Human Hrd1 is an E3 ubiquitin ligase involved in degradation of proteins from the Endoplasmic Reticulum.
Summary

The ubiquitin system plays an important role in ER-associated degradation (ERAD) of proteins that are misfolded, those 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 *S. cerevisiae* ER-resident E3 ligase Hrd1p/Der3p functions in the metabolically regulated degradation of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), 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 hsHrd1. We show that hsHrd1 is a non-glycosylated, stable ER protein with a cytosolic RING-H2 finger domain. In the presence of the ubiquitin-conjugating enzyme hsUbc7, the RING-H2 finger has *in vitro* ubiquitination activity for K48-specific polyubiquitin linkage, suggesting that human Hrd1 is an E3 ubiquitin ligase involved in protein degradation. HsHrd1 appears to be involved in the basal degradation of HMGR, but not in the degradation that is regulated by sterols. Additionally, we show that hsHrd1 is involved in the elimination of two model ERAD substrates, TCR-α and CD3-δ.
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 (1). 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 ΔF508 in cystic fibrosis, serve as an example in this context (2). In addition, proteins that lack their oligomerization partner(s) are prone to degradation, e.g. individual subunits of the T-cell receptor like TCR-α and CD3-δ (3). 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-CoA reductase (HMGR) (4), which is further described below, and apolipoprotein B (5).

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 sec61α (6-8), as well as a complex of proteins designated CDC48/p97-Ufd1-Npl4 (9-11). Ubiquitination also plays an essential role in dislocation, as illustrated by the inhibition of protein dislocation when the ubiquitination machinery is disrupted (12-16). The coupling of ubiquitin chains to proteins involves three enzymes. An E1 enzyme 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 (1,17). 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 (1,16). 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 (18-21). 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 catalyses 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 (22), also designated Der3p (23). This protein was identified by Hampton and co-workers in search for factors that take part in the degradation of \textit{S. cerevisiae} Hmg2p, one of the yeast isozymes of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR). HMGR is the rate-limiting enzyme in the mevalonate (MVA) 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 MVA-derived sterol and nonsterol metabolites (24-26). This feedback control involves alteration of enzyme stability (24,27). 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 26S proteasome (22,28). Hrd1p/Der3p, as an E3 ubiquitin ligase, was shown to be involved in this metabolically regulated degradation of yeast HMGR (29). Moreover, it has been demonstrated that Hrd1p, which was independently isolated by Wolf et al as Der3p, is also involved in ERAD of other ER proteins, including CPY* and sec61-2p (23).

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

Another yeast E3 ligase involved in ERAD is Doa-10 (32), which contains a RING finger of the RING-CH type at its N-terminus, and is ER-localized. It degrades the transcription factor MATα2, 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 (33), 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-δ and Apolipoprotein B100 (34,35). CHIP is a cytosolic U-box-containing E3 ligase, which can target CFTR for degradation from the ER in an hsp/hsc70-dependent way (36). It also catalyzes degradation of glucocorticoid hormone receptor (37) 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 (38).

In this study, we characterized the structure and function of the recently identified human homologue of yeast Hrd1p/Der3p E3 ligase, designated hsHrd1. Expression and subcellular localization of hsHrd1 were addressed, and the overall membrane topology of human Hrd1 was determined using deglycosylating enzymes and proteinase K digestions. *In vitro* ubiquitination assays were performed to establish whether ubiquitin linkage by hsHrd1 is K48 specific. The anticipated function of hsHrd1 in degradation of HMGR was investigated, as well as its role in degradation of other ERAD substrates.
Experimental procedures

Materials

Unless noted otherwise, all reagents were obtained from Sigma (St. Louis, MO). Geneticin (G-418 sulphate) was procured from Invitrogen Life Technologies. 25-hydroxycholesterol was purchased from Steraloids. Immobilized recombinant Protein A was obtained from RepliGen and Protein A- and G-Sepharose from Amersham Biosciences. Proteinase K was purchased from Invitrogen. MicroBCA protein reagent and SuperSignal® chemiluminescent substrate were from Pierce, and ECL+ chemiluminescence 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 (LPDS; d ≥1.25) was prepared by ultracentrifugation as described (39).

Plasmids

Several entries comprising the human homologue of S. cerevisiae Hrd1p are present in the NCBI gene database (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 foetal brain tissue, misses two exons compared to the rest of the entered sequences. Entries NP_115807, XP_045498, AAL26903 on one hand and NP_757385, 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 paper was performed with the short isoform (identical to entries NP_115807, XP_045498, AAL26903), which was
cloned as described below.

The KIAA1810 cDNA, cloned in pBluescript vector, was obtained from the HUGE sequencing project (40). To produce an expression construct, the hsHrd1 encoding ORF 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 ORF misses two exons (encoding 51 amino acids) relative to other hsHrd1 entries in the NCBI database (accession numbers: AAL26903, AAH30530, XP_045498, NP_757385 and NP_115807). The missing region was isolated from HeLa cells cDNA by PCR, and cloned into the KIAA1810 ORF. A mutation of the first or second cysteine of the RING finger into an alanine was accomplished with the Quick Change Site Directed Mutagenesis kit (Stratagene), resulting in a product designated hsHrd1C1A or hsHrd1C2A respectively. The hsHrd1 ORF 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 Laboratories Inc., Palo Alto, CA). The resulting constructs contained a C-terminal myc- and his-tag or a FLAG-tag respectively. pTCR-α-Neo was kindly provided by Dr. Ron Kopito (Stanford University), and pLZRS based retroviruses that express TCR-α were a kind gift from Dr. Mirjam Heemskerk (LUMC Leiden, The Netherlands). Expression plasmids pCIneo-gp78 and pCIneo-gp78R2m were a kind gift from Dr. Allan Weissman (National Cancer Institute, Bethesda). The human CD3-δ gene was isolated from pCD1-CD3-δ (a gift from Dr. Peter van den Elsen (LUMC Leiden, The Netherlands)) by PCR and cloned into pcDNA3.1/hygro(+) vector using XhoI and XbaI sites.

Antibodies

Polyclonal rabbit antiserum against hsHrd1 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 (Immunoblot and IP) or Invitrogen (IP). Anti-FLAG monoclonal antibodies, clone M2, were purchased from Sigma. Anti-GFP antibodies were from Invitrogen, or a kind gift from Dr. Jaques Neefjes (Netherlands Cancer Institute, Amsterdam). Anti-US11 antiserum was produced in rabbits as described previously (14). Monoclonal antibodies against human transferrin receptor (clone H68.4) were purchased from Zymed Laboratories Inc. (San Francisco, CA). HMGR was immunoprecipitated with specific antiserum, which was described earlier (41), directed against peptides derived from the HMGR membrane domain. Antibodies against TCR-α (H28-710 monoclonal antibody) were kindly provided by Dr. Ron Kopito (Stanford University), and polyclonal rabbit antibodies against CD3-δ and TCR-α (42) were a generous gift from Dr. Frits Koning (LUMC, Leiden, The Netherlands). Polyclonal antibodies against gp78 (34) were kindly provided by Dr. Allan Weissman (National Cancer Institute, Bethesda). Ubiquitin was detected with a monoclonal antibody from Santa Cruz (clone P4D1). Horseradish peroxidase (HRP)-conjugated goat anti-mouse or anti-rabbit antibodies were from Jackson ImmunoResearch Laboratories.

Cells

NIH-3T3 cells were grown in Medium A (DMEM supplemented with 10% FCS, 10mM Na-HEPES, pH 7.4, 2mM glutamine, 100U/ml penicillin, 100µg/ml streptomycin). In order to obtain stably transfected cells, a 293 GP packaging cell line was transfected with pLNC-hsHrd1 or pLNC-hsHrd1C2A. Cells were selected with 1mg/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 250µg/ml Geneticin. HeLa cells were grown in RPMI 1640 medium supplemented with 10% FCS, 2mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin.

Metabolic labelling and immunoprecipitation
Cells were transfected using jetPEI (Qbiogene molecular biology) or Lipofectamine Plus™ (Invitrogen) according to the manufacturer’s instructions. Where indicated, HMGR expression was up-regulated 14-16 hours prior to radioactive labeling by re-feeding the cells with medium B (DMEM supplemented with 10% lipoprotein-deficient serum, 2mM glutamine, 2µM compactin and 100µM MVA). At 24 or 48 hours after transfection cells were starved and metabolically labeled with 35S-amino acids as described (14) and chased for the times indicated. Lysates were made in Nonidet P-40 lysis mix (50 mM Tris HCl pH 7.4, 5 mM Mg Cl₂, and 0.5% v/v Nonidet P-40) and proteins were immunoprecipitated using sepharose A and G beads as described (14). Dried poly-acrylamide gels were analyzed using phosphor-imaging technology.

**PAGE and immunoblotting**

Transfected cells were harvested 24 or 48 hours after transfection, lysed in a small volume of NP-40 lysis mix, incubated for 30 min on ice, and centrifuged for 10 minutes at 14,000 g. One volume of 2x sample buffer (40 mM Tris/HCl, pH8.0; 4 mM EDTA; 8% (w/v) SDS; 40% (w/v) glycerol; 0.1% Bromophenol Blue) was added to the supernatant, and the samples were incubated at 95°C for five minutes. Proteins were separated on poly-acrylamide gels and blotted onto Optitran BAS-83 reinforced nitrocellulose membranes (Schleicher & Schuell; Dassel, Germany) or PVDF membrane (PerkinElmer). Immuno-detected proteins were visualized using chemiluminescence. Quantifications were done using Quantity-One software (Bio-Rad).

**In vitro ubiquitination assay**

The GST-HsHrd1-RING fusion protein was obtained from bacterial expression of a plasmid in which the nucleotide sequence coding for residues 272-343 of HsHrd1 was inserted downstream of the BamH1 site of the vector pGEX-4T1. The expressed protein was purified by affinity chromatography on glutathione-coupled gel beads. The GST-ScHrd1-RING fusion protein was similarly obtained by replacing the HsHrd1 sequence.
with that coding for residues 331-413 of the *Saccharomyces cerevisiae* Hrd1p. The ubiquitin-conjugating enzymes (E2) ScUbc7, ScUbc4, ScUbc2, HsUbc7, HsUbcH5b, and HsUbc2 were obtained as previously described (43-45). Purified ubiquitin-activating enzyme (E1) was obtained by expression of a *Saccharomyces cerevisiae* N-terminally polyHis-tagged UBA1 coding sequence in a Δuba1 strain (kindly provided by J. Dohlman) and subsequent purification by sequential Ni-affinity and ubiquitin-affinity chromatography. The concentrations of E1 and E2 were determined by measuring the amount of ubiquitin that forms a thiolester bond with the enzymes. The concentration of GST fusion proteins was obtained from absorbance measured at 280 nm in 8M 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, pH7.5, 1 mM ATP, 10 mM MgCl2, 10 nM E1 enzyme, 1 μM GST-RING fusion protein, 0.1 μM of an E2 enzyme, and 50 μM of 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. (14). In *vitro* translation and proteinase K digestion

HsHrd1 was transcribed *in vitro* with T7 RNA polymerase using an *in vitro* transcription system (Gibco BRL), and the resulting RNA was translated using a Promega *in vitro* translation kit in the presence of 35S labelled methionine (Amersham), and canine pancreatic microsomal membranes. Translation reactions were performed at 30 °C for 90 minutes.

For proteinase K digestions, microsomal membranes were spun down at 14,000 g and 4 °C for 15 minutes and washed with 100 ¼l KMH buffer (110 mM KAc, 2 mM MgAc and
20 HEPES-KOH pH 7.2). Proteinase K digestions were performed in 50 ¼l KMH or NP-40 lysis mix for 30 minutes on ice at concentrations indicated. After digestion, 1 ¼l 500 mM PMSF was added to the NP-40 samples, and 200 ¼l KMH containing 4 mM PMSF was added to the KMH samples. The microsomes were spun down at 14,000 g at 4 °C for 15 minutes and resuspended in 60 ¼l NP40 lysis mix containing 1¼l 500 mM PMSF. After lysis for 20 minutes, samples were cleared by centrifugation at 14,000 g and 4 °C for 15 minutes. The supernatant was split and used for either direct loads or immunoprecipitation. Immunoprecipitations, SDS/PAGE and phosphor-imaging were performed as described (14).
Results

Expression, N-linked glycosylation and topology of hsHrd1

In order to gain information on the expression of hsHrd1, a polyclonal antiserum was developed against hsHrd1 by immunising a rabbit with the C-terminal 228 amino acids of the hsHrd1 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 four-hour treatment with tunicamycin resulted in a 3-4 fold increase in the amount of endogenous hsHrd1 protein in HeLa cells (Fig. 1A, lane 2). This is in agreement with the observation that elevated hsHrd1 mRNA levels occur under ER stress conditions (46). As judged from pulse chase experiments, both endogenous (Fig. 1B) and transiently transfected hsHrd1 protein (Fig. 1C) display a half-life of approximately 15 hours in HeLa cells (Fig. 1B, C), which renders hsHrd1 a protein of relatively high stability. Figure 1D shows subcellular localization of transfected hsHrd1 in HeLa cells. Most hsHrd1 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 hsHrd1 (indicated by arrows in Fig 1D). 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 hsHrd1 is a stable protein localized in the ER.

Figure 2A shows a schematic representation of the predicted membrane topology of hsHrd1, based on its hydrophobicity plot (according to Kyte and Doolittle (47)) and predictions through several algorithms from the Expasy internet site (SOSUI (TUAT; Tokyo Univ. of Agriculture & Technology), TMHMM (CBS; 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 (N\(\text{xT/S}\)) were found in the sequence, which 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 hsHrd1 (Fig. 1A, B). Indeed, Fig. 2B shows that treatment of transfected hsHrd1 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 hsHrd1 is not N-glycosylated. These results are, however, inconclusive in establishing the topology of hsHrd1, and we therefore addressed hsHrd1 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 \(^{35}\text{S}\) labelled amino acids. The reaction mixture was then digested with proteinase K. Only cytosolic parts of hsHrd1 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 hsHrd1, 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 hsHrd1 (data not shown). Taken together, the data shown in Fig. 2 strongly suggest that the topology of hsHrd1 corresponds to the model presented in Fig. 2A, and is therefore comparable to the topology that has been proposed for yeast Hrd1p/Der3p (30, 48).

The HsHrd1 RING-H2 domain has in vitro ubiquitination activity.

To evaluate the ability of the RING-H2 domain in hsHrd1 to interact with specific E2 enzymes, we fused the RING-H2 domain to the C-terminus of glutathione S-transferase (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. The specific E2 enzymes tested included the yeast and human Ubc2 (Rad6), Ubc7 and Ubc4. ScHrd1p is known to cooperate with ScUbc7 in ubiquitin-mediated degradation of HMGcoA reductase (29), and our analysis to be described below is consistent with the model that the RING domain in HsHrd1 interacts specifically with HsUbc7.

Incubation of GST-HsHrd1-RING with HsUbc7 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 K48 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 K48-linked ubiquitin dimer was also formed by incubating yeast Ubc7 and GST-ScHrd1-RING in similar reactions (Fig. 3A, lanes 7-8). 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 is also conjugated to lysines within the human and yeast GST-
RING fusions (Fig. 3A; Ub-GST-RING) although this reaction is less robust than ubiquitin dimer formation.

The formation of K48-linked ubiquitin dimer is apparently specific for Ubc7 since this product was not formed when Ubc7 was replaced by either Ubc2/Rad6 (data not shown) or Ubc4/UbcH5b (Fig. 3B). Neither ubiquitin dimer or ubiquitin-GST-RING fusion products could be detected by replacing Ubc7 with either human or yeast Ubc2/Rad6 (data not shown). Replacing Ubc7 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 lysine-less UbK0 mutant, ubiquitin can be linked to as much as six lysine sites in the GST-RING fusions (Fig. 3B, lanes 4,8). In addition, ubiquitin-ubiquitin linkages on the GST-RING fusion could also form, resulting in dense smears (compare lanes 2 to 4, and 6 to 8). These ubiquitin-ubiquitin linkages are not confined to K48 in ubiquitin since they occurred even by replacing ubiquitin with UbK48R in the reactions (lanes 3,7). Thus, while Ubc4/UbcH5b can interact with the Hrd1-RING domain and produce ubiquitinated products efficiently in vitro, it does not support the specific K48 ubiquitin-ubiquitin linkage in a polyubiquitin chain that targets proteins to the proteasome (49,50).

*Over-expression of hsHrd1 accelerates basal but not metabolically regulated degradation of endogenous HMGR.*

Since yeast Hrd1p is implicated in the degradation of Hmg2p, we examined whether hsHrd1 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 (17,34,46,51). 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, which provides an instrument to study involvement of E3 ligases in
degradation of particular substrate proteins.

Sterol-depleted naïve NIH-3T3 cells, or cells stably over-expressing either the wild-type hsHrd1, or the RING finger mutant, hsHrd1C2A, were pulse-labelled, 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 (25,28), 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} = 8.9h) from 8.9h (Fig. 4 top panel, lanes 1-5) to 1.7h (Fig. 4 top panel, lanes 6-10, note the different chase times in the presence of sterols). In the hsHrd1-overproducing cells, degradation of HMGR proceeded with markedly faster kinetics (t_{1/2} = 2.9h; 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} = 1.6h; Fig. 4 middle panel, lanes 6-10).

Over-expression of the hsHrd1C2A mutant reversed the phenotype of over-expressed hsHrd1 in sterol-depleted cells and the basal half-life of HMGR returned to its value in naïve cells (t_{1/2} = 9.5h; Fig. 4 bottom panel, lanes 1-5). Yet again, there was no effect of mutant hsHrd1 on the sterol-accelerated degradation of HMGR (t_{1/2} = 1.9h; Fig. 4 bottom panel, lanes 6-10). These results demonstrate that hsHrd1 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 hsHrd1 that take 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 hours with sterols, or 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 hsHrd1- and mutant hsHrd1C2A-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 hsHrd1. 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 hsHrd1-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 over-expressing mutant hsHrd1 the amounts of ubiquitinated HMGR recovered upon treatment with MG-132 were much lower than in cells expressing wild type hsHrd1 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 hsHrd1-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
over-express wild type hsHrd1 is the result of enhanced ubiquitination of the enzyme. Thus, hsHrd1 may function \textit{in vivo} as a protein-ubiquitin ligase for HMGR degradation in sterol-depleted cells.

\textit{Involvement of hsHrd1 in ERAD of other proteins}

We next tested whether hsHrd1 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 partners (3). To study the role of hsHrd1 in the degradation of TCR-\(\alpha\), 3T3 cells were transiently transfected with TCR-\(\alpha\) alone, or co-transfected with TCR-\(\alpha\) and wild-type hsHrd1, or TCR-\(\alpha\) and hsHrd1C2A. Fig. 6A shows rapid degradation of TCR-\(\alpha\) in wild-type and hsHrd1-transfected 3T3 cells (lanes 1-6, and 7-12 respectively). Expression of mutant hsHrd1 along with TCR-\(\alpha\) markedly delayed the degradation of TCR-\(\alpha\), exhibiting a pronounced dominant negative effect of the mutated RING finger of hsHrd1 (Fig. 6A). Effect on degradation of TCR-\(\alpha\) was also examined in 3T3 cells that stably express wild-type or mutant hsHrd1, by transducing these cells with a retrovirus encoding TCR-\(\alpha\). Compared to wild-type hsHrd1, hsHrd1C2A 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 hsHrd1 plays a role in the degradation of TCR-\(\alpha\), a \textit{bona fide} ERAD substrate.

A similar series of experiments was performed to analyse the role of hsHrd1 in degradation of CD3-\(\delta\). HeLa cells were transfected with CD3-\(\delta\) alone, or with CD3-\(\delta\) and variants of the hsHrd1 protein (Fig. 7A). Again, GFP was co-transfected to serve as a loading control. Steady state levels of CD3-\(\delta\), in equally transfected HeLa cells, were analysed by Western blotting (Fig. 7A). Co-transfection of RING finger mutant variants of hsHrd1 resulted in increased amounts of CD3-\(\delta\) (Fig. 7A; compare lanes 2 to 3 and 4.
to 5), indicating an inhibited degradation of the substrate by the dominant negative form of hsHrd1. Gp78/AMFR, another known RING finger E3 ligase involved in mammalian ERAD, has been shown also to be involved in CD3-δ degradation (34). Co-expression of CD3-δ and a RING finger mutant of gp78/AMFR indeed resulted in increased steady state levels of CD3-δ (Fig. 7A, lanes 6 and 7). The effect of gp78 and its RING finger mutant on the degradation of CD3-δ was comparable to what we have found for the hsHrd1 variants. Pulse chase experiments confirmed the role of hsHrd1 in the degradation of CD3-δ (Fig. 7B). Expression of wild type hsHrd1 increased the degradation rate of CD3-δ (Fig. 7B compare lanes 1-3 and 4-6), and expression of the RING finger mutant slowed down degradation of CD3-δ (Fig. 7B compare lanes 1-3 to 7-9).

The results indicate that degradation of a single substrate, CD3-δ, may be catalyzed by different E3 ligases. This observation may explain the moderate effect of mutant hsHrd1 on the degradation of CD3-δ, since endogenous gp78 (in addition to endogenous hsHrd1) may compete for binding to CD3-δ and catalyze its ubiquitination.

In support of our conclusion that hsHrd1 variants influence CD3-δ degradation, is the co-precipitation of mutant hsHrd1 with CD3-δ and vice-versa (Fig. 7C). Since mutant hsHrd1 does not possess E3 ligase activity due to the disruption of its RING finger motif, its association with the substrate is prolonged compared to wild-type hsHrd1, 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, hsHrd1, 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 hsHrd1 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 (50,52). 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 nonsterol isoprenoid farnesyl pyrophosphate (FPP) (27). It appears that degradation of mammalian HMGR is mechanistically more complex, since it is regulated by both sterol and nonsterol signals (25). Additionally, recent studies have pointed out to a key role of the ER resident Insig protein(s) in the regulated turnover of HMGR in mammalian cells (53,54), while no counterparts of such proteins have been found yet in yeast.

The hsHrd1 described here, hastens the turnover of mammalian HMGR only when over-expressed in sterol-depleted cells. HsHrd1 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 MVA-derived nonsterol metabolite(s) such as FPP. This would conform to the role of yeast Hrd1p in nonsterol 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 hsHrd1-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. Over-expression of the hsHrd1 mutant does not slow down the degradation of HMGR in sterol-depleted cells (Fig. 4, lower panel compared to upper panel). This result may be explained by the possibility that hsHrd1 is not the sole E3 ligase serving the basal degradation of HMGR, but is shunted to this process when over-expressed, a situation that may be achieved in vivo by ER stress. Alternatively, the lack of a dominant negative effect on the basal degradation by over-expression of the RING-finger mutant of hsHrd1 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 (30). In humans, SEL1L is a proposed homologue of Hrd3p (55), and it remains to be investigated whether SEL1L interacts with hsHrd1. Since we show here that both endogenous and transfected hsHrd1 are relatively stable proteins (Fig. 1B, C), it seems unlikely that SEL1L ensures the stability of hsHrd1 in a similar manner as observed in yeast.

We showed that HsHrd1 influences the degradation of individual subunits of the T-cell receptor complex, TCR-α and CD3-δ, two “classic” ERAD substrates. Over-expression of wild-type hsHrd1 did not increase the rate of TCR-α degradation (Fig. 6). This suggests that, for this substrate, the endogenous level of hsHrd1 may not be limiting. Possibly, other components of the ER quality control machinery that "decide" whether
and when TCR-α is to be degraded are rate limiting, and the degradative rate for TCR-α may not increase by the addition of more wt hsHrd1. However, over-expression of the RING-finger mutant hsHrd1 had a marked inhibitory effect, indicating that the mutant can exert a dominant-negative function. This suggests that hsHrd1 has high affinity for components of the ERAD machinery that take part in the elimination of TCR-α.

Moreover, unlike HMGR in sterol-depleted cells, TCR-α has a very short half-life. Thus, its stabilization by a dominant-negative hsHrd1 will be readily observed.

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-δ (34). This finding of a common substrate may be explained by the observation that gp78/AMFR and hsHrd1 exhibit considerable homology in their N-terminal and RING finger regions (see Fig 8; 28% overall similarity and 18% identity; 69% similarity and 54% identity within the RING finger domain). Additionally, both E3 ligases seem to use Ubc7 as an E2 conjugating enzyme ((34) and Fig. 3A). Gp78/AMFR and hsHrd1 may have evolved from the same ancestor, from which yeast Hrd1p may have originated as well.

Although gp78/AMFR and hsHrd1 are likely to serve diverged populations of ERAD substrates, some overlap may be expected due to their homology, and CD3-δ 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 hsHrd1 levels are upregulated upon ER stress (Fig. 1A, B), one might hypothesize that hsHrd1 is an E3 ligase that supports the degradation of all ERAD substrates upon ER stress in order to increase the general degradative capacity of the cell. However, while it is likely that the three substrates identified in this paper belong to only a subset of substrates that are handled by hsHrd1, 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 CFTRΔF508 (2) is not influenced by hsHrd1 (51). Additionally, we evaluated the possible role of hsHrd1 in the rapid ER degradation of MHC class I molecules in the presence of human cytomegalovirus (HCMV) US11 or US2 proteins (6). Transient over-expression of wild type and RING finger mutant forms of hsHrd1 in this system did not affect MHC class I degradation (data not shown). Also, degradation of α1-antitrypsin Hong-Kong null mutant protein (56) was found not to be influenced by hsHrd1 (data not shown). These observations indicate that hsHrd1 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.

Acknowledgements

We thank Dr. Theo van Laar, Dr. Shoshana Bar-Nun and Dr. Randy Hampton for helpful discussions. We are grateful to Dr. Allan Weissman for providing gp78 expression plasmid and specific antiserum, and to Dr. Ron Kopito for the TCR-α expression plasmid and antibodies. Peter van den Elsen is gratefully acknowledged for providing the CD3-δ plasmid. J. Dohlman is thanked for providing a yeast strain to purify E1 enzyme. Dr. Mirjam Heemskerk and Dr. Frits Koning are thanked for providing us with several useful materials. We are thankful to Dr. Jacques Neefjes for helpful discussions and for the anti-GFP antiserum. This study was supported by a grant from the Dutch Cancer Society (KWF/RUL 98-179) to G.H. and by an NIH grant (GM62194) to V.C.
Reference List


**Figure Legends**

Fig. 1 *Endogenous hsHrd1 protein is upregulated by ER stress*

A, HeLa cells were either left untreated (None), or treated with 10 μg/ml tunicamycin (Tun) for 4 hours. The cells were metabolically labeled with $^{35}$S-promix. Cell lysates were subjected to immunoprecipitation with anti-hsHrd1 polyclonal antiserum and anti-transferrin receptor (Tfr) antibodies. B, Pulse-chase analysis of endogenous hsHrd1 in HeLa cells, with or without tunicamycin treatment. Cells were metabolically labeled for one hour and chased for the times indicated. HsHrd1 was isolated from denatured cell lysates by immunoprecipitation.
with anti-hsHrd1 polyclonal antiserum. C, Pulse chase analysis of transfected hsHrd1 and hsHrd1C1A (hsHrd1*) in HeLa cells; method same as in (B).

Transferrin receptor (Tfr) and hsHrd1 were isolated from denatured cell lysates by immunoprecipitation with anti-hsHrd1 polyclonal antiserum and anti-transferrin receptor (Tfr) antibodies. Asterisks on the side of the gels in panels (A), (B) and (C) indicate a nonspecific band that precipitates with the protein A or protein G beads used. D, Immunofluorescence analysis of transfected hsHrd1 in HeLa cells. Cells were transfected with hsHrd1 expressing plasmid, and stained for hsHrd1 (left panel) and calnexin (middle panel) using respective specific polyclonal antibodies, and Cy3 (left panel) or fluorescein isothiocyanate (FITC, middle panel) –conjugated anti-immunoglobulin second antibodies. The right panel shows an overlay. Clusters of protein are indicated with arrowheads.

Fig. 2 Membrane topology and N-linked glycosylation of hsHrd1

A, Schematic representation of the predicted membrane topology of hsHrd1. N-linked glycosylation motifs (NxT/S) in the protein are indicated, as well as the RING-H2 motif. B, HsHrd1 was expressed in HeLa cells that were also transfected with HCMV US11 protein. The cells were metabolically labeled with 35S-promix and HsHrd1 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 hsHrd1. HsHrd1, tagged at the extreme C-terminus with a myc-epitope, was translated in the presence of microsomes and 35S 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.
**Fig. 3** The RING domain of hsHrd1 has in vitro E3 ligase activity

*In vitro* ubiquitination assays were performed with purified E1 enzyme, E2 enzyme (HsUbc7, ScUbc7, UbcH5b or ScUbc4, as indicated), purified GST-hsHrd1-RING-H2 (derived from human Hrd1) or GST-ScHrd1p-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 on SDS-PAGE gels, and stained with Coomassie Brilliant Blue. *A*, Assays with hsUbc7 or scUbc7 as an E2, and wt and K48R ubiquitin as indicated. *B*, Assays with UbcH5b or ScUbc4 as an E2, and different ubiquitin variants as indicated.

**Fig. 4** Effect of hsHrd1 on degradation of HMGR

Naïve 3T3 cells and 3T3 cells stably transfected with hsHrd1 or hsHrd1C2A (hsHrd1*) were treated with sterols, or mock-treated. A pulse-chase analysis was performed as indicated, and HMGR was immunoprecipitated from lysed cells.

**Fig. 5** Effect of hsHrd1 on ubiquitination of HMGR

Naïve 3T3 cells and 3T3 cells stably transfected with hsHrd1 and hsHrd1C2A (hsHrd1*) were mock-treated, or treated with sterols, or proteasome inhibitor MG-132, or both. Cells were lysed and HMGR was immunoprecipitated. The immunoprecipitate was analysed on Western blot using anti-ubiquitin antiserum (upper panel), or anti-HMGR antiserum (lower panel). Arrow in upper panel indicates migration level of HMGR protein.

**Fig. 6** Effect of hsHrd1 on degradation of TCR-α

*A*, 3T3 cells were transiently transfected with TCR-α, or TCR-α and hsHrd1
(FLAG-tagged), or TCR-α and hsHrd1C2A (hsHrd1*; FLAG-tagged). A pulse-chase experiment was performed with and without the addition of proteasome inhibitor MG-132. HsHrd1 (hsHrd1*) and TCR-α were immunoprecipitated with anti-FLAG and anti-TCR-α HA28-710 monoclonal antibodies respectively. B, 3T3 cells stably transfected with hsHrd1 or hsHrd1C2A (hsHrd1*) were transduced with retrovirus expressing TCR-α and GFP. A pulse-chase analysis was performed as indicated, and TCR-α and GFP were immunoprecipitated from cell lysates using polyclonal antiserum against TCR, and anti-GFP antiserum respectively.

**Fig. 7** Effect of hsHrd1 on degradation of CD3-δ, and co-precipitation of hsHrd1 with CD3-δ

**A,** HeLa cells were co-transfected with CD3-δ (1 ¼g) and GFP (0.2 ¼g), and different hsHrd1 or gp78 variants (1 ¼g) as indicated. Asterisk indicates RING finger mutants, (myc) indicates C-terminally myc-tagged proteins. 24 hours after transfection cell lysates were analyzed on Western blot using polyclonal antiserum against CD3-δ and GFP respectively. Quantifications relative to cells transfected with CD3-δ and GFP only (mock) are shown in the lower panel. **B,** HeLa cells were co-transfected with CD3-δ and hsHrd1 variants as indicated. 24 hours after transfection cells were metabolically pulse-labeled with 35S-promix and chased for the times indicated. CD3-δ was immunoprecipitated with specific polyclonal antiserum. **C,** HeLa cells were co-transfected with CD3-δ and hsHrd1 or CD3-δ and hsHrd1 RING finger mutant as indicated. The left panel shows immunoprecipitation with anti-CD3-δ antibodies; hsHrd1C1A mutant co-precipitates with CD3-δ. The right panel shows immunoprecipitation with anti-hsHrd1 antibodies, and co-immunoprecipitation of CD3-δ with hsHrd1C1A mutant protein is indicated.
Fig. 8 Alignment of protein sequences of *S. cerevisiae* (sc), *H. sapiens* (hs) Hrd1, and gp78/AMFR.

RING-H2 motifs are underlined.
Figure 1
Figure 2
Figure 3
<table>
<thead>
<tr>
<th>Cells</th>
<th>Addition:</th>
<th>None</th>
<th>Sterols</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive 3T3</td>
<td>0</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>3T3 hsHrd1</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>3T3 hsHrd1+</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

Chase time (hrs): 0 2 4 7 10 0 .5 1 2 4

IP: anti-HMGR

Figure 4
Figure 5
Figure 6
Figure 7
Figure 8
Human Hrd1 is an E3 ubiquitin ligase involved in degradation of proteins from the endoplasmic reticulum

Marjolein Kikkert, Ram Doolman, Min Dai, Rachel Avner, Gerco Hassink, Sjaak van Voorden, Swapna Thanedar, Joseph Roitelman, Vincent Chau and Emmanuel Wiertz

*J. Biol. Chem.* published online October 30, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M307453200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts