1.39 Unfolded Protein Response

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Glossary

AARE (amino acid response element) An enhancer element to which a transcription factor ATF4 bind in response to amino acid starvation and endoplasmic reticulum stress.
C/EBP (CCAAT/enhancer-binding protein) A transcription factor that binds to a CCAAT sequence and forms a heterodimer with CHOP.
CHIP (carboxy terminus of HSP70-interacting protein) A co-chaperone of HSP70.
CHOP (C/EBP-homologous protein) A transcription factor that is similar to C/EBP and regulates endoplasmic reticulum stress-induced apoptosis.
CTA1 (catalase) An enzyme that converts hydrogen peroxide to water and oxygen.
DVE-1 (defective proventriculus-1) A transcription factor regulating the mitochondrial unfolded protein response.
FBX (F-box protein) A series of proteins containing the F-box motif.
GCN2 (general control nondepressible) A kinase activated by amino acid starvation and involved in translational attenuation.
GRP (glucose-regulated protein) A series of proteins of which expression is increased in response to glucose starvation.
HRD (HMG-CoA reductase degradation protein) A series of proteins involved in endoplasmic reticulum-associated degradation.
OASIS (old astrocyte specifically induced substance) A transcription factor similar to ATF6.
ORP (oxygen-regulated protein) Proteins of which expression is increased upon hypoxia.
OS-9 (osteosarcoma 9) A glycoprotein that is localized in the endoplasmic reticulum (ER) and involved in ER-associated degradation.
PERK (PKR-like endoplasmic reticulum kinase) A transmembrane protein that is located in the endoplasmic reticulum and regulates the endoplasmic stress response.
PEX (peroxisomal matrix) A series of proteins involved in protein transport from the cytosol to the peroxisomes.
RMA (RING membrane-anchor) A membrane protein in the endoplasmic reticulum (ER) regulating ER-associated degradation with a RING finger motif.
RPN (regulatory particle non-ATPase) A series of proteins contained in the 19S subunit of the proteasome with no ATPase activity.
SEC (secretion) A series of proteins involved in protein secretion.
VIP (vesicular integral membrane protein) A vesicular integral membrane protein that recognizes glycans and regulates transport of glycoproteins.
VIPL (VIP36-like protein) A transmembrane protein similar to VIP36, and involved in transport of glycoproteins from the endoplasmic reticulum to the Golgi apparatus.

1.39.1 Introduction

The mass production of useful proteins, such as peptide hormones and enzymes, is one of the important issues in biotechnology. Although recombinant proteins can be produced either in the cytosol or in the secretory organelles, recombinant proteins used in humans for medical purposes are often secretory proteins, and need to be synthesized in the secretory organelles, that is, the endoplasmic reticulum (ER) and the Golgi apparatus in order to undergo appropriate processing and modifications for maturation. For instance, they should be correctly folded with the assistance of specialized chaperones (ER chaperones), form proper disulfide bonds in collaboration with protein disulfide isomerases (PDIs) in the oxidative environment (see Chapter **1.17**), and undergo various modifications, including N-glycosylation, O-glycosylation (see Chapters **1.07** and **3.41**), phosphorylation, sulfation, and cleavage by proteinases (**Figure 1**). Although recombinant proteins can be produced in either prokaryotes or eukaryotes, it is necessary to produce them in the original organisms if post-translational modification of the recombinant proteins is critical for their activities, as the modification pattern is quite diverse among species. For instance, N-linked and O-linked oligosaccharides are quite different among yeast, insect, and mammals, while little glycosylation of proteins occurs in bacteria, such as *Escherichia coli*.

However, there is one major problem in the production of recombinant proteins in the ER. Too much production of secretory proteins easily overwhelms the ER, as its capacity is very limited. In that case, most secretory proteins are unfolded or malfolded, and detained in the ER, resulting in degradation of recombinant proteins. Moreover, unfolded proteins tend to form aggregates, and such accumulation of unfolded proteins is very harmful to cells (ER stress), and often causes ER stress-induced cell death. Fortunately, eukaryotic cells have an adaptive mechanism to deal with ER stress, which is called the unfolded protein response (UPR) or the ER stress response (**Figure 2**) [1–7]. The UPR activates the transcription of ER chaperone genes as well as genes involved in ER-associated degradation (ERAD), which degrades unfolded or malfolded proteins by the ubiquitin–proteasome system. In the UPR, the capacity of the ER is greatly upregulated and can sustain the production of a large number of secretory proteins; however, the capacity of the UPR is not limitless. If we can artificially enhance the capacity of the UPR, it will greatly improve the efficiency of recombinant protein production. The molecular mechanism of the UPR in mammalian cells will be described in detail, as well as in other organisms, such as *Saccharomyces cerevisiae* (baker's yeast), *Caenorhabdtis elegans* (nematodes), and *Drosophila melanogaster* (fruit fly).

Understanding the mechanism of the UPR is not sufficient. Organelles other than the ER also have regulatory mechanisms similar to the UPR, that is, the mitochondrial UPR, lysosomal stress response, peroxisome stress response, and Golgi stress response, to maintain proteostasis in their compartments [2]. Understanding the molecular mechanism regulating the capacity of organelles will make it possible to enhance the quality and yield of recombinant proteins, and contribute to devising a 'suprasecretory cell' that efficiently produces an enormous number of recombinant proteins (see Chapter **1.31**).

In this article, the basics of the UPR, ER chaperones, and ERAD machinery will be described, as well as recent progress in UPR research. In the final section, the UPR-like mechanisms observed in other organelles will be described. Only essential references are cited due to space limitations, and sincere apologies are sent to the many researchers in this field whose papers could not be cited here.

1.39.2 Molecular Mechanism of the UPR

In eukaryotic cells, secretory and membrane proteins are synthesized in the ER. Nascent proteins produced in the ER are mostly unfolded or malfolded, and a team of ER chaperones assists them to form their proper conformation. Only correctly folded proteins are transported to the Golgi apparatus, while misfolded proteins are detained in the ER by the function of ER chaperones, and, finally degraded by the ERAD system. If cells increase the production of secretory proteins and the capacity of the ER chaperones and ERAD machinery, unfolded nascent proteins accumulate in the ER and form aggregates toxic to cells, resulting in the induction of ER stress. As ER stress-induced apoptosis is one of the major causes of various diseases, including Alzheimer's and Parkinson's diseases

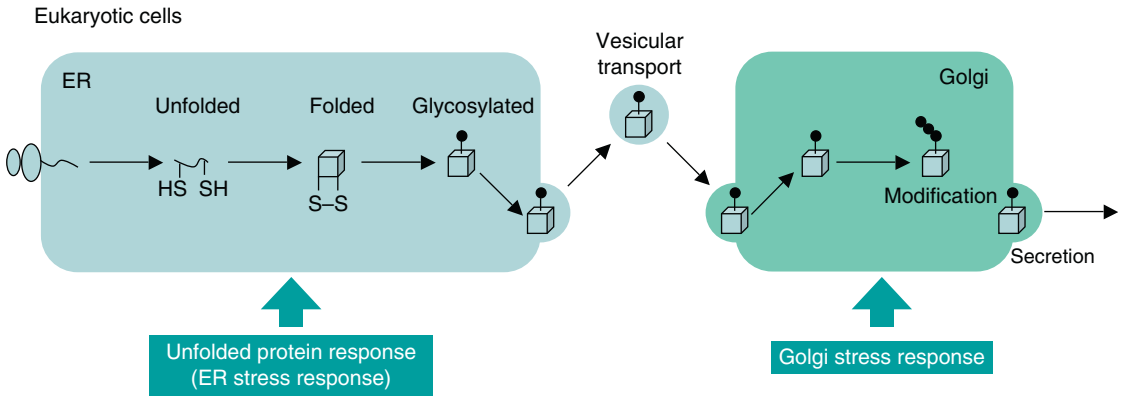

Figure 1 Maturation of secretory proteins in eukaryotic cells.Secretory proteins are correctly folded and undergo various modifications in the ER and the Golgi apparatus, which are important for their functions. The capacity of the ER and the Golgi apparatus to deal with secretory proteins is regulated by the unfolded protein response and the Golgi stress response, according to cellular needs.

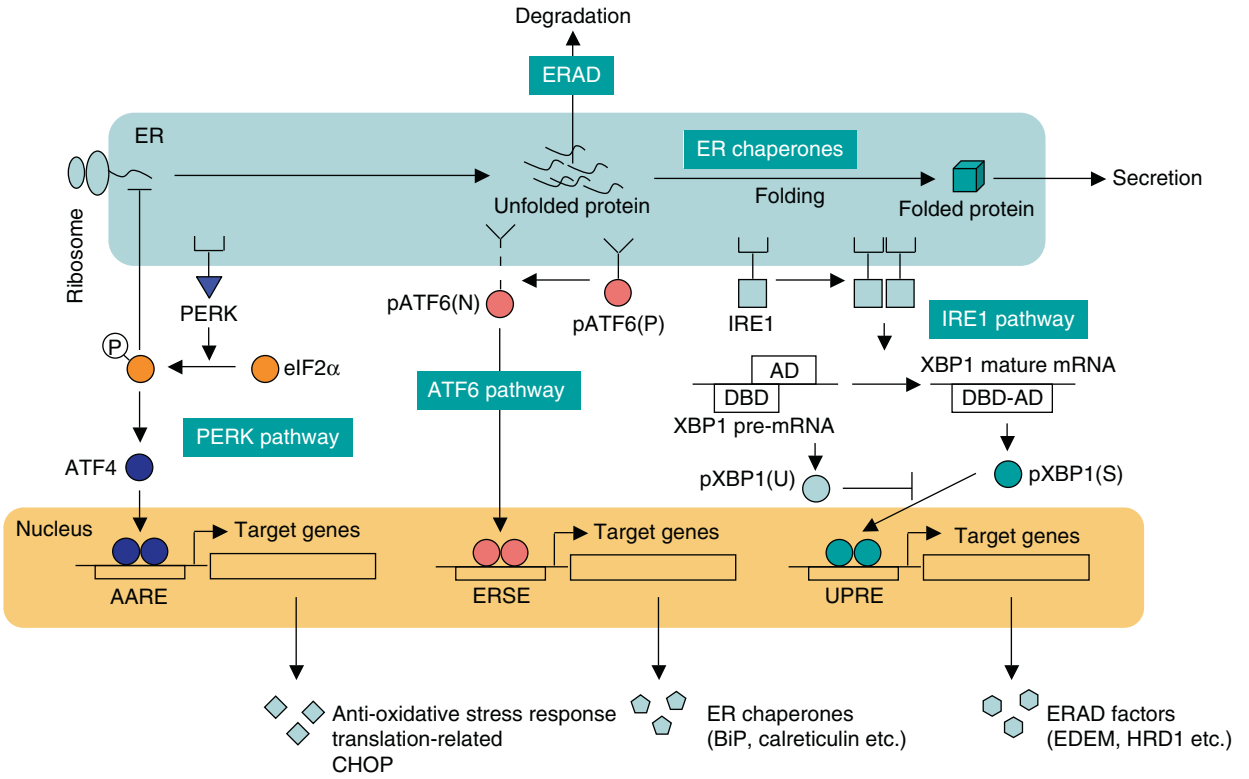

Figure 2 Molecular mechanism of the mammalian unfolded protein response. The unfolded protein response is a defensive mechanism to cope with unfolded proteins accumulated in the ER, which are toxic to cells. Mammalian cells have three response pathways to regulate the response, that is, the ATF6, IRE1, and PERK pathways.

[8], research on ER stress is also important in medicine. To alleviate ER stress, cells activate the UPR to enhance the expression of ER chaperones and ERAD components as well as to reduce the amount of influx of nascent proteins into the ER by attenuating translation (Figure 2). In this article, the basics of ER chaperones and the ERAD system will be explained, followed by an explanation of the molecular mechanism of the UPR.

1.39.2.1 ER Chaperones

A variety of molecular chaperones and folding enzymes[9], collectively called ER chaperones, of which BiP/GRP78 is the most important, exist in the ER. BiP was first identified as a binding protein of immunoglobulin in the ER, and Gething and colleagues revealed that the expression of BiP is induced by the accumulation of unfolded proteins in the ER [10]. BiP belongs to the heat shock protein 70 (HSP70) family, and preferably binds to the hydrophobic region of client proteins. BiP is involved in various processes in the ER. It assists in the folding of nascent proteins in the ER, aids in the translocation of nascent polypeptides through the translocation channel, and in the degradation of malfolded proteins by the ERAD system. BiP exerts these functions using the energy produced by the hydrolysis of adenosine triphosphate (ATP), and by collaborating with various HSP40 family proteins (J-proteins). GRP170/ORP150 is another HSP70 family chaperone, while GRP94 is a HSP90 family chaperone. ORP150/GRP170 and GRP94 participate in protein folding in the ER, although functional differences, such as substrate specificity among BiP, ORP150, and GRP94, have not been clarified. As ORP150 knockout mice become sensitive to ER stress and GRP94 knockout mice are embryonically lethal, their function is not redundant. The ER also contains various ER chaperones belonging to the HSP40 family, including SEC63/ERdj2, P58IPK, ERdj1/MTJ1, ERdj3/DNAJB11, ERdj4, and ERdj5. These HSP40 proteins are thought to function in association and collaboration with BiP and ORP150 through their J domains. SEC63/ERdj2 is an ER membrane protein, forms a complex with SEC61 (a core component of the translocon) and SEC62, and is involved in the translocation of secretory proteins into the ER lumen. p58IPK is involved in the dephosphorylation of eIF2α as well as the preemptive quality control of secretory proteins (see below). ERdj1/MTJ1 and ERdj3/DNAJB11 are thought to increase the ATPase activity of BiP and to assist in the folding of secretory proteins in collaboration with BiP, whereas ERdj4 and ERdj5 are involved in ERAD (see below).

Calnexin (CNX) and calreticulin (CRT) are lectin-type ER chaperones specialized in the folding of glycoproteins [11]. Most secretory proteins receive *N*-linked glycans in the ER, which consist of two *N*-acetyl-glucosamine residues, nine mannose residues, and three glucose residues (G3M9) (Figure 3). When two glucose residues are removed by glucosidase I and II (G1M9), CNX and CRT bind to client proteins and facilitate their folding process. When the last glucose residue is removed by glucosidase II (M9), client

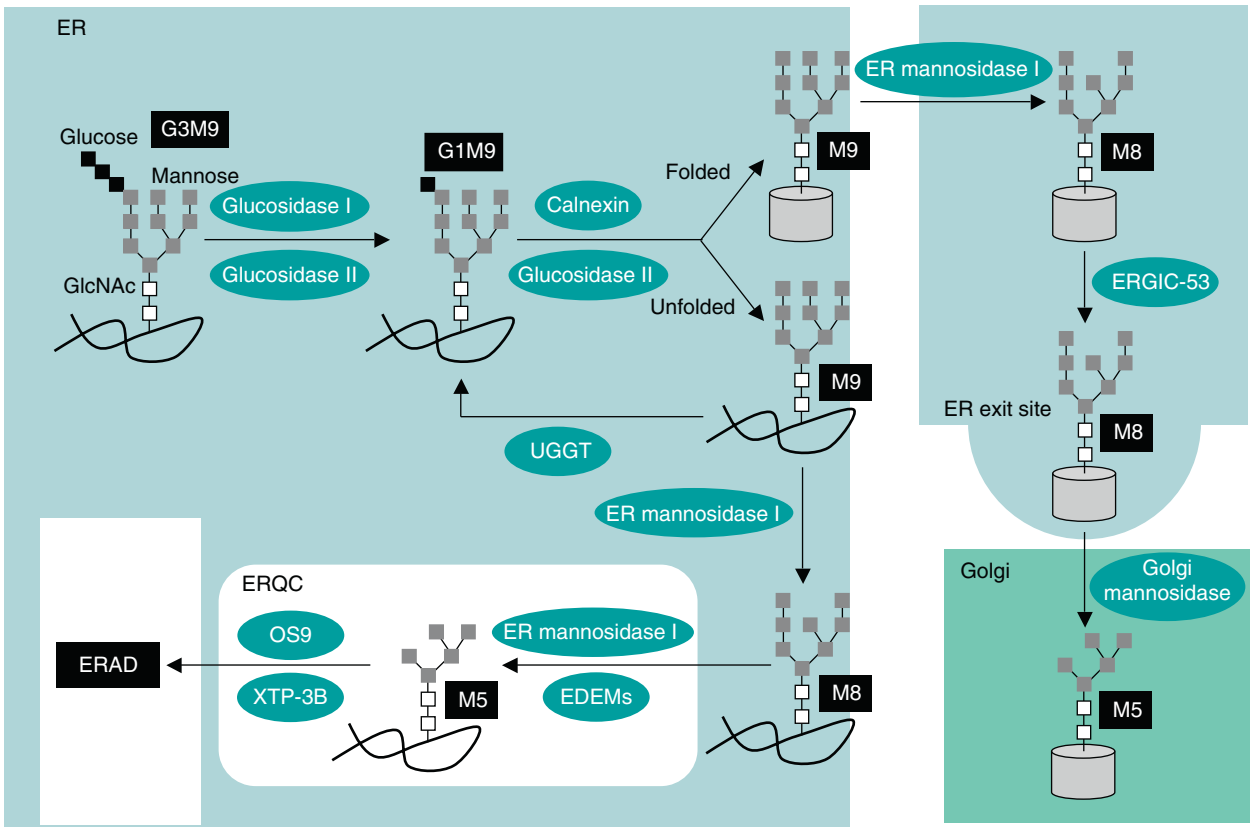

Figure 3 Processing of *N*-glycans in the ER. *N*-glycans of glycoproteins are sequentially trimmed by a set of glycosidases, and the resultant glycans are recognized by specific lectins. These various forms of glycans are utilized to discriminate the proteins to be secreted from those to be degraded.

proteins are released from CNX and CRT, and bind to uridine diphosphate (UDP)–glucose–glycoprotein glucosyltransferase (UGGT). If client proteins are correctly folded, they are released from UGGT, recognized by cargo receptors such as ER–Golgi intermediate compartment (ERGIC-53), VIP36-like protein (VIPL), and VIP36, recruited to the ER exit site and translocated to the Golgi apparatus, where three more mannose residues are removed by the Golgi mannosidase to be released from the cargo receptor (M5). If the client proteins have not correctly folded, UGGT adds one glucose residue to the clients (G1M9), and CNX and CRT again try to fold them. Thus, UGGT is a folding sensor that inspects the folding status of client proteins. This folding process is called the CNX cycle.

Secretory proteins often contain several disulfide bonds formed between their cysteine residues to stabilize their structure. The ER is a considerably oxidative compartment suitable for the formation of disulfide bonds. The formation of disulfide bonds (oxidation of cysteine residues) in secretory proteins is facilitated by the family of PDIs residing in the ER [12]. As disulfide bonds have to be formed between properly selected cysteine residues, the formation of disulfide bonds seems to be a very complex process. Actually, mammalian cells have a number of PDI-like proteins, including ERp57/GRP58, PDIp, ERp72, PDI-like protein of the testis (PDILT), ERp27, PDIr, ERp28, ERdj5, P5, ERp18, ERp44, ERp46, transmembrane Trx-related protein (TMX), TMX2, TMX3, and TMX4. These PDI family proteins usually contain several CXXC motifs, with a thioredoxin-like structure, and which are important for their activities. PDI contains four thioredoxin-like domains, called a, b, b′ and a′ domains. The a and a′ domains, which contain CXXC motifs, are thought to be catalytic domains, whereas the b′ domain is the major site for substrate binding. ERp57 and ERp72 are well known to be involved in the process of protein folding as well as ERAD, but the involvement of other PDI-like proteins in these processes has been elucidated only recently. The TMX subfamily members are thioredoxin-related transmembrane proteins, and Nagata and colleagues recently revealed that TMX4 has reductase activity instead of oxidase activity. TMX4 might be involved in the folding process and the retrotranslocation process of the ERAD by reducing and extending its substrates. ERdj5 has four CXXC motifs and the J-domain, which is shared among the HSP40 family proteins and is involved in interaction with HSP70 proteins, suggesting that ERdj5 may function in collaboration with BiP. As the cysteine residues of PDI are reduced after disulfide bond formation of the client, they have to be oxidized to perform another round of disulfide bond formation. This oxidation of PDI is carried out by the ER membrane protein ER oxidoreductin 1 (ERO1). ERO1 itself is oxidized by molecular oxygen.

1.39.2.2 ER-Associated Degradation

ERAD is another quality control system in the ER. Unfolded or malfolded proteins accumulated in the ER are recognized by ERAD sensors, extended by ER chaperones, retrotranslocated from the ER to the cytosol, and degraded by the ubiquitin–proteasome

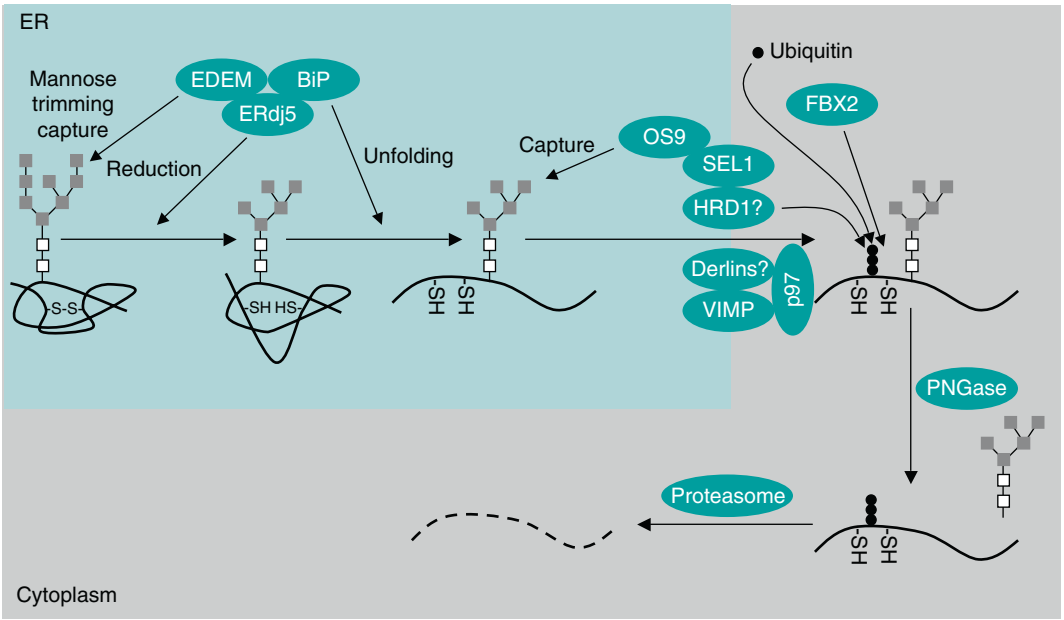

Figure 4 Mechanism of mammalian ERAD system. The mechanism of ERAD consists of several steps, including the recognition of ERAD substrates, unfolding, retrotranslocation, ubiquitination, deglycosylation, and degradation by the proteasome.

system [11, 13–22]. The process of ERAD can be separated into several steps. First, ERAD substrates have to be specifically recognized by the ERAD machinery (Figure 4). ER lectins, including EDEMs, OS9, and XTP3B, are thought to be involved in this recognition step. During the CNX cycle, N-glycans of glycoproteins consist of two *N*-acetylglucosamines, nine mannose, and one or none glucose residues (G1M9 or M9) (Figure 3). If the client proteins are difficult to fold and the CNX cycle is prolonged, one mannose residue of the N-glycan is removed by ER mannosidase I (M8), to which EDEM binds with its mannosidase-like domain, and recruits the client to the ER quality control compartment (ERQC), where components of the ERAD machinery are concentrated. As EDEM cannot bind to *N*-glycans containing nine mannose residues, the mannose residues seem to function as timers counting the time allowed for secretory proteins to fold correctly, and EDEM is a gatekeeper determining the fate of secretory proteins. EDEM recognizes not only the *N*-glycan but also the polypeptide portion of glycoproteins, and discriminates unfolded from folded polypeptides, although the mechanism of this discrimination process is unknown. Mammalian cells contain three EDEM proteins. EDEM1 is an ER transmembrane protein, while EDEM2 and EDEM3 are luminal ER proteins, although their functional differences have not been identified.

In the ERQC, more mannose residues are removed from the N-glycans of misfolded glycoproteins (M6-5) by ER mannosidase I and EDEM (Figures 3 and 4). It is debatable whether EDEM has mannosidase activity or functions as a cofactor of ER mannosidase I. As cargo receptors, such as ERGIC-53, bind only large high-mannose chains (M9-8), trimming to Man6-5 by ER mannosidase means that the client is now destined for degradation. Other ER lectins, OS9 and XTP3B, containing a mannose 6-phosphate receptor (MPR)-like domain, specifically recognize small *N*-glycans containing five to six mannose residues (Man 6–5) through their MPR-like domain, and present them to the core ERAD machinery containing E3 ubiquitin ligases, such as HRD1, although the precise mechanism remains unclear. It is interesting that the transcription of EDEM, OS9, and XTP3B is upregulated by the UPR.

In the second step, ERAD substrates have to be unfolded and extended enough to be able to be retrotranslocated to the cytosol through the retrotranslocation channel. ERAD substrates are unfolded mainly by BiP collaborating with HSP40 family proteins, including ERdj3/DNAJB11, ERdj4, and ERdj5, using the energy of ATP hydrolysis catalyzed by the ATPase activity of BiP; however, BiP can disrupt only noncovalent bonds, such as hydrogen bonds, while covalent bonds, such as disulfide bonds, must be reduced and cleaved by PDI family proteins. Nagata and colleagues recently reported that ERdj5, which contains the J-domain as well as the four CXXC motifs (see above), associates with EDEM and BiP (Figure 4). ERAD substrates are recognized and captured by EDEM and BiP, efficiently unfolded by BiP, and reduced by ERdj5, resulting in a retrotranslocation-ready state. Interestingly, Nagata and colleagues also reported that EDEM interacts with CNX, suggesting that the factors involved in ERAD form a supramolecular ERAD complex to enhance the ERAD process.

Extended ERAD substrates are retrotranslocated to the cytosol through the retrotranslocon (Figure 4). It is still debatable as to which proteins constitute the retrotranslocon. Previously, it was reported that the translocon (SEC61) is used as the retrotranslocon, whereas Rapoport and colleagues proposed that the retrotranslocon may contain derlin-1, derlin-2, and derlin-3. Recently, growing evidence has suggested that the retrotranslocon includes E3 ubiquitin ligases, such as HRD1. ERAD substrates are pulled into the cytoplasm with the force of AAA-ATPase p97 and its cofactors, UFD1 and NPL4. In the cytosol, ERAD substrates are ubiquitinated by E3 ubiquitin ligases specialized for ERAD substrates, such as HRD1, Doa10, gp78, carboxy termimus of HSP70-interacting protein

(CHIP), RMA1, FBX1, FBX2, and Parkin. Although the substrate specificity of these E3 ligases has not been fully clarified, it has been proposed that HRD1 is an E3 ligase specific to ERAD substrates whose luminal or membrane domain is unfolded (ERAD-L and ERAD-M substrates), whereas Doa10 is specific to those whose cytosolic domain is unfolded (ERAD-C substrates). FBX1 and FBX2 specifically recognize any N-glycans on retrotranslocated glycoproteins and ubiquitinate them. The expression of FBX2 is ubiquitous, whereas that of FBX1 is neuron specific. Parkin is involved in the onset of Parkinson's disease and its well-known substrate is the Pael receptor, a G-protein-coupled transmembrane polypeptide. Ubiquitinated ERAD substrates are deprived of *N*-glycans by a peptide, *N*-glycanase (PNGase), ubiquitin moieties are removed by RPN11 in the 26S subunit of the proteasome, and their polypeptide portion is degraded by the proteasome. The above ERAD mechanism deals with glycosylated proteins, while the degradation of nonglycosylated secretory proteins by ERAD has been less studied. Hendershot and colleagues reported that BiP and Herp are involved in the ERAD of nonglycosylated proteins [23]. It is possible that unfolded nonglycosylated proteins are recognized by ER chaperones, such as BiP and GRP94, and recruited to E3 ubiquitin ligases.

In addition to the conventional ERAD, eukaryotic cells have another quality control system called pre-emptive quality control (pre-QC) [24]. With ER stress, the translocation of secretory proteins is transiently attenuated in a signal sequence-selective manner. Substrates of pre-QC are rerouted to the cytosol and degraded by the proteasome to alleviate the burden of misfolded proteins entering the ER. Ron and colleagues reported that p58IPK associates with the translocon, recruits HSP70 to pre-QC substrates, and helps pre-QC substrates to be extracted to the cytoplasm; p58IPK knockout mice become susceptible to ER stress. Not all secretory proteins are equally degraded by pre-QC, but they are degraded in a signal sequence-dependent manner. Thus, secretory proteins that tend to aggregate during the UPR are selectively degraded by pre-QC, while ER chaperones are not.

1.39.2.3 Mechanism of Mammalian UPR

1.39.2.3.1 ATF6 pathway

Mammalian cells have three pathways of the UPR, that is, the activating transcription factor 6 (ATF6), inositol requiring enzyme 1 (IRE1), and PKR-like endoplasamic reticulum (PERK) pathways (**Figure 2**) [1–7]. The sensor molecule detecting ER stress in the ATF6 pathway is pATF6(P), a transmembrane protein residing in the ER membrane and surveying the accumulation of unfolded protein (**Figure 2**). With ER stress, pATF6(P) is transported to the Golgi apparatus via the vesicular transport machinery, and sequentially cleaved by S1P and S2P proteases in the Golgi apparatus (**Figure 5**). These proteases are also involved in the cleavage and activation of another ER-anchored transcription factor, sterol regulatory element binding protein (SREBP), which is translocated from the ER to the Golgi apparatus when the cellular level of sterol drops, but never activated during the UPR. Thus, ATF6 and SREBP are specifically regulated at the level of vesicular transport. The cytoplasmic domain of pATF6(P) contains the basic-leucine zipper (bZIP) motif essential for DNA binding and dimer formation. When released from the Golgi membrane by S2P cleavage, the resultant cytoplasmic portion (pATF6(N)) is translocated into the nucleus, by the function of the basic region, and activates the transcription of various ER chaperone genes, including BiP, GRP94, and CRT, through binding to an common enhancer element, ER stress response element (ERSE), whose consensus sequence is CCAAT(N9)CCACG (**Figure 2**). Thus, ATF6 is a sensor molecule for ER stress as well as a transcription factor.

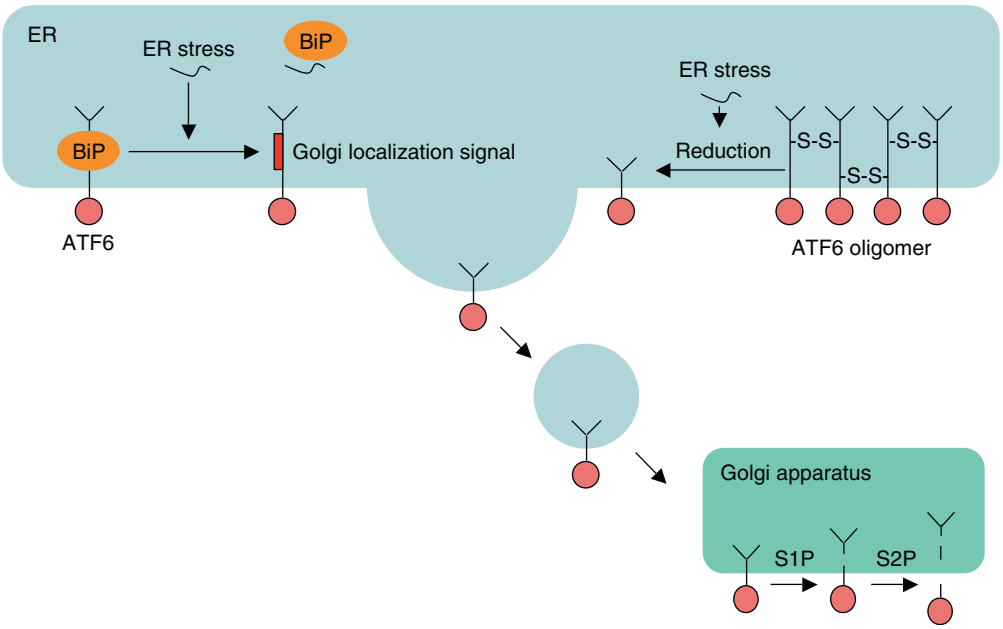

Figure 5 Activation mechanisms of ATF6. Transportation of ATF6 to the Golgi apparatus is negatively regulated by BiP binding (left) and oligomerization through disulfide bond formation (right). In the Golgi apparatus, ATF6 is sequentially cleaved by S1P and S2P proteases.

The mechanism of how ATF6 detects ER stress is still controversial. Prywes and colleagues reported that BiP binds to the luminal domain of ATF6 and retains ATF6 in the ER by masking the Golgi-localization signals (GLSs) of ATF6 (**Figure 5**) [25]. With ER stress, BiP is released from ATF6 and binds to unfolded proteins, resulting in exposure of the GLSs and translocation to the Golgi apparatus. By contrast, Mori and colleagues found that ATF6 forms an oligomer through disulfide bonds in the absence of ER stress. In response to ER stress, the disulfide bonds are cleaved by reduction, and the resultant ATF6 monomer is translocated to the Golgi apparatus [26], suggesting that ATF6 is anchored to the ER by forming oligomers, and that the reduction of ATF6 is a key for its activation. These two regulatory mechanisms are not mutually exclusive, and activation of ATF6 may be securely regulated by these dual systems.

Vertebrate cells have two ATF6 genes, ATF6α and ATF6β. Although both ATF6α and ATF6β are ubiquitously expressed, ATF6α is a more potent transcriptional activator than ATF6β. Although gene knockout mice of either ATF6α or ATF6β show no significant phenotype, BiP induction in response to artificial ER stress inducers, such as tunicamycin and thapsigargin, was severely diminished in murine embryonic fibroblasts (MEFs) prepared from ATF6α knockout mice, whereas MEFs obtained from ATF6β responded normally. When both ATF6α and ATF6β genes were disrupted, the embryos died at an early stage of embryogenesis.

Mammalian cells have several ATF6 homolog genes. Kaufman and colleagues revealed that CREB-H is a liver-specific ATF6 homolog that activates the transcription of serum amyloid P-component and C-reactive protein in response to ER stress, and is involved in the induction of the systemic inflammatory response. Lu and colleagues reported that Luman is a cellular counterpart of Herpes simplex virus VP16, and activates the transcription of an ERAD component, Herp. Imaizumi and colleagues found that old astrocyte specifically-induced substance (OASIS) is expressed in osteoblasts, activates the transcription of type 1 collagen, and is involved in bone formation, whereas BBF2H7 is expressed in chondrocytes, activates the transcription of Sec23a, and is essential for chondrogenesis. Nojima and colleagues reported that Tisp40 is a testis-specific ATF6 homolog, important for spermatogenesis. These cell-type-specific homologs may be beneficial to deal with the unfolded proteins produced in specific cell types, and are also involved in specific differentiation processes.

1.39.2.3.2 IRE1 pathway

The second pathway of mammalian UPR is the IRE1 pathway (**Figure 2**). IRE1 is the second sensor molecule detecting ER stress, and is a transmembrane RNase localized in the ER. With ER stress, IRE1 forms a homo-oligomer and converts pre-mRNA of X-box binding protein (XBP1) (XBP1(U) mRNA) to mature mRNA (XBP1(S) mRNA), from which a potently active transcription factor, pXBP1(S), is translated, which activates the transcription of ERAD genes, including HRD1 and EDEM [27, 28]. Interestingly, Walter, Ron, Kaufman, and Yoshida and their colleagues revealed that the mechanism of splicing XBP1(U) mRNA (and yeast homolog HAC1 mRNA) is completely distinct from conventional mRNA splicing, and takes place in the cytoplasm. Thus, the splicing of metazoan XBP1 and yeast HAC1 is called cytoplasmic splicing.

It is unknown why the IRE1 pathway has to be regulated by cytoplasmic splicing, but not conventional splicing, but it is speculated that there are two reasons. First, the mechanism of cytoplasmic splicing is simple compared with that of conventional nuclear splicing. In nuclear splicing, the ER stress signal is received in the cytosol, and transduced into the nucleus, where XBP1(U) mRNA is spliced. As translation cannot be carried out in the nucleus, spliced XBP1 mRNA has to be transported to the cytosol, where XBP1(S) mRNA is translated. The resultant pXBP1(S) is a transcription factor containing the bZIP motif and has to be translocated into the nucleus to regulate gene expression. Thus, molecules have to cross the nuclear membrane 3 times in nuclear splicing. By contrast, splicing and translation can occur in the cytosol, and only pXBP1(S) is translocated to the nucleus in cytoplasmic splicing. Second, in nuclear splicing, XBP1(U) mRNA has to be transcribed as cytosolic XBP1(U) mRNA cannot be spliced during ER stress, which is time- and energy-consuming. Moreover, XBP1(U) mRNA persists in the cytosol, resulting in the prolonged production of unnecessary products. By contrast, cytoplasmic splicing can utilize XBP1(U) mRNA accumulated in the cytosol, and convert it to XBP1(S) mRNA, which is time- and energy efficient, and waste free.

Interestingly, a protein is efficiently translated from XBP1(U) mRNA to produce a protein called pXBP1(U). Yoshida and colleagues revealed that pXBP1(U) contains a degradation-enhancing domain as well as the nuclear exclusion signal, and its expression is induced during the recovery phase of the UPR. pXBP1(U) binds to pXBP1(S), sequesters it from the nucleus to the cytosol, and enhances its degradation, leading to suppression of the UPR. Thus, the XBP1 gene encodes both an active transcription factor (pXBP1(S)) and a negative regulator of the UPR (pXBP1(U)); and XBP1 splicing in response to ER stress switches XBP1 expression from pXBP1(U) to pXBP1(S).

Walter and colleagues reported that, in the absence of ER stress, translation of Hac1p (the yeast counterpart of XBP1) is suppressed by the interaction between the intron and its 5′-untranslated region (UTR) (**Figure 6**). With ER stress, the intron is removed and the translation of spliced HAC1 mRNA is increased. Interestingly, Walter and colleagues revealed that HAC1 mRNA is recruited to the IRE1 oligomer in response to ER stress by means of a conserved bipartite targeting element contained in the 3′ UTR, to enhance the splicing of HAC1 mRNA by Ire1p (**Figure 6**). By contrast, Kenji Kohno and colleagues reported that, in mammalian cells, XBP1(U) mRNA is tethered to the ER membrane even in the absence of ER stress. They revealed that the hydrophobic region of pXBP1(U) (the HR2 region) binds to the ER membrane and anchors XBP1(U) mRNA to the ER membrane. Thus, IRE1 substrates are localized in the ER membrane in both yeasts and mammals through distinct mechanisms.

The mechanism of how IRE1 senses the accumulation of unfolded proteins in the ER has been highly debated. Ron and colleagues reported that binding BiP to the luminal domain of mammalian IRE1 suppresses its activation, and that the accumulation of unfolded proteins sequesters BiP from IRE1 and activates it (**Figure 7**). By contrast, Kohno and colleagues proposed that the core sensing domain of yeast Ire1p is different from the BiP binding site, and that BiP is not the principal determinant of Ire1

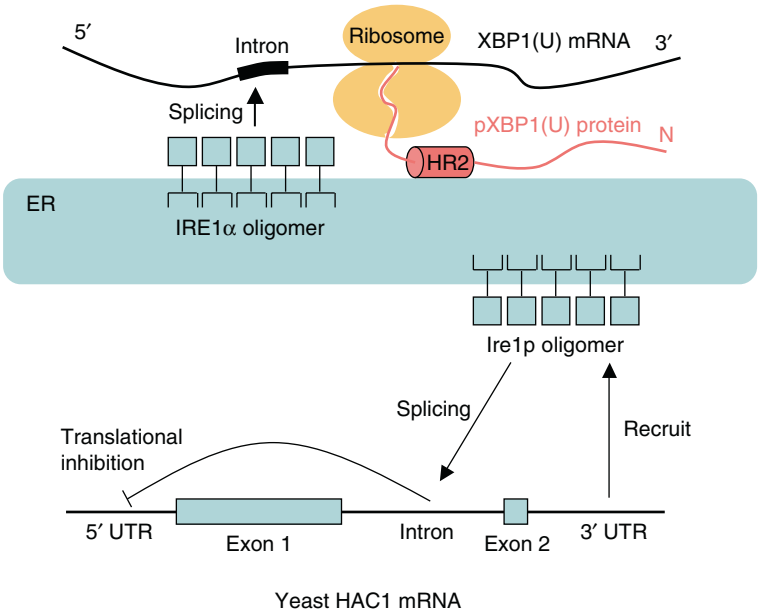

Figure 6 Recruitment of XBP1 and HAC1 mRNA to IRE1. Yeast HAC1 mRNA is recruited to Ire1p oligomers through the 3′-UTR (below), while mammalian XBP1(U) mRNA is anchored to the ER membrane through the interaction of pXBP1(U) protein with ER lipids to facilitate the splicing reaction.

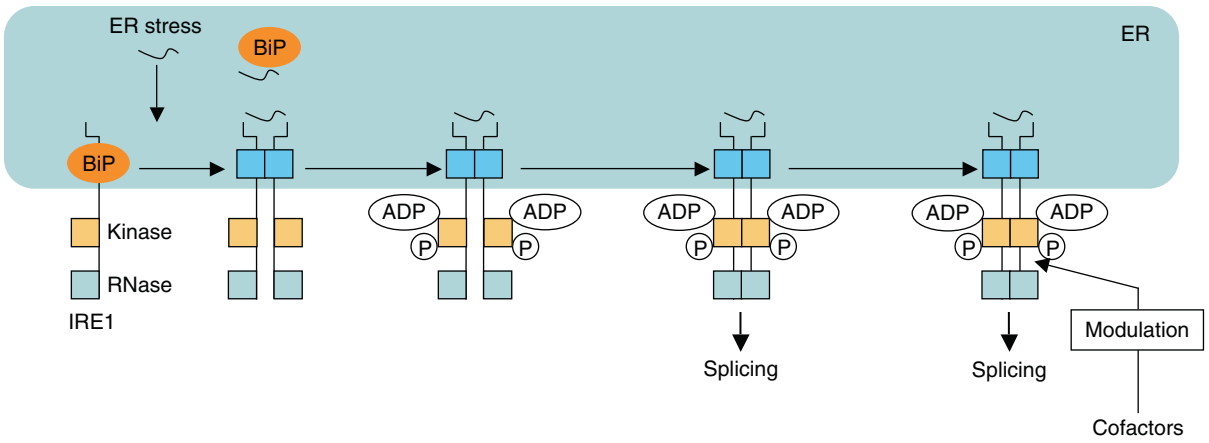

Figure 7 The current model for the activation mechanism of IRE1. Yeast and mammalian IRE1 are activated by multiple steps, that is, the release of BiP, oligomerization of the luminal domain, binding of unfolded proteins, phosphorylation of the kinase domain, binding of ADP, oligomerization of the cytosolic domain, and modulation by cytosolic cofactors.

activity, but an adjustor of sensitivity to various stresses. Recently, Walter and colleagues analyzed the crystal structure of yeast Ire1p, and found that the luminal domain of Ire1p can form an oligomer, and has a structure similar to the antigen-peptide-binding domain of mammalian major histocompatibility complex class I (MHC-I) in its gross architecture, and proposed that Ire1p is activated by the direct binding of unfolded proteins. By contrast, Kaufman and colleagues analyzed the crystallographic structure of human IRE1, and revealed that the luminal domain of mammalian IRE1 cannot accommodate unfolded proteins, proposing that the activation of mammalian IRE1 solely depends on BiP dissociation rather than on the direct binding of unfolded proteins. Thus, it is difficult to reach a consensus on the IRE1 activation process. The most plausible working hypothesis at present is shown in Figure 7. With ER stress, BiP bound to IRE1 is sequestered by unfolded proteins, and oligomerization of the luminal domain of IRE1 is initiated. At the dimerization surface, the MHC-like peptide-binding cleft is formed. Direct binding of unfolded proteins to the IRE1 luminal domain causes structural changes to the overall structure of the IRE1 molecule, resulting in transphosphorylation of the activation loop in the kinase domain domain, and ADP binding to the nucleotide-binding site, resulted in oligomerization of the cytoplasmic domain. After these laborious processes, RNase in the cytoplasmic domain is finally activated. Recently, Ron and colleagues revealed that flavonoids, such as quercetin, bind to a site distinct from the nucleotide-binding domain, and activate RNase by facilitating oligomer formation of the cytosolic domain, suggesting that signals from the cytosol, such as nutrient signals, modulate IRE1 activity.

Mammalian cells have two IRE1 genes, IRE1α and IRE1β. IRE1α is ubiquitously expressed, and gene knockout of IRE1α resulted in lethality in the early phase of embryogenesis. Iwawaki and colleagues revealed that IRE1α is predominantly activated in the placenta, and that extraembryonic function of IRE1α rescued the lethality of an IRE1α-defficient embryo. By contrast, the expression of IRE1β is restricted to the epithelial cells of the gastrointestinal tract. Ron and colleagues reported that IRE1β knockout mice show no significant phenotype, but when administered with dextran sodium sulfate, the IRE1β knockout mice developed colitis earlier than wild-type mice, possibly due to inflammation caused by ER stress.

Besides XBP1 splicing, IRE1 has a role in translational attenuation in response to ER stress. Weissman and colleagues found that *Drosophila* and mammalian IRE1 cleave ER-localized mRNAs encoding secretory proteins during the UPR to alleviate the burden of protein folding in the ER, which is called regulated IRE1-dependent decay of mRNAs (RIDD). Kohno and colleagues also reported that mammalian IRE1β cleaves 28S ribosomal RNA in response to ER stress to attenuate translation. These protective mechanisms not only suppress the overload of client proteins in the ER lumen, but also ensure that translational and translocational machinery is readily available to mRNAs encoding ER chaperones and ERAD components.

IRE1 also functions as a platform of intracellular signaling, to which factors involved in various processes bind, including ERAD and apoptosis. For instance, Ron and colleagues revealed that IRE1 transmits an apoptotic signal to the TRAF2–ASK1–JNK cascade (tumor necrosis factor receptor-associated factor 2– apoptosis signal-regulating kinase 1–cJUN NH_2-terminal kinase (TRAF2–ASK1–JNK). By contrast, Reed and colleagues reported that Bax Inhibitor-1 (BI-1) associates with IRE1 and attenuates its activity in mammals and *D. melanogaster*, but not in yeasts. Glimcher and colleagues named the IRE1 signaling macromolecular complex 'UPRosome'. The complex transmits intracellular signals in a highly regulated manner, according to a tissue-specific context.

Glimcher and colleagues reported that XBP1 knockout mice are embryonically lethal due to anemia. They also revealed that XBP1 is crucial to the differentiation of plasma cells, pancreatic and salivary gland acinar cells, and dendritic cells, and innate immunity in macrophages. Kim and colleagues also reported that XBP1 expression is induced by pathogen infection, and that XBP1 is important for the induction of the innate immunity of *C. elegans*. XBP1 deletion in intestinal epithelial cells resulted in spontaneous enteritis and increased susceptibility to induced colitis, as in IRE1β knockout mice, suggesting a link between ER stress and inflammation. Glimcher and Ling Qi and their colleagues revealed that XBP1 was also essential for hepatic lipogenesis and adipocyte differentiation. Adipocyte differentiation may result in the induction of ER stress, as well as the transcriptional induction of XBP1 by C/EBPβ. Expression of pXBP1(S) induced by adipocyte differentiation activates the transcription of C/EBPα, leading to adipogenesis. Dynlacht and colleagues reported that muscle differentiation also depends on XBP1, suggesting the important role of XBP1 in differentiation.

The IRE1–XBP1 pathway also upregulates the synthesis of membrane lipid, possibly to accommodate an increased number of secretory proteins. Brewer and colleagues reported that pXBP1(S) enhances the activity of choline cytidylyltransferase (CCT) and cholinephosphotransferase 1 (CPT1), which are key enzymes in the cytidine diphosphocholine pathway of phosphatitylcholine biosynthesis. Overexpression of pXBP1(S) in cells increases the surface area and volume of the ER. ATF6 is also involved in ER expansion by mechanisms at least partially distinct from those of XBP1. As the transcription of neither CCT nor CPT1 is induced upon pXBP1(S) overexpression, pXBP1(S) may upregulate the transcription of unknown factor(s) regulating the activity of CCT and CPT1.

1.39.2.3.3 PERK pathway

The third pathway regulating mammalian UPR is the PERK pathway (**Figure 2**). Ron and colleagues reported that PERK is a sensor molecule as well as a transmembrane kinase residing in the ER membrane. The mechanism by which PERK senses ER stress is thought to be similar to that of IRE1, as the structures of their luminal domains resemble each other, and their luminal domains are interchangeable. In response to ER stress, PERK forms a homo-oligomer, like IRE1, and phosphorylates the α subunit of the eukaryotic translational initiation factor 2 (eIF2α), leading to translational attenuation. Translation of a transcription factor, ATF4, is enhanced upon general translational attenuation, and ATF4 containing the bZIP motif activates the transcription of anti-oxidative stress proteins as well as a pro-apoptotic transcription factor, C-EBP-homologous protein (CHOP). Thus, prolonged activation of the PERK pathway leads to apoptosis. Although the precise mechanism of ER stress-induced apoptosis remains unclear, the PERK–CHOP pathway is thought to be one of major pathways of ER stress-induced apoptosis, in addition to the IRE1–TRAF2 pathway, p53-upregulated modulation of apoptosis (PUMA) pathway and caspase-12 pathway.

Translational attenuation by the phosphorylation of eIF2α is shared by other signaling pathways. The cytosolic kinase PKR, which is structurally similar to PERK, phosphorylates eIF2α when PKR is activated by virus infection, to suppress viral propagation. The phosphorylation of eIF2α also occurs when HRI is activated in response to heme deficiency in erythrocytes, to balance the production of heme and hemoglobin. GCN2 phosphorylates eIF2α when amino acids are starved in yeast and mammalian cells, to limit the overall consumption of amino acids. These signaling pathways are thought to be evolutionarily linked and are collectively called the integrated stress response.

Why do mammalian cells have three branches of the UPR pathway? Mori and colleagues proposed that the ATF6, IRE1, and PERK pathways do not function simultaneously, but sequentially (**Figure 8**). A small number of ER chaperones, as well as ERAD components, are constitutively expressed with unfolded proteins in normal cells that do not suffer ER stress. With ER stress, mammalian cells transiently attenuated translation by the PERK pathway, and cells tried to refold unfolded proteins. If unfolded proteins are not refolded, then cells activate the ATF6 pathway and upregulate the capacity of protein folding in the ER. If unfolded proteins persist, the IRE1 pathway is activated to increase the capacity of the ERAD as well as the refolding machinery. If the cells cannot deal with unfolded proteins at this stage, the apoptotic pathway is activated to dispose off damaged cells to ensure the

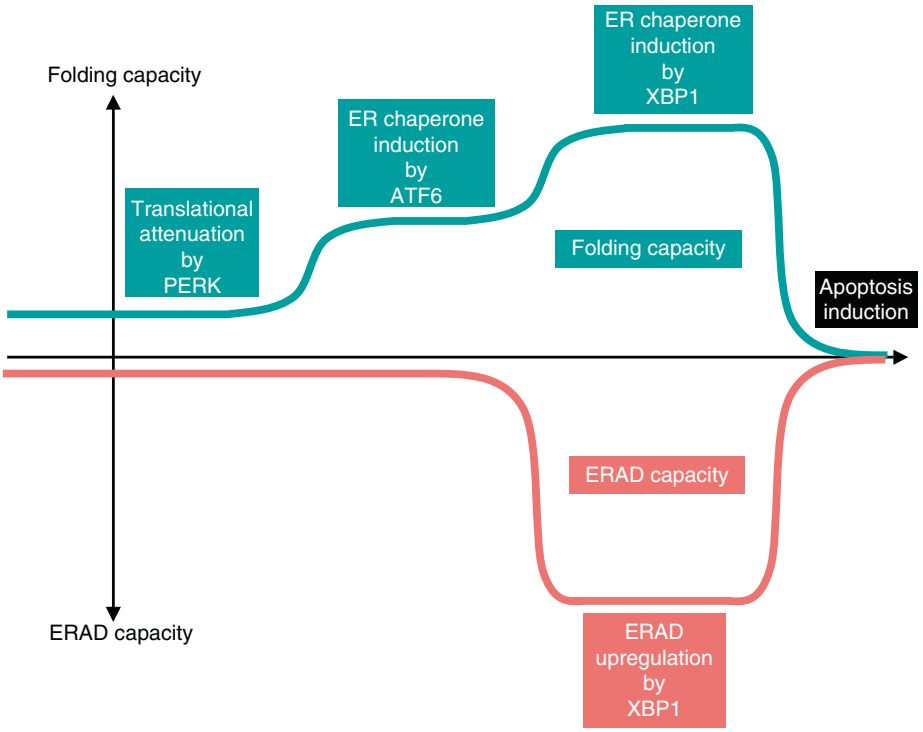

Figure 8 Defense in-depth strategy of the mammalian UPR. The first layer of defense is translational attenuation, in which constitutively expressed ER chaperones deal with a surprise attack of unfolded proteins while further influx of secretory proteins into the ER is temporarily halted by the PERK pathway. The second layer of defense is exerted by ER chaperones reinforced by the ATF6 pathway to upregulate the folding capacity. If the second defense is broken, the last and most robust response is launched, in which all-out attack by reinforced ER chaperones as well as ERAD components is accomplished. If these attempts all fail, a cell defeated by ER stress undergoes apoptosis to secure the survival of the organism, to which all cells pledge their allegiance.

survival of the organism. Yeast cells have only the IRE1 pathway, which increases both the refolding capacity and the ERAD machinery, and degrades unfolded proteins that can be refolded. Thus, the mammalian system is more sophisticated than that of yeasts.

Although it seems to be theoretically possible to increase the capacity of the ER by engineering genes encoding ER chaperones and ERAD components for efficient production of recombinant proteins, it is practically difficult as there are many ER chaperones and ERAD components. For instance, ER chaperones include BiP, GRP94, CRT, CNX, ERdjs, various PDIs, and so on, whereas ERAD components include EDEM, osteosarcoma 9 (OS-9), HRDs, Derlins, Herp, VCP, and so forth. Moreover, several unidentified factors critical to folding and ERAD seem to exist. The best way to enhance ER capacity is to engineer regulators of the UPR, such as pATF6(N), pXBP1(S), and ATF6. These UPR regulators may enhance the expression of ER factors immediately, although they might also turn on the apoptotic pathway. It would be beneficial to shut off the apoptotic pathway by disrupting the genes involved in apoptosis signaling.

1.39.2.3.4 UPR in other organisms

The mechanism of the UPR in yeast, *S. cerevisiae*, is very simple and consists of only the IRE1 pathway, which was revealed by Gething and Walter and their colleagues. HAC1 pre-mRNA is cleaved by Ire1p, a yeast homolog of mammalian IRE1, and is ligated by Rlg1p, a transfer RNA (tRNA) ligase. HAC1 pre-mRNA is not translated because of translational suppression by the association between the intron and the 5′UTR. Hac1p translated from HAC1 mature mRNA is a potent transcriptional activator. Although the primary structure of Hac1p is not similar to that of pXBP1(S), Hac1p is a functional homolog of pXBP1(S). As for *C. elegans* and *D. melanogaster*, the IRE1–XBP1 and PERK pathways regulate their UPR. A single ATF6 gene is found in these organisms, although its role in the UPR is unclear.

1.39.2.3.5 Apoptotic pathways

The pathways regulating ER stress-induced apoptosis are very complicated and controversial [8, 29]. The most important pathway seems to be the CHOP pathway. The promoter of the mammalian CHOP gene contains amino acids response element (AARE) as well as ERSE, and the transcription of CHOP is upregulated by ATF4 and pATF6(N). Thus, the CHOP pathway is under the regulation of the PERK and ATF6 pathways. CHOP activates the transcription of Bim, a proapoptotic BH3-only member of the Bcl-2

family, to induce ER stress-mediated apoptosis. Bim expression is also upregulated by protein phosphatase 2A-mediated dephosphorylation of Bim during ER stress, which prevents its ubiquitination and degradation by the proteasome.

Another major pathway of ER stress-induced apoptosis is the IRE1–TRAF2–ASK1–JNK pathway. Ron and colleagues revealed that the cytoplasmic portion of mammalian IRE1 binds TNF receptor-associated factor 2 (TRAF2), an adaptor protein coupling the ligand receptors, to the activation of JNK/stress-activated protein kinase (SAPK). TRAF2 associates with ASK1, which is a member of the mammalian mitogen-activated protein kinase kinase kinase (MAPKKK), and ASK1 transmits an apoptosis signal to JNK. Ichijo and colleagues revealed that polyglutamine polypeptides causing polyQ diseases, including Huntington's disease, activate the IRE1–TRAF2–ASK1–JNK pathway and induce ER stress-mediated apoptosis.

The third pathway regulating ER stress-mediated apoptosis is the caspase-12 pathway. Tohyama and colleagues reported that caspase-12 is a member of the caspase family and is specifically involved in ER stress-induced apoptosis in murine cells; however, the caspase gene is an inactive pseudogene in human cells. Thus, it is still debatable whether the caspase-12 pathway is involved in ER stress-induced apoptosis in human cells.

1.39.3 Stress Responses in Other Organelles

Accumulated evidence suggests that organelles other than the ER also have specific stress response mechanisms to regulate their capacity. In this article, the stress response mechanisms observed in the Golgi apparatus, mitochondria, lysosomes, and peroxisomes will be briefly described.

1.39.3.1 Golgi Stress Response

Enhancement of the capacity of the ER seems insufficient to sustain the increased production of secretory proteins. When the ER capacity is upregulated and a large number of secretory proteins are correctly folded, these secretory proteins are transported to and inundate the Golgi apparatus, resulting in insufficient Golgi capacity. Thus, it is necessary to augment the capacity of the Golgi apparatus and the ER simultaneously to efficiently produce a large number of secretory proteins. Indeed, secretory cells, such as secretory mucous cells in Brunner's gland, contain well-developed Golgi structures as well as extended ER membranes. Eukaryotic cells may have a regulatory mechanism similar to the UPR to adjust the Golgi capacity to cellular demands. We named this regulatory mechanism the Golgi stress response, and started to analyze its molecular mechanism (Yoshida *et al.*, unpublished results). Studies of the Golgi stress response and the ER stress response would enable the creation of suprasecretory cells useful for the production of recombinant proteins.

1.39.3.2 Lysosome Stress Response

Lysosomes are organelles where proteins, carbohydrates, lipids, and nucleotides are degraded by proteases, such as cathepsins, amylases, lipase, and nuclease. Lysosomes digest not only nutrients incorporated by endocytosis or pinocytosis for energy synthesis, but also worn-out proteins and organelles engulfed by autophagosomes for recycling, and pathogens such as viruses and bacteria for defense. Inefficient degradation results in the accumulation of lysosomal substrates and finally causes lysosomal storage diseases, including Tay–Sachs disease and Pompe's disease. To enhance the expression of proteins involved in the lysosomal function, mammalian cells activate the mechanism of the lysosomal stress response. Ballabio and colleagues reported that when the capacity of lysosomes becomes insufficient, the transcription factor TFEB is translocated from the cytosol to the nucleus, binds to the *cis*-acting element CLEAR whose consensus sequence is GTCACGTGAC, and increases the transcription of lysosomal genes, including cathepsins. As the production of a large number of recombinant proteins often results in an increase of flawed products, it would be beneficial to enhance the capacity of lysosomes.

1.39.3.3 Mitochondrial Unfolded Protein Response

The mitochondria are organelles that procure most of the ATP and nicotinamide adenine dinucleotide (NADH) produced in eukaryotic cells. Various proteins involved in mitochondrial function, including tricarboxylic acid (TCA) cycle enzymes as well as electron transport chain factors, reside in the mitochondrial matrix and mitochondrial outer and inner membranes. As mitochondria have a double membrane, mitochondrial matrix proteins have to be unfolded and extended for efficient transport from the cytosol to the matrix. In addition, mitochondria have their own translational machinery, and nascent unfolded polypeptides are produced in the matrix. Moreover, environmental stress, such as oxidative stress, often results in the malfolding of mitochondrial proteins. These unfolded or malfolded proteins are correctly folded by mitochondrial chaperones, including miHSP70, mtHSP40, and mtHSP60, and flawed proteins are degraded by mitochondrial proteases. Although the expression of these HSPs was previously thought to be regulated by a transcription factor, HSF, and an enhancer, HSE, recent studies have revealed the existence of specific regulatory mechanisms to upregulate mitochondrial chaperones and proteases, and to adjust the protein folding and degradation capacity in the mitochondria to cellular needs, called the mitochondrial UPR. Ron and colleagues revealed that, when unfolded proteins are accumulated in the mitochondrial matrix of *C. elegans*, an AAA-protease CLPP-1 cleaves unfolded proteins into small peptides, which are transported to the cytoplasm through matrix peptide transporter hematopoiesis-associated factor (HAF-1)

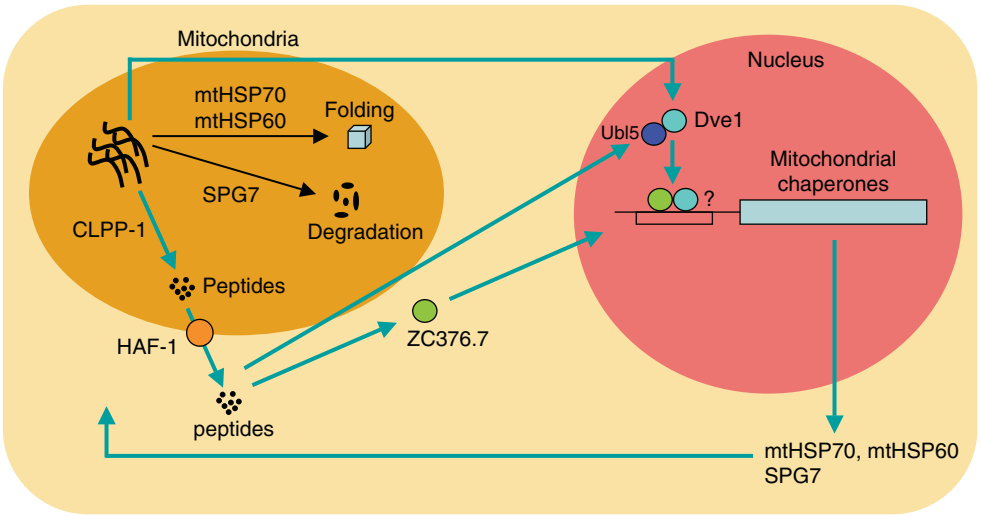

Figure 9 Mechanism of the mitochondrial unfolded protein response in *C. elegans*. Mitochondrial UPR is regulated by at least two transcription factors, Dve1 and ZC376.7. The sensor molecule CLPP-1 detects the accumulation of unfolded proteins in the mitochondrial matrix.

(Figure 9) [30]. HAF-1 activates a transcription factor ZC376.7 and the small ubiquitin-like protein UBL-5. DVE-1, another transcription factor containing a homeodomain, is also activated by CLPP-1 function. Both DVE-1, collaborating with UBL-5, and ZC376.7 finally activate the transcription of target genes of the mitochondrial UPR. Hoogenraad and colleagues reported that the transcription factor, CHOP, is involved in the transcriptional induction of mammalian mitochondrial chaperone genes during mitochondrial UPR, although the precise regulatory mechanism remains to be clarified. Since the efficient production of recombinant proteins needs an increased amount of ATP, artificial enhancement of the mitochondrial function by the mitochondrial UPR may be important for recombinant protein production.

1.39.3.4 Peroxisome Stress Response

Peroxisomes are organelles where the metabolism of cellular metabolites occurs, including the beta-oxidation of very long chain fatty acids. Peroxisomes contain oxidative enzymes, such as catalase, and deficiencies of the peroxisome function result in peroxisomal disorders, such as Zellweger syndrome. Fujiki and colleagues revealed the mechanism of peroxisome biogenesis by identifying a set of PEX genes involved in the process. Tabak, Small, and Picard and their colleagues revealed that fatty acids directly bind and activate a zinc-finger type transcription factor, Oaf1p, in *S. cerevisiae*. Oaf1p dimerizes with Pip2p/Oaf2, and the resultant heterodimer binds to an enhancer element, called ORE (oleate response element), whose consensus sequence is CGG(N_{15-18})CCG, and activates the transcription of genes involved in peroxisomal function, including CTA1 encoding catalase A and POX1 encoding acyl oxidase. The regulatory mechanism of the peroxisome capacity is well conserved between yeast and mammals. Semenkovich and colleagues revealed that in mammals, long chain fatty acids, such as 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine (16:0/18:1-GPC), directly bind to and activate a transcription factor, peroxisome proliferator-activated receptor (PPARα). Activated PPARα heterodimerizes with another transcription factor, retinoid X receptor (RXR), binds to an enhancer called PPRE (peroxisome proliferator response element) whose consensus sequence is AGGTCA(N)AGGTCA, and activates the transcription of genes encoding peroxisome genes, such as lipid-metabolizing genes. Oaf1p, Pip2p/Oaf2p, PPARα, and RXR all belong to the nuclear hormone receptor family.

1.39.4 Concluding Remarks

Eukaryotic cells contain various organelles, and each organelle exerts its own function. As the capacity of each organelle is tightly regulated in accordance with cellular needs, by the signaling between the organelle and the nucleus, mass-producing recombinant proteins in eukaryotic cells requires ample knowledge of the mechanism regulating the capacity of each organelle. In particular, the UPR in the ER and the Golgi stress response is critical for the production of secretory proteins. By revealing the underlying mechanisms regulating the capacity of each organelle, it would become possible to regulate organelle function at will.

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