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## **The role of the ubiquitin system in human cytomegalovirus-mediated degradation of MHC class I heavy chains**

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**CHAPTER 4 HUMAN HRD1 IS AN E3 UBIQUITIN  
LIGASE INVOLVED IN DEGRADATION  
OF PROTEINS FROM THE  
ENDOPLASMIC RETICULUM**

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## Abstract

The ubiquitin system plays an important role in endoplasmic reticulum (ER)-associated degradation of proteins that are misfolded, that fail to associate with their oligomerization partners, or whose levels are metabolically regulated. E3 ubiquitin ligases are key enzymes in the ubiquitination process as they recognize the substrate and facilitate coupling of multiple ubiquitin units to the protein that is to be degraded. The *Saccharomyces cerevisiae* ER-resident E3 ligase Hrd1p/Der3p functions in the metabolically regulated degradation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase and additionally facilitates the degradation of a number of misfolded proteins from the ER. In this study we characterized the structure and function of the putative human orthologue of yeast Hrd1p/Der3p, designated human HRD1. We show that human HRD1 is a non-glycosylated, stable ER protein with a cytosolic RING-H2 finger domain. In the presence of the ubiquitin-conjugating enzyme UBC7, the RING-H2 finger has in vitro ubiquitination activity for Lys48-specific polyubiquitin linkage, suggesting that human HRD1 is an E3 ubiquitin ligase involved in protein degradation. Human HRD1 appears to be involved in the basal degradation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase but not in the degradation that is regulated by sterols. Additionally we show that human HRD1 is involved in the elimination of two model ER-associated degradation substrates, TCR- $\alpha$  and CD3- $\delta$ .

## Introduction

When a newly synthesized protein molecule is translocated into the ER, there is a fair chance that it may never reach its final destination as a functional molecule, since a significant proportion of newly synthesized proteins is degraded via the endoplasmic reticulum-associated degradation (ERAD) pathway <sup>1</sup>. In particular, proteins that misfold along the folding pathway or cannot be appropriately folded as a result of mutations are degraded via this route. The cystic fibrosis transmembrane conductance regulator (CFTR) and its common mutation  $\Delta F508$  in cystic fibrosis serve as an example in this context <sup>2</sup>. In addition, proteins that lack their oligomerization partner(s) are prone to degradation, e.g. individual subunits of the T-cell receptor like TCR- $\alpha$  and CD3- $\delta$  <sup>3</sup>. Finally, ERAD also functions in the homeostatic regulation of metabolic pathways to degrade proteins whose activity needs to be attenuated at a certain metabolic state. Examples include

3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR) <sup>4</sup>, which is further described below, and apolipoprotein B <sup>5</sup>.

Degradation of proteins from the ER requires dislocation of the substrate from the ER to the cytosol followed by proteolysis via the ubiquitin-proteasome pathway. The dislocation process is thought to require components of the translocon channel, including Sec61 $\alpha$  <sup>6-8</sup>, as well as a complex of proteins designated CDC48/p97-Ufd1-Npl4 <sup>9-11</sup>. Ubiquitination also plays an essential role in dislocation as illustrated by the inhibition of protein dislocation when the ubiquitination machinery is disrupted <sup>9,12-16</sup>.

The coupling of ubiquitin chains to proteins involves three enzymes. A ubiquitin-activating enzyme (E1) activates ubiquitin in an ATP-dependent manner. Subsequently one of a second set of enzymes, designated E2, conjugates the activated ubiquitin through a thiol ester bond to its essential cysteine residue. Finally, with the aid of a third set of enzymes, E3 ubiquitin ligases, the ubiquitin molecules are successively transferred from the E2 onto one or more lysine residues or the N terminus of the protein destined for degradation <sup>1,17</sup>. It is thought that E3 ubiquitin ligases or the combinations of E2/E3 enzymes provide specificity to the ubiquitination of protein targets. Thus, the identification of E3 ubiquitin ligases that are involved in the elimination of proteins associated with the ER should greatly advance our understanding of the regulation of this process.

At present, three classes of E3 ligases are recognized. The first group has a HECT domain, named after the E6-AP C terminus, and the second group, to which the CHIP E3 ligase belongs, contains a so-called U-box <sup>1,16</sup>. The third group that seems to expand the fastest is that of the RING finger-containing E3 ligases. The RING motif consists of a series of eight conserved cysteines and histidines, which bind two zinc atoms and form a structure of "cross-braced" rings. The middle two residues in the motif comprise either one or two histidines, resulting in three subclasses of RING finger motifs: classical or RING-HC, RING-CH, and RING-H2. To date, all characterized examples of these variants have been shown to possess E3 ligase activity in vitro <sup>18-21</sup>. For some RING finger E3 ligases, it was found that the RING finger structure binds the E2. However, the exact mechanism by which the RING finger-containing E3 ligase catalyzes the transfer of ubiquitin to the target proteins is yet unknown.

One of the best characterized RING-H2 finger-containing E3 ligases involved in ERAD in yeast is Hrd1p <sup>22</sup>, also designated Der3p <sup>23</sup>. This protein was identified by Hampton and co-workers <sup>22</sup> in search for factors that take part in the degradation of *Saccharomyces cerevisiae* Hmg2p, one of the yeast isozymes of HMGR. HMGR is the rate-limiting enzyme in the mevalonate pathway in which sterols and a myriad of essential isoprenoids are

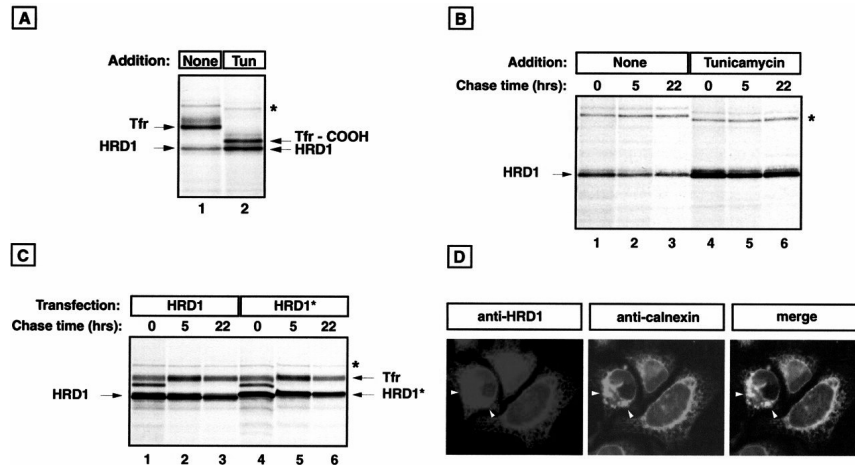
synthesized. In mammalian as well as in yeast cells, the intracellular levels of HMGR are tightly regulated by the cellular demands for mevalonate-derived sterol and non-sterol metabolites<sup>24-26</sup>. This feedback control involves alteration of enzyme stability<sup>24-27</sup>. Thus, when the demands are high, HMGR protein is stable. When the requirements for these metabolites have been satisfied, the enzyme is rapidly degraded. Studies in yeast, as well as more recent experiments in mammalian cells, have unequivocally shown that the degradation of HMGR involves its regulated ubiquitination and eventual elimination by the 26 S proteasome<sup>22,28</sup>. Hrd1p/Der3p, as an E3 ubiquitin ligase, was shown to be involved in this metabolically regulated degradation of yeast HMGR<sup>29</sup>. Moreover, it has been demonstrated that Hrd1p, which was independently isolated by Wolf et al.<sup>23</sup> as Der3p, is also involved in ERAD of other ER proteins, including CPY\* and Sec61-2p.

Hrd1p/Der3p is a multispanning membrane protein with its C-terminal RING-H2 finger domain located in the cytoplasm. In yeast, Hrd1p is found in a stoichiometric complex with Hrd3p, a lumen-oriented ER membrane protein that stabilizes Hrd1p and modulates its ligase activity<sup>29</sup>. The enzyme predominantly uses Ubc7p as an E2 but also cooperates with Ubc6p and Ubc1p in ERAD<sup>30,31</sup>.

Another yeast E3 ligase involved in ERAD is Doa-10<sup>32</sup>, which contains a RING finger of the RING-CH type at its N terminus, and is ER-localized. It degrades the transcription factor MAT $\alpha$ 2 and a number of other ERAD substrates that are not served by the Hrd1p/Der3p complex. A third yeast E3 ligase implicated in degradation of ER quality control substrates is the HECT domain-containing Rsp5p<sup>33</sup>, which also seems to assist in degradation of proteins in the ER, especially in times of ER stress.

In the mammalian cell, the number of E3 ligases involved in ERAD is also rapidly expanding. gp78, previously known as the autocrine motility factor receptor (AMFR), was identified as an ER-localized E3 ubiquitin ligase that can mediate the degradation of the ERAD substrates CD3- $\delta$  and apolipoprotein B100<sup>34,35</sup>. CHIP is a cytosolic U-box-containing E3 ligase that can target CFTR for degradation from the ER in an Hsp/Hsc70-dependent way<sup>36</sup>. CHIP also catalyzes degradation of glucocorticoid hormone receptor<sup>37</sup> via a process that requires Hsp90. Finally, F-box2 protein, the substrate-recognizing subunit of an SCF (Skp, Cullin, F-box) E3 ligase complex localized in the cytosol, binds to N-glycans of proteins in the ER and assists in their degradation<sup>38</sup>.

In this study, we characterized the structure and function of the recently identified human homologue of yeast Hrd1p/Der3p E3 ligase, designated HRD1. Expression and subcellular localization of human HRD1 were addressed, and the overall membrane topology of human HRD1 was



**Figure 1. Endogenous human HRD1 protein is up-regulated by ER stress.**

(A) HeLa cells were either left untreated (None) or treated with 10  $\mu$ g/ml tunicamycin (Tun) for 4 h. The cells were metabolically labeled with <sup>35</sup>S Promix. Cell lysates were subjected to immunoprecipitation with anti-HRD1 polyclonal antiserum and anti-transferrin receptor (Tfr) antibodies. (B) pulse-chase analysis of endogenous HRD1 in HeLa cells with or without tunicamycin treatment. Cells were metabolically labeled for 1 h and chased for the times indicated. HRD1 was isolated from denatured cell lysates by immunoprecipitation with anti-HRD1 polyclonal antiserum. (C) pulse-chase analysis of transfected HRD1 and HRD1-C1A (HRD1\*) in HeLa cells; the method was the same as in B. Transferrin receptor and HRD1 were isolated from denatured cell lysates by immunoprecipitation with anti-HRD1 polyclonal antiserum and anti-transferrin receptor antibodies. Asterisks on the side of the gels in A, B, and C indicate a nonspecific band that precipitates with the Protein A or Protein G beads used. (D) immunofluorescence analysis of transfected HRD1 in HeLa cells. Cells were transfected with human HRD1-expressing plasmid and stained for HRD1 (left panel) and calnexin (middle panel) using respective specific polyclonal antibodies and Cy3- (left panel) or fluorescein isothiocyanate (middle panel)-conjugated anti-immunoglobulin second antibodies. The right panel shows an overlay. Clusters of protein are indicated with arrowheads.

determined using deglycosylating enzymes and proteinase K digestions. In vitro ubiquitination assays were performed to establish whether ubiquitin linkage by HRD1 is Lys48-specific. The anticipated function of human HRD1 in degradation of HMGR was investigated as well as its role in degradation of other ERAD substrates.

## Results

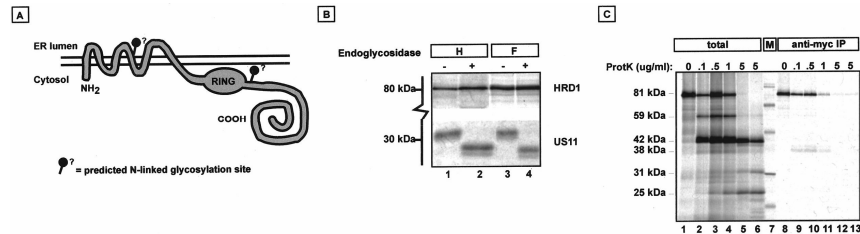
### Expression, N-Linked Glycosylation, and Topology of Human HRD1

To gain information on the expression of human HRD1, a polyclonal antiserum was developed against human HRD1 by immunizing a rabbit with the C-terminal 228 amino acids of the HRD1 protein fused at the N terminus to maltose-binding protein. This antiserum specifically recognized a single protein of 81 kDa in HeLa cells (Fig. 1A, lane 1). We found that a 4-h

treatment with tunicamycin resulted in a 3-4-fold increase in the amount of endogenous HRD1 protein in HeLa cells (Fig. 1A, lane 2). This is in agreement with the observation that elevated HRD1 mRNA levels occur under ER stress conditions<sup>39</sup>. As judged from pulse-chase experiments, both endogenous (Fig. 1B) and transiently transfected human HRD1 protein (Fig. 1C) display a half-life of ~15 h in HeLa cells (Fig. 1, B and C), which renders HRD1 a protein of relatively high stability. Fig. 1D shows subcellular localization of transfected human HRD1 in HeLa cells. Most HRD1 is localized in a typical lacy pattern, characteristic for the ER. This is confirmed by the co-localization with calnexin, an ER-resident chaperone (Fig. 1D). Besides the ER pattern, clustered protein was detected in some of the HeLa cells transiently transfected with human HRD1 (Fig. 1D, arrows). These clusters also co-localized with calnexin, suggesting that they may be ER membrane-derived (Fig. 1D). Staining with anti-vimentin antibodies did not show any vimentin "cages" characteristic of aggresomes (data not shown). We conclude that human HRD1 is a stable protein localized in the ER.

Fig. 2A shows a schematic representation of the predicted membrane topology of human HRD1 based on its hydrophobicity plot (according to Kyte and Doolittle<sup>40</sup>) and predictions through several algorithms from the ExPASy internet site (SOSUI (TUAT, Tokyo University of Agriculture and Technology), TMHMM (CBS, Copenhagen, Denmark), and others). The resulting model contains six putative transmembrane domains and a RING finger-containing C-terminal domain positioned in the cytosol. Two potential N-linked glycosylation sites (NX(T/S)) were found in the sequence that are indicated in the model as well. Since one N-linked glycosylation site is very close to a putative transmembrane region and the other is predicted to be cytosolic (Fig. 2A), it is anticipated that neither of the two will actually be glycosylated if this model is correct. This notion was already indicated by the lack of effect of tunicamycin, which inhibits N-linked glycosylation, on the electrophoretic mobility of human HRD1 (Fig. 1, A and B). Indeed, Fig. 2B shows that treatment of transfected HRD1 with either endoglycosidase H or F did not result in a shift in migration of the protein, while in the same samples, the single N-linked glycan of US11 (a human cytomegalovirus protein present in the cells used) was cleaved off. This demonstrates that human HRD1 is not N-glycosylated.

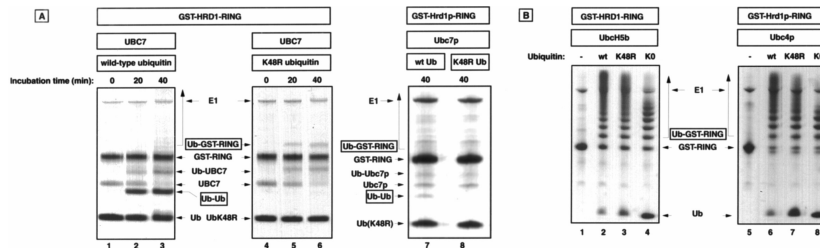




**Figure 2. Membrane topology and N-linked glycosylation of human HRD1.**

(A) schematic representation of the predicted membrane topology of human HRD1. N-Linked glycosylation motifs (NX(T/S)) in the protein are indicated as well as the RING-H2 motif. (B) human HRD1 was expressed in HeLa cells that were also transfected with human cytomegalovirus US11 protein. The cells were metabolically labeled with <sup>35</sup>S Promix, and HRD1 and US11 were immunoprecipitated from cell lysates. Samples were incubated with endoglycosidase H or F or were mock-treated. (C) proteinase K digestion of in vitro translated HRD1. HRD1, tagged at the extreme C terminus with a MYC epitope, was translated in the presence of microsomes and [<sup>35</sup>S]methionine. Subsequently the microsomes were incubated with increasing amounts of proteinase K (ProtK) as indicated. The left side of the panel shows total protein samples; the samples on the right side are immunoprecipitated with antiserum against the MYC tag. M, marker; IP, immunoprecipitation.

These results are, however, inconclusive in establishing the topology of human HRD1, and we therefore addressed HRD1 membrane topology more specifically. The protein, which was MYC-tagged at its C terminus, was translated in vitro in the presence of canine pancreatic microsomes and <sup>35</sup>S-labeled amino acids. The reaction mixture was then digested with proteinase K. Only cytosolic parts of HRD1 will be degraded, thus providing information on the topology when antibodies against the MYC tag are used for immunoprecipitation. Proteinase K digestion of HLA-A2, a type I transmembrane protein, was used as a control. This resulted in the removal of the HLA-A2 cytoplasmic tail (not shown), indicating that proteins are inserted correctly into the membranes of the microsomes. Fig. 2C shows that discrete protein fragments are produced upon proteinase K treatment of in vitro translated human HRD1 as evident from direct load of the digestion mixtures (Fig. 2C, lanes 1-6). When digested protein was immunoprecipitated with antiserum against the C-terminal MYC tag, only the full-length protein could be recovered from the digestion mixtures (Fig. 2C, lanes 8-13). This indicates that all of the smaller protein fragments seen in the directly loaded samples lack the C-terminal epitope, and therefore the C terminus must have been on the cytosolic side of the membranes where it was gradually digested by proteinase K. Similar results were obtained when the experiment was performed with antiserum against the C-terminal 228 amino acids of human HRD1 (data not shown). Taken together, the data shown in Fig. 2 strongly suggest that the topology of human HRD1 corresponds to the model presented in Fig. 2A and is therefore comparable to the topology that has been proposed for yeast Hrd1p/Der3p<sup>29,41</sup>.



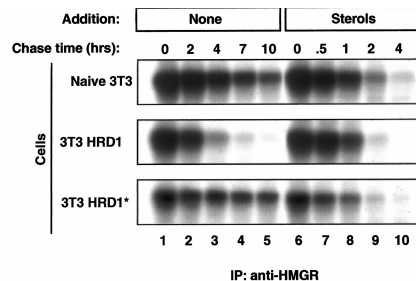
**Figure 3. The RING domain of human HRD1 has in vitro E3 ligase activity.** In vitro ubiquitination assays were performed with purified E1 enzyme, E2 enzyme (human UBC7, yeast Ubc7p, human UbcH5b, or yeast Ubc4p as indicated), purified GST-HRD1-RING-H2 (derived from human HRD1), or GST-Hrd1p-RING (derived from *S. cerevisiae* Hrd1p) as indicated and either wild-type ubiquitin (Ub), K48R mutant ubiquitin (K48R), or a lysineless ubiquitin mutant (K0). Reaction mixtures were separated by SDS-PAGE and stained with Coomassie Brilliant Blue. (A) assays with human UBC7 or yeast Ubc7p as an E2 and wild-type and K48R ubiquitin as indicated. (B) assays with human UbcH5b or yeast Ubc4p as an E2 and different ubiquitin variants as indicated. wt, wild-type.

#### The Human HRD1 RING-H2 Domain Has in Vitro Ubiquitination Activity

To evaluate the ability of the RING-H2 domain in human HRD1 to interact with specific E2 enzymes, we fused the RING-H2 domain to the C terminus of GST and tested for the ability of E2 enzymes to ubiquitinate the GST fusion protein. For comparison, we tested a similar GST fusion protein that contains the RING-H2 domain from *S. cerevisiae* Hrd1p. Yeast Hrd1p is known to cooperate with yeast Ubc7p in ubiquitin-mediated degradation of HMGR<sup>30</sup>, and our analysis to be described below is consistent with the model that the RING domain in human HRD1 interacts specifically with human UBC7.

Incubation of GST-HRD1-RING with human UBC7 in the presence of the ubiquitin-activating enzyme E1, ubiquitin, and ATP resulted in the formation of a ubiquitin dimer (Fig. 3A, lanes 1-3, Ub-Ub), which is apparently formed by the linkage of the C-terminal carboxyl group of one ubiquitin to Lys48 of another ubiquitin since this dimer is not formed when ubiquitin was replaced with the ubiquitin mutant UbK48R (Fig. 3A, lanes 4-6). Similarly a Lys48-linked ubiquitin dimer was also formed by incubating yeast Ubc7p and GST-Hrd1p-RING in similar reactions (Fig. 3A, lanes 7 and 8). The formation of this dimer required the presence of all components in the reaction mixture, although the yeast and human components were interchangeable (data not shown). In addition to ubiquitin dimer, ubiquitin was also conjugated to lysines within the human and yeast GST-RING fusions (Fig. 3A, Ub-GST-RING), although this reaction was less robust than ubiquitin dimer formation.

The formation of Lys48-linked ubiquitin dimer is apparently specific



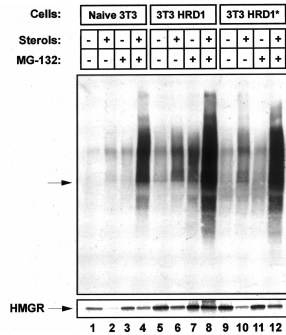
**Figure 4. Effect of human HRD1 on degradation of HMGR.**

Naive 3T3 cells and 3T3 cells stably transfected with HRD1 or HRD1-C2A (HRD1\*) were treated with sterols or were mock-treated. A pulse-chase analysis was performed as indicated, and HMGR was immunoprecipitated from lysed cells. IP, immunoprecipitation.

for UBC7/Ubc7p since this product was not formed when UBC7/Ubc7p was replaced by either Ubc2/Rad6 (data not shown) or Ubc4p/UbcH5b (Fig. 3B). Neither ubiquitin dimer or ubiquitin-GST-RING fusion products could be detected by replacing UBC7/Ubc7p with either human or yeast Ubc2/Rad6 (data not shown). Replacing UBC7/Ubc7p with Ubc4/UbcH5b resulted in the robust linkage of multiple ubiquitin moieties to the GST-RING fusions (Fig. 3B). Judging by the products formed with the lysineless UbK0 mutant, ubiquitin can be linked to as many as six lysine sites in the GST-RING fusions (Fig. 3B, lanes 4 and 8). In addition, ubiquitin-ubiquitin linkages on the GST-RING fusion could also form, resulting in dense smears (compare lane 2 with 4 and lane 6 with 8). These ubiquitin-ubiquitin linkages are not confined to Lys48 in ubiquitin since they occurred even by replacing ubiquitin with UbK48R in the reactions (lanes 3 and 7). Thus, while Ubc4/UbcH5b can interact with the HRD1/Hrd1p-RING domain and produce ubiquitinated products efficiently *in vitro*, it does not support the specific Lys48 ubiquitin-ubiquitin linkage in a polyubiquitin chain that targets proteins to the proteasome<sup>42,43</sup>.

#### **Overexpression of Human HRD1 Accelerates Basal but Not Metabolically Regulated Degradation of Endogenous HMGR**

Since yeast Hrd1p is implicated in the degradation of Hmg2p, we examined whether HRD1 might be similarly involved in the turnover of mammalian HMGR. By mutating one of the crucial cysteines of the RING finger, E3 ligase activity of RING finger E3 ligases can be disrupted as was shown *in vitro* and *in vivo* before<sup>17,34,39,44</sup>. It is thought that substrate binding of such RING finger mutants will not be disturbed since such binding is attributed to other domains of the E3 ligase. In this way, a dominant negative effect can be achieved, providing an instrument to study involvement of E3 ligases in degradation of particular substrate proteins.



**Figure 5. Effect of human HRD1 on ubiquitination of HMGR.**

Naïve 3T3 cells and 3T3 cells stably transfected with human HRD1 and HRD1-C2A (HRD1\*) were mock-treated or treated with sterols, proteasome inhibitor MG-132, or both. Cells were lysed, and HMGR was immunoprecipitated. The immunoprecipitate was analyzed on Western blot using anti-ubiquitin antiserum (upper panel) or anti-HMGR antiserum (lower panel). The arrow in the upper panel indicates the migration level of HMGR protein.

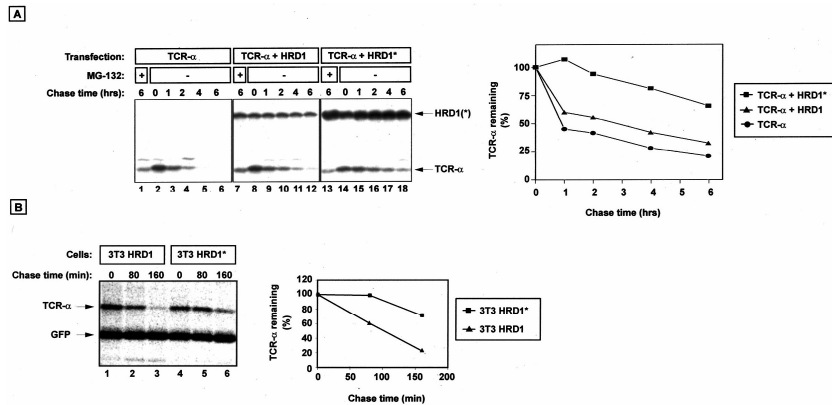
Sterol-depleted naïve NIH-3T3 cells or cells stably overexpressing either the wild-type HRD1 or the RING finger mutant HRD1-C2A were pulse-labeled and chased in the absence or presence of a mixture of sterols (25-hydroxycholesterol plus cholesterol). Endogenous HMGR was precipitated with antibodies directed against its membrane domain (Fig. 4). Consistent with previous reports<sup>25,28</sup>, addition of sterols during the chase in naïve cells accelerated the basal rate of HMGR degradation 5-fold, decreasing its half-life ( $t_{1/2}$ ) from 8.9 h (Fig. 4, top panel, lanes 1-5) to 1.7 h (Fig. 4, top panel, lanes 6-10; note the different chase times in the presence of sterols). In the HRD1-overproducing cells, degradation of HMGR proceeded with markedly faster kinetics ( $t_{1/2} = 2.9$  h; Fig. 4, middle panel, lanes 1-5), but there was no noticeable effect on the rate of HMGR degradation in the presence of sterols ( $t_{1/2} = 1.6$  h; Fig. 4, middle panel, lanes 6-10). Overexpression of the HRD1-C2A mutant reversed the phenotype of overexpressed HRD1 in sterol-depleted cells, and the basal half-life of HMGR returned to its value in naïve cells ( $t_{1/2} = 9.5$  h; Fig. 4, bottom panel, lanes 1-5). Yet again, there was no effect of mutant HRD1 on the sterol-accelerated degradation of HMGR ( $t_{1/2} = 1.9$  h; Fig. 4, bottom panel, lanes 6-10). These results demonstrate that human HRD1 participates in the basal turnover of HMGR in sterol-depleted cells but not in the sterol-accelerated degradation of the enzyme, suggesting that these two processes are distinct. Alternatively, the endogenous level/activity of HRD1 that takes part in HMGR degradation in sterol-treated cells is not limiting.

To examine whether the observed effects on HMGR degradation correlate with its ubiquitination, we immunoprecipitated HMGR from control

cells or from cells that were treated for 2 h with sterols, the proteasome inhibitor MG-132, or both. The immune complexes were blotted onto nitrocellulose and probed sequentially with monoclonal antibodies against ubiquitin and HMGR (Fig. 5). In untreated cells (Fig. 5, upper panel, lanes 1, 5, and 9) there was very little high molecular weight material that reacted with the ubiquitin antibody. The amount of this material, relative to the amount of HMGR, substantially increased in all cells upon addition of sterols (Fig. 5, lanes 2, 6, and 10), but noticeably, similar amounts of polyubiquitin-decorated material were detected in wild-type HRD1- and mutant HRD1-C2A-expressing cells (compare lanes 6 and 10). This confirms that the sterol-enhanced production of ubiquitinated HMGR, and thereby degradation of HMGR, is not significantly influenced by the expression of either wild-type or mutant HRD1. Ubiquitinated proteins can be more readily detected in the presence of proteasome inhibitors, which prevent their degradation and allow a better evaluation of their amounts. In sterol-depleted naïve 3T3 cells that were treated with the proteasome inhibitor MG-132 (Fig. 5, lane 3) there was a low amount of ubiquitinated HMGR protein detected, which corresponds to a slow rate of HMGR degradation (Fig. 4, upper panel, lanes 1-5). A marked increase in the amounts of ubiquitinated HMGR was observed in HRD1-overexpressing cells that were treated with MG-132 (Fig. 5, lane 7). This corresponds to the rapid turnover of HMGR in these cells even in the absence of added sterols (Fig. 4, middle panel, lanes 1-5). In cells overexpressing mutant HRD1 the amounts of ubiquitinated HMGR recovered upon treatment with MG-132 were much lower than in cells expressing wild-type HRD1 and nearly returned to the levels seen in naïve cells (Fig. 5, compare lanes 3, 7, and 11). This corresponds to the lower rate of HMGR degradation in the sterol-depleted mutant HRD1-expressing cells (Fig. 4, bottom panel, lanes 1-5). Treatment with both sterols and MG-132 caused a massive accumulation of ubiquitinated HMGR in all cell types (Fig. 5, lanes 4, 8, and 12), a combined effect of enhanced degradation by sterols and improved recovery of ubiquitin conjugates due to proteasome inhibition. Taken together with Fig. 4, these data demonstrate that the increased rate of HMGR degradation in sterol-depleted cells that overexpress wild-type human HRD1 is the result of enhanced ubiquitination of the enzyme. Thus, human HRD1 may function *in vivo* as a protein-ubiquitin ligase for HMGR degradation in sterol-depleted cells.

#### **Involvement of Human HRD1 in ERAD of Other Proteins**

We next tested whether HRD1 is involved in the degradation of other substrates. The TCR- $\alpha$  and CD3- $\delta$  subunits of the T-cell receptor complex are ERAD substrates when expressed in the absence of their oligomerization

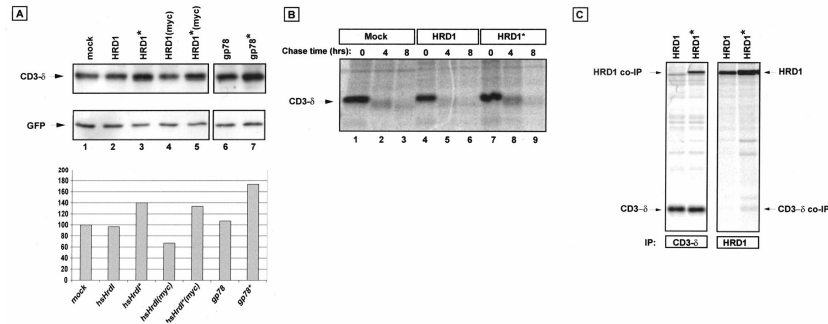


**Figure 6. Effect of human HRD1 on degradation of TCR- $\alpha$ .**

(A) 3T3 cells were transiently transfected with TCR- $\alpha$ , TCR- $\alpha$  and HRD1 (FLAG-tagged), or TCR- $\alpha$  and HRD1-C2A (HRD1\*, FLAG-tagged). A pulse-chase experiment was performed with and without the addition of proteasome inhibitor MG-132. HRD1 and HRD1-C2A (HRD1\*) and TCR- $\alpha$  were immunoprecipitated with anti-FLAG and anti-TCR- $\alpha$  HA28-710 monoclonal antibodies, respectively. (B) 3T3 cells stably transfected with HRD1 or HRD1-C2A (HRD1\*) were transduced with retrovirus expressing TCR- $\alpha$  and GFP. A pulse-chase analysis was performed as indicated, and TCR- $\alpha$  and GFP were immunoprecipitated from cell lysates using polyclonal antiserum against TCR and anti-GFP antiserum, respectively.

partners<sup>3</sup>. To study the role of human HRD1 in the degradation of TCR- $\alpha$ , 3T3 cells were transiently transfected with TCR- $\alpha$  alone or co-transfected with TCR- $\alpha$  and wild-type HRD1 or TCR- $\alpha$  and HRD1-C2A. Fig. 6A shows rapid degradation of TCR- $\alpha$  in wild-type and HRD1-transfected 3T3 cells (lanes 1-6 and lanes 7-12, respectively). Expression of mutant HRD1 along with TCR- $\alpha$  markedly delayed the degradation of TCR- $\alpha$ , exhibiting a pronounced dominant negative effect of the mutated RING finger of HRD1 (Fig. 6A). The effect on degradation of TCR- $\alpha$  was also examined in 3T3 cells that stably express wild-type or mutant HRD1 by transducing these cells with a retrovirus encoding TCR- $\alpha$ . Compared with wild-type HRD1, HRD1-C2A retarded the degradation of retrovirus-encoded TCR- $\alpha$  (Fig. 6B). GFP, which was expressed by the same recombinant retrovirus from an internal ribosomal entry site, was expressed at similar levels in all cells and remained stable throughout the chase period (Fig. 6B). Taken together, these results indicate that human HRD1 plays a role in the degradation of TCR- $\alpha$ , a bona fide ERAD substrate.

A similar series of experiments was performed to analyze the role of human HRD1 in degradation of CD3- $\delta$ . HeLa cells were transfected with CD3- $\delta$  alone or with CD3- $\delta$  and variants of the HRD1 protein (Fig. 7A). Again GFP was co-transfected to serve as a loading control. Steady state levels



**Figure 7. Effect of human HRD1 on degradation of CD3- $\delta$  and co-precipitation of HRD1 with CD3- $\delta$ .**

(A) HeLa cells were co-transfected with CD3- $\delta$  (1  $\mu$ g) and GFP (0.2  $\mu$ g) and different HRD1 or gp78 variants (1  $\mu$ g) as indicated. The asterisk indicates RING finger mutants; (myc) indicates C-terminally MYC-tagged proteins. 24 h after transfection cell lysates were analyzed on Western blot using polyclonal antiserum against CD3- $\delta$  and GFP, respectively. Quantifications relative to cells transfected with CD3- $\delta$  and GFP only (mock) are shown in the lower panel. (B) HeLa cells were co-transfected with CD3- $\delta$  and HRD1 variants as indicated. 24 h after transfection cells were metabolically pulse-labeled with <sup>35</sup>S Promix and chased for the times indicated. CD3- $\delta$  was immunoprecipitated with specific polyclonal antiserum. (C) HeLa cells were co-transfected with CD3- $\delta$  and HRD1 or CD3- $\delta$  and HRD1 RING finger mutant as indicated. The left panel shows immunoprecipitation with anti-CD3- $\delta$  antibodies; HRD1-C1A mutant co-precipitates with CD3- $\delta$ . The right panel shows immunoprecipitation with anti-HRD1 antibodies, and co-immunoprecipitation of CD3- $\delta$  with HRD1-C1A mutant protein is indicated. IP, immunoprecipitation.

of CD3- $\delta$ , in equally transfected HeLa cells, were analyzed by Western blotting (Fig. 7A). Co-transfection of RING finger mutant variants of HRD1 resulted in increased amounts of CD3- $\delta$  (Fig. 7A, compare lane 2 with 3 and lane 4 with 5), indicating an inhibited degradation of the substrate by the dominant negative form of HRD1. gp78/AMFR, another known RING finger E3 ligase involved in mammalian ERAD, has also been shown to be involved in CD3- $\delta$  degradation (34). Co-expression of CD3- $\delta$  and a RING finger mutant of gp78/AMFR indeed resulted in increased steady state levels of CD3- $\delta$  (Fig. 7A, lanes 6 and 7). The effect of gp78 and its RING finger mutant on the degradation of CD3- $\delta$  was comparable to what we found for the HRD1 variants. Pulse-chase experiments confirmed the role of human HRD1 in the degradation of CD3- $\delta$  (Fig. 7B). Expression of wild-type HRD1 increased the degradation rate of CD3- $\delta$  (Fig. 7B, compare lanes 1-3 and 4-6), and expression of the RING finger mutant slowed down degradation of CD3- $\delta$  (Fig. 7B, compare lanes 1-3 with 7-9).

The results indicate that degradation of a single substrate, CD3- $\delta$ , may be catalyzed by different E3 ligases. This observation may explain the moderate effect of mutant HRD1 on the degradation of CD3- $\delta$  since endogenous gp78 (in addition to endogenous HRD1) may compete for binding to CD3- $\delta$  and catalyze its ubiquitination.

In support of our conclusion that human HRD1 variants influence CD3- $\delta$  degradation is the co-precipitation of mutant HRD1 with CD3- $\delta$  and vice versa (Fig. 7C). Since mutant HRD1 does not possess E3 ligase activity due to the disruption of its RING finger motif, its association with the substrate is prolonged compared with wild-type HRD1, allowing the two binding partners to be readily co-precipitated. These results also show that the catalytic activity of the RING finger is dispensable for substrate binding.

## Discussion

At present, it is generally accepted that the ubiquitin system plays a crucial role in the degradation of ER proteins through the ERAD pathway. Not only is the recognition by the proteasome of ER degradation substrates achieved by their tagging with ubiquitin molecules, but also the dislocation of substrate from the ER to the cytosol depends on the ubiquitination machinery. This mandates that specialized ubiquitination enzymes should be involved in degradation of ER proteins. Yeast Hrd1p, an integral ER membrane E3 ligase, may fulfil such a specialized function, and indeed it was shown to be involved in the degradation of a number of yeast ER proteins. Here we characterized a human homologue of yeast Hrd1p, human HRD1, and we conclude that it has a similar function in the degradation of ERAD substrates. Our results indicate that the *in vitro* E3 ligase activity of human HRD1 is restricted to the linkage of ubiquitin molecules through their lysine at position 48 when UBC7 is present in the assay as an E2, supporting a putative role in protein degradation<sup>43,45</sup>. We showed that human HRD1 does not carry N-linked glycans, and the overall subcellular localization and membrane topology of the yeast and human proteins were found to be similar.

In yeast, Hrd1p was shown to be involved in the degradation of HMGR. The regulation of this degradation in yeast is mainly dependent on the non-sterol isoprenoid farnesyl pyrophosphate<sup>27</sup>. It appears that degradation of mammalian HMGR is mechanistically more complex since it is regulated by both sterol and non-sterol signals<sup>25</sup>. Additionally recent studies have pointed to a key role of the ER-resident Insig protein(s) in the regulated turnover of HMGR in mammalian cells<sup>46,47</sup>, while no counterparts of such proteins have been found yet in yeast.

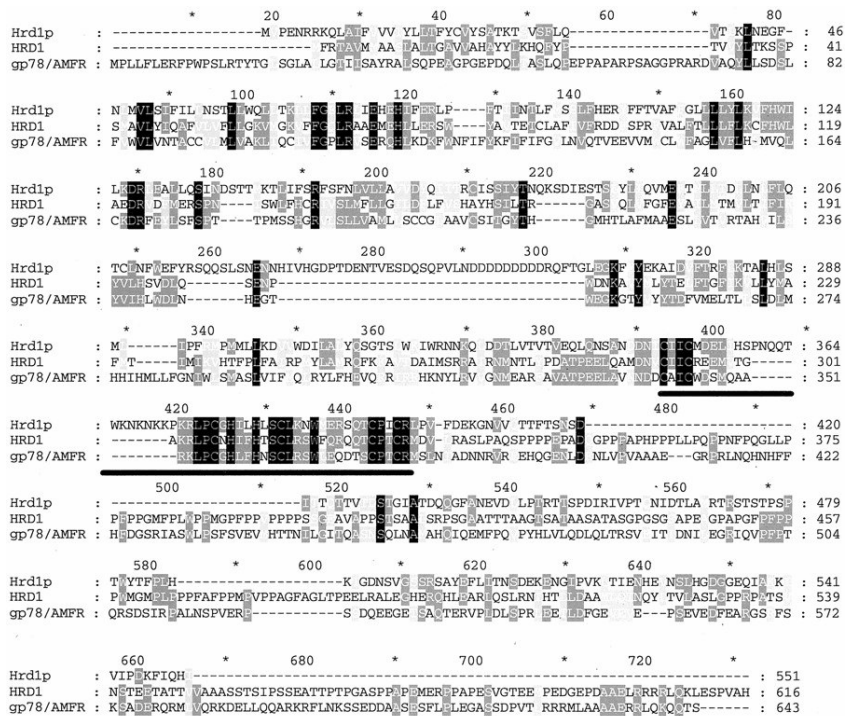
The human HRD1 described here hastens the turnover of mammalian HMGR only when overexpressed in sterol-depleted cells. Human HRD1 thus seems to have a role in the basal degradation of mammalian HMGR. It is possible that this basal rate is dictated by the intracellular levels of mevalonate-



derived non-sterol metabolite(s) such as farnesyl pyrophosphate. This would conform to the role of yeast Hrd1p in non-sterol-regulated turnover of yeast Hmg2p. Additionally, the basal rate of HMGR degradation in sterol-depleted mammalian cells may also include the elimination of newly synthesized but aberrant HMGR. Thus, the acceleration of this basal rate of degradation in HRD1-overexpressing cells may conform to the role of this E3 ligase in ERAD of aberrant proteins in general. The sterol-regulated degradation of HMGR in mammalian cells should involve another, yet unidentified E3 ubiquitin ligase. Overexpression of the HRD1 mutant does not slow down the degradation of HMGR in sterol-depleted cells (Fig. 4, lower panel compared with upper panel). This result may be explained by the possibility that HRD1 is not the sole E3 ligase serving the basal degradation of HMGR but is shunted to this process when overexpressed, a situation that may be achieved in vivo by ER stress. Alternatively the lack of a dominant negative effect on the basal degradation by overexpression of the RING finger mutant of HRD1 may be due to the significantly longer half-life of HMGR in sterol-depleted cells, causing further stabilization not readily measurable.

In yeast, Hrd1p associates with Hrd3p, and deletion of the latter causes Hrd1p to be unstable<sup>29</sup>. In humans, SEL1L is a proposed homologue of Hrd3p<sup>48</sup>, and it remains to be investigated whether SEL1L interacts with HRD1. Since we show here that both endogenous and transfected human HRD1 are relatively stable proteins (Fig. 1, B and C), it seems unlikely that SEL1L ensures the stability of human HRD1 in a manner similar to that observed in yeast.

We showed that human HRD1 influences the degradation of individual subunits of the T-cell receptor complex, TCR- $\alpha$  and CD3- $\delta$ , two "classic" ERAD substrates. Overexpression of wild-type HRD1 did not increase the rate of TCR- $\alpha$  degradation (Fig. 6). This suggests that, for this substrate, the endogenous level of HRD1 may not be limiting. Possibly other components of the ER quality control machinery that "decide" whether and when TCR- $\alpha$  is to be degraded are rate-limiting, and the degradative rate for TCR- $\alpha$  may not increase by the addition of more wild-type HRD1. However, overexpression of the RING finger mutant HRD1 had a marked inhibitory effect, indicating that the mutant can exert a dominant negative function. This suggests that HRD1 has high affinity for components of the ERAD machinery that take part in the elimination of TCR- $\alpha$ . Moreover, unlike HMGR in sterol-depleted cells, TCR- $\alpha$  has a very short half-life. Thus, its stabilization by a dominant negative HRD1 will be readily observed.



**Figure 8. Alignment of protein sequences of *S. cerevisiae* Hrd1p, Homo sapiens HRD1, and H. sapiens gp78/AMFR. RING-H2 motifs are underlined.**

It is noteworthy that gp78/AMFR, another RING-H2 finger-containing E3 ligase involved in mammalian ERAD, has also been shown to aid in the degradation of CD3- $\delta$  <sup>34</sup>. This finding of a common substrate may be explained by the observation that gp78/AMFR and human HRD1 exhibit considerable homology in their N-terminal and RING finger regions (see Fig. 8, 28% overall similarity and 18% identity and 69% similarity and 54% identity within the RING finger domain). Additionally both E3 ligases seem to use UBC7 as an E2-conjugating enzyme (Ref. 34 and Fig. 3A). gp78/AMFR and HRD1 may have evolved from the same ancestor from which yeast Hrd1p may have originated as well. Although gp78/AMFR and HRD1 are likely to serve diverged populations of ERAD substrates, some overlap may be expected due to their homology, and CD3- $\delta$  seems to be one of those common substrates. While different E3 ligases are available, each of which may be specific for a subpopulation of substrates, a certain degree of redundancy may be a way to assure flexible and robust ERAD machinery with the ability to adjust to different cellular needs.

Since human HRD1 levels are up-regulated upon ER stress (Fig. 1, A and B), one might hypothesize that HRD1 is an E3 ligase that supports the degradation of all ERAD substrates upon ER stress, to increase the general degradative capacity of the cell. However, while it is likely that the three substrates identified in this study are only a subset of the substrates that are handled by human HRD1, this E3 ligase, on the other hand, is not able to direct the degradation of some other ERAD substrates. This is illustrated by the observation that degradation of CFTR $\Delta$ F508<sup>2</sup> is not influenced by human HRD1<sup>44</sup>. Additionally, we evaluated the possible role of HRD1 in the rapid ER degradation of major histocompatibility complex class I molecules in the presence of human cytomegalovirus US11 or US2 proteins<sup>6</sup>. Transient overexpression of wild-type and RING finger mutant forms of human HRD1 in this system did not affect major histocompatibility complex class I degradation (data not shown). Also degradation of  $\alpha$ 1-antitrypsin Hong-Kong null mutant protein<sup>12</sup> was found not to be influenced by human HRD1 (data not shown). These observations indicate that human HRD1 is involved in the degradation of a particular subset of proteins. The information on ubiquitination enzymes, which undoubtedly will become available in the future, may shed more light on the regulation of the ERAD machinery under normal and ER stress conditions.

## Materials & Methods

### Materials

Unless noted otherwise, all reagents were obtained from Sigma. Geneticin (G418 sulfate) was procured from Invitrogen. 25-Hydroxycholesterol was purchased from Steraloids. Immobilized recombinant Protein A was obtained from RepliGen, and Protein A- and G-Sepharose were from Amersham Biosciences. Proteinase K was purchased from Invitrogen. MicroBCA protein reagent and SuperSignal® chemiluminescence substrate were from Pierce, and the ECL+ chemiluminescence kit was from Amersham Biosciences. MG-132 proteasome inhibitor was purchased from Calbiochem or Peptide Institute (Osaka, Japan). Compactin was a kind gift from Robert Simoni, Stanford University, and mevalonolactone was bought from Fluka (Buchs, Switzerland). Lipoprotein-deficient fetal calf serum (d  $\geq$  1.25) was prepared by ultracentrifugation as described previously<sup>49</sup>.

### Plasmids

Several entries comprising the human homologue of *S. cerevisiae* Hrd1p, HRD1, are present in the National Center for Biotechnology Information (NCBI) gene data base (i.e. accession numbers AAL26903

AAH30530 NM\_032431 , XP\_045498, NP\_757385 , and NP\_115807 ), resulting from independent cloning and sequencing of the gene by different researchers. Some major and minor variations in the gene can be identified: the KIAA1810 clone (protein accession number BAB47439 , for which the cDNA was produced from human fetal brain tissue, lacks two exons compared with the rest of the entered sequences. Entries NP\_115807 , XP\_045498, and AAL26903 on one hand and NP\_757385 and AAH30530 on the other represent two other splice variants, designated Isoform a ("short") and Isoform b ("long"), respectively. This splice variation results in one additional codon in the long isoform, encoding an alanine residue at amino acid position 413. The work described in this report was performed with the short isoform (identical to entries NP\_115807 , XP\_045498, and AAL26903 , which was cloned as described below.

The KIAA1810 cDNA, cloned in pBluescript vector, was obtained from the HUGE sequencing project<sup>50</sup>. To produce an expression construct, the HRD1-encoding open reading frame was cut out using KpnI and BspLU11I restriction enzymes. The BspLU11I-cut side was made blunt, and the fragment was cloned into KpnI/EcoRV sites of a pcDNA3.1/hygro(+) vector. The KIAA1810 open reading frame lacks two exons (encoding 51 amino acids) relative to other human HRD1 entries in the NCBI data base (accession numbers AAL26903 AAH30530 XP\_045498, NP\_757385 , and NP\_115807 ). The missing region was isolated from HeLa cell cDNA by PCR and cloned into the KIAA1810 open reading frame. A mutation of the first or second cysteine of the RING finger into an alanine was accomplished with the QuikChange site-directed mutagenesis kit (Stratagene), resulting in a product designated HRD1-C1A or HRD1-C2A, respectively. The human HRD1 open reading frame and its RING finger mutant were also cloned into the EcoRI/KpnI sites of the pcDNA3.1 Myc/His A(-) vector and into a FLAG tag-containing pLNCX vector (Clontech). The resulting constructs contained a C-terminal Myc and His tag or a FLAG tag, respectively. pTCR- $\alpha$ -Neo was kindly provided by Dr. Ron Kopito (Stanford University), and pLZRS-based retroviruses that express TCR- $\alpha$  were a kind gift from Dr. Mirjam Heemskerk (Leiden University Medical Center, Leiden, The Netherlands). Expression plasmids pCIneo-gp78 and pCIneo-gp78R2m were a kind gift from Dr. Allan Weissman (NCI, National Institutes of Health, Bethesda, MD). The human CD3- $\delta$  gene was isolated from pCD1-CD3- $\delta$  (a gift from Dr. Peter van den Elsen, Leiden University Medical Center) by PCR and cloned into pcDNA3.1/hygro(+) vector using XhoI and XbaI sites.

#### **Antibodies**

Polyclonal rabbit antiserum against human HRD1 was produced using

a purified fragment of the C-terminal 228 amino acids of the protein fused N-terminally to maltose-binding protein. Anti-Myc antibodies were from Roche Applied Science (immunoblot and immunoprecipitation) or Invitrogen (immunoprecipitation). Anti-FLAG monoclonal antibodies, clone M2, were purchased from Sigma. Anti-GFP antibodies were from Invitrogen or were a kind gift from Dr. Jaques Neefjes (Netherlands Cancer Institute, Amsterdam). Anti-US11 antiserum was produced in rabbits as described previously<sup>14</sup>. Monoclonal antibodies against human transferrin receptor (clone H68.4) were purchased from Zymed Laboratories Inc. HMGR was immunoprecipitated with specific antiserum, which was described earlier<sup>51</sup>, directed against peptides derived from the HMGR membrane domain. Antibodies against TCR- $\alpha$  (H28-710 monoclonal antibody) were kindly provided by Dr. Ron Kopito (Stanford University), and polyclonal rabbit antibodies against CD3- $\delta$  and TCR- $\alpha$ <sup>52</sup> were a generous gift from Dr. Frits Koning (Leiden University Medical Center). Polyclonal antibodies against gp78<sup>34</sup> were kindly provided by Dr. Allan Weissman (NCI, National Institutes of Health). Ubiquitin was detected with a monoclonal antibody from Santa Cruz Biotechnology (clone P4D1). Horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit antibodies were from Jackson ImmunoResearch Laboratories.

#### **Cells**

NIH-3T3 cells were grown in Medium A (Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 10 mM Na-HEPES, pH 7.4, 2 mM glutamine, 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin). To obtain stably transfected cells, an HEK 293 packaging cell line was transfected with pLNC-HRD1 or pLNC-HRD1-C2A. Cells were selected with 1 mg/ml Geneticin (Invitrogen), and recombinant retrovirus was collected from the supernatant. NIH-3T3 cells were transduced with the recombinant retroviruses, and expressing clones were isolated by limiting dilution. These cells were maintained in Medium A supplemented with 250  $\mu$ g/ml Geneticin. HeLa cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin.

#### **Metabolic Labeling and Immunoprecipitation**

Cells were transfected using jetPEI (Qbiogene Molecular Biology) or LipofectAMINE Plus<sup>TM</sup> (Invitrogen) according to the manufacturer's instructions. Where indicated, HMGR expression was up-regulated 14-16 h prior to radioactive labeling by refeeding the cells with Medium B (Dulbecco's modified Eagle's medium supplemented with 10% lipoprotein-deficient serum, 2 mM glutamine, 2  $\mu$ M compactin, and 100  $\mu$ M mevalonate). At 24 or

48 h after transfection cells were starved and metabolically labeled with <sup>35</sup>S-amino acids as described previously <sup>14</sup> and chased for the times indicated. Lysates were made in Nonidet P-40 lysis mixture (50 mM Tris/HCl, pH 7.4, 5 mM MgCl<sub>2</sub>, and 0.5% (v/v) Nonidet P-40), and proteins were immunoprecipitated using Protein A- and G-Sepharose beads as described previously <sup>14</sup>. Dried polyacrylamide gels were analyzed using phosphorimaging technology.

#### **PAGE and Immunoblotting**

Transfected cells were harvested 24 or 48 h after transfection, lysed in a small volume of Nonidet P-40 lysis mixture, incubated for 30 min on ice, and centrifuged for 10 min at 14,000 x g. One volume of 2x sample buffer (40 mM Tris/HCl, pH 8.0, 4 mM EDTA, 8% (w/v) SDS, 40% (w/v) glycerol, 0.1% bromphenol blue) was added to the supernatant, and the samples were incubated at 95 °C for 5 min. Proteins were separated on polyacrylamide gels and blotted onto Optitran BAS-83 reinforced nitrocellulose membranes (Schleicher & Schuell) or polyvinylidene difluoride membrane (PerkinElmer Life Sciences). Immunodetected proteins were visualized using chemiluminescence. Quantifications were done using Quantity-One software (Bio-Rad).

#### **In Vitro Ubiquitination Assay**

The glutathione S-transferase (GST)-HRD1-RING fusion protein was obtained from bacterial expression of a plasmid in which the nucleotide sequence coding for residues 272-343 of human HRD1 was inserted downstream of the BamHI site of the vector pGEX-4T1. The expressed protein was purified by affinity chromatography on glutathione-coupled gel beads. The GST-Hrd1p-RING fusion protein was similarly obtained by replacing the human HRD1 sequence with that coding for residues 331-413 of the *S. cerevisiae* Hrd1p. The ubiquitin-conjugating enzymes (E2) yeast Ubc7p, yeast Ubc4p, yeast Ubc2p, human UBC7, human UbcH5b, and human UBC2 were obtained as described previously <sup>53-55</sup>. Purified E1 was obtained by expression of a *S. cerevisiae* N-terminally poly-His-tagged UBA1 coding sequence in a  $\Delta$ uba1 strain (kindly provided by J. Doolman) and subsequent purification by sequential nickel affinity and ubiquitin affinity chromatography. The concentrations of E1 and E2 were determined by measuring the amount of ubiquitin that forms a thiol ester bond with the enzymes. The concentration of GST fusion proteins was obtained from absorbance measured at 280 nm in 8 M urea using a molar extinction coefficient based on their tryptophan, tyrosine, and phenylalanine content.

In vitro ubiquitination assays were carried out at 30 °C in a reaction

mixture containing 25 mM HEPES, pH 7.5, 1 mM ATP, 10 mM MgCl<sub>2</sub>, 10 nM E1 enzyme, 1 μM GST-RING fusion protein, a 0.1 μM concentration of an E2 enzyme, and 50 μM ubiquitin. Reactions were terminated by the addition of SDS gel sample buffers, and protein components were separated by SDS-PAGE and visualized by Coomassie Brilliant Blue staining.

#### **Immunofluorescence Assay**

Immunofluorescence of transfected protein was performed as described in Kikkert et al.<sup>14</sup>.

**In Vitro Translation and Proteinase K Digestion**—Human HRD1 was transcribed in vitro with T7 RNA polymerase using an in vitro transcription system (Invitrogen), and the resulting RNA was translated using a Promega in vitro translation kit in the presence of 35S-labeled methionine (Amersham Biosciences) and canine pancreatic microsomal membranes. Translation reactions were performed at 30 °C for 90 min.

For proteinase K digestions, microsomal membranes were centrifuged at 14,000 x g at 4 °C for 15 min and washed with 100 μl of KMH buffer (110 mM KAc, 2 mM MgAc, and 20 HEPES-KOH, pH 7.2). Proteinase K digestions were performed in 50 μl of KMH buffer or Nonidet P-40 lysis mixture for 30 min on ice at concentrations indicated. After digestion, 1 μl of 500 mM phenylmethylsulfonyl fluoride was added to the Nonidet P-40 samples, and 200 μl of KMH buffer containing 4 mM phenylmethylsulfonyl fluoride was added to the KMH buffer samples. The microsomes were centrifuged at 14,000 x g at 4 °C for 15 min and resuspended in 60 μl of Nonidet P-40 lysis mixture containing 1 μl of 500 mM phenylmethylsulfonyl fluoride. After lysis for 20 min, samples were cleared by centrifugation at 14,000 x g at 4 °C for 15 min. The supernatant was split and used for either direct loads or immunoprecipitation. Immunoprecipitations, SDS-PAGE, and phosphorimaging were performed as described previously<sup>14</sup>.

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