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Chapter

Endoplasmic Reticulum-Associated Degradation (ERAD)

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Abstract

The newly synthesized proteins are kept in the endoplasmic reticulum (ER) until their maturation is completed. The accurate protein folding is vital for homeostasis, but this process is error-prone since it is chemically complicated. Aberrant folding may result in aggregates having a toxic gain of function or may lead to a loss of protein function; therefore, protein misfolding can lead to several pathologies. The ER protein quality control mechanism monitors the fidelity of protein folding. Those proteins that fail to fold or assemble properly are subjected to degradation via a process known as ER-associated degradation (ERAD). Besides clearing proteins having folding problems, ERAD is also known to regulate the levels of some physiological proteins including 3-hydroxy-3-methylglutaryl-coenzymeA reductase (HMGR) catalyzing the rate-limiting step of cholesterol biosynthesis. ERAD is a complex, multistep process starting with the recognition and targeting of substrates, followed by ubiquitination, retrotranslocation and proteasomal degradation. A large number of ERAD factors functioning in different molecular machineries increases the complexity of mammalian ERAD. ERAD is fundamental for human health and there is increasing evidence linking ERAD with various diseases. Here, the different modules/machineries of the ERAD process together with its tight regulation will be discussed.

Keywords: endoplasmic reticulum-associated degradation (ERAD), protein misfolding, ubiquitin-mediated degradation, proteasomal degradation

1. Introduction

The endoplasmic reticulum (ER) is an extensive network of flattened, membrane-enclosed tubes or sacs that extends throughout the cytosol [1]. ER has important roles in many biochemical processes required for cell survival and normal cellular functions. ER regulates these cellular processes through proteins that are localized in its complex network structures [1–3]. In addition to protein synthesis, significant cellular activities such as protein transport and folding, lipid and steroid synthesis, carbohydrate metabolism, calcium storage and protein quality control processes occur in the ER [1–4].

Approximately one-third of all newly synthesized proteins are targeted to the ER and traffic to other organelles of secretory pathway, plasma membrane or the extracellular space [5]. Protein translocation to the ER occurs through Sec61 complex [6, 7]. As synchronized with translocation, protein is exposed to the ER's oxidizing and calcium-rich environment, which is suitable for protein folding and

co- and post-translational modifications such as glycosylation, disulfide bond formation and glycosylphosphatidylinositol (GPI) anchoring [8]. During this folding process, many proteins such as lectin-type molecular chaperones (e.g., calnexin (CNX) or calreticulin (CLR)), HSP70-like chaperone BiP) and enzymes like protein disulfide isomerases (PDI) work in association with each other [4, 9, 10]. Conformational maturation and folding of the proteins in the ER are instantly controlled through the added N-glycan groups to decide whether the proteins are directed to distant compartments via the secretory pathway or included in the refolding cycle [11, 12].

The folding process is not completely accurate. In mammals, 30% of all newly synthesized proteins are estimated to be incorrectly folded [13]. However, genetic mutations, errors in transcription and translation, toxic compounds and cellular stresses such as defects in cellular redox regulation due to hypoxia, oxidants and reducing agents that interact with disulfide bonds in the ER lumen, glucose starvation and abnormalities in calcium regulation lead to a significant increase in the ratio of incorrectly folded proteins [4, 11, 14]. Adequate removal of these unwanted proteins is crucial for protecting cells from proteotoxicity caused by the formation of protein aggregates through the re-opening of hydrophobic residues as well as by unfolded or misfolded proteins that may compete with their properly folded counterparts for substrate binding or for complex formation with partners. Even though the primary damage of these unwanted proteins is restricted to the cell they reside, the damage gets wider if it is a secretory protein [11]. Therefore, there is a robust control via "Protein Quality Control Mechanisms" for the removal of defective proteins in living cells, and thus, only properly folded proteins are allowed to exit from ER lumen to the secretory pathway [11, 15–18]. When the folding process fails, the terminal mannose residues from the core glycan chain are gradually removed, allowing the proteins to be recognized by mannose-specific lectins and defective proteins are transferred to the 26S proteasome for degradation through the protein quality control mechanism called "ER-associated degradation (ERAD)" [19–21].

In addition to misfolding proteins, ERAD also targets some proteins that might fold into their native structures under the right conditions and also orphan subunits of oligomeric complexes. The chloride channel protein CFTR (cystic fibrosis transmembrane conductance regulator) is the best example, where it is targeted to ERAD as a consequence of its complex and inefficient folding pathway. The low folding efficiency is further decreased upon mutation as seen in CFTRΔF508. CFTRΔF508 is the most common mutation found in cystic fibrosis patients, can fold and function in plasma membrane; thus, degradation of CFTR via ERAD is obtrusive. ERAD also functions in supporting the correct stoichiometry of multimeric protein complexes by degrading components that are produced in excess of the limiting monomer [22]. For example, the unassembled subunits of T cell receptor-like TCRα and CD3δ are also well-known ERAD substrates [23]. These proteins contain charged residues in the intramembrane sections promoting the assembly of complexes. However, when oligomerization is not proper, these residues might initiate degradation via recruiting specific ERAD factors [23].

ERAD also functions in cell homeostasis by regulating the endogenous levels of many enzymes and signal molecules especially those localized to the ER membrane or plasma membrane under physiological conditions [24]. For instance, ERAD plays a homeostatic role in the regulation of HMG-CoA reductase (HMGR), which is the key enzyme of cholesterol metabolism; apolipoprotein B, an essential secreted protein member of triacylglycerol-rich lipoproteins responsible for the export of lipids, triglycerides and cholesterol; hepatic cytochrome P450 enzyme 3A4 metabolizing endo- and xenobiotics; IP3 receptor, an ER-localized protein allowing Ca^{2+} release

by binding seconder messenger inositol 1,4,5-triphosphate (IP3); type II iodothyronine deiodinase, an ER-localized enzyme converting thyroxin (T4) to the biologically active hormone triiodothyronine (T3) and GABA neurotransmitter receptor responsible for the reduction of neuronal excitability and the tumor metastasis suppressor KAI1 levels [22, 25–28].

Some viruses hijack the ERAD system through encoding effectors by serving as adaptors that redirect correctly folded molecules towards degradation. US2 and US11, the human cytomegalovirus gene products, induce degradation of major histocompatibility complex (MHC) class I heavy chain, which enables virus-infected cell to avoid detection by the immune system [29]. Similarly, Vpu is a glycoprotein encoded in the human immunodeficiency virus (HIV-1) genome and binds and targets newly synthesized CD4 for degradation [30], allowing them to evade immunosurveillance. Moreover, toxins like diphtheria, cholera and ricin enter the cell by endocytosis and move to the ER. They use the ERAD system to escape from the ER lumen and gain access to their enzymatic substrates in the cytoplasm [31].

ERAD is a highly complicated and regulated mechanism in which the diversity and combination of components change according to the protein to be destroyed [19–21, 32]. Maturation-defective proteins are removed from the ER lumen or lipid bilayer by retrotranslocation through the ERAD pathway and degraded by proteasome. The ubiquitin system is an integral part of the ERAD and is composed of factors necessary for the recruitment, processing and binding of ubiquitin chains to substrates [24]. In other words, ERAD is composed of steps that include substrate selection, modification with ubiquitin chain, retrotranslocation and 26S proteasomal degradation. Several key molecules such as E1, E2, and E3 enzymes responsible for ubiquitin transfer, channel components responsible for retrotranslocation, chaperones and cofactor proteins function in a synchronized manner during ERAD pathway [12, 19–21].

This critical role of ERAD in the regulation of cell homeostasis is an evident that ERAD disorders will have important effects on cell survival. Furthermore, it has been shown that aberrations in ERAD function play a role in the pathology of nearly 70 diseases such as cystic fibrosis, α1-antitrypsin (AAT) insufficiency, diabetes, neurodegenerative diseases (Parkinson, Alzheimer's and Huntington's diseases), viral infection and albinism [4, 33].

In this section, the knowledge related to the basic mechanism and regulation patterns of the ERAD will be summarized and presented.

2. Molecular mechanisms of ERAD

2.1 Protein folding process and recognition of misfolded proteins

About 30% of the total proteins and all transmembrane proteins of the cell are synthesized in the ER, which acts as a portal for entry into the secretory pathway via the Sec61 channel [7–8]. As being translocated, the N terminal hydrophobic signal sequence of newly synthesized protein is cleaved by a peptidase complex [34]. Co- and post-translational modifications such as disulfide bond formation, initial steps of N-glycosylation, and glycophosphatidylinositol (GPI) anchorage take place in the ER.

The oxidizing environment of ER assists the formation of disulfide bonds, which stabilizes tertiary protein structure and facilitates protein assembly. During the folding process, disulfide bonds are formed through the oxidation of pairs of free thiols on cysteine residues by protein disulfide isomerases (PDIs). PDIs act as

cycles, and after initial oxidation, disulfide bonds are sometimes isomerized by PDI and ERp57, which is a thiol oxidoreductase, in order to stabilize the correct folding of protein [35]. Conversely, the reduction of disulfide bonds of misfolded proteins is necessary for retrotranslocation step of ERAD. Indeed, PDI enables the retrotranslocation of the simian virüs-40 (SV-40) and cholera toxin [36, 37]. ERdj5, an ER oxidoreductase, reduces disulfide bonds and interacts with EDEM (ER-degradation enhancing mannosidase-like protein) and also accelerates the step of retrotranslocation of SV-40 [37]. ERDJ5 also regulates the degradation of disease-causing α1-antitrypsin variant (null Hong Kong) [38].

Folding is aided by molecular chaperones shepherding against misfolding and unfolding. Chaperone-like glycans bind to N-glycans playing a crucial role in protein folding and degradation. It is apparent that N-glycosylation, quality control of protein folding and ERAD are functionally linked. After entering to the ER, a large majority of the newly synthesized polypeptide chain are being N-linked glycosylated. The oligosaccharyltransferase enzyme recognizes the Asn-X-Ser/Thr consensus sequence in the most of the nascent protein molecule and covalently integrates a high mannose containing core glycan groups ($Glc_3Man_9GlcNAc_2$) from dolichol localized on the ER membrane to the protein [39]. Due to the very short half-life of triglycosylated form of protein-bound oligosaccharide, glycan processing starts immediately after the transfer of precursor glycan groups through glucosidase enzymes. Following cleavage of two of three glucose residues, the nascent protein could interact with quality control lectins like CNX and CLR. This interaction is preserved until cleavage of remaining glucose residue. After releasing the glycoprotein from CNX/CLR cycle, final glucose is also trimmed creating unglycosylated substrate. This compromises the interaction of substrate with the lectin chaperones. At this stage, if protein is properly folded, it could exit the ER for their final destination. However, if glycoprotein is still unfolded, it is retained in the ER and reglucosylated by UDP-glucose:glycoprotein glucosyltransferase and rebound with CNX and CLR giving protein more time for proper folding [40, 41]. It is not yet understood the mechanisms involved in the termination of reglycosylation/deglycosylation cycles. However, it is clear that, if the polypeptide chain cannot reach its mature form after repeated folding attempts, terminal mannose residues from the core glycan chain are gradually removed by ER α1,2-mannosidase I (ERMan1). ERMan1 produces $Man_8GlcNAc_2$ isomer by removing a mannose residue from the middle branch of N-glycans. By this trimming, glycoprotein becomes poorer substrates for reglycosylation and exit from the CNX cycle [11].

The hydrophobic patches of properly folded proteins are usually buried within the interior of soluble proteins. However, those patches could be exposed in misfolded proteins. If a protein has exposed hydrophobic surfaces, BiP binds to it in order to hide these aggregation-prone surfaces for proper folding attempts by preventing aggregation. However, if folding does not succeed or delayed, extended chaperone-misfolding protein interaction serve for a sophisticated process where protein is transferred to other chaperones and/or to the ERAD process [27, 42].

It is well accepted that the first step of ERAD is selection of misfolded proteins by chaperones. As early as 1999, it was found that yeast ERAD substrates strikingly differed in their requirement for the ER-luminal Hsp70, BiP [43]. The degradation of soluble substrates such as pαF and a mutant form of the vacuolar protease carboxypeptidase Y* (CPY*) were dependent on BiP, while degradation of transmembrane proteins Pdr5*p, Ste6-166p, Sec61-2p and Hmg2p occurred in a BiP-independent manner. In 2004, it has been shown that substrates with cytosolic domain such as Ste6-166p were degraded BiP-independently, while proteins with luminal defects required BiP, suggesting that depending on the topology of

misfolded lesion (ER lumen, ER membrane and cytoplasm) cytosolic or luminal chaperones function in the recognition and targeting for the degradation [44].

It is possible to study substrate recognition during ERAD using model misfolded proteins. It is clear that de-mannosylation is required for degradation of misfolded glycoproteins since inhibition of this mannose trimming stabilizes misfolded glycoproteins in the ER [45]. Overexpression of ERMan1 accelerates the degradation of N-glycosylated proteins [39, 46]. The resulting Man_8-$GlcNAc_2$ containing glycoprotein after this trimming becomes a substrate for EDEM1 (ER-degradation enhancing mannosidase-like protein 1, Htm1p in yeast)—a mannosidase-related lectin in the ER. It was further proposed that misfolded glycoproteins interact with ERManI and EDEM1 for their ERAD, and lectin-carbohydrate interaction found to be crucial for EDEM substrate recognition [47]. Although ERMan1 was suggested to be a biological timer initiating the ERAD of misfolded proteins [48], recent studies revealed that mannosidases are not solely responsible for intensive demannosylation during ERAD, especially under non-basal conditions. Under ER stress (unfolded protein response active) conditions, the transcriptional elevation of EDEM1 enhances the ERAD efficiency by suppressing proteolytic downregulation of ERMan1 [49]. It appeared that EDEMs also play an important role in demannosylation of substrates [50]. EDEM1 also prevents reglycosylation and promotes retrotranslocation and degradation of some ERAD substrates [51]. On the other hand, while mannosidase homology domain (MHD) of Htm1p is necessary for substrate binding, mammalian EDEM1 binds misfolded proteins independent of MHD domain, and therefore, EDEM1 substrate binding may not require mannose trimming or even glycosylation [52]. Thus, in addition to N-linked oligosaccharide moieties of glycoproteins, EDEM1 can recognize the folding lesions of misfolded proteins. In summary, EDEMs are directly or indirectly involved in demannosylation of glycoproteins and/or serve as receptors that bind and target mannose-trimmed proteins for ERAD (**Figure 1**).

Truncation of terminal mannose from branch C exposes α terminal α1,6-bonded mannose residues functioning as a recognition signal for ERAD lectins such as OS9 (Yos9 in yeast) and XTP3-B (**Figure 2**). Through their mannose-6-phosphate receptor homology (MRH) domain, both proteins primarily recognize α1,6-linked mannose j. Additionally, OS-9 also recognizes α1,6-linked mannose e and c [53].

Several reports suggest that factors (EDEMs, OS9 and XTP3-B) required for substrate recognition and targeting reside within supramolecular complexes and/or interact with important ERAD regulators [54]. For example, EDEM1 interacts with CNX, receives substrates from CNX cycle and facilitates ERAD substrate

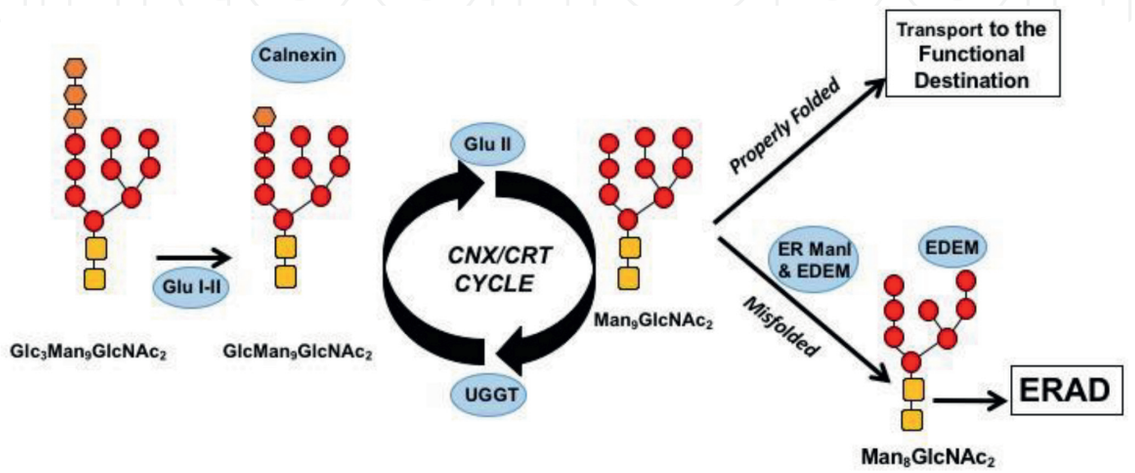

Figure 1.
Protein quality control and targeting misfolding proteins to the ERAD.

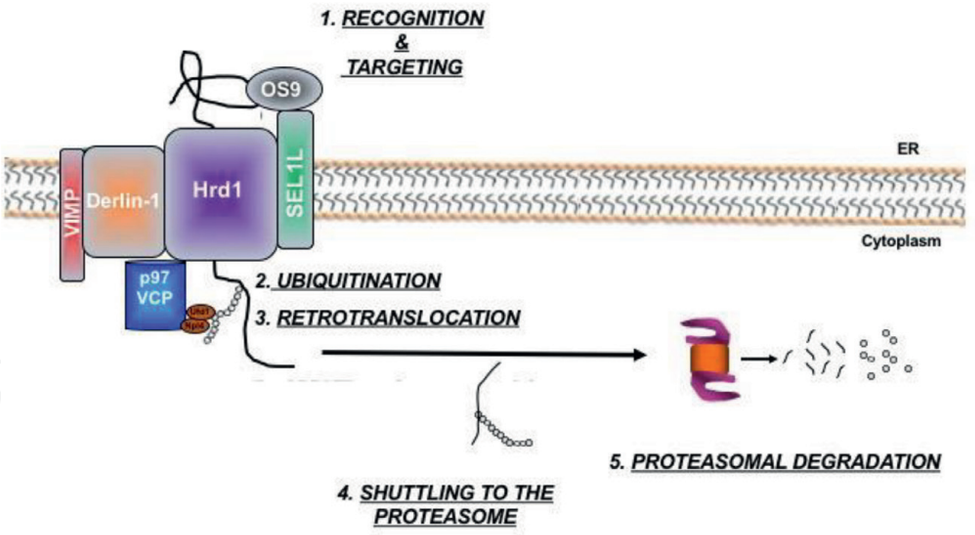

Figure 2.
Schematic representation of ERAD using the Hrd1 complex as model.

degradation such as NHK-α1-antitrypsin mutant [55–57]. EDEM1 also associates with the components of ER retrotranslocation machinery. It is suggested that EDEM1 binds misfolded proteins and uses its MHD domain to target aberrant proteins to the ER-resident glycoprotein SEL1L protein of the Hrd1-SEL1L ubiquitin ligase complex [58]. SEL1L scaffolds several luminal substrate recognition factors and links them to Hrd1. OS9 and XTP3-B also associate with Hrd1-SEL1L complex, which also includes BiP and GRP94 [59, 60]. Furthermore, XTP3-B is proposed to link BiP with Hrd1 complex [60]. According to a hypothesis, these three chaperones (EDEM1, OS9 and XTP3-B) function as oligomers, where one monomer interacts with substrate and another with Hrd1-SEL1L complex [61]. Additionally, EDEM1 also interacts with Derlins, a transmembrane protein, which is a candidate for translocon [62]; furthermore, Derlin2 is shown to enhance the interaction of EDEM1 with a cytosolic AAA-ATPase p97, which couples ATP hydrolysis to the retrotranslocation of misfolded proteins [50].

It is clear that substrate recognition step of ERAD is a complicated mechanism, in which several different enzymes and chaperones having distinct but concerted roles in the ERAD are involved. Moreover, depending on substrates, the number and features of involved proteins vary. For example, concerted roles of EDEM, ERdj5 and BiP in the degradation of misfolded proteins have been suggested [63]. After exiting CNX-CLR cycle, EDEM1 further trims the Man_8-$GlcNAc_2$ glycan structure and ERdj5 reduces disulfate bonds. Concomitantly, ERdj5 activates BiP's ATPase activity. ADP-bound BiP binds to the misfolded protein and holds it in a retrotranslocation component form until it transfers to the retrotranslocation complex [63].

ERAD is also involved in the quality control of non-glycosylated proteins, which is independent of lectin-like proteins. Immunoglobulin light chain (Ig-K-LC), a non-glycosylated ERAD substrate, is degraded in a BiP-dependent manner. Okuda-Shimizu and Hendershot have characterized an ERAD pathway for this non-glycosylated BiP substrate [64] and different protein interaction dynamics seen to play a role in this process. Ig-K-LC has two intramolecular disulfide bonds, and its fully oxidized form does not have ability to pass from the ER to the cytoplasm. BiP interacts with only partially oxidized form of the Ig, preventing the full oxidation of Ig-K-LC and thereby facilitating its release from the ER [64]. Furthermore, a transmembrane UBL domain-containing protein, homoCys-responsive ER-resident protein (HERP), has been implicated as a receptor for non-glycosylated BiP

substrates [64]. HERP interacts with Derlin1, and the partially oxidized Ig-K-LC is transferred from BiP to the HERP-Derlin1-Hrd1 complex and subsequently directed to proteasomal degradation [65]. Besides BiP, ERdj5 as disulfide reductase is also indicated to be important for ERAD of non-glycosylated proteins [63]. The non-glycosylated substrates captured by BiP are transferred to ERdj5 for the cleavage of disulfide bonds. Then, these substrates are transferred to SEL1L by the help of BiP for retrotranslocation [63]. Besides BiP, both OS9 and XTP3-B have been implicated in the ERAD of non-glycosylated proteins [12].

2.2 Ubiquitination

Ubiquitin is a 76 amino acid polypeptide encoded on multiple genes. It is ubiquitously expressed in all eukaryotic cells and highly conserved from yeast to human. Ubiquitin can be covalently conjugated to other proteins as monomers or as chains through a complex, highly regulated process called ubiquitination. Although there are reports for evidence of Ser- and Thr-linked ubiquitination, ubiquitin chain is generally attached on the Lys residue on misfolded protein. Lys-6, -11, -27, -29, 33, -48 and -63 are the residues used for ubiquitin linkage. Both the type of ubiquitination (mono/poly) and the linkages of ubiquitin chains affect the fate, localization, stability and activity of target proteins [9].

Ubiquitination has a regulatory role in almost all cellular processes by altering the fate and function of the proteins. The most well-established role of ubiquitination is targeting proteins for degradation by the 26S proteasome, and the most efficient way of targeting proteins to the proteasome is by tagging them with chains of ubiquitin [66]. This targeting requires modification of proteins with chains of four or more ubiquitins attached through lysine 48 (K48) and the specific recognition of these chains by the 19S cap of the 26S proteasome [67]. Mainly Lys-48 but rarely Lys-11-based polyubiquitin chains are reported to bind onto ERAD substrates [68].

Ubiquitination regulates several critical cellular functions, often by mediating the selective degradation of important regulatory proteins. Antigen presentation, inflammatory response induction and cell cycle progression are few examples. As expected, malfunctioning of ubiquitin-dependent proteolysis has implications for cancer and several inherited diseases, such as Angelman syndrome, Parkinson's disease and Alzheimer's disease [69].

The role of ubiquitination, however, is not limited to proteasomal targeting. The type of residue that the chain is built is critical for the fate of the ubiquitinated protein. Monoubiquitination has effects in protein trafficking, including endocytosis and lysosomal targeting. Polyubiquitin chains conjugated through K48 or other lysines (often K63) also have effect on proteasome-independent mechanisms, such as DNA repair, regulation of transcription factor activity and protein kinase activation [70].

Ubiquitination is a multi-enzyme process. Three enzymes are involved: E1-ubiquitin activating enzyme, E2-ubiquitin conjugating enzyme and E3-ubiquitin ligase. During ubiquitination, E1 forms a thiol-ester bond between its active cysteine and C-terminal glycine of ubiquitin in an adenosine triphosphate (ATP)-dependent manner. Ubiquitin on E1 is now activated and transferred to the active cysteine of E2 by a trans-thiolation reaction. E3 binds both to E2 and substrate and facilitates the formation of an isopeptide linkage between C-terminal glycine of ubiquitin and an internal lysine residue on substrate. Ubiquitin modification is dynamic and could be removed by deubiquitination enzymes (DUBs).

Today only 2 E1 enzymes and 35 E2 enzymes have been identified in mammals, but there are approximately 100 E3 in yeast and at least 600 in humans [71, 72]. E3s

catalyzing the transfer of active ubiquitin moieties on the substrate are responsible for substrate specificity. There are two large families of E3s: (1) HECT [homologous to E6-associated protein (E6AP) C-terminus] domain E3s and (2) RING [really interesting new gene] domain E3s. HECT domain E3s share a 350-residue region harboring a strictly conserved cysteine residue that forms an essential thiol-ester intermediate during catalysis. That is why ubiquitin is transferred to the active-site cysteine of the HECT domain followed by transfer to substrate or to a substrate-bound multi-ubiquitin chain. The RING finger defines the largest family of E3s. RING fingers range from 40 to 100 amino acids and are defined by eight conserved cysteine and histidine residues that coordinate two zinc ions stabilizing a characteristic cross-braced conformation. For RING E3s, current evidence indicates that ubiquitin is transferred directly from E2 to substrate [69, 70].

Ubiquitination step marks ERAD substrates for proteasomal degradation. In yeast, Doa10p and Hrd1p ligases are mainly responsible for ubiquitination of ERAD substrates, but additional E3s shown to contribute to the ERAD under special circumstances [9]. Depending on the topology of misfolded lesion, factors required for ERAD vary. In yeast, three ERAD pathways have been proposed. ERAD-C, ERAD-L and ERAD-M target proteins with lesions in the cytoplasmic, luminal and membrane domains, respectively [44, 73, 74]. ERAD-L substrates use the Hrd1p ubiquitin ligase complex containing Hrd1p, Hrd3p, Usa1p, Der1p, and Yos9p, whereas ERAD-M substrates use Hrd1p and Hrd3p, only in some cases Usa1p [68]. Hrd3p is specifically important for structural integrity of Hrd1p complex. Hrd3p stabilizes Hrd1p, and when it is absent, Hrd1p is auto-ubiquitinated and rapidly degraded. Hrd3p and its mammalian homolog SEL1L also function as an adaptor bridging substrate recognition, ubiquitination and retrotranslocation in Hrd1-mediated ERAD. On the other hand, ERAD-C substrates interact with the Doa10p ubiquitin ligase complex. These three pathways have been identified only in yeast and mammalian has more complicated machinery. Even in yeast, some membrane proteins require both Doa10p and Hrd1p E3s; thus, these pathways could overlap [42].

Although Hrd1p and Doa10p are conserved evolutionary (mammalian homologs: Hrd1 and TEB4, respectively), the number of ERAD E3s in mammals is highly expanded. Besides Hrd1 and TEB4, gp78, RNF5/RMA1, RNF170, RNF185, Trc8, RNF103, RFP2, Fbx2, Fbx6, Parkin, CHIP and UBE4a are other characterized ERAD E3s [9, 27]. Hrd1 and gp78, both homologues to yeast Hrd1p, are the most studied ERAD E3 indicated for degradation of several substrates, some of which are associated with the quality of disease-related proteins. HMG-CoA reductase, apolipoprotein B, cytochrome P450 CYP3A4, CFTRΔF508, z-variant antitrypsin, CD3δ and KAI1 are shown to be degraded via gp78-mediated ERAD, whereas studies have been suggested that Hrd1 is important for the ERAD of GABAb receptor, Nrf2, Pael-receptor mutant tyrosinase, z-variant antitrypsin and gp78 [22, 75–78]. Only a couple of substrates are known for other E3 ligases. It is also interesting that multiple E3s often function in the degradation on same substrate either in parallel or in tandem.

As Hrd1p in yeast, Hrd1 in mammals functions in a multi-protein complex. While it is complex with EDEM1, Derlins, OS9, XTP-3B and SEL1-L have been linked with degradation of glycosylated substrates (**Figure 2**), and another Hrd1 complex utilizing BiP, HERP and Derlin1 functions in the degradation of non-glycosylated substrates. Other ERAD factors have also been shown to interact with Hrd1 including UBXD2 and UBXD8 that interact with p97/VCP and recently identified chaperones such as ubiquilin and BAG6. Similarly, gp78, the second major mammalian ERAD E3 enzyme, functions in multiprotein complex in conjunction with E2 enzyme UBE2G2. Besides its diversity on substrate specificity, gp78 also has

variety of different partners allowing its communication with proteins on both sites of ER membrane. gp78 uses a VIM (VCP-interacting motif) segment to bind p97/VCP [77] and CUE domain recruiting a multiprotein complex composed of Bag6 and its cofactors [79].

After initial E3-mediated ubiquitin attachment, ubiquitin chain extension ("polyubiquitination") occurs by the covalent modification of additional ubiquitin monomers onto a Lys residue in a previously linked ubiquitin. This forms an extended isopeptide-linked polyubiquitin chain. In some selected cases, the cooperative extension of a polyubiquitin chain is by the E4s, ubiquitin chain extension enzymes, that facilitate ERAD [80–82].

2.3 Retrotranslocation and shuttling substrates to the proteasome

The ERAD substrates must be retrotranslocated to the cytosol for proteasomal degradation and the cytoplasmic AAA+ ATPase p97 (VCP or Cdc48p in yeast) is the main retrotranslocation protein providing the mechanical force required for removal of proteins from the ER. It is an essential protein having many roles in diverse biological processes, such as endoplasmic reticulum-associated degradation (ERAD), homotypic membrane fusion, transcriptional control, cell cycle regulation, autophagy, endosomal sorting and regulating protein degradation at the outer mitochondrial membrane [83–85].

p97/VCP has a multidomain structure including N domain, D1 weak ATPase, D2 major ATPase and C domain [86–88]. p97/VCP functions as a homohexamer and D1 domain is responsible for oligomerization independent of nucleotide binding. The change in the conformation of hexameric ring by ATP hydrolysis is persistent with its function in retrotranslocation [88, 89].

The diversity in cellular functions of p97/VCP is dictated by the variety of its partner proteins that interact with its N domain. p97/VCP associates with several E3s like Hrd1 and gp78, DUBs like ataxin3 and YOD1 and ERAD accessory factors such as UbxD2 and VIMP. Moreover, many p97/VCP interacting proteins (Ufd1-Npl4 dimer, gp78 etc.) bind directly to ubiquitin. p97/VCP functions as a segregase using the energy from ATP hydrolysis to segregate ubiquitinated proteins from large immobile complexes of ER to the cytosol. This cytosolic protein is recruited to the ER membrane through its interaction with membrane-embedded ERAD components. There are at least seven different ERAD members that could interact with p97/VCP via certain motifs such as VIM motif (gp78 and SVIP), UBX domains (UBXD2 and UBXD8), SHP boxes (Derlin1 and Derlin2) and uncharacterized cytosolic regions of Hrd1 and VIMP that found to have p97/VCP-binding motif [12, 42, 90].

Retrotranslocation is tightly coupled with both ubiquitination and proteasomal degradation. In most cases, inhibiting ubiquitination prevents both degradation and retrotranslocation. The interaction of p97/VCP/CDC48p with its cofactor Ufd1-Npl4 dimer enhances its affinity to ubiquitin (**Figure 2**). However, it has been also suggested that Hrd1-mediated ERAD requires well-established retrotranslocation machinery, the p97/VCP–Ufd1–Npl4 complex, whereas the gp78 pathway needs only p97/VCP and Npl4 [75].

Many deubiquitinating enzymes (DUBs) in mammalian cells, including Ataxin3, USP13, USP25 and YOD1, are also implicated in the ERAD through physical interaction with ERAD core machinery [72, 91, 92]. Several studies revealed that p97/VCP interacts with DUBs. However, the function of DUBs in the ERAD is still not fully characterized. Otu1p (yeast homolog of YOD1) binds to CDC48p and trims the polyubiquitin chain, resulting oligoubiquitin chains with up to 10 ubiquitin molecules. It has been further suggested that releasing substrates from CDC48p requires DUBs [93]. Consistently, catalytically inactive YOD1 inhibits retrotranslocation of

ERAD substrates [91]. In conclusion, many p97-associated DUBs serve as positive regulators of ERAD.

Several putative retrotranslocation channels have been proposed such as the Sec61 complex, members of Derlin family and polytopic E3s such as Hrd1 and gp78. Sec61 is one of the proposed channel protein mutants, which prevented degradation of some ERAD substrates in yeast [94, 95]. Cholera toxin also translocates from ER by utilizing Sec61 [96]. On the other hand, retrotranslocation of some other ERAD substrates has been suggested to depend on Derlins [97, 98], a family of polytopic transmembrane ER proteins linked to some ERAD substrates. Moreover, Derlin1 recruits p97/VCP [99], a key protein of retrotranslocation, which provides energy for the process. Derlin1 also interacts with some E3s like Hrd1, gp78 and RNF5 forming large complexes on the ER membrane [9]. Recently, Hrd1 ubiquitin ligase has been suggested to be the top candidate for retrotranslocation channel [9]. Auto-ubiquitination of Hrd1p in its RING finger domain triggers conformational change allowing the misfolded luminal domain of a substrate to move across the membrane. Thus, it was suggested that Hrd1 forms an ubiquitin-gated protein-conducting channel [33]. It has also been suggested that proteins might exit the ER via the formation of lipid droplets or lipid droplets serve as an intermediate step for substrates en route to the proteasome [100]. However, studies in yeast suggested that lipid droplet formation is dispensable for ERAD-L and ERAD-M [101].

Once retrotranslocated from ER to the cytosol, ERAD substrates should be rapidly targeted to the proteasome for degradation in order to avoid accumulation of aggregates in the cytosol. Consistently, proteasomal inhibition also stabilizes ERAD substrates in the ER lumen. For the coupling of retrotranslocation with degradation, ubiquitinated substrates must be recognized by cytosolic proteins functioning as ubiquitin receptors. Ubiquitin-binding domain containing proteins has ability to shuttle ubiquitinated proteins from retrotranslocation complex at the ER membrane to the proteasome since these proteins interact both with proteasome and p97/VCP. Indeed, it has been suggested that p97/VCP bridges the ER to the proteasome by forming a complex with mHR23B (homolog of yeast Rad23p)-PNGase [102] (**Figure 2**). In yeast, the substrates are probably transferred from CDC48p to the proteasome indirectly via ubiquitin- and proteasome-binding domains containing shuttling factors Rad23p and Dsk2p [103, 104]. Recently, Bag6/Bat3/Scythe has been characterized as a novel chaperone system with regulatory functions in protein degradation [79]. The chaperone holdase activity of this system keeps some retrotranslocated substrates in a soluble state for proteasome degradation. Bag6, also a partner protein of gp78 E3 enzyme, interacts with proteasome, and proteins like ubiquilin that known as proteasome adaptor proteins suggesting Bag6 might act between p97/VCP and proteasome to hand substrates off from retrotranslocation machinery to the proteasome.

3. Regulation of ERAD

Regulation of ERAD in normal and pathological conditions is also of great importance since hyper-ERAD may cause in loss-of-function phenotypes upon unnecessary degradation of folding intermediates as seen in CFTR and hypo-ERAD may result in gain-of-function phenotypes upon accumulation and/or aggregation of misfolded and unassembled proteins. Several studies suggested different regulation paths for ERAD activity via ubiquitin ligases and their dynamic ERAD complexes, UPR and endogenous ERAD inhibitors.

It is thought that ERAD functions at relatively low levels under basal conditions, but under proteotoxic stress its activity is enhanced. Accumulation of the unfolding

or misfolding proteins in the ER lumen triggers "ER stress" by decreasing free chaperone levels [105]. In response to this cellular stress, the pathway known as the "Unfolded Protein Response (UPR)" is activated and results in specific cellular functions classified as adaptation, alarm and apoptosis [4]. Three transmembrane proteins with luminal domains that sense the changes in the ER environment function as UPR sensor proteins are inositol requiring enzyme-1 (IRE1), activating transcription factor 6 (ATF6) and protein kinase RNA-like endoplasmic reticulum kinase (PERK). PERK is a serine/threonine kinase, and IRE1 possesses both kinase and endoribonuclease domains [27, 50]. These sensors initiate signal transduction by sensing the presence of unfolded proteins in the ER lumen and thus control the UPR pathway [15, 18, 106]. All these transmembrane proteins interact with BIP under basal conditions. However, when unfolded proteins are present, BIP dissociates from the UPR sensor proteins. After dissociation of BIP, PERK and IRE1 dimerize and become activated by auto-phosphorylation, whereas ATF6 become translocated to the Golgi and proteolytically cleaved [27, 50]. Activated PERK phosphorylates translation factor eIF2α attenuating protein synthesis to limit protein load. IRE1 activates XBP-1 that enhances transcription of ERAD factors [27, 50]. On the other pathway, ATF6 upregulates many genes that encode ER-resident chaperones and folding assistants like BIP, CNX, CLR and PDI. To summarize, with the induction of UPR in the cell, the overall translation is inhibited for several hours primarily to slow down the entry of newly synthesized proteins to the ER, the amount of chaperones and ER protein folding capacity is increased for proper folding of accumulated unfolded proteins, and thus, the normal ER function and homeostasis are protected [4, 107]. UPR also enhances ERAD capacity by upregulating some of the ERAD genes to ensure that defective proteins are degraded when the folding attempts fail [21–23]. EDEM proteins, Hrd1, SVIP, OS9 and gp78, are only some of the targets of the ER stress-induced Ire1/Xbp1 pathway [62, 108–111]. If the cellular stress is consistently increasing, UPR induces cell death mechanisms such as apoptosis or autophagy [4, 14, 112].

It has been suggested that large or prolonged variations such as change in Ca^{2+} or redox homeostasis, exposure to pathogens and large-scale accumulation of misfolded proteins may induce UPR to adapt ERAD activity. However, smaller or more transient fluctuations on ER load may be overcome rapidly by post-translational pathways that control stability, localization and assembly of ERAD components [23]. For example, reversible ADP ribosylation adapts BIP response for short-term fluctuations [113]. Reversible palmitoylation changes the sub-organelle distribution of CNX [114, 115]. Moreover, many ERAD factors/enhancers, including EDEM1, ER Man1, HERP, OS9, SEL1L and gp78, have fast turnover. This is important since when protein misfolding crisis is over, ERAD activity should rapidly turn back to the basal levels. Many ERAD factors then rapidly degraded via a process called ERAD tuning [23]. ERAD tuning does not require signal transduction from the ER to the nucleus [23]. Hrd1 was suggested to be a central regulator of ERAD tuning. It has been shown that Hrd1 ubiquitinates gp78 E3 enzyme and enhances its degradation, which in turn causes inhibition of gp78-mediated ERAD. Very recently, Hrd1 was also found to regulate the stability of OS9 [116]. Hrd1 also undergoes auto-ubiquitination to induce its own proteasomal degradation [117]. Another homeostatic control mechanism, in which ERAD activity itself is regulated post-translationally and independent of UPR, is degradation of EDEM1, OS9 and SEL1L by the E2 enzyme UBC6e, a component of Hrd1 supramolecular complex [118].

Another type of ERAD regulation occurs via substrate-specific adaptor, as reported for HMGR. The adaptor proteins, Insig1 or Insig2, bind to HMGR only in the presence of 24,25-dihydrolanosterol, an intermediate molecule in sterol

biosynthesis. Under low sterol levels, HMGR is stable; however, when sterol levels are high, Insig-HMGR interaction become favored, leading delivery of HMGR to E3 complex following by its proteasomal degradation [119]. Likewise, ERAD-mediated degradations of apolipoprotein and IP3R are initiated when lipid levels are low and calcium levels are high, respectively [23].

DUBs are also proposed as factors that regulate ERAD. As explained above, several DUBs have been reported to interact with p97/VCP and function as positive regulators of retrotranslocation. Additionally, some DUBs are linked with the regulation of E3 enzyme stability. For example, USP19, an ER-anchored DUB, rescues HRD1 from proteasomal degradation and thereby regulates HRD1 stability [120]. Similarly, USP19 enhances the stability and activity of another E3 MARCH6 [121].

SVIP (small VCP interacting protein), a VCP-interaction motif (VIM) containing protein, is the first identified endogen ERAD inhibitor. SVIP interacts with p97/VCP and Derlin1 and inhibits the ubiquitination and degradation of gp78-dependent ERAD substrates [111]. Another endogen ERAD inhibitor is SAKS1. SAKS1 binds to the polyubiquitin chain of the substrate and p97/VCP and attenuates the ERAD process [122].

ERAD activity can also be controlled by hormonal regulation. Glucocorticoids have been suggested to ameliorate ER stress by promoting correct folding of secreted proteins and enhancing removal of misfolded proteins from the ER probably through induction of UPR. Recently, androgen-mediated regulation of ERAD has been reported. Androgen treatment upregulated the expression of Os9, p97/VCP, Ufd1, Npl4, Hrd1 and gp78, but downregulated ERAD inhibitor SVIP, which in turn enhanced the proteolytic activity of ERAD in androgen-sensitive prostate cancer cells [123]. Furthermore, the regulation of ERAD by androgen is mediated via AR and is partially or fully independent on the androgen-mediated induction of IRE1α branch [123].

Conflict of interest

The authors declare no conflict of interest.

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