



Targeting unfolded protein response signaling pathways to ameliorate protein misfolding diseases

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Protein homeostasis (or proteostasis) within the endoplasmic reticulum (ER) is regulated by the unfolded protein response (UPR). The UPR consists of three integrated signaling pathways activated by the accumulation of misfolded proteins within the ER lumen. Activation of the UPR alters ER proteostasis through translational attenuation of new protein synthesis and transcriptional remodeling of ER proteostasis pathways, providing a mechanism to adapt ER proteostasis in response to cellular stress. The capacity of the UPR to alter ER proteostasis suggests that exogenous manipulation of UPR signaling pathways offers therapeutic promise to alter the fate of pathologic proteins associated with human protein misfolding diseases. Here, we discuss the therapeutic potential of exogenous UPR activation to treat human disease and highlight specific small molecule approaches for regulating UPR signaling that could be beneficial to treat protein misfolding diseases.

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Endoplasmic reticulum proteostasis and protein misfolding diseases

Nearly one-third of the human proteome is targeted to the endoplasmic reticulum (ER) for folding and trafficking to downstream environments of the secretory pathway, such as the plasma membrane and the extracellular space. These proteins are involved in numerous essential biological processes including cell–cell communication, inflammatory signaling, and immunological signaling.

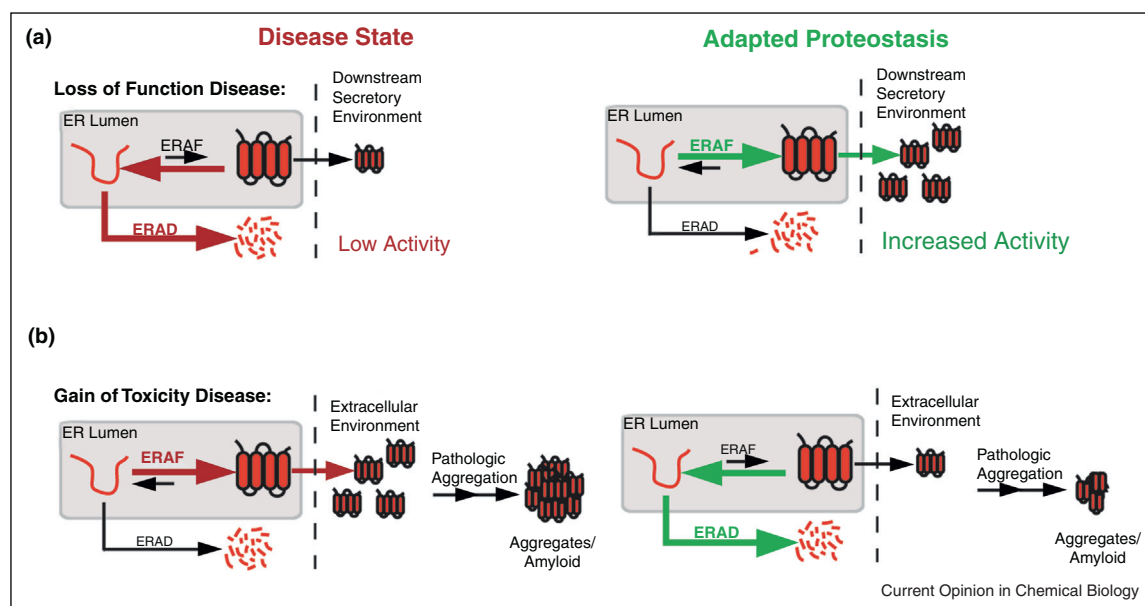
Thus, the maintenance of ER protein homeostasis (or proteostasis) is a critical factor in organismal physiology.

ER proteostasis is maintained by the partitioning of polypeptide clients between biological pathways involved in ER-assisted folding (ERAF) and ER-associated degradation (ERAD) [1–3]. Newly synthesized proteins, cotranslationally translocated into the ER lumen as unfolded polypeptides, interact with an ER-localized network of chaperones and folding enzymes (ERAF pathways) that facilitate the proper folding of proteins into their native three-dimensional structures. Once folded, proteins are incorporated into COPII vesicles for trafficking to downstream environments of the secretory pathway. Proteins unable to obtain a folded conformation in the ER are recognized by components of the ERAD pathway and retro-translocated to the cytosol where they are degraded by the cytosolic proteasome. Thus, the partitioning of proteins between ERAF and ERAD is a critical determinant in defining the integrity of proteostasis in the ER and throughout the secretory pathway.

Imbalances in the partitioning of destabilized mutant proteins between ERAD and ERAF can result in pathology associated with numerous human protein misfolding diseases [4,5]. Loss-of-function protein misfolding diseases, such as cystic fibrosis and the lysosomal storage diseases, can be attributed to the excessive degradation of destabilized proteins, preventing their proper folding and trafficking (Figure 1a). Thus, pathology results directly from the low activity of these proteins in their downstream functional environments. Alternatively, gain-of-toxicity protein misfolding diseases, including the amyloidoses, often result from the efficient folding and trafficking of destabilized, aggregation-prone protein variants (Figure 1b). This efficient trafficking leads to high extracellular concentrations of misfolding-prone proteins, facilitating extracellular formation of large insoluble aggregates and amyloid fibrils that appear to cause post-mitotic tissue loss in these disorders.

These imbalances have prompted many researchers to speculate about whether exogenous manipulation of ER proteostasis would offer a potential therapeutic strategy to prevent the aberrant degradation or ER protein folding/trafficking intricately involved in these disorders [4,5]. For example, increasing the pro-folding ERAF pathway/activity(ies) offers a potential strategy to prevent the aberrant ERAD of disease-associated mutants involved in loss-of-function protein misfolding diseases (Figure 1a).

Figure 1



Adapting the proteostasis capacity of ERAF and ERAD pathways can attenuate the aberrant ER protein folding, trafficking or degradation processes involved in human protein misfolding disease pathology. **(a)** Left, illustration showing the excessive ERAD for destabilized, mutant proteins involved in loss-of-function protein misfolding diseases. Premature ERAD reduces trafficking of these mutants to their downstream functional environments, resulting in pathology that stems from low protein activities in their native environments. Right, illustration showing that increasing ERAF activity could attenuate the premature degradation of destabilized mutant proteins and increase trafficking to their downstream functional environment, allowing for increased protein activity. **(b)** Left, illustration showing the efficient folding and trafficking of destabilized, mutant proteins involved in gain-of-toxicity protein misfolding diseases. Efficient trafficking leads to high extracellular concentrations that facilitate pathologic concentration-dependent aggregation. Right, illustration showing that increasing ERAD activity could attenuate the secretion of these destabilized mutant proteins, reducing extracellular concentrations and decreasing concentration-dependent aggregation.

Alternatively, increasing ERAD activity could potentially reduce the secretion of destabilized, aggregation-prone proteins linked to degenerative disorders (Figure 1b). While many strategies to target specific aspects of ER proteostasis have been proposed, here we focus on evaluating the potential for activating the unfolded protein response (UPR), the endogenous stress-responsive signaling pathway responsible for regulating ER proteostasis, to attenuate the aberrant ER protein folding, trafficking or degradation associated with protein misfolding pathology.

UPR activation and ER proteostasis

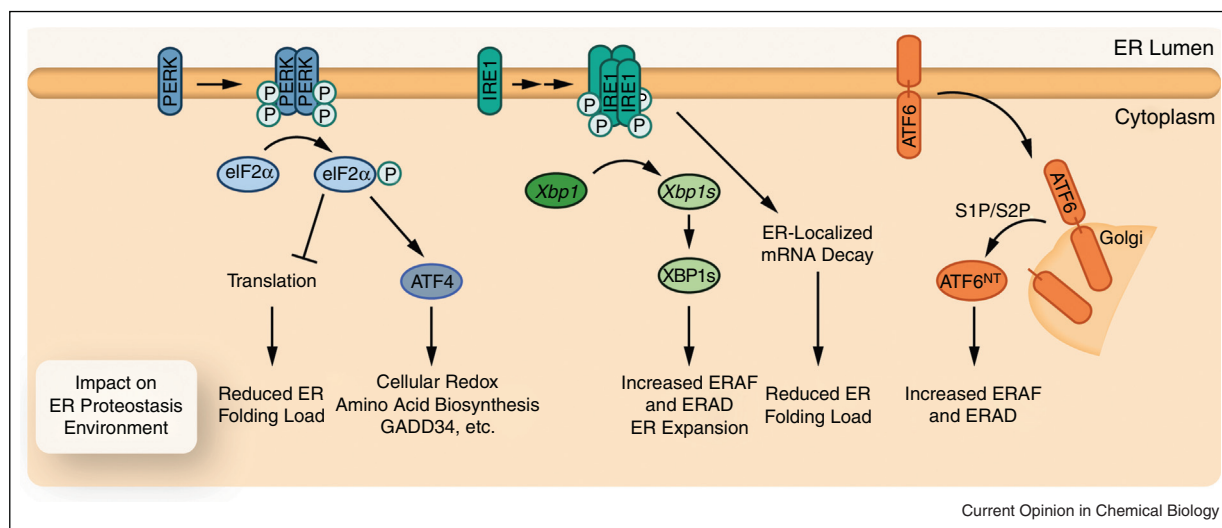
The UPR comprises three integrated signaling pathways that emanate from the ER-localized transmembrane proteins: inositol-requiring protein-1 (IRE1), PKR-like ER kinase (PERK), and activating transcription factor 6 (ATF6) (Figure 2) [6–9]. These three proteins act as sensors of the ER proteostasis environment and are activated by the accumulation of misfolded proteins within the ER lumen. Misfolding can be induced by environmental, genetic, developmental or aging-related stress. Activation of the UPR adapts ER proteostasis through translational attenuation and transcriptional remodeling of the ER proteostasis network. While many good reviews have highlighted the mechanisms of UPR

activation and the involvement of UPR activation in the context of human disease (e.g. [6–9]), here we focus on highlighting the functional implications for UPR activation to adapt ER proteostasis in the context of human protein misfolding diseases.

PERK-mediated translational attenuation promotes ER proteostasis by reducing the protein folding load

UPR-dependent translational attenuation is primarily mediated through the activation of PERK, by a well-described mechanism involving dimerization and *trans*-autophosphorylation [6–9] (Figure 2). Once activated, a cytosolic PERK kinase domain selectively phosphorylates the α subunit of eukaryotic initiation factor 2 (eIF2 α). Phosphorylated eIF2 α inhibits the activity of the eIF2B translation initiation complex, attenuating ribosomal translation and reducing the population of newly synthesized proteins entering the ER [6–9]. eIF2 α phosphorylation also leads to the translation of a selective set of stress-responsive transcription factors such as activating transcription factor 4 (ATF4) that increases expression of genes involved in basic aspects of cellular proteostasis, including cellular redox regulation, amino acid biosynthesis, and the eIF2 α phosphatase regulatory subunit GADD34 — a protein involved

Figure 2



Activation of the three UPR signaling arms differentially influences ER proteostasis capacity. Illustration of downstream consequences of PERK, IRE1, or ATF6 activation on ER proteostasis. PERK activation leads to a reduced protein folding load mediated through translational attenuation and an increase in global proteostasis capacity mediated by the ATF4-dependent induction of genes involved in general cellular proteostasis maintenance including cellular redox regulation, amino acid biosynthesis, and negative feedback regulation of translational attenuation (i.e. GADD34). IRE1 activation increases ERAF/ERAD activity and induces ER expansion through the downstream activation of XBP1s. Furthermore, IRE1 can potentially attenuate ER protein folding load through the degradation of ER-localized mRNA. ATF6 activation leads to the cleavage of the active ATF6^{NT} transcription factor that primarily induces genes involved in ERAF and ERAD pathways.

in the suppression of eIF2α phosphorylation in a negative feedback signaling loop [6–9].

PERK-dependent translational attenuation directly impacts ER proteostasis by reducing the concentration of newly synthesized, unfolded protein clients entering the ER (Figure 2). This reduced translation in turn attenuates the burden on ERAF and ERAD pathways, freeing components of these pathways to influence the folding, trafficking and degradation of unfolded or misfolded proteins in the ER by increasing the proteostasis network component/polypeptide client ratio. This capacity to reduce the protein folding and degradation load offers a potential mechanism to restore the aberrant ER proteostasis associated with human protein misfolding diseases. For example, preemptive PERK activation increases secretion of a destabilized fibulin-3 mutant involved in the pathology of the rare macular dystrophy malattia leventinese [10]. Presumably this results from increasing the ERAF component/client ratio in the ER lumen, although the specific molecular mechanism(s) remains to be determined. Activation of PERK also appears to be required for the UPR-dependent increased secretion of destabilized mutants of β-glucocerebrosidase involved in Gaucher disease, a loss-of-function disorder [11]. These results and others indicate that promoting PERK-mediated translation attenuation could be beneficial for adapting ER proteostasis in the context of ER protein misfolding diseases. However, there is one

significant drawback to therapeutic PERK activation, as eIF2α phosphorylation also leads to activation of CHOP — a pro-apoptotic transcription factor that increases the probability of cellular apoptosis following prolonged ER stress [6–9].

Activation of IRE1 and/or ATF6 results in distinct ER proteostasis network environments that can be therapeutically accessed to ameliorate ER misfolding diseases

Activating the IRE1 and/or ATF6 stress sensors primarily influences ER proteostasis through the activation of downstream transcription factors that remodel the ER proteostasis network via a transcriptional program. Activation of IRE1, the most evolutionarily conserved arm of the UPR, involves oligomerization and *trans*-autophosphorylation, resulting in the activation of a cytosolic IRE1 endoribonuclease domain that is required for the splicing of *Xbp1* mRNA [6–9] (Figure 2). Spliced *Xbp1* encodes the bZIP transcription factor, XBP1s, that induces the expression of genes involved in nearly all aspects of ER proteostasis, including translocation, ERAF, COPII-trafficking, and ERAD [12,13]. IRE1-dependent XBP1s splicing is also required for other aspects of ER proteostasis maintenance, including the ER expansion essential for the development of plasma cells [13–15]. IRE1 activation can also lead to regulated IRE1-dependent decay (RIDD), a promiscuous mRNA ribonuclease activity that targets mRNAs encoding proteins

directed to the ER [16–19]. RIDD activity may provide an alternative mechanism to reduce the folding and degradation load in the ER, analogous to that observed for PERK-mediated translational attenuation. While the mechanism of this divergent, multi-tiered IRE1 response requires further study, it has been suggested that IRE1 phosphorylation and oligomerization states are important for the relative *XBP1* splicing and RIDD activities [16,19].

ATF6 also influences ER proteostasis through direct remodeling of the ER proteostasis network. ATF6 is activated by trafficking full-length ATF6 from the ER to the Golgi, where it is proteolytically processed by the Site 1 and Site 2 proteases [6–9] (Figure 2). This processing releases the N-terminal, cytosolic domain of ATF6 (ATF6^{NT}) comprising an active bZIP transcription factor, which induces expression of ER proteostasis genes primarily involved in ERAF and ERAD [20,21]. While both *XBP1*s and ATF6^{NT} function to remodel ER proteostasis pathways, the gene sets induced by these transcription factors are distinct, suggesting that activation of *XBP1*s or ATF6^{NT} uniquely impacts the activity of ER proteostasis pathways [12,13,20,21]. These transcription factors can also heterodimerize. The heterodimer is predicted to selectively increase expression of genes involved in specific aspects of ER proteostasis maintenance, such as ERAD [22]. Thus, the independent or combined activation of IRE1–*XBP1*s and/or ATF6 could result in three distinct ER proteostasis environments that could be therapeutically accessed to ameliorate the aberrant ER protein folding, trafficking or degradation of destabilized mutant proteins involved in human protein misfolding diseases.

A challenge for evaluating the potential of IRE1–*XBP1*s and/or ATF6 activation to influence ER proteostasis is that each disease-associated protein has a unique dependence on specific ERAF and ERAD pathways, sometimes differentially influenced by arm-selective UPR signaling. Thus, the impact of activating IRE1–*XBP1*s and/or ATF6 on ER proteostasis for a specific disease-associated protein client cannot be easily determined *a priori*. Regardless, significant evidence is emerging that demonstrates targeting IRE1–*XBP1*s or ATF6 influences the ER proteostasis of mutant disease-associated proteins. Activation of either IRE1–*XBP1*s or ATF6 reduces the intracellular aggregation of disease-associated rhodopsin mutants, potentially indicating that activating these pathways can attenuate the photoreceptor cell death associated with autosomal dominant retinitis pigmentosa [23^{••},24^{••}]. IRE1, but not ATF6, is required for the UPR-dependent increase in β -glucocerebrosidase mutant trafficking in cellular models of Gaucher disease [11[•]]. Furthermore, ATF6^{NT} overexpression increases ERAD of the destabilized α 1-antitrypsin (A1AT) mutant, PiZZ, and attenuates PiZZ aggregation, indicating that ATF6

activation could be an approach to ameliorate the pathologic aggregation of this protein involved in A1AT-related liver disease [25^{••}]. Collectively, these results highlight the unique sensitivity of destabilized mutant proteins to *XBP1*s-dependent and/or ATF6^{NT}-dependent remodeling of the ER proteostasis environment and demonstrate the potential for activating these transcription factors to influence ER proteostasis in the context of protein misfolding diseases.

Therapeutic targeting of UPR signaling arms to ameliorate protein misfolding diseases

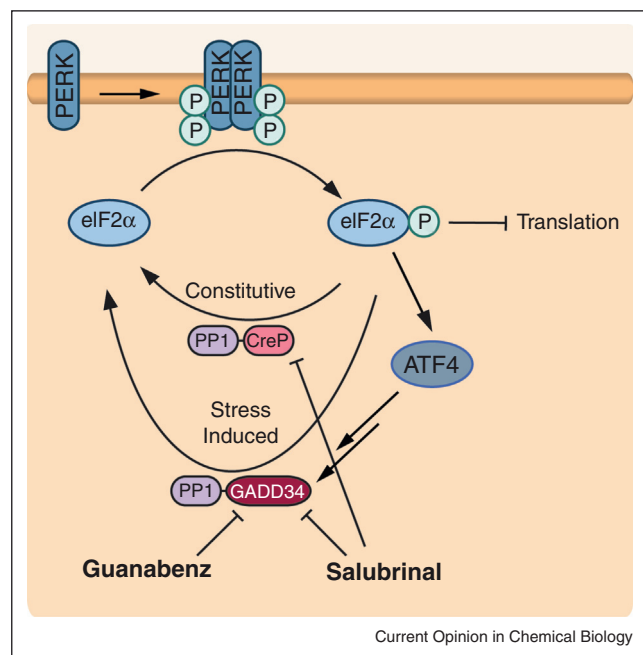
Evidence for the therapeutic relevance of arm-selective UPR activation has resulted in a significant effort to identify small molecule regulators of the UPR signaling arms as potential therapeutic strategies to treat protein misfolding and other human diseases. We highlight some of the most relevant pharmacologic or chemical biological approaches to modulate UPR signaling pathways below.

Targeting PERK-mediated translational attenuation

To date, no small molecule activator of PERK has been identified, although small molecule PERK inhibitors have been recently described [26,27]. Small molecule regulation of PERK-dependent translational attenuation has been achieved by targeting the two eIF2 α phosphatase complexes, which are composed of protein phosphatase 1 (PP1) and either the constitutively expressed regulatory subunit, CreP, or the stress-induced regulatory subunit, GADD34 [7,9] (Figure 3). The first described inhibitor was salubrinal, which was found to inhibit both PP1–CreP and PP1–GADD34 phosphatase complexes [28[•]]. Inhibition of the constitutive PP1–CreP complex allows salubrinal to induce eIF2 α -mediated translational attenuation in the absence of stress. Salubrinal-dependent continuance of eIF2 α phosphorylation decreases viral replication, suggesting an increased capacity to regulate ER proteostasis and viral production through translational attenuation.

A potential limitation of the increase in eIF2 α phosphorylation afforded by salubrinal is the potential for prolonged eIF2 α phosphorylation to increase expression of pro-apoptotic genes. Interestingly, guanabenz, an α 2-adrenergic receptor agonist used in the treatment of hypertension, avoids this potential pitfall by *selectively* inhibiting the stress-induced PP1–GADD34 eIF2 α phosphatase complex [29^{••}]. The selective inhibition of PP1–GADD34 means that guanabenz alone does not induce eIF2 α phosphorylation in the absence of stress, but instead prolongs the translational attenuation observed following stress-dependent activation of PERK or other eIF2 α kinases. This allows guanabenz to selectively modulate stress-responsive PERK signaling, potentially providing a mechanism to manipulate ER proteostasis in the context of ER stress-induced UPR activation. For example, guanabenz treatment increased survival of

Figure 3



Small molecule modulation of PERK signaling can be mediated by targeting eIF2 α phosphatase complexes. PERK activation increases eIF2 α phosphorylation, which in turn attenuates translation and increases expression of stress-responsive transcription factors. This pathway is negatively regulated by a phosphatase complex between protein phosphatase 1 and the constitutively expressed regulatory subunit CreP (PP1-CreP) and/or a stress-induced regulatory subunit GADD34 (PP1-GADD34). Small molecules that target these complexes can modulate eIF2 α -dependent signaling, effectively mimicking PERK activation. Salubrinal inhibits both the PP1-CreP and PP1-GADD34 phosphatase complexes, allowing for increased eIF2 α phosphorylation in the absence of stress. Guanabenz selectively targets PP1-GADD34, providing a mechanism to prolong PERK-dependent eIF2 α phosphorylation signaling activation in response to ER stress. Figure adapted from Wiseman *et al.* [39].

Min6 and Ins1 cells overexpressing the Akita insulin mutation, suggesting that modulating stress-induced translational attenuation may provide a therapeutic approach to attenuate pancreatic beta cell death involved in diabetes [29^{**}].

Therapeutic targeting of the IRE1-XBP1s or ATF6 signaling arms

The influence of IRE1 signaling on numerous human diseases has resulted in a significant focus on identifying small molecule modulators of IRE1 signaling, both activators and inhibitors, the latter having the potential to ameliorate the clonal expansion of plasma B cells pathologically linked to cancers such as multiple myeloma [30^{*}]. Much of this work has focused on targeting the IRE1 nucleotide binding pocket to regulate IRE1 activity. Historically, focus on the nucleotide binding pocket stems from experiments showing that mutating the pocket using ‘bump-hole’ technology allowed the

identification of an IRE1 mutant that could be activated by the addition of an exogenous complementary nucleotide analog, clearly demonstrating that IRE1 activation could be induced by binding to the kinase binding site [31^{**},32^{**}]. Kinase inhibitors that bind to the nucleotide binding pocket can activate the IRE1 endoribonuclease domain, although the selectivity of these kinase inhibitors for IRE1 remains to be clarified [19,33,34^{**}]. Interestingly, recent evidence suggests that type I and type II kinase inhibitors that stabilize different active site kinase conformations have distinct effects on IRE1 activity [34^{**}], although the underlying molecular mechanism of these diverse effects remains to be determined. Type I kinase inhibitors appear to activate the IRE1 RNase activity while type II inhibitors inhibit IRE1 RNase activity, indicating that therapeutic targeting of the IRE1 nucleotide binding pocket could allow for either the activation or inhibition of IRE1.

Apart from the nucleotide binding pocket, other sites on IRE1 have been identified that could be targeted to influence IRE1 RNase activity. The small molecule quercetin activates yeast Ire1 by binding to a small molecule allosteric regulatory site on the dimeric interface of the IRE1 RNase domain [35^{*}]. This unique small molecule binding pocket could be used to modulate IRE1 activity *in vivo*, although a similar site has yet to be characterized in mammalian IRE1. Alternatively, small molecule inhibitors of IRE1 have been found to bind directly to the RNase active site through the formation of reversible covalent interactions [30^{*},36^{**},37^{*}]. The capacity to target the RNase active site or the quercetin binding sites offers the potential for developing highly selective small molecule modulators of IRE1 signaling — a difficult challenge when targeting the more widely conserved nucleotide binding pocket.

The lack of identifiable small molecule binding sites on ATF6 has significantly challenged the pharmacologic inhibition or activation of ATF6. Currently, we are aware of only one small molecule that appears to activate the ATF6 arm of the UPR, BIX [38]. The addition of BIX increases expression of ATF6 target genes in an ATF6-dependent manner and does not appear to activate the IRE1-XBP1s or PERK arms of the UPR. However, the mechanism of BIX-dependent induction of ATF6 target genes remains unknown and the potential for this molecule to adapt ER proteostasis through ATF6 activation has not been explored. We anticipate that as more studies demonstrate the therapeutic potential of ATF6 activation to adapt ER proteostasis of disease associated proteins, more effort will be directed toward identifying selective, small molecule ATF6 activators.

Concluding remarks

While there is emerging evidence suggesting that arm-selective UPR activation is a viable therapeutic approach

to alter the aberrant ER protein folding, trafficking or degradation involved in a variety of human protein misfolding diseases, many critical questions still remain that must be addressed before aggressively applying this therapeutic strategy to treat these maladies. For example, how does activation of IRE1, ATF6, or PERK influence the folding and trafficking of the endogenous secreted proteome? While destabilized misfolding-prone proteins appear to be sensitive to activation of these stress-responsive signaling pathways, it is unclear how activation of these pathways will influence the folding and trafficking of the nearly one-third of the endogenous human proteome targeted to the ER. Furthermore, additional studies need to be focused on characterizing the transcriptional output of these pathways on the activity of the ER proteostasis network. While transcriptional profiling has identified specific transcriptional targets induced by these pathways, how this transcriptional remodeling alters the composition, and thus activity, of ER proteostasis pathways remains to be determined. Finally, more studies are required to explore the advantages of IRE1, ATF6 or PERK activation for altering the folding, trafficking or degradation of destabilized pathologic proteins in the ER lumen. Identifying classes of protein misfolding diseases that are potentially treatable by IRE1, PERK, and/or ATF6 activation will further motivate the identification of small molecule activators of these pathways, as one pharmacologic agent could be used for treating multiple maladies of similar origin. Currently, we are just beginning to understand the power of activating individual UPR signaling arms in the context of protein misfolding diseases, and we believe that in the coming years more studies will continue to emerge that highlight the potential for this approach to treat these devastating disorders.

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