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Review

ER stress and the unfolded protein response

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Abstract

Conformational diseases are caused by mutations altering the folding pathway or final conformation of a protein. Many conformational diseases are caused by mutations in secretory proteins and reach from metabolic diseases, e.g. diabetes, to developmental and neurological diseases, e.g. Alzheimer's disease. Expression of mutant proteins disrupts protein folding in the endoplasmic reticulum (ER), causes ER stress, and activates a signaling network called the unfolded protein response (UPR). The UPR increases the biosynthetic capacity of the secretory pathway through upregulation of ER chaperone and foldase expression. In addition, the UPR decreases the biosynthetic burden of the secretory pathway by downregulating expression of genes encoding secreted proteins. Here we review our current understanding of how an unfolded protein signal is generated, sensed, transmitted across the ER membrane, and how downstream events in this stress response are regulated. We propose a model in which the activity of UPR signaling pathways reflects the biosynthetic activity of the ER. We summarize data that shows that this information is integrated into control of cellular events, which were previously not considered to be under control of ER signaling pathways, e.g. execution of differentiation and starvation programs.
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Keywords: Conformational diseases; Endoplasmic reticulum storage diseases; Protein folding; Molecular chaperones; Unfolded protein response

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1. Endoplasmic reticulum stress

In its broadest definition, stress is the response of any system to perturbations of its normal state. For a cell or organism these can be either life-enhancing changes, e.g. feeding, or life-threatening changes, e.g. starvation [1–3]. To apply this definition of stress to an organelle, e.g. the ER, we have to address the following questions: what are the physiological functions of the ER and how are they perturbated? Furthermore, we have to understand how these perturbations are sensed and how signals are transduced to initiate countermeasures to restore the original state.

In eukaryotic cells the ER is the first compartment in the secretory pathway. It is responsible for the synthesis, modification and delivery of proteins to their proper target sites within the secretory pathway and the extracellular space. All secretory proteins enter the secretory pathway through the ER. In the ER, proteins fold into their native conformation and undergo a multitude of post-translational modifications, including asparagine-linked glycosylation [4,5], and the formation of intra-

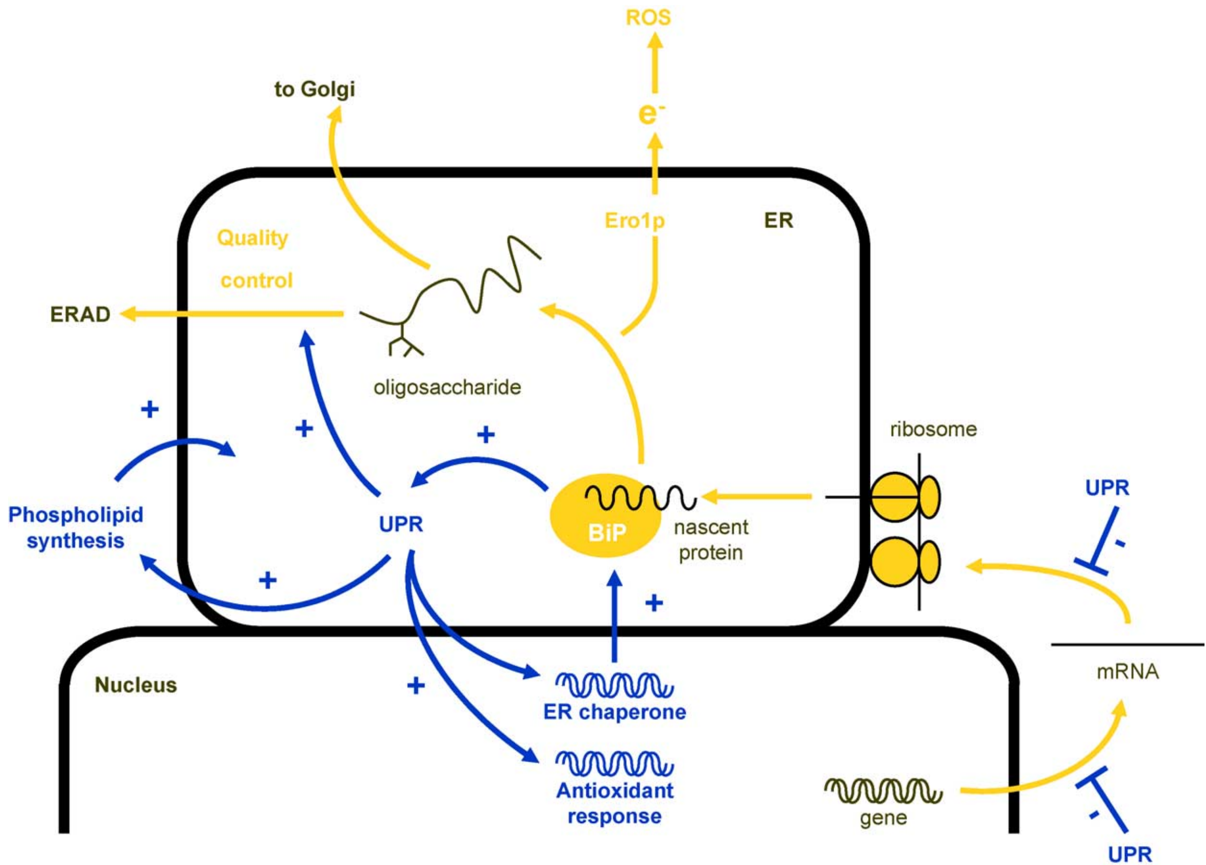

Fig. 1. Protein flux through the ER (orange) and principal activities of the UPR to couple the ER protein folding capacity with its protein folding burden (blue). Abbreviations: ER – endoplasmic reticulum, ROS – reactive oxygen species.

and intermolecular disulfide bonds [6]. In a process termed quality-control only correctly folded proteins are exported to the Golgi complex (Fig. 1), while incompletely folded proteins are retained in the ER to complete the folding process or to be targeted for degradation [7]. In addition, the ER is the site for the synthesis of sterols and lipids [8]. In lower eukaryotes a major portion of the cell wall is synthesized in the ER [9].

Disruption of any of these processes causes ER stress. Historically, the focus is on ER stress caused by disruption of protein folding, and little is currently known about ER stress caused, for example, by aberrations in lipid metabolism, or disruption of cell wall biogenesis. Proof of principle experiments established that expression of mutant, folding-incompetent proteins causes ER stress and an ER stress response, called the unfolded protein response (UPR) [10–13]. This is the biochemical basis for many ER storage diseases, in which folding-incompetent proteins accumulate in the ER [14,15]. In vivo protein folding requires a complex ER-resident protein folding machinery. Exhaustion of the capacity of this protein folding machinery by over-expression of wild-type proteins, e.g. blood coagulation factor VIII [16,17], or antithrombin III [18,19] results in the accumulation of unfolded, aggregated proteins in the ER and activation of the UPR. Recently, many physiological conditions were identified in which the demand on the ER-resident protein folding machinery exceeds its capacity, e.g. differentiation of B-cells into plasma cells, a cell type highly specialized in secretion [20–22], viral infection [23,24], and, in plants, the host's response to a microbial infection [25,26].

Two simple adaptive mechanisms are employed to bring the folding capacity of the ER and its unfolded protein burden into line and return the ER to its normal physiological state (Fig. 1): (1) upregulation of the folding capacity of the ER through induction of ER-resident molecular chaperones and foldases and an increase in the size of the ER, and (2) down-regulation of the biosynthetic load of the ER through shut-off of protein synthesis on a transcriptional [27,28] and translational level [29] and increased clearance of unfolded proteins from the ER through upregulation of ER associated degradation (ERAD; [30–32]). When these mechanisms do not remedy the stress situation, apoptosis is initiated in higher eukaryotic organisms, presumably to eliminate unhealthy or infected cells [33,34].

UPR activity is also detected in cells that are considered "unstressed" [35,36], that is, have not been subjected to experimental manipulations that disrupt protein folding in the ER. This basal UPR activity was implicated in nutrient sensing and control of cellular responses to fluctuations in nutrient levels [35,37,38]. These observations extend the physiological functions of the UPR and are summarized at the end of this review.

2. Principles of protein folding

To understand, why protein folding is very easily disrupted, e.g. by the over-expression of WT proteins, and why this is so detrimental to the ER, we briefly summarize our current understanding of protein folding with focus on the ER. The ER is a major protein folding compartment in an eukaryotic cell, second only to the cytosol. Many principles governing protein folding in the cytosol also apply to the ER and are summarized in this chapter.

2.1. Thermodynamics

For any given protein the number of possible conformations, as defined by the number of native and total interactions of its residues, is determined by its amino acid sequence. Each conformation has a certain free energy. Plotting of all free energies versus their corresponding conformations yields a distinctive energy surface or landscape for the protein. On this energy landscape the protein folds along several competing pathways leading to conformations with ever decreasing free energies until a transition state is crossed [39]. Folding stops when the conformation with the lowest free energy is reached. In many cases this conformation is identical to the native conformation of the protein [39]. Thus, the primary amino acid sequence of a protein is the major determinant for the folding of the protein, a phenomenon first summarized in Anfinsen's dogma [40].

2.2. Kinetics

Kinetically, protein folding is initiated by a hydrophobic collapse, in which several hydrophobic side chains shield each other from surrounding water [41]. Burial of electrostatic interactions, such as salt bridges or hydrogen bonds, in the hydrophobic core limits the number of possible conformations for the folding protein, and is a major determinant in the folding pathway [41]. Individual structures, e.g. α-helices or β-turns fold within 0.1–1 μs [42,43]. Small proteins fold in less than 50 μs [44,45] without significantly populating intermediate states [46]. Compared to the rate of protein folding, translation of mRNAs is slow and proceeds at ≈4–6 amino acid residues/s [47]. To form secondary and tertiary structural elements in which residues far apart in the amino acid sequence interact, e.g. β-sheets or disulfide bonds, the preceding residues must be maintained in a folding competent state until the interacting partners are added to the polypeptide chain. This problem is exacerbated by the high protein concentration in vivo. For example, the protein concentration in the ER is ≈100 g/l (≈2 mM), and even the assembly of IgG heavy and light chains, whose concentration in the ER of an antibody secreting plasma cell is ≈4–6 μM, can in principle be a diffusion-controlled process [41]. Thus, it is necessary to shield folding proteins displaying hydrophobic patches on their surface from inadvertently colliding and interacting with other maturing and mature proteins.

3. Protein folding in the ER

The ER differs significantly from the cytosol topologically, in its chemical composition, and in its protein folding machinery. All these differences can significantly affect protein folding in the ER.

3.1. Topology

The ER is a membrane surrounded compartment, and its luminal space is topologically equivalent to the extracellular space. Proteins destined for the ER are directed to the ER through a predominantly hydrophobic signal sequence and have to, either co- or post-translationally, traverse the ER membrane through the Sec61p complex [48–50]. The presence and timing of cotranslocational signal sequence cleavage with folding of the polypeptide chain affects the folding pathway [51,52].

3.2. Chemical composition

As in the cytosol, the pH in the ER is near neutral [53]. In mammalian cells the ER is the major site for Ca^{2+} storage. ER luminal Ca^{2+} concentrations reach 5 mM, compared to 0.1 μM in the cytosol [54]. ER luminal Ca^{2+} concentrations rapidly and frequently fluctuate as the ER Ca^{2+} pool is mobilized during intracellular signaling [55]. Ca^{2+} can participate in electrostatic interactions in proteins and through these alters hydrophobic interactions. Thus, the effect of fluctuations in the ER Ca^{2+} pool on protein folding depends on the protein [56,57]. More importantly, the majority of the ER-resident molecular chaperones and foldases are vigorous Ca^{2+} binding proteins. Perturbation of the ER Ca^{2+} pool affects their folding, activity [58–60], and interactions with other chaperones [61].

The major redox buffer in the cell is glutathione. In the cytosol the ratio of reduced (GSH) to oxidized glutathione (GSSG) is 30:1 to 100:1. In contrast, in the ER this ratio is 1:1 to 3:1 [62]. Disulfide bond formation in the ER is catalyzed by protein disulfide isomerases (PDI) (Fig. 2). Reduced PDI is recycled by the FAD-dependent oxidases Ero1p [63–66] and Erv2p [67,68]. A third FAD-dependent oxidase, Fmo1p also contributes to disulfide bond formation [69]. The final electron acceptor for Ero1p and Erv2p is O_2 [65,66,68]. Peroxide and superoxide are minor electron acceptors for Ero1p [66]. Further, Ero1p is essential under anaerobic conditions in yeast, suggesting that an alternative electron acceptor for Ero1p exists [66]. Thus, uncoupling of Ero1p from its physiologic electron acceptor, e.g. during ER stress, may result in generation of reactive oxygen species (Fig. 1).

3.3. N-linked glycosylation

A multitude of post-translational modifications occur in the ER: *N*-linked glycosylation, disulfide bond formation, lipidation, hydroxylation, oligomerization, etc. We will focus on general post-translational modifications common to the majority of secreted proteins, *N*-linked glycosylation and formation of disulfide bonds (see above). *N*-linked glycosylation

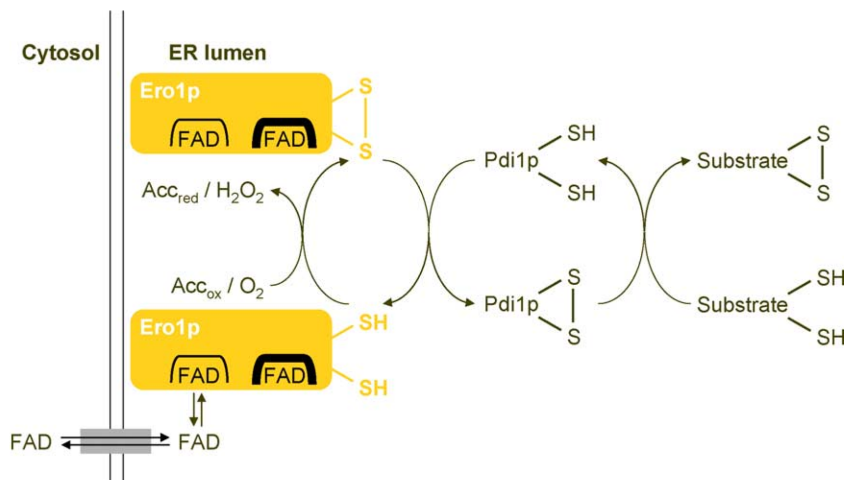

Fig. 2. Disulfide bond formation and generation of reactive oxygen species by protein folding. Abbreviation: Acc – electron acceptor. Substrate = unfolded or folded protein, or glutathione.

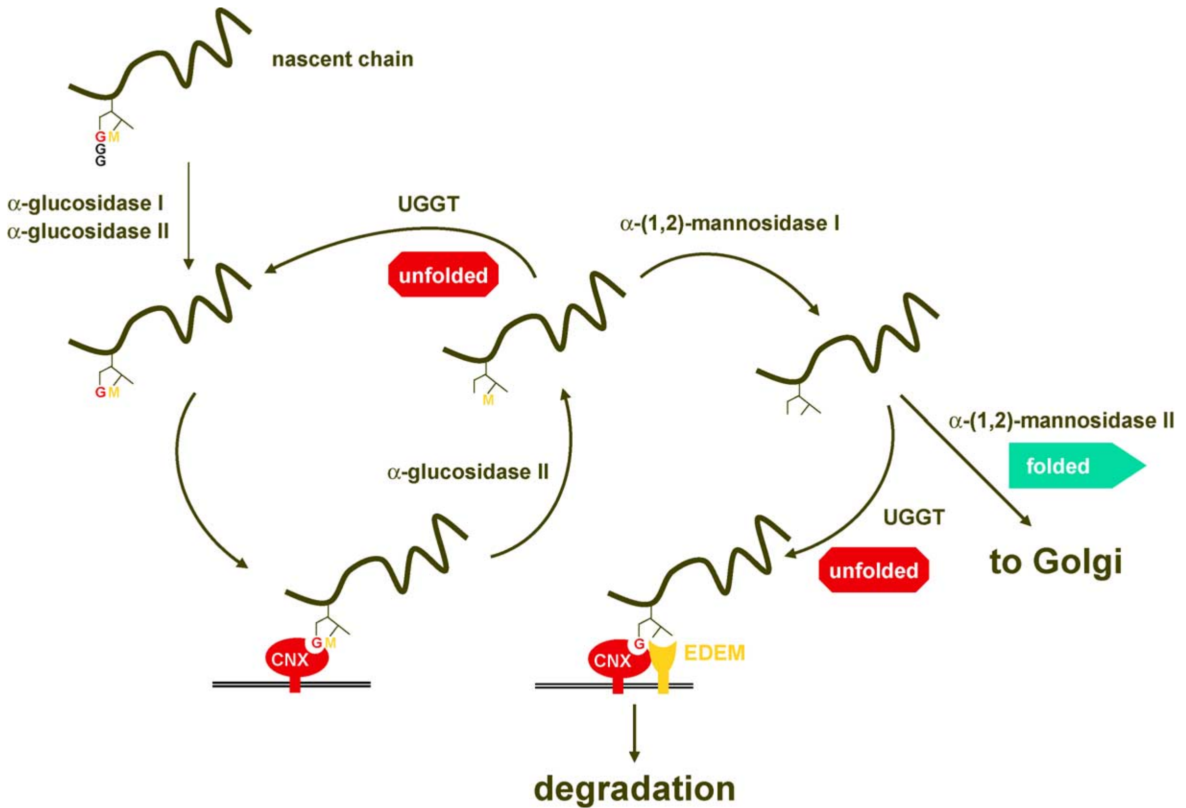

Fig. 3. Quality-control by the calnexin/calreticulin cycle. Abbreviations: CNX – calnexin, EDEM – ER degradation-enhancing α-mannosidase-like protein, G – glucose, M – mannose, and UGGT – uridine diphosphate (UDP)-glucose:glycoprotein glucosyl transferase.

is initiated by transfer of a core oligosaccharide from a membrane-bound dolichol phosphate anchor to consensus Asn-X-Ser/Thr residues in the polypeptide chain (Fig. 3; [4,5]). Glycosylation serves several purposes in protein folding: first, due to the hydrophilic nature of carbohydrates, glycosylation increases the solubility of glycoproteins and defines the attachment area for the surface of the protein. Second, due to their large hydrated volume oligosaccharides shield the attachment area from surrounding proteins. Third, oligosaccharides interact with the peptide backbone and stabilize its conformation [70]. Lastly, sequential trimming of sugar residues is monitored by a lectin machinery to report on the folding status of the protein (Fig. 3; [7]). This calnexin/calreticulin cycle is one arm of the quality-control machinery in the ER that monitors protein conformations and dictates whether a molecule is exported to the Golgi or targeted for ERAD. Briefly, the monoglucosylated form of a folding protein shuttles through several cycles of de- and reglucosylation by α-glucosidase II and uridine diphosphate (UDP)-glucose:glycoprotein glucosyl transferase (UGGT) activities (Fig. 3; [7]). The monoglucosylated form is retained in the ER via interaction with the lectins calnexin (CNX) and calreticulin (CRT). UGGT preferentially recognizes the unfolded conformation. Proteins are extracted from this cycle after demannosylation by α(1,2)-mannosidase I (Fig. 3). Compared to other oligosaccharide trimming reactions in the ER, this reaction is slow [71], giving the protein time to go through several folding cycles. If folded correctly, the mannose-trimmed protein is exported to the Golgi complex. If improperly folded, reglucosylation by UGGT initiates interaction with calnexin, transfer to the lectin Mnl1p/Htm1p/EDEM (ER degradation-enhancing α-mannosidase-like protein; [72–75]) and retrograde translocation to the cytosol for degradation by the proteasome (Fig. 3).

3.4. Protein folding machinery

The protein folding machinery of the ER consists of three classes of proteins: foldases, molecular chaperones, and the lectins calnexin, calreticulin, and EDEM (Table 1). Foldases are enzymes that catalyze steps in protein folding to increase their rate. Prominent examples are *cis–trans* peptidyl–prolyl isomerases

Table 1
ER-resident molecular chaperones, foldases, and lectins

Class and name	Function and reference
Chaperones, HSP70 class	
BiP/GRP78/Kar2p	Chaperone [270], translocation, folding sensor [127,129,130]
Lhs1p/Cer1p/Ssi1p/GRP170	Chaperone [76–79]
	Chaperone [79]
Chaperones, DNA-J-like, HSP40 class	
ERdj1/MTJ1	Co-chaperone regulating ATPase activity of BiP [92]
ERdj3/HEDJ/Scj1p	Co-chaperone regulating ATPase activity of BiP [93–96]
ERdj4	Co-chaperone regulating ATPase activity of BiP [97]
ERdj5	Co-chaperone regulating ATPase activity of BiP [98]
Jem1p	Co-chaperone regulating ATPase activity of BiP [102]
Sec63p	Co-chaperone regulating ATPase activity of BiP, translocation [99–101]
Chaperones, GrpE-like	
BAP	Nucleotide exchange factor for BiP [103]
Sls1p/Sil1p	Nucleotide exchange factor for BiP [105]
Chaperones, HSP90 class	
GRP94/endoplasmin	Chaperone [80]
Lectins	
Calnexin	Glycoprotein quality-control [271,272]
Calreticulin	Glycoprotein quality-control [273]
Mnl1p/Htm1p/EDEM	Glycoprotein degradation [72–75]
Carbohydrate processing enzymes	
UGGT	Folding sensor [7]
α-Glucosidase I	Removal of terminal glucose residues from glycoproteins [274,275]
α-Glucosidase II	Removal of terminal glucose residues from glycoproteins, release of glycoproteins from calnexin [274,275]
α-Mannosidase I	Removal of terminal mannose residues, extraction of glycoproteins from calnexin cycle
α-Mannosidase II	Removal of terminal mannose residues, extraction of glycoproteins from calnexin cycle
Foldases, subclass disulfide isomerases	
PDI	Oxidoreductase [276]
ERp72	Oxidoreductase [277]
ERp61	Oxidoreductase [278]
ERp57	Oxidoreductase [278]
ERp44	Retention of Ero1α in ER [279]
Ero1p/Ero1α, Ero1β	Oxidoreductase for PDI [63,64]
Erv2p	Oxidoreductase for PDI [67,68]
Foldases, subclass FAD-dependent oxidases	
Fmo1p	FAD-dependent oxidase [69]
Foldases, peptidyl-prolyl isomerases	
FKBP13	[280]
FKBP65	[281]
S-Cyclophilin	[282]
CCYLP	[283]
Cyclophilin B	[284]

(PPI/immunophilins) which catalyze the *cis–trans* isomerization of peptidyl–prolyl bonds and PDIs (see above). Molecular chaperones facilitate protein folding by shielding unfolded regions from surrounding proteins. They do not enhance the rate of protein folding. According to their cytosolic counterparts they are classified into several groups: class HSP70 chaperones in the ER are BiP/GRP78/Kar2p, Lhs1p (Cer1p/Ssi1p) [76–78], and GRP170 [79]. BiP also participates in the translocation of nascent polypeptide chains into the ER. The HSP90 class chaperone GRP94/endoplasmin [80] recognizes a subset of peptide substrates, in a manner coordinated with other chaperones, e.g. BiP [81], and facilitates the display of immunogenic peptides on MHC class I complexes [82]. In addition, PDI has disulfide-dependent and -independent chaperone activity [83,84]. Preferential interaction of unfolded proteins with ER-resident molecular chaperones constitutes the second arm of the quality-control machinery in the ER.

4. Recognition of unfolded proteins

Thermodynamically, any conformation with a higher free-energy than the native conformation is unfolded. This is due to hydrophobic regions exposed on the surface in the non-native conformation. Contact of these residues with surrounding water increases the free surface energy. In the native conformation these regions are buried in the protein core. However, this definition of an unfolded protein is difficult to access experimentally. Therefore, biochemically, conformations that interact with molecular chaperones are regarded as unfolded. However, different chaperones recognize different client proteins and many client–protein specific chaperones evolved to facilitate the folding of just one or a few proteins (Table 2). Thus, protein folding status depends on the chaperone under investigation. Despite these drawbacks there is large agreement between both definitions of an unfolded protein where the mechanism of unfolded protein recognition by individual molecular chaperones has been studied in detail.

4.1. Recognition of unfolded proteins by BiP

BiP has an *N*-terminal ATPase and a *C*-terminal substrate binding domain. In the ADP-bound form

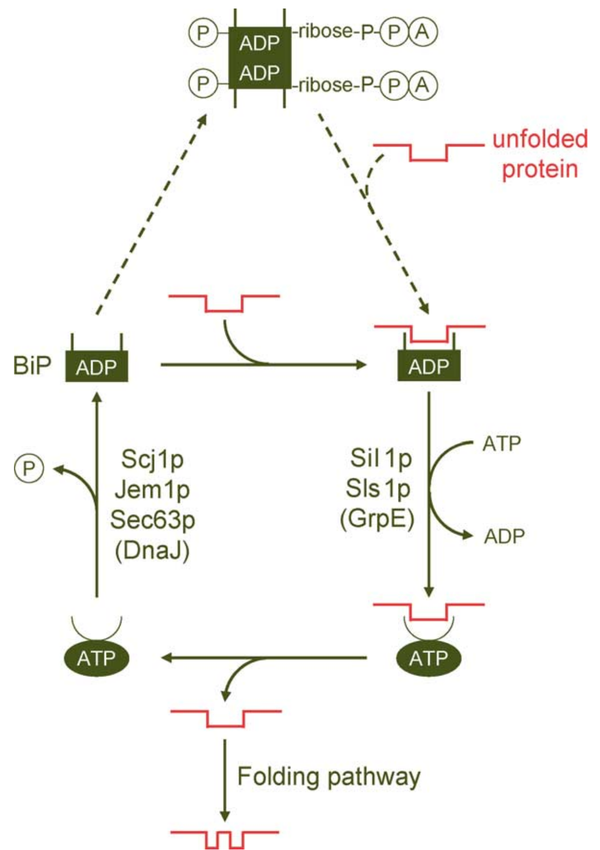

Fig. 4. Quality-control by the BiP ADP–ATP-cycle.

BiP has high affinity for protein substrates (Fig. 4). Substrates bound to BiP are locked in their conformation and stimulate the ATPase activity of BiP [85,86]. Affinity panning and binding assays with random peptide libraries demonstrated that short hydrophobic peptides, such as those forming β-strands deeply buried in the protein core, are preferentially bound by BiP [86,87]. The affinity for these peptides is low (1–100 mM), allowing for a wide substrate spectrum. Exchange of ADP with ATP releases the substrate from BiP [85], which then progresses on its folding pathway. Subsequent ATP-hydrolysis returns BiP into the ADP, high affinity state (Fig. 4). Thus, by cycling through the BiP ADP–ATP cycle a folding polypeptide chain consumes ATP. Indeed, the folding of many secretory proteins can be inhibited by depleting cellular ATP levels [88–91]. Both reactions, nucleotide exchange and ATP-hydrolysis are regulated by co-chaperones.

Table 2
Specialized client–protein chaperone pairs in the ER

Factor	Function	Client–protein	Organism/cell type	Ref.
HSP47	Chaperone	Procollagen	Mammalian collagen producing cells	[285]
Prolyl 4-hydroxylase	Enzyme, chaperone	Procollagen	Mammalian cells	[286]
Microsomal triglyceride transfer protein	Assists translocation, assembly, and secretion	Apolipoprotein B (apoB)	Primarily liver cells and intestinal cells	[287]
NinaA: *cis–trans* peptidyl–prolyl isomerase	Possibly chaperone and/or escort; promotes ER secretion competence through direct interaction	Rh1 and Rh2 rhodopsins	*Drosophila* photoreceptor cells	[288]
BOCA and Mesd	Assembly and transport	LDL-receptor	Mammalian cells	
Invariant chain	Escort to prevent aggregation and premature ligand binding and to direct endosomal targeting	MHC class II	Mammalian antigen presenting cells	[289]
RAP	Escort to prevent aggregation and premature ligand binding	LDL receptor family	Mammalian cells	[290]
Neurophysin	Escort	Arginine vasopressin	Magnocellular neurons of the hypothalamus	[14]
β-Catenin	Targeting to the basal-lateral membrane	E-cadherin	Epithelial cells	[291]
p24 Family	Potential cargo receptors	Invertase, Gas1p (*S. cerevisiae*), many others	*S. cerevisiae*, *C. elegans*, and mammalian cells	[292]
LMAN1/ERGIC-53	Potential cargo receptor for glycoproteins	Cathepsin C, blood clotting factors V and VIII	Mammalian cells	[293]
Lst1p	Potential cargo receptor	Plasma membrane H^+-ATPase Pma1p	*S. cerevisiae*	[294]
Erv14p	Potential cargo receptor	Plasma membrane protein Axl2p	*S. cerevisiae*	[295]
Vma12p–Vma22p complex	Promotes complex assembly	Vacuolar H^+-ATPase subunit Vph1p	*S. cerevisiae*	[296]
Chs7p	Promotes ER secretion competence	Chs3p, catalytic subunit of chitin synthetase III	*S. cerevisiae*	[297]
Gsf2p	Promotes ER secretion competence	Hexose transporters Hxt1p and Gal2p	*S. cerevisiae*	[298]
Lag1p and Dgt1p	Promotes ER secretion of GPI-anchored proteins	GPI-anchored proteins Gas1p and Yap3p	*S. cerevisiae*	[299]
Shr3p	Promotes ER secretion competence	Amino acid permeases, e.g. Hip1p and Gap1	*S. cerevisiae*	[300]
ODR-4 and -8	Promotes ER secretion competence	Odorant receptors ODR-10 and STR-2	*C. elegans* olfactory neurons	[301]
BAP31	Promotes ER secretion competence	Cellubrevin	Mammalian cells	[289]
Protective protein/cathepsin A	Promotes ER secretion competence through direct interaction and directs lysosomal targeting	Neuraminidase and β-galactosidase	Mammalian cells	[302]
Tapasin	Prevents ER exit of MHC class I without bound antigenic peptide	MHC class I	Mammalian cells	[303]
Egasyn	Mediates ER retention of target molecule through KDEL-like ER retention signal	β-Glucuronidase	Mammalian cells	[304]
Carboxylesterase	Mediates ER retention of target molecule through KDEL-like ER retention signal	C-reactive protein	Hepatocytes	[305]
SCAP	Retention of SREBP	SREBP	Mammalian cells	[306]

The DnaJ-like proteins MTJ1/ERdj1 [92], ERdj3/HEDJ [93,94]/Scj1p [95,96], Erdj4 [97], Erdj5 [98], Sec63p [99–101], and Jem1p [102] stimulate the ATPase activity of BiP, and the GrpE-like protein BiP-associated protein (BAP) [103], and Sls1p [105] stimulate the nucleotide exchange reaction. In vitro the K_m for ATP-binding by bovine HSP70 is 1–2 μM in the presence and absence of unfolded proteins. Since the cytosolic ATP concentration is in the mM range, nucleotide binding is not rate-limiting for the function of cytosolic HSP70s. ATP is imported into the ER via antiport with ADP and AMP [106]. ATP-import may be limiting for the function of ER luminal HSP70 chaperones. The in vitro rate constants of nucleotide exchange and ATP-hydrolysis by BiP are similar [107]. Thus, differential regulation of nucleotide exchange and ATP-hydrolysis by co-chaperones in vivo may be important for the regulation of BiP function.

BiP, as other HSP70s [107], cycles between a monomeric and oligomeric state (Fig. 4; [108–111]). In the oligomeric state BiP is post-translationally modified by phosphorylation [108,112–114] and ADP-ribosylation [115–117]. Only monomeric unmodified BiP associates with unfolded proteins [108,109]. Induction of unfolded proteins increased the monomeric, unmodified BiP pool [109,118]. Therefore, it was suggested that modified oligomeric BiP constitutes a storage pool from which BiP is recruited to the monomeric pool by interaction with unfolded proteins [119]. These events are the first events in signal transduction in response to the accumulation of unfolded proteins in the ER lumen.

4.2. *Recognition of unfolded proteins by UGGT*

In contrast to BiP, UGGT simultaneously recognizes two features in an unfolded protein: exposed hydrophobic sequences and the oligosaccharide moiety [120]. UGGT recognizes the innermost *N*-acetylglucosamine residue of an linked oligosaccharide [121]. The structural flexibility of this residue and neighboring amino acid residues may be a key determinant in recognition of unfolded proteins by UGGT, since this residue extensively interacts with the polypeptide backbone of the protein [70]. However, the region judged as being misfolded by UGGT and the oligosaccharide glucosylated by UGGT can be up to 4 nm apart [122].

5. Transduction of the unfolded protein signal across the ER membrane

Three transmembrane proteins transduce the unfolded protein signal across the endoplasmic reticulum membrane (Fig. 5). The ER luminal domains of the type I transmembrane proteins IRE1 (inositol requiring 1)/ERN1 (ER to nucleus signaling 1) and PERK [double-stranded RNA-activated protein kinase (PKR)-like endoplasmic reticulum kinase]/PEK [pancreatic eukaryotic initiation factor 2α (eIF2α) kinase] are ER stress regulated oligomerization domains [123–125]. However, the cytosolic domain of IRE1 also possesses, albeit weaker, potential for homodimerization [124]. The type II transmembrane protein activating transcription factor 6 (ATF6) contains two independent ER stress regulated Golgi localization sequences (GLS) [126]. The luminal domains of IRE1 and PERK show a small degree of homology conserved throughout all eukaryotes, but no homology exists with the luminal domain of ATF6. Functional studies in yeast revealed that the ER luminal domains of IRE1 and PERK are interchangeable and that their function is evolutionarily conserved [127,128]. Surprisingly, their function can be completely substituted for by a non-homologous dimerization domain in the bZIP proteins MafL and JunL [127]. In an inactive state the luminal domains of IRE1 and PERK are associated with BiP [129–131]. Upon ER stress, BiP is competitively titrated from the luminal domains of IRE1 and PERK by the huge excess of unfolded proteins in the ER lumen, resulting in oligomerization of IRE1 and PERK [129,130] and activation of these proximal signal transducers. Consistent with this model is that interactions of BiP with its substrates are transient. Further, the huge excess of BiP over IRE1 and PERK is set-off by the low affinity of BiP for its substrates. Thus, only small fluctuations in the free BiP pool should be required for its release from IRE1 and PERK. In IRE1α the domains required for signaling, oligomerization, and BiP-binding partially overlap (Fig. 5; [131]) and BiP may actually mask an important oligomerization motif in IRE1α to keep it in its monomeric, inactive state. However, in PERK the domains required for oligomerization and BiP are distinct (Fig. 5; [132]). Here BiP indirectly interferes with oligomerization either sterically or through induction of a conformational change in the luminal do-

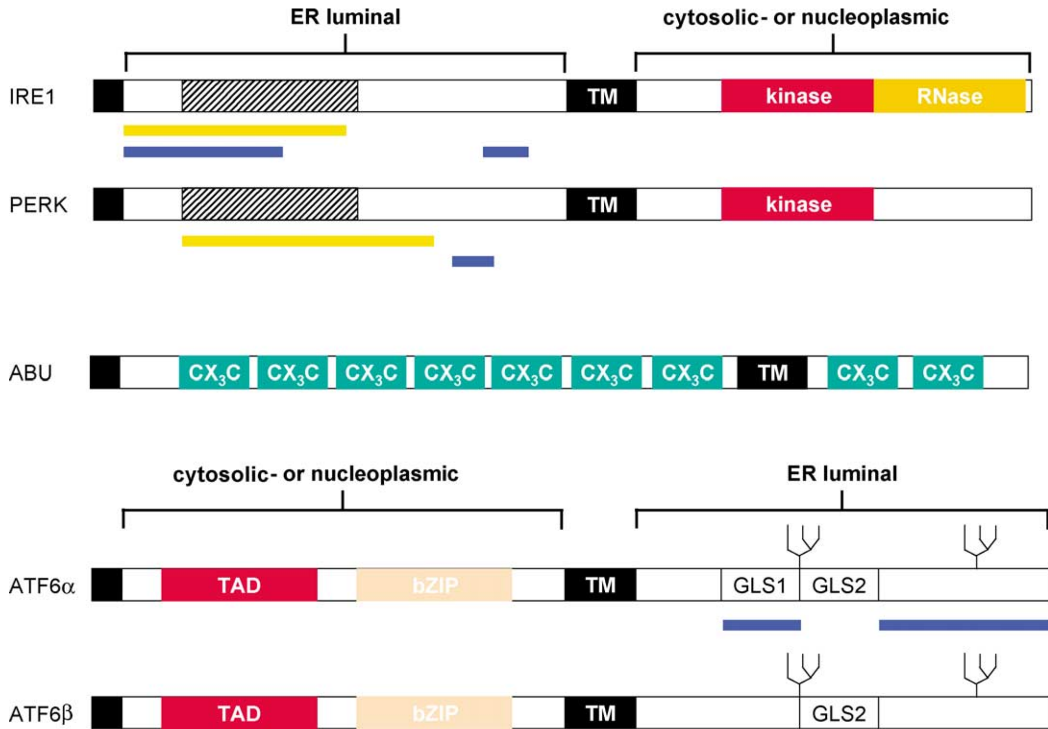

Fig. 5. Principal architecture of the ER stress sensors IRE1, PERK, ABU, and ATF6. Orange bars represent regions sufficient for signal transduction or oligomerization. Blue bars represent regions binding to BiP. A black box represents the signal peptide and the hatched box depicts the region of limited homology between IRE1 and PERK. Abbreviations: bZIP – basic leucine zipper, CX_3C – $CX_3CX_3CX_3C$ domain (pfam 02363), GLS1 and GLS2 – Golgi localization sequences 1 and 2, TAD – transcriptional activation domain, and TM – transmembrane domain. Drawings are not to scale.

main of PERK that inactivates the oligomerization domain.

ATF6 is regulated in a similar way by BiP as PERK or IRE1 [126]. The major difference is that BiP does not regulate the activity of oligomerization domains in ATF6, but rather the activity of two independent and redundant Golgi localization sequences, GLS1 and GLS2. BiP binds to GLS1, but not to GLS2. In the absence of BiP-binding GLS2 is dominant, resulting in constitutive translocation of ATF6 to the Golgi and ATF6 activation [126]. In addition, ATF6 is retained in the ER by interaction with the lectin calreticulin [133]. Under ER stress conditions, newly synthesized ATF6 is underglycosylated, which abrogates its interaction with calreticulin. Consistent with this model is the observation that ATF6α mutants in which some of its three glycosylation sites were destroyed are more potent transcriptional activators than WT ATF6α in a site-2 protease (S2P) dependent manner [133]. These are the first data, albeit indirect, that support involvement of the calnexin/calreticulin cycle in activation of the proximal ER stress transducers. Both quality-control mechanisms operating in the ER, the calnexin/calreticulin cycle and recognition of unfolded proteins by BiP, regulate the activity of the proximal stress transducer ATF6. However, the conserved *N*-linked glycosylation site in yeast Ire1p was completely dispensable for its function [127]. This suggests that differential regulation of the three arms of the UPR, ATF6, IRE1, and PERK exists to fine tune UPR signaling to specific folding demands in the ER [134].

Candidates for additional metazoan ER stress sensors are the activated in blocked UPR (*ABU*) genes, a family of homologous type I transmembrane proteins up-regulated in xbp-1 mutant *Caenorhabditis elegans* [135,136].

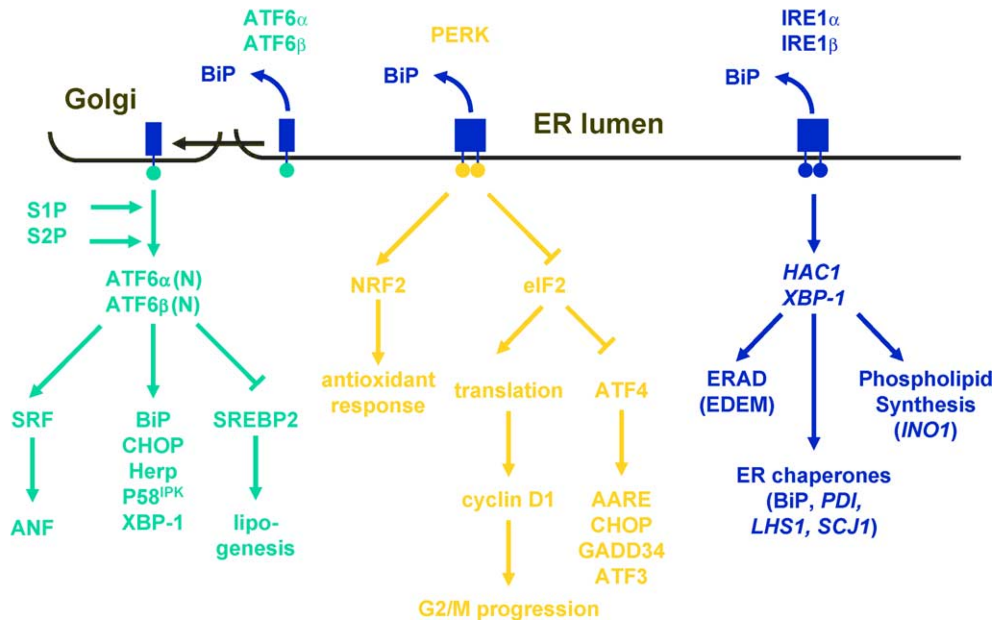

Fig. 6. Overview of protective ER stress signaling pathways.

6. Activation of protective responses by the UPR

6.1. ATF6

Two homologous proteins, ATF6α and ATF6β/CREB-RP/G13 exist in mammals (Fig. 5; [137]). ATF6 translocates to the Golgi complex after release of the molecular chaperone BiP from its ER luminal domain (Fig. 6; [138]). Site-1 protease (S1P) cleaves ATF6 in the luminal domain. The *N*-terminal, membrane anchored half is then cleaved by S2P [139–141]. These proteolytic reactions release the cytosolic *N*-terminal portion of ATF6 encoding a basic leucine zipper (bZIP) transcription factor. ATF6 binds to the ATF/CRE element [142] and to the ER stress response elements I and II (ERSE-I, CCAAT-N_9-CCACG [143], and ERSE-II, ATTGG-N-CCACG [144]). Binding of ATF6 to ERSE-I requires NF-Y/CBF [145]. Important targets regulated by ATF6 are *BiP/GRP78, XBP-1, CHOP/GADD153* [141], *P58^{IPK}* [146], and the membrane protein Herp [144]. Gene profiling analysis using overexpression of ATF6α(1-373) [147] and ATF6β(1-392) [137] revealed that ATF6α and ATFβ positively regulate transcription of ER-resident molecular chaperones and foldases. However, heterodimeric complexes between ATF6α and ATF6β are a transcriptional repressor of the BiP promoter [148]. In RNAi gene knock-down experiments no specific targets for ATF6α or ATF6β were identified, suggesting that pathways redundant to the ATF6 pathway exist [149]. Further, ATF6α interacts with the transactivation domain of serum response factor (SRF) and antisense ATF6α reduced serum induction of reporter constructs [150]. Activation of the gene atrial natriuretic factor (*ANF*) by ATF6 and SRF was proposed to be dependent on phosphorylation of ATF6 by the stress response kinase p38 [151]. ATF6 forms a heterodimeric complex with the basic helix-loop-helix (bHLH) transcription factor sterol response element (SRE) binding protein 2 (SREBP2). This complex counters the lipogenic effects of SREBP2 by recruiting the histone deacetylase complex 1 (HDAC1) to the SRE to repress transcription [152].

6.2. IRE1

The IRE1 pathway regulates chaperone induction, ERAD, and expansion of the ER in response to ER stress (Fig. 6). Further, this pathway is evolutionarily

the oldest pathway, since it is present in all eukaryotes, and distinguished by unique features transducing the stress signal.

6.2.1. Mechanism of signal transduction

IRE1 encodes an atypical type I transmembrane protein kinase endoribonuclease [153–155], consisting of an ER luminal dimerization domain, and cytosolic kinase and endoribonuclease domains. After dissociation of BiP from the ER luminal domain, IRE1 oligomerizes [123–125] and activates its RNase domain by autophosphorylation [123,124]. However, occupancy of the ATP-binding pocket by ADP is sufficient for activation of the RNase domain after oligomerization [156]. Mutations in the RNase domain of Ire1p abolished activation of an ERP72 CAT reporter construct [157,158]. Transient transfection experiments with kinase- and RNase-defective Ire1p indicate that two functional RNase domains are required for signaling by Ire1p [157]. The substrate for the Ire1p endoribonuclease was first identified in yeast and is the mRNA for the bZIP transcription factor *HAC1* [159–161]. *HAC1* mRNA is unusual for yeast as it has a large intron of 252 bp located in the 3′-end of the mRNA. Activated Ire1p cleaves both 5′- and 3′-exon–intron junctions in *HAC1* mRNA [162–165] and generates 5′-OH and 3′-cyclic PO_4 ends (Fig. 7; [166]). tRNA ligase (Rlg1p/Trl1p) joins both exons (Fig. 7; [167]). The ligase leaves a 2′-phosphate on the 5′-end of the joined junction [166] that is removed by the NAD^+-dependent phosphatase Tpt1p [168,169]. NAD^+ serves as phosphate acceptor in an unusual reaction that generates nicotinamide and ADP-ribose 1“-2”-cyclic phosphate (App-ribose > *P*) [170]. In summary, the mechanism of *HAC1* mRNA splicing is similar to pre-tRNA splicing [171]. In contrast to mRNA splicing or the self-splicing of groups I and II introns, this mechanism does not provide an explanation for how the ligase distinguishes between exons and introns. In vitro, the HAC1 exons remain associated after cleavage of both exon–intron junctions by Ire1p [166]. *HAC1* mRNA splicing is different from tRNA splicing in that it is likely to be cytoplasmic. *HAC1* mRNA can be spliced in polysomes, but association with polysomes is not a prerequisite for splicing [36]. The majority of *HAC1* mRNA is located in the cytoplasm [172]. By inhibiting de novo transcription with temperature-sensitive alleles of RNA polymerase II Walter and coworkers showed that this cytoplasmic pool can be spliced [173]. However, since tRNA splicing is nuclear [174,175], either a low level of cytoplasmic tRNA is sufficient for *HAC1* mRNA splicing, tRNA ligase shuttles in stress conditions into the cytoplasm, or the cytoplasmic *HAC1* mRNA pool relocates into the nucleus.

The *HAC1* mRNA splicing reaction has two consequences: expression of an alternative *C*-terminus with increased transcriptional activation potential [176] and removal of a translational attenuator from *HAC1* mRNA [173]. Base pairing between the 5′-UTR of unspliced *HAC1* mRNA and the intron represses translation of the unspliced mRNA [173]. Unspliced *HAC1* mRNA is found in association with polysomes [172]. mRNAs are exported with their 5′-end first in higher eukaryotes, which would allow for loading of the mRNA with polyribosomes before secondary structure elements are formed, which then trap the loaded polyribosomes on *HAC1* mRNA [173]. These observations raise the interesting questions how the endonuclease accesses the splice junctions in polysomal *HAC1* mRNA and if this recognition process is controlled by ER stress. *LHP1*, the yeast gene encoding the eukaryotic RNA-binding protein La implicated in the metabolism and translation of RNA polymerase III transcripts, e.g. tRNAs, was recently implicated to facilitate translation of spliced *HAC1* mRNA [177].

Spliced Hac1p ($Hac1^ip$, i for induced) then binds to the unfolded protein response element (CAGCGTG, [13,160,178–180]). $Hac1^ip$ interacts in vitro with components of the SAGA histone acetyltransferase complex [181]. Activation of *KAR2* and *PDI1* by ER stress, but not heat shock, is partially dependent on a functional SAGA [182]. Thus, activation of ER chaperone genes by $Hac1^ip$ involves, at least in part, recruitment of SAGA to their promoters by $Hac1^ip$. It has not been investigated if spliced and unspliced Hac1p interact differently with SAGA components, which may provide an explanation for the increased transcriptional activation potential of the spliced version over the unspliced version.

6.2.2. Regulation of Ire1p

Yeast Ire1p is negatively regulated by the phosphatase Ptc2p [183]. Whether this negative regulation is a constitutive, or ER stress responsive activity, is not known.

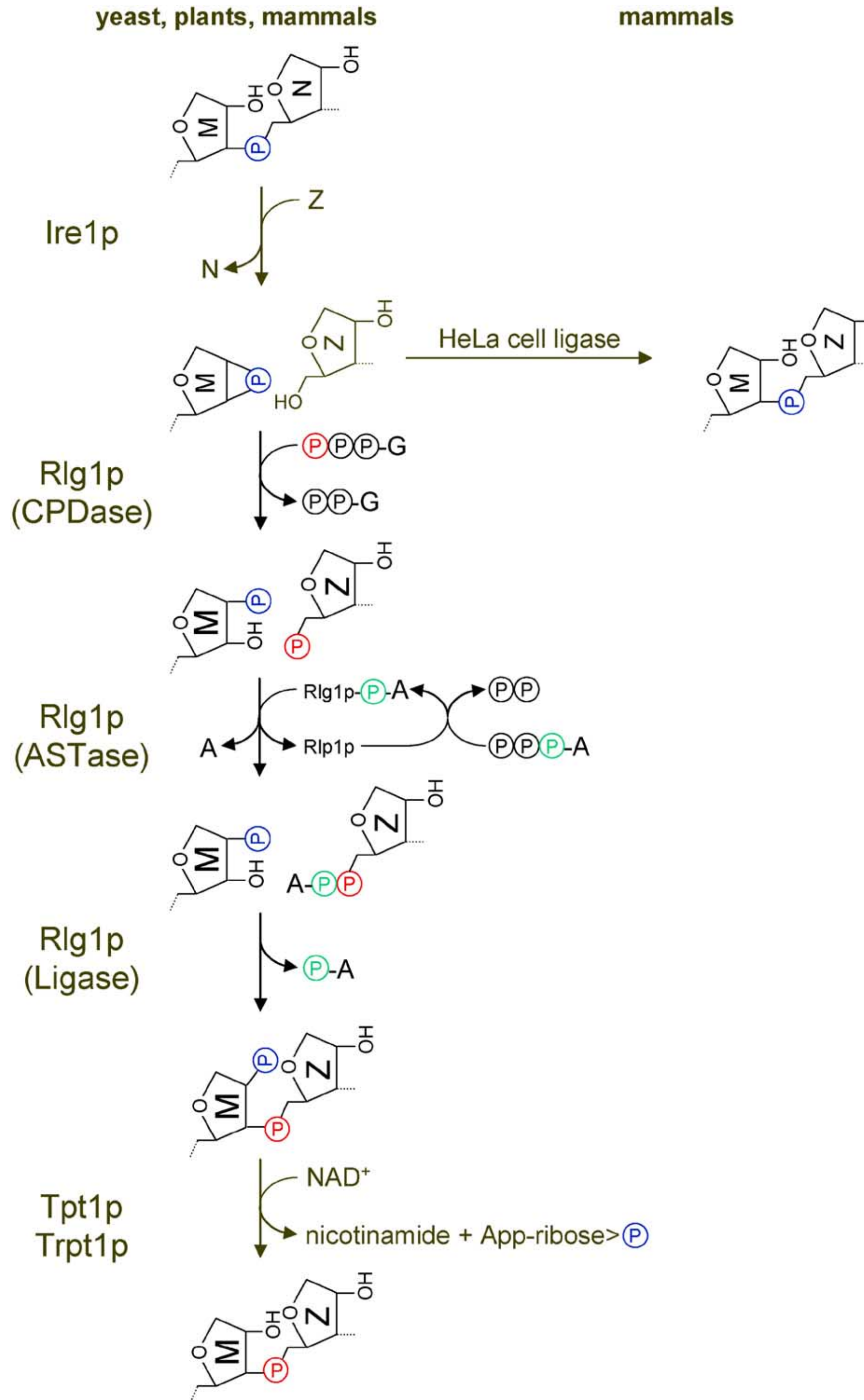

Fig. 7. Mechanism of *HAC1* mRNA and tRNA splicing in yeast, plants and mammals. Abbreviations: CPDase – cyclic phosphodiesterase, ASTase – adenylyl synthetase.

6.2.3. Role of IRE1 and HAC1 in regulation of membrane proliferation

In cell types with high secretory capacity, e.g. the pancreas, the liver, or plasma cells, a dramatic proliferation of the ER and other membrane compartments is observed. Based on the following three observations a role for the UPR in coordination of the unfolded protein load in the ER and membrane proliferation was proposed. First, *ire1Δ* and *hac1Δ* yeast strains are inositol auxotrophs [153,161]. Second, treatment of yeast with ER stress inducers such as tunicamycin induces transcription of *INO1* encoding inositol-1-phosphate synthase, a key enzyme in phospholipid biosynthesis (Fig. 8), in an *IRE1*- and *HAC1*-dependent manner [32,184]. Third, induction of membrane proliferation by expression of membrane proteins is in some, but not all [185], cases dependent on a functional UPR pathway [184,186,187]. Based on these data, the UPR can (a) have a specialized function in increasing phospholipid biosynthesis and ER proliferation in response to acute and/or severe ER stress, or (b) generally, that is under all conditions, monitor and regulate phospholipid biosynthesis and the biogenesis of membrane compartments, e.g. through transcriptional regulation of *INO1* [184]. This latter view has recently been challenged. First, in *ire1Δ* yeast grown on glucose induction of two membrane proteins, the integral peroxisomal membrane protein Pex15p and the inner mitochondrial membrane protein Acr1p was lethal. Both lethalities were rescued by growth on a fatty acid, oleate [188]. This observation suggested that an indirect mechanism is the cause for the lethal phenotype. Second, activation of *INO1* by inositol starvation was only modestly defective in *ire1Δ* or *hac1Δ* strains [184,189]. Upon 4 h inositol starvation CDP-diacylglycerol levels in *ire1Δ* and *hac1Δ* strains were increased compared to WT, and phosphatidic acid and phosphatidylinositol levels decreased. In a strain

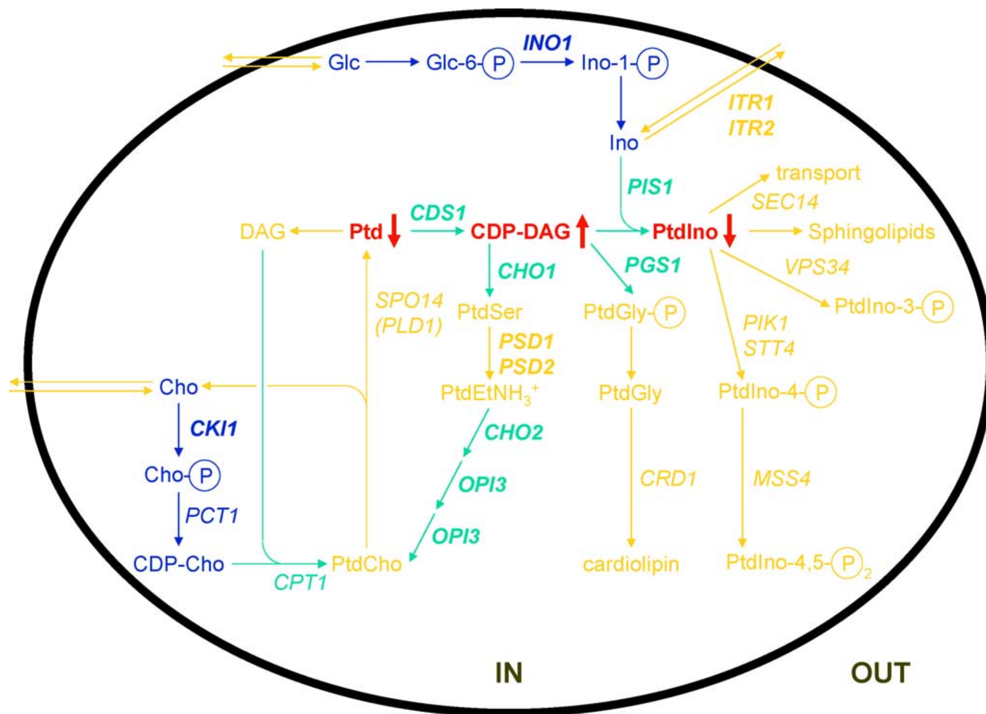

Fig. 8. Key reactions in phospholipid biosynthesis are catalyzed at the ER membrane. Water soluble molecules, enzymes, and enzymatic reactions in the cytosol are in blue, membrane-bound molecules, enzymes, and reactions at membrane compartments are in orange or red. Enzymes and reactions at the ER membrane are in green. Phospholipids whose levels are altered in *ire1Δ* and *hac1Δ* strains during inositol starvation are in red, and arrows (↓ or ↑) indicate if their levels are decreased or increased [189]. Enzymes whose genes are repressed by inositol and choline in the growth medium are in bold [8]. Abbreviations: Cho – choline, DAG – diacylglycerol, $EtNH_3^+$ – ethanolamine, Glc – glucose, Gly – glycerol, Ino – inositol, Ptd – phosphatidic acid or phosphatidyl, Ser – serine.

with an overexpression of inositol (Opi$^-$) phenotype these changes were reversed and activation of the *INO1* promoter by inositol starvation not affected by a *HAC1* deletion [189]. Thus, the UPR does not directly control expression of *INO1* in response to inositol starvation. The changes in phospholipid levels seen in *ire1Δ* and *hac1Δ* strains suggest a role for the UPR in regulation of key metabolic reactions in phospholipid metabolism at the ER membrane (Fig. 8).

6.2.4. The IRE1 pathway in higher eukaryotes

IRE1 is conserved throughout all eukaryotic kingdoms [127,128,153,155,190–192]. Mammals have two copies of *IRE1*, *IRE1α* [191] and *IRE1β* [193]. *IRE1α* is ubiquitously expressed [135,191], and deletion of *IRE1α* results in an embryonic lethal phenotype between days 9.5 and 11.5 in mice [135]. Expression of *IRE1β* is limited to the gut. *Ire1β*$^{-/-}$ mice are viable, but are more susceptible to dextran sodium sulfate induced colitis [194]. In plants, *Arabidopsis thaliana* has two functional copies, *IRE1-1* and *-2*, and a gene lacking the ER luminal domain [128], but there is only one copy in *Oryza sativa* (rice). This suggests that *IRE1-1* and *-2* have overlapping functions in *A. thaliana*. The cytosolic domains of yeast Ire1p are functional and can oligomerize [124]. It is therefore possible that the truncated gene in *A. thaliana* encodes a functional protein that may be involved in host defenses, by analogy to RNase L [195].

The bZIP transcription factor downstream of *IRE1* shows a large degree of divergence. For example, even between yeast and filamentous fungi [196] only the bZIP domain is conserved. In metazoans the functional homologue for *HAC1* is *XBP-1* [20,197–199]. XBP-1 is a bZIP transcription factor of the ATF/CREB family and controls genes containing a CRE (cAMP response element)-like element [GATGACGTG(T/G)NNN(A/T)T] [200]. XBP-1 is essential for terminal B-cell differentiation [21]. Compounds that stimulate terminal B-cell differentiation, e.g. lipopolysaccharides, also induced XBP-1 splicing [20,22]. These observations raise the possibility that the UPR regulates B-cell differentiation. As in *HAC1*, *XBP-1* splicing introduces a frame-shift and an alternative *C*-terminus with increased transcriptional activation potential. However, there is no differential translational control of unspliced and spliced *XBP-1* mRNAs, *XBP-1*^{u} and *XBP-1*^{s}, respectively. Thus, XBP-1^{u}, in analogy to the small bZIP Maf proteins, acts as a dominant negative for XBP-1^{s} through occupying the CRE-like element or through formation of less potent and therefore inhibitory XBP-1^{u}–XBP-1^{s} heterodimers. Degradation of XBP-1^{u} by the proteasome is necessary for efficient activation of the UPR [149]. There is little known about the mechanism of *XBP-1* splicing. Despite their divergence, the splice junctions in *XBP-1* and *HAC1* mRNA are conserved [197,199]. However, it has not been directly demonstrated that IRE1α or -β generate 2′,3′-cyclic PO_4 ends in *XBP-1* mRNA. Consistent with nuclear localization of *XBP-1* splicing in mammalian cells is the convincing localization of mammalian Ire1α to the inner leaflet of the nuclear envelope [199].

A ligase with similar properties as yeast Rlg1p was characterized in wheat germ [201–204] and in *Chlamydomonas* [205,206]. Mammals have at least two ligase activities, termed yeast-like tRNA ligase and HeLa cell ligase (Fig. 7). In the yeast tRNA ligase-like reaction the junction phosphate is derived from the γ-phosphate of GTP, whereas in the reaction catalyzed by HeLa cell ligase the junction phosphate is derived from the phosphate backbone of the RNA substrate (Fig. 7; [207]). This HeLa cell ligase has an approximate molecular weight of 160 kDa and ligates several RNAs bearing 5′ hydroxyl and 2′,3′ cyclic phosphate termini in an ATP-dependent reaction [208,209]. It should be straightforward to test if this ligase can join *XBP-1* exons. Based on labeling experiments a yeast-like tRNA ligase is present in mammalian cells [210]. The 2′-NAD$^+$-dependent phosphatase Tpt1p is conserved in bacteria, yeast, plants, and mammals [211,212]. In fact, human TRPT1 can complement a defect in yeast Tpt1p [212]. Interestingly, TRPT1 is primarily expressed in heart and skeletal muscle [212].

6.2.5. Targets of the IRE1 pathway

Genome profiling in yeast [32] and *A. thaliana* [27] and a genetic analysis in yeast [213] revealed that the IRE1 pathway, as the only major pathway in these organisms, coordinates multiple aspects of the secretory pathway including chaperone induction, upregulation of ERAD genes [31,32,134], membrane biogenesis, and ER quality-control. In mammals, XBP-1 regulates a subset of ER-resident molecular chaperones [149]. It was recently shown that moderate, *IRE1*- and *HAC1*-independent transcriptional induc-

tion from a core promoter happens in response to ER stress in yeast [214], suggesting that a second signal transduction pathway exists in yeast that modulates and augments activation of ER chaperone genes by the *IRE1–HAC1* pathway in response to ER stress.

6.3. PERK

As IRE1, PERK is a type I transmembrane kinase and activated by release of BiP from its ER luminal domain. PERK then oligomerizes and phosphorylates substrate proteins, eIF2α [29,215,216] and the bZIP Cap'n'Collar transcription factor Nrf2 (Fig. 6; [217]). Phosphorylation of eIF2α by PERK shuts-off general translation [29]. $Perk^{-/-}$ cells are sensitive to ER stress and are partially rescued by translation inhibitors, e.g. cycloheximide [218]. Short-lived proteins are cleared from the cell during inhibition of translation. An important example is cyclin D1. Loss of cyclin D1 during ER stress arrests mammalian cells in G_1 [219–221]. In the absence of PERK, eukaryotic cells, e.g. tunicamycin-treated yeast cells, arrest in G_2/M [222] dependent on the function of the morphogenesis and pachytene checkpoint kinase Swe1p [223].

Besides eIF2α, PERK also phosphorylates Nrf2 which contributes to survival of ER stress in mammalian cells. In unstressed cells, Nrf2 is found in an inactive cytoplasmic complex with the cytoskeletal anchor Keap1 [217]. Upon ER stress PERK phosphorylates Nrf2 resulting in dissociation of the Nrf2–Keap1 complex, nuclear localization of Nrf2 and activation of transcription by Nrf2 through the antioxidant response element (ARE) [224–226]. The ARE controls expression of genes involved in the phase II metabolism of xenobiotics, e.g. electrophilic thiol-reactive substances that mimic an oxidative insult. Genes regulated by the ARE include the A1 and A2 subunits of glutathione S-transferase, NAD(P)H:quinone oxidoreductase, γ-glutamylcysteine synthetase, and UDP-glucuronosyl transferase. $Perk^{-/-}$ cells accumulate reactive oxygen species when exposed to ER stress [38] which suggests that the sensitivity of $Nrf2^{-/-}$ cells to ER stress [217] results from their impaired ability to respond to an oxidative insult. The idea that an imbalance in the cell's redox status is caused by ER stress is further supported by the observation that the redox-sensitive transcription factor nF-κB is activated in response to ER stress, and that this activation was inhibited by antioxidants [227]. It is interesting to speculate here that this imbalance is caused by uncoupling of the disulfide isomerase Ero1p from its yet to be identified physiological substrate by an elevated unfolded protein load of the ER.

Phosphorylation of eIF2α allows for preferential translation of mRNAs encoding several short upstream open reading frames (uORF, [228]). The mRNA for ATF4 [37,218] is regulated in this way in mammalian cells. ATF4 binds to the amino acid response element [218]. Targets of ATF4 are *CHOP*, *GADD34* [218,229,230] and *ATF3* [230]. ATF4 is also required for expression of genes involved in amino acid import, glutathione biosynthesis, and resistance to oxidative stress [38]. ATF3 contributes to expression of *CHOP* and *GADD34* [230].

6.3.1. Regulation of PERK signaling

Translational inhibition by PERK is transient to allow for recovery from ER stress, and to mount an efficient protective response to prolonged periods of ER stress. Expression of the HSP40 co-chaperone $P58^{IPK}$ is activated by ATF6 late in ER stress. $P58^{IPK}$ inhibits PERK by binding to its kinase domain [146,231]. Nck-1 is an eIF2α phosphatase, however, regulation of Nck-1 expression or activity in response to ER stress still has to be determined [232]. GADD34 and CreP regulate the phosphatase activity of protein phosphatase 1 (PP1) through their homologous *C*-terminal domains. PP1 accepts eIF2α as substrate. CreP is a constitutive regulator of PP1 [233], whereas expression of *GADD34* is induced by ATF4 late in ER stress [234–236]. The *N*-terminal 180 residues of GADD34 target the α isoform of PP1 to the ER [237]. Thus, activation of $P58^{IPK}$ and *GADD34* late in ER stress is a negative feedback mechanism that limits shut-off of translation through phosphorylation of eIF2α by PERK to the early phase of ER stress. It still has to be determined how and if the action of ATF6 on the $P58^{IPK}$ promoter is delayed, and how and if the action of GADD34 on PP1 is delayed.

6.4. Modulation of a network of bZIP transcription factors by the UPR

Yeast [214] and plants, e.g. *A. thaliana* and rice [27], lack ATF6 and PERK. In these organisms the UPR regulates the activity of one bZIP transcription factor, *HAC1* in yeast and its homologue in plants. The

absence of ATF6 and PERK from plants shows that these pathways are not required for multicellularity. In mammalian cells the situation is strikingly different and more complex. All three arms of the UPR, ATF6, IRE1, and PERK feed into a complex network of bZIP transcription factors. Extensive crosstalk exists at this level through the ability of bZIP proteins to regulate each others activity through formation of activating or repressing homo- and heterodimers (Table 3). In addition, the activity of a given bZIP dimer is influenced

Table 3
bZIP transcription factors in the mammalian UPR (compiled from references [238,307–311])

bZIP protein	Alias	Preferred DNA binding site	Interaction partners	Post-translational modification	Targets
ATF3	LRF-1, LRG-21, CRG-5, TI-241	ATF/CRE	**ATF2** (−/+), **ATF3** (−), **ATF4**, **ATF7**, **C/EBPγ**, **CHOP**, **CREBPA**, **c-Jun** (−/+ on ATF/CRE), **JunB** (−/+) on CRE, **JunD** (−/+ on CRE), hepatitis B virus X protein (−), HTLV-Tax, NF-κBp50		***ATF3*** (−), ***CHOP*** (−), E-selectin (−), *GADD34* (+), phosphoenolpyruvate carboxylase (−)
ATF4	C/ATF, CREB2, mTR67, TAXREB67	ATF/CRE, ARE (**ATF4-Nrf2**)	**ATF3**, **ATF7**, **B-ATF**, **C/EBPα**, **C/EBPβ**, **C/EBPγ**, **C/EBPδ**, **C/EBPε**, **CHOP**, **CREBPA**, **Fos**, **FosB**, **HLF**, **Jun**, **JunD**, **cMaf**, **NFE2L1**, **Nrf2** (+), **p21SNFT**, **ZF**, **Zip kinase**, CBP, TBP, TFIIB, RAP30 subunit of TFIIF, Tax, βTrCp (F-box protein) (−), Cdc34 (E2 ubiquitin ligase) (−)	PKA? (−)	***ATF3*** (+), ***CHOP*** (+), *GADD34* (+), amino acid transport (+), glutathione biosynthesis (+), resistance to oxidative stress (+)
ATF6α		ATF/CRE (+), ERSE-I (+), ERSE-II (+)	**ATF6α** (+), **ATF6β** (−)?, **XBP-1** (+), NF-Y/CBF (+), SRF (+), SREBP2 (−)	p38 P (+)	*ANF* (+), *BiP* (−/+), ***CHOP*** (+), ER chaperones, *Herp* (+) *P58^IPK^* (+), ***XBP-1*** (+)
ATF6β	CREB-RP G13	ERSE-I (−/+) ?	**ATF6α** (−)?, **ATF6β**, (−/+) ?, NF-Y/CBF (+)		*BiP* (−/+), ER chaperones (−/+)
CHOP	CHOP-10, Gadd153		**ATF2**, **ATF3**, **ATF4**, **ATF7**, **B-ATF**, **C/EBPα** (−), **C/EBPβ** (−), **C/EBPγ** (−/+), **C/EBPδ** (−), **C/EBPε** (−), **CHOP**?, **CREBPA**, **DBP**, **Fos**, **HLF**, **MafG**, **MafK**, **p21SNFT**, **TEF**, **LAP** (−)	p38 P	*DOC1* (carbonic anhydrase VI), *DOC4* (similar to *Drosophila melanogaster Tenm/Odz*), *DOC6*, apoptosis
Nrf2	NFE2LE	ARE (**Nrf2-ATF4**, **Nrf2-MafK**), MARE (heterodimer with small Maf)	**ATF4** (+), **c-Jun** (+), **JunB** (+), **JunD** (+), **MafG** (−), **MafK** (−/+), Keap (−)	ERK P (+), p38 P (+), PERK P (+), PKC P (+)	Oxidative stress, inducible genes, phase II xenobiotics response genes
XBP-1^u^	TREB5	CRE-like (−)	**ATF6**?		ER chaperones (−)
XBP-1^s^	TREB5	CRE-like (+)	**ATF6** (+)		ER chaperones (+)

ATF/CRE – TGACGT(C/A)(G/A), ARE – (G/C)TGAC/TN$_3$GC(A/G), ERSE-I – CCAAT-N$_9$-CCACG, ERSE-II – AATTGG-N-CCACG, Maf recognition element (MARE) – TGCTGAC(G)TCAGCA, and CRE-like – GATGACGTG(T/G)N$_3$(A/T)T. Activating and repressing activities are indicated with a "+" or "−", respectively. bZIP proteins are in bold. A question mark indicates conflicting data reported in the literature. Abbreviations: P – phosphorylation.

by the promoter element to which it is bound. A comprehensive leucine zipper protein array has identified most of the potentials for heterodimer formation [238]. Many of the homo- or heterodimeric bZIP protein complexes are either activators or competitive repressors of transcription. However, the identity of individual complexes formed in ER stressed cells, their activity, and the influence of individual promoter elements on their activity, are only incompletely known to date. Further, it is reasonable that the consequences of the UPR are adjusted to the needs of individual cell types, e.g. plasma cells or pancreatic β-cells, through cell-type dependent modulation of the bZIP protein network.

Information on the cooperation of the ATF6, IRE1, and PERK pathways has been collected in gene profiling studies. Cells deficient in XBP-1 and ATF6α were significantly impaired in induction of UPR target genes, suggesting at least partial redundancy in function for these bZIP transcription factors [149]. Interestingly, the ATF6 pathway is activated before the XBP-1 pathway, thus creating a time window in which the ATF6-mediated response tries to remedy the stress situation in the ER solely through chaperone induction. Upon prolonged stress the XBP-1 pathway then further augments chaperone induction and also up-regulates the capacity of ERAD [134].

7. Signal transduction by the UPR – apoptosis

Two major pathways control apoptosis – an intrinsic pathway responding to intracellular insults, e.g. DNA damage, and an extrinsic pathway responding to extracellular stimuli (Fig. 9). The extrinsic pathway is triggered by self-association of cell surface receptors, recruitment of caspases, mainly caspase-8, and initiation of a caspase cascade. The intrinsic pathway is controlled by a balance between proapoptotic BH3-only proteins, e.g. Bad, Bak, and Bax, and anti-apoptopic proteins, e.g. Bcl-2 proteins. The BH3-only proteins Bak and Bax act on the mitochondrial membrane resulting in release of cytochrome *c*. Cytochrome *c* then facilitates formation of a complex between Apaf-1 and procaspase-9, subsequent activation of a caspase cascade, and activation of the executioner caspase caspase-3 [34]. Apoptosis in response to ER stress is a response specific to metazoan cells. Topologically, the ER lumen is equivalent to the extracellular space. Thus, it is

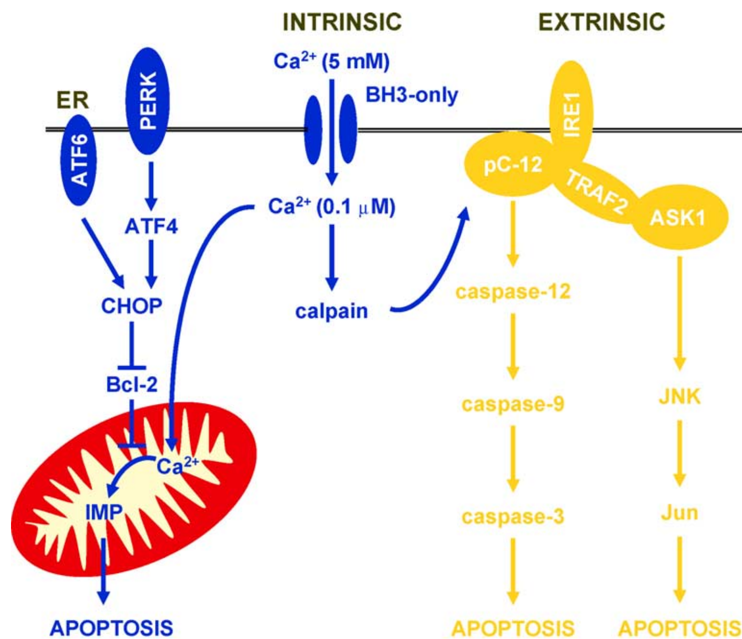

Fig. 9. Apoptotic pathways regulated by the UPR. Abbreviation: IMP – inner mitochondrial membrane potential.

not surprising that ER stress activates a combination of intrinsic and extrinsic apoptotic pathways.

7.1. Intrinsic pathways

In response to ER stress Bak and Bax undergo conformational changes and/or oligomerization at the ER membrane [239], resulting in Ca^{2+} release from the ER lumen by ER-localized Bak. Perturbation of Ca^{2+} pools activates calpain in the cytosol, which converts ER-localized procaspase-12 to caspase-12 [54]. Activated caspase-12 then initiates a caspase cascade through cleavage of procaspase-9 and -3 by caspase-9 [240,241]. Surprisingly, this pathway is independent of Apaf-1 [241] and mitochondrial cytochrome *c* release [240]. Ca^{2+} released from the ER is rapidly taken up by mitochondria [242], where it may lead to collapse of the inner membrane potential, and subsequent initiation of apoptosis. Overexpression of Bcl-X_L and viral mitochondrial inhibitor of apoptosis (vMIA) blocked depolarization of the inner mitochondrial membrane in response to ER stress [243]. The antiapoptotic effects of Bcl-2 [244,245] are suppressed by downregulation of *Bcl-2* transcription by the transcription factor *CHOP* [246]. Expression of *CHOP* in ER stress is up-regulated by ATF6 [229], and preferential synthesis of ATF4 [218,229] after phosphorylation of eIF2α by PERK [37,218]. Supporting the importance of these pathways in ER stress initiated apoptosis are the observations that *caspase-12*$^{-/-}$ cells [247] and *chop*$^{-/-}$ cells [246,248] are partially resistant to apoptosis.

Tumor necrosis factor receptor-associated factor 2 (TRAF2) promotes clustering of and is released from procaspase-12 upon ER stress, presumably by sequestering IRE1 [249]. Clustering of procaspase-12 was proposed to be a prerequisite for its subsequent activation [249]. Procaspase-12 expression is upregulated by ER stress [250] and caspase-12 can activate procaspase-12 in overexpression experiments [240]. Further, procaspase-12 is activated by caspase-7 after its relocation from the cytosol to the ER [250]. Activation of procaspase-12 by ER stress is inhibited by binding to the microsome-associated protein MAGE-3 to procaspase-12 [240]. It remains to be established how caspase-7, and association of procaspase-12 with MAGE-3 are regulated by ER stress. In addition, the tyrosine kinase c-Abl localizes to the ER and translocates to mitochondria upon ER stress, resulting in release of cytochrome *c*. ER stress induced apoptosis is attenuated in c-Abl deficient cells [251].

7.2. Extrinsic pathways

In response to ER stress IRE1 forms a heterotrimeric complex with TRAF2 and the apoptosis signal-regulating kinase 1 (ASK1) and activates c-Jun amino-terminal kinase [135] and cell death [252]. In addition, c-Jun *N*-terminal inhibitory kinase (JIK) associates with IRE1 and promotes phosphorylation and association of TRAF2 with IRE1 [249]. The utilization of both intrinsic and extrinsic pathways to execute apoptosis in response to insults to the ER indicates that not all insults are equal, and that the ER organelle has intra- and extracellular properties. Indeed, different insults on the ER cause apoptosis through preferential activation of extrinsic and intrinsic pathways [253].

8. Endoplasmic reticulum storage diseases

Diseases caused by malfunction of any aspect of the ER fall into one of the following classes (Table 4).

I. *Mutant cargo molecules*: Mutations affecting the fold of cargo molecules result in retention of the cargo in the ER. Four subclasses exist, depending on whether the mutants are functional or non-functional, and if they are susceptible to ERAD. Mutants not susceptible to ERAD can exhibit dominant properties, e.g. disruption of the formation of multimeric complexes, or disruption of the ER [14]:
   - I.A. The mutants are functional, retained in the ER because they do not pass quality-control criteria, and susceptible to ERAD. A prominent example is cystic fibrosis caused by a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) [254]. Therapeutic approaches to treat these diseases include expression of the wild-type (WT) protein, manipulation of the ER quality-control machinery, and the development of chemical chaperones tailored towards the mutant protein [255].
   - I.B. The mutants are non-functional, retained in the ER, but susceptible to ERAD. An

example is α_1-antitrypsin deficiency [256]. Therapeutic approaches include the expression of the WT protein and the development of chemical chaperones.

I.C. The mutants are functional, retained in the ER and not susceptible to ERAD. Diseases that would fall into this class are currently not known.

Table 4
Endoplasmic reticulum storage diseases

Type/disease	Class	Affected protein	Ref.
Cystic fibrosis	I.A.	CFTR	[254]
Diabetes mellitus	I.A.	Insulin receptor	[312]
Albinism/tyrosinase deficiency	I.B.	Tyrosinase	[15]
α_1-Antitrypsin deficiency without liver disease	I.B.	α_1-Antitrypsin	[15]
Cardiovascular diseases	I.B.	Lipoprotein(a)	[313]
Congenital hypothyroidism	I.B.	Thyroglobulin	[14,15]
	I.B.	Thyroid peroxidase	[314–316]
	I.B.	Thyroxine binding globulin	[317,318]
Familial hyperchylomicronemia	I.B.	Lipoprotein lipase	[14]
Familial isolated hypoparathyroidism	I.B.	Preproparathyroid hormone	[319]
Global polyendocrinopathy associated with obesity and infertility (*fat/fat* mouse)	I.B.	Carboxypeptidase E	[320,321]
Hemophilia A	I.B.	Factor VIII	[322]
Hypercholesterolemia	I.B.	LDL receptor	[14]
Laron dwarfism	I.B.	Growth hormone receptor	[323]
Diabetes insipidus	I.B./I.D.	Arginine vasopressin (AVP)	[14,15,257]
	I.B.	AVP receptor 2	[14,15]
	I.B.	aquaporin-2	[14,15]
Obesity	I.B.	Prohormone convertase 1	[15]
Osteogenesis imperfecta	I.B./I.D.	Type I procollagen	[14,257]
	I.B.	Decorin	[324]
Parkinsonism, autosomal recessive juvenile	I.B.	Pae I receptor	[325]
Protein C deficiency	I.B.	Protein C	[15]
Spondyloepiphyseal dysplasia due to hypochondrogenesis	I.B.	Type II procollagen	[326]
von Willebrand disease	I.B.	von Willebrand factor	[327]
Spondyloperipheral dysplasia	I.B./I.D.	Type II collagen	[328]
β-Amyloid toxicity	I.D.	β-Amyloid	[247]
α_1-Antitrypsin deficiency with liver disease	I.D.	α_1-Antitrypsin	[256]
Charcot-Marie-Tooth disease	I.D.	Peripheral myelin protein PMP22	[257]
Diabetes mellitus in the Akita mouse	I.D.	Insulin 2	[258]
Pelizaeus-Merzbacher leukodystrophy	I.D.	Proteolipid protein	[257]
Pre-senile dementia/myoclonus	I.D.	Neuroserpin	[257]
Abetalipoproteinemia	II.	Apolipoprotein B/microsomal triglyceride transfer protein	[14]
Combined coagulation factors V and VIII deficiency	II.	Factor V, factor VIII/LMAN1	[260]
	II.	Factor V, factor VIII/MCFD2	[261]
Bipolar disorder	III.	XBP-1	[264]
Colitis (mouse model)	III.	IRE1β	[194]
Diabetes mellitus (mouse model)	III.	PERK	[263]
Hypoglycemia (mouse model)	III.	eIF2α	[37]
Wollcott-Rallison syndrome	III.	PERK	[262]
Polyglutamine diseases (dentatorubral-pallidoluysian atrophy, Huntington's disease, spinobulbar muscular atrophy, spinocerebellar ataxia)	IV.	Proteasome	[252]

Classes are defined in the text. For class II diseases the WT proteins whose loss of expression is the primary cause for the disease are listed first, followed by the mutated protein responsible for the loss of expression of the aforementioned proteins.

I.D. The mutants are non-functional, retained in the ER and not susceptible to ERAD. Loss-of-function of the protein in its usual cellular compartment or the extracellular space is eclipsed by disruption of the ER and subsequent initiation of apoptosis [257]. These are usually dominant diseases and associated with increased ER-chaperone levels. Examples are β-amyloid toxicity [247] and autosomal dominant diabetes in the Akita mouse [258,259].

II. *A defective ER folding and transport machinery prevents wild-type proteins from reaching their destination*: These can be very specific defects affecting just a single protein due to the fact that many specific client–chaperone pairs have evolved (Table 2). A prominent example is a combined factors V and VIII deficiency in patients with mutations in the LMAN1–MCFD2 lectin complex [260,261] required for the transport of factors V and VIII from the ER to the Golgi complex.

III. *Defective UPR signaling*: These diseases are caused by loss of one arm of the UPR, e.g. through mutation of a proximal or downstream gene involved in the UPR. Early-infancy insulin-dependent diabetes (Wolcott-Rallison syndrome) is caused by a kinase defective mutation in PERK [262]. In addition, $Perk^{-/-}$ mice develop diabetes mellitus [263]. A mouse model in which a Ser51Ala mutation in eIF2α that abolishes phosphorylation of eIF2α by PERK, and other eIF2α kinases, displays a pancreatic β cell defect and defective gluconeogenesis leading to lethal hypoglycemia [37]. Mutations in the *XBP-1* promoter which affect its autostimulation are associated with bipolar disorder [264].

IV. *Inhibition of adaptive responses regulated by the UPR*: Polyglutamine repeats cause proteasomal dysfunction, thus eliminating one arm of the UPR and subsequent activation of apoptosis through ASK1 [252]. Examples for polyglutamine diseases are neurodegenerative diseases, e.g. Huntington's disease, spinobulbar muscular atrophy, dentatorubral-pallidoluysian atrophy, and six spinocerebellar ataxias (SCAs 1, 2, 6, 7, 17, and SCA3/Machado-Joseph disease) [252]. Lastly, the same concept, inhibition of proteasomal function with small inhibitors, e.g. the dipeptidyl boronic acid proteasome inhibitor bortezomib, can be employed as a therapeutic concept for certain cancers derived from secretory cell types, e.g. myelomas and lymphomas derived from B-cells [265].

9. The UPR in "unstressed" cells

There is nothing such as a totally unstressed cell. There is only a minimal level of stress [1]. Indeed, yeast defective in the UPR and ERAD are synthetic lethal [31,32]. The level of *HAC1* mRNA splicing in yeast in exponentially growing cultures ranges from 3 to 30% [35,36]. Both observations suggest that an unfolded protein load sufficient to activate the UPR exists in otherwise "unstressed" cells. Minor changes in the unfolded protein load or stress level should be an informative tool for a cell to access its overall metabolic state (Fig. 10). This is illustrated by the observation that, in yeast, the level of *HAC1* splicing in exponentially growing cells correlates with the quality of the carbon source. It is low on preferred, fermentable carbon sources, e.g. D-glucose, high on non-fermentable C-sources, e.g. acetate or ethanol, and intermediate on disaccharides such as D-maltose [35,36]. Furthermore, *HAC1*-splicing is also regulated by nitrogen. In nitrogen-rich conditions *HAC1* mRNA is processed, whereas *HAC1* splicing stops very rapidly after induction of complete nitrogen starvation [35]. This information is integrated into decision making of yeast cells in response to their nutritional status (Fig. 10A). Diploid budding yeast enter one of two developmental programs in response to nitrogen starvation, pseudohyphal growth or sporulation [266]. Pseudohyphal growth is a directional growth form of yeast allowing this organism to forage for nutrients during starvation [267]. Sporulation yields a long-lived and stress-resistant metabolically quiescent ascus containing four haploid spores [268]. Genetic and pharmacological experiments with drugs that disrupt protein folding in the ER, e.g. tunicamycin, demonstrated that $Hac1^{i}p$ is a negative regulator of both nitrogen starvation induced differentiation programs (Fig. 10A) [35]. Thus, increased synthesis of $Hac1^{i}p$ in nitrogen-rich conditions represses nitrogen starvation responses in yeast. In mammals, activation of ATF4 through the PERK pathway, and subsequent activation of amino acid biosynthetic genes by ATF4 may constitute a feedback loop to anticipate the loss of amino acids from the

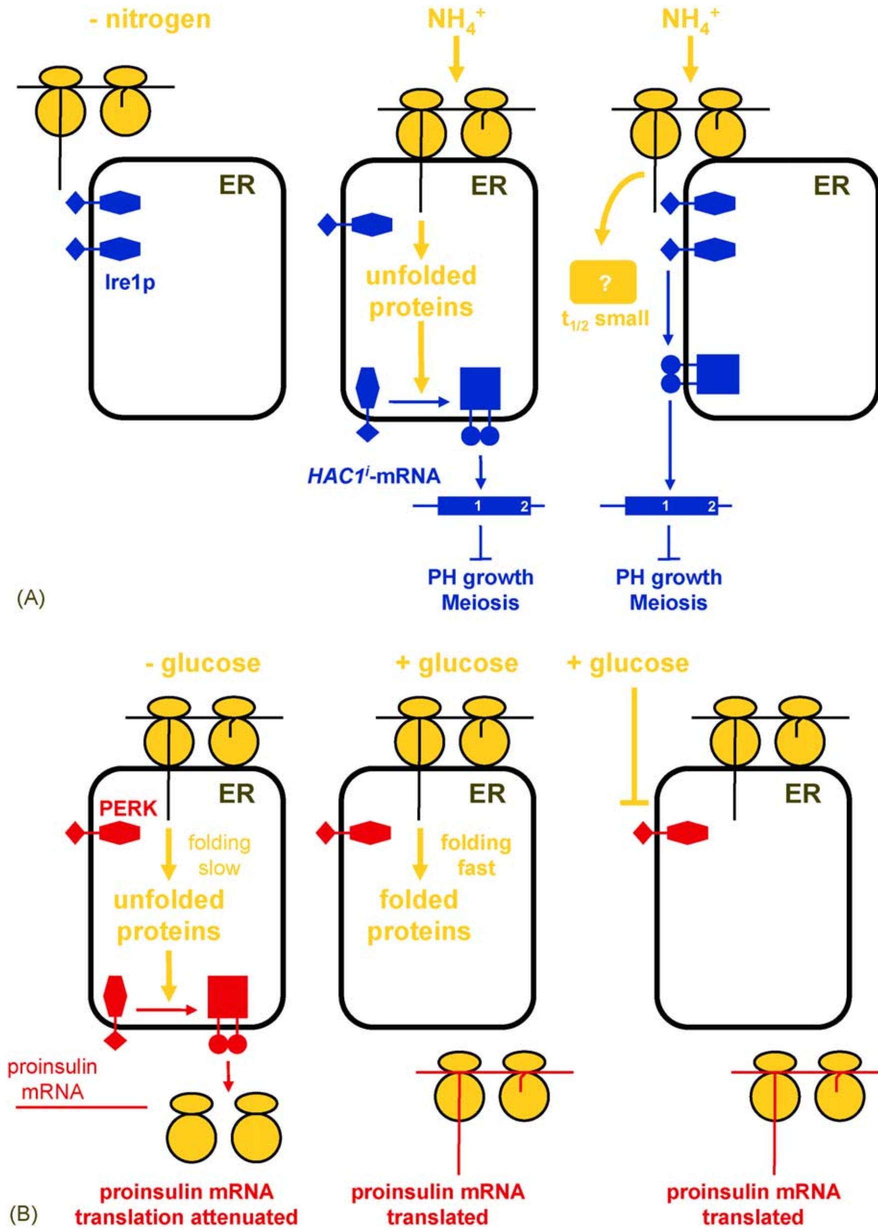

Fig. 10. The balance between ER protein folding capacity and folding need as a sensor for the nutritional state of the cell. (A) Nutrient sensing in yeast and (B) glucose sensing in β-cells. Two models (center and right) can explain how nutrients regulate *HAC1* mRNA splicing (A) or PERK (B). In the first model (center), high nitrogen concentrations (center) are responsible for an increased influx of nascent unfolded polypeptide chains into the ER and activation of Ire1p (A). High glucose concentrations stimulate protein folding through increased synthesis of ATP and oligosaccharides, resulting in inactivation of PERK and stimulation of protein synthesis (B). In the second model (right) the activity of Ire1p and PERK is directly modulated in response to nutrient availability. Abbreviation: PH – pseudohyphal.

cell through secretion [38]. Thus, a role for the UPR in regulation of nitrogen metabolism and nitrogen starvation responses is conserved from yeast to mammals.

The same concept, monitoring small fluctuations in the unfolded protein load as an indicator of the metabolic state of the cell, was proposed to work in glucose-regulated synthesis of proinsulin in pancreatic β-cells [37]. In low glucose, protein folding in the ER is inefficient, since ATP-generation from glucose and synthesis of the core oligosaccharide are impaired [269]. This activates the UPR and PERK shuts-off translation, including translation of proinsulin mRNA. When glucose levels rise ATP generation and glycosylation become more efficient resulting in inactivation of the UPR, resumption of translation, again including proinsulin mRNA. Thus, the UPR would contribute to glucose sensing in pancreatic β-cells (Fig. 10B) [37]. β-cells may be predisposed to this sensing mechanism due to increased levels of IRE1 [129] and PERK [215], which allow for the detection of smaller fluctuations in the free BiP pool by IRE1 and PERK. Consistent with this model is the β cell defect observed in Ser51Ala/Ser51Ala eIF2α mice [37] and the observation that *Perk*$^{-/-}$ mice develop diabetes mellitus [263]. Both observations show that glucose sensing by β-cells is perturbated in these animals. However, proinsulin translation in response to glucose in islets isolated from WT PERK and *Perk*$^{-/-}$ mice was very similar in vitro [263]. Thus, it still needs to be determined if PERK activity responds to fluctuations in the glucose level. Taken together, these observations in yeast and mammals uncover a second physiological function for the UPR. In addition to keeping the biosynthetic burden and biosynthetic capacity of the ER in line, the UPR also monitors the biosynthetic activity of the ER to inform the cell about its overall metabolic state. This information, provided by the UPR, is then integrated into decision making to changes in the nutritional environment of the cell, e.g. severe starvation in yeast.

10. Future directions

The regulation of signaling pathways and mechanisms of signal transduction from the ER to the nucleus have been characterized in considerable detail. However, only limited information and even less understanding is available for how the signal generated by the UPR remodels the network of bZIP transcription factors downstream of its proximal ER membrane resident signal transducers. An interesting aspect here is that cell-type specific bZIP transcription factor expression patterns may modulate downstream signaling events in the UPR to adjust these to the specific needs of individual cell types. Further, in the metazoan UPR all three arms of the UPR transduce protective and apoptotic signals. Are both signals transduced at the same time or do slightly different stimuli, or cell-type specific modulations of signal generation at the ER membrane, generate different signals? How would signaling specificity in the UPR then be achieved, maintained, and regulated? Finally, UPR signaling has for a long time been thought of to be only responsible to balance the folding capacity of the ER with its biosynthetic load. Recent observations show that signaling by the UPR extends beyond this limited scope [35,37]. In addition to its well recognized function the UPR also monitors the biosynthetic activity of the ER as an indicator for the overall metabolic state of the cell. Thus, UPR signaling is integrated into the regulation of physiological events not previously associated with the ER, e.g. the regulation of starvation and differentiation responses [35,37]. Here, the next critical step clearly is to identify the points of signal integration, and to show that loss-of-function mutations at this point abolish regulation of these responses by the UPR.

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