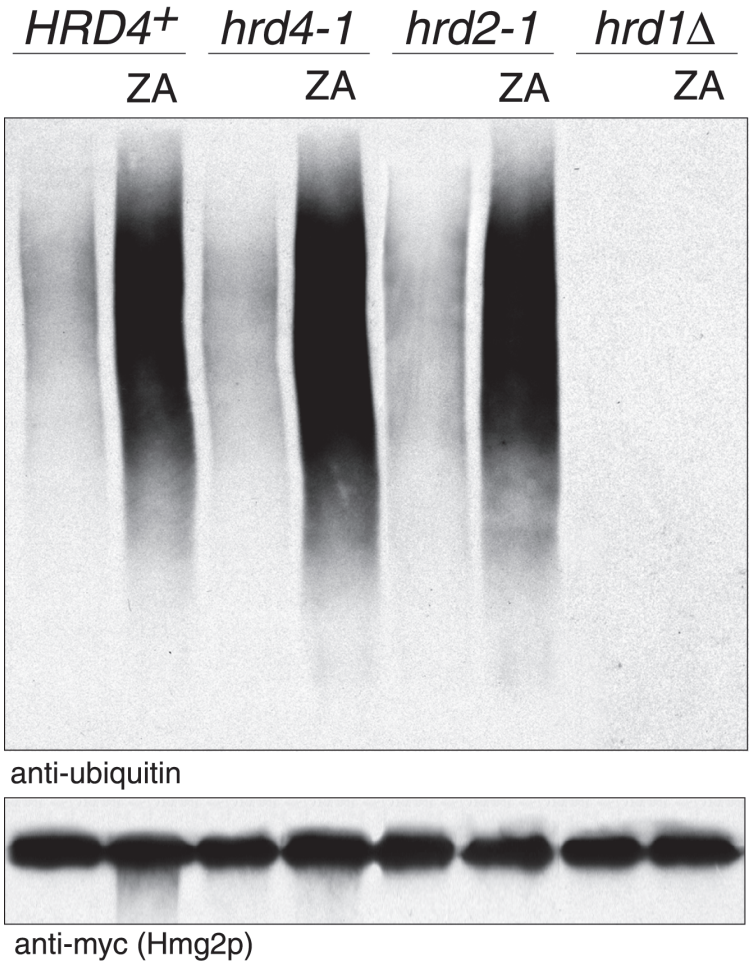


Figure 4-10

Hmg2p is fully ubiquitinated in a *hrd4-1* mutant strain

Ubiquitination of 1myc-Hmg2p was assayed by immunoprecipitation of 1myc-Hmg2p from lysates obtained from log-phase cells using anti-Hmg2p antisera. Immunoprecipitated material was separated by SDS-PAGE and immunoblotted for ubiquitin and the myc epitope tag as indicated.

actual ubiquitination of Hmg2p, but instead like Hrd2p/Rpn1p, Hrd4p/Npl4p was required for the degradation of fully-ubiquitinated Hmg2p.

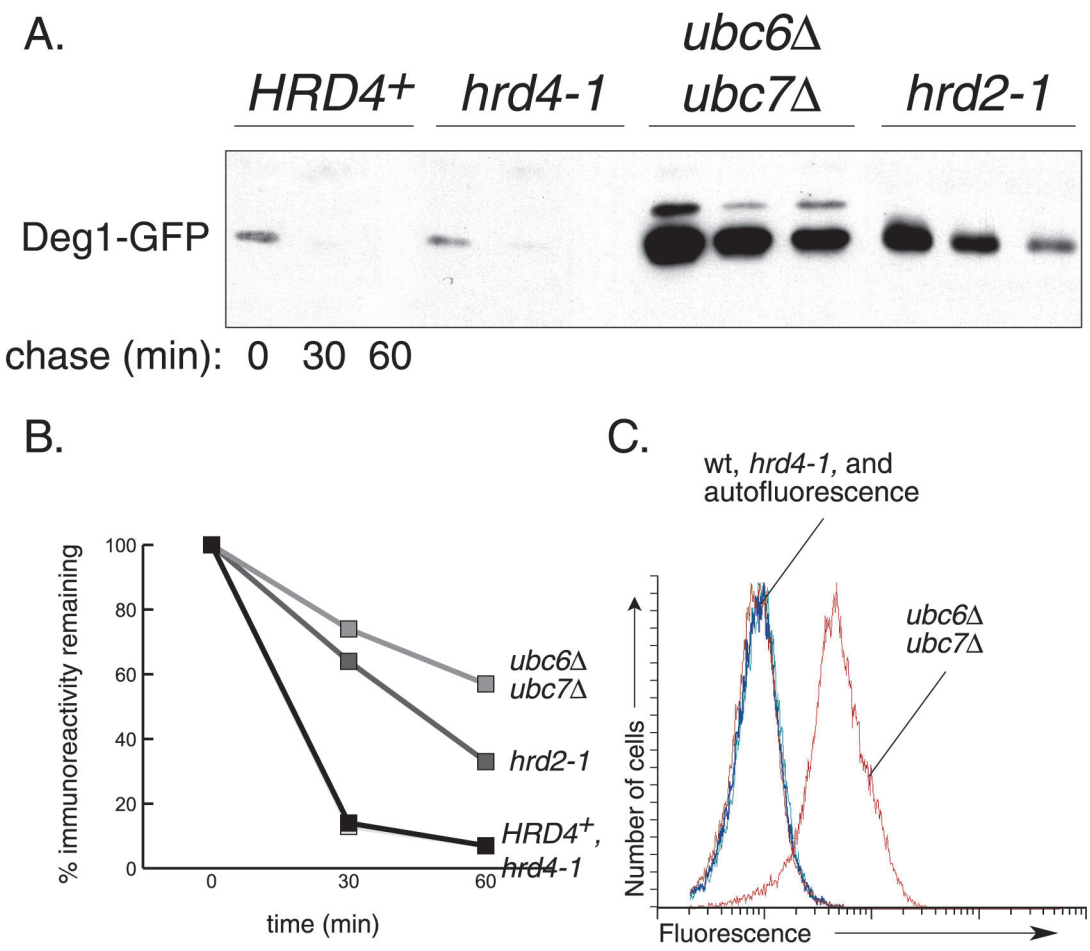


Figure 4-11

Degradation of Deg1-GFP is unaffected by the *hrd4-1* mutation

(A) Cycloheximide chase of Deg1-GFP was performed by the addition of cycloheximide to log-phase cells at the indicated times prior to collection. Equal numbers of cells were lysed and analyzed by SDS-PAGE followed by immunoblotting for GFP using anti-GFP antibody. (B) Loss of Deg1-GFP immunoreactivity during cycloheximide chase. Densitometric analysis of data obtained in (A) was used to determine loss of GFP immunoreactivity in each indicated strain at 0, 30, and 60 min. (C) Steady-state fluorescence of the Deg1-GFP protein was determined for the indicated strains by flow cytometry. Fluorescence was identical for the following strains: *HRD4*$^{+}$ *Deg1-GFP, hrd4-1 Deg1-GFP* and a *HRD4*$^{+}$ strain transformed with no GFP construct at all (cell autofluorescence). All experiments were performed at the permissive temperature of 30°C.

Since both *hrd4* and the proteasomal *hrd2/rpn1* mutants allowed ubiquitination but not degradation of Hmg2p, *hrd4/npl4* mutants were tested for their affect on general proteasomal degradation by measuring the degradation of several cytosolic proteins. The first protein, Deg1-GFP, is a variant of the green fluorescent protein (GFP) bearing the Deg1 sequence at its N terminus (Deg1-GFP). The Deg1 sequence originates from the rapidly degraded Matα2 protein and meets the classic definition of a "degron" in that it can be transferred to a number of different stable proteins to target them for degradation by the ubiquitin-proteasome pathway (Chen et al., 1993; Hochstrasser et al., 1991). Indeed, the addition of the Deg1 sequence to GFP caused the protein to be rapidly degraded (Figure 4-11 on page 221 and data not shown). In fact, this Deg1-GFP fusion was so rapidly degraded that its steady-state fluorescence in flow cytometry was barely detectable above normal cellular autofluorescence of wild-type cells (Figure 4-11 on page 221 and data not shown). Only when Deg1-GFP degradation was impaired by the *ubc6*Δ *ubc7*Δ double mutation were Deg1-GFP expressing cells bright (Figure 4-11 on page 221). No difference in fluorescence was seen between *HRD4*$^{+}$ and *hrd4-1* cells indicating that *HRD4* was not required for degradation of Deg1-GFP (Figure 4-11 on page 221). This was confirmed by examining Deg1-GFP stability with a cycloheximide chase followed by immunoblotting rather than flow cytometry. Deg1-GFP was indeed rapidly degraded (Figure 4-11 on page 221). This degradation was impaired in a *ubc6*Δ *ubc7*Δ strain (Figure 4-11 on page 221), an observation consistent with the requirement for these *UBC* genes in Deg1-mediated degradation (Chen et al., 1993). Similarly, loss

of function in the 26S proteasome gene *HRD2/RPN1* also impaired degradation of Deg1-GFP (Figure 4-11 on page 221), which is also consistent with the previously described requirement for the 26S proteasome in Deg1-mediated degradation (DeMarini et al., 1995). In striking contrast, loss of Hrd4p/Npl4p function had no detectable effect on the degradation rate of Deg1-GFP (Figure 4-11 on page 221). It could therefore be concluded that proteasome function was not impaired in *hrd4-1* strains because the degradation of the proteasome-dependent substrate Deg1-GFP was unaffected by the *hrd4-1* mutation. This observation was extended by testing the affect of *hrd4-1* on two other ubiquitin-mediated routes to proteasomal degradation: the N-end rule and UFD (ubiquitin-fusion degradation) pathways (Johnson et al., 1995; Varshavsky, 1996). To test effects on the N-end rule pathway, ubiquitin-R-GFP, ubiquitin-L-GFP, and ubiquitin-P-GFP fusions were expressed in *HRD*$^{+}$, *hrd4-1* and *hrd2-1* strains. As expected, ubiquitin-R-GFP was destabilized by the presence of the arginine amino-terminal residue resulting from *in vivo* cleavage of the ubiquitin moiety (Figure 4-12 on page 224 and data not shown). The degradation of ubiquitin-R-GFP was equally rapid in both *HRD*$^{+}$ and *hrd4-1* strains, but was severally impaired in a *hrd2-1* strain (Figure 4-12 on page 224 and data not shown). The same result was seen for the other N-end rule substrates, ubiquitin-L-GFP and ubiquitin-P-GFP (Figure 4-12 on page 224 and data not shown; note that ubiquitin-P-GFP is also subject to UFD degradation due to slow cleavage of ubiquitin moiety).

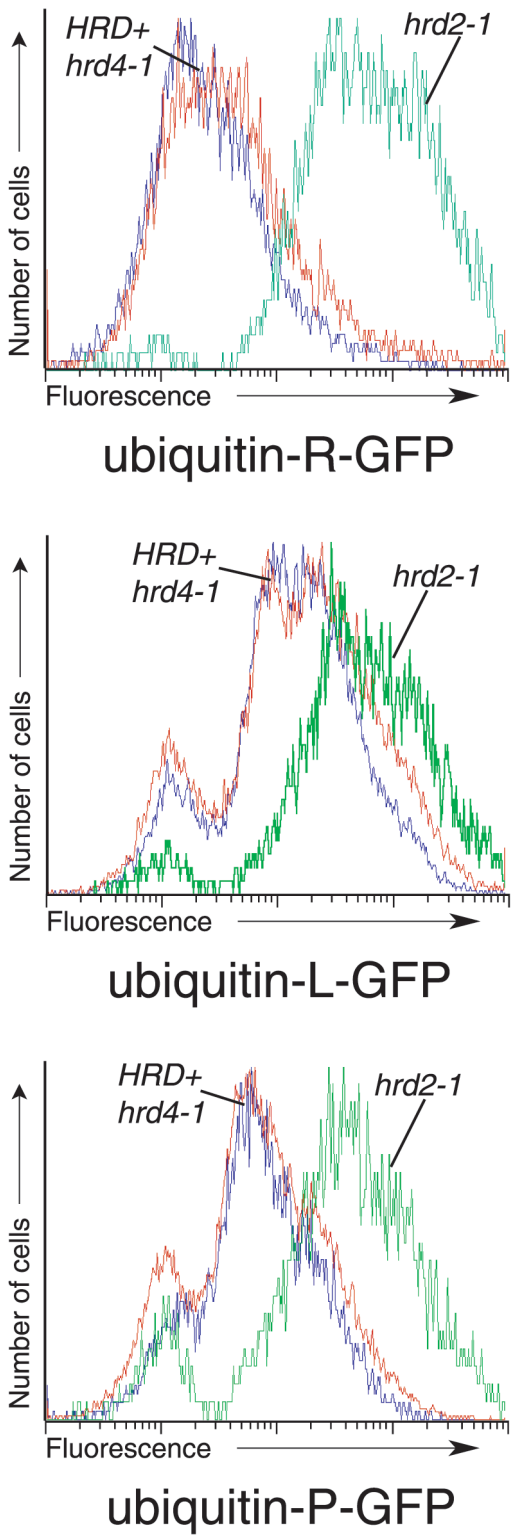


Figure 4-12

Degradation of several cytosolic proteins requires the 26S proteasome, but not *HRD4/NPL4*

The steady-state fluorescence of GFP fusions bearing different N-terminal amino acids was measured by flow cytometry in the indicated strains grown at the permissive temperature of 30°C. Each histogram represents 20,000 cells.

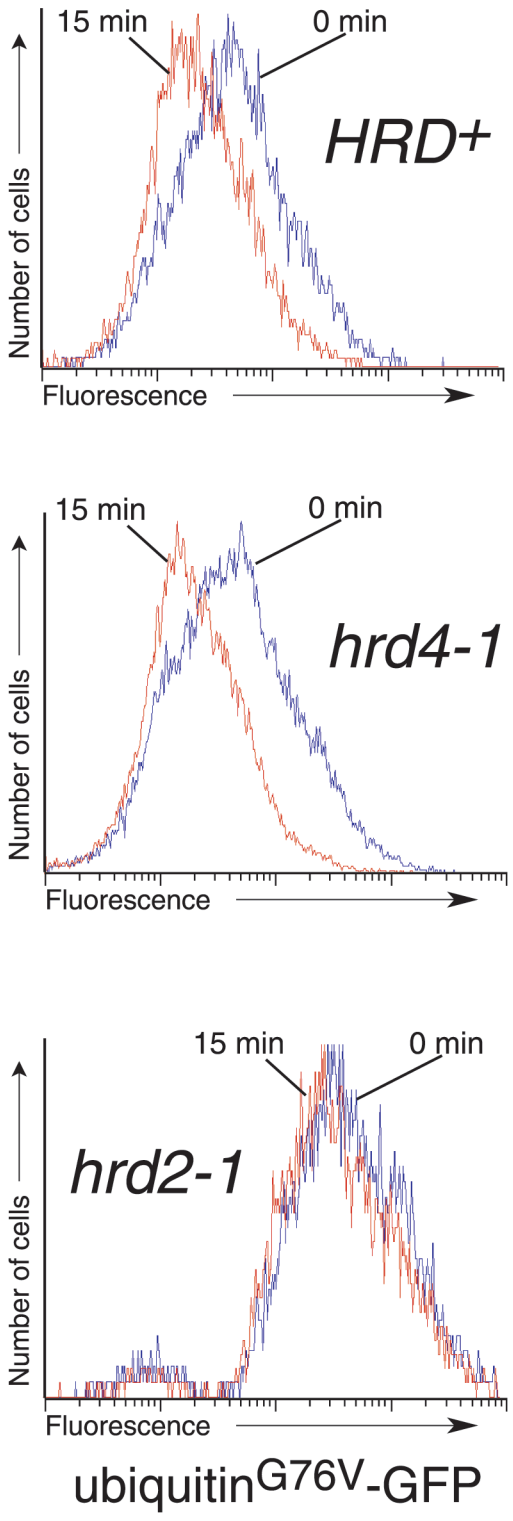


Figure 4-13

The rapid degradation of a UFD pathway substrate, ubiquitin^{G76V}-GFP, is unaffected by the *hrd4-1* mutation.

Degradation of the cytosolic UFD pathway substrate, ubiquitin^{G76V}-GFP, was examined by cycloheximide chase followed by flow cytometry. Identical loss of fluorescence was seen in both wild-type and *hrd4-1* cells during the 15 min chase period. Each histogram represents 20,000 cells. All strains were grown and assayed at the permissive temperature of 30°C.

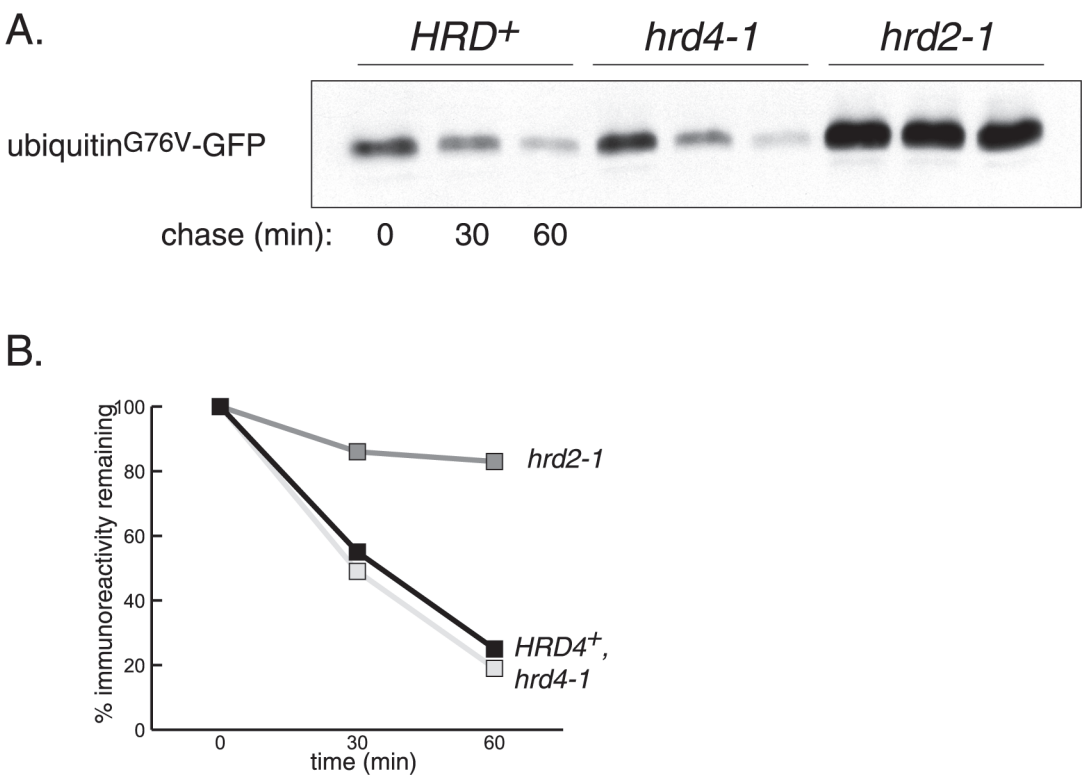


Figure 4-14

Biochemical analysis of ubiquitin^{G76V}-GFP degradation

(A) Cycloheximide chase of ubiquitin^{G76V}-GFP followed by immunoblotting using anti-GFP antibody. (B) Loss of ubiquitin^{G76V}-GFP immunoreactivity during cycloheximide chase was determined by densitometric analysis of data obtained in (A)

Along with ubiquitin-P-GFP, the degradation of another UFD substrate, ubiquitin^{G76V}-GFP, was examined. (The mutation of the carboxy-terminal glycine in ubiquitin blocks cleavage of the ubiquitin moiety and renders the entire fusion unstable.) Ubiquitin^{G76V}-GFP was rapidly degraded in both *HRD*$^{+}$ and *hrd4-1* strains (Figure 4-13 on page 225 and Figure 4-14 on page 226), but its degradation was dramatically

slowed by the *hrd2-1* mutation. Although ubiquitin^{G76V}-GFP degradation was quite sensitive to a defect in the 26S proteasome, its degradation was not affected by loss of Hrd4p/Npl4p function. Taken together, these studies on three distinct classes of soluble proteasomal substrates indicated that loss of Hrd4p/Npl4p does not affect general proteasome function. Furthermore, *hrd4-1* strains showed no altered sensitivity to the amino acid analog canavanine (data not shown), unlike most proteasome mutants previously tested (Fu et al., 1998; Heinemeyer et al., 1994; Hilt et al., 1993; Rubin et al., 1998).

CDC48, *UFD1*, and *HRD4/NPL4* in ERAD

Hrd4p/Npl4p is present in a complex with Ufd1p and Cdc48p in the cell (Meyer et al., 2000). Cdc48p is a AAA ATPase capable of interacting with an impressive array of proteins, including three proteins in the Ufd degradation pathway: Ufd1p, Ufd2p, and Ufd3p/Doa1p (Ghislain et al., 1996b; Koegl et al., 1999; Meyer et al., 2000). Cdc48p also physically interacts with the 26S proteasome in an ATP-dependent manner (Verma et al., 2000). *CDC48* was tested for a role in ERAD. *CDC48*$^{+}$ and *cdc48-2* mutant strains were transformed with Hmg2-GFP, and cycloheximide chase analysis of Hmg2-GFP degradation was performed. Although Hmg2-GFP was degraded in wild-type *CDC48*$^{+}$ cells, *cdc48-2* showed little degradation over the chase period Figure 4-15 on page 228, performed at the permissive temperature of 30°C). This result indicated that *CDC48* was indeed required for ERAD.

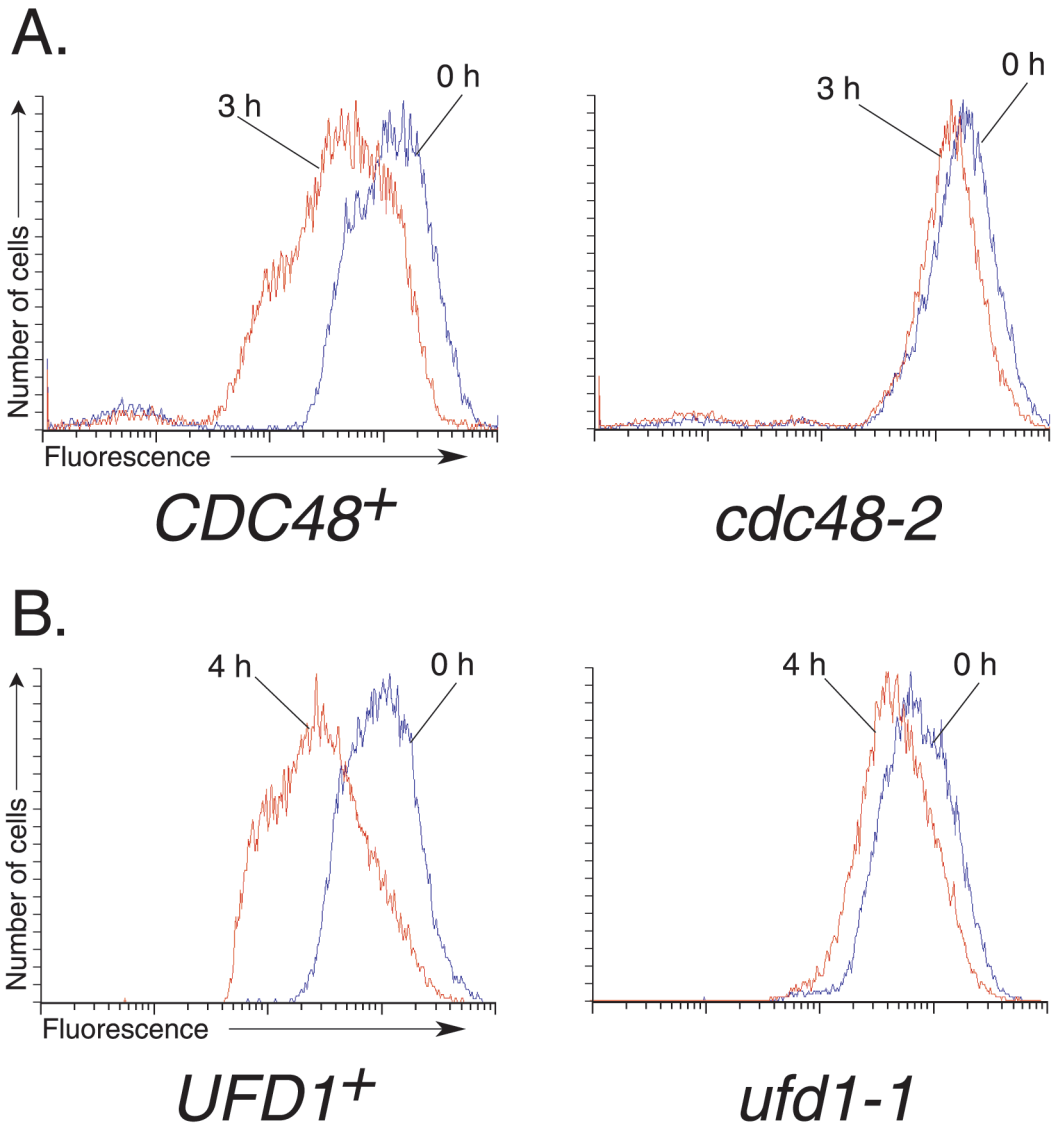


Figure 4-15

***CDC48* and *UFD1* are required for the degradation of Hmg2p**

Hmg2-GFP was transformed into strains with the indicated *CDC48* (A) and *UFD1* (B) alleles. Degradation of Hmg2-GFP was then measured by cycloheximide chase followed by flow cytometry. All experiments were performed at the permissive temperature of 30°C.

The requirement for the Cdc48p-interacting protein, Ufd1p, in ERAD was also tested. The Hmg2-GFP reporter protein was expressed in both *UFD1*$^{+}$ and *ufd1-1* strains. The loss of fluorescence during a cycloheximide chase of Hmg2-GFP protein was measured by flow cytometry. *ufd1-1* strains showed only a minor loss of fluorescence over the chase period while *UFD1*$^{+}$ strains displayed typical Hmg2-GFP degradation (Figure 4-15 on page 228). Therefore, *UFD1* was required for ERAD. Furthermore, each member of the Hrd4p/Npl4p-Ufd1p-Cdc48p complex was required for ERAD, but not for the degradation of UFD pathway substrates.

Discussion

Hrd4p/Npl4p and the Endoplasmic Reticulum

Hrd4p/Npl4p is required for ERAD of diverse substrates including 6myc-Hmg2p, native Hmg2p, CPY* and UP*. *HRD4/NPL4*-dependent substrates include at least one protein, UP*, whose degradation does not require the Hrd1p ubiquitin ligase (Wilhovsky et al., 2000). The processing of the ER-membrane-bound transcription factors Spt23p and Mga2p also requires *NPL4*: *hrd4/npl4* mutant strains fail to liberate the ER-membrane bound Spt23 or Mga2 proteins when cells are deprived of unsaturated fatty acids (Hitchcock et al., 2001). This processing of Spt23p and Mga2p is a ubiquitin- and proteasome-dependent process requiring the E3 Rsp5p (Hoppe et al., 2000). Thus, loss of Hrd4p/Npl4p function appears to create a broad lesion in the ER-localized process-

ing of ubiquitinated proteins as the defect in *hrd4/npl4* mutant cells crosses several E3 and E2 boundaries.

The accumulation of misfolded proteins at the ER leads to an elevated gene expression known as the Unfolded Protein Response (UPR). Genes elevated in the UPR include those coding for protein folding machinery like the chaperone Kar2p as well as ERAD machinery like the E3 Hrd1p (Travers et al., 2000). The UPR serves a sensitive and useful indicator of the protein folding state at the ER because the loss of genes required for protein folding and protein degradation at the ER leads to a constitutively elevated UPR (Friedlander et al., 2000; Travers et al., 2000). Using a P_{KAR2}-*GFP* reporter for the unfolded protein response, *hrd4⁻/npl4⁻* cells showed an elevated unfolded protein response, indicating that Hrd4p/Npl4p is centrally involved in ER protein maintenance.

These instances of Hrd4p/Npl4p function at the endoplasmic reticulum do not preclude the involvement of Hrd4p/Npl4p in cytoplasmic processes. It is possible that Hrd4p/Npl4p exerts some cytosolic effect as the majority of Npl4 protein is found in the cytosol (Hitchcock et al., 2001). Nevertheless, the "ER-centric" behavior of Hrd4p/Npl4p is striking and prompts further study into Hrd4p/Npl4p function at the endoplasmic reticulum. Given the striking simliarity of Hrd4p/Npl4p sequence among divergent species (Figure 4-16 on page 236), these studies will undoubtedly illuminate an essential process conserved among eukaryotes.

Hrd4p/Npl4p functions at a novel step in ERAD

Previous genetic studies in ERAD have identified two broad classes of mutants: mutants that block the actual ubiquitination of a target protein and mutants affecting proteasome function. For example, Hrd1p and Hrd3p are components of an ERAD-dedicated ubiquitin ligase, whereas Hrd2p/Rpn1p is a subunit of the 26S proteasome (Bays et al., 2001; Gardner et al., 2000; Hampton et al., 1996). *HRD4/NPL4* is not required for the ubiquitination of Hmg2p, consistent with the observation that *HRD4/NPL4* is required for the degradation of substrates with different E3 and E2 requirements. Because *npl4* mutants did not fall into the first class of mutants, *hrd4/npl4*mutants were tested for a defect in proteasome function. These experiments indicated that the proteasome could function normally in *hrd4/npl4* mutant cells, as the degradation of a variety of cytosolic, proteasome-dependent substrates was normal in *hrd4/npl4*mutant cells. *hrd4/npl4*mutants also exhibited no altered sensitivity to canavanine unlike other proteasome mutants (Fu et al., 1998; Heinemeyer et al., 1994; Hilt et al., 1993; Rubin et al., 1998). Furthermore, several ambitious and thorough efforts, biochemical and genetic, to identify all of the components of the 26S proteasome have never implicated Hrd4p/Npl4p as a proteasome subunit or even a proteasome-interacting protein (Heinemeyer et al., 1994; Glickman et al., 1998; Verma et al., 2000). It appears that neither ubiquitination or proteasomal function are deficient in *hrd4/npl4* mutant cells indicating that *hrd4/npl4* mutants are blocked in degradation at a step between ER ubiquitination of the target protein and its degradation by the 26S protea-

some. These results suggest that Hrd4p/Npl4p has an interesting and novel role in proteasomal processing of ubiquitinated proteins at the ER.

The results in this chapter argue for the placement of function Hrd4p/Npl4p function between ubiquitination and proteasomal degradation, but the possibility does exist that Hrd4p/Npl4p may act at the ubiquitination step in the construction and/or editing of the multiubiquitin chains added to target proteins. In this case, the loss of Hrd4p/Npl4p function would somehow causes the formation of multiubiquitin chains that are not competent for recognition by the 26S proteasome. The observation that the rate and pattern of Hmg2p ubiquitination is not noticeably affected in a *hrd4-1* mutant strain may somewhat argue against this model, but it cannot rule out the possibility that Hrd4p/Npl4p may somehow affect the structure of the multiubiquitin chain.

One continuing area of investigation in protein degradation concerns whether the proteasome alone can directly recognize ubiquitinated proteins. Although a fundamental question, a definitive answer has remained elusive. In *npl4*$^-$ cells, the 26S proteasome fails to recognize ubiquitinated ER proteins although the proteasome is fully capable of degrading a model cytosolic substrates. This suggests, at least for ERAD substrates, that the proteasome *alone* is not capable of recognizing ubiquitinated proteins and requires some function that is lost in *hrd4/npl4* mutant cells. Whether Hrd4p/Npl4p directly presents ubiquitinated ER proteins to the proteasome is not yet clear, but further study of the function lost in *hrd4*$^-$*/npl4*$^-$ cells will likely provide much needed insight into how ubiquitinated proteins are recognized by the proteasome.

hrd4⁻/npl4⁻ phenotypes result from a defect in protein degradation

Since Hrd4p/Npl4p is required for protein degradation, nuclear transport, and unsaturated fatty acid biosynthesis, I was compelled to ask which primary function was actually being lost in an *hrd4/npl4* mutant and whether loss of that one function could explain the other observed *hrd4/npl4* phenotypes. Nuclear transport was first considered as the primary function lost in *hrd4/npl4* mutants, but this model is fairly unconvincing as *hrd4/npl4* mutants show a profound defect in protein degradation at the *permissive* temperature where nuclear transport functioned normally. Furthermore, an equally severe block in nucleocytoplasmic traffic caused by the *nup116*Δ mutant had no effect on ERAD. Thus, loss of nuclear transport did not cause general ERAD defects. Conversely, several reports have indicated a requirement for protein degradation in nuclear transport. For example, a mutation in the gene coding for the proteasome subunit Rpn2p causes a profound block in nuclear transport, as do mutations in the ubiquitin-protein ligase Tom1p (Utsugi et al., 1999; Yokota et al., 1996). Taken together, the reasonable model is that the original nuclear defects of *npl4* mutants are the result of the degradation defect in these cells.

Recent studies of Hrd4p/Npl4p involvement in unsaturated fatty acid (UFA) biosynthesis prompted another investigation into the cause of *hrd4/npl4* phenotypes. Hitchcock *et al.* (2001) show that *npl4* mutant strains are deficient in the UFA-regulated processing of two transcription factors (Spt23p and Mga2p) required for the production of Ole1p, a Δ9-fatty acid desaturase (Zhang et al., 1999). Since unsaturated fatty acid

levels can have profound effects on functions throughout the cell and especially at cell membranes like the ER membrane, (Stewart and Yaffe, 1991; Stukey et al., 1990; Zhang et al., 1999) the affect of unsaturated fatty acids on *hrd4-1* strains was tested, but only a slight suppression of the Ts^- phenotype by UFA supplementation was found. More importantly, there was no suppression of the *hrd4-1* lovastatin-resistance phenotype by unsaturated fatty acids. Although Hrd4p/Npl4p clearly plays an important role in the processing of the transcription factors Spt23p and Mga2p, processing of Spt23p and/or Mga2p cannot be the sole function of Hrd4p/Npl4p because even *mga2*Δ *spt23*^{ts} mutants are completely suppressed by overexpression of *OLE1* at their restrictive temperature. *hrd4/npl4* mutants are not completely suppressed by *OLE1,* and thus are not phenocopies of cells that simply lack functional Mga2p and/or Spt23p. Furthermore, no role for unsaturated fatty acids or *OLE1* in the ERAD defect of *hrd4/npl4* mutants could be found.

A defect in proteasomal processing of ubiquitinated proteins at the ER best explains the reported *hrd4/npl4* phenotypes: *hrd4/npl4* mutants are deficient in the proteasome-dependent processing of ER-bound transcription factors required for unsaturated fatty acid synthesis. This reduction in cell unsaturated fatty acid levels leads to some growth defect and appears to be at least partially responsible for the nuclear transport defect in *hrd4/npl4* mutants (Hitchcock et al., 2001). The *npl4/hrd4* defect in proteasomal processing also affects the degradation of ER proteins because ERAD proceeds by action of the ubiquitin-proteasome pathway. Therefore, this work offers that protein

degradation is the primary function lost in *hrd4/npl4* mutants and that the reported *hrd4/npl4* phentoypes are best explained by this loss of ubiquitin-mediated degradation.

The mechanism of Hrd4p/Npl4p function

In *hrd4/npl4* mutants, ubiquitinated ER proteins fail to be processed by a functional 26S proteasome. This phenotype suggests several models of Hrd4p/Npl4p function. In one model, Hrd4p/Npl4p may physically mediate interaction between the 26S proteasome and ERAD substrates. This mediation may well also require Cdc48p, which has been shown to physically associate with Npl4p via Ufd1p (Meyer et al., 2000). Cdc48p has also been shown to associate with the proteasome in an ATP-dependent manner (Verma et al., 2000). It is intriguing to speculate that the Cdc48p-Ufd1p-Hrd4p complex actually anchors or recruits the 26S proteasome to the ER. Because a specific population of ER-bound proteasomes exists in the cell (Hori et al., 1999), it will be enlightening to determine whether *cdc48* and/or *npl4* mutants have any effect on the abundance or distribution of these ER-bound proteasomes. As Cdc48p is required for protein degradation at the both the cytosol (Ghislain et al., 1996a) and the endoplasmic reticulum (this report), Cdc48p may mediate the physical association of ubiquitinated proteins and the proteasome at both locations with Hrd4p/Npl4p acting as the "ER-specific adapter." This model of Cdc48p function is consistent with recent data suggesting that Cdc48p acts in the recognition of multiubiquitin chains (Dai and Li, 2001). Determining the effect of Hrd4p/Npl4p on the recognition of multiubiquitinated pro-

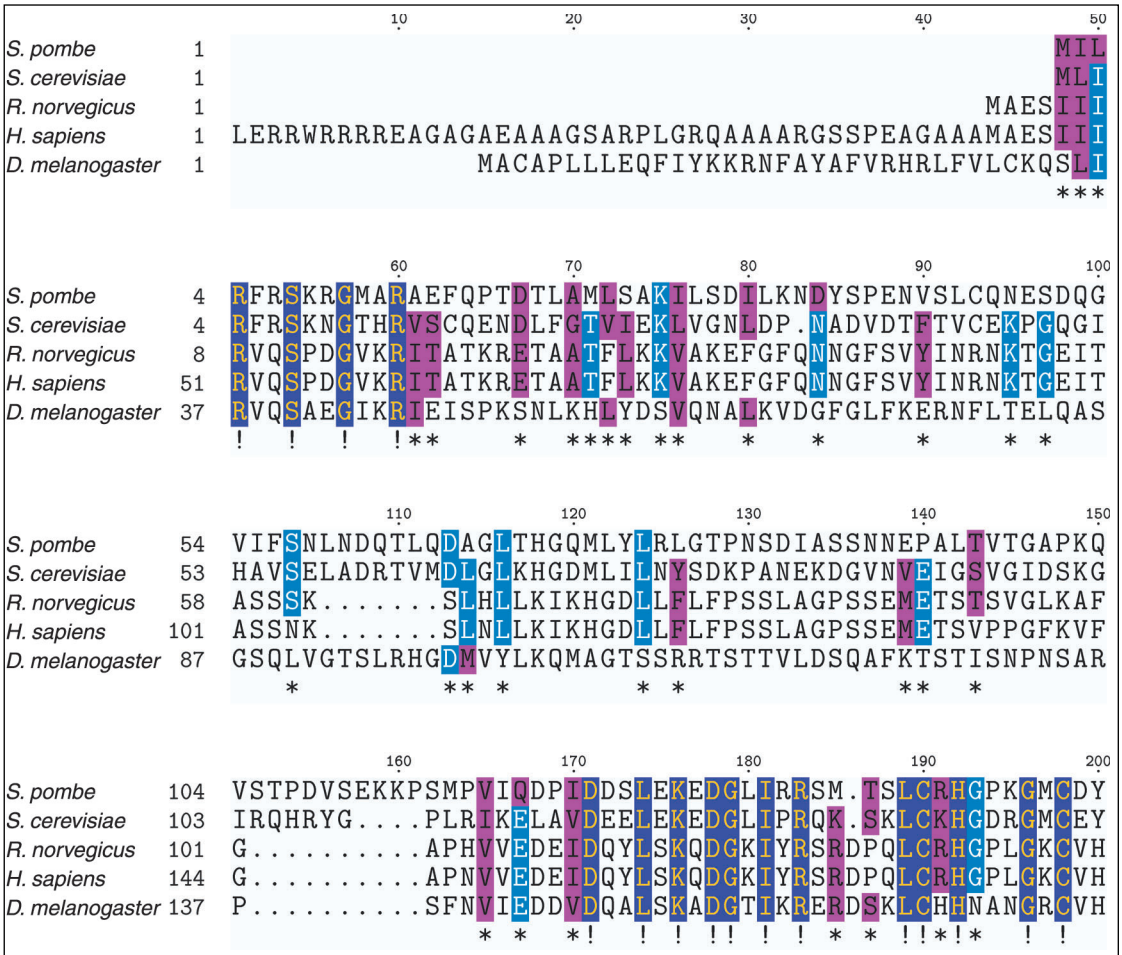


Figure 4-16

Hrd4p/Npl4p is highly conserved throughout eukaryotes

Sequence alignment of Hrd4/Npl4 proteins from the indicated species was performed using the CLUSTALW algorithm (Thompson et al., 1994).

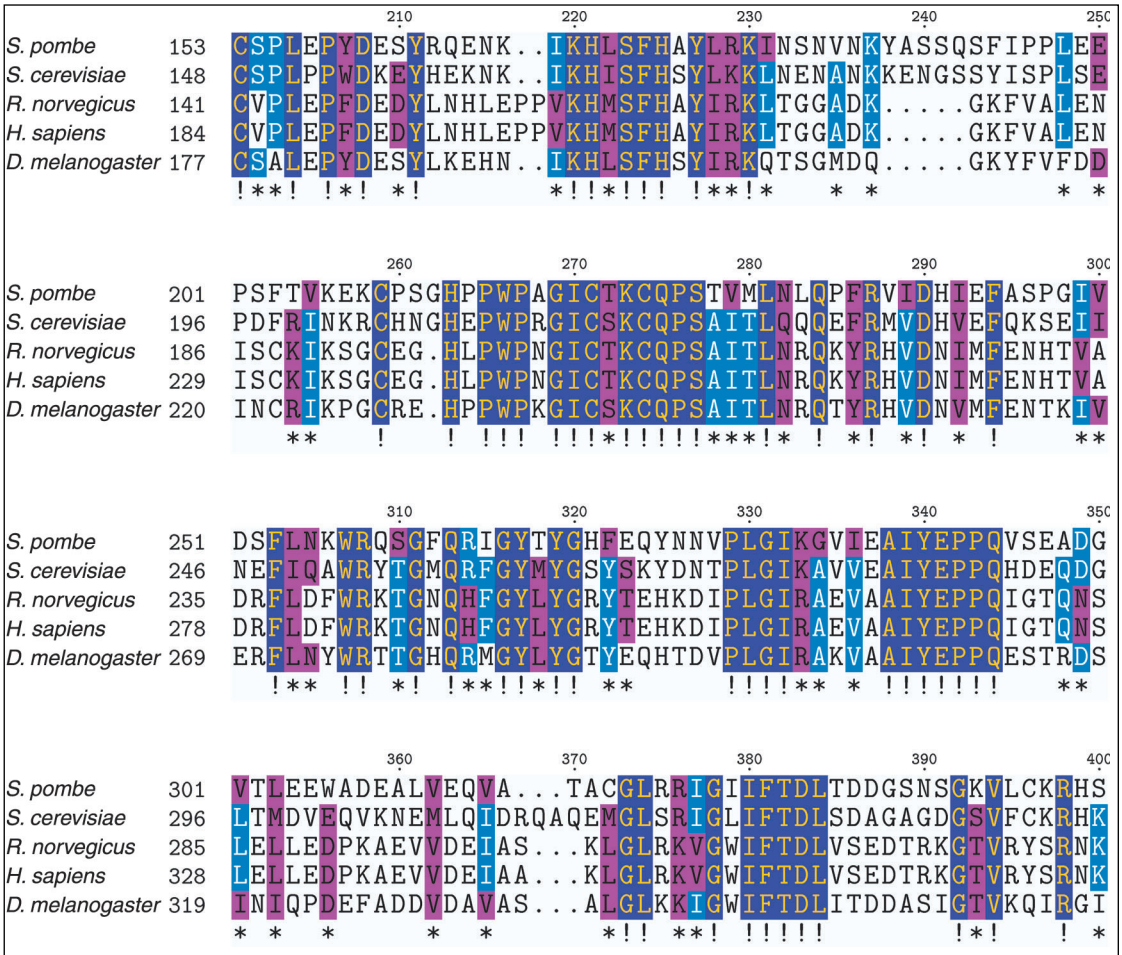


Figure 4-16 (continued)

Hrd4p/Npl4p is highly conserved throughout eukaryotes

Sequence alignment of Hrd4/Npl4 proteins from the indicated species was performed using the CLUSTALW algorithm (Thompson et al., 1994).

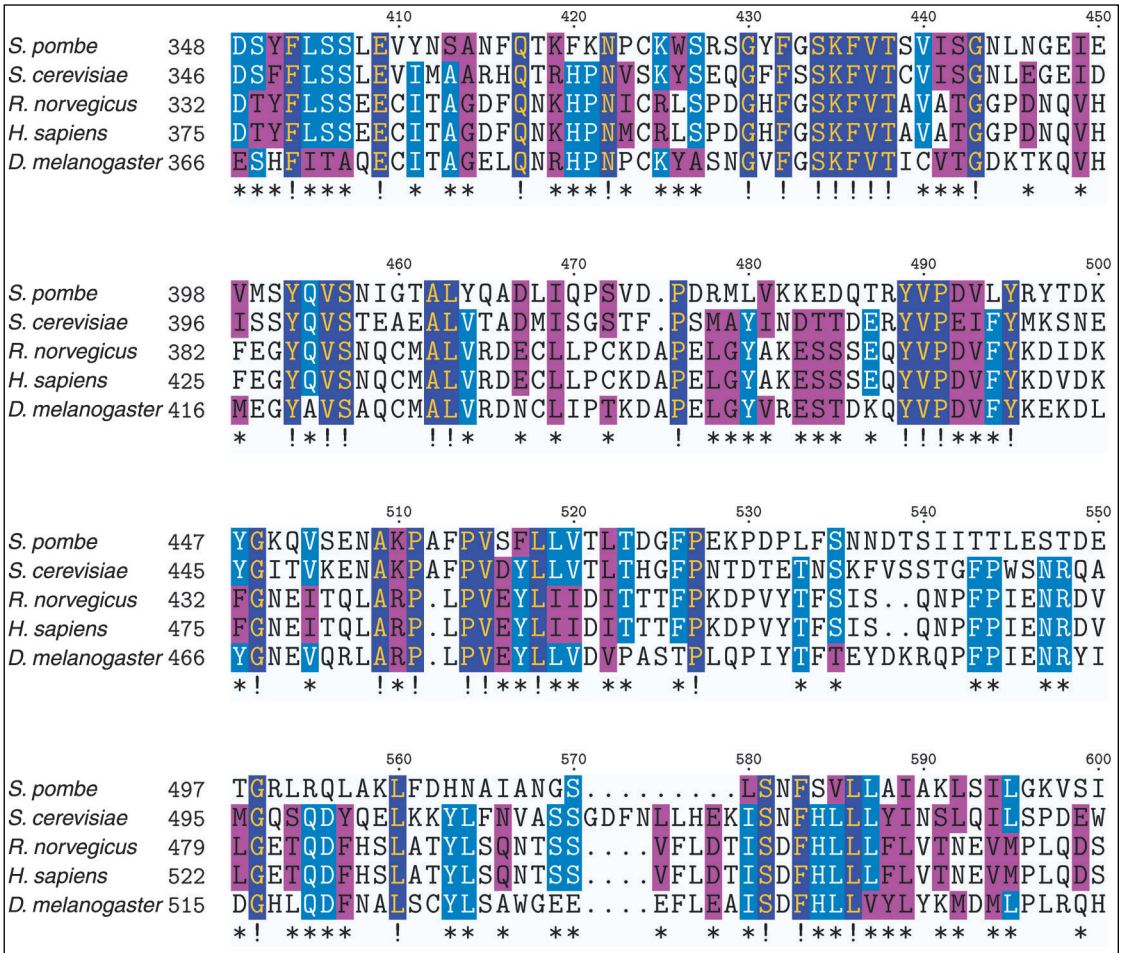


Figure 4-16 (continued)

Hrd4p/Npl4p is highly conserved throughout eukaryotes

Sequence alignment of Hrd4/Npl4 proteins from the indicated species was performed using the CLUSTALW algorithm (Thompson et al., 1994).

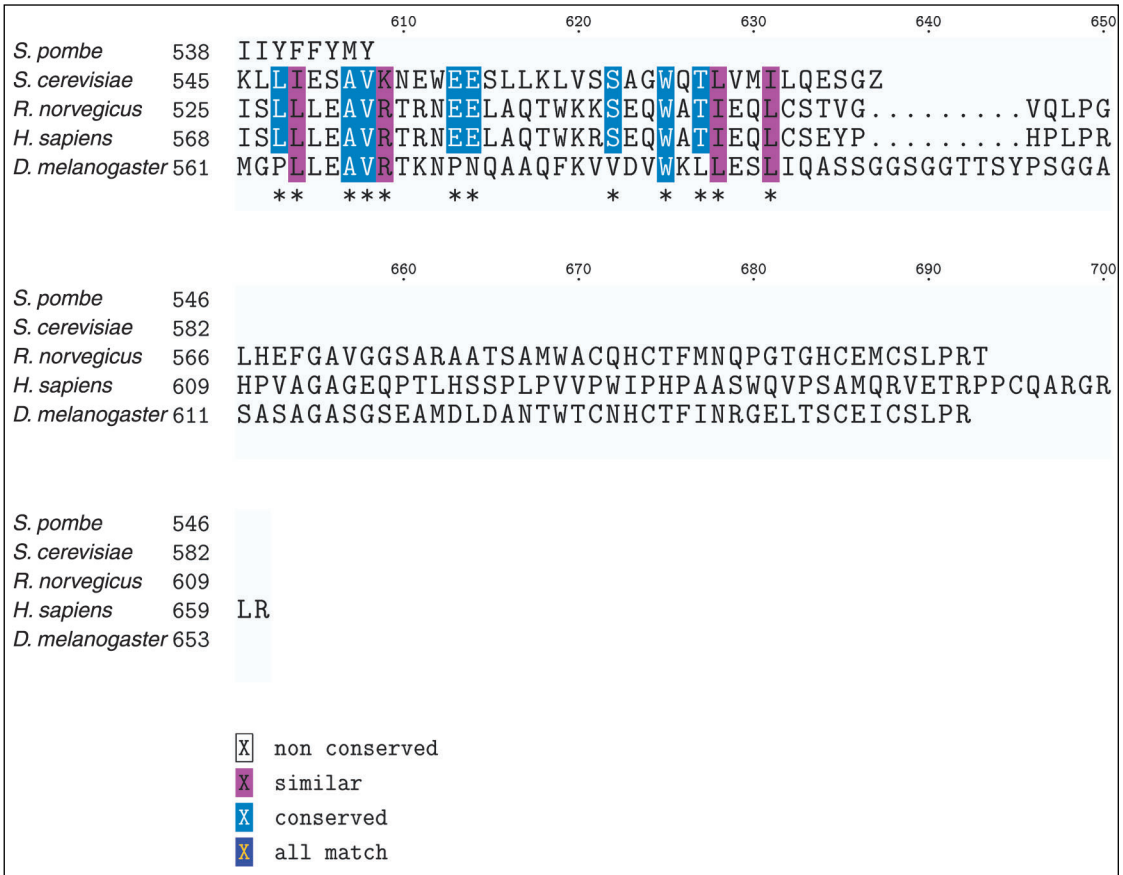


Figure 4-16 (continued)

Hrd4p/Npl4p is highly conserved throughout eukaryotes

Sequence alignment of Hrd4/Npl4 proteins from the indicated species was performed using the CLUSTALW algorithm (Thompson et al., 1994).

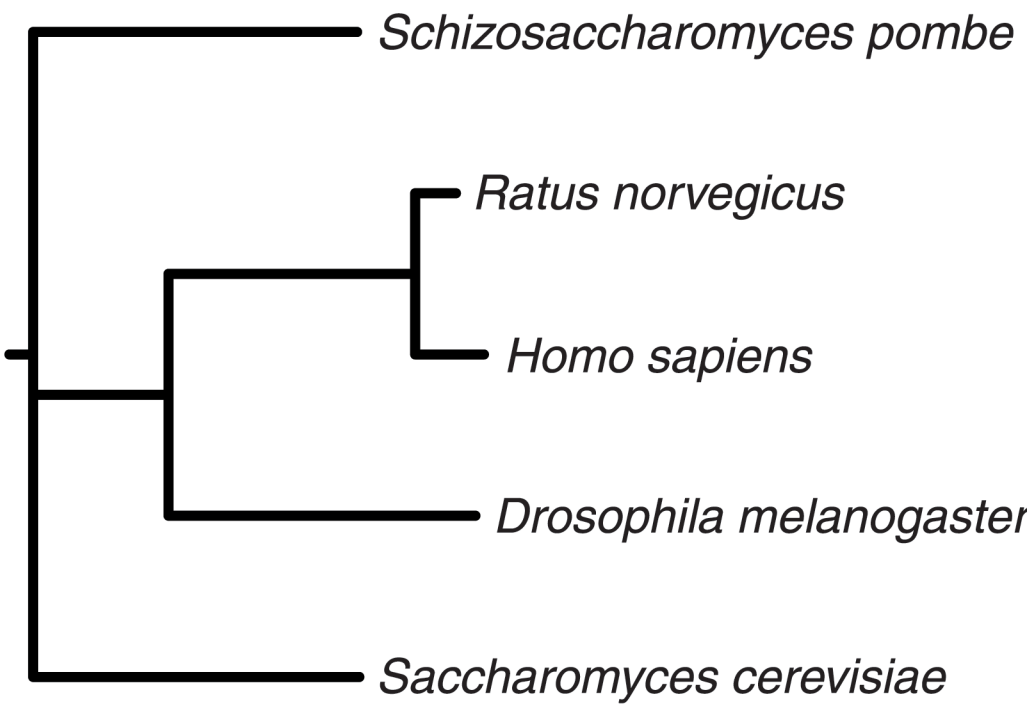


Figure 4-17

Phylogenetic tree depicting Hrd4 protein sequence divergence

The indicated proteins were aligned using the CLUSTALW algorithm (Thompson et al., 1994) and phylogentic tree based on the Hrd4 alignment was constructed using the PHYLIP package (Phylogeny Inference Package, J. Felsenstein, University of Washington, Seattle).

teins may well lend important insight into Hrd4p/Npl4p function as well as the "ER-centric" behavior of Hrd4p/Npl4p.

Materials and Methods

Identification of *HRD4* as *NPL4*

The *hrd4-1* mutant allele was recovered from a lovastatin-resistant colony isolated in the *hrd* selection described previously (Hampton et al., 1996). The *hrd4-1* allele yielded a yeast strain that grew well at 30° but was inviable at 35°C – failing to complete any further cell division after the temperature shift. This strain bred true for lovastatin resistance (Lov^r) and so was then crossed to the wild-type parent strain. A wild-type diploid resulted from the cross, indicating recessive allelle(s) were conferring lovastatin-resistance. This diploid was then sporulated. Tetrad analysis revealed that the Lov^r and Ts^- phenotypes were the result of a single recessive mutant allele, *hrd4-1*. The *hrd4-1* allele defined a new *HRD* complementation group as crosses to other haploid *hrd* mutant strains always resulted in a Hrd^+ Ts^+ diploid. When sporulated and dissected, these diploids yielded progeny with Hrd and Ts phenotypes in the expected ratios for two unlinked *hrd* alleles.

The wild-type allele for *HRD4* was cloned using a yeast genomic library bearing the *URA3* prototrophy marker (Rose et al., 1987). A Ura^- Ts^- Lov^r *hrd4-1* strain was transformed with library DNA, and Ura^+ Ts^+ colonies were then selected from the transformants by incubation at 35°C. Ts^+ colonies were analyzed for lovastatin resistance.

Those colonies that showed both Ts^+ and Lov^s phenotypes were subjected to *URA3* counterselection using 5-fluoroorotic acid (Guthrie and Fink, 1991). This selection for loss of the library plasmid resulted in $Ura^- Ts^- Lov^r$ colonies displaying the original *hrd4-1* phenotypes. Plasmid DNA was extracted from the original $Ura^+ Ts^+ Lov^s$ transformants, bacterially amplified, and re-transformed into a *hrd4-1* mutant strain. Plasmids capable of reversing the *hrd4-1* $Ts^- Lov^r$ phenotypes were sequenced. Two different plasmids containing a common region of yeast chromosome II were isolated. Further subcloning of the library plasmids revealed that only one orf (open reading frame) was required for complementation. That orf corresponded to the previously identified gene *NPL4*. To test whether *HRD4* was indeed *NPL4*, the *NPL4* gene was cloned into a yeast integrating plasmid bearing the *URA3* gene. This *URA3*-marked *NPL4* was integrated at the *NPL4* locus in both *HRD4*$^+$ and *hrd4-1* Ura^- strains. After transformation, both strains were $Ura^+ Ts^+ Lov^s$ (Figure 4-1 on page 203). These strains were then crossed to a Ura^- *hrd4-1* strain. The resulting diploid was sporulated. Every tetrad showed 2:2 segregation of the Ura^+/Ura^-, Ts^+/Ts^-, and Lov^r/Lov^s phenotypes and no Ura^+ segregant was ever Ts^- or Lov^r. These results indicated that *hrd4-1* was indeed a mutant allele of *NPL4*.

Yeast Strains

Genotypes of yeast strains used in this study are listed in table 1. The following alleles were described previously: *hrd1-1, hrd2-1, hrd1Δ::TRP1, ubc6Δ::KanMX,*

*ubc7*Δ*::HIS3*. (Hampton and Bhakta, 1997; Hampton et al., 1996; Wilhovsky et al., 2000) The *nup116*Δ allele was introduced into RHY400 by transformation with a *nup116*Δ deletion construct described previously to create RHY877 (Wente and Blobel, 1993). Deletion of *NUP116* was verified by immunoblotting with anti-Nup116p antibody. *OLE1*$^{+}$ and *ole1*Δ strains were described previously as were the *NPL4*$^{+}$*/npl4-1/npl4-2* , *CDC48*$^{+}$*/cdc48-2,* and *UFD1/ufd1-1* strains (DeHoratius and Silver, 1996; Johnson et al., 1995; Latterich et al., 1995; Stukey et al., 1990). All strains were constructed according to standard techniques (Guthrie and Fink, 1991). Yeast strains were grown in yeast minimal media as described previously (Hampton and Rine, 1994) except plates in Figures 7-9 which used synthetic complete media (-uracil in Figure 9) prepared as decscribed (Guthrie and Fink, 1991). Oleic and palmitoleic acids were added to media where indicated from 10% stocks in absolute ethanol to a final concentration of 0.5 mM each UFA. 1% Tergitol NP-40 was added to plates containing UFA for solubilization. (Tergitol NP-40 is not the same as Nonidet NP-40 which is somewhat toxic to yeast [data not shown and personal communication, Charles Martin])

Plasmids

The yeast centromeric plasmid pRH590 (YCp*NPL4*) was constructed by PCR amplification of the *NPL4* locus from the genomic library plasmid pRH562 using Vent™ DNA polymerase and the following primers oRH571:5'-AAGCTTATGTGATTTTTGGTAAGGGGACG-3' and oRH572:5'-GGTACCGGCAAACT-

CAAGTAGTTGTGCGTAC-3'. The product was then digested with HindIII and KpnI and ligated into pRS416 digested with the same enzymes. The integrating form of p*NPL4* (pRH617) was created by ligating the KpnI-SacI fragment of pRH590 containing *NPL4* into pRS406. The P_{TDH3}-*Deg1-GFP* fusion was created by PCR amplification of the gene coding for GFP^{S65T} (contained in pRH465) with the following primers: oRH1219 5'-CGCGGGGATCCAAATGGGTAAAGGAGAAGAACTT-3' and oRH1220 5'-CAAATGTGGTATGGCTGAT-3'. The product was digested with BamHI and SalI and ligated into pRH421 (containing the *Deg1* sequence) cut with BglII and SalI.

N-end rule and UFD pathway substrates were constructed as follows. Ubiquitin-X-GFP fusions (where X indicates amino acid residue following Glycine 76 of ubiquitin) were previously constructed for expression in mammalian cells (Dantuma et al., 2000). The pEGFP-N1-based (commercial vector: Clontech) ubiquitin-M-GFP construct was digested with NheI and BsaHI. The 2.4 kb fragment containing the ubiquitin-M-GFP orf was then ligated into SpeI-ClaI digested pRH1556, a previously described ARS/CEN plasmid driving expression of cloned genes from the *TDH3* promoter (Mumberg et al., 1995). The resulting plasmid, pRH1561, served as the recipient vector for the ubiquitin-R-GFP, ubiquitin-L-GFP, ubiquitin-P-GFP, and $ubiquitin^{G76V}$-GFP orfs previously described (Dantuma et al., 2000). Each orf was extracted from the mammalian expression vector by digestion with EcoRI and NotI. The 1000 bp fragment was then ligated into the 7.3 kbp backbone of pRH1561 to form pRH1562 (ubiquitin-

R-GFP), pRH1563 (ubiquitin-L-GFP), pRH1564 (ubiquitin-P-GFP), and pRH1565 (ubiquitin^{G76V}-GFP). The following plasmids were described previously: CPY*-HA, P_{KAR2}-*GFP*, *nup116*Δ*::URA3,* and 2μ *OLE1* (Ng et al., 2000; Pollard et al., 1998; Wente and Blobel, 1993; Zhang et al., 1999).

Assays for protein degradation

Stationary chase and cycloheximide chases were performed as previously described (Cronin and Hampton, 1999; Hampton and Rine, 1994) and methods are summarized in figure legends. Flow cytometry was performed using a BectonDickinson FACScalibur™ instrument. Statistical analysis of flow cytometry data was performed as described (Young, 1977). The graph for figures 11B and 14B was created to quantify loss of immunoreactivity in the experiments shown in Figure 11A and 14A, respectively. Different exposures of x-ray film were obtained after immunoblotting and scanned at 600 dpi resolution using NIH Image 1.61 software for MacOS, a UMAX PowerLook 1100 scanner, and a Power Macintosh G4 computer. These scanned images were then subjected to densitometric analysis using the program NIH Image 1.61 for MacOS according to the supplied instructions. This analysis determines the shade of gray (degree of x-ray film exposure) for each pixel and determines the total degree of exposure for a single band. A "threshold" test was employed for each band to ensure that no pixel was saturated and therefore that a valid linear comparison could be made between timepoints.

These data were then expressed as loss of immunoreactivity over time with the exposure at time 0 set as 100% immunoreactivity.

Antibodies, Immunoprecipitation and Immunoblotting

Monoclonal anti-myc antibodies were produced as cell-culture supernatant from 9e10 hybridomas obtained from ATCC. Monoclonal anti-HA antibodies (clone 12CA5) were obtained from Babco as purified antibody derived from mouse ascites fluid. Anti-GFP antisera was a gift from Charles Zuker (UCSD). Anti-Nup116p antisera (to verify deletion of *NUP116*) was a gift from Susan Wente (Washington University, St. Louis). Anti-Hmg2p antisera was described previously (Hampton and Rine, 1994). Anti-ubiquitin antibodies were purchased from Zymed (So. San Francisco, CA). SDS-PAGE was performed using 8% Tris-glycine gels except the experiment in Figure 10 which used 3-8% Tris-acetate gels (Invitrogen: Carlsbad, CA). Immunoprecipitation was performed as described in Hampton and Rine, 1994 but with additional protease inhibitors (*n*-ethylmaleimide, AEBSF, E-64, benzamidine and ε-amino-*n*-caproic acid). Immunoblotting was also performed as described in Hampton and Rine, 1994 except that tris-buffered saline contained 0.45% Tween 20, and 20% heat-inactivated bovine calf serum was used as the blocking agent. Anti-ubiquitin blots were also processed as previously described (Swerdlow et al., 1986).

Strain	Genotype
RHY244	*MATa ade2-101 lys2-801 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 met2 ura3-52::P_{TDH3}-6MYC-HMG2::URA3*
RHY400	*MATa ade2-101 lys2-801 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 met2 ura3-52::P_{TDH3}-6MYC-HMG2*
RHY554	*MATa ade2-101 lys2-801 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 met2 ura3-52::P_{TDH3}-6MYC-HMG2 hrd4-1*
RHY587	*MATa ade2-101 lys2-801 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 met2 ura3-52::P_{TDH3}-6MYC-HMG2 hrd1Δ::URA3*
RHY877	*MATa ade2-101 lys2-801 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 met2 ura3-52::P_{TDH3}-6MYC-HMG2 nup116Δ::URA3*
RHY1172	*MATalpha ade2-101 lys2-801 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 met2 ura3-52::P_{TDH3}-1MYC-HMG*
RHY1216	*MATa ade2-101 lys2-801 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 met2 ura3-52::P_{TDH3}-6MYC-HMG2* pRH696[a] *[fur4-430::URA3]*
RHY1220	*MATa ade2-101 lys2-801 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 met2 ura3-52::P_{TDH3}-6MYC-HMG2 hrd4-1* pRH696[a] *[fur4-430::URA3]*
RHY1221	*MATalpha ade2-101 lys2-801 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 met2 ura3-52::P_{TDH3}-6MYC-HMG2 ubc7Δ::HIS3* pRH696[a] *[fur4-430::URA3]*
RHY1234	*MATalpha ade2-101 lys2-801 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 met2 trp1::hisG ura3-52::P_{TDH3}-1MYC-HMG2 hrd1Δ::TRP1*
RHY1246	*MATa ade2-101 lys2-801 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 met2 ura3-52::P_{TDH3}-1MYC-HMG2 hrd4-1*

Table 4-1

Yeast Strains

RHY1252	*MATa ade2-101 lys2-801 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 met2 ura3-52::P_{TDH3}-6MYC-HMG2::URA3 hrd4-1*
RHY1389	*MATa ade2-101 lys2-801 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 met2 ura3-52::P_{TDH3}-1MYC-HMG2 hrd2-1*
RHY1628	*MATa ade2-101 lys2-801 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 met2 ura3-52::P_{TDH3}-1MYC-HMG2::URA3 hrd2-1*
RHY2458	*MATalpha leu2-3,112 ura3-52::hmg2-GFP::URA3*
RHY2461	*MATalpha ura3-52::hmg2-GFP::URA3 cdc48-2*
RHY2472	*MATa ade2-101 lys2-801 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 met2 ura3-52::P_{TDH3}-1MYC-HMG2::URA3::P_{KAR2}-GFP*
RHY2473	*MATa ade2-101 lys2-801 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 met2 trp1::hisG ura3-52::P_{TDH3}-1MYC-HMG2::URA3::P_{KAR2}-GFP hrd1Δ::TRP1*
RHY2474	*MATa ade2-101 lys2-801 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 met2 ura3-52::P_{TDH3}-1MYC-HMG2::URA3::P_{KAR2}-GFP hrd4-1*
RHY2476	*MATa ade2-101 lys2-801 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 leu2Δ met2 trp1::hisG ura3-52::P_{TDH3}-1MYC-HMG2::URA3::P_{TDH3}-Deg1-GFP*
RHY2477	*MATa ade2-101 lys2-801 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 leu2Δ met2 trp1::hisG ura3-52::P_{TDH3}-1MYC-HMG2::URA3::P_{TDH3}-Deg1-GFP hrd4-1*
RHY2478	*MATa ade2-101 lys2-801 his3Δ200 hmg1Δ::LYS2 hmg2Δ-4 leu2Δ met2 trp1::hisG ura3-52::P_{TDH3}-1MYC-HMG2::URA3::P_{TDH3}-Deg1-GFP ubc6Δ::KanMX ubc7Δ::HIS3*
RHY2479	*MATa ade2-101 lys2-801 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 met2 ura3-52::P_{TDH3}-1MYC-HMG2::URA3::P_{TDH3}-Deg1-GFP hrd2-1*
RHY2485	*MATa ade2-101 lys2-801 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 leu2Δ met2 trp1::hisG ura3-52::P_{TDH3}-1MYC-HMG2* pRH1377[a] *[HA-prc1-1::URA3]*

Table 4-1 (continued)

Yeast Strains

RHY2486	*MATa ade2-101 lys2-801 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 leu2Δ met2 trp1::hisG ura3-52::P_{TDH3}-1MYC-HMG2* pRH1377[a] *[HA-prc1-1::URA3] hrd4-1*
RHY2488	*MATa ade2-101 lys2-801 his3Δ200 hmg1Δ::LYS2 hmg2Δ-4 leu2Δ met2 trp1::hisG ura3-52::P_{TDH3}-1MYC-HMG2* pRH1377[a] *[HA-prc1-1::URA3] ubc6Δ::KanMX ubc7Δ::HIS3*
RHY2494	*MATa ade2-101 lys2-801 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 met2 ura3-52::P_{TDH3}-6MYC-HMG2::URA3*
RHY2495	*MATa ade2-101 lys2-801 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 met2 ura3-52::P_{TDH3}-6MYC-HMG2::URA3 hrd4-1*
RHY2496	*MATa ade2-101 lys2-801 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 met2 ura3-52::P_{TDH3}-6MYC-HMG2::URA3::NPL4 hrd4-1*
RHY2497	*MATa ade2-101 lys2-801 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 leu2Δ met2 trp1::hisG ura3-52::P_{TDH3}-1MYC-HMG2::URA3*
RHY2498	*MATa ade2-101 lys2-801 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 leu2Δ met2 trp1::hisG ura3-52::P_{TDH3}-1MYC-HMG2::URA3 hrd4-1*
RHY2499	*MATa ade2-101 lys2-801 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 leu2Δ met2 trp1::hisG ura3-52::P_{TDH3}-1MYC-HMG2 hrd4-1::URA3::NPL4*
RHY2659	*MATa ade2-101 lys2-801 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 met2 ura3-52::P_{TDH3}-6MYC-HMG2 hrd4-1* pRH540[b] *[URA3]*
RHY2660	*MATa ade2-101 lys2-801 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 met2 ura3-52::P_{TDH3}-6MYC-HMG2 hrd4-1* pRH1443[b] *[OLE1::URA3]*
RHY2661	*MATa ade2-101 lys2-801 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 met2 ura3-52::P_{TDH3}-6MYC-HMG2* pRH540[b] *[URA3]*
RHY2662	*MATa ade2-101 lys2-801 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 met2 ura3-52::P_{TDH3}-6MYC-HMG2* pRH1443[b] *[OLE1::URA3]*

Table 4-1 (continued)

Yeast Strains

RHY2669/ DTY11A	*MATalpha ade2-1 can1-100 leu2-3,112 trp1-1*
RHY2670	*MATalpha ade2-1 can1-100 leu2-3,112 trp1-1 ole1Δ::LEU2*
RHY2673	*MATalpha ade2-1 can1-100 leu2-3,112 trp1-1* pRH540[b] *[URA3]*
RHY2674	*MATalpha ade2-1 can1-100 leu2-3,112 trp1-1 ole1Δ::LEU2* pRH1443[b] *[OLE1::URA3]*
RHY2675	*MATalpha his3Δ200 leu2Δ1 ura3-52::hmg2-GFP::URA3*
RHY2676	*MATa leu2Δ1 ura3-52::hmg2-GFP::URA3 npl4-1*
RHY2677	*MATa his3Δ200 ura3-52::hmg2-GFP::URA3 npl4-2*
RHY2679/ BWG-7a	*MATa ade1-100 his4-519 leu 2-3,112 ura3-52 ufd1-1*
RHY2680	*MATa ade1-100 his4-519 leu 2-3,112 ura3-52*
RHY2681	*MATa ade2-101 lys2-801 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 met2 ura3-52::P_{TDH3}-6MYC-HMG2 hrd4-1* pRH590[a] *[NPL4::URA3]*
RHY2702	*MATa ade1-100 his4-519 leu 2-3,112 ura3-52::hmg2-GFP::URA3 ufd1-1*
RHY2703	*MATa ade1-100 his4-519 leu 2-3,112 ura3-52::hmg2-GFP::URA3*
RHY2704	*MATalpha ade2-101 lys2-801 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 met2 ura3-52::P_{TDH3}-1MYC-HMG* pRH1562[a] *[ubiquitin-R-GFP::URA3]*
RHY2705	*MATa ade2-101 lys2-801 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 met2 ura3-52::P_{TDH3}-1MYC-HMG hrd4-1* pRH1562[a] *[ubiquitin-R-GFP::URA3]*
RHY2706	*MATa ade2-101 lys2-801 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 met2 ura3-52::P_{TDH3}-1MYC-HMG hrd2-1* pRH1562[a] *[ubiquitin-R-GFP::URA3]*
RHY2707	*MATalpha ade2-101 lys2-801 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 met2 ura3-52::P_{TDH3}-1MYC-HMG* pRH1563[a] *[ubiquitin-L-GFP::URA3]*

Table 4-1 (continued)

Yeast Strains

RHY2708	*MATa ade2-101 lys2-801 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 met2 ura3-52::P_{TDH3}-1MYC-HMG hrd4-1* pRH1563[a] *[ubiquitin-L-GFP::URA3]*
RHY2709	*MATa ade2-101 lys2-801 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 met2 ura3-52::P_{TDH3}-1MYC-HMG hrd2-1* pRH1563[a] *[ubiquitin-L-GFP::URA3]*
RHY2710	*MATalpha ade2-101 lys2-801 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 met2 ura3-52::P_{TDH3}-1MYC-HMG* pRH1564[a] *[ubiquitin-P-GFP::URA3]*
RHY2711	*MATa ade2-101 lys2-801 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 met2 ura3-52::P_{TDH3}-1MYC-HMG hrd4-1* pRH1564[a] *[ubiquitin-P-GFP::URA3]*
RHY2712	*MATa ade2-101 lys2-801 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 met2 ura3-52::P_{TDH3}-1MYC-HMG hrd2-1* pRH1564[a] *[ubiquitin-P-GFP::URA3]*
RHY2713	*MATalpha ade2-101 lys2-801 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 met2 ura3-52::P_{TDH3}-1MYC-HMG* pRH1565[a] *[ubiquitin^{G76V}-GFP::URA3]*
RHY2714	*MATa ade2-101 lys2-801 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 met2 ura3-52::P_{TDH3}-1MYC-HMG hrd4-1* pRH1565[a] *[ubiquitin^{G76V}-GFP::URA3]*
RHY2715	*MATa ade2-101 lys2-801 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 met2 ura3-52::P_{TDH3}-1MYC-HMG hrd2-1* pRH1565[a] *[ubiquitin^{G76V}-GFP::URA3]*

[a] ARS/CEN plasmid, [b] 2μ plasmid

Table 4-1 (continued)

Yeast Strains

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A

Phenotypes for a second repetition of the *hrd* selection

Table A-1 **Phenotypes for a second repetition of the *hrd* selection**

Col-ony#	Stabi-lizes 6myc-Hmg2p?	Breeds true for Lov(r)?	Temperature sensitive?	Colony size on original plate	Growth rate
1.1		no	yes	≈ 1 mm	slow
1.2		yes	no	≥ 2 mm	fast (normal yeast growth)
1.3		no	yes	1 mm<size<2 mm	slow
1.4		no	yes	≈ 1 mm	slow
1.5		yes	no	≥ 2 mm	fast (normal yeast growth)
2.1	yes	yes	yes	≈ 1 mm	fast (normal yeast growth)
2.2	no	yes	yes	≥ 2 mm	medium
2.3	yes	yes	yes	< 1 mm	fast (normal yeast growth)
3.1		no	yes	< 1 mm	medium
3.2	yes	yes	yes	< 1 mm	fast (normal yeast growth)
3.3		no	yes	≈ 1 mm	fast (normal yeast growth)

Table A-1 **Phenotypes for a second repetition of the *hrd* selection**

Colony#	Stabilizes 6myc-Hmg2p?	Breeds true for Lov(r)?	Temperature sensitive?	Colony size on original plate	Growth rate
4.1		no	no	< 1 mm	very slow
4.2	yes	yes	yes	1 mm<size<2 mm	fast (normal yeast growth)
4.3		yes	no	≥ 2 mm	fast (normal yeast growth)
5.1	yes	yes	yes	≥ 2 mm	fast (normal yeast growth)
5.2		yes	no	≈ 1 mm	fast (normal yeast growth)
6.1		yes	no	< 1 mm	fast (normal yeast growth)
6.2		no	yes	≈ 1 mm	medium
6.3		yes	slightly	≥ 2 mm	fast (normal yeast growth)
7.1		yes	no	< 1 mm	fast (normal yeast growth)
7.2		yes	no	1 mm<size<2 mm	fast (normal yeast growth)

Table A-1 **Phenotypes for a second repetition of the *hrd* selection**

Colony#	**Stabilizes 6myc-Hmg2p?**	**Breeds true for Lov(r)?**	**Temperature sensitive?**	**Colony size on original plate**	**Growth rate**
7.3		yes	no	1 mm<size<2 mm	fast (normal yeast growth)
8.1	slightly	yes	yes	< 1 mm	medium
8.2		no	yes	< 1 mm	very slow
8.3	yes	yes	yes	≈ 1 mm	fast (normal yeast growth)
9.1	slightly	yes	yes	≈ 1 mm	medium
9.2	yes	yes	yes	≥ 2 mm	medium
10.1	yes	yes	yes	< 1 mm	medium
10.2	yes	yes	yes	1 mm<size<2 mm	medium
10.3		yes	slightly	≥ 2 mm	fast (normal yeast growth)
11.1	yes	yes	yes	≥ 2 mm	medium
11.2		no	no	< 1 mm	very slow
12.1		no	no	< 1 mm	”very
12.2		yes	no	< 1 mm	medium
12.3		no	no	≈ 1 mm	very slow

Table A-1 **Phenotypes for a second repetition of the *hrd* selection**

Colony#	Stabilizes 6myc-Hmg2p?	Breeds true for Lov(r)?	Temperature sensitive?	Colony size on original plate	Growth rate
13.1		no	yes	< 1 mm	medium
13.2	no	yes	yes	< 1 mm	medium
13.3	no	yes	yes	≥ 2 mm	fast (normal yeast growth)
14.1		yes	no	< 1 mm	fast (normal yeast growth)
14.2	no	yes	slightly	≈ 1 mm	fast (normal yeast growth)
14.3		yes	no	≥ 2 mm	fast (normal yeast growth)
15.1	no	yes	yes	1 mm<size<2 mm	medium
16.1		no	yes	≈ 1 mm	slow
16.2		no	yes	1 mm<size<2 mm	slow
17.1		no	yes	≈ 1 mm	fast (normal yeast growth)
17.2		no	no	≈ 1 mm	medium
18.1		no	slightly	1 mm<size<2 mm	slow

Table A-1 **Phenotypes for a second repetition of the *hrd* selection**					
Col-ony#	**Stabi-lizes 6myc-Hmg2p?**	**Breeds true for Lov(r)?**	**Temperature sensitive?**	**Colony size on original plate**	**Growth rate**
18.2		yes	slightly	≥ 2 mm	fast (normal yeast growth)
19.1		no	no	< 1 mm	”very
19.2	no	yes	yes	< 1mm	medium
20.1		yes	slightly	≥ 2 mm	fast (normal yeast growth)
20.2		no	yes	≈ 1 mm	medium
20.3	no	yes	yes	1 mm<size<2 mm	fast (normal yeast growth)
20.4		yes	yes	1 mm<size<2 mm	slow

B

Complementation group assignment and phenotypes for a third repetition of the *hrd* selection

↓# indicates candidate number

Table B-1

Complementation group assignment for third repetition mutants

◆= Member of complementation group (strong noncomplementation in diploid with indicated mutant),

◆=Potential member of complementation group (some noncomplementation in diploid)

#	*hrd5*	*hrd6*	*hrd7*	*hrd8*	*hrd9*	*hrd10*	*hrd11*	*hrd12*	*hrd13*	*hrd14*	*hrd15*	*hrd16*	*hrd17*	*hrd18*	*hrd19*	*hrd20*
test strain>	h220	h116.1	h32.1	h420.1	h476.1	h502.1	h508.1	h535.1	h87.1	h460.1	h3.1	h504.1	h583.1	h550.1	h553.1	h447.1
3											◆					
5		◆			◆											
7											◆					
13											◆					
29									◆		◆					
32			◆													
37											◆					
53						◆										
56										◆						
64				◆												
87									◆							
96											◆					

Table B-1

Complementation group assignment for third repetition mutants

◆= Member of complementation group (strong noncomplementation in diploid with indicated mutant),

◆=Potential member of complementation group (some noncomplementation in diploid)

#	*hrd5*	*hrd6*	*hrd7*	*hrd8*	*hrd9*	*hrd10*	*hrd11*	*hrd12*	*hrd13*	*hrd14*	*hrd15*	*hrd16*	*hrd17*	*hrd18*	*hrd19*	*hrd20*
116		◆														
140											◆					
145																
162											◆					
168									◆							
173											◆					
175											◆					
181		◆		◆			◆									
186											◆					
191											◆					
195											◆					
196						◆										
220	◆															
221									◆							

Table B-1

Complementation group assignment for third repetition mutants

◆= Member of complementation group (strong noncomplementation in diploid with indicated mutant),

◆=Potential member of complementation group (some noncomplementation in diploid)

#	*hrd5*	*hrd6*	*hrd7*	*hrd8*	*hrd9*	*hrd10*	*hrd11*	*hrd12*	*hrd13*	*hrd14*	*hrd15*	*hrd16*	*hrd17*	*hrd18*	*hrd19*	*hrd20*
230			◆													
403		◆	◆		◆											
405		◆				◆										
410				◆												
411									◆							
412		◆			◆											
419				◆												
420				◆												
422								◆								
423					◆											
425				◆												
427																
429																
436								◆								

Table B-1

Complementation group assignment for third repetition mutants

◆= Member of complementation group (strong noncomplementation in diploid with indicated mutant),

◆=Potential member of complementation group (some noncomplementation in diploid)

#	*hrd5*	*hrd6*	*hrd7*	*hrd8*	*hrd9*	*hrd10*	*hrd11*	*hrd12*	*hrd13*	*hrd14*	*hrd15*	*hrd16*	*hrd17*	*hrd18*	*hrd19*	*hrd20*
439				◆												
440				◆												
442				◆												
444				◆												
447																◆
448					◆			◆								
449				◆												
453				◆												
455					◆											
456				◆												
457				◆							◆					
460										◆						
464				◆												
465				◆												

Table B-1

Complementation group assignment for third repetition mutants

◆= Member of complementation group (strong noncomplementation in diploid with indicated mutant),

◆=Potential member of complementation group (some noncomplementation in diploid)

#	*hrd5*	*hrd6*	*hrd7*	*hrd8*	*hrd9*	*hrd10*	*hrd11*	*hrd12*	*hrd13*	*hrd14*	*hrd15*	*hrd16*	*hrd17*	*hrd18*	*hrd19*	*hrd20*
466																
470								◆								
476					◆											
478				◆												
486		◆			◆											
488					◆											
489		◆										◆				
495		◆			◆											
497		◆				◆	◆									
498		◆			◆											
501		◆					◆									
502						◆										
503							◆									
504		◆										◆				

Table B-1

Complementation group assignment for third repetition mutants

◆= Member of complementation group (strong noncomplementation in diploid with indicated mutant),

◆=Potential member of complementation group (some noncomplementation in diploid)

#	*hrd5*	*hrd6*	*hrd7*	*hrd8*	*hrd9*	*hrd10*	*hrd11*	*hrd12*	*hrd13*	*hrd14*	*hrd15*	*hrd16*	*hrd17*	*hrd18*	*hrd19*	*hrd20*
505																
508							◆									
511					◆											
513	◆															
514		◆			◆											
515	◆															
518					◆											
522								◆								
523																
525		◆														
527								◆								
528				◆												
530		◆					◆									
533				◆												

Table B-1

Complementation group assignment for third repetition mutants

◆= Member of complementation group (strong noncomplementation in diploid with indicated mutant),

◆=Potential member of complementation group (some noncomplementation in diploid)

#	*hrd5*	*hrd6*	*hrd7*	*hrd8*	*hrd9*	*hrd10*	*hrd11*	*hrd12*	*hrd13*	*hrd14*	*hrd15*	*hrd16*	*hrd17*	*hrd18*	*hrd19*	*hrd20*
535								◆								
537										◆						
538		◆														
539				◆												
540																
541																
543					◆											
548					◆											
550														◆		
551									◆							
553															◆	
554		◆														
555								◆								
557				◆												

Table B-1

Complementation group assignment for third repetition mutants

◆= Member of complementation group (strong noncomplementation in diploid with indicated mutant),

◆=Potential member of complementation group (some noncomplementation in diploid)

#	*hrd5*	*hrd6*	*hrd7*	*hrd8*	*hrd9*	*hrd10*	*hrd11*	*hrd12*	*hrd13*	*hrd14*	*hrd15*	*hrd16*	*hrd17*	*hrd18*	*hrd19*	*hrd20*
560					◆											
561				◆												
562						◆										
565					◆				◆							
567					◆											
569					◆											
574				◆												
575						◆										
578					◆											
579				◆												
581		◆	◆		◆											
582					◆											
583													◆			
586				◆												

Table B-1

Complementation group assignment for third repetition mutants

◆= Member of complementation group (strong noncomplementation in diploid with indicated mutant),

◆=Potential member of complementation group (some noncomplementation in diploid)

#	*hrd5*	*hrd6*	*hrd7*	*hrd8*	*hrd9*	*hrd10*	*hrd11*	*hrd12*	*hrd13*	*hrd14*	*hrd15*	*hrd16*	*hrd17*	*hrd18*	*hrd19*	*hrd20*
591					◆			◆								
#	3	19	4	25	24	7	6	9	7	3	14	2	1	1	1	1

↓# indicates candidate number

Table B-2

Phenotypes for third repetition mutants

#	Comp Group 1	Comp Group 2	Mutagenesis	Grows on YM?	Lov(r)?	Grows at 35°C?	Grows in glycerol?	Hrd(-) by plate chase?
1			EMS	yes	yes	yes	yes	no
2			EMS	yes	yes	no	yes	no
3	hrd15		EMS	yes	yes	no	yes	yes
4			EMS	yes	yes	no	yes	no
5	hrd6	hrd9	EMS	yes	yes	yes	yes	yes
6			EMS	yes	yes	no	yes	no
7	hrd15		EMS	yes	no	no	yes	yes
8			EMS	yes	yes	no	yes	no
9			EMS	yes	yes	no	yes	no
10	ubc7		EMS	yes	yes	no	yes	yes
11			EMS	yes	yes	no	yes	no
12			EMS	yes	yes	no	yes	no
13			EMS	yes	no	no	yes	yes
14			EMS	yes	yes	no	yes	no
15			EMS	yes	yes	yes	yes	no
16			EMS	yes	no	no	yes	no
17			EMS	yes	yes	no	yes	no
18			EMS	yes	yes	yes	yes	no
19			EMS	yes	yes	some-what	yes	yes
20			EMS	yes	yes	some-what	yes	no
21			EMS	yes	yes	no	yes	no

Table B-2

Phenotypes for third repetition mutants

#	Comp Group 1	Comp Group 2	Mutagenesis	Grows on YM?	Lov(r)?	Grows at 35°C?	Grows in glycerol?	Hrd(-) by plate chase?
22			EMS	yes	yes	no	yes	yes
23			EMS	yes	yes	some-what	yes	no
24			EMS	yes	yes	yes	yes	no
25			EMS	yes	yes	yes	yes	some-what
26	hrd1	hrd4	EMS	yes	yes		yes	yes
27			EMS	yes	yes	no	yes	no
28			EMS	yes	yes	no	yes	no
29	hrd15	hrd13	EMS	yes	yes	yes	yes	yes
30			EMS	yes	yes	no	yes	no
31			EMS	yes	yes	yes	yes	no
32	hrd7		EMS	yes	yes	no	yes	yes
33			EMS	yes	yes	no	yes	no
34			EMS	yes	yes	some-what	yes	no
35	ubc7		EMS	yes	yes	yes	yes	yes
36			EMS	yes	no	no	no	no
37			EMS	yes	yes	no	yes	yes
38			EMS	yes	yes	no	yes	no
39			EMS	yes	yes	yes	yes	no
40			EMS	yes	some-what	yes	yes	no
41			EMS	yes	some-what	no	yes	no

Table B-2

Phenotypes for third repetition mutants

#	Comp Group 1	Comp Group 2	Mutagenesis	Grows on YM?	Lov(r)?	Grows at 35°C?	Grows in glycerol?	Hrd(-) by plate chase?
42			EMS	yes	yes	some-what	yes	no
43			EMS	yes	yes	no	yes	yes
44			EMS	yes	yes	yes	yes	no
45	hrd4		EMS	yes	yes	no	yes	no
46	hrd1		EMS	yes	no	no	yes	yes
47			EMS	yes	yes	yes	yes	no
48			EMS	yes	yes	no	yes	yes
49			EMS	yes	yes	yes	yes	no
50			EMS	yes	yes	no	yes	no
51			EMS	no		no	yes	no
52	ubc7		EMS	yes	yes	no	yes	no
53	hrd10		EMS	yes	yes	yes	yes	yes
54			EMS	no		no	yes	no
55			EMS	yes	yes	no	yes	no
56	hrd14		EMS	yes	yes	no	yes	yes
57	ubc7		EMS	yes	yes	yes	yes	yes
58			EMS	yes	yes	yes	yes	no
59			EMS	yes	yes	no	yes	no
60	hrd1	hrd4	EMS	yes	yes	yes	yes	yes
61			EMS	no		no	no	no
62			EMS	yes	yes	yes	yes	some-what
63			EMS	yes	yes	yes	yes	no
64	hrd8		EMS	yes	yes	no	yes	yes

Table B-2

Phenotypes for third repetition mutants

#	Comp Group 1	Comp Group 2	Mutagenesis	Grows on YM?	Lov(r)?	Grows at 35°C?	Grows in glycerol?	Hrd(-) by plate chase?
65	hrd1	hrd4	EMS	yes	yes	no	yes	yes
66	ubc7		EMS	no		no	no	yes
67			EMS	no		no	yes	no
68			EMS	some-what	some-what	no	yes	no
69			EMS	yes	yes	no	yes	no
70	ubc7		EMS	yes	yes	no	yes	yes
71			EMS	yes	yes	some-what	yes	no
72			EMS	yes	yes	yes	yes	no
73			EMS	no		no	no	no
74			EMS	yes	yes	yes	yes	yes
75	ubc7		EMS	yes	yes	no	yes	yes
76			EMS	yes	yes	no	yes	no
77			EMS	yes	yes	yes	yes	no
78			EMS	yes	yes	yes	yes	yes
79			EMS	yes	yes	yes	yes	no
80			EMS	yes	yes	yes	yes	no
81			EMS	yes	yes	yes	yes	no
82			EMS	no		no	no	yes
83	ubc7		EMS	yes	yes	yes	yes	yes
84	hrd4	ubc7	EMS	yes	yes	no	yes	yes
85			EMS	yes	yes	some-what	yes	some-what
86			EMS	yes	yes	no	yes	no

Table B-2

Phenotypes for third repetition mutants

#	Comp Group 1	Comp Group 2	Mutagenesis	Grows on YM?	Lov(r)?	Grows at 35°C?	Grows in glycerol?	Hrd(-) by plate chase?
87	hrd13		EMS	yes	yes	no	yes	yes
88			EMS	no		no	yes	no
89			EMS	yes	yes	yes	yes	no
90			EMS	no		no	no	no
91	hrd4		EMS	yes	yes	yes	yes	yes
92	hrd4		EMS	yes	yes		yes	yes
93			EMS	some-what	some-what	no	yes	no
94			EMS	yes	yes	yes	yes	no
95			EMS	yes	yes	no	no	no
96	hrd15		EMS	yes	yes	no	yes	yes
97			EMS	yes	yes	no	yes	yes
98			EMS	yes	some-what	no	no	no
99			EMS	yes	yes	no	yes	no
100			EMS	yes	yes	some-what	yes	no
101			EMS	yes	yes	yes	yes	no
102			EMS	yes	yes	some-what	yes	no
103			EMS	yes	yes	no	yes	no
104			EMS	yes	yes	no	yes	no
105			EMS	yes	yes	yes	yes	no
106			EMS	yes	yes	yes	yes	no
107			EMS	yes	yes	no	yes	no

Table B-2

Phenotypes for third repetition mutants

#	Comp Group 1	Comp Group 2	Mutagenesis	Grows on YM?	Lov(r)?	Grows at 35°C?	Grows in glycerol?	Hrd(-) by plate chase?
108			EMS	yes	yes	yes	yes	somewhat
109			EMS	yes	yes	yes	yes	no
110			EMS	yes	somewhat	no	yes	no
111			EMS	yes	yes	yes	yes	no
112			EMS	yes	yes	no	yes	no
113			EMS	yes	yes	yes	yes	no
114	ubc7		EMS	yes	yes	somewhat	yes	somewhat
115			EMS	yes	yes	no	yes	no
116	hrd6		EMS	yes	yes	yes	yes	yes
117			EMS	yes	yes	yes	yes	no
118			EMS	yes	somewhat	somewhat	no	no
119			EMS	yes	somewhat	no	yes	no
120			EMS	yes	yes	no	yes	no
121			EMS	yes	yes	no	yes	yes
122			EMS	yes	yes	yes	yes	yes
123	ubc7		EMS	yes	yes	yes	yes	yes
124			EMS	yes	somewhat	no	no	no
125	hrd4		EMS	yes	yes	yes	yes	yes
126	ubc7		EMS	yes	yes	no	yes	yes
127			EMS	yes	yes	no	no	no

Table B-2

Phenotypes for third repetition mutants

#	Comp Group 1	Comp Group 2	Mutagenesis	Grows on YM?	Lov(r)?	Grows at 35°C?	Grows in glycerol?	Hrd(-) by plate chase?
128			EMS	yes	yes	no	yes	no
129	hrd1		EMS	yes	yes	no	yes	yes
130			EMS	yes	yes	yes	yes	no
131			EMS	yes	yes	yes	yes	no
132			EMS	yes	yes	yes	yes	no
133			EMS	yes	yes	no	yes	no
134			EMS	yes	yes	no	yes	no
135	hrd4		EMS	yes	yes	yes	yes	yes
136	hrd1		EMS	yes	yes	no	yes	yes
137			EMS	yes	yes	yes	yes	no
138			EMS	yes	yes	no	yes	no
139			EMS	yes	yes	no	yes	no
140	hrd15		EMS	yes	yes	no	yes	yes
141			EMS	yes	yes	no	yes	no
142			EMS	yes	yes	no	yes	no
143			EMS	yes	yes	no	yes	no
144			EMS	yes	yes	no	yes	no
145	h145		EMS	yes	yes	no	no	yes
146			EMS	yes	yes	no	yes	no
147			EMS	yes	yes	some-what	yes	no
148			EMS	yes	yes	no	yes	no
149			EMS	yes	yes	yes	yes	no
150			EMS	yes	yes	no	no	no

Table B-2

Phenotypes for third repetition mutants

#	Comp Group 1	Comp Group 2	Mutagenesis	Grows on YM?	Lov(r)?	Grows at 35°C?	Grows in glycerol?	Hrd(-) by plate chase?
151			EMS	yes	yes	no	no	no
152			EMS	yes	yes	yes	yes	no
153			EMS	yes	yes	yes	yes	no
154			EMS	yes	yes	yes	yes	no
155			EMS	yes	yes	no	yes	no
156			EMS	yes	yes	no	yes	no
157			EMS	yes	yes	no	yes	no
158			EMS	yes	yes	yes	yes	no
159			EMS	yes	yes	yes	yes	no
160			EMS	yes	yes	no	yes	no
161			EMS	yes	yes	no	yes	no
162	hrd15		EMS	yes	yes	no	yes	yes
163			EMS	yes	yes	yes	yes	no
164			EMS	yes	yes	no	yes	no
165			EMS	yes	yes	no	yes	no
166	ubc7		EMS	yes	yes	yes	yes	yes
167			EMS	yes	yes	no	yes	no
168	hrd13		EMS	yes	yes	yes	yes	yes
169			EMS	yes	yes	no	yes	no
170			EMS	yes	yes	yes	yes	no
171			EMS	yes	yes	no	yes	no
172			EMS	yes	yes	no	yes	no
173	hrd15		EMS	yes	yes	no	yes	yes
174	ubc7		EMS	yes	yes	yes	yes	yes

Table B-2

Phenotypes for third repetition mutants

#	Comp Group 1	Comp Group 2	Mutagenesis	Grows on YM?	Lov(r)?	Grows at 35°C?	Grows in glycerol?	Hrd(-) by plate chase?
175	hrd15		EMS	yes	yes	no	yes	yes
176			EMS	yes	yes	yes	yes	no
177			EMS	yes	yes	yes	yes	no
178			EMS	no		no	no	no
179			EMS	yes	yes	no	yes	no
180			EMS	yes	yes	no	yes	no
181	hrd6	hrd8	EMS	yes	yes	no	yes	yes
182			EMS	yes	yes	no	yes	no
183			EMS	yes	yes	no	yes	some-what
184			EMS	yes	yes	no	no	no
185			EMS	yes	yes	yes	yes	some-what
186	hrd15		EMS	yes	yes	no	yes	yes
187			EMS	no		no	yes	no
188			EMS	yes	yes	no	yes	no
189			EMS	yes	yes	no	yes	no
190			EMS	yes	yes	no	yes	no
191	hrd15		EMS	yes	yes	no	yes	yes
192			EMS	yes	yes	no	yes	no
193			EMS	yes	yes	yes	yes	no
194			EMS	yes	yes	some-what	yes	no
195	hrd15		EMS	yes	yes	no	yes	yes
196	hrd10		EMS	yes	yes	yes	yes	yes

Table B-2

Phenotypes for third repetition mutants

#	Comp Group 1	Comp Group 2	Mutagenesis	Grows on YM?	Lov(r)?	Grows at 35°C?	Grows in glycerol?	Hrd(-) by plate chase?
197			EMS	yes	yes	no	yes	no
198			EMS	yes	yes	yes	yes	no
199			EMS	yes	yes	no	yes	no
200			EMS	yes	yes	no	yes	no
201			EMS	no		no	yes	no
202			EMS			no	yes	no
203			EMS			yes	yes	no
204			EMS			yes	yes	no
205			EMS			no	yes	no
206			EMS			no	yes	no
207			EMS			yes	yes	no
208			EMS			no	yes	no
209			EMS			no	yes	no
210			EMS			no	yes	yes
211			EMS			no	yes	no
212			EMS			no	yes	no
213			EMS			no	yes	no
214			EMS			no	yes	yes
215			EMS			yes	yes	no
216			EMS			no	yes	no
217			EMS			no	yes	yes
218			EMS			yes	yes	no
219			EMS			no	yes	no
220	hrd5		EMS			no	yes	yes

Table B-2

Phenotypes for third repetition mutants

#	Comp Group 1	Comp Group 2	Mutagenesis	Grows on YM?	Lov(r)?	Grows at 35°C?	Grows in glycerol?	Hrd(-) by plate chase?
221	hrd13		EMS			yes	yes	yes
222			EMS			no	yes	some-what
223			EMS			no	yes	no
224			EMS			no	yes	no
225			EMS			yes	yes	yes
226			EMS			no	yes	no
227			EMS			no	yes	no
228			EMS			no	yes	no
229			EMS			some-what	yes	no
230	hrd7		EMS			yes	yes	yes
231			EMS			no	yes	no
232			EMS			yes	yes	no
233			EMS			no	yes	no
234			EMS			yes	yes	no
235			EMS			no	yes	no
236			EMS			no	yes	no
237			EMS			no	yes	no
238			EMS					
239			EMS					
240			EMS	yes	yes	no	no	
241			EMS	yes	no	yes	yes	
242			EMS	yes	yes	yes	yes	
243			EMS	yes	yes	no	yes	

Table B-2

Phenotypes for third repetition mutants

#	Comp Group 1	Comp Group 2	Mutagenesis	Grows on YM?	Lov(r)?	Grows at 35°C?	Grows in glycerol?	Hrd(-) by plate chase?
244			EMS	yes	yes	no	no	
245			EMS	yes	yes	no	yes	
246			EMS	yes	yes	no	yes	
247			EMS	yes	no	yes	yes	
248			EMS	yes	yes	yes	yes	
249			EMS	yes	no	yes	yes	
250			EMS	yes	some-what	yes	no	
251			EMS	yes	yes	no	yes	
252			EMS	yes	yes	no	yes	
253			EMS	yes	yes	no	yes	
254			EMS	yes	yes	yes	yes	
255			EMS	yes	yes	yes	no	
256			EMS	yes	yes	some-what	no	
257			EMS	yes	yes	yes	yes	
258			EMS	yes	yes	some-what	no	
259			EMS	yes	yes	yes	yes	
260			EMS	yes	no	yes	yes	
261			EMS	yes	no	no	yes	
262			EMS	yes	yes	no	yes	
263			EMS	yes	yes	yes	no	
264			EMS	yes	yes	no	yes	
265			EMS	yes	yes	yes	yes	

Table B-2

Phenotypes for third repetition mutants

#	Comp Group 1	Comp Group 2	Mutagenesis	Grows on YM?	Lov(r)?	Grows at 35°C?	Grows in glycerol?	Hrd(-) by plate chase?
266			EMS	yes	yes	yes	yes	
267			EMS	yes	no	yes	yes	
268			EMS	yes	some-what	no	yes	
269			EMS	yes	yes	yes	no	
270			EMS	yes	no	yes	no	
271			EMS	yes	no	yes	no	
272			EMS	yes	yes	no	yes	
273			EMS	yes	yes	no	yes	
274			EMS	no	no	no	no	
275			EMS	yes	yes	yes	yes	
276			EMS	yes	yes	yes	yes	
277			EMS	yes	yes	no	yes	
278			EMS	yes	yes	yes	yes	
279			EMS	yes	yes	yes	yes	
280			EMS	yes	yes	yes	yes	
281			EMS	yes	yes	yes	yes	
282			EMS	yes	yes	yes	yes	
283			EMS	yes	yes	no	yes	
284			EMS	yes	yes	yes	yes	
285			EMS	yes	yes	yes	yes	
286			EMS	yes	yes	yes	yes	
287			EMS	yes	yes	yes	yes	
288			EMS	yes	yes	yes	yes	

Table B-2

Phenotypes for third repetition mutants

#	Comp Group 1	Comp Group 2	Mutagenesis	Grows on YM?	Lov(r)?	Grows at 35°C?	Grows in glycerol?	Hrd(-) by plate chase?
289			EMS	yes	yes	yes	yes	
290			EMS	yes	yes	no	yes	
291			EMS	no	no	no	yes	
292			EMS	yes	yes	yes	yes	
293			EMS	no	no	no	no	
294			EMS	yes	yes	yes	yes	
295			EMS	yes	yes	yes	yes	
296			EMS	yes	yes	no	yes	
297			EMS	yes	no	yes	no	
298			EMS	yes	yes	yes	yes	
299			EMS	yes	no	yes	yes	
300			EMS	yes	yes	no	yes	
301			EMS	yes	yes	yes	yes	
302			EMS	yes	yes	yes	yes	
303			EMS	yes	yes	yes	yes	
304			EMS	yes	yes	yes	yes	
305			EMS	yes	no	some-what	yes	
306			EMS	yes	yes	yes	yes	
307			EMS	yes	no	no	yes	
308			EMS	yes	some-what	no	yes	
309			EMS	yes	yes	no	yes	
310			EMS	yes	no	no	yes	

Table B-2

Phenotypes for third repetition mutants

#	Comp Group 1	Comp Group 2	Mutagenesis	Grows on YM?	Lov(r)?	Grows at 35°C?	Grows in glycerol?	Hrd(-) by plate chase?
311			EMS	yes	yes	some-what	yes	
312			EMS	yes	yes	yes	yes	
313			EMS	yes	no	no	no	
314			EMS	yes	no	no	no	
315			EMS	yes	no	no	yes	
316			EMS	yes	yes	yes	no	
317			EMS	yes	yes	yes	yes	
318			EMS	yes	yes	yes	yes	
319			EMS	yes	no	yes	some-what	
320			EMS	yes	no	yes	no	
321			EMS	yes	yes	no	yes	
322			EMS	yes	yes	yes	some-what	
323			EMS	yes	yes	no	no	
324			EMS	yes	yes	yes	yes	
325			EMS	yes	yes	yes	yes	
326			EMS	yes	yes	no	yes	
327			EMS	yes	no	no	some-what	
328			EMS	yes	yes	yes	yes	
329			EMS	yes	no	no	no	
330			EMS	yes	yes	yes	yes	
331			EMS	yes	yes	no	yes	

Table B-2

Phenotypes for third repetition mutants

#	Comp Group 1	Comp Group 2	Mutagenesis	Grows on YM?	Lov(r)?	Grows at 35°C?	Grows in glycerol?	Hrd(-) by plate chase?
332			EMS	yes	yes	no	yes	
333			EMS	yes	yes	yes	yes	
334			EMS	yes	yes	no	yes	
335			EMS	yes	yes	yes	yes	
336			EMS	yes	yes	no	yes	
337			EMS	yes	yes	no	yes	
338			EMS	yes	yes	yes	yes	
339			EMS	no	no	no	yes	
340			EMS					
341			EMS	yes	yes	yes	yes	
342			EMS	yes	yes	yes	yes	
343			EMS	yes	yes	yes	yes	
344			EMS	yes	yes	yes	yes	
345			EMS	yes	yes	yes	yes	
346			EMS	yes	some-what	yes	yes	
347			EMS	yes	yes	yes	yes	
348			EMS	yes	yes	some-what	some-what	
349			EMS	yes	yes	no	yes	
350			EMS	yes	yes	no	yes	
351			EMS	yes	yes	yes	yes	
352			EMS	yes	yes	yes	yes	
353			EMS	yes	yes	yes	yes	
354			EMS	yes	no	yes	no	

Table B-2

Phenotypes for third repetition mutants

#	Comp Group 1	Comp Group 2	Mutagenesis	Grows on YM?	Lov(r)?	Grows at 35°C?	Grows in glycerol?	Hrd(-) by plate chase?
355			EMS	yes	some-what	no	yes	
356			EMS	yes	yes	yes	yes	
357			EMS	yes	yes	yes	yes	
358			EMS	yes	yes	no	yes	
359			EMS	yes	yes	yes	yes	
360			EMS	yes	some-what	yes	yes	
361			EMS	yes	yes	yes	yes	
362			EMS	yes	some-what	yes	yes	
363			EMS	yes	yes	some-what	yes	
364			EMS	yes	yes	yes	yes	
365			EMS	yes	no	no	yes	
366			EMS	yes	yes	yes	yes	
367			EMS	yes	yes	yes	yes	
368			EMS	yes	no	no	no	
369			EMS	yes	yes	yes	yes	
370			EMS	yes	yes	yes	yes	
371			EMS	yes	yes	some-what	yes	
372			EMS	yes	yes	yes	yes	
373			EMS	yes	no	yes	some-what	

Table B-2

Phenotypes for third repetition mutants

#	Comp Group 1	Comp Group 2	Mutagenesis	Grows on YM?	Lov(r)?	Grows at 35°C?	Grows in glycerol?	Hrd(-) by plate chase?
374			EMS	yes	yes	some-what	yes	
375			EMS	yes	some-what	some-what	yes	
376			EMS	yes	no	yes	yes	
377			EMS	yes	yes	yes	yes	
378			EMS	yes	yes	yes	yes	
379			EMS	some-what	no	no	some-what	
380			EMS	no	no	no	no	
381			EMS	yes	yes	yes	yes	
382			EMS	yes	yes	yes	yes	
383			EMS	yes	no	yes	yes	
384			EMS	yes	yes	yes	yes	
385			EMS	yes	yes	yes	yes	
386			EMS	yes	yes	yes	yes	
387			EMS	yes	yes	yes	yes	
388			EMS	yes	yes	no	yes	
389								
390								
391								
392								
393								
394								
395								

Table B-2

Phenotypes for third repetition mutants

#	Comp Group 1	Comp Group 2	Mutagenesis	Grows on YM?	Lov(r)?	Grows at 35°C?	Grows in glycerol?	Hrd(-) by plate chase?
396								
397								
398								
399								
400								
401			UV	yes	yes	some-what	yes	yes
402			UV	yes	yes	yes	yes	some-what
403	hrd7	hrd8	UV	yes	yes	yes	yes	yes
404			UV	no	no	no	no	no
405	hrd10		UV	yes	yes	yes	yes	yes
406			UV	yes	yes	yes	yes	no
407			UV	yes	yes	yes	yes	yes
408			UV	yes	yes	yes	yes	some-what
409			UV	yes	yes	no	yes	yes
410	hrd8		UV	yes	yes	yes	yes	yes
411	hrd13	h145	UV	yes	yes	yes	yes	yes
412	hrd9	hrd6	UV	yes	yes	yes	yes	yes
413			UV	yes	yes	yes	yes	some-what
414			UV	no	no	no	no	no
415			UV	yes	yes	yes	no	no
416			UV	yes	yes	yes	no	yes

Table B-2

Phenotypes for third repetition mutants

#	Comp Group 1	Comp Group 2	Mutagenesis	Grows on YM?	Lov(r)?	Grows at 35°C?	Grows in glycerol?	Hrd(-) by plate chase?
417			UV	yes	yes	some-what	yes	yes
418			UV	yes	yes	some-what	no	yes
419	hrd8		UV	yes	yes	yes	yes	yes
420	hrd8		UV	yes	yes	yes	yes	yes
421			UV	yes	yes	yes	no	some-what
422	hrd12		UV	yes	yes	yes	yes	yes
423	hrd9		UV	yes	yes	yes	yes	yes
424			UV	yes	yes	yes	no	yes
425	hrd8		UV	yes	yes	yes	yes	yes
426			UV	no	no	no	no	no
427	h145		UV	yes	yes	yes	yes	yes
428			UV	yes	yes	yes	no	yes
429			UV	yes	yes	yes	yes	yes
430			UV	yes	some-what	yes	no	yes
431			UV	yes	yes	yes	no	some-what
432			UV	yes	yes	yes	yes	some-what
433			UV	yes	yes	yes	yes	some-what
434			UV	yes	yes	yes	no	yes

Table B-2

Phenotypes for third repetition mutants

#	Comp Group 1	Comp Group 2	Mutagenesis	Grows on YM?	Lov(r)?	Grows at 35°C?	Grows in glycerol?	Hrd(-) by plate chase?
435			UV	yes	no	some-what	no	no
436	hrd12		UV	yes	yes	yes	yes	yes
437			UV	yes	yes	yes	yes	some-what
438			UV	yes	yes	no	yes	yes
439	hrd8		UV	yes	yes	yes	yes	yes
440	hrd8		UV	yes	yes	yes	yes	yes
441			UV	yes	yes	yes	yes	some-what
442	hrd8		UV	yes	yes	yes	yes	yes
443			UV	yes	yes	yes	no	some-what
444	hrd8		UV	yes	yes	yes	yes	yes
445			UV	some-what	no	yes	yes	no
446			UV	yes	yes	some-what	yes	yes
447	hrd20		UV	yes	yes	yes	yes	yes
448	hrd9	hrd12	UV	yes	yes	yes	yes	yes
449	hrd8		UV	yes	yes	yes	yes	yes
450			UV	yes	yes	yes	yes	some-what
451			UV	yes	yes	yes	yes	no
452			UV	yes	some-what	yes	no	no

Table B-2

Phenotypes for third repetition mutants

#	Comp Group 1	Comp Group 2	Mutagenesis	Grows on YM?	Lov(r)?	Grows at 35°C?	Grows in glycerol?	Hrd(-) by plate chase?
453	hrd8		UV	yes	yes	yes	yes	yes
454			UV	some-what	no	some-what	no	yes
455	hrd9		UV	yes	yes	yes	yes	yes
456	hrd8		UV	yes	yes	yes	yes	yes
457	hrd15	hrd8	UV	yes	yes	yes	yes	yes
458			UV	yes	yes	yes	yes	no
459			UV	yes	yes	yes	yes	no
460	hrd14		UV	yes	yes	yes	yes	yes
461			UV	yes	some-what	yes	no	no
462			UV	no	no	no	some-what	yes
463			UV	no	no	no	no	no
464	hrd8		UV	yes	yes	yes	yes	yes
465	hrd8		UV	yes	yes	yes	yes	yes
466	h145		UV	yes	yes	yes	yes	yes
467			UV	yes	yes	yes	yes	some-what
468			UV	yes	yes	yes	yes	no
469			UV	yes	some-what	yes	some-what	yes
470	hrd12		UV	yes	yes	yes	yes	yes
471			UV	yes	some-what	yes	no	no

Table B-2

Phenotypes for third repetition mutants

#	Comp Group 1	Comp Group 2	Mutagenesis	Grows on YM?	Lov(r)?	Grows at 35°C?	Grows in glycerol?	Hrd(-) by plate chase?
472			UV	yes	yes	yes	yes	somewhat
473			UV	somewhat	no	no	no	yes
474			UV	yes	yes	yes	yes	no
475			UV	yes	somewhat	yes	no	no
476	hrd9		UV	yes	yes	yes	yes	yes
477			UV	somewhat	somewhat	somewhat	no	somewhat
478	hrd8		UV	yes	yes	yes	yes	yes
479			UV	yes	yes	yes	yes	no
480			UV	yes	somewhat	somewhat	yes	somewhat
481			UV	yes	yes	yes	yes	no
482			UV	somewhat	no	no	somewhat	yes
483			UV	yes	yes	yes	no	no
484			UV	yes	yes	yes	yes	no
485			UV	somewhat	somewhat	somewhat	no	somewhat
486	hrd6	hrd9	UV	yes	yes	yes	yes	yes
487			UV	yes	somewhat	yes	no	somewhat
488			UV	yes	yes	yes	yes	yes
489			UV	yes	yes	yes	yes	yes

Table B-2

Phenotypes for third repetition mutants

#	Comp Group 1	Comp Group 2	Mutagenesis	Grows on YM?	Lov(r)?	Grows at 35°C?	Grows in glycerol?	Hrd(-) by plate chase?
490			UV	yes	yes	yes	yes	some-what
491			UV	some-what	no	yes	no	yes
492			UV	yes	yes	yes	yes	some-what
493			UV	yes	yes	some-what	yes	yes
494			UV	yes	yes	yes	yes	some-what
495	hrd9	hrd8	UV	yes	yes	yes	yes	yes
496			UV	yes	yes	yes	yes	some-what
497	hrd10	hrd11	UV	yes	yes	yes	yes	yes
498	hrd9	hrd8	UV	yes	yes	yes	yes	yes
499			UV	yes	yes	some-what	yes	yes
500			UV	yes	no	yes	some-what	no
501	hrd11		UV	yes	yes	yes	yes	yes
502	hrd10		UV	yes	yes	yes	yes	yes
503	hrd11		UV	yes	yes	yes	yes	yes
504	hrd16		UV	yes	yes	yes	yes	yes
505			UV	yes	yes	yes	yes	yes
506			UV	yes	yes	some-what	yes	some-what

Table B-2

Phenotypes for third repetition mutants

#	Comp Group 1	Comp Group 2	Mutagenesis	Grows on YM?	Lov(r)?	Grows at 35°C?	Grows in glycerol?	Hrd(-) by plate chase?
507			UV	yes	yes	yes	yes	some-what
508	hrd11		UV	yes	yes	yes	yes	yes
509			UV	yes	some-what	yes	no	yes
510			UV	yes	yes	yes	no	some-what
511	hrd9		UV	yes	yes	yes	yes	yes
512			UV	yes	yes	yes	yes	some-what
513	in cis		UV	yes	yes	yes	yes	yes
514	hrd9	hrd6	UV	yes	yes	yes	yes	yes
515	in cis		UV	yes	yes	yes	yes	yes
516			UV	yes	yes	yes	no	yes
517			UV	yes	yes	yes	no	some-what
518	hrd9		UV	yes	yes	yes	yes	yes
519			UV	yes	yes	yes	yes	no
520			UV	yes	yes	yes	yes	some-what
521			UV	yes	yes	yes	yes	no
522	hrd12		UV	yes	yes	yes	yes	yes
523	h145		UV	yes	yes	yes	yes	yes
524			UV	yes	yes	yes	yes	some-what
525	hrd6		UV	yes	yes	yes	yes	yes

Table B-2

Phenotypes for third repetition mutants

#	Comp Group 1	Comp Group 2	Mutagenesis	Grows on YM?	Lov(r)?	Grows at 35°C?	Grows in glycerol?	Hrd(-) by plate chase?
526			UV	yes	yes	yes	no	yes
527	hrd12		UV	yes	yes	yes	yes	yes
528	hrd8		UV	yes	yes	yes	yes	yes
529			UV	yes	yes	some-what	yes	yes
530	hrd11		UV	yes	yes	yes	yes	yes
531			UV	yes	yes	yes	yes	some-what
532			UV	yes	yes	yes	yes	some-what
533	hrd8		UV	yes	yes	some-what	yes	yes
534			UV	yes	yes	some-what	yes	yes
535	hrd12		UV	yes	yes	yes	yes	yes
536			UV	yes	some-what	yes	yes	some-what
537	hrd14		UV	yes	yes	yes	yes	yes
538	hrd6		UV	yes	yes	yes	yes	yes
539	hrd8		UV	yes	yes	yes	yes	yes
540			UV	yes	yes	yes	yes	yes
541			UV	yes	yes	yes	yes	yes
542			UV	yes	yes	some-what	yes	yes
543	hrd9		UV	yes	yes	yes	yes	yes

Table B-2

Phenotypes for third repetition mutants

#	Comp Group 1	Comp Group 2	Mutagenesis	Grows on YM?	Lov(r)?	Grows at 35°C?	Grows in glycerol?	Hrd(-) by plate chase?
544			UV	yes	yes	yes	no	some-what
545			UV	yes	yes	yes	yes	no
546			UV	yes	some-what	some-what	yes	yes
547			UV	yes	some-what	some-what	yes	yes
548	hrd9		UV	yes	yes	yes	yes	yes
549			UV	some-what	some-what	no	yes	yes
550	hrd18		UV	yes	yes	yes	yes	yes
551	hrd13		UV	yes	yes	yes	yes	yes
552			UV	yes	yes	some-what	yes	yes
553	hrd19		UV	yes	yes	yes	yes	yes
554	hrd6		UV	yes	yes	yes	yes	yes
555	hrd12		UV	yes	yes	yes	yes	yes
556			UV	some-what	no	some-what	no	yes
557	h145	hrd8	UV	yes	yes	yes	yes	yes
558			UV	yes	some-what	some-what	yes	some-what
559			UV	yes	yes	some-what	yes	yes
560	hrd9		UV	yes	yes	yes	yes	yes
561	hrd8		UV	yes	yes	yes	yes	yes

Table B-2

Phenotypes for third repetition mutants

#	Comp Group 1	Comp Group 2	Mutagenesis	Grows on YM?	Lov(r)?	Grows at 35°C?	Grows in glycerol?	Hrd(-) by plate chase?
562	hrd10		UV	yes	yes	yes	yes	yes
563			UV	yes	yes	yes	yes	some-what
564			UV	yes	yes	yes	no	yes
565	hrd13		UV	yes	yes	yes	yes	yes
566			UV	yes	yes	yes	yes	some-what
567	hrd9		UV	yes	yes	yes	yes	yes
568			UV	yes	some-what	some-what	yes	yes
569	hrd9		UV	yes	yes	yes	yes	yes
570			UV	yes	yes	yes	some-what	some-what
571			UV	yes	yes	yes	yes	some-what
572			UV	yes	yes	some-what	yes	yes
573			UV	yes	yes	yes	yes	some-what
574	hrd8		UV	yes	yes	yes	yes	yes
575	hrd10		UV	yes	yes	yes	yes	yes
576			UV	yes	yes	some-what	yes	yes
577			UV	yes	yes	yes	yes	no
578	hrd9		UV	yes	yes	yes	yes	yes
579	hrd8		UV	yes	yes	yes	yes	yes

Table B-2

Phenotypes for third repetition mutants

#	Comp Group 1	Comp Group 2	Mutagenesis	Grows on YM?	Lov(r)?	Grows at 35°C?	Grows in glycerol?	Hrd(-) by plate chase?
580			UV	yes	yes	yes	no	yes
581	hrd6	hrd7	UV	yes	yes	yes	yes	yes
582	hrd9		UV	yes	yes	yes	yes	yes
583	hrd17		UV	yes	yes	yes	yes	yes
584			UV	yes	yes	yes	no	no
585			UV	some-what	some-what	yes	yes	yes
586	hrd8		UV	yes	yes	yes	yes	yes
587			UV	yes	yes	no	yes	yes
588			UV	yes	yes	yes	some-what	yes
589			UV	yes	yes	yes	yes	some-what
590			UV	some-what	some-what	no	some-what	yes
591	hrd9	hrd12	UV	yes	yes	yes	yes	yes
592			UV	yes	some-what	yes	some-what	no

C

Additional Hrd1 *in vitro* reactions

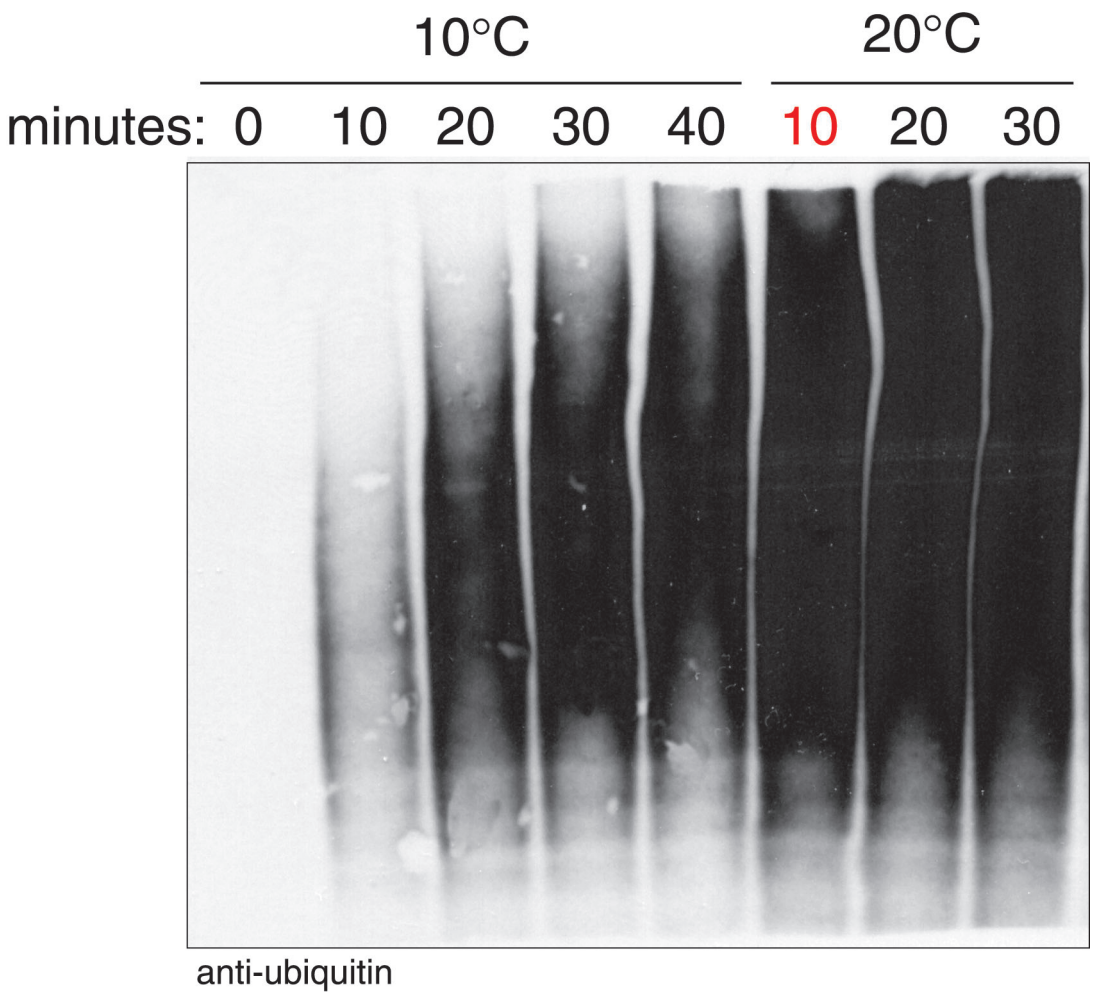

Figure C-1

Hrd1 autoubiquitination proceeds rapidly

Profound autoubiquitination of Hrd1 occurs by 10 minutes at 20°C.

in vitro ubiquitination reactions were performed as in Chapter 3, except Hrd1 added to reactions was entire soluble COOH terminal portion of Hrd1p (as a GST fusion protein with the last 500 amino acids of Hrd1p).

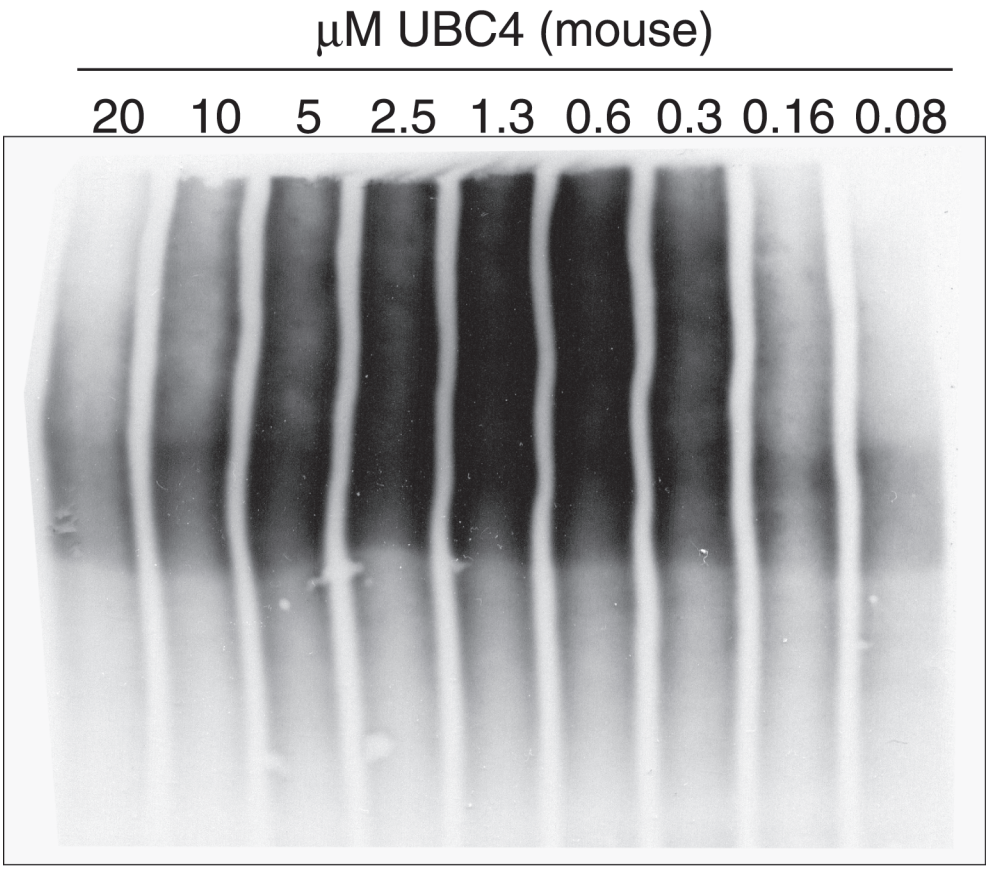

Figure C-2

Autoubiquitination activity of Hrd1 with differing E2 concentrations

in vitro ubiquitination reactions were performed as in Chapter 3, except Hrd1 added to reactions was entire soluble COOH terminal portion of Hrd1p (as a GST fusion protein with the last 500 amino acids of Hrd1p). Differing amounts of UBC4 were added to reactions as indicated.

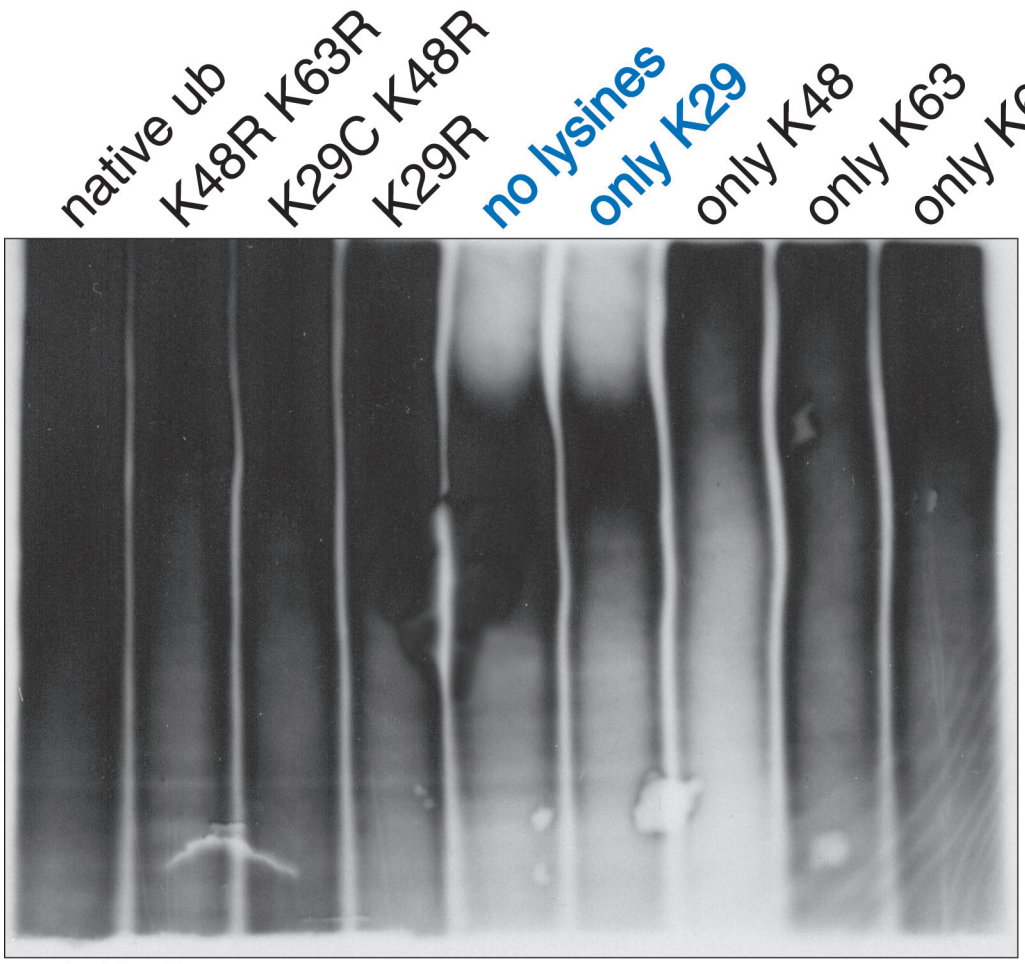

Figure C-3

Effect of different ubiquitin proteins on autoubiquitination of Hrd1

Different ubiquitin proteins with the indicated mutations were added to *in vitro* reactions perfomed as in the preceding figures. “only K48,” etc. indicates that ubiquitin protein added to the reaction contained only one lysine at the indicated position. Hrd1 can catalyze autoubiquitination using several lysines except for lysine 29.