The ubiquitylation machinery of the endoplasmic reticulum

Christian Hirsch[1], Robert Gauss[1], Sabine C. Horn[1], Oliver Neuber[1,2] & Thomas Sommer[1]

As proteins travel through the endoplasmic reticulum (ER), a quality-control system retains newly synthesized polypeptides and supports their maturation. Only properly folded proteins are released to their designated destinations. Proteins that cannot mature are left to accumulate, impairing the function of the ER. To maintain homeostasis, the protein-quality-control system singles out aberrant polypeptides and delivers them to the cytosol, where they are destroyed by the proteasome. The importance of this pathway is evident from the growing list of pathologies associated with quality-control defects in the ER.

About 20% of the proteins encoded by the human genome are predicted to be secretory proteins[1]. These proteins pass through the endoplasmic reticulum (ER) on the way to their destination in membranes, exocytotic and endocytotic compartments or the cell exterior. But far from being a passive conduit, the ER is home to an array of molecular chaperones that help proteins to fold and guide their maturation. Despite this support, protein biogenesis is prone to errors. Approximately one-third of all newly synthesized proteins are degraded co-translationally or destroyed within minutes of their synthesis[2], suggesting that these polypeptides fail to attain their native conformation owing to mutations, transcriptional and translational errors, folding defects or the imbalanced manufacture of subunits. Mature proteins can be damaged by environmental conditions, such as high-energy radiation, chemical insults or metabolic by-products. The malfunction or aggregation of defective proteins challenges the homeostasis of the ER and the cell as a whole. As a result, evolution has produced an ER-resident protein-quality-control system that operates on several levels to maintain the integrity of the ER.

When polypeptides are first synthesized, they are protected from degradation by a specific N-linked glycan structure to allow their maturation. Later, potentially misfolded substrates are flagged up by a unique glycan code generated by mannosidases in the ER (Fig. 1). This signal is decoded by a ubiquitin ligase anchored in the ER membrane. Proteins committed for degradation are transported across the ER membrane, ubiquitylated and degraded by the 26S proteasome, by a process referred to as ER-associated degradation (ERAD). Misfolded proteins are not the only substrates to fall victim to this system; it also regulates sterol synthesis by eliminating the pathway's rate-limiting enzyme when sterols are abundantly available[3].

Initially, biochemical and genetic screens allowed researchers to identify the components that constitute the ERAD system. Now, the field has moved to functional aspects of this system, as exemplified by recent discoveries on the breakdown of misfolded glycoproteins. Here we review how the ER's protein-quality-control system selectively disposes of aberrant proteins without jeopardizing nascent polypeptides. Because the ERAD pathways seem to be conserved from yeast to mammals, we will use the yeast *Saccharomyces cerevisiae* to outline the fundamental processes and extend our findings with examples from mammalian systems. Figure 2 gives an overview of the relevant yeast and mammalian ERAD factors in their respective cellular compartments.

Glycans and protein folding

The prime function of the ER's protein quality-control system is to retain unfolded polypeptides in the ER. Hsp70-type and glycan-dependent chaperones bind non-native proteins to prevent their export to the Golgi, and act jointly with oxidoreductases to remodel proteins that have incorrect conformations. The canonical view suggests that the export machinery accepts only proteins that have attained their native fold on the basis of exit signals embedded in the primary sequences of the polypeptide[4]. An alternative model suggests that there is a flexible standard for protein export[5] based on the energetics of a protein's fold and a cell-specific folding environment (Fig. 3 and Box 1).

Most polypeptides synthesized in the ER are modified by N-linked oligosaccharides. Oligosaccharyltransferase covalently attaches glucose$_3$-mannose$_9$-*N*-acetylglucosamine$_2$ oligosaccharides to asparagines in Asn-X-Ser/Thr motifs of polypeptides that enter the ER (Fig. 4). Initially, N-glycans render proteins more hydrophilic and direct the co-translational folding of proteins[6]. Moreover, modifications in the oligosaccharide structure provide information about the current folding state of a protein. The two outermost glucose moieties are then immediately removed by α-glucosidases present in the ER to label polypeptides as in the process of folding (Fig. 4). At this stage, glycan-dependent chaperones associate with the protein. In mammalian cells, calnexin or calreticulin bind to monoglucosylated glycoproteins and promote their maturation[7]. When liberated from these chaperones, ER α-glucosidase-II trims the residual glucose moiety to prevent a reassociation with calnexin or calreticulin. Glycoproteins that have successfully attained their native structure are allowed to leave the ER[8].

But what about polypeptides that require more assistance to mature? Being the main folding sensor of the mammalian ER, UDP-glucose:glycoprotein glucosyltransferase (GT, also termed UGGT) recognizes non-native polypeptides and reglucosylates the A branch of oligosaccharides to initiate their reassociation with calnexin or calreticulin for further folding[9] (Fig. 4b). This calnexin–calreticulin cycle serves as a first layer of protein quality control in mammalian cells. However, if a polypeptide has exhausted its chances to mature, further trimming of the oligosaccharide can trigger its destruction.

Moulding the signal for destruction

Polypeptides that are unable to acquire their native structure must be degraded to prevent fruitless folding attempts and stop the ER from being

[1]Max-Delbrück Center for Molecular Medicine, Robert-Rössle-Strasse 10, 13125 Berlin, Germany. [2]Present address: Institute for Genetics, University of Cologne, Zülpicher Strasse 47, 50674 Cologne, Germany.

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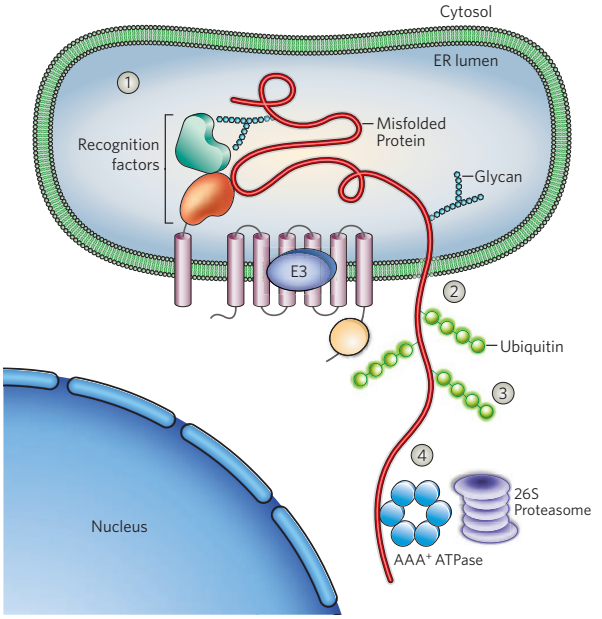

Figure 1 | Protein degradation at the endoplasmic reticulum (ER). The degradation of misfolded protein in the ER involves several distinct steps. In step 1, ubiquitin ligases embedded in the ER membrane cooperate with accessory recognition factors to recognize misfolded proteins. Next, in the dislocation phase (step 2), proteins are exported into the cytosol via a so far unidentified channel. At the cytosolic face of the ER, the substrate is ubiquitylated by an E3 ligase (step 3). Finally, the substrate is removed from the membrane by the AAA+ ATPase Cdc48 and escorted to the 26S proteasome for degradation (step 4).

congested with misfolded polypeptides. The 'mannose timer' model proposes that the removal of mannose residues terminates the time a protein is granted to acquire its native conformation[10]. Indeed, members of the glycosylhydrolase-47 (GH47) family in the ER initiate the disposal of misfolded polypeptides. In yeast, the GH47 family encompasses two proteins found in the ER: Mns1 (an α1,2-mannosidase) and the mannosidase-like protein Htm1 (homologue to mannosidase 1, also termed Mnl1 for mannosidase-like 1). Mns1 removes the outermost mannose residue from the B branch of N-glycans[11] (Fig. 4). This trimming step affects virtually all glycoproteins in the ER without contributing to glycoprotein maturation or yeast viability[12,13]. Deletion of the *MNS1* gene, however, delays the breakdown of aberrant proteins[13], suggesting that glycoproteins are protected from degradation until Mns1 converts a mannose$_9$-*N*-acetylglucosamine$_2$ glycan to a mannose$_8$-*N*-acetylglucosamine$_2$ glycan.

Because Mns1 processes proteins regardless of their folding status, the mannose$_8$ structure alone is not sufficient to trigger glycoprotein breakdown; an additional signature must distinguish defective polypeptides from mature proteins. Htm1 was initially proposed to function as a lectin[14,15], but although direct evidence is still missing, it seems to partake in the removal of the capping α1,2-mannose of the C branch from N-glycans that have been processed by Mns1 (ref. 16). The sequential action of Mns1 and Htm1 therefore generates a unique protein-bound mannose$_7$-*N*-acetylglucosamine$_2$ structure that flags potential ERAD substrates. This idea also explains why deletion of the gene encoding α1,3-mannosyltransferase (Alg3) bypasses the need for Htm1 during glycoprotein degradation[13]. The mannose$_5$-*N*-acetylglucosamine$_2$ structure found on N-glycoproteins of Δalg3 cells is already equipped with the terminal α1,6-mannosyl residue generated by Htm1. How Htm1 selects its substrates is not known. It forms a complex with the protein disulphide isomerase Pdi1 (ref. 16), so the action of Htm1 may be restricted to those substrates of Pdi1 that associate with the oxidoreductase for a prolonged time.

In mammalian cells, the GH47 family has diversified into four members found in the ER: ER α1,2-mannosidase-I (ERManI) and three mannosidase-like proteins, EDEM1, EDEM2 and EDEM3 (ref. 17). Like its yeast orthologue, mammalian ERManI hydrolyses mannose-α1,2-mannose bonds, liberating a mannose residue from the B branch[18]. The abundance of ERManI influences the stability of ERAD substrates. Knockdown of ERManI by short interfering RNA (siRNA) impairs degradation of the uncleaved precursor of asialoglycoprotein receptor H2a[19]. Overexpression of ERManI coincides with enhanced de-mannosylation and breakdown of the null Hongkong (NHK) variant of α1-antitrypsin[20,21].

As in yeast, the action of ERManI alone is not sufficient to flag a glycoprotein for degradation. Presumably, ERManI must cooperate with members of the EDEM family to trigger the disposal of aberrant glycoproteins. Although once believed to be lectins, data now suggest that the EDEMs are mannosidases like Htm1. Overexpression of EDEM1 and EDEM3 coincides with mannose trimming of glycans attached to substrates such as NHK, BACE451 and the α-subunit of the T-cell receptor[22,23]. Moreover, structural modelling of the EDEM proteins unravels striking similarities to ERManI and reveals that all the residues important for the catalytic activity of ERManI are conserved[24]. This suggests that at least EDEM1 and EDEM3 also function as mannosidases. How these enzymes select their substrates remains to be established, however. EDEM1 interacts with calnexin, where it may target glycoproteins that are trapped in the calnexin–calreticulin cycle for degradation[25,26].

The ERAD system sorts it out

Misfolded glycoproteins that have been processed by ER mannosidase I and Htm1 must ultimately arrive at a ubiquitin ligase for ubiquitylation. Aberrant substrates that are not glycosylated share this fate, although the events that initiate their degradation are poorly understood. An overview of ERAD substrates is given in Box 2. The heterogeneity of the defects that can occur and the notion that errors may arise in the luminal, transmembrane or cytosolic domain of a protein necessitate distinct E3 ligases, which harness accessory factors to recruit terminally misfolded proteins.

Different defects require different pathways

In yeast, there are two E3 ligases that target largely discrete substrates. The ligase Doa10 (degradation of Mat-α2–10) acts on substrates with lesions in their cytosolic domain, whereas substrates of the HRD (HMG-CoA reductase degradation) ligase typically have defects in their transmembrane or luminal domain. To emphasize these differences, an extension of the ERAD term was proposed: ERAD-C degrades proteins with defective cytosolic domains; ERAD-M recognizes lesions in membrane domains; and ERAD-L targets luminal substrates[27,28]. All three pathways commence at different branches and converge in the cytosol. Proteins with multiple defects are preferentially processed by ERAD-C, which seems to operate faster than the other pathways[28]. It should be noted, however, that this terminology is based on a limited set of substrates in yeast and may not be generally applicable.

Keynotes from ERAD in yeast

The HRD ligase is a membrane-anchored complex comprising at least five distinct subunits. Hrd1/Der3 features six transmembrane domains and a cytosolic RING-finger domain that ubiquitylates target proteins of the ligase[29,30]. Hrd1 interacts with Usa1 (U1-Snp1 associating-1)[27], the adapter for Der1 (degradation in the ER-1)[31]. The luminal domain of the HRD complex is composed of two proteins: Hrd3 and Yos9 (yeast OS-9 homologue)[27,32–34]. The latter is equipped with a mannose-6 phosphate receptor homology (MRH) domain that recognizes N-glycans containing a terminal α1,6-linked mannose[35], a structure generated with the help of Htm1 (ref. 16). Mutations in the MRH domain abolish binding of N-glycans and thus the breakdown of CPY* (CPY with a mutation of glycine to arginine at position 255) and other glycosylated polypeptides[35], suggesting that Yos9 links the recognition of misfolded glycoproteins to the ubiquitin–proteasome system. However, the

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deletion of Yos9 or removal of the glycosylation sites in CPY* does not disrupt the interaction between substrates and the HRD ligase[33,34]. So what is the function of Yos9 or the glycans on CPY* if they are not required for substrate binding? The MRH domain of Yos9 might scan client proteins for the presence of a mannose$_7$-*N*-acetylglucosamine$_2$ glycan without engaging in a stable interaction. Instead, other components of the complex may adhere to misfolded polypeptides. Indeed, Hrd3 binds aberrant proteins and has a preference for hydrophobic polypeptides[34]. Also associated with Yos9 is the Hsp70 chaperone Kar2 (karyogamy 2)[33], suggesting that the luminal domain of the HRD ligase could interact with misfolded proteins by multiple modes.

Although speculative, the following model may explain how unwanted polypeptides are selected by the HRD complex for clearance from the ER. Escorted by Kar2, non-native polypeptides arrive at the HRD ligase. These polypeptides can either be terminally misfolded species or proteins that will eventually mature. Next, Hrd3 binds the potential substrate, perhaps mediated by hydrophobic patches within the substrate. Meanwhile, Yos9 inspects client proteins for the presence of a terminal α1,6-linked mannose. Only substrates that are concurrently recognized by Yos9 and Hrd3 will be ubiquitylated by Hrd1, with glycoproteins that bear mannose$_{8-9}$ glycans being released for further refolding.

Overexpression of Hrd1 in the absence of Yos9 and Hrd3 results in the promiscuous degradation of ER-resident proteins[33], confirming that Hrd3 and Yos9 may act as gatekeepers to ensure that only legitimate substrates arrive in the cytosol for ubiquitylation. To this end, Hrd1 acts jointly with the soluble E2 enzyme Ubc1 (ubiquitin-conjugating enzyme 1) and with Ubc7, which is recruited to the ER membrane through interaction with Cue1 (coupling of ubiquitin conjugation to ER degradation 1)[29,36].

The second E3 ligase present in the ER membrane in yeast is Doa10. This protein was first isolated in a screen for factors that degrade the soluble transcriptional repressor Matα2 (ref. 37). With the identification of several membrane-anchored substrates, Doa10 was also linked to the ERAD pathway. Doa10 features an amino-terminal RING-finger and 14 transmembrane domains[38]. Despite this intricate membrane topology, the subunit composition of the complex is relatively simple: the only identified cofactors of Doa10 are the E2 enzymes Ubc6 and Ubc7. The latter is recruited to the ER membrane by Cue1 (ref. 36).

Mammalian ligases

Perhaps not unexpectedly, many more ligases have been characterized that participate in ERAD in mammals than in yeast. Two mammalian proteins with homology to yeast Hrd1 are known: HRD1 (or Synoviolin) and gp78 (also termed AMFR, for tumour autocrine motility factor receptor)[39–41]. HRD1 is part of a complex related to the yeast HRD ligase as it involves the human Hrd3 orthologue SEL1L (suppressor of Lin12-like protein)[42], HERP (Usa1 in yeast)[43,44], Derlin 1–3 (a family of Der1 representatives)[45,46] and OS-9 (amplified in osteosarcoma, the human orthologue of Yos9)[47,48]. Mammalian cells express two forms of OS-9: OS-9.1 and a splice variant, OS-9.2, which lacks 55 amino-acid residues. Both proteins interact with SEL1L and bind the misfolded NHK variant of α1-antitrypsin[47,48]. Consistent with reports on their yeast counterpart Yos9, binding does not require N-linked glycans, as the interaction persists when a non-glycosylated variant of NHK (NHK-QQQ) is used as substrate[47]. However, a lack of glycans does not result in the degradation of the client protein. Reduction of OS-9 levels by siRNA impairs NHK degradation and increases the amount of

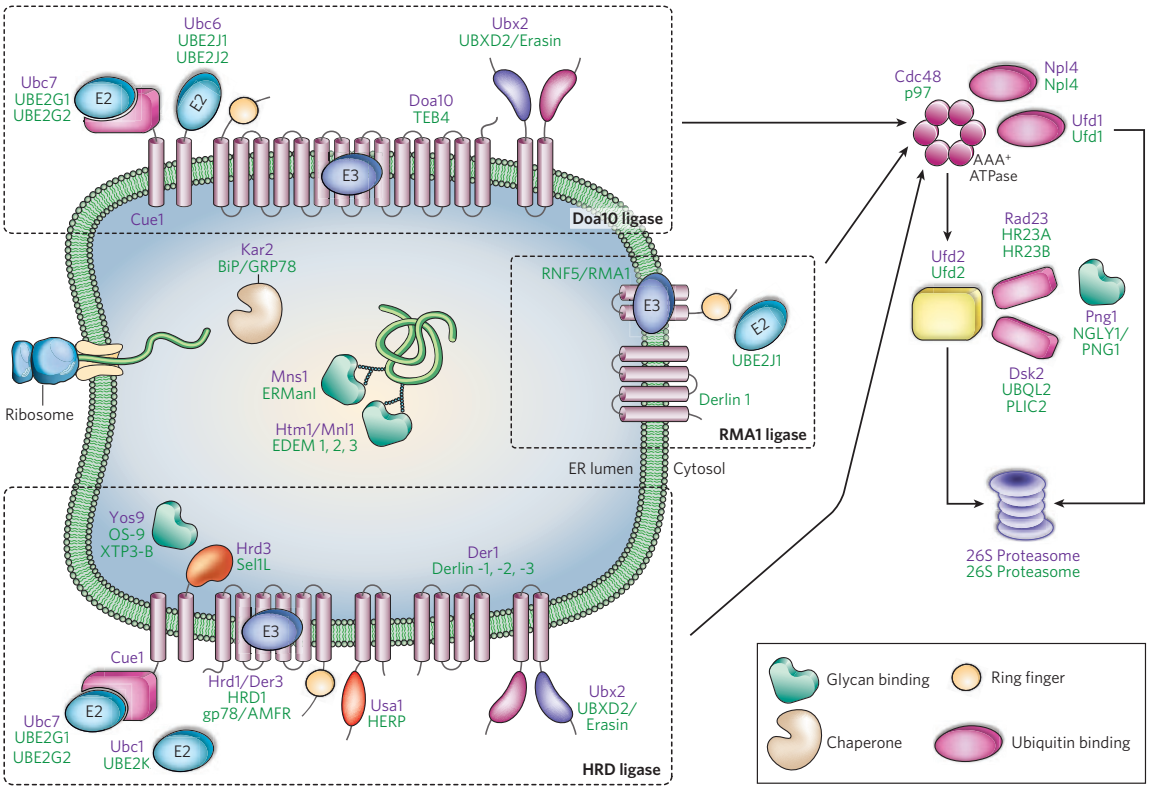

Figure 2 | Mechanism of protein degradation in yeast and mammalian cells. Molecular chaperones and proteins of the glycosylhydrolase-47 family (Mns1 and Htm1) detect misfolded polypeptides and direct them to membrane-bound ligases (Doa10, RMA1, HRD). After dislocation to the cytosolic side of the ER membrane, substrates are ubiquitylated by an E3 ligase. All ligase complexes comprise a central, catalytic RING-finger protein (E3), ubiquitin-conjugating enzymes (E2) and additional factors. The AAA$^+$ ATPase Cdc48 releases ubiquitylated molecules from the ER membrane. The adaptor proteins Rad23 and Dsk2 escort the ubiquitylated molecules to the 26S proteasome for degradation; concurrently Png1 deglycosylates glycoproteins through its association with Rad23. Yeast proteins are labelled in purple, their mammalian counterparts in green.

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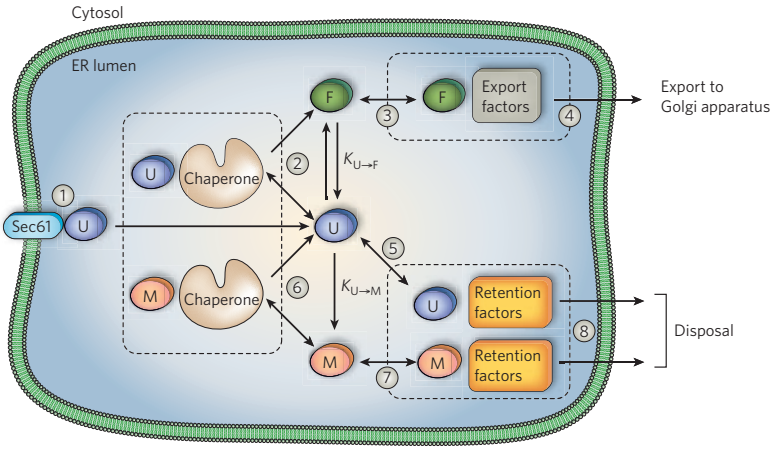

Figure 3 | Protein homeostasis in the ER. Proteins are translocated into the ER by Sec61 (step 1). Unfolded proteins (U) can engage chaperones, which help them fold into their native conformation (F, step 2). Export factors select correctly folded proteins (step 3) and transport them to the Golgi (step 4). Retention factors prevent the exit of unfolded proteins (step 5). Chaperones try to remodel any misfolded proteins (M) into a folding-competent, unfolded state (step 6). Retention factors keep misfolded proteins in the ER (step 7). Eventually, defective proteins, along with a fraction of folding intermediates (U), are exported to the cytosol for disposal (step 8). The equilibrium constants for the folding ($K_{U\to F}$) and misfolding ($K_{U\to M}$) reactions are indicated.

NHK secreted. Overexpression of OS-9 primarily reduces the secretion of NHK without enhancing degradation[47]. Thus OS-9 might perform two tasks: at physiological concentrations, it cooperates with SEL1L to facilitate the disposal of misfolded proteins; when overexpressed, excess OS-9 is unable to contact the HRD ligase and retains non-native proteins in the lumen of the ER.

Another mammalian orthologue of Yos9 is XTP3-B (XTP3-transactivated protein B, or Erlectin)[47,49]. This protein has two MRH domains and interacts with SEL1L. Like OS-9, XTP3-B binds NHK and its unglycosylated variant. Overexpression of XTP3-B delays the degradation of both substrates, whereas siRNA against XTP3-B has no influence on NHK stability[49]. Suitable substrates for XTP3-B may still await identification; alternatively, XTP3-B may retain immature proteins in the ER and prevent their aggregation, much like overexpressed OS-9.

HRD1 protects cells from stress-induced apoptosis[50], suggesting a broader role in the ubiquitylation of damaged ER proteins. Among the substrates of the HRD ligase identified are a truncated and misfolded form of Ribophorin termed RI332 (ref. 42), unassembled secretory Igμ chains[51] and a non-glycoslated variant of the Igκ light chain[52]. In the ER, this Igκ light chain exists in a fully oxidized form equipped with two disulphide bonds and a partly reduced variant that has one disulphide bond[52]. Only the partly reduced molecule interacts with both Derlin-1 and HERP, suggesting that one disulphide bond in the oxidized form of the Igκ light chain is reduced, before the light chain is recruited to the ligase[52]. The human cytomegalovirus (HCMV) gene product US11 (unique short region protein 11) also uses components of the HRD ligase as it requires SEL1L and Derlin-1 to target MHC-class-I heavy chains for destruction[42,45,46].

Gp78 and human HRD1 have more than 50% sequence homology in their transmembrane domains. Unlike Hrd1, which requires Cue1 to associate with Ubc7, gp78 is equipped with a G2BR (UBE2G2-binding region) domain distal to its RING-finger domain that recruits the E2 UBE2G2 (ubiquitin-conjugating enzyme E2G 2, the orthologue of yeast Ubc7) to the ligase[53]. Among the targets of gp78 is the Pi Z variant of α1-antitrypsin[54] and orphan subunits of the T-cell receptor, such as TCRα[53] and Cd3δ[39]. Furthermore, gp78 ubiquitylates HMG-CoA reductase[55], implying that the role of the ERAD system in sterol metabolism is broadly conserved. Another gp78 substrate of medical importance is KAI1, a tetraspanning protein that suppresses cancer metastasis[56]. Downregulation of gp78 by siRNA increases KAI1 levels and coincides

Box 1 | A flexible standard for protein export

It is commonly believed that the export machinery accepts only properly folded proteins, with aberrant polypeptides being degraded. This suggests that the endoplasmic reticulum (ER) uses a fixed quality-control standard based on the conformation of the wild-type protein. But this idea cannot explain observations such as cell-specific secretion efficiencies of mutant proteins[97,98]. What global principles govern protein quality control in the ER?

In an attempt to understand the basis of tissue-specific amyloid diseases caused by transthyretin (TTR), Sekijima *et al.* demonstrated that the secretion of TTR mutants correlates with a 'folding stability score'[99]. This variable integrates thermodynamic stability, defined as the Gibbs free-energy difference between the elongated polypeptide chain and the folded state, and kinetic parameters, such as the rate of TTR tetramer assembly. Accordingly, discrepancies in secretion efficiency in different tissues seem to be based on the varying activities of chaperones, export machineries and ERAD components that set cell-specific standards for export. For example, a particular protein might be exported in one cell type despite being degraded in another tissue because decreased chaperone activity lowers the 'folding stability score' threshold applied for export.

On the basis of this study, Wiseman *et al.* developed a quantitative model of folding for export (FoldEx)[5] that reduced the complex homeostasis network of the ER to a few basic pathways (translocation, chaperone-independent and chaperone-assisted folding, protein export and ERAD), each considered as an individual unit (Fig. 3). Treated like enzymes, the function of each pathway was described with Michaelis–Menten kinetics. Combining the different equations, the authors described the export efficiency mathematically depending on the folding equilibrium constant, which is related to the thermodynamic stability. This theoretical approach correctly describes the observation that in a given cell-specific environment the absolute thermodynamic stability of the protein's fold defines its export efficiency. It also explains why even very stable proteins, including multidomain proteins such as CTFR or multi-subunit complexes, can be degraded if they fold slowly. The model reveals that export efficiency depends on the ratio of ERAD and export activities and the rate of misfolding; that is, export efficiency decreases as protein misfolding becomes more likely.

The FoldEx model emphasizes that an 'adaptable standard' determines the export efficiency of each individual protein in a given cell type. This standard is established by the complex interplay of the protein's primary sequence and the local environment of the folding compartment (for example, chaperones and folding enzymes; metabolites; export and ERAD machineries), and can be adjusted to counterbalance conditions of cellular stress. Different proteins will compete for these machineries and, consequently, the export efficiency of a particular protein depends on the proteome that is expressed at a certain time.

The FoldEx model is the first approach to define a theoretical framework that combines the thermodynamic and kinetic parameters of protein folding and the adjustable activities of ER folding, ERAD and transport machineries. It is likely to prove a useful tool for further experiments.

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with a reduced metastatic aggressiveness of the tumour cells[56].

A likely candidate for the mammalian orthologue of Doa10 is TEB4 (ref. 57). Both proteins share the same transmembrane topology[38] and an N-terminal RING-finger domain. Although TEB4 ubiquitylates itself, substrates of this ligase are yet not known[57].

Apart from these ligases, which share obvious similarities with yeast enzymes, several mammalian ERAD ligases have been characterized that have no equivalent in fungi. The ER protein RMA1 (RING-finger protein with membrane anchor 1) is a ubiquitin ligase conserved from *Arabidopsis* to humans[58]. RMA1 interacts with UBE2J1, and overexpression of the ligase reduces the abundance of the cystic fibrosis transmembrane conductance regulator (CFTR)[58]. RMA1 primarily monitors the folding of the N-terminal domain of CFTR during or soon after translation[58]. Post-translationally, the cytosolic E3 ligase CHIP (carboxyl terminus of Hsp70-interacting protein) relieves RMA1 and monitors the folding of CFTR[58]. CHIP encompasses a U-box that is structurally related to the RING-finger motif and a tetratricopeptide repeat domain that interacts with Hsp70 and Hsp90 (ref. 59). The combination of chaperone binding and ubiquitin–protein ligase activity suggests that CHIP supervises Hsp70-mediated protein folding and triggers the proteasomal degradation of substrates that are irreversibly misfolded.

Another factor that cooperates with RMA1 is BAP31 (B-cell receptor-associated protein 31). This integral membrane protein is involved in the sorting of a diverse set of ER membrane proteins. BAP31 interacts with components of the Sec61 pore, the E3 protein RMA1 and Derlin-1 (ref. 60). Because siRNA-mediated knockdown of BAP31 stabilizes CFTR, BAP31 may chaperone newly synthesized CFTR polypeptides and divert variants that are refractory to folding through RMA1 and Derlin-1 for degradation.

Mutations in the ubiquitin ligase Parkin are associated with juvenile Parkinson's disease[61]. Expression of defective Parkin triggers the accumulation of Pael-R in the ER, followed by a degeneration of dopaminergic neurons, a hallmark of Parkinson's disease. Parkin ubiquitylates Pael-R *in vitro*, albeit with low efficiency. In combination with CHIP, this ubiquitylation is significantly enhanced[62].

The cytosolic SCF complex is a ubiquitin ligase comprising Skp1, Cul1, Roc1 (also called Rbx1) and an F-Box protein. Among the large set of F-Box proteins that determine the substrate specificity of the SCF ligase are two neuron-specific factors, Fbs1 and Fbs2 (F-box protein that recognizes sugar chains). Both proteins have a sugar-binding domain that binds to the innermost portion of N-linked glycans[63]. The substrates of the SCF^{Fbs1} complex have not yet been characterized, but it may clear the cytosol of glycoproteins that escaped the classical ERAD pathways or leaked from the ER through damage in the lipid bilayer.

Crossing the membrane

Before misfolded proteins can be ubiquitylated and degraded, they must cross the ER membrane. This export step is referred to as dislocation or retrotranslocation. How proteins are extracted from the ER is not known, but ideas proposed range from leaving via the Sec61 translocon[64,65], in a reaction that resembles a reversal of the import process, to the formation of lipid droplets that facilitate the escape of polypeptides from the ER[66].

The HRD ligase links important events on both sides of the ER membrane: substrate recruitment at the luminal side, and protein ubiquitylation at its cytosolic face[33,34,67]. It follows that the conduit for protein export should be in the proximity of the ligase. Perhaps the HRD complex itself discharges aberrant proteins into the cytosol. Associated with the HRD ligase are members of the Derlin family, which are considered prominent candidates for components of the translocation channel. The three proteins of this group show weak homology to yeast Der1 and form hetero-oligomers with each other[45,46]. Derlin-1 interacts with the HCMV protein US11, and MHC class I heavy chains are also present in this complex[45,46]. When the proteasome is inhibited pharmacologically, deglycosylated heavy chains occur in association with Derlin-1 (ref. 45). N-glycanase, the enzyme that deglycosylates class I heavy chains, is restricted to the cytosol[65,68,69]. The association between Derlin-1 and the heavy-chain molecules must therefore occur either during or immediately after dislocation of the substrate. Additional evidence that Derlin proteins may channel aberrant ER proteins to the cytosol comes from the observation that the ATPase associated with various cellular activities, the AAA^{+}-ATPase p97 (Cdc48 in yeast), interacts with Derlin-1 and Derlin-2 (refs 70, 71). As discussed below, Cdc48 contributes to the driving force that mobilizes ERAD substrates from the ER membrane. Derlin-1 is not required for the US2-mediated degradation of heavy chains[45], so it may serve a different function altogether, or alternative exits from the ER must exist.

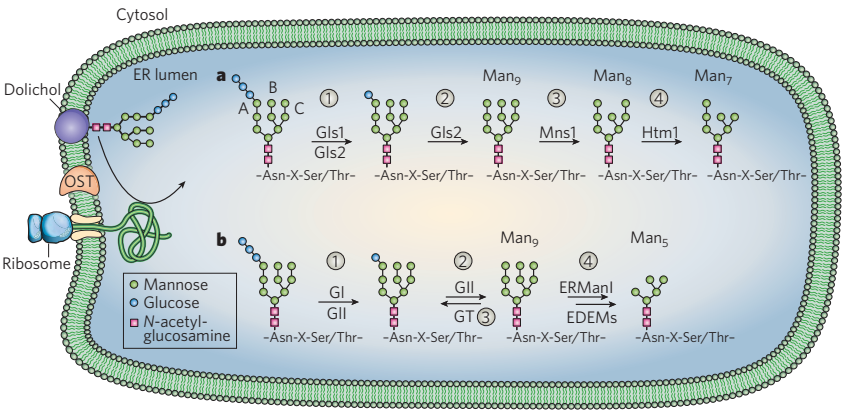

Figure 4 | Processing of N-linked glycans in the yeast and mammalian ER. a, In yeast and mammals, oligosaccharyltransferase (OST) transfers preassembled glucose$_3$-mannose$_9$-*N*-acetylglucosamine$_2$ oligosaccharide from a dolichol carrier to asparagine residues in Asn-X-Ser/Thr motifs in the emerging peptide. In yeast, Gls1 and Gls2 (glucosidases 1 and 2) remove the two outermost glucose residues and generate a glucose$_1$-mannose$_9$-*N*-acetylglucosamine$_2$ sugar (step 1). Further processing by Gls2 results in a mannose$_9$-*N*-acetylglucosamine$_2$ glycan that protects the glycoprotein from disposal (step 2). Mns1 trims the outermost mannose moiety of the B (middle) branch, yielding a mannose$_8$-*N*-actelyglucosamine$_2$ oligosaccharide, which indicates a protein that is retained in the ER for a prolonged period of time (step 3). Htm1 then processes the C (right-hand) branch, yielding a mannose$_7$-*N*-actelyglucosamine$_2$ oligosaccharide (step 4). **b**, In mammalian cells, steps 1 and 2 resemble those in the yeast system, yet removal of the last glucose residue from the A branch by glucosidase-II (GII, step 2) is reversible. Glucosyltransferase (GT) reglucosylates glycoproteins that have not attained their native fold (step 3). Extensive demannosylation by ERManI and probably the ER degradation enhancing EDEM proteins yield a mannose$_5$-*N*-acetylglucosamine$_2$ glycan that is a signal for disposal (step 4).

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Membrane release and destruction

The movement of aberrant proteins across the ER membrane requires a force to provide directionality. Additionally, substrates must be released from the membrane to become accessible to the proteasome. Both transport and release require energy and may be coupled. The energy is in part required for the ubiquitylation of the substrate, which is a prerequisite for dislocation[72,73]. Another energy-dependent step is mediated by Cdc48 (p97 in mammals)[73–77]. This AAA-ATPase forms homohexamers that associate with the cofactors Ufd1 (ubiquitin fusion degradation 1) and Npl4 (nuclear protein localization 4) to mobilize aberrant ER proteins from the ER membrane. The molecular mechanism of this process is unclear. According to the 'molecular ratchet' model[78], coordinated cycles of ATP hydrolysis induce conformational changes within the complex and propel the membrane release of substrates that attach to the N terminus of p97. In a different model, the guanidyl-rich environment of an arginine-rich ring at the base of the complex forms a 'denaturation collar' that unfolds substrates through denaturation[79]. To exert mechanical force on substrates, the Cdc48 complex must be fastened to the ER membrane. Consequently, Cdc48 interacts with the UBX (ubiquitin regulatory X) domain of the membrane-anchored protein Ubx2 by a mechanism that depends on a ubiquitylated substrate[80,81]. The mammalian candidate protein for this function is UBXD2 (or ERASIN)[82].

When the substrates are polyubiquitylated and extracted from the membrane, they are forwarded to the proteasome by the action of Rad23 (radiation sensitive 23) and Dsk2 (dominant suppressor of Kar1)[83,84]. These homologous adaptor proteins have a C-terminal UBA (ubiquitin-associated) motif that binds polyubiquitin chains linked by a lysine at ubiquitin residue 48 and an N-terminal UBL (ubiquitin-like) domain to guide adaptor and substrate to the proteasome, which recognizes polyubiquitylated substrates through its subunits Rpn10 (regulatory particle non-ATPase 10)[85] and Rpn13 (refs 86, 87). In addition, Dsk2 and Rad23 may shield the polyubiquitin chain from the action of ubiquitin hydrolases. In this canonical pathway, the E3 ligase alone polyubiquitylates substrates. In yeast there is a second route, in which the substrate is first mono- or diubiquitylated by the ligase[84]. Further chain elongation is catalysed by the U-Box protein Ufd2, which interacts with Cdc48 (ref. 84). Cdc48 limits the action of Ufd2 to the attachment of a short oligoubiquitin chain of four to six molecules, which is large enough for recognition by Rad23 and Dsk2 (ref. 84). This incremental increase of the ubiquitin chain may provide the directionality required to target substrates to the proteasome.

Because N-glycanase interacts with Rad23 (ref. 88) and possibly with other components of the ERAD machinery, glycoproteins are liberated from their oligosaccharides when they reach the cytosol. This trimming step is not crucial for degradation[89,90], but removal of the bulky side groups may facilitate the threading of substrates into the channel that leads to the active-site chamber of the proteasome. Before proteasomal degradation, deubiquitylating enzymes remove the polyubiquitin chain from the substrate and the ubiquitin moieties are recycled[91–93].

Box 2 | ERAD substrates

α1-Antitrypsin (α1-AT)
A soluble, glycosylated serine-protease inhibitor secreted by hepatocytes. The NHK (null Hong Kong) variant harbours a premature stop codon. Although folding-defective, a fraction of the NHK variant escapes the ER quality control and is secreted. NHK-QQQ is an unglycosylated, engineered variant. In the Pi Z variant, a mutation of glutamic acid to lysine at position 342 interferes with folding and prevents secretion. In certain cases, Pi Z accumulates in the ER, leading to liver cirrhosis. The lack of α1-AT triggers lung emphysema, owing to increased activity of neutrophile elastase, which disrupts the connective tissue.

ASGPR H2a
A subunit of the asialoglycoprotein receptor. This receptor clears desialylated glycoproteins from the circulation by receptor-mediated endocytosis.

BACE457
A pancreatic isoform of the β-secretase (BACE) that does not contribute to the processing of the amyloid precursor protein. Instead, BACE457 is degraded.

CD3δ and TCRα
Members of the T-cell receptor, which is assembled in the ER. Orphan subunits are degraded. Likewise, secretory Ig-μ is degraded in the absence of the light chain.

CFTR (cystic fibrosis conductance regulator)
A chloride channel expressed by epithelial cells. The ΔF508 mutation is the most common cause of cystic fibrosis, an inherited autosomal recessive disease. The ΔF508 deletion only marginally impairs the biological activity of the channel, yet maturation of mutant protein is highly ineffective. The lack of osmotically active chloride ions results in the reduced hydration of the mucus produced by epithelial cells. The high viscosity of the mucus impairs clearance of the airways and causes persistent microbial infections, which result in lung fibrosis and failure.

CPY (carboxypeptidase Y)
A soluble serine protease active in the vacuole of the yeast *Saccharomyces cerevisiae*. CPY carries four N-linked glycans. CPY* has a mutation of glycine to arginine at position 255. CPY* that lacks a C-terminal oligosaccharide is not recognized by ERAD.

HMG-CoA reductase (3-hydroxy-3-methyl-glutaryl-CoA reductase)
A membrane-anchored protein. It is the rate-limiting enzyme of the mevalonate pathway. Its stability is tightly linked to the abundance of sterols. When sterol levels are sufficient, the enzyme is degraded.

Igκ LC NS
A transport-incompetent immunoglobulin light chain that is degraded by ERAD.

KAI1 (also known as CD82)
A member of the tetraspanin family. In several human cancers, the loss of KAI1 expression correlates with the increased metastatic potential of the tumour.

MHC (major histocompatibility complex) class I molecules
Molecules that present peptides derived from the intracellular protein pool to T cells that eradicate cells displaying foreign peptides. Human cytomegalovirus (HCMV) uses the ERAD system to target MHC class I heavy chains (subunits of the MHC class I molecule) for destruction, to prevent the presentation of virus-derived peptides.

Pael-R (Pael receptor)
A substrate of the ubiquitin ligase Parkin. When Parkin is inactivated, unfolded Pael-R accumulates in the ER and causes neuronal death, suggesting that Pael-R has an important role in the pathogenesis of autosomal recessive juvenile Parkinson's disease.

Pdr5
An ATP-binding cassette transporter that confers multidrug resistance to the yeast *Saccharomyces cerevisiae*.

Ribophorin I
A membrane-anchored subunit of the oligosaccharyltransferase complex. Deletion of the C-terminal transmembrane domain yields the misfolded, soluble RI322 variant, which bears a single N-linked glycan.

Transthyretin (TTR)
A non-glycosylated serum protein secreted by the liver that functions as a carrier of the hormone thyroxine. Mutations that destabilize the homotetramer result in the formation of amyloid fibrils, causing neurodegeneration and organ failure.

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Adjusting the capacity of folding and disposal

Environmental changes and developmental processes can drastically change the workload of the ER. To maintain ER homeostasis, a coordinated programme known as the unfolded protein response (UPR) adjusts the folding capacity of the ER to meet demand and engages the destructive pathway if necessary[94]. The sensor proteins Ire1 (inositol requiring enzyme 1) in yeast and IRE1, PERK (dsRNA-activated protein kinase-like ER kinase) and ATF6 (activating transcription factor 6) in mammals detect elevated levels of unfolded polypeptides in the ER lumen and signal for increased expression of folding promoters and destructive modules. Moreover, cells have evolved additional strategies to save resources during times of stress. When the UPR is induced, selected messenger RNAs are degraded by a mechanism that requires IRE1 (ref. 95). Targets of this pathway include proteins that increase the workload of the ER without contributing to its fidelity. Removal of the signal sequence or the introduction of frame shifts stabilize mRNAs that are otherwise degraded, suggesting that the mRNA is processed at the ER membrane, perhaps by IRE1 itself. An additional pathway denies the entry of certain secretory proteins into the ER[96]. During acute stress, cargo proteins such as PrP (prion protein) are rerouted to the cytosol for immediate degradation by the proteasome, although BiP continues to enter the ER. A chimaeric PrP equipped with the BiP signal sequence can enter the ER of stressed cells, suggesting that this process is largely regulated by the protein's signal sequence, although the underlying structural differences are not clear. The mechanisms that regulate which proteins are admitted to the ER are collectively referred to as 'pre-emptive quality control'.

Future perspectives

More and more pieces of the ERAD pathway are coming to light, yet even some basic questions remain unresolved. How do Htm1 and members of the EDEM family recognize their targets? Does Htm1 use Pdi1 for this purpose, and does calnexin confer specificity to the actions of EDEM1? Are other members of the EDEM family also associated with chaperones? Which mechanisms govern the recognition of misfolded proteins that bear no glycans? Perhaps an ERAD component binds to non-native proteins, and the probability of their breakdown increases with the duration or frequency of the interaction? The HRD ligase binds substrates through Hrd3. Is this contact critical for substrate selection? How are substrates handled after Yos9 has recognized the correct glycan code, and how does the HRD ligase release proteins that are not suitable for degradation? Which factors make up the pore that shuttles aberrant proteins into the cytosol? What is the function of the ubiquitin-binding domains found in several of the cytosolic ERAD components? Perhaps these domains contribute to the directionality of the ERAD pathway. This could be accomplished if downstream components have a higher affinity for ubiquitin than factors that act earlier in the pathway. With these questions in mind, it would be useful to have an *in vitro* system that recapitulates the breakdown of a misfolded glycoprotein. Now that most of the ERAD factors have been identified, as well as the glycan detected by Yos9, it might be possible to establish such a system. ■

1. Lander, E. S. *et al.* Initial sequencing and analysis of the human genome. *Nature* **409,** 860–921 (2001).
2. Schubert, U. *et al.* Rapid degradation of a large fraction of newly synthesized proteins by proteasomes. *Nature* **404,** 770–774 (2000).
3. Hampton, R. Y. Proteolysis and sterol regulation. *Annu. Rev. Cell Dev. Biol.* **18,** 345–378 (2002).
4. Gurkan, C., Stagg, S. M., Lapointe, P. & Balch, W. E. The COPII cage: unifying principles of vesicle coat assembly. *Nature Rev. Mol. Cell Biol.* **7,** 727–738 (2006).
5. Wiseman, R. L., Powers, E. T., Buxbaum, J. N., Kelly, J. W. & Balch, W. E. An adaptable standard for protein export from the endoplasmic reticulum. *Cell* **131,** 809–821 (2007).
6. Daniels, R., Kurowski, B., Johnson, A. E. & Hebert, D. N. N-linked glycans direct the cotranslational folding pathway of influenza hemagglutinin. *Mol. Cell* **11,** 79–90 (2003).
7. Molinari, M. N-glycan structure dictates extension of protein folding or onset of disposal. *Nature Chem. Biol.* **3,** 313–320 (2007).
8. Solda, T., Galli, C., Kaufman, R. J. & Molinari, M. Substrate-specific requirements for UGT1-dependent release from calnexin. *Mol. Cell* **27,** 238–249 (2007).
This study shows that some substrates are released from the calnexin cycle after one binding event, whereas others undergo several binding rounds.
9. Hebert, D. N., Foellmer, B. & Helenius, A. Glucose trimming and reglucosylation determine glycoprotein association with calnexin in the endoplasmic reticulum. *Cell* **81,** 425–433 (1995).
10. Helenius, A. & Aebi, M. Roles of N-linked glycans in the endoplasmic reticulum. *Annu. Rev. Biochem.* **73,** 1019–1049 (2004).
11. Camirand, A., Heysen, A., Grondin, B. & Herscovics, A. Glycoprotein biosynthesis in *Saccharomyces cerevisiae*. Isolation and characterization of the gene encoding a specific processing α-mannosidase. *J. Biol. Chem.* **266,** 15120–15127 (1991).
12. Jelinek-Kelly, S. & Herscovics, A. Glycoprotein biosynthesis in *Saccharomyces cerevisiae*. Purification of the α-mannosidase which removes one specific mannose residue from $Man_9GlcNAc$. *J. Biol. Chem.* **263,** 14757–14763 (1988).
13. Jakob, C. A., Burda, P., Roth, J. & Aebi, M. Degradation of misfolded endoplasmic reticulum glycoproteins in *Saccharomyces cerevisiae* is determined by a specific oligosaccharide structure. *J. Cell Biol.* **142,** 1223–1233 (1998).
14. Jakob, C. A. *et al.* Htm1p, a mannosidase-like protein, is involved in glycoprotein degradation in yeast. *EMBO Rep.* **2,** 423–430 (2001).
15. Hosokawa, N. *et al.* A novel ER α-mannosidase-like protein accelerates ER-associated degradation. *EMBO Rep.* **2,** 415–422 (2001).
16. Clerc, S. *et al.* Htm1 protein generates the N-glycan signal for glycoprotein degradation in the endoplasmic reticulum. *J. Cell Biol.* **184,** 159–172 (2009).
This study identified Htm1 as a factor involved in the generation of $mannose_7$ oligosaccharides on ERAD substrates.
17. Olivari, S. & Molinari, M. Glycoprotein folding and the role of EDEM1, EDEM2 and EDEM3 in degradation of folding-defective glycoproteins. *FEBS Lett.* **581,** 3658–3664 (2007).
18. Tremblay, L. O. & Herscovics, A. Cloning and expression of a specific human α1,2-mannosidase that trims $Man_9GlcNAc_2$ to $Man_8GlcNAc_2$ isomer B during N-glycan biosynthesis. *Glycobiology* **9,** 1073–1078 (1999).
19. Avezov, E., Frenkel, Z., Ehrlich, M., Herscovics, A. & Lederkremer, G. Z. Endoplasmic reticulum (ER) mannosidase I is compartmentalized and required for N-glycan trimming to $Man_{5-6}GlcNAc_2$ in glycoprotein ER-associated degradation. *Mol. Biol. Cell* **19,** 216–225 (2008).
20. Hosokawa, N. *et al.* Enhancement of endoplasmic reticulum (ER) degradation of misfolded Null Hong Kong α1-antitrypsin by human ER mannosidase I. *J. Biol. Chem.* **278,** 26287–26294 (2003).
21. Wu, Y., Swulius, M. T., Moremen, K. W. & Sifers, R. N. Elucidation of the molecular logic by which misfolded α1-antitrypsin is preferentially selected for degradation. *Proc. Natl Acad. Sci. USA* **100,** 8229–8234 (2003).
22. Hirao, K. *et al.* EDEM3, a soluble EDEM homolog, enhances glycoprotein endoplasmic reticulum-associated degradation and mannose trimming. *J. Biol. Chem.* **281,** 9650–9658 (2006).
23. Olivari, S. *et al.* EDEM1 regulates ER-associated degradation by accelerating de-mannosylation of folding-defective polypeptides and by inhibiting their covalent aggregation. *Biochem. Biophys. Res. Commun.* **349,** 1278–1284 (2006).
24. Moremen, K. W. & Molinari, M. N-linked glycan recognition and processing: the molecular basis of endoplasmic reticulum quality control. *Curr. Opin. Struct. Biol.* **16,** 592–599 (2006).
25. Molinari, M., Calanca, V., Galli, C., Lucca, P. & Paganetti, P. Role of EDEM in the release of misfolded glycoproteins from the calnexin cycle. *Science* **299,** 1397–1400 (2003).
26. Oda, Y., Hosokawa, N., Wada, I. & Nagata, K. EDEM as an acceptor of terminally misfolded glycoproteins released from calnexin. *Science* **299,** 1394–1397 (2003).
27. Carvalho, P., Goder, V. & Rapoport, T. A. Distinct ubiquitin-ligase complexes define convergent pathways for the degradation of ER proteins. *Cell* **126,** 361–373 (2006).
28. Vashist, S. & Ng, D. T. Misfolded proteins are sorted by a sequential checkpoint mechanism of ER quality control. *J. Cell Biol.* **165,** 41–52 (2004).
29. Bays, N. W., Gardner, R. G., Seelig, L. P., Joazeiro, C. A. & Hampton, R. Y. Hrd1p/Der3p is a membrane-anchored ubiquitin ligase required for ER-associated degradation. *Nature Cell Biol.* **3,** 24–29 (2001).
30. Deak, P. M. & Wolf, D. H. Membrane topology and function of Der3/Hrd1p as a ubiquitin–protein ligase (E3) involved in endoplasmic reticulum degradation. *J. Biol. Chem.* **276,** 10663–10669 (2001).
References 29 and 30 characterize Hrd1 as an E3 ligase that ubiquitylates several ERAD substrates.
31. Knop, M., Finger, A., Braun, T., Hellmuth, K. & Wolf, D. H. Der1, a novel protein specifically required for endoplasmic reticulum degradation in yeast. *EMBO J.* **15,** 753–763 (1996).
32. Buschhorn, B. A., Kostova, Z., Medicherla, B. & Wolf, D. H. A genome-wide screen identifies Yos9p as essential for ER-associated degradation of glycoproteins. *FEBS Lett.* **577,** 422–426 (2004).
33. Denic, V., Quan, E. M. & Weissman, J. S. A luminal surveillance complex that selects misfolded glycoproteins for ER-associated degradation. *Cell* **126,** 349–359 (2006).
34. Gauss, R., Jarosch, E., Sommer, T. & Hirsch, C. A complex of Yos9p and the HRD ligase integrates endoplasmic reticulum quality control into the degradation machinery. *Nature Cell Biol.* **8,** 849–854 (2006).
References 33 and 34 show that Yos9 and Hrd3 act as a surveillance platform that binds to aberrant proteins and decodes the glycosylation status of a protein.
35. Quan, E. M. *et al.* Defining the glycan destruction signal for endoplasmic reticulum-associated degradation. *Mol. Cell* **32,** 870–877 (2008).
This study characterizes N-glycans containing terminal α1,6-mannose as the preferred ligand of Yos9.
36. Biederer, T., Volkwein, C. & Sommer, T. Role of Cue1p in ubiquitination and degradation at the ER surface. *Science* **278,** 1806–1809 (1997).
37. Swanson, R., Locher, M. & Hochstrasser, M. A conserved ubiquitin ligase of the nuclear envelope/endoplasmic reticulum that functions in both ER-associated and Matα2 repressor degradation. *Genes Dev.* **15,** 2660–2674 (2001).
38. Kreft, S. G., Wang, L. & Hochstrasser, M. Membrane topology of the yeast endoplasmic reticulum-localized ubiquitin ligase Doa10 and comparison with its human ortholog TEB4 (MARCH-VI). *J. Biol. Chem.* **281,** 4646–4653 (2006).
39. Fang, S. *et al.* The tumor autocrine motility factor receptor, gp78, is a ubiquitin protein ligase implicated in degradation from the endoplasmic reticulum. *Proc. Natl Acad. Sci. USA* **98,** 14422–14427 (2001).

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Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00009

40. Kikkert, M. *et al.* Human HRD1 is an E3 ubiquitin ligase involved in degradation of proteins from the endoplasmic reticulum. *J. Biol. Chem.* **279,** 3525–3534 (2004).
41. Nadav, E. *et al.* A novel mammalian endoplasmic reticulum ubiquitin ligase homologous to the yeast Hrd1. *Biochem. Biophys. Res. Commun.* **303,** 91–97 (2003).
42. Mueller, B., Lilley, B. N. & Ploegh, H. L. SEL1L, the homologue of yeast Hrd3p, is involved in protein dislocation from the mammalian ER. *J. Cell Biol.* **175,** 261–270 (2006).
43. Kokame, K., Agarwala, K. L., Kato, H. & Miyata, T. Herp, a new ubiquitin-like membrane protein induced by endoplasmic reticulum stress. *J. Biol. Chem.* **275,** 32846–32853 (2000).
44. Schulze, A. *et al.* The ubiquitin-domain protein HERP forms a complex with components of the endoplasmic reticulum associated degradation pathway. *J. Mol. Biol.* **354,** 1021–1027 (2005).
45. Lilley, B. N. & Ploegh, H. L. A membrane protein required for dislocation of misfolded proteins from the ER. *Nature* **429,** 834–840 (2004).
46. Ye, Y., Shibata, Y., Yun, C., Ron, D. & Rapoport, T. A. A membrane protein complex mediates retro-translocation from the ER lumen into the cytosol. *Nature* **429,** 841–847 (2004).
References 45 and 46 show that Derlin-1 is required for the membrane extraction of certain aberrant proteins from the ER.
47. Bernasconi, R., Pertel, T., Luban, J. & Molinari, M. A dual task for the Xbp1-responsive OS-9 variants in the mammalian endoplasmic reticulum: inhibiting secretion of misfolded protein conformers and enhancing their disposal. *J. Biol. Chem.* **283,** 16446–16454 (2008).
48. Christianson, J. C., Shaler, T. A., Tyler, R. E. & Kopito, R. R. OS-9 and GRP94 deliver mutant α1-antitrypsin to the Hrd1–SEL1L ubiquitin ligase complex for ERAD. *Nature Cell Biol.* **10,** 272–282 (2008).
49. Hosokawa, N. *et al.* Human XTP3-B forms an ER quality control scaffold with the HRD1–SEL1L ubiquitin ligase complex and BiP. *J. Biol. Chem.* **283,** 20914–20924 (2008).
50. Kaneko, M., Ishiguro, M., Niinuma, Y., Uesugi, M. & Nomura, Y. Human HRD1 protects against ER stress-induced apoptosis through ER-associated degradation. *FEBS Lett.* **532,** 147–152 (2002).
51. Cattaneo, M. *et al.* SEL1L and HRD1 are involved in the degradation of unassembled secretory Ig-μ chains. *J. Cell. Physiol.* **215,** 794–802 (2008).
52. Okuda-Shimizu, Y. & Hendershot, L. M. Characterization of an ERAD pathway for nonglycosylated BiP substrates, which require Herp. *Mol. Cell* **28,** 544–554 (2007).
53. Chen, B. *et al.* The activity of a human endoplasmic reticulum-associated degradation E3, gp78, requires its Cue domain, RING finger, and an E2-binding site. *Proc. Natl Acad. Sci. USA* **103,** 341–346 (2006).
54. Shen, Y., Ballar, P. & Fang, S. Ubiquitin ligase gp78 increases solubility and facilitates degradation of the Z variant of α1-antitrypsin. *Biochem. Biophys. Res. Commun.* **349,** 1285–1293 (2006).
55. Song, B. L., Sever, N. & DeBose-Boyd, R. A. Gp78, a membrane-anchored ubiquitin ligase, associates with Insig-1 and couples sterol-regulated ubiquitination to degradation of HMG CoA reductase. *Mol. Cell* **19,** 829–840 (2005).
56. Tsai, Y. C. *et al.* The ubiquitin ligase gp78 promotes sarcoma metastasis by targeting KAI1 for degradation. *Nature Med.* **13,** 1504–1509 (2007).
57. Hassink, G. *et al.* TEB4 is a C4HC3 RING finger-containing ubiquitin ligase of the endoplasmic reticulum. *Biochem. J.* **388,** 647–655 (2005).
58. Younger, J. M. *et al.* Sequential quality-control checkpoints triage misfolded cystic fibrosis transmembrane conductance regulator. *Cell* **126,** 571–582 (2006).
59. McDonough, H. & Patterson, C. CHIP: a link between the chaperone and proteasome systems. *Cell Stress Chaperones* **8,** 303–308 (2003).
60. Wang, B. *et al.* BAP31 interacts with Sec61 translocons and promotes retrotranslocation of CFTRΔF508 via the Derlin-1 complex. *Cell* **133,** 1080–1092 (2008).
61. Kitada, T. *et al.* Mutations in the Parkin gene cause autosomal recessive juvenile parkinsonism. *Nature* **392,** 605–608 (1998).
62. Imai, Y. *et al.* CHIP is associated with Parkin, a gene responsible for familial Parkinson's disease, and enhances its ubiquitin ligase activity. *Mol. Cell* **10,** 55–67 (2002).
63. Yoshida, Y. *et al.* E3 ubiquitin ligase that recognizes sugar chains. *Nature* **418,** 438–442 (2002).
64. Plemper, R. K., Bohmler, S., Bordallo, J., Sommer, T. & Wolf, D. H. Mutant analysis links the translocon and BiP to retrograde protein transport for ER degradation. *Nature* **388,** 891–895 (1997).
65. Wiertz, E. J. *et al.* Sec61-mediated transfer of a membrane protein from the endoplasmic reticulum to the proteasome for destruction. *Nature* **384,** 432–438 (1996).
66. Ploegh, H. L. A lipid-based model for the creation of an escape hatch from the endoplasmic reticulum. *Nature* **448,** 435–438 (2007).
67. Gauss, R., Sommer, T. & Jarosch, E. The Hrd1p ligase complex forms a linchpin between ER-lumenal substrate selection and Cdc48p recruitment. *EMBO J.* **25,** 1827–1835 (2006).
68. Hirsch, C., Blom, D. & Ploegh, H. L. A role for N-glycanase in the cytosolic turnover of glycoproteins. *EMBO J.* **22,** 1036–1046 (2003).
69. Suzuki, T., Park, H., Hollingsworth, N. M., Sternglanz, R. & Lennarz, W. J. *PNG1*, a yeast gene encoding a highly conserved peptide:N-glycanase. *J. Cell Biol.* **149,** 1039–1052 (2000).
70. Lilley, B. N. & Ploegh, H. L. Multiprotein complexes that link dislocation, ubiquitination, and extraction of misfolded proteins from the endoplasmic reticulum membrane. *Proc. Natl Acad. Sci. USA* **102,** 14296–14301 (2005).
71. Oda, Y. *et al.* Derlin-2 and Derlin-3 are regulated by the mammalian unfolded protein response and are required for ER-associated degradation. *J. Cell Biol.* **172,** 383–393 (2006).
72. de Virgilio, M., Weninger, H. & Ivessa, N. E. Ubiquitination is required for the retro-translocation of a short-lived luminal endoplasmic reticulum glycoprotein to the cytosol for degradation by the proteasome. *J. Biol. Chem.* **273,** 9734–9743 (1998).
73. Shamu, C. E., Flierman, D., Ploegh, H. L., Rapoport, T. A. & Chau, V. Polyubiquitination is required for US11-dependent movement of MHC class I heavy chain from endoplasmic reticulum into cytosol. *Mol. Biol. Cell* **12,** 2546–2555 (2001).
74. Bays, N. W., Wilhovsky, S. K., Goradia, A., Hodgkiss-Harlow, K. & Hampton, R. Y. HRD4/NPL4 is required for the proteasomal processing of ubiquitinated ER proteins. *Mol. Biol. Cell* **12,** 4114–4128 (2001).
75. Braun, S., Matuschewski, K., Rape, M., Thoms, S. & Jentsch, S. Role of the ubiquitin-selective CDC48(UFD1/NPL4) chaperone (segregase) in ERAD of OLE1 and other substrates. *EMBO J.* **21,** 615–621 (2002).
76. Jarosch, E. *et al.* Protein dislocation from the ER requires polyubiquitination and the AAA-ATPase Cdc48. *Nature Cell Biol.* **4,** 134–139 (2002).
77. Ye, Y., Meyer, H. H. & Rapoport, T. A. The AAA ATPase Cdc48/p97 and its partners transport proteins from the ER into the cytosol. *Nature* **414,** 652–656 (2001).
References 74–77 demonstrate that yeast Cdc48, or p97 in mammals, mobilizes ERAD substrates from the ER membrane.
78. Zhang, X. *et al.* Structure of the AAA ATPase p97. *Mol. Cell* **6,** 1473–1484 (2000).
79. DeLaBarre, B., Christianson, J. C., Kopito, R. R. & Brunger, A. T. Central pore residues mediate the p97/VCP activity required for ERAD. *Mol. Cell* **22,** 451–462 (2006).
80. Neuber, O., Jarosch, E., Volkwein, C., Walter, J. & Sommer, T. Ubx2 links the Cdc48 complex to ER-associated protein degradation. *Nature Cell Biol.* **7,** 993–998 (2005).
81. Schuberth, C. & Buchberger, A. Membrane-bound Ubx2 recruits Cdc48 to ubiquitin ligases and their substrates to ensure efficient ER-associated protein degradation. *Nature Cell Biol.* **7,** 999–1006 (2005).
82. Liang, J. *et al.* Characterization of erasin (UBXD2): a new ER protein that promotes ER-associated protein degradation. *J. Cell Sci.* **119,** 4011–4024 (2006).
83. Medicherla, B., Kostova, Z., Schaefer, A. & Wolf, D. H. A genomic screen identifies Dsk2p and Rad23p as essential components of ER-associated degradation. *EMBO Rep.* **5,** 692–697 (2004).
84. Richly, H. *et al.* A series of ubiquitin binding factors connects CDC48/p97 to substrate multiubiquitylation and proteasomal targeting. *Cell* **120,** 73–84 (2005).
This study demonstrates how components of the ubiquitin–proteasome system cooperate to deliver ubiquitylated proteins to the proteasome.
85. Deveraux, Q., Jensen, C. & Rechsteiner, M. Molecular cloning and expression of a 26S protease subunit enriched in dileucine repeats. *J. Biol. Chem.* **270,** 23726–23729 (1995).
86. Husnjak, K. *et al.* Proteasome subunit Rpn13 is a novel ubiquitin receptor. *Nature* **453,** 481–488 (2008).
87. Schreiner, P. *et al.* Ubiquitin docking at the proteasome through a novel pleckstrin-homology domain interaction. *Nature* **453,** 548–552 (2008).
88. Suzuki, T., Park, H., Kwofie, M. A. & Lennarz, W. J. Rad23 provides a link between the Png1 deglycosylating enzyme and the 26S proteasome in yeast. *J. Biol. Chem.* **276,** 21601–21607 (2001).
89. Blom, D., Hirsch, C., Stern, P., Tortorella, D. & Ploegh, H. L. A glycosylated type I membrane protein becomes cytosolic when peptide:N-glycanase is compromised. *EMBO J.* **23,** 650–658 (2004).
90. Misaghi, S., Pacold, M., Blom, D., Ploegh, H. L. & Korbel, G. A. Using a small molecule inhibitor of peptide:N-glycanase to probe its role in glycoprotein turnover. *Chem. Biol.* **11,** 1677–1687 (2004).
91. Hu, M. *et al.* Structure and mechanisms of the proteasome-associated deubiquitinating enzyme USP14. *EMBO J.* **24,** 3747–3756 (2005).
92. Verma, R. *et al.* Role of Rpn11 metalloprotease in deubiquitination and degradation by the 26S proteasome. *Science* **298,** 611–615 (2002).
93. Yao, T. & Cohen, R. E. A cryptic protease couples deubiquitination and degradation by the proteasome. *Nature* **419,** 403–407 (2002).
94. Ron, D. & Walter, P. Signal integration in the endoplasmic reticulum unfolded protein response. *Nature Rev. Mol. Cell Biol.* **8,** 519–529 (2007).
95. Hollien, J. & Weissman, J. S. Decay of endoplasmic reticulum-localized mRNAs during the unfolded protein response. *Science* **313,** 104–107 (2006).
96. Kang, S. W. *et al.* Substrate-specific translocational attenuation during ER stress defines a pre-emptive quality control pathway. *Cell* **127,** 999–1013 (2006).
97. Hammarstrom, P. *et al.* D18G transthyretin is monomeric, aggregation prone, and not detectable in plasma and cerebrospinal fluid: a prescription for central nervous system amyloidosis? *Biochemistry* **42,** 6656–6663 (2003).
98. Sekijima, Y. *et al.* Energetic characteristics of the new transthyretin variant A25T may explain its atypical central nervous system pathology. *Lab. Invest.* **83,** 409–417 (2003).
99. Sekijima, Y. *et al.* The biological and chemical basis for tissue-selective amyloid disease. *Cell* **121,** 73–85 (2005).

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