Early events in the endoplasmic reticulum unfolded protein response (UPR)

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Abstract:

The physiological consequences of the unfolded protein response are mediated by changes in gene expression. Underlying them are rapid processes involving pre-existing components. We review recent insights gained into the regulation of the ER Hsp70 chaperone BiP, whose incorporation into inactive oligomers and reversible AMPylation and de-AMPylation present a first line of response to fluctuating levels of unfolded proteins. BiP activity is tied to the regulation of the UPR transducers by a recently discovered cycle of ER-localized J-protein-mediated formation of a repressive IRE1-BiP complex, whose working we contrast to an alternative model for UPR regulation that relies on direct recognition of unfolded proteins. We conclude with a discussion of mechanisms that repress mRNA translation to limit the flux of newly synthesized proteins into the ER, a rapid adaptation that does not rely on new macromolecule biosynthesis.

Introduction:

In eukaryotes, the synthesis of most proteins destined for secretion or membrane insertion occurs at the endoplasmic reticulum (ER), where their folding and maturation is assisted by compartment-specific molecular chaperones. Thus, protein homeostasis in the secretory pathway relies on the ability of cells to continuously adjust their complement of chaperones to match the load of newly-synthesized proteins. An imbalance between folding load and capacity due to excess of non-native ER clients – a condition known as ER stress – can cause proteotoxic misfolding and aggregation, whereas a surplus of chaperones may slow down productive folding and favor aberrant degradation.

The dynamic adjustments required to defend ER protein homeostasis are governed by a negative feedback-regulated signaling pathway, the unfolded protein response (UPR) (Kozutsumi et al. 1988). The UPR depends on the activation of transmembrane signal transducers during ER stress to evoke mainly two downstream effects: (i) limiting the influx of proteins into the ER to cap the folding load and (ii) increasing the expression of effector molecules with functions in ER protein quality control (ERQC) to expand the folding capacity (Figure 1).

In unicellular eukaryotes, such as yeast, the adaptation to the burden of unfolded proteins in the ER is dominated by a transcriptional program that centres on the regulated activity of a single nuclear protein, HAC1 (Cox and Walter 1996). HAC1 abundance is co-ordinated with the level of ER stress by the activity of the transmembrane signalling protein IRE1 via a linear pathway (Karagoz and Walter 2019). The targets of the yeast UPR include ER-localized chaperones, such as the Hsp70 BiP, that directly bind and buffer unfolded proteins, but also genes that operate throughout the secretory pathway by enhancing membrane lipid synthesis and the ability to degrade unfolded ER proteins (Cox et al. 1997; Travers et al. 2000).

These features of the UPR as a latent transcriptional program by which cells gradually develop the capacity to cope with the demands of protein secretion is conserved in higher eukaryotes: the animal counterpart to HAC1, XBP1, was originally identified as a developmental gene required for immunoglobulin secretion from plasma cells (Reimold et al. 2001). Animal-cell-specific UPR regulated transcription factors ATF6 and BBF2H7 are required for the developmental-stage specific secretion of type II collagen (Ishikawa et al. 2017). However, animals have also acquired reversible mechanisms that rapidly match the ER's capacity to physiological fluctuations in ER stress, independent of transcription and new protein

synthesis. The context for this is likely to be the highly dynamic nature of secretion in animals. For example, in response to fluctuating blood glucose levels the quantity of proinsulin entering the ER of a pancreatic beta cell can change by 10-fold over a time frame of less than 1 hour (Itoh and Okamoto 1980). A purely yeast-like transcriptional UPR is ill equipped to cope with such dynamism.

This review will address the early post-translational events in the animal cell UPR. We will begin by discussing AMPylation, an animal-specific mechanism for rapid, reversible inactivation of the major ER chaperone BiP. This will lead to a critical discussion of BiP's role in regulating the UPR transducers. We shall end by discussing another animal-specific adaptation to ER stress – control over the rate of new protein synthesis.

Main Text:

Emerging role of post-translational mechanisms for short-term regulation of chaperone availability in the ER

The mammalian UPR is multi-branched and depends mainly on the activity of three transmembrane signaling molecules IRE1, ATF6 and PERK. The transcriptional output of each is mediated through pathway-specific transcription factors XBP1, ATF6-N and ATF4, respectively (Figure 1). The inherent latency of the UPR limits its responsiveness to changes in the burden of unfolded proteins. In particular, dedicated secretory cells experience recurring physiological fluctuations in the unfolded protein load that appear too transient for alterations of the transcriptional program, alone, to handle, hinting at the need for other compensatory mechanisms to adjust the folding capacity (Figure 2).

Among the effector proteins that are targets of the UPR is the Hsp70-type chaperone BiP, an essential ERQC component involved in protein import, guiding polypeptide folding, assembly of protein complexes, and channeling misfolded polypeptides to degradation (reviewed in, Behnke et al. 2015). BiP also has important roles as a folding load sensor and negative feedback regulator of UPR signaling (see below), making it a key player in the control of ER protein homeostasis.

BiP, which has a relatively long half-life in cultured cells (≥24 hrs) (Satoh et al. 1993) and in tissues (≥3 days) (Price et al. 2010; Lau et al. 2016), is present at high concentrations in the ER basally (~0.5 mM) and can increase several-fold during prolonged ER stress (Bakunts et al. 2017). While shortage of BiP corrupts central aspects of ERQC, excess of BiP also impairs secretion (Dorner et al. 1992). The cost of excess BiP suggests that a UPR based solely on changes in levels of gene expression would be ill-equipped to cope with short-term physiological changes in the demand for a highly-abundant chaperone with slow turnover rates.

Recent discoveries brought to attention two reversible, post-translational mechanisms that regulate the quantity of active BiP in response to changes in the concentration of unfolded substrates. The first entails the sequestration of client-free BiP into homomeric complexes. That BiP forms oligomers has been long known (Carlino et al. 1992; Freiden et al. 1992; Blond-Elguindi et al. 1993), but recently their potential physiological role came into focus. In vitro experiments revealed that oligomerization is based on canonical substrate interactions between individual BiP molecules and thus in direct competition with binding to unfolded clients (Blond-Elguindi et al. 1993; Preissler et al. 2015a). Furthermore, BiP oligomers diminish in

cells treated with ER stress-inducing agents (Freiden et al. 1992; Preissler et al. 2015a). Together these observations suggest that BiP oligomers constitute a dynamic inactive pool into which free BiP is deposited to prevent non-productive interactions with native proteins and from which active chaperone can be recruited when the concentration of unfolded proteins rises (Figure 3A).

A second mechanism of BiP regulation involves its reversible inactivation by post-translational modification. As the modification entailed labeling of BiP from both cellular phosphate and adenosine pools, it was long believed to represent ADP-ribosylation (Carlsson and Lazarides 1983; Ledford and Jacobs 1986; Hendershot et al. 1988; Chambers et al. 2012). However, the discovery of an ER-localized enzyme, FICD (or HYPE), that uses ATP to catalyze the covalent attachment of AMP via a phosphodiester bond to BiP, suggested an alternative scenario – AMPylation (Ham et al. 2014; Sanyal et al. 2015). Indeed, disruption of the *FICD* gene in mammalian cells abolishes all detectable BiP modification, indicating that AMPylation (not ADP-ribosylation) is the most prominent detectable modification of BiP (Preissler et al. 2015b).

Early observations that BiP modification correlates inversely with the unfolded protein load in the ER (Carlsson and Lazarides 1983; Ledford and Jacobs 1986; Laitusis et al. 1999) and that only unmodified BiP binds substrates (Hendershot et al. 1988; Freiden et al. 1992) suggested an inhibitory effect on BiP's chaperone activity. AMPylation occurs at a single residue in BiP's substrate binding domain, threonine 518. Reconstitution of BiP AMPylation with purified components revealed that BiP, in its ATP-bound conformation, is the favored substrate for AMPylation (Preissler et al. 2015b). Furthermore, AMPylation biases BiP conformationally towards the ATP-bound state (Preissler et al. 2017b; Wieteska et al. 2017), which is characterized by fast substrate dissociation rates. AMPylation also impairs stimulation of BiP's ATPase activity by J-protein co-factors (Preissler et al. 2017b), which is required for Hsp70s to attain high-affinity for their substrates. Thus, like oligomerization, AMPylation creates a dynamic pool of inactive BiP to match the concentration of active chaperone to the amount of unfolded proteins (Figure 3B).

Because AMPylation affects BiP's ability to engage in substrate interactions, modified BiP is excluded from oligomers. The two mutually exclusive inactivating processes regulate BiP with different kinetics. Oligomerization is in direct competition with substrate binding and thus is a mass action-driven, fast process, whereas enzyme-catalyzed BiP modification (and de-modification) is slower (Ham et al. 2014; Preissler et al. 2015a; Preissler et al. 2015b). Indeed, physiological changes of

modified BiP levels occur over a few hours in the murine pancreas and correlate with the rates of secreted protein synthesis during alternating fasting and feeding intervals (Chambers et al. 2012). Therefore, it is plausible that both mechanisms operate side-by-side to regulate BiP availability on short (seconds or few minutes) and intermediate (several minutes to hours) time scales.

BiP AMPylation is especially prominent during the recovery after prolonged ER stress when, due to its long half-life, BiP levels remain elevated whilst unfolded proteins decrease (Preissler et al. 2015b). BiP inactivation by modification restores ER functionality and secretory efficiency without degrading excess BiP. Indeed, *FICD* itself is transcriptionally upregulated during the UPR (Ham et al. 2014; Preissler et al. 2015b; Sanyal et al. 2015), which presumably enhances the capacity for BiP inactivation upon ER stress resolution. Thus, post-translational regulation of BiP activity provides a means to fine-tune chaperone-client protein balance, obviating a costly and slow adjustment of BiP protein levels by de novo synthesis and degradation (Figure 2B).

How is AMPylated BiP reactivated? Surprisingly, it was found that FICD is a bifunctional enzyme that uses the same active site both to modify BiP and to catalyze the hydrolytic removal of AMP from the chaperone (Casey et al. 2017; Preissler et al. 2017a). In fact, while the AMPylation activity of FICD is strongly autoinhibited (Engel et al. 2012; Bunney et al. 2014) the default activity is de-AMPylation (Casey et al. 2017; Preissler et al. 2017a). This implies that AMPylation of BiP in vivo requires a functional switch in FICD that is subordinate to changes in the unfolded protein load. The molecular basis of this switch remains to be uncovered.

The early steps in UPR activation

Downstream events in signaling by UPR transducers and the resultant changes in gene expression have been studied intensely (Karagoz and Walter 2019). However, the upstream molecular mechanisms that monitor the balance between folding load and capacity in the ER are less well understood. By definition these processes constitute early events in the UPR.

Any information about proteostatic imbalance must ultimately converge on the signal transducers in the ER membrane and evoke a response proportional to the magnitude of stress. In principle, activation (or repression) of the signal transducers can involve the physical recognition of unfolded polypeptides or either free or occupied effector molecules that interact with the unfolded proteins (i.e. ERQC components such as chaperones).

The UPR signal transducers IRE1 and PERK are both ER-localized type-I transmembrane proteins. They consist of a structurally (Credle et al. 2005; Zhou et al. 2006; Carrara et al. 2015a; Wang et al. 2018) and functionally-related (Bertolotti et al. 2000; Liu et al. 2000) stress-sensing luminal domain, a transmembrane domain, and a functionally-divergent cytosolic kinase/effector domain. It is widely accepted that dimerization/oligomerization in the plane of the membrane, which brings together two or more effector domains, initiates IRE1 and PERK activation. However, the molecular principles that govern this dimerization remain unresolved with two models receiving experimental attention: activation by direct binding of unfolded proteins and chaperone inhibition. Much of the research in this area has been carried out on IRE1, which is conserved amongst all eukaryotes, however, it is assumed that the metazoan-restricted PERK functions similarly.

Direct activation of IRE1 by unfolded polypeptides

The direct binding model was boosted by the crystal structure of the yeast IRE1 (yIRE1) luminal domain (LD). It revealed a symmetric dimer traversed by a groove with similarity to the peptide-binding groove of major histocompatibility complexes (MHCs) (Credle et al. 2005), suggesting that binding of extended polypeptides (i.e. unfolded proteins) stabilizes the LD dimer and favors IRE1 activation (Figure 4A) – a model supported by the observation that mutations in residues that line the groove adversely affect the ability of yIRE1 to respond to stress in vivo (Credle et al. 2005) and by the observation that the yIRE1-LD possesses chaperone activity, indicative of its ability to bind unfolded proteins in vitro (Kimata et al. 2007). Indeed, the yIRE1-LD interacts with peptides in a ligand-blot assay and a binding motif could be extracted from analyzing their sequences (Gardner and Walter 2011). A solution-phase assay was developed for the highest affinity peptides yielding a dissociation rate in the micromolar range and addition of peptide to dilute solutions of yIRE1-LD enhanced the population of higher order species, though a clear shift from monomers to dimers, predicted by the engagement of peptide in MHC-like groove, was not readily apparent.

Human IRE1α-LD also crystalized as a dimer, with structural features similar to yIRE1 (Zhou et al. 2006). However, the MHC-like groove is less hydrophobic and appears too narrow to accommodate a peptide (although it was argued that flexibility in solution may allow peptide binding). Furthermore, the purified human IRE1α-LD does not possess chaperone activity in vitro (Kimata et al. 2007; Oikawa et al. 2009). Nevertheless, peptides have recently been identified that bind the human IRE1α-LD with micromolar affinity and a NMR analysis revealed important structural

rearrangements to the IRE1α-LD wrought by exposure to such peptides (Karagoz et al. 2017). Unfortunately, unlike MHC molecules that invariably co-crystalize with a bound peptide, no such structure of the IRE1-LD is available. Whilst the recent detailed NMR study did show peptide-induced perturbation of residues near the MHC-like groove, this cannot be unambiguously interpreted as peptide engagement inside the groove. Furthermore, although the binding of peptide was apparently saturable, it is unclear if it featured competitive-binding at a single site, consistent with the MHC groove, or if the effects of high concentration of peptide observed in the NMR spectra of the IRE1α-LD arose through multiple, less specific interactions (Karagoz et al. 2017). Resolving this issue seems a paramount consideration for the direct binding model.

Unlike the chaperone inhibition model, described below, the direct binding model lacks an explicit kinetic component (beyond the association and dissociation parameters of peptide binding to the LD). Does peptide release from the LD, which is critical for the system to respond dynamically to changes in ER stress, occur passively or is it an assisted process requiring other agents? This seems especially important for peptides that associate cooperatively with several IRE1 molecules and are predicted to bind with high affinity.

Regulation of IRE1 by chaperones

The chaperone inhibition model for ER stress sensing is based on an inverse correlation between the amount of BiP recovered in co-immunoprecipitation with IRE1 and PERK and the level of ER stress (Bertolotti et al. 2000; Kimata et al. 2003; Kimata et al. 2004). In the simplest scenario, the interaction between BiP and the LDs occurs by the same mechanism governing BiP's interaction with unfolded clients. The model posits that BiP association somehow favors the monomeric, inactive state of IRE1 (and PERK) and that competition for BiP by client proteins titrates BiP away from the LDs, favoring dimerization and activation. This model is analogous to the cytosolic counterpart of the UPR, the heat shock response, in which chaperones associate with the transcription factor Hsf1, in eukaryotes, and σ^{32}, in bacteria, and interfere with their activity (Abravaya et al. 1992; Shi et al. 1998a; Tomoyasu et al. 1998).

Indeed, typical substrate-like interactions between BiP and IRE1 – i.e. via BiP's substrate binding domain and with affinities dictated by its nucleotide-dependent conformational cycle – have been observed (Bertolotti et al. 2000; Kimata et al. 2003; Liu et al. 2003). A different type of interaction (involving BiP's ATPase domain) and a

non-competitive mechanism of BiP-mediated IRE1 regulation has also been proposed, according to which binding of unfolded proteins to LD-associated BiP allosterically triggers BiP dissociation (Todd-Corlett et al. 2007; Carrara et al. 2015b). However, this mechanism appears to operate independently of BiP's nucleotide state and its acceptance is marred by the difficulty of reconciling it to fundamental principles of nucleotide-dependent allosteric regulation of Hsp70-type chaperones.

Repression of Hsf1 by cytosolic Hsp70 and σ^{32} by bacterial DnaK is directed by J-domain co-chaperones, Hdj1 and DnaJ, respectively (Shi et al. 1998a; Tomoyasu et al. 1998). The ER-localized J-protein, ERdj4, was recently found to contribute to IRE1 repression in mammalian cells. In vitro characterization indicated that ERdj4 promotes substrate interactions between BiP and the IRE1α-LD that disrupt LD dimers (Amin-Wetzel et al. 2017). J-proteins act as adaptors that facilitate high-affinity binding of Hsp70s to specific substrates by recruiting the chaperone in the ATP-bound state with high substrate association rates and converting it to the ADP state with low substrate dissociation rates by stimulation of ATP hydrolysis. Consistent with this canonical J-protein function, ERdj4-mediated binding of BiP to IRE1 was strictly dependent on stimulation of BiP's ATPase activity. Furthermore, in the absence of BiP/ERdj4/ATP activity the IRE1-LD was mostly dimeric, a feature emphasized by early work from the Kaufman lab (Liu et al. 2003; Zhou et al. 2006). These observations imply a chaperone inhibition model whereby a non-equilibrium cycle of ERdj4-mediated, ATP hydrolysis-driven BiP-IRE1 complex formation and ATP binding-induced BiP-IRE1 complex dissociation dynamically establishes a pool of inactive, monomeric IRE1 against its intrinsic propensity to dimerize (Figure 4B).

Many details remain to be worked out: whilst, ERdj4 can stimulate BiP binding to IRE1-LD dimers, it is unclear if the target of binding is indeed the dimer or the monomeric species, which co-exist in some equilibrium. Likewise, if BiP binds the dimer, it is not known whether J-protein-mediated loading of BiP onto IRE1 dimers causes their dissociation entropically or allosterically. The biophysical details, in terms of the structural changes to the IRE1-LD remain to be determined. They may resemble auxillin/Hsc70-driven disassembly of clathrin coats (Xing et al. 2010), or the Hsp40/Hsp70-mediated partial unfolding of the glucocorticoid receptor (Kirschke et al. 2014). The apparent parallels to repression of the bacterial heat shock response, which relies on destabilization and degradation of the σ^{32} transcription factor by J-protein-mediated interaction with the Hsp70 chaperone DnaK (Rodriguez et al. 2008), suggest that mechanisms for sensing unfolded protein stress evolved several

times independently exploiting the same functional principles of the highly conserved Hsp70 system.

BiP's concentration vastly exceeds the UPR transducers' (Kim et al. 2014; Kulak et al. 2014); an observation seemingly at odds with the sensitivity of UPR signaling to small changes in client protein abundance. However, the fraction of ATP-bound free BiP, which can effectively respond to J-proteins, is likely rather small (Freiden et al. 1992; Preissler et al. 2015a; Preissler et al. 2015b): most BiP molecules either engage substrates, are sequestered in oligomers, or inactivated by AMPylation. Indeed, the full functional scope of BiP AMPylation may be revealed in the context of UPR regulation by chaperone inhibition: consistent with its inactivating character, modification of BiP abolishes ERdj4-dependent disruption of IRE1-LD dimers in vitro (Amin-Wetzel et al. 2017). J-protein (e.g., ERdj4)-mediated loading onto IRE1 (leading to UPR repression) thus faces fierce competition for the small pool of active BiP by J-proteins directing the formation of client-BiP complexes. The constant cycling of BiP onto and off the IRE1-LD provides a sensitive system that can respond rapidly and bi-directionally to changes in the unfolded protein load. The transient accumulation of AMPylated BiP during recovery from ER stress may thus not only counteract over-chaperoning of secretory proteins but also set the tone of the UPR to enable longer-term gene expression programs that build secretory capacity.

ERdj4's role in IRE1 regulation is partially redundant, as knockout cells retain a measure of responsiveness to ER stress (despite higher basal level of IRE1 signaling). Recent studies implicated a physical interaction between mammalian IRE1α and Sec63 (ERdj2) in IRE1 signaling (Plumb et al. 2015; Adamson et al. 2016; Sundaram et al. 2017). Sec63 is a J-protein that associates with the Sec61 translocon channel in all eukaryotes and loads BiP onto polypeptides to support their unidirectional import into the ER (Matlack et al. 1999; Misselwitz et al. 1999). By analogy with ERdj4, Sec63/ERdj2 may promote inhibitory IRE1-BiP complexes when translocation rates are low, to establish an antagonistic crosstalk between protein import and IRE1 repression.

It has been noted that yeast IRE1 mutants and human-yeast chimeras, that associate with less BiP in co-immunoprecipitation assays, were not constitutively activate and retained responsiveness to ER stress (Kimata et al. 2004; Pincus et al. 2010; Mai et al. 2018). In contrast, mutating the corresponding region in IRE1α (which also affects BiP association) strongly impairs repression in mammalian cells (Oikawa et al. 2009). However, we believe conclusions based on quantifying the association of BiP with IRE1 by co-immunoprecipitation must be drawn with caution.

Such studies tend to ignore that most physiological BiP interactions are kinetically-driven by its nucleotide-dependent chaperone cycle and thus highly dynamic and prone to post-lysis binding artifacts. The distinction between regulatory and non-regulatory interactions may be further confounded by association of BiP with nascent IRE1 when the latter is overexpressed.

These mechanisms proposed for IRE1 regulation are not mutually exclusive and, despite the overall structural conservation of IRE1-LD's architecture, the contribution of different mechanisms to IRE1 regulation may vary between organisms and cell types. In vitro, mammalian J-protein-mediated chaperone inhibition targets the IRE1 monomer-dimer equilibrium to control dimerization and thus the initial step of IRE1 activation (Amin-Wetzel et al. 2017), while high concentration of unfolded proteins promotes further oligomerization (Karagoz et al. 2017). A similar two-pronged regulatory mechanism was also proposed in yeast (Kimata et al. 2007), but the relative contribution of BiP repression and unfolded protein binding remains to be established experimentally (Pincus et al. 2010).

Translational repression as an early response to ER stress

In animal cells, most secreted proteins are translocated into the ER co-translationally as unfolded nascent polypeptides. It follows therefore that the rate of mRNA translation influences the burden of unfolded proteins in the ER. Concordantly, in animal cells a conserved mechanism is found that attenuates protein synthesis in response to ER stress. Its short latency and inherent reversibility qualifies this adaptation as an early event in the UPR.

The PERK arm of the UPR

The ER-localized transmembrane protein PERK is upstream of another UPR pathway found in metazoans. Though PERK's stress-sensing luminal domain is only distantly related in primary sequence to IRE1, it assumes a very similar fold (Carrara et al. 2015a; Wang et al. 2018), suggesting that similar principles govern its activation. The cytosolic, effector domain of PERK resembles kinases that phosphorylate the α subunit of translation initiation factor 2 (eIF2α) on serine 51 (Shi et al. 1998b). This phosphorylation event negatively affects the rate at which translation is initiated on most mRNAs (Wek 2018). Thus, PERK couples ER stress to repression of global protein synthesis (Harding et al. 1999).

Such repression develops rapidly. Within minutes of exposing cells to agents that cause ER stress, PERK is autophosphorylated, levels of phosphorylated eIF2α increase, and global rates of protein synthesis decline. PERK has a non-redundant

role in this chain of events. Mammalian cells that lack PERK (Harding et al. 2000b) or cells treated with the potent PERK kinase inhibitor, GSK2606414 (Axten et al. 2012), maintain normal levels of protein synthesis in face of ER stress. PERK's role in modulating the rate of protein synthesis is observed not only under severe, pharmacologically-imposed ER stress, but also in the context of physiological signals that affect the rate of ER client protein synthesis. In cultured insulin-secreting beta cells acute inhibition of PERK signaling deregulates metabolically-entrained proinsulin biosynthesis (Harding et al. 2012) and in the tissues of PERK-deficient mice heightened activity of the parallel IRE1 pathway was observed, suggesting loss of an important feedback (Harding et al. 2001) (Figure 1A).

In animal cells, IRE1's RNAse activity is not limited to the *XBP1* mRNA. Rather IRE1 has the capacity to degrade other ER membrane-associated mRNAs in a process known as RIDD (Hollien and Weissman 2006; Hollien et al. 2009) (Figure 1A). The quantitative impact of RIDD on protein folding homeostasis in the ER remains to be determined. Confounding this issue is the observation that PERK-mediated translational repression contributes to RIDD (Moore and Hollien 2015). Thus, the roles of PERK-mediated eIF2α phosphorylation and IRE1-mediated RIDD cannot be readily apportioned by genetic criteria. Similar considerations apply to other processes that may influence ER client protein synthesis, such as the observed ER stress-induced mRNA dissociation from the ER (Reid et al. 2014).

These observations are consistent with a PERK-imposed restrain on the rate of ER client protein synthesis contributing to ER protein folding homeostasis under physiological circumstances. PERK deficiency, which in mammals is associated with rapid decline in the function of major secretory tissues (Delepine et al. 2000; Harding et al. 2001; Zhang et al. 2002), may be understood to exert its consequences through the disruption of this short-term adaptation to ER stress. However, this might be an oversimplification (Cavener et al. 2010).

Phosphorylated eIF2 (eIF2(αP)) attenuates the initiation step in mRNA translation indirectly, by inhibiting eIF2B, the guanine nucleotide exchange factor (GEF) that recycles eIF2 from its inactive GDP-bound to its active GTP-bound form (Panniers and Henshaw 1983) (Figure 5). PERK and other eIF2α kinases limit the stock of active eIF2 in the cell, attenuating translation of most mRNAs, and with it global protein synthesis. However, a small subset of mRNAs with specialized features in their 5' untranslated region are translated more efficiently in a regime of limited eIF2. This phenomenon, originally described in yeast (Abastado et al. 1991; Dever et al. 1992), is conserved in animal cells (Harding et al. 2000a; Lu et al. 2004; Vattem and

Wek 2004). Some of the mRNAs implicated in this divergent response encode potent transcription factors that couple eIF2α phosphorylation to a gene expression program. As eIF2α is phosphorylated under diverse conditions, this program integrates otherwise unrelated stress signals giving rise to an integrated stress response (ISR) (Harding et al. 2003). The ISR's target genes affect the capacity to synthesize and secrete proteins, as well as important cell fate decisions (Pakos-Zebrucka et al. 2016).

In theory, it should be possible to separate PERK's early and direct role in protein folding homeostasis from its effects on ISR target genes by enforced expression of ATF4, CHOP, and other ISR activating transcription factors. However, mimicking the dosage and temporal profile of a physiological ISR, required for such genetic rescue, is impractical. Therefore, at present we cannot gauge the relative contribution of these two linked aspects of PERK action to its role in preserving the function of secretory cells.

Dephosphorylation of eIF2(αP):

PERK-mediated translational repression, as a short-term adaptation to the threat of protein misfolding in the ER, is counteracted by eIF2(αP) dephosphorylation. In mammals, this process is known to be directed by two structurally-related regulatory subunits of a PP1-containing holophosphatase. PPP1R15A/GADD34 is expressed at low levels but is rapidly upregulated by the ISR, closing a negative feedback loop that contributes to the restoration of protein synthesis (Novoa et al. 2001; Brush et al. 2003; Novoa et al. 2003); whereas PPP1R15B/CReP is constitutively present (Jousse et al. 2003).

Whilst combined deficiency of both regulatory subunits severely compromises cells, lack of the inducible PPP1R15A is well tolerated (Harding et al. 2009). Furthermore, cells and tissues of mice lacking PPP1R15A exhibit a measure of resistance to certain circumstances associated with higher levels of ER stress (Marciniak et al. 2004; Lin et al. 2008; D'Antonio et al. 2013). These findings speak to a failure of homeostasis, whereby the enhanced ability of the PPP1R15A-posessing (wildtype) cells to reverse the consequences of PERK activation, places them at a disadvantage compared to the PPP1R15A mutant cells, when both are exposed to the same challenge. It is tempting to interpret the consequences of eliminating PPP1R15A solely in light of its effect on protein synthesis and thus on proteostasis (Marciniak et al. 2004; Han et al. 2013), however, here too we must be reminded that

the consequences of altered levels of eIF2(αP) are played out both in terms of its short-term effect on unfolded protein load, and also in terms of gene expression.

Either way, the benefit of attenuated PPP1R15A activity to certain mouse models of human diseases associated with ER stress has engendered interest in the details of eIF2(αP) dephosphorylation and in the prospects of targeting the process for inhibition. The crystal structure of the complex between the C-terminus of the PPP1R15 regulatory subunits and catalytic PP1 subunit explains their tight binding, but not the selectivity for eIF2(αP) (Chen et al. 2015; Choy et al. 2015). Further observations indicate that substrate specificity is imparted by G-actin joining the PPP1R15-PP1 complex as a third component (Chambers et al. 2015; Chen et al. 2015). However, a recent study reported on dramatic acceleration of eIF2(αP) dephosphorylation by either regulatory subunit (in the absence of G-actin) and on the selective susceptibility of the PPP1R15A-PP1 complex to inhibition by the drug Guanabenz and its close derivative, Sephin1 (Carrara et al. 2017). The latter biochemical observations have been greeted enthusiastically, as they suggest the possibility of tuning ER proteostasis by targeting the rate of eIF2(αP) dephosphorylation (Das et al. 2015). Unfortunately, the proposed inhibitory effect of Guanabenz/Sephin1 on eIF2(αP) dephosphorylation in vitro or in cultured cells could not be confirmed in subsequent studies (Crespillo-Casado et al. 2017; Crespillo-Casado et al. 2018). Thus, the promise of targeting the PPP1R15 holophosphatase to restore proteostasis by affecting the early adaptation to ER stress likely has yet to be realized.

Concluding Remarks

This review has focused on early events in the UPR that do not require new macromolecule synthesis. However, as they act upstream of the extensively-studied changes in gene expression and cellular phenotypes brought about by physiological and pathological fluctuations in protein folding homeostasis, they stand to influence the organismal response and the implications of the UPR in a variety of disease conditions (Karagoz and Walter 2019).

These early events seem especially important when considering the prospects of pharmacologic modulation of the UPR. The aforementioned attempts to target eIF2(αP) dephosphorylation are an interesting case in point, as it seems likely that further progress will depend on assays that reproduce the relevant enzymatic reactions in vitro. But it is also possible to consider how small molecules that shift the

equilibrium between LD monomers and dimers might affect signaling in the IRE1 or PERK branches of the UPR. Such small molecules may be discovered by direct biophysical assays that exploit the recent in vitro models for IRE1 activity. Thus, the question of how the UPR is activated and therefore the validity of the in vitro assays that aim to recapitulate relevant aspects of that process may assume practical significance, beyond the curiosity of scholars.

Acknowledgments:

We thank Niko Amin-Wetzel for comments on the manuscript. Supported by a Wellcome Trust Principal Research Fellowship to D.R. (Wellcome 200848/Z/16/Z) and a Wellcome Trust Strategic Award to the Cambridge Institute for Medical Research (Wellcome 100140)

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Figure Legends:

Figure 1: Overview of the mammalian UPR.

A) The central elements of the UPR signalling network. Each of the three signal transducers: IRE1, PERK, and ATF6 constitutes a separate branch of the UPR with a specific output to counteract proteostatic perturbations in the ER.

B) Basic components and downstream events of the UPR signalling pathways. ER stress induces dimerization/oligomerization of IRE1 and its trans-autophosphorylation, which leads to non-conventional *XBP1* mRNA splicing and production of the functional XBP1 transcription factor to induce expression of genes involved in ERQC and ER-associated degradation (ERAD) of misfolded proteins. Activated, oligomeric IRE1 may also degrade ER-targeted mRNAs (via RIDD). PERK-mediated phosphorylation of eIF2α causes general translation attenuation, to relieve the load of newly-synthesized proteins, and preferred synthesis of the ATF4 transcription factor (see Figure 5). Production of the downstream transcription factor CHOP feeds back negatively on the PERK pathway (by enhancing expression of the eIF2 (αP)-specific regulatory phosphatase subunit GADD34) and induction of pro-apoptotic genes to favour cell death at later stages of unrectified stress. ER stress also induces ATF6 translocation to the Golgi where it is processed by S1P and S2P proteases to liberate the ATF6(N) transcription factor element. At the transcriptional level all branches primarily facilitate expansion of the ER folding capacity and crosstalk exists between them (e.g. dotted line).

Figure 2: Latency of the negative-feedback-regulated transcriptional UPR.

A) Adaptation of the UPR by negative-feedback regulation via ERQC components. Apart from stress-denatured proteins, newly-synthesized secretory proteins constitute the majority of unfolded species in the ER and their accumulation activates the UPR transducers. The response entails rapid attenuation of translation to alleviate the unfolded protein load (see Figure 5), rapid post-translational regulation of the pool of active chaperone in the ER (see Figure 3) and the slower induction of ERQC genes to expand the folding capacity and mediate negative-feedback regulation of the UPR (see Figure 4).

B) Theoretical, time-resolved response profiles to illustrate the inherent latency of the UPR. Recurring physiological short-term fluctuations in the unfolded protein load do not cause a significant transcriptional response, suggesting that they are buffered by rapid, non-transcriptional adjustments of the folding capacity. During acute stress the fast rise of the folding load induces UPR signalling but expansion of the folding capacity is delayed due to the inherent latency of transcriptional reprogramming. Negative-feedback inhibition attenuates the transcriptional response as the folding capacity builds up to establish homeostasis at a new level. During the recovery from ER stress the folding load drops rapidly. Were the adjustment in ERQC capacity to rely exclusively on the decay of the ERQC mRNAs and encoded proteins, ERQC capacity would likely exceed the unfolded protein load in this recovery phase (dotted blue line). However, post-translational mechanisms that inactivate chaperones rapidly adjust the effective folding capacity and prevent over-chaperoning (arrow).

Figure 3: Post-translational regulation of BiP.

A) Dynamic partitioning of BiP amongst different pools. Unfolded substrate polypeptides directly compete with oligomerization for a limited pool of free, ATP-bound BiP. Free BiP also interacts with UPR signal transducers and contributes to repression of the UPR (see Figure 4). J-proteins promote both high-affinity substrate interactions and competing BiP oligomerization (with yet-to-be characterized relative kinetics), whereas nucleotide exchange factors (NEFs) induce substrate release and oligomer disassembly. When the folding load declines ATP-bound BiP becomes reversibly modified and inactivated by FICD-mediated AMPylation. Calcium depletion from the ER strongly induces BiP oligomerization by an unknown mechanism.

B) Chaperone and AMPylation cycles of BiP. ATP-bound BiP can be recruited to its substrates by J-proteins, which stimulate ATP hydrolysis by BiP to achieve high-affinity interactions with its substrates. Nucleotide exchange factors (NEFs) promote ADP release from BiP to enable ATP re-binding and dissociation of substrates. When the folding load is low, ATP-bound BiP becomes AMPylated by FICD (magenta). This locks BiP in a low-affinity state for substrates and renders the chaperone insensitive to stimulation by J-proteins, thereby causing its functional inactivation. The same enzyme, FICD, de-modifies BiP when the unfolded protein load increases to rapidly recruit pre-existing inactive BiP into the pool of active chaperones. The different functional states of FICD, default de-AMPylation and yet-to-be defined AMPylation, are indicated in light blue and magenta, respectively.

Figure 4: Role of BiP in regulating IRE1.

A) Direct binding model. Accumulating unfolded proteins bind directly to a groove on the luminal domain (LD) of IRE1 to promote its dimerization/oligomerization and activation. In this model sequestration of unfolded proteins by chaperones such as BiP counteracts their binding to IRE1, repressing the UPR and establishing a measure for the balance between unfolded proteins and available chaperones.

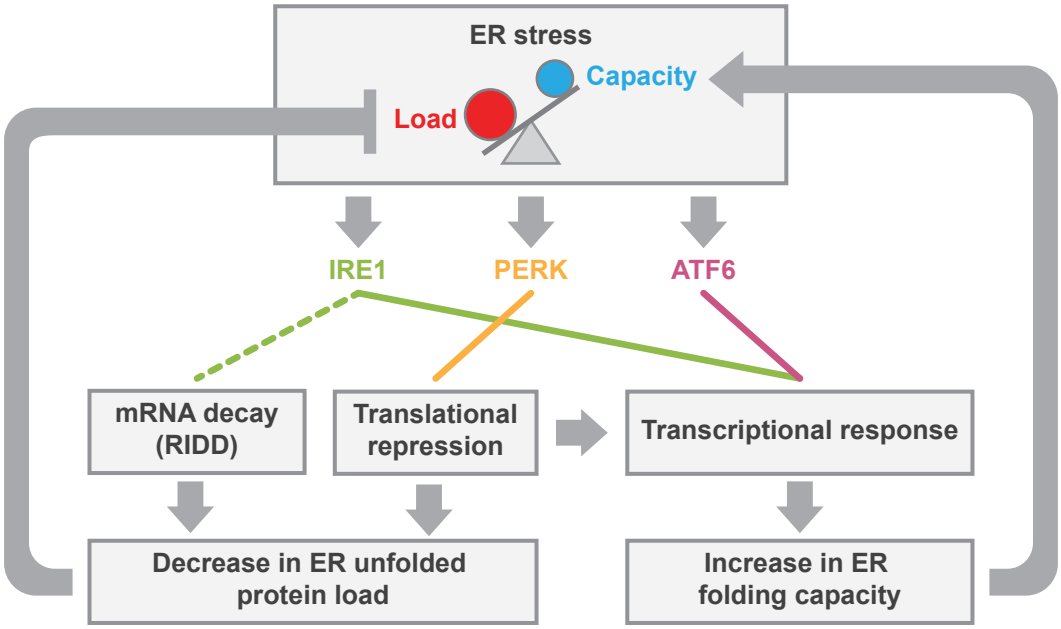
B) Chaperone inhibition model. Step 1: ER-localized J-proteins (exemplified by ERdj4) associate with the LD of (dimeric) IRE1. Step 2: The J-protein recruits BiP and stimulates its ATPase activity to promote a canonical substrate interaction between BiP and IRE1. Step 3. BiP binding triggers J-protein release and disrupts IRE1 dimers causing IRE1 inactivation. BiP dissociates from IRE1 upon nucleotide exchange. Step 4: Chaperone-free IRE1 has an intrinsic propensity to dimerize and become active. J-protein-mediated loading of BiP onto IRE1 competes with loading of BiP onto unfolded substrates. This directly couples the availability of BiP (and J-proteins) to establish a dynamic pool of monomeric, inactive IRE1 to the load of unfolded proteins in the ER.

Figure 5: The eIF2(αP)-dependent Integrated Stress Response (ISR)

Translational control over client protein load is imposed by the ER stress-mediated PERK activation and phosphorylation of eIF2 on its α subunit. This figure highlights the inhibition the GEF, eIF2B, by phosphorylated eIF2 and the effects on mRNA translation of the resultant decline in availability of eIF2•GTP•$tRNA_i^{Met}$ ternary complexes. Also cartooned are the phosphatase complexes that dephosphorylate eIF2 to terminate signalling in the ISR. Targeting this phosphatase complexes for pharmacological inhibition has emerged as a potentially useful way to combat certain diseases of protein misfolding, by enhancing ISR activity.

Figure 1: Overview of the mammalian UPR

A)



B)

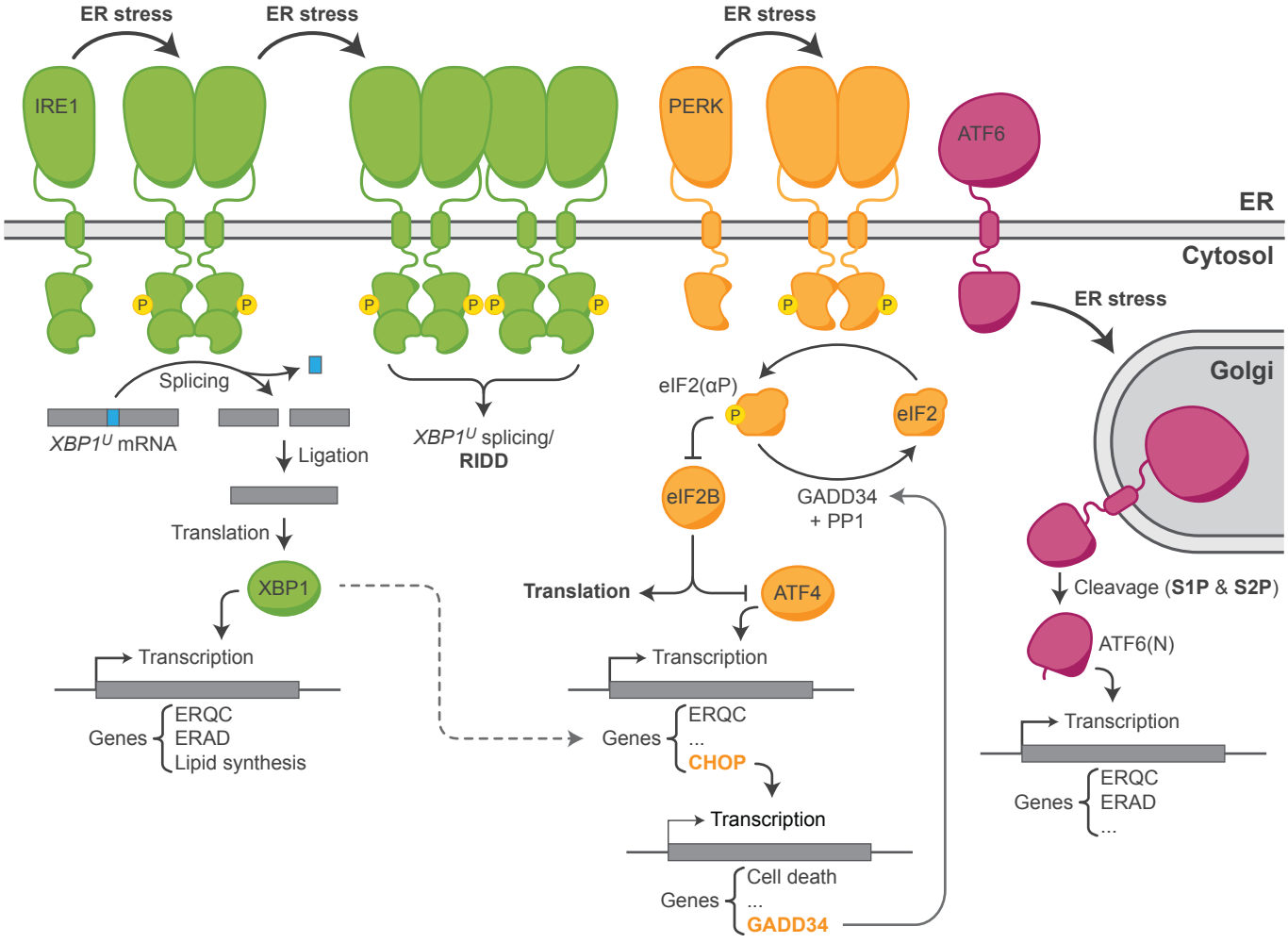
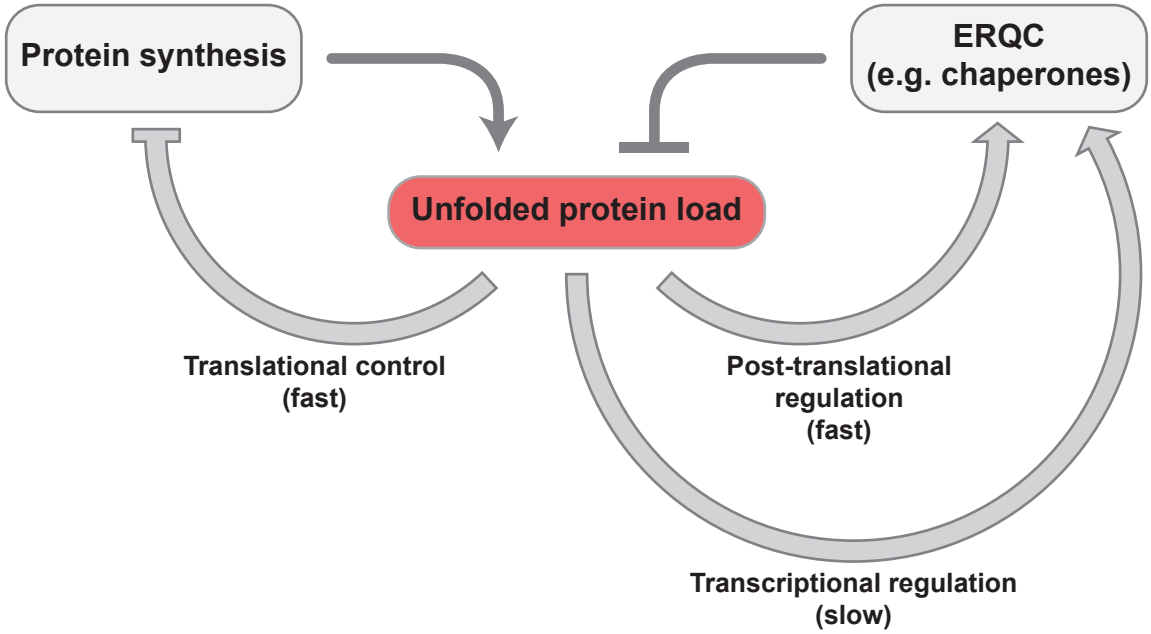


Figure 2: Latency of the negative-feedback regulated transcriptional UPR

A)



B)

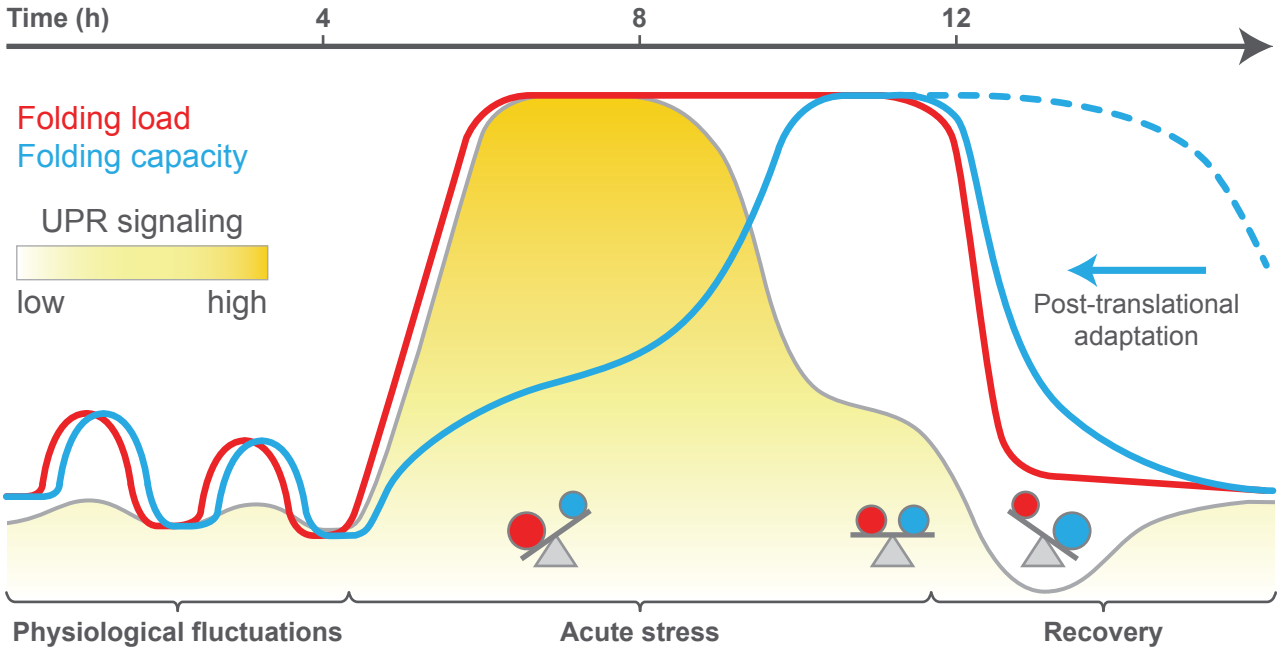
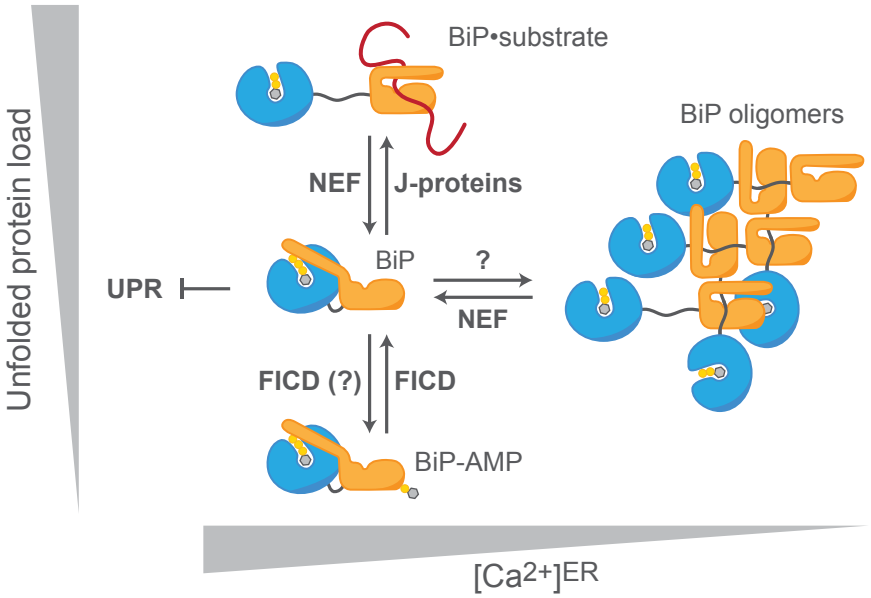


Figure 3: Post-translational regulation of BiP

A)



B)

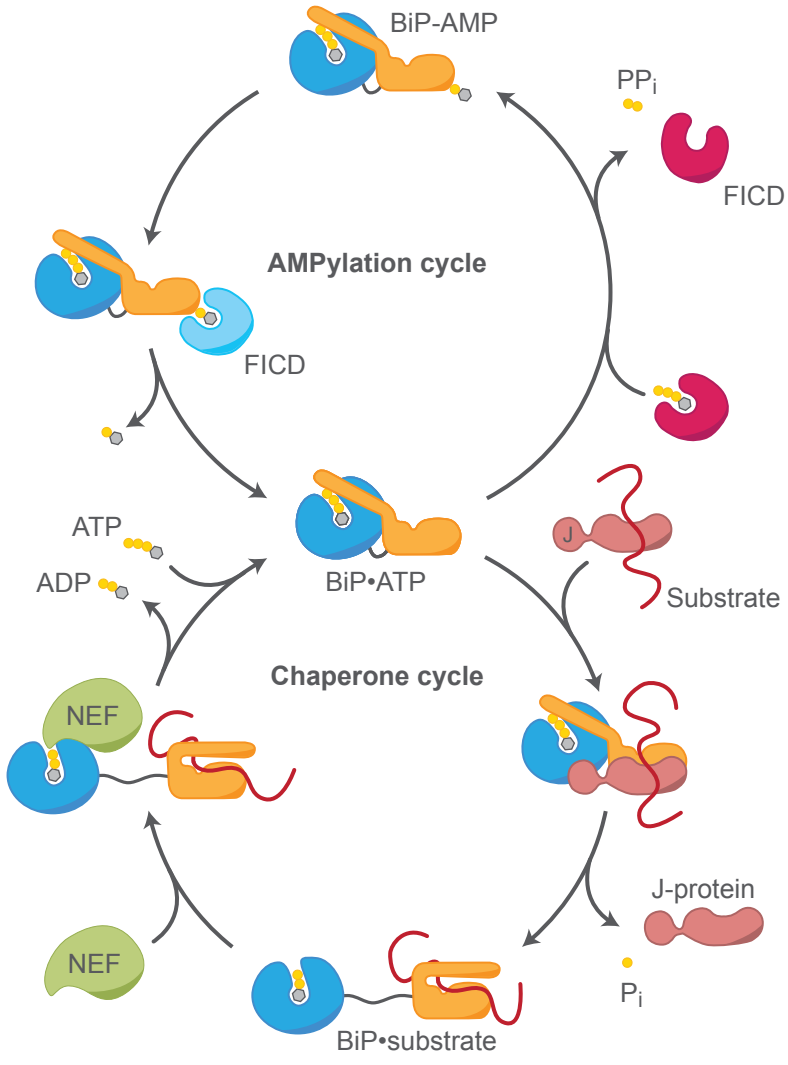
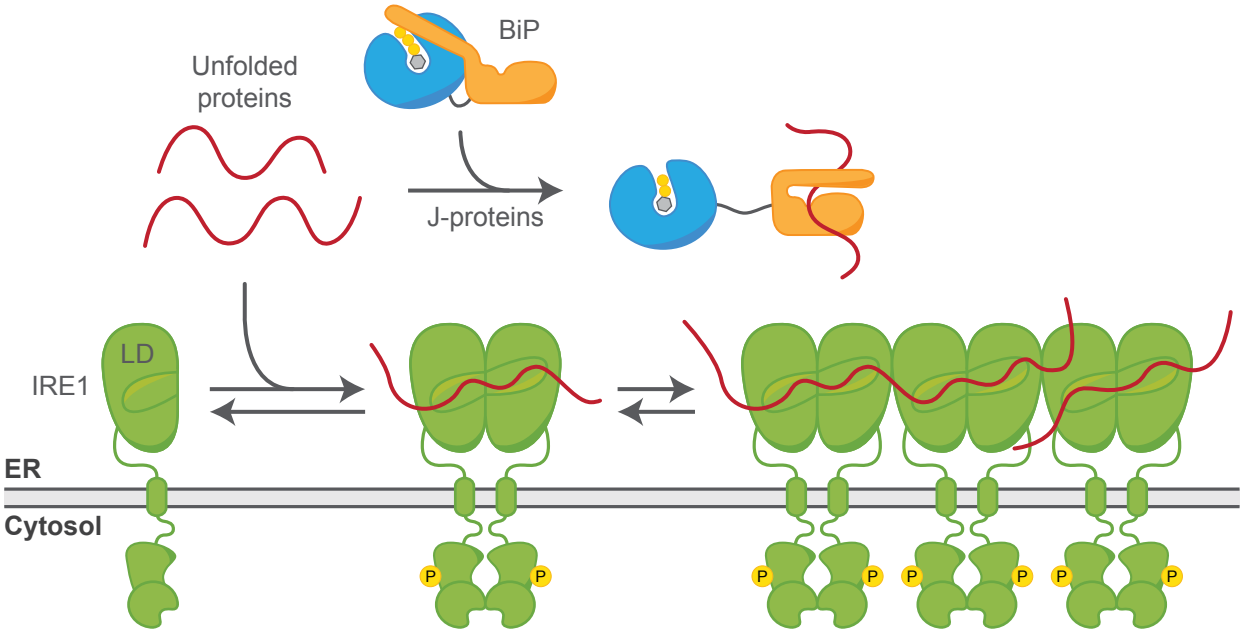


Figure 4: Role of BiP in regulating IRE1

A)



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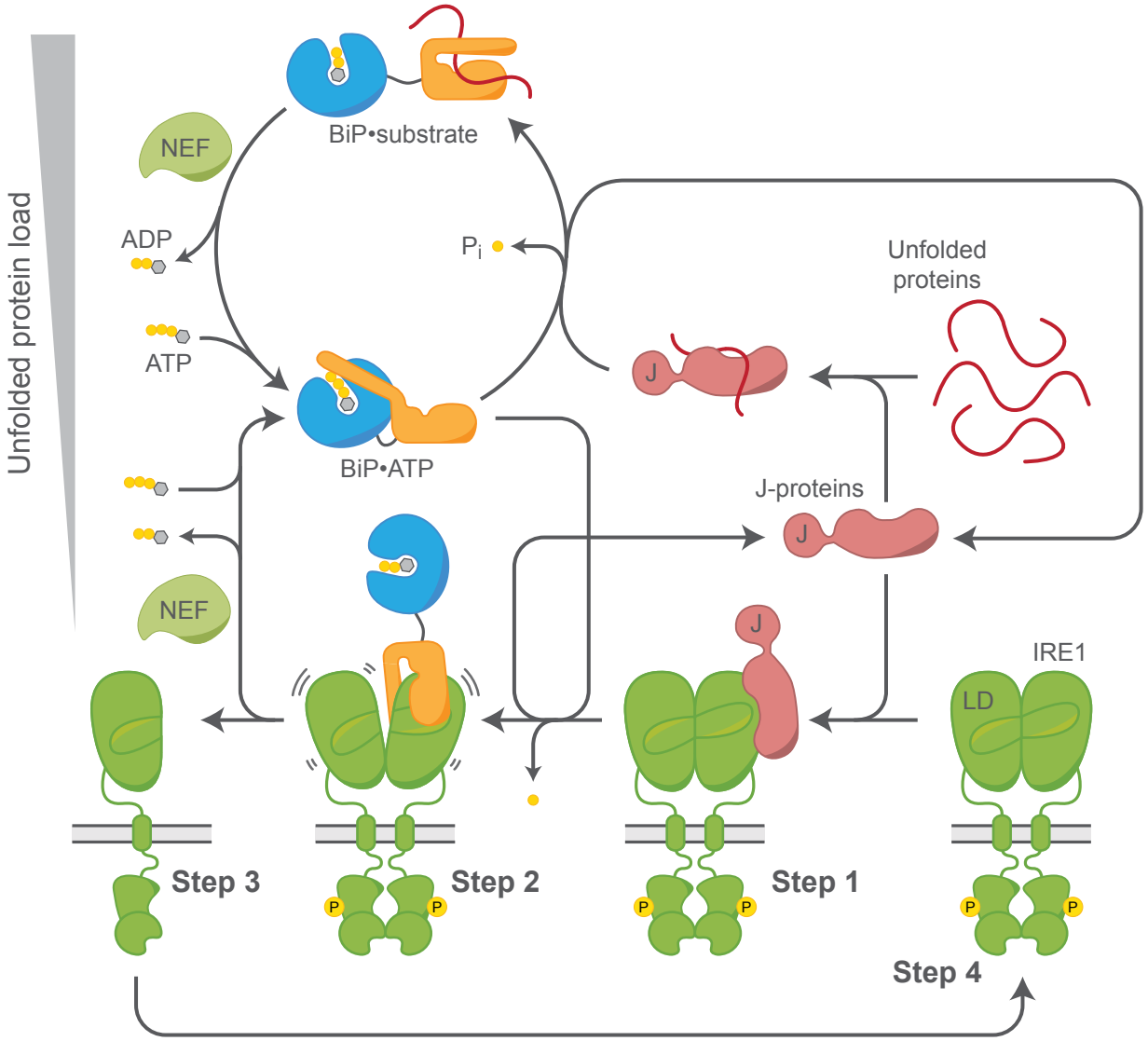
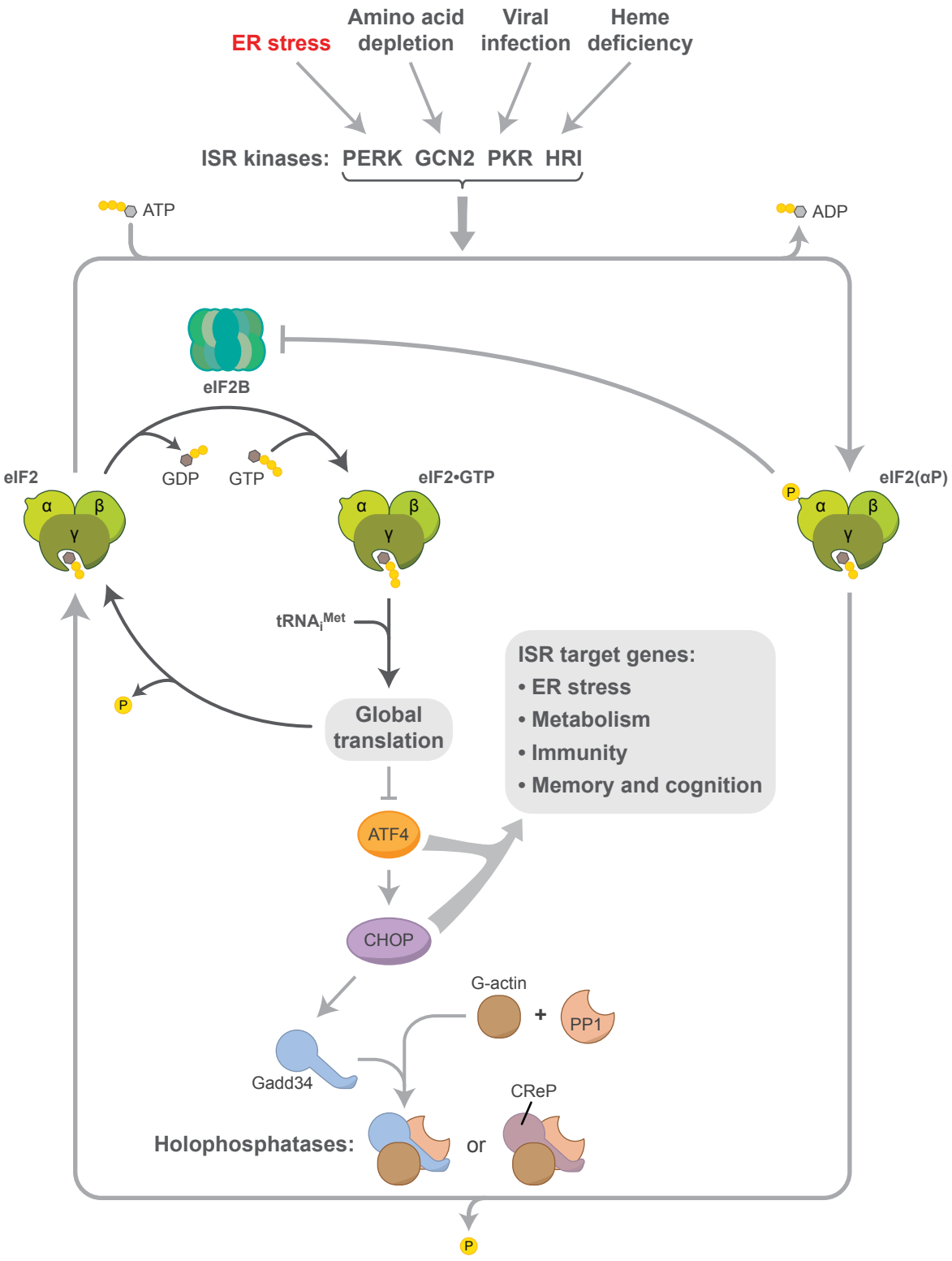


Figure 5: ISR


ER stress
Amino acid depletion
Viral infection
Heme deficiency
ISR kinases: PERK GCN2 PKR HRI
ATP
ADP
eIF2B
eIF2
GDP
GTP
eIF2•GTP
eIF2(αP)
α
β
γ
P
tRNA$_i^{Met}$
Global translation
ISR target genes:
• ER stress
• Metabolism
• Immunity
• Memory and cognition
ATF4
CHOP
G-actin
+
PP1
Gadd34
CReP
Holophosphatases:
or