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Protein misfolding and ER stress

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Abstract

The endoplasmic reticulum (ER) is a major site of protein synthesis, most strikingly in the specialised secretory cells of metazoans, which can produce their own weight in proteins daily. Cells possess a diverse machinery to ensure correct folding, assembly and secretion of proteins from the ER. When this machinery is overwhelmed, the cell is said to experience ER stress, a result of the accumulation of unfolded or misfolded proteins in the lumen of the organelle. Here we discuss the causes of ER stress and the mechanisms by which cells elicit a response, with an emphasis on recent discoveries.

Introduction

The biogenesis of secretory and cell surface proteins begins at the endoplasmic reticulum (ER). Newly synthesized polypeptides enter the ER via a proteinaceous pore called the translocon (33, 46, 55, 151). ER-resident enzymes guide protein folding towards the native state by chaperoning and post-translational modification. Proteins that fail to adopt their native conformation are retained in the ER and eventually targeted for ER-associated degradation (ERAD) (Figure 1). The processes governing protein production, quality control and degradation help to maintain protein homeostasis (proteostasis).

Efficient protein folding within the ER requires tight matching of the load of new proteins entering the organelle with the capacity of its folding machinery. When the load of client proteins outweighs the capacity, the cell is said to experience 'ER stress', which represents a threat to accumulate unfolded aggregation-prone species. In the face of ER stress, an unfolded protein response (UPR) is employed to restore proteostasis. In metazoans, three UPR signal transducers, IRE1, PERK and ATF6, govern distinct but overlapping transcriptional programs to increase folding capacity, while measures to attenuate the rate of secretory protein translation simultaneously reduce the protein load (Figure 1). In this review we will highlight recent advances in the field of ER stress.

Protein folding in the ER

Whilst some denatured proteins can refold *in vitro* without auxiliary factors, in the crowded molecular environment of the cell a folding machinery is required.

Protein folding in the ER is supported by a variety of chaperones and folding factors. These include enzymes that facilitate post-translational modifications, such as N-linked glycosylation and disulfide bond formation (141, 164) (14).

Chaperones of the heat shock protein 70 (HSP70) and HSP90 families are present in abundance within the ER. BiP (also known as GRP78) is the major HSP70, binding to short stretches of hydrophobic residues exposed by non-native proteins. It functions to reduce the effective concentration of aggregation prone sequences. The process of aggregation is highly concentration-dependent and so by shielding aggregation prone sequences BiP discourages aggregation and promotes native folding, i.e. its so-called holdase function. In order to allow the progression of folding, BiP cycles through release and rebinding to its substrates, whilst consuming ATP in the process. With each release cycle the client protein has the opportunity to fold. In this sense, folding competes with chaperone binding, both processes offering thermodynamic stability. Whether BiP and other HSP70s actively catalyze the folding process (a foldase function) remains unclear.

Two classes of auxiliary proteins contribute to the HSP70 chaperone cycle. J-domain proteins (J-DP), which contain a conserved domain from the ancestral DnaJ co-chaperone of bacteria, play two major roles in the HSP70 chaperone cycle (68). Firstly, some J-DPs can interact with unfolded substrates, which they deliver to the ATP-bound, substrate-receptive conformation of the HSP70. Secondly, interaction with both substrate and J-domain induces the HSP70 to hydrolyze its bound ATP to promote a tight substrate binding conformation.

This latter function is conserved amongst all J-DPs and is essential for HSP70 chaperoning. Following J-DP dissociation, a nucleotide exchange factor (NEF) associates with the HSP70 to facilitate ADP release. Subsequent rebinding of ATP induces substrate release and a return to the 'open' substrate-receptive conformation (94). In humans there are at least 41 J-DPs, of which 7 are thought to have J-domains in the ER lumen (68). Different J-DPs display a variety of substrate specificities and interact with their partner HSP70 through specific contact residues (159) and both the identity of the interacting J-DP and NEF determine HSP70 chaperone activity *in vitro* (128). It has been estimated that BiP clients may constitute up to 50% of protein within the ER lumen (34) and consequently BiP is essential for embryonic growth (139). In addition to BiP, non-classical HSP70s, such as GRP170, are also present within the ER, but are less well characterised. While GRP170 binds to unfolded client proteins in a classical chaperone fashion, it lacks the expected requirement for nucleotide binding (123). This may relate to a large unstructured loop in its putative substrate binding site that may destabilize substrate interaction and facilitate client exchange (4).

Following their interaction with HSP70s, a subset of ER client proteins requires interaction with an HSP90 called GRP94. Within the cytosol, HSP90 client proteins are frequently kinases involved in mitogenic signalling and so are currently being targeted by novel anti-cancer agents (92, 99, 127, 146). Within the ER, the clients of GRP94 are less easy to classify including, for example, insulin-like growth factors, α_1-antitrypsin and apoprotein B (91). However, the existing inhibitors of HSP90 are relatively non-selective, also inhibiting GRP94,

and so it is unclear to what extent their effects are mediated by altered protein folding within the ER.

The ability to increase the availability of chaperones to match the demand of client proteins is critical for an effective UPR. Classically, this is achieved by transcriptional reprogramming of the cell triggered during ER stress (29, 52, 172) (Figure 1). Whilst it is clear that insufficient chaperone activity would be toxic, it is less well appreciated that excessive chaperoning would have deleterious effects through limiting protein secretion (39, 169). Consequently, the mechanisms by which chaperone availability is reduced with declining stress have, until recently, been neglected. The chaperone BiP has a half-life of between 6 and 48 hours and does not appear to be rapidly degraded when ER stress subsides (56, 64). Instead, its activity has recently been shown to be regulated by ADP-ribosylation of its substrate-binding domain (17, 56, 74, 80). This reversible modification reduces interaction with client proteins and thus enables rapid down-regulation of chaperone activity (19) (Figure 2). To date, the ADP-ribosylation of BiP has only been observed in vertebrates, which may reflect the complex secretory requirements and longer lifespans of higher organisms.

Efficient protein folding during ER stress is further promoted by increasing the volume of the ER (7). Accordingly, components of the membrane lipid biogenesis machinery are induced as targets of the UPR (28). In yeast, this process requires the transcription factors Ino2 and Ino4, which induce expression of lipid synthetic enzymes in response to ER stress, causing the expansion of the ER membrane (138). In mammalian cells, overexpression of

spliced XBP1 is sufficient to promote ER expansion and leads to increased production of the membrane precursor lipids phosphotidylcholine and phosphotidylethanolamine (140, 149). ATF6α signalling is thought to elicit a distinct but complementary mechanism involving up-regulation of choline kinase to promote production of phosphatidylcholine via the cytidine diphosphocholine pathway (13, 88). In addition, maturation of sterol regulatory element binding protein 1 (SREBP1), a transcription factor that controls lipogenic enzyme expression, has been found to be compromised in *Perk* knockout fibroblasts, and is completely blocked by mutating eIF2α to a non-phosphorylatable form (10).

Ablation of the lipid synthetic response to ER stress in yeast can be compensated for by up-regulation of chaperones, but equally, expansion of the ER can protect cells unable to induce ER chaperones, suggesting that reduced protein crowding is sufficient to relieve ER stress (138). In pancreatic β-cells the volume of the rough ER can increase by 3-fold in response to glucose infusion and the resultant increased synthesis of insulin (150). It is likely that reduced protein crowding may promote correct folding over aggregation simply by reducing client concentration within the lumen. The morphology of the newly synthesised ER membranes seems unimportant, since in the yeast model the promotion of lamellar or reticular structures does not impact on the benefits of membrane expansion (138). Interestingly, during the expansion of the ER, the UPR simultaneously induces a number of autophagy genes which promote autophagic degradation of the ER (7). While this may appear counter intuitive, this may assist in the removal of luminal aggregates of misfolded proteins

Oxidative protein folding

A characteristic feature of protein folding in the ER is the ability to generate disulfide bonds. These bonds promote proper folding and stabilize native protein conformations; consequently, perturbations of oxidative folding leads to ER stress (41). The formation of disulfide bonds is driven by an oxidative folding machinery and in contrast to the reducing environment of the cytosol, is supported by an oxidizing glutathione buffer system within the ER lumen (66). For many years, the ER oxidase 1 proteins, ERO1α and the pancreatic isoform ERO1β, were presumed to drive the generation of disulfide bonds, since yeast Ero1p is an essential protein (41, 126). ERO1 forms an inter-molecular disulfide bond with protein disulfide isomerase (PDI) with molecular oxygen as the ultimate electron acceptor. This bond is rearranged and subsequently used to oxidized ER client proteins (42). The pancreatic ß-cell is dependent upon efficient oxidative protein folding, since its primary secretory protein insulin possesses three disulfide bonds per molecule. Unexpectedly, deletion of both *Ero1α* and *Ero1β* within the whole animal causes only a mild diabetic phenotype (176). This observation led to the identification of peroxiredoxin 4 (PRDX4) as an alternative source of disulfide bonds; PRDX4 utilizes hydrogen peroxide as the source of oxidizing equivalents (47, 152, 178). Remarkably, when a mouse lacking PRDX4 and both ERO1 isoforms was generated, it was able to form disulfide bonds albeit with abnormalities of collagen folding (177). This result points to the existence of yet more unidentified components of the oxidizing machinery within the mammalian ER.

In mammalian cells it appears that the redox balance of the ER is well buffered during physiological fluctuations in protein load (2), although fluctuations in ER calcium concentration can cause dramatic changes in the redox state (2, 9). Since the ER is the major store for calcium within the cell and many signalling and metabolic pathways can trigger calcium release, such a link between ER calcium and redox state may have far reaching implications. The inositol 1,4,5 triphosphate receptors (IP3R) and ryanodine receptor (RyR) are ER membrane protein channels that control the efflux of calcium ions from the ER in response to physiological stimuli; this efflux of calcium ions functions as a second messenger. The activity of both channels can be modulated by protein thiol redox state, suggesting the intriguing possibility that fluctuations in ER redox could potentially help to define the intensity and duration of cellular calcium signalling (1, 40, 69, 98). Further research in this area is eagerly awaited.

Pathology of ER protein folding

Perturbations of ER protein folding manifest as disease through a variety of mechanisms. In their simplest form, such perturbations arise from loss-of-function mutations of the folding machinery or toxic gain-of-function mutations of the client protein. For example, mutants of the ER client protein collagen or of its selective ER chaperone HSP47 each lead to the connective tissue disorder osteogenesis imperfecta (22). Similarly, failure to fold the epithelial chloride conductance CFTR within the ER underlies the human disease cystic fibrosis (165). Many of the pathogenic mutants of CFTR fail to fold sufficiently well to exit the ER and instead are degraded (20, 165). But not all mutated ER proteins

205 can be degraded as efficiently. The P23H and K296E mutants of rhodopsin are
206 extracted from the ER but subsequently form ubiquitinated aggregates in the
207 cytosol (135). These may inhibit normal functioning of the proteasome leading
208 to cell death, which has been suggested as the pathogenic mechanism for P23H
209 autosomal dominant retinitis pigmentosa (67). Accordingly, in P23H-expressing
210 rats, viral delivery of BiP reduced UPR signalling and photoreceptor apoptosis
211 without affecting rhodopsin localisation, supporting a for ER dysfunction in the
212 pathogenesis of the disease (45). However, even without genetic defects, protein
213 translation is sufficiently error-prone to permit a missense mutation of the
214 protein every 1,000 to 10,000 amino acid, resulting in defects in between 4% to
215 36% of all new proteins made (15, 108). This can be tolerated if these proteins
216 can be degraded, but when the load is excessive, as occurs during ß-cell
217 exhaustion in type II diabetes, cell death can ensue (65, 75).
218
219 In contrast to diseases caused by aberrant protein folding and ER stress, many
220 patients who are homozygous for the Z variant of α_1-antitrypsin accumulate large
221 quantities of ordered protein polymers within the lumen of their hepatocytes
222 without high levels of ER stress (59, 60, 114). These patients typically develop
223 pulmonary emphysema at an accelerated rate owing to the loss of circulating α_1-
224 antitrypsin, which normally functions as an inhibitor of neutrophil elastase.
225 When unopposed, elastase degrades the extracellular matrix causing alveolar
226 destruction (95). Moreover, the failure of airway epithelial cells to secrete α_1-
227 antitrypsin appears also to deprive them of an important autocrine anti-
228 inflammatory signal, leading to elevated ERK signalling and cytocine secretion
229 (158). In addition, the accumulation of Z α_1-antitrypsin as large protein

polymers within the ER of hepatocytes, while failing to trigger ER stress directly, increases the sensitivity of these cells to activation of the UPR when faced with a second insult (59, 60, 114). This may explain the apparently stochastic nature of liver failure observed in some paediatric cases of α_1-antitrypsin.

Quality control and ER associated Degradation (ERAD)

In addition to expanding the folding capacity of the ER, the UPR increases the cell's ability to dispose of terminally misfolded proteins (49, 112). One mechanism takes advantage of the N-linked glycosylation found on many ER client proteins (37). Calnexin and calreticulin are lectin-like ER chaperones that interact periodically with glycosylated clients. This is governed by the removal and re-addition of glucose moieties to the glycans of their substrates. Clients that continue to cycle for prolonged periods without achieving their native state are eventually targeted for ERAD. This involves the time-dependent trimming of a mannose residue from the glycan, which is monitored by ER degradation enhancer alpha-mannosidase-like protein 1 (EDEM1) (27, 63). The difference in the kinetics of glucose vs mannose trimming is believed to determine the time allotted to folding a protein before it is deemed terminally misfolded. Data suggest that variation in the efficiency of this machinery may impact on the pathogenesis of protein folding related disease states. A single nucleotide polymorphism was identified in ER mannosidase I (ERManI), an enzyme responsible for mannose trimming in ERAD (3), was suggested to increase the likelihood of fulminant liver disease in children homozygous for Z α_1-antitrypsin (121).

Both calnexin and calreticulin associate with ERp57, a PDI family member that isomerizes non-native disulfides during the folding process. Recent work in *C. elegans* suggests that for many substrates N-glycosylation may be rate-limiting in their quality control (32). Gain of function mutants in the *gfat*-1 gene, a component of the hexosamine synthetic pathway required for production of N-linked glycans, enhance ERAD, reduce ER stress, and extend lifespan. Similarly, supplementation of growth medium with the glycan precursor N-acetylglucosamine had similar beneficial effects, implicating glycan-dependent quality control as a crucial determinant of proteostasis and aging (32).

Proteins destined for ERAD are removed from the lumen of the ER in a process that results in their ubiquitination by membrane-associated E3 ligases that include HRD1 and GP78. The substrate is ubiquitinated on the cytosolic side of the ER membrane before its complete extraction and proteasomal degradation (18, 23). Many components have been implicated in the retrotranslocation and ubiquitination events with evidence for significant substrate specificity (25). Disulfide bonded proteins require the reductase activity of the PDI family member ERdj5 prior to their retrotranslocation (156). This enzyme is not dedicated solely to ERAD as it also facilitates native disulfide bond formation in low-density lipoprotein receptor (LDLR) (110). In a manner analogous to the interaction between ERp57 and the lectin-chaperones, ERdj5 requires BiP for substrate selection. Thus, in addition to its role in preventing aggregation, BiP delivers terminally misfolded proteins to the ERAD machinery (54). Unlike the

lectin chaperones, however, there is no obvious mechanism by which BiP can distinguish partially folded intermediates from terminally misfolded species.

The ERAD of a number of non-glycosylated substrates of BiP has been shown also to utilise the E3-ligasse HRD1 and HERP, which interacts both with the proteasome and ubiquitinated ERAD substrates (113). Although glycosylated mutants of α_1-antitrypsin have been suggested not to require HERP during ERAD (113), it has been suggested more recently that HERP is required for the degradation of both glycosylated and non-glycosylated transmembrane proteins (79). Similarly, EDEM1 was shown recently to interact with non-glycosylated ERAD substrates as well as misfolded glycoproteins (143). This suggests significant overlap between glycan-dependent and independent ERAD pathways.

ER quality control and ERAD ensure that newly synthesised proteins are afforded sufficient opportunity to achieve their native conformation whilst preventing the accumulation of unstable folding intermediates. Indeed, the removal of unfolded proteins by ERAD appears to be essential for maintaining the synthetic output of professional secretory cells (38). Processes that cause a dramatic increase in secretion, for example glucose stimulation of insulin production or differentiation of B lymphocytes in to more secretory plasma cells, can briefly overwhelm quality control leading to activation of the UPR as part of normal cellular homeostasis (51, 129). Conversely, destabilising mutations and stressful insults can saturate ERAD leading to pathological ER stress and cell death (104, 116, 148). Thus, during short-term 'physiological' stress, UPR signalling acts to promote efficient protein folding though enlargement of the ER,

increased synthesis of chaperones and more effective ERAD, but during chronic ER stress, through mechanisms that are yet to be fully understood, the UPR can activate cell death pathways and contribute to pathogenesis, The mechanisms by which cells detect and respond to ER stress will now be discussed.

ER stress signal transduction

IRE1

IRE1 governs the most evolutionary conserved arm of the UPR, being found in all eukaryotes. In the absence of stress, IRE1 is thought to be held inactive by the binding of BiP to its luminal domain. Current models suggest that during ER stress, BiP is titrated away by increased levels of unfolded proteins leading to UPR signalling (8, 142). However, structural studies have suggested that the luminal domain of yeast Ire1p might be capable of interacting directly with unfolded polypeptides by forming a peptide-binding groove reminiscent of that found in MHC class I molecules (30), although the equivalent structure of mammalian IRE1 does not appear to support this model (175). More recent work suggests that these models are not mutually exclusive, whereby BiP binding desensitizes Ire1 to activation by the direct binding of unfolded proteins in the absence of stress. During stress, BiP dissociation then lowers the energy barrier of Ire1 activation by unfolded proteins (125).

Detection of ER stress by the luminal domain of Ire1p leads to trans-autophosphorylation of its cytosolic kinase domain, promoting back-to-back dimer stabilising salt bridges (72, 78). The resulting conformational changes

lead to a rearrangement of the RNase domain of Ire1p to promote binding and cleavage of a single mRNA substrate, XBP1 in mammals or HAC1 in yeast, to initiate an unconventional splicing event. For XBP1, splicing causes a shift in reading frame resulting in a change in the sequence of the C-terminal portion of the protein. For HAC1, by contrast, splicing leads to the removal of a translational repression structure (131). When spliced mRNA is translated and an active transcription factor is generated, downstream targets mediate increased ER folding capacity, membrane biogenesis and autophagy (76, 107).

In recent years, an additional role for the endonuclease domain of IRE1 has emerged in the form of ‘regulated IRE1 dependent decay’ (RIDD) (61, 62, 93). Under conditions of ER stress, IRE1 cleaves a subset of mRNAs. This appears to be a non-random process, with a degree of transcript sequence specificity (93, 109) and preferential degradation of mRNAs localised to the ER membrane (62, 109). In this manner, the cell can reduce the number of mRNA transcripts being translated at the surface of ER and thus reduce the rate of secretory protein biosynthesis. Engineered variants of Ire1 such as the Shokat mutant have been designed to allow activation of XBP1 splicing by the addition of an exogenous ligand (122). Remarkably, activation of XBP1-directed nuclease activity in this way appears to be insufficient to trigger RIDD. Instead, *bona fide* ER stress was required to enable IRE1 to degrade mRNAs, suggesting a more complex mechanism for the activation of IRE1 than was first anticipated (61). Moreover, evidence suggests that basal RIDD may also occur under conditions in which XBP1 splicing is not activated (recently reviewed in (93)). In addition to back-to-back dimers, higher order oligomers of IRE1 have also been observed in both

yeast and mammalian cells during ER stress (72, 82). It is tempting to speculate that the different degrees of oligomerisation, for example single dimers vs higher order oligomers, of active IRE1 might differ in their functional output, but further evidence is required.

Recent studies have begun to clarify the physiological and pathological relevance of RIDD. If unchecked, RIDD can impair the synthesis of secreted and membrane proteins that impact on immune function. Deletion of XBP1 in murine B□lymphocytes leads to hyperactivation of IRE1 and degradation of mRNA encoding μ heavy chains. This appears to be mediated by RIDD, since the deficiency of IgM can be rescued partially by also ablating IRE1 (5). The incompleteness of this rescue reflects the requirement for XBP1 in the efficient maturation of plasma cells. Silencing of either XBP1 or IRE1 impairs the production of immunoglobin through de-commitment from full plasma cell differentiation (81). Interestingly, loss of XBP1 and its target genes have been observed in bortezomib-resistant myeloma cells (81). Bortezomib is chemotherapeutic agent that kills myeloma cells by blocking ERAD and thereby worsening ER stress. The loss of XBP1 in this setting may protect these cells by reverting them to a less secretory, and consequently ER stress-resistant, phenotype.

RIDD appears to play a role in innate immunity, specifically through the initiation of inflammation. Binding of cholera toxin to the luminal portion of IRE1 induces an inflammatory response in a manner dependent upon the nuclease activity of IRE1 but independent of XBP1 (21). Fragments of cleaved

mRNA generated by RIDD appear to activate RIG-I, which in turn induces effectors of the innate immune response. Since RNase-L activates RIG-I by the production of chemically similar fragments of mRNA during viral infection, this may hint at a common evolutionary ancestry for these responses.

The RIDD component of the UPR has been implicated in modulating lipid metabolism. Ablation of XBP1 in the liver was previously shown to impair hepatic lipid metabolism leading to hypocholesterolemia and hypotriglyceridemia (77). Although it was initially thought that ER stress due to the deficiency of XBP1 might impair the secretion of very low-density lipoprotein (VLDL), it is now believed that increased RIDD observed in XBP1-ablated cells degrades transcripts encoding components of lipid metabolic pathways (145). Indeed, ablation of XBP1 in leptin-deficient *ob/ob* obese mice reduces the accumulation of hepatic triglycerides, while XBP1-deficient animals are protected against developing hypercholesterolemia when fed a high fat diet (145).

The failure to observe RIDD in the budding yeast *S. cerevisiae* led to a presumption that RIDD was a metazoan innovation, but now similar activities have been reported in plants and fission yeast. In plants, IRE1 cleaves mRNA encoding bZIP60 to generate an active transcription factor and initiate the plant UPR (102). But when ER stress is induced in bZIP60 deficient *Arabidopsis,* the degradation of secretory protein mRNAs has been observed in an IRE1-dependent manner (97). Moreover, in fission yeast IRE1 initiates an ER stress response related to RIDD. The UPR in *S. pombe* has long presented a paradox

because although it possesses IRE1, no mRNA substrate encoding a transcription factor could be detected. Recently, RIDD-like IRE1-dependent reduction of secretory pathway mRNAs was observed during ER stress in fission yeast (71). Strikingly, IRE1 was observed to cleave the mRNA of *Bip1,* the major ER HSP70 of this organism, at a conserved RIDD target sequence; however, unlike all other known RIDD substrates, which are destabilized when cleaved by IRE1, the *Bip1* transcript was stabilized leading to its increased expression. Thus, in *S. pombe* this UPR target mRNA is regulated post-transcriptionally. It is tempting to speculate that such RIDD-like processing may be an ancient mechanism acquired from the ancestral IRE1, but it was subverted in budding yeast to process HAC1 transcripts and persists both as degradation and selective processing in multicellular organisms.

PERK (protein kinase RNA-like endoplasmic reticulum kinase)

PERK regulates a younger arm of the UPR, which evolved in metazoans. Whilst simple eukaryotes maintain ER proteostasis through transcriptional programming and depleting the mRNAs of secretory proteins by RIDD, animals directly regulate the rate of protein translation during ER stress (Figure 1). The accumulation of unfolded proteins in the ER triggers the dimerization of the luminal domains of two PERK protomers due to the dissociation of BiP, similar to the activation of IRE1. The PERK dimer undergoes *trans*-autophosphorylation to activate its cytosolic kinase domain, which phosphorylates the α subunit of eukaryotic initiation factor 2 (eIF2). Unusually for kinases, activated PERK binds its substrate avidly via a heavily phosphorylated insert-loop domain, but has

much less affinity for the phosphorylated product which is subsequently released (89). During the initiation of protein translation, eIF2α participates in the recruitment of the initiator methionine-tRNA forming a ternary complex of eIF2:GTP:Met-tRNA that binds to the ribosome. During this process GTP is hydrolyzed and subsequent cycles require exchange of GDP for GTP catalysed by the guanine nucleotide exchange factor (GEF) eIF2B. Phosphorylated eIF2α forms a non-productive complex with eIF2B, blocking its GEF activity and attenuating cap-dependent translation (120). The resultant downturn in global translation reduces the load of new proteins entering the ER. In addition, a small subset of transcripts is translated more efficiently, most notably that of activating transcription factor 4 (ATF4) (Figure 3). ATF4 induces another transcription factor, CCAAT/Enhancer-Binding Protein Homologous Protein (CHOP), and in combination these transcription factors lead to the up-regulation of a regulatory subunit of protein phosphatase 1 (PP1) named GADD34 (or PPP1R15a). GADD34 directs PP1 specificity to dephosphorylate eIF2α allowing the recovery of translation (90). Other targets of ATF4 and CHOP contribute to a number of cytoprotective mechanisms, including amino acid import and synthesis. CHOP additionally promotes disulfide bond formation in the ER through induction of ERO1α. Whilst ERO1α generates disulfide bonds required for ER protein folding, it is also a source of reactive oxygen species (ROS) that contribute to cytotoxicity in prolonged stress. The effects of these ROS are in part ameliorated by ATF4-driven induction of enzymes that combat oxidative stress.

In addition to RIDD and the phosphorylation of eIF2α, other mechanisms enable the cell to regulate protein translation during ER stress. The initiation factor eIF4E forms another control point, both by regulation of its expression and phosphorylation of its inhibitory binding partner 4E-BP by mammalian target of rapamycin (mTOR) complex 1 (6, 86, 124). Induction of ATF4 driven by PERK has been shown to induce 4E-BP in a number of cell types and promotes pancreatic β-cell survival during ER stress (166). The long half-life of 4E-BP may afford protective inhibition of translation long after GADD34-mediated dephosphorylation of eIF2α.

The durations of IRE1 and PERK signalling are important in determining the fate of cells during prolonged stress (84, 85). While the mechanisms governing these processes are still unclear, in prolonged stress the UPR switches from a protective mechanism to cytotoxicity. For example, β-cell death in type 2 diabetes results at least in part from the downstream effects of PERK signalling in response to prolonged ER stress (115). Conversely, the highly secretory nature of many tumors makes them potential targets for therapeutics that sensitize cells to ER stress and exaggerate the cytotoxic effects of the UPR. Cell type and disease context must be key considerations in such strategies, exemplified by the differential effects of GADD34 induction. Although the downstream effects of GADD34 are considered to be toxic in the face of prolonged stress (and its antagonism has been shown to be cytoprotective) (155), in the context of prion disease GADD34 overexpression leads to increased survival of hippocampal neurons by the virtue of increased translation rates (100).

Research now points towards additional roles for PERK outside of the classical UPR. Cross talk between the ER and mitochondria is generating much interest and PERK has been implicated as a potential facilitator of this. Mitofusin 2 (Mfn2) is a small GTPase that resides in the outer mitochondrial membrane. It participates in mitochondrial fusion and was recently shown to bind directly to PERK (101). Ablation of Mfn2 led to induction of all three arms of the UPR as well as to mitochondrial calcium overload. This suggested that the interaction with PERK is potentially important for ER-mitochondrial signalling. Indeed, the deletion of PERK ameliorated mitochondrial dysfunction and ROS production in Mfn2-deficient cells, while PERK overexpression induced mitochondrial fragmentation. In a separate study, PERK-mediated interactions were shown to be important for ROS-driven mitochondrial apoptotic mechanisms (160). ROS production in both the ER and mitochondria contribute to ER stress, and the interplay between these compartments deserves further study.

ATF6

ATF6 performs dual roles as both a sensor and a direct effector of the UPR. Upon ER stress, BiP dissociates from the luminal domain of ATF6, allowing it to exit the ER and traffic to the Golgi apparatus (Figure 1). Within the Golgi, ATF6 is cleaved by site-1 and site-2 proteases (S1P and S2P) to yield a soluble cytosolic fragment that functions as a transcription factor. This migrates to the nucleus where it up-regulates factors involved in protein folding, lipid biogenesis and ERAD (171). The transcriptional targets of ATF6 overlap only partially with

those of XBP1 (76, 111, 168). A recent study employed artificial activation of either XBP1 or ATF6 in the absence of stress to define these targets further (144). This identified three groups of genes that were specific either to XBP1 and ATF6 or common to both; thirty-one genes displaying cooperative regulation by these two arms of the UPR.

Experimental evidence has linked ATF6 functionality to the susceptibility to diabetes. β-cells harvested from Akita mice show higher levels of ATF6 activation and enhanced expression of ATF6 target genes compared with controls consistent with the heightened levels of ER stress found in this model of diabetes (105). ATF6, in fact, exists as two isoforms, ATF6α and ATF6β (157, 173) of which ATF6α is 200-times more active than ATF6β (154). Whilst individual loss of either isoform is tolerated, ATF6α/ATF6β double knockout in a mouse model caused embryonic lethality (167), likely due to enfeebled induction of BiP (87). *Atf6α-/-* mice are euglycaemic and have normal levels of circulating insulin, but when made obese by feeding with a high-fat diet are more prone to hyperinsulinaemia than wildtype controls (157). This suggests that impaired function of ATF6α might increase an animal's vulnerability to diabetes, perhaps through increased ER stress-induced insulin resistance. In humans, genetic evidence also implicates ATF6 in the susceptibility to diabetes. Polymorphisms of the *ATF6* gene have been shown to associate with type 2 diabetes in different ethnic (24, 96, 153). Three of these variants of *ATF6* display complete linkage disequilibrium with type 2 diabetes in Pima Indians and have a marginal impact on insulin levels (153).

A number of other ATF6-like members of the CREB/ATF family have been identified. One such protein, BBF2H7/CREB3L2, is preferentially expressed in chondrocytes of developing cartilage and was recently shown to possess a novel function in addition to its role as a transcription factor (132). Upon ER stress, the luminal domain of BB2H7 is released from the membrane and subsequently secreted by the cell. It appears to function as an activator of hedgehog signaling, promoting chondrocyte proliferation and inhibiting hypertrophic differentiation (133). While ATF6-like proteins share significant N-terminal homology, their luminal C-terminal domains vary greatly, with the potential to support a wide variety of hitherto unknown functions if others are shown to generate secreted factors.

The role of ER stress in disease

While the UPR is cytoprotective in the face of acute ER stress, chronic UPR signalling promotes cell death (90). As a result, both insufficient and hyperactive UPR signalling is pathogenic. PERK signalling is necessary for β-cell function and development, with *Perk* knockout mice developing diabetes mellitus due to loss of β–cell mass (51, 174). These animals mirror the disease seen in humans with Wolcott-Rallison syndrome, a recessive genetic disorder caused by loss-of-function mutations of the *PERK* gene, which results in the failure to phosphorylate eIF2α during physiological levels of ER stress (31). Indeed, in cultured cells, inhibition of PERK leads to increased proinsulin synthesis and its rapid accumulation in the ER (53). It has been suggested that the toxicity associated with loss of PERK function may not be driven by uncontrolled protein

548 synthesis, as translation rates of wild-type and *Perk*-/- mice were comparable
549 (48). Instead, *Perk*-/- mice displayed reduced ER to Golgi transport and
550 impaired protein retrotranslocation in ERAD, leading to accumulation of
551 proinsulin in the ER lumen. Moreover, loss of PERK led to constitutive ATF6
552 signalling and a concomitant up-regulation of a number of ER folding factors.
553 Intriguingly, the onset of the diabetic phenotype in mice carrying the pathogenic
554 Akita *Ins2* mutation was delayed in response to *Perk* haploinsufficiency and was
555 hastened by over-expression of PERK (48). Somewhat surprisingly, Akita *Ins2*
556 mice showed an expanded β-cell mass and islet size at the time of progression to
557 frank diabetes. Another study has attributed reduced glucose-stimulated insulin
558 secretion in cultured PERK-deficient β-cells to aberrations in calcium signalling.
559 PERK was implicated as a positive regulator of the SERCA pump, which
560 maintains the high concentration calcium stores of the ER. Inhibition of PERK led
561 to reduced exocytosis of insulin in a manner dependent both on ER calcium and
562 the calcium-dependent protein phosphatase calcineurin (163), which has
563 previously been shown to interact with PERK (12). These data suggest
564 additional layers of complexity in the role of PERK signalling in the diabetic
565 phenotype.
566
567 By contrast, type 2 diabetes (T2D) is associated early in the disease with
568 elevated levels of insulin production in response to peripheral insulin resistance,
569 which results in β-cell exhaustion and apoptosis mediated by chronic PERK
570 activation (115, 147). Ablation of the downstream PERK effector CHOP was
571 shown to be protective in mouse models of T2D, leading to increased β-cell mass
572 and improved glycemic control (147). ER stress has been implicated in the

peripheral resistance to insulin seen in T2D, particularly that of adipose tissue in obesity (11, 118), while ER stress in the liver is a hallmark of T2D (103, 119). The causative mechanisms of ER stress in obesity are incompletely understood, but recent work revealed impaired autophagy in the livers of *ob/ob* mice due to reduced expression of ATG7, leading to ER stress and hepatic insulin resistance (170). Restoration of ATG7 to levels of lean controls ameliorated the ER stress and rescued the defect in insulin signalling thus reducing the expression of genes involved in gluconeogenesis. Disruption of ER calcium signalling within the liver of obese mice appears also to induce ER stress via an additional mechanism (43). Obese mice express some genes involved in lipid biosynthesis more strongly than lean controls, which increases the ratio of phosphatidylcholine (PC) to phosphatidylethanolamine (PE). Aberrant ER stress signalling could be corrected by increasing the capacity for ER-calcium uptake through overexpression of the sarco/endoplasmic reticulum calcium ATPase (SERCA) pump, or by redressing the altered PC to PE ratio (43). Previous studies have demonstrated that SERCA activity is sensitive to the lipid composition of the ER membrane. For example, cholesterol loading of the ER membrane impairs SERCA activity (83). However, the precise mechanism by which PC/PE ratio alters SERCA activity remains unclear.

ER stress has also been linked to the metabolic syndrome through its hypothalamic effects. In mice with diet-induced obesity, elevated ER stress in hypothalamic neurons impairs the response to the anorexigenic hormone leptin, contributing further to obesity (117). Recently, diet-induced obesity was shown to decrease Mfn2-mediated mitochondrial to ER contacts in anorexigenic pro-

598 opiomelanocortin (POMC) neurons of the hypothalamus (137). Mice lacking
599 Mfn2 in POMC neurons developed early onset leptin resistance with elevated ER
600 stress and mitochondrial ROS production. Intriguingly, the obese phenotype of
601 these animals could be reversed by ER stress-ameliorating chemical chaperones,
602 suggesting a potential for therapeutic intervention in leptin resistance (137).
603
604 Many neurodegenerative diseases are associated with aberrant protein folding
605 and the formation of insoluble protein aggregates. Often the proteins involved
606 are located in compartments other than the ER, but disturb proteostasis
607 throughout the cell to cause ER stress leading to induction of the UPR (recently
608 reviewed in (36, 57, 130). The accumulation of protein aggregates within
609 neurons appears to overload the proteasomal and autophagic degradation
610 machinery to cause ER stress (36, 50). Pathogenic mutations in the superoxide
611 dismutase 1 gene (SOD1) are causative in some inherited cases of familial
612 amyotrophic lateral sclerosis (FALS). Although SOD1 is primarily expressed in
613 the cytosol, in some cell types it is secreted, requiring its maturation in the ER.
614 Pathogenic mutations lead to the ER accumulation of misfolded SOD1 causing ER
615 stress by saturation of the ERAD pathway (104). A *Perk*+/- mouse expressing
616 the pathogenic G85R mutant of SOD1 showed more rapid loss of motor neurons
617 (162). This likely reflects the loss of cytoprotective ER stress-induced
618 translational repression and failure to induce ATF4 and CHOP in the *Perk*
619 haploinsufficient animals (162). This suggests that phosphorylation of eIF2α is
620 protective in this disorder. Accordingly, treatment with the drug salubrinal,
621 which promotes eIF2α phosphorylation, delayed the onset of FALS in a separate
622 study (136) and inactivating mutations of *Gadd34*, one of the eIF2α phosphatases,

also ameliorated this disease (161). Intriguingly, loss of XBP-1 or IRE1 was protective against mutant SOD1 (58). However, this appeared to be mediated by increased autophagy that helped clear aggregates of SOD1. This finding is surprising given that autophagy is believed to be induced by ER stress (107). Mechanisms have been described for both IRE1 and PERK-mediated induction of autophagy (35, 73, 107, 134), however, in the context of FALS, it seems likely that the downstream effects of PERK signalling form the dominant mechanism.

Our understanding of the role of ER stress in cancer is far from complete, but has now reached a level where it has produced viable drug targets and therapeutic strategies (26). These studies are also uncovering exciting new concepts in the field, such as the potential for transmissible ER stress between cells. The pro-apoptotic factor Par-4 was recently shown to be secreted into the extracellular milieu in response to ER stress, where it caused apoptosis of surrounding cells (16). Somewhat surprisingly, the authors of this study showed that the ER chaperone BiP acted as the pro-apoptotic receptor for prostate apoptosis response-4 (Par-4) at the cell surface. Other studies have also suggested secretion of ER factors, including BiP, in response to ER stress (44, 70, 106), making this an attractive subject for future research.

Concluding Comments

Current understanding of ER stress and the resultant cellular response mechanisms are providing a gateway for drug discovery and therapeutic strategies in human disease. However, a number of long standing questions

647 regarding the basic biology of ER stress still remain unanswered. For example,
648 how are non-glycosylated targets of ERAD recognized and what is the precise
649 mechanism by which chronic ER stress brings about toxicity? In addition, many
650 of the recent advances in this field have raised new questions, such as what is the
651 physiological role of RIDD and what are the implications of ER-mitochondrial
652 communication for cellular physiology? Our growing understanding of these
653 concepts will no doubt continue to inform translational medicine.
654

Figure 1

Proteostasis in the endoplasmic reticulum

Polypeptide chains enter the ER co-translationally (white arrow) and rapidly associate with ER chaperones and resident factors that promote folding such as BiP, PDI and calreticulin (CRT). Upon adopting the native state, proteins are released from the ER to pass down the secretory pathway via the Golgi (blue arrow). Proteins which fail to adopt their native conformation are retained in the ER and eventually targeted for retro-translocation and proteasomal degradation by ERAD (yellow arrow). Accumulation of unfolded proteins in the lumen leads to activation of the unfolded protein response (UPR) signalling molecules IRE1, ATF6, and PERK (red arrows). IRE1 activation up-regulates UPR target genes by unconventional splicing of the transcription factor XBP1, whereas ATF6 activation causes it to traffic to the Golgi where proteolytic processing releases a nuclear targeted transcription factor domain. Both XBP1 and cleaved ATF6 up-regulate components of the ER folding and degradation machineries. PERK activation phosphorylates eIF2α, leading to translational attenuation that prevents further accumulation of unfolded proteins. Phosphorylation of eIF2α paradoxically induces translation of the transcription factor ATF4, promoting oxidative folding, amino acid synthesis, and recovery of normal translation rates. XBP1 is also thought to reduce translation during stress, by degrading ER-localized mRNA transcripts in a process termed regulated IRE1-dependent decay (RIDD).

Figure 2

ADP-ribosylation of BiP modulates the size of the active chaperone pool in line with ER client load.

The amount of active BiP in the ER is tightly coupled to the load of unfolded proteins. BiP chaperone activity is driven by rounds of ATP binding and hydrolysis in the nucleotide binding domain (light blue), which cycles the substrate binding domain (dark blue) between high and low substrate affinity conformations. An increase in ER unfolded protein load (as seen in ER stress) leads to up-regulation of BiP through the transcriptional reprogramming by the unfolded protein response (UPR). A reduction in unfolded protein load leads to deactivation of BiP by ADP-ribosylation of its substrate-binding domain. This modification converts both the ATP and ADP bound forms of BiP to a low substrate affinity conformation, creating a latent chaperone pool. This modification is thought to be catalysed by an ADP-ribosyl transfease and removed by an ADP-ribosyl hydrolase. The identities of these enzymes and the exact mechanism of their regulation are currently unknown.

Figure 3

Translation of ATF4 is up-regulated by ER stress induced eIF2α phosphorylation.

(a) The ATF4 transcript contains two upstream open reading frames (uORFs), the most 3' uORF (uORF2) overlaps out of frame with the ATF4 open reading frame. (b) The GTP loaded 43S pre-initiation complex (43S-PIC) scans along the transcript until it reaches uORF1, where the 60S ribosomal subunit is recruited and translation is initiated. After uORF1 translation is complete, the ribosome disassembles and the 40S ribosomal subunit continues to scan the transcript for subsequent ORFs. Prior to a second round of translation, GTP-loaded eIF2 must bind the 40S subunit. In the absence of stress, the relative abundance of eIF2-GTP promotes this interaction before the scanning subunit reaches uORF2, resulting in uORF2 translation, which prohibits translation of ATF4. (c) During ER stress, phosphorylation of eIF2α phosphorylation leads to a relative depletion of eIF2-GTP. This reduces the probability of eIF2-GTP interaction with the scanning 40S subunit prior to uORF2 and increases the likelihood of initiation at the ATF4 ORF, thus increasing ATF4 expression.

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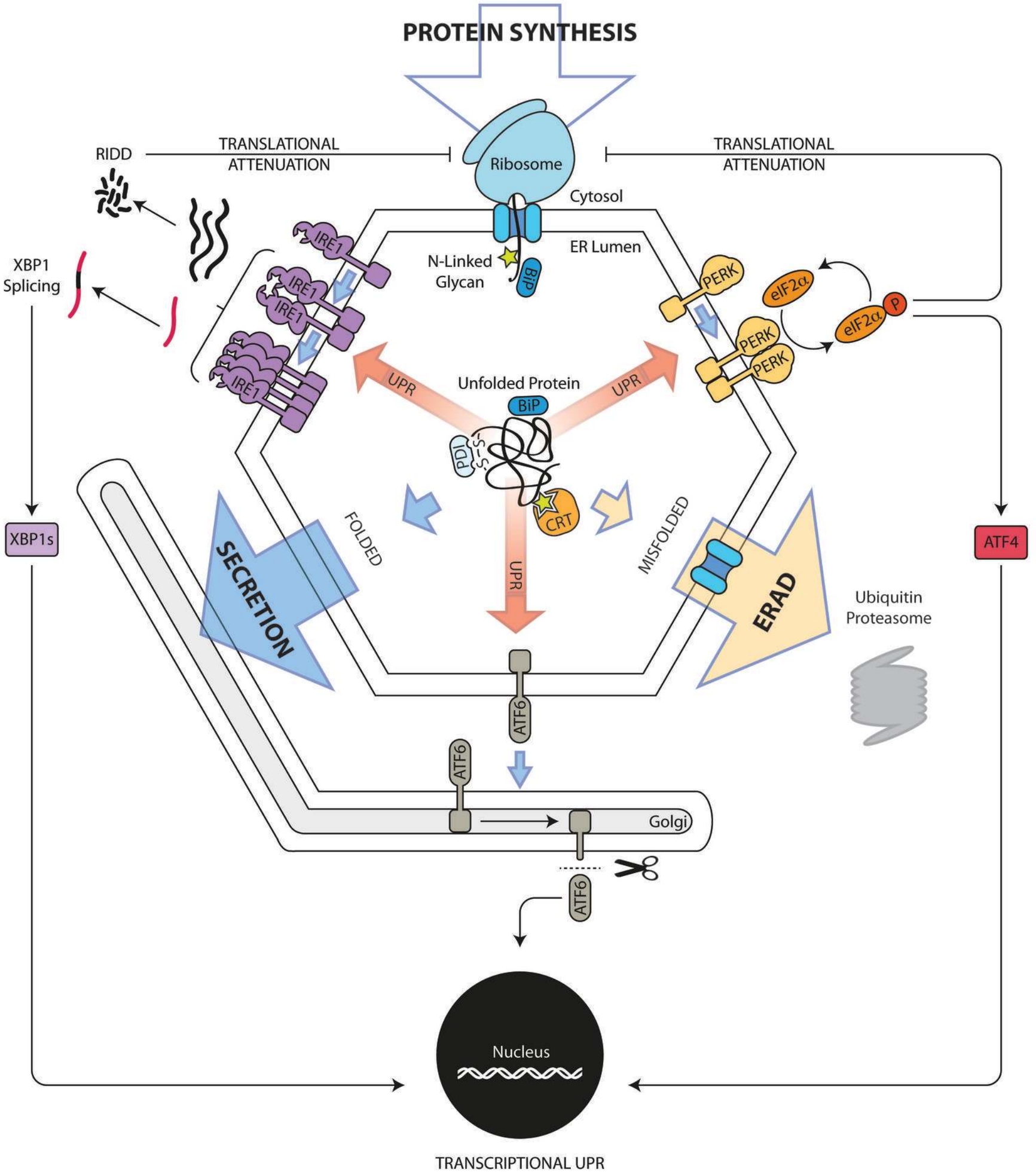
PROTEIN SYNTHESIS
RIDD
TRANSLATIONAL ATTENUATION
Ribosome
TRANSLATIONAL ATTENUATION
Cytosol
ER Lumen
XBP1 Splicing
IRE1
IRE1
IRE1
IRE1
N-Linked Glycan
BiP
PERK
PERK
PERK
eIF2α
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UPR
Unfolded Protein
UPR
BiP
PDI
CRT
FOLDED
MISFOLDED
UPR
XBP1s
SECRETION
ERAD
ATF4
Ubiquitin Proteasome
ATF6
ATF6
Golgi
ATF6
Nucleus
TRANSCRIPTIONAL UPR

UNFOLDED PROTEIN LOAD

ACTIVE CHAPERONE POOL

Transcriptional
UPR

Substrate
Binding
Domain

Nucleotide
Binding
Domain

ADP

Substrate

ATP

ADP-Ribosyl
Hydrolase

ADP-Ribosyl
Transferase

ADP-Ribosyl
Hydrolase

ADP-Ribosyl
Transferase

LATENT CHAPERONE POOL

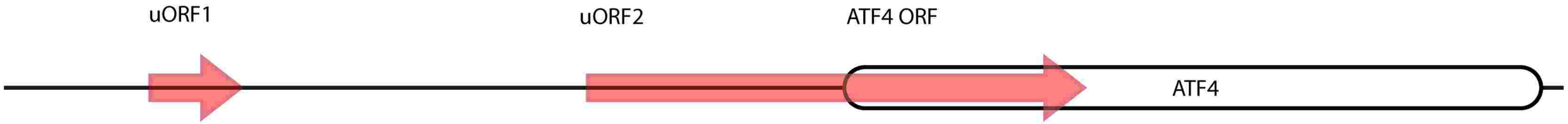
ADPR

ADP

ADPR

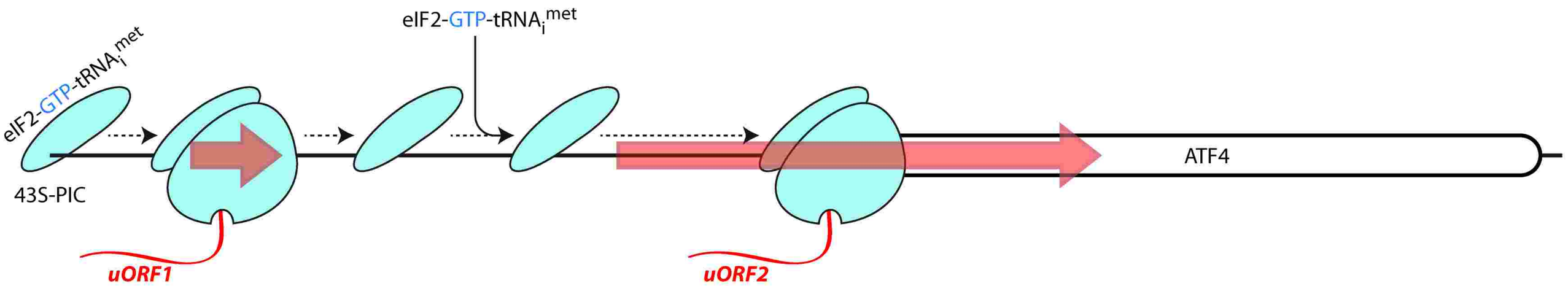
ATP

A.



B. Unstressed → Low eIF2a-P → **eIF2-GTP**

eIF2-GDP



C. Stressed → High eIF2a-P → eIF2-GDP

eIF2-GTP