

## JB Minireview—Quality Control of the Cellular Protein Systems Mechanism and components of endoplasmic reticulum-associated degradation

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**The folding of secretory and membrane proteins takes place in the endoplasmic reticulum (ER). The quality of the proteins folded in the ER is carefully monitored by an ER quality control mechanism that allows only correctly folded proteins to be transported to their final destination, and misfolded or unassembled proteins to be retained in the ER and subsequently degraded in a process termed ‘ER-associated degradation’ (ERAD). The ERAD pathway is conserved from yeast to mammals, and plays an essential role in the maintenance of ER homeostasis, as well as in the prevention of various diseases that arise from the accumulation of aberrant proteins in the ER. In the ERAD pathway, molecular chaperones and lectin-like proteins are involved in the identification of misfolded proteins, ER-resident reductases cleave disulfide bonds in these proteins to facilitate retrograde transport to the cytosol and AAA<sup>+</sup> adenosine triphosphatase withdraws them from the retrotranslocation channel to the cytosol where they are degraded by the ubiquitin/proteasome system. The possible mechanisms that underlie ERAD and the various factors involved in this process are discussed in this article.**

**Keywords:** J domain/lectin/molecular chaperone/non-glycosylated proteins/reductase.

**Abbreviations:** CNX, Calnexin; CRT, calreticulin; ER, endoplasmic reticulum; ERAD, ER-associated degradation; ER ManI, ER  $\alpha$ -mannosidase I; Glc, glucose; GlcNAc, *N*-acetylglucosamine; Ig  $\kappa$  LC, immunoglobulin light chain; Man, mannose; M5, Man<sub>5</sub>GlcNAc<sub>2</sub>; M7, Man<sub>7</sub>GlcNAc<sub>2</sub>; M8B, Man<sub>8</sub>GlcNAc<sub>2</sub> isomer B; MHC, major histocompatibility complex; UPR, unfolded protein response.

The folding of proteins is a complex and error-prone process. Genetic mutations, viral infection and various other cellular alterations, such as heat shock and

oxidative stress, frequently cause the misfolding of newly synthesized as well as pre-existing proteins. Accumulation of misfolded or unfolded proteins is often toxic to cells, resulting in the development of protein folding diseases such as Parkinson’s, Alzheimer’s and prion diseases. To combat the cellular damage caused by improperly processed proteins, cells have developed effective protein quality control mechanisms.

The endoplasmic reticulum (ER) is a ‘protein factory’ where the secretory and membrane proteins that account for about one-third of all cellular proteins are folded. The ER is equipped with a stringent quality control system that monitors the proteins that are synthesized and folded in the ER. This ER quality control system is able to discern between the correctly folded proteins that exit the ER *en route* to their final destinations and the misfolded or unfolded proteins that are retained and refolded in the ER (1). Proteins that are terminally misfolded are selectively transported from the ER into the cytosol, and subsequently ubiquitinated and degraded by the proteasome, a process called ER-associated degradation (ERAD) (2). The accumulation of misfolded or unfolded polypeptides in the ER activates the unfolded protein response (UPR), which induces the expression of molecular chaperones and ERAD components that increase the folding capacity of the ER and activate the clearance of accumulating misfolded proteins (3). The identities and activities of various components involved in ERAD have been disclosed by numerous elaborate studies. This review describes the most recent findings on the molecular mechanisms of ERAD.

### Lectin-like ERAD Factors

Most newly synthesized polypeptides are co-translationally modified with *N*-glycans in the ER. When nascent polypeptides enter the ER lumen, Glc<sub>3</sub>Man<sub>9</sub>GlcNAc<sub>2</sub> (Glc: glucose, Man: mannose, GlcNAc: *N*-acetylglucosamine) oligosaccharides are attached to the asparagine residues of the polypeptides by the oligosaccharide transferase complex, and the two outer glucoses are removed immediately by glucosidase I and II (4). The resulting monoglucosylated *N*-glycans on the polypeptides are recognized by the ER-resident lectin-like chaperones, calnexin (CNX) and calreticulin (CRT), which promote folding of the glycoproteins (4). After removal of the third glucose by glucosidase II, the substrates dissociate from CNX or CRT, and are transported to the Golgi apparatus through vesicular transport. However, if the

polypeptides are incorrectly or immaturely folded, they are re-glycosylated by UDP-glucose glycosyl transferase, resulting in a monoglucose form that is again recognized and folded by CNX (or CRT). This pathway is called the ‘calnexin cycle’ (4).

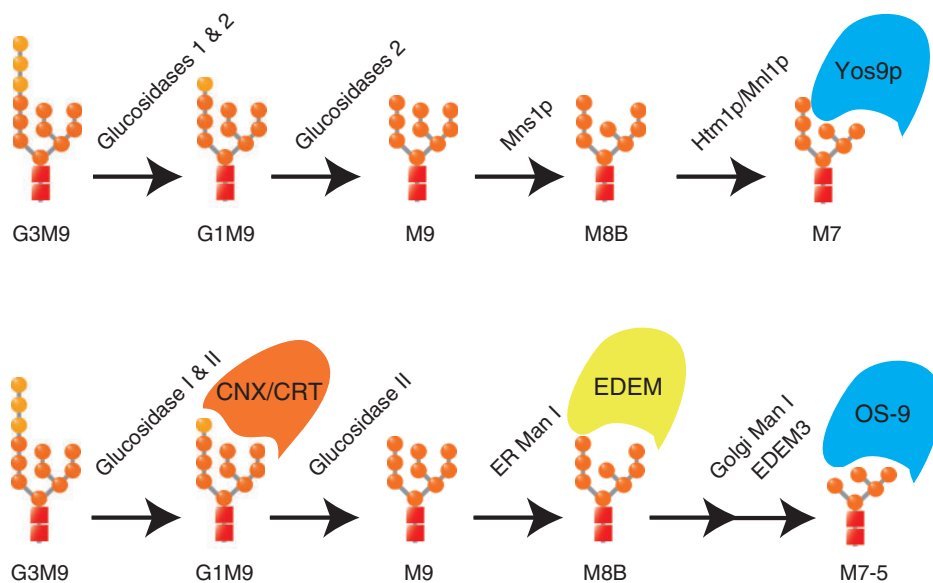
However, if the polypeptides are terminally misfolded, they are degraded by ERAD. ER  $\alpha$ -mannosidase I (ER ManI) produces Man<sub>8</sub>GlcNAc<sub>2</sub> isomer B (M8B) from Man<sub>9</sub>GlcNAc<sub>2</sub> by removing the mannose residue from the middle branch of the *N*-glycans. Inhibition of this mannose trimming has been shown to stabilize misfolded glycoproteins in the ER (5), suggesting that M8B may act as a degradation signal for ERAD. Some lectin-like proteins that recognize the M8B forms of *N*-glycans are believed to exist in the ER. EDEM, or the yeast homologue Htm1p/Mnl1p, is a candidate for a lectin-like protein that promotes ERAD of misfolded glycoproteins in a mannose-trimming-dependent manner (6–8). The transfer of terminally misfolded glycoproteins from CNX to EDEM for ERAD has been reported (9, 10).

EDEM and EDEM family proteins, including EDEM2 and EDEM3, have  $\alpha$ -mannosidase-like domains with conserved catalytic residues for glycolytic activity. EDEM has very weak, if any, mannosidase activity that trims mannose from the A branch or the C branch of *N*-glycans on misfolded proteins (11, 12). However, an EDEM mutant that lacks the putative active site for mannosidase was still able to accelerate ERAD, suggesting that the ERAD-accelerating activity of this mutant is not dependent on enzymatic activity (8, 11). In addition, EDEM2 was reported to have no enzymatic activity although it is still able to promote ERAD (13, 14). Conversely, EDEM3 and Htm1p/Mnl1p exhibit mannosidase activity, and this activity has been shown to be a prerequisite

for ERAD-accelerating activity by these proteins (15, 16). The functional redundancy between EDEM family proteins in the ER remains to be revealed. Due to the difficulties in preparation and purification of EDEM family proteins, it is still not known whether EDEM proteins may also possess lectin-like activity.

A genome-wide screen of yeast mutants identified Yos9p (yeast osteosarcoma 9), another lectin-like protein that is able to accelerate ERAD of misfolded glycoproteins (17). Yos9p contains a mannose-6-phosphate homologue (MRH) domain, which mediates the specific binding of the protein with the ERAD substrate CPY\* that bears M8B and Man<sub>5</sub>GlcNAc<sub>2</sub> (M5) glycans (18–20). *In vitro* study using frontal affinity chromatography revealed a direct interaction between Yos9p and *N*-glycans that contain a terminal  $\alpha$ 1,6-linked mannose, such as Man<sub>7</sub>GlcNAc<sub>2</sub> (M7) and M5 (21). Interestingly, immunoprecipitation with chemical cross-linker revealed that Yos9p was able to bind to a CPY\* variant with no sugar chain, suggesting that Yos9p recognizes both the specific sugar chain and the polypeptide portion of misfolded proteins (18). Htm1p is required for binding of Yos9p with misfolded substrates (6). Figure 1 shows a recent model illustrating substrate recognition in the ER; Htm1p enhances trimming of mannose from M8B glycans of misfolded glycoproteins, thereby producing M7 glycans that are recognized by Yos9p and promoting the dislocation of misfolded proteins (16).

Recent studies revealed that a mammalian orthologue of Yos9p, OS-9, which contains an MRH domain, is involved in ERAD. Christianson *et al.* (22) first showed that OS-9 binds to the sugar chain of SEL1L, a scaffold protein in the ERAD complex, but recent work demonstrated that OS-9 enhances



**Fig. 1 Quality control by lectin-like proteins.** Oligosaccharyltransferase transfers Glc<sub>3</sub>Man<sub>9</sub>GlcNAc<sub>2</sub> (G3M9) oligosaccharides to asparagine residues in the Asn-X-Ser/Thr motif in nascent polypeptides. The two outer glucoses are removed immediately by glucosidases I and II. The resulting monoglucosylated form is recognized by CNX (or CRT), which promote folding of the glycoproteins. In yeast, the ER  $\alpha$  mannosidase Mns1p trims the outermost mannose moiety of the B branch, yielding M8B. Htm1p processes the C branch, yielding M7, which Yos9p recognizes as a degradation signal. In mammals, ER ManI trims the outermost mannose moiety of the B branch, yielding M8B, which EDEM recognizes for ERAD. Extensive demannosylation by Golgi mannosidase I and EDEM3 yields M7-M5, which OS-9 recognizes as a degradation signal.

the clearance of misfolded proteins by binding directly to *N*-glycans that lack the terminal mannose from the C branch (M5, M6, M7 and M8C) (12). Taken together, a model similar to the one presented for yeast in Fig. 1 might be proposed for mammalian ERAD. First, terminally misfolded proteins are transferred from CNX to EDEM after the trimming of glucose and mannose by glucosidase II and ER ManI, respectively. OS-9 recognizes the misfolded glycoproteins lacking mannoses in the C branch resulting from the mannosidase activity of EDEM3 and Golgi mannosidase I, and promotes redirection of the misfolded proteins from the ER to the cytosol for ERAD. XTP3-B, another MRH domain-containing protein in the mammalian ER, is also likely to be an ERAD component (22, 23), although the lectin activity of XTP3-B has not been shown and the role of XTP3-B in ERAD remains elusive.

### Requirement for Redox Reactions in ERAD

The oxidative environment of the ER is suitable for the formation of disulfide bonds, which stabilize tertiary protein structure and facilitate protein assembly (24). Most secretory and membrane proteins are co-translationally oxidized at the cysteine residues, resulting in the formation of disulfide bonds within the polypeptide by PDI that is coupled to the PDI oxidase ERO1L $\alpha$  and ERp57 that is coupled to with CNX. After initial oxidation, disulfide bonds are sometimes isomerized by PDI and ERp57 to stabilize the correct folding of nascent proteins (25). The redox activities of PDI and ERp57 as an oxidase and/or isomerase are exerted through CXXC motifs in these proteins. In some cases, such as immunoglobulin production, disulfide bond formation between subunits leads to the assembly of multiple proteins into a functional protein complex.

Conversely, the reduction of disulfide bonds of immature and misassembled proteins is thought to be necessary for retrotranslocation into the ERAD pathway. Treatment of cells with oxidizing agents or cysteine blocking reagents inhibits the degradation of misfolded proteins in the ER, while addition of dithiothreitol promotes ERAD, supporting a requirement for the reduction of disulfide bonds (26–28). However, reduction itself rarely occurs in the oxidative environment of the ER, and it is highly likely that a currently unidentified reductase is responsible for the cleavage of disulfide bonds.

Recently, we have identified an EDEM-binding ER resident protein, ERdj5, as a disulfide reductase that participates in ERAD (29). ERdj5 is composed of a J-domain able to interact with BiP (an ER-resident member of the Hsp70 family) and four thioredoxin-like domains with CXXC motifs. Overexpression of ERdj5 promotes the degradation of the misfolded glycoprotein NHK by cleaving its intermolecular disulfide bonds and preventing dimer formation. Mutation of the CXXC motifs in ERdj5 to inactive forms, such as SXXS or AXXA, or knockdown of endogenous ERdj5 renders ERdj5 unable to accelerate ERAD. *In vitro*

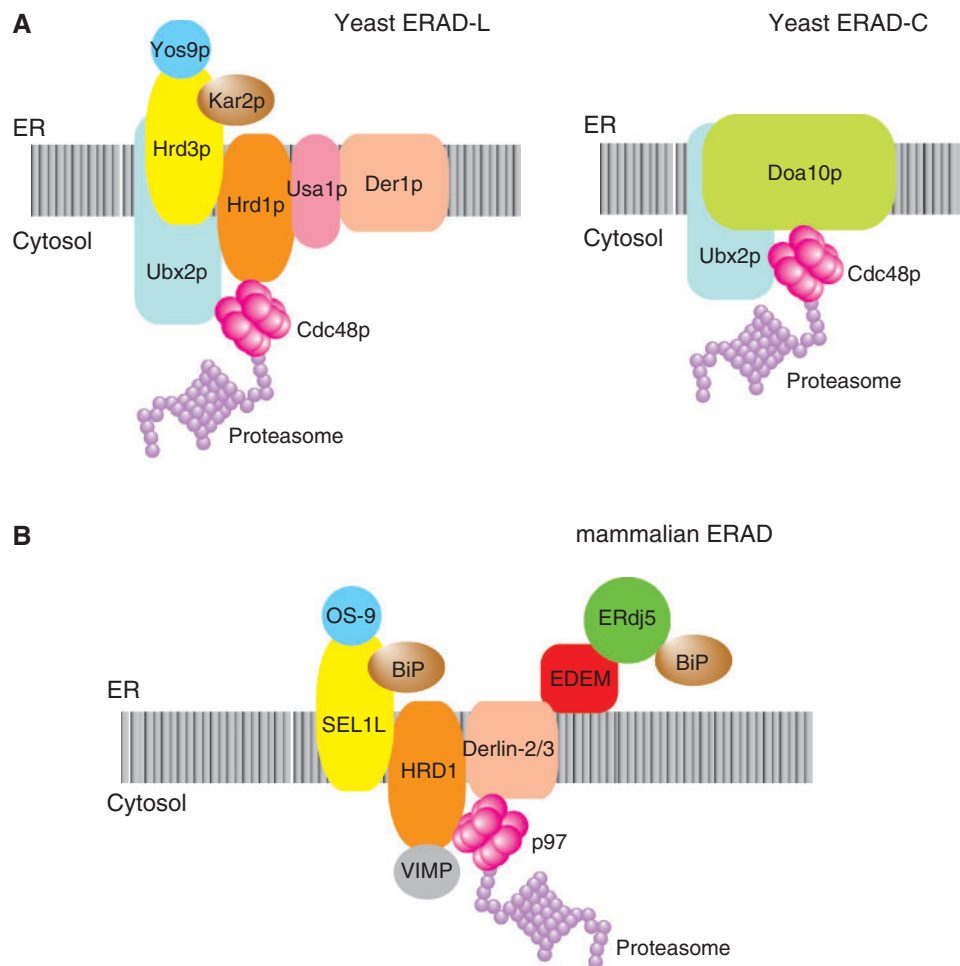
analysis of the redox activities of recombinant ERdj5 confirmed that it possesses reductase activity in the oxidative ER environment. Thus, ERdj5 is the first disulfide reductase reported in the mammalian ER so far. It will be important to identify the electron donor for the reductase activity of ERdj5 in future studies. Interestingly, ERdj5 has been shown to cooperate with EDEM and BiP in ERAD acceleration. When cells were treated with kifunensine, an ER mannosidase inhibitor, ERdj5 was unable to accelerate the degradation of NHK, suggesting that functional cooperation with EDEM is necessary for ERdj5-mediated acceleration of ERAD of glycoproteins. Thus, a complex composed of EDEM, ERdj5 and BiP is a novel regulator of glycoprotein ERAD.

ERdj5 is highly conserved in mammals, vertebrates and nematodes, but does not have a direct homologue in yeast. Interestingly, Pdi1p, a yeast PDI homologue, interacts specifically with the yeast EDEM homologue, Htm1p/Mnl1p, and the interaction has been shown to be required for ERAD acceleration activity of Htm1p/Mnl1p (30). Pdi1p might be involved in ERAD of glycoproteins as a reductase in a manner similar to ERdj5 in mammals.

### Components of the ERAD Complex

Most ERAD components are upregulated by UPR to facilitate the degradation of misfolded proteins. To ensure efficient recognition, dislocation and ubiquitination of misfolded proteins, ERAD components form multi-protein complexes on the ER membrane, and physically and functionally interact with the 26S proteasome in the cytosol. In yeast, three distinct membrane protein complexes that define different ERAD pathways (ERAD-L, ERAD-M and ERAD-C) have been proposed (31–33). When proteins are misfolded in the ER lumen, they are degraded by the ERAD-L pathway in yeast. Similarly, when membrane proteins are misfolded in the membrane spanning domain or the cytosolic domain, they are degraded through the ERAD-M and ERAD-C pathways, respectively.

The ubiquitin ligase, Hrd1p, forms a complex with Hrd3p, an ER membrane protein that has a luminal domain with a tetratricopeptide repeat (TPR) motif, and this complex is involved in degradation of luminal misfolded proteins (ERAD-L pathway, Fig. 2A, left). Yos9p interacts with Hrd3p and Kar2p (a yeast homologue of BiP) and recruits proteins destined for degradation to the Hrd1p/Hrd3p complex. Other components, including Ubc7p/Cue1p (E2 complex), Der1p, Ubx2p and Usa1p, are also found in the Hrd1p/Hrd3p complex. Der1p is a yeast homologue of Derlin-1 that is believed to be a part of the dislocation channel (34, 35), which transports terminally misfolded proteins out of the ER. Sec61p, which is component of the translocation channel for newly synthesized proteins, is also believed to be a component of the dislocation channel for ERAD-L substrates (36, 37). Determination of what components comprise a dislocation channel remains to be elucidated. Usa1p

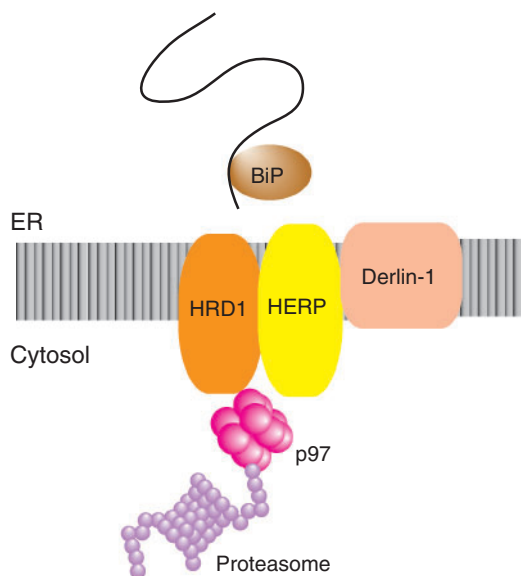


**Fig. 2 ERAD machinery.** (A) Distinct ERAD complexes in yeast. The left panel shows the Hrd1p/Hrd3p complex in the ERAD-L pathway. The right panel shows the Doa10p complex of the ERAD-C pathway. Yos9p recognizes *N*-glycans on misfolded proteins, which are then ubiquitinated by Hrd1p in the cytosol and degraded by the proteasome. (B) The mammalian HRD1/SEL1L ERAD complex. OS-9 (and possibly XTP3-B) recognizes *N*-glycans on misfolded proteins, which are then ubiquitinated by HRD1 in the cytosol and degraded by the proteasome. EDEM also recognizes *N*-glycans on misfolded proteins and recruits them to Derlin-2/3 and p97. ERdj5 interacts with EDEM and BiP and promotes dislocation of misfolded proteins from the ER by cleaving their incorrect disulfide bonds.

and Ubx2p, both of which are transmembrane proteins, link Der1p and the cytosolic AAA<sup>+</sup> ATPase Cdc48p, responsible for withdrawal of substrates, to the Hrd1p/Hrd3p complex, respectively. Proteins with misfolded portions that lie within the membrane are also retrogradely transported into the cytosol *via* the ERAD-M pathway, which contains the Hrd1p/Hrd3p complex (31). Whether additional factors are required for the ERAD-M pathway is unknown, although Der1p and Usa1p are not believed to be involved (31). The component of the ERAD-C pathway necessary for the degradation of ER membrane proteins with misfolded cytosolic regions is Doa10p ubiquitin ligase, instead of the Hrd1p/Hrd3p complex (Fig. 2A, right).

In mammalian cells, similar but distinct ERAD components direct the translocation of misfolded proteins from the ER to the cytosol for degradation *via* the ubiquitin–proteasome system. Derlin-1, a multi-spanning membrane protein, was identified as an ERAD component required for dislocation of the human cytomegalovirus protein US11-mediated major

histocompatibility complex class I (MHC class I) heavy chain (34, 35). Derlin-1 and its homologue, Derlin-2, interact with the p97, a homologue of yeast Cdc48p, and its co-factors *via* the connector protein, VIMP (38, 39). p97 then directly binds to a ubiquitin–ligase complex similar to the yeast ERAD-L complex that consists of HRD1 (Hrd1p homologue) and SEL1L (Hrd3p homologue) (Fig. 2). Another mammalian homologue of yeast Hrd1p, gp78, interacts with p97 and cooperates with RMA, an E3 ubiquitin ligase, in polyubiquitination of mutant CFTR (cystic fibrosis transmembrane conductance regulator) to accelerate degradation (40). OS-9 and XTP3-B, both of which are orthologues of Yos9p, associate with BiP and Grp94 in addition to interacting with the HRD1/SEL1L complex (22, 23). While the function of Grp94 in ERAD is unclear, BiP is known to recruit non-glycosylated misfolded proteins to the ERAD machinery [(23), also see below and Fig. 3]. HERP is a novel ubiquitin-like membrane protein induced by ER stress (41). A complex consisting of Derlin-1, HRD1, p97 and HERP was shown to associate with



**Fig. 3 ERAD pathway for non-glycosylated proteins.** BiP recognizes misfolded non-glycoproteins and then recruits them to the HERP complex that contains p97 and Derlin-1.

non-glycosylated misfolded proteins [(42), see below and Fig. 3]. In addition, the interaction of EDEM with p97 *via* Derlin-2 and Derlin-3 has also been identified as an ERAD complex (43). Finally, a recent study showed that ERFAD, a newly identified ERAD promoter with a FAD and NADPH binding motif, interacts with ERdj5, SEL1L and OS-9 (44), although the biological significance of this interaction is not well understood.

### Mechanism of ERAD of Non-Glycosylated Proteins

As described above, ER quality control of glycosylated proteins is monitored by lectin-like proteins. However, the ER quality control system for non-glycosylated proteins is independent of the lectin-like proteins involved in ERAD. Previous yeast studies showed that Kar2p is responsible for ERAD of immature non-glycosylated proteins, such as pro- $\alpha$  factor and CPY\* (45–47). In mammalian cells, unassembled immunoglobulin light chain (Ig  $\kappa$  LC), a non-glycosylated ERAD substrate, is degraded in a BiP-dependent manner (48). Recently, a report that clearly detailed the mechanism of degradation of Ig  $\kappa$  LC was published (42). Ig  $\kappa$  LC can form two intra molecular disulfide bonds, but the fully oxidized form of Ig  $\kappa$  LC is not dislocated from the ER to the cytosol. Interestingly, BiP interacts only with the partially oxidized form of Ig  $\kappa$  LC, thereby preventing the formation of the fully oxidized form and facilitating its dislocation. The partially oxidized Ig  $\kappa$  LC is transferred from BiP to a complex with HERP and Derlin-1 and subsequently degraded with the assistance of p97 and HRD1 (Fig. 3).

In addition to BiP/Kar2p, ERdj family proteins are involved in the clearance of misfolded non-glycosylated proteins. The J domain in these proteins

is an  $\alpha$ -helical domain with an HPD motif (the binding motif for Hsp70s) located at the turn between helices II and III (49). In mammals, the J domain activates ATP hydrolysis of HSP70, which induces strong binding of Hsp70 with unfolded or misfolded substrates (50). Yeast deletion mutants of Jem1p and Scj1p proteins that contain the J domain were reported to exhibit retarded degradation of non-glycosylated ERAD substrates (47). Recently, it was reported that ERdj4 and ERdj5 cooperate in facilitating the degradation of misfolded surfactant protein C, in which mutations are associated with the occurrence of human interstitial lung disease (51). Recently, we have also established a role for ERdj5 in ERAD of non-glycosylated proteins. With respect to the degradation of glycosylated substrates, ERdj5 interacts with substrates recruited by EDEM and accelerates their clearance through reductive activity (29). ERdj5 also accelerates the degradation of non-glycosylated misfolded proteins (Ushioda *et al.*, unpublished data). Interestingly, misfolded substrates may be recruited to ERdj5 by BiP, independent of the binding of ERdj5 to EDEM, suggesting that misfolded substrates are recruited to ERdj5 by two distinct pathways, the CNX-EDEM pathway and the BiP pathway. In both of these pathways, ERdj5 plays a key role in facilitating the processing of a wide range of aberrant proteins for ERAD.

### Concluding Remarks

Elaborate studies have identified and characterized various components that contribute to the ERAD pathway, which has significantly advanced the understanding of the molecular mechanisms of ERAD. However, numerous basic questions remain unanswered. What comprises of the retrotranslocation channel? What is the mechanism of retrotranslocation of the substrates to the cytosol? How are misfolded proteins recognized? How are the disulfide bonds of misfolded proteins cleaved in the yeast ER? What is the reductive source for ERdj5? Once these questions are answered, the entire picture of ERAD will be clarified and new therapeutic strategies and approaches to many diseases related to ERAD can be developed.

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### Conflict of interest

None declared.

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