Targeting Phosphorylation of Eukaryotic Initiation Factor-2α to Treat Human Disease

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The unfolded protein response, also known as endoplasmic reticulum (ER) stress, has been implicated in numerous human diseases, including atherosclerosis, cancer, diabetes, and neurodegenerative disorders. Protein misfolding activates one or more of the three ER transmembrane sensors to initiate a complex network of signaling that transiently suppresses protein translation while also enhancing protein folding and proteasomal degradation of misfolded proteins to ensure full recovery from ER stress. Gene disruption studies in mice have provided critical insights into the role of specific signaling components and pathways in the differing responses of animal tissues to ER stress. These studies have emphasized an important contribution of translational repression to sustained insulin synthesis and β-cell viability in experimental models of type-2 diabetes. This has focused attention on the recently discovered small-molecule inhibitors of eIF2α phosphatases that prolong eIF2α phosphorylation

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to reduce cell death in several animal models of human disease. These compounds show significant cytoprotection in cellular and animal models of neurodegenerative disorders, highlighting a potential strategy for future development of drugs to treat human protein misfolding disorders.

I. Introduction

Cells face frequent fluctuations in the availability of nutrients and other environmental factors. Thus, mammalian cells have developed complex signaling mechanisms to detect such environmental disturbances and adjust their physiology appropriately to survive these stresses. Common to all eukaryotes is their ability to attenuate protein translation in response to stress. As protein synthesis places a high demand on their energy supply, this allows cells to temporarily focus their attention on the task of overcoming the stress. Analyzing the cell's response to misfolded proteins, a common everyday occurrence, can elucidate the signaling pathways that oversee protein synthesis and quality control. These stress response pathways are greatly exacerbated in diseases associated with protein overload, expression of folding-defective proteins, or errors in protein processing. In this review, we discuss these signaling mechanisms and, in particular, highlight the phosphorylation of the eukaryotic translation initiation factor eIF2α, which results in translational attenuation, an early event triggered by many different forms of cell stress. Genetic evidence in mice and humans suggest that the inability to catalyze eIF2α phosphorylation may contribute to diseases such as diabetes. Finally, we discuss the recent discovery of small molecules that enhance eIF2α phosphorylation and protect cells from the damaging effects of protein misfolding. These discoveries raise new hopes for the development of novel treatments for human diseases, including diabetes, cancer, and neurodegenerative disorders.

II. Control of Protein Translation

Timely synthesis of proteins and their folding into active three-dimensional structures are absolutely critical for the normal functioning and growth of all eukaryotic cells. Mammalian cells utilize a complex array of regulatory mechanisms, including the reversible phosphorylation of several initiation and elongation factors, as well as many ribosomal proteins, to control protein synthesis.[1]

Iron deficiency is one the most prevalent nutritional deficiencies globally and is a major cause of life-threatening anemias. Nearly 40 years ago, Irving London and colleagues studying the synthesis of the globin polypeptides *in vitro* in rabbit reticulocyte lysates established the presence of a heme-regulated inhibitor (HRI) that is activated by iron deficiency or the absence of heme, a member of the porphyrin family and a cofactor for hemoglobin.[2] Subsequent work showed that HRI was a protein kinase that phosphorylated the translational initiation factor eIF2α and prevented the assembly of an initiation complex comprised of mRNA, ribosomal subunits, and methionyl-tRNA, thereby inhibiting the synthesis of α- and β-globins. When the rabbit HRI cDNA was cloned,[3] it was noted that the primary sequence encoded by this cDNA bore remarkable homology to the yeast GCN2 (general control nonderepressible) protein. The *Saccharomyces cerevisiae* GCN2 gene also encoded a protein kinase, which was activated by amino acid starvation. The buildup of uncharged tRNAs that occurs as a result of amino acid deprivation activates Gcn2, which phosphorylates the yeast eIF2α (also known as Sui2) to inhibit general protein synthesis and elicit cell cycle arrest. At the same time, the increased eIF2α phosphorylation enhances the translation of the transcription factor Gcn4 and the transcription of yeast enzymes that promote amino acid biosynthesis.[4,5] In this manner, eIF2α phosphorylation allows yeast to overcome the deficiency in amino acids in the external media. Later studies have established that GCN2 is also present in mammalian cells and is activated by nutrient deprivation and other stresses.[6,7]

The primary structure of the HRI protein also shares sequence homology with an inhibitory protein factor termed dsI, which is found in cells infected with viruses. This factor also displays the properties of a protein kinase and is activated by double-stranded RNAs present in the virus-infected cells. The dsI protein is also induced by interferons, cytokines released by host cells to communicate the presence of a viral (or bacterial) infection to recruit immune cells, and aids the eradication of these pathogens. The primary role of dsI, also known as double-stranded RNA-dependent eIF2α kinase (or PKR), is to phosphorylate eIF2α and inhibit protein synthesis in the virus-infected cells.[8] Thus, PKR functions as a component of the host surveillance system that inhibits viral replication and limits the spread of infection to other cells and tissues. Interferons and PKR also play a role in fighting cancers.[7] Facing this challenge, viruses have evolved multiple strategies to circumvent PKR, including the expression of pseudosubstrate inhibitors, degradation of PKR protein, expression of proteins that sequestrate dsRNA, as well as the expression of a component of eIF2α phosphatase (discussed below) that overrides PKR function.[8] Interestingly, PKR can substitute for HRI in inhibiting globin synthesis in reticulocyte lysates, and both mammalian HRI and PKR can functionally replace GCN2 in budding yeast,[9] highlighting the common role of these protein kinases in translational repression.

Nearly 30 years after the discovery of HRI, the fourth eIF2α kinase was identified and termed PEK or pancreatic eIF2α kinase because of its high expression in pancreas and, more specifically, islets. Like HRI and PKR, PEK could also inhibit globin synthesis in reticulocyte lysates and mediate GCN2 signaling in yeast.[10] More recently renamed double-stranded RNA-activated protein kinase-like endoplasmic reticulum kinase or PERK, this kinase responds to the presence of unfolded proteins in the endoplasmic reticulum (ER)[11] and is a major focus of discussion in this review.

In summary, while more than a dozen protein components undergo reversible phosphorylation to regulate protein translation, either initiation or elongation, in mammalian cells, only one, namely the phosphorylation of eIF2α at serine-51, is evolutionarily conserved from yeast to humans. Moreover, in contrast to budding yeast, which responds to nutritional deprivation by activating a single eIF2α kinase, namely Gcn2, higher eukaryotes utilize four distinct eIF2α kinases (also discussed in Chapter 8) to repress protein translation in response to a wide variety of physiological and pathological stresses. Moreover, increase in eIF2α phosphorylation plays a critical role in the response of eukaryotic cells to environmental stress.

III. Cellular Mechanisms for Protein Folding

Newly synthesized proteins must be folded into their native macromolecular structure to acquire their full biological function. In this regard, the stochastic folding of a linear polypeptide chain is dictated partly by the sequence of amino acids. The primary sequence of all polypeptides confers some propensity for folding, but, in general, the energetics of protein folding is too high to permit most proteins to spontaneously adopt their native structures. The inherent folding capability of the polypeptide chain is further enhanced by molecular crowding, achieved by the high concentration of proteins in cytoplasm of all eukaryotic cells, which demands that all proteins occupy minimal space to function. The process of protein folding is, however, intrinsically inefficient and must be aided by the activity of protein chaperones present in all eukaryotic cells. Many of these are heat shock proteins whose levels increase in response to elevated temperature and other physiological stresses. Cells express a multitude of heat shock proteins, collectively described as Hsp60, Hsp70, Hsp90, and Hsp100 based on their apparent molecular size. These chaperones do not carry any specific information to fold the diverse array of cellular proteins into their native state. Rather, most chaperones associate with the hydrophobic surfaces of newly synthesized proteins to prevent their premature aggregation. Following the complete folding of their substrates, the chaperones hydrolyze adenosine triphosphate (ATP) to release the folded

proteins.[12] Thus, both protein translation and folding make heavy demands on the cell's energy. In addition, cells express lectin chaperones, protein disulfide isomerases, and protein prolyl isomersases, which also participate in the correct folding of cellular proteins.[13] The energy used for protein translation and folding, as well as the oxidative stress created by the insertion of disulfide bridges, can accumulate mitochondrial damage or dysfunction and trigger programmed cell death.[14,15]

When faced with the task of synthesizing mutant proteins that are inherently defective in protein folding, under conditions of protein overload (e. g., viral infection), or if the expression of cellular chaperones fails to keep pace with ongoing protein synthesis, intracellular protein aggregates and oxidative stress or mitochondrial dysfunction are frequently observed. These protein aggregates are often polyubiquitinated and can potentially overwhelm the protein degradation machinery, comprising the 26S proteasome, to further enhance protein aggregation.[16] The accumulation of intracellular protein aggregates are readily observed in histological samples and are a hallmark of many human diseases, including diabetes and neurodegenerative disorders.[17] When faced with elevated levels of unfolded proteins, cells activate a complex cellular response, known as the "unfolded protein response" or UPR, to promote protein folding, reduce oxidative stress, and facilitate cell survival. However, if this process is unsuccessful and excessive cell damage occurs, UPR also activates programmed cell death or apoptosis to eliminate the dysfunctional or damaged cell.[18]

IV. The Unfolded Protein Response

Proteins are the workhorses of all cellular processes and are synthesized by both cytosolic and membrane-bound ribosomes. The membrane-bound ribosomes stud the outer surface of the ER, described as the "rough" ER, which is readily visible by electron microscopy. These ribosomes are tethered to the ER via signal peptides present at the N-termini of secreted or transmembrane proteins, which are folded and further processed in the ER. The signal peptides bind signal recognition particles that transport the associated ribosome to ER. Here, the signal peptide crosses the ER membrane and is proteolytically cleaved in the lumen. Following complete synthesis of the secreted or transmembrane protein, the ribosome is released from the ER membrane and returns to the cytosol.[19]

The presence of ER and distribution of membrane-bound and cytosolic ribosomes vary widely from cell to cell. For example, erythrocytes synthesize hemoglobin, a cytoplasmic protein, and possess an abundance of ribosomes but very little ER. By contrast, plasma cells synthesize and secrete antibodies and

contain an extensive, densely packed rough ER. The traditional view that cytosolic (and nuclear) proteins are translated on cytosolic ribosomes while membrane (and secreted) proteins are translated on membrane-bound ribosomes has been challenged with the recent findings that, in addition to synthesizing secreted and transmembrane membrane proteins, the ER-bound ribosomes also translate mRNAs encoding cytoplasmic and nuclear proteins.[20] Even more remarkable is the observation that ER binding significantly enhances the efficiency of mRNA translation by ribosomes.[21] Together, this suggests that ER-bound ribosomes are a major site of protein synthesis in many mammalian cells. The smooth ER lacks ribosomes and plays a key role in the metabolism of lipids, steroids, and carbohydrates, as well as in controlling calcium homeostasis and drug metabolism. Although the molecular basis is still poorly understood, increasing evidence points to the modulation of these unrelated biochemical processes also contributing to altered protein synthesis and quality control in the ER.

The ER is a highly dynamic organelle, so that drugs that alter the cellular levels of ER-bound proteins also modify the content and morphology of the ER. For example, statins inhibit the ER-bound enzyme HMG-CoA reductase to inhibit cholesterol biosynthesis and activate the ER-associated sterol response element-binding protein (SREBP) transcription factors to induce further expression of HMG-CoA reductase. This is turn induces UPR and expansion of a crystalloid ER in many tissues.[22,23] Similarly, problems in protein misfolding lead to dramatic changes in ER content and morphology. For example, pancreatic β-cells in both diabetic mice and humans with type-2 diabetes display an extensively dilated ER with the presence of amorphous protein aggregates in the lumen.[24,25] Thus, both protein misfolding and changes in ER-localized metabolic processes induce UPR. For these reasons, the unfolded protein response is also referred to as "ER stress."

Recent studies have highlighted a membranous connection between ER and mitochondria. This mitochondrial-associated ER-membrane compartment, known as MAMs or mitochondrial-associated membranes, may transduce signals from the ER to mitochondria to dictate cell fate or the decision to initiate cell death or ensure cell survival.[26] This is a very active area of research and future studies will likely provide a better understanding of the contribution of MAMs to UPR.

V. UPR Signaling Pathways

BiP or binding immunoglobulin protein (also known as GRP78, a glucose-regulated protein) is a major ER luminal protein and among the first sensors to recognize newly synthesized or unfolded proteins in the ER lumen.[27] BiP is a

member of the Hsp70 family of chaperone proteins. Although an abundant ER protein, BiP levels are further increased by UPR and other forms of stress. This increases the overall protein folding capacity in the cell. It is noteworthy that BiP serves many other functions in the ER besides binding to unfolded proteins. For example, BiP aids the entry or translocation of proteins newly synthesized by the ER-bound ribosomes into the ER lumen. BiP recruits PDIs (protein disulfide reductases) to catalyze the formation of disulfide bridges. Elevated BiP levels facilitate the retrograde transport of the misfolded proteins back across the ER membrane to facilitate their degradation by the cytoplasmic 26S proteasome.[28] Finally, Bip functions as a regulator of three ER transmembrane proteins that serve as cellular sensors of UPR. These proteins, PERK, ATF6, and IRE1, all share homology in their luminal domains that bind BiP (Fig. 1). When there is a buildup of misfolded proteins in the ER, the disordered proteins compete for BiP binding. Thus, BiP is sequestered away from the three signal transducers,[29] activating UPR signaling to initiate the complex translational and transcriptional program, sometimes referred to as the "integrated stress response".[30,31]

A. Transient Repression of Protein Translation

One of the earliest events that follow UPR activation is the dimerization and autophosphorylation of PERK. This activates PERK, which then phosphorylates eIF2α at serine-51 and attenuates protein translation. In all eukaryotes, eIF2α associates with eIF2β and eIF2γ to form an eIF2 complex. In the presence of GTP, the eIF2 complex assembles Met-tRNA, the large and small ribosomal subunits at the ATG start site on mRNA, to form the translation initiation complex.[32] Subsequently, GTP is hydrolyzed to GDP, and the recycling of GDP to GTP catalyzed by eIF2B is critical for new rounds of translation initiation. The phosphorylation of serine-51 on eIF2α increases its affinity for eIF2B and inhibits GDP–GTP exchange.[33] Thus, the covalent modification of eIF2α attenuates general protein translation. However, translation of a subset of mRNAs remains unhindered. Previous studies have shown that the mRNA encoding the transcription factor ATF4 (activating transcription factor 4) contains upstream open reading frames (uORFs) and is more efficiently translated following eIF2α phosphorylation.[34] Under conditions of low eIF2α phosphorylation, the presence of uORFs slows the ribosome scanning, and ATF4 mRNA is either poorly translated or not at all. In the presence of elevated eIF2α phosphorylation, however, ribosomes are preferentially recruited to the downstream ATG and mRNAs containing uORFs, which include ATF4 and its downstream targets, CHOP (CAAT/enhancer binding protein homologous transcription factor, also known as GADD153 or growth arrest and DNA damage-inducible gene 153)[35] and GADD34,[36] and are more efficiently translated.

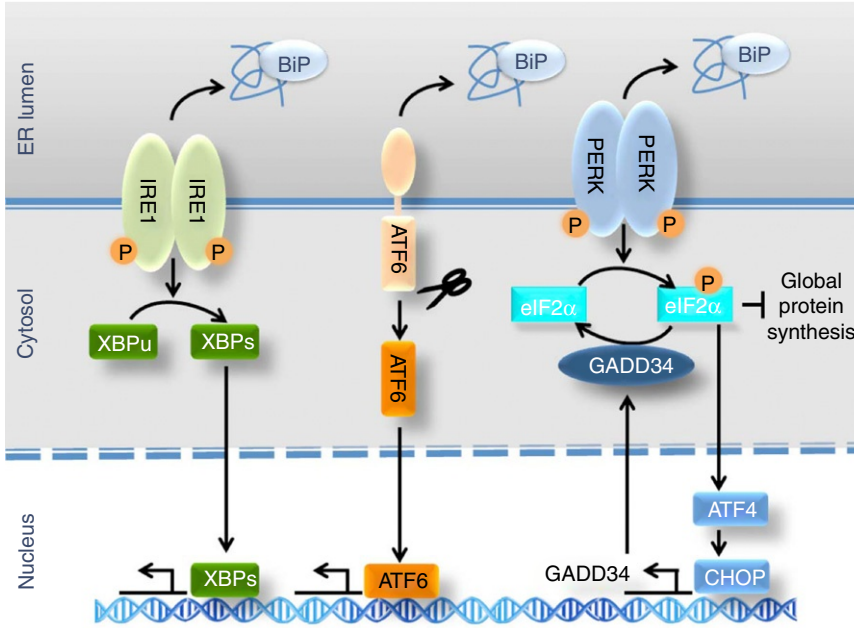

FIG. 1. The unfolded protein response (UPR). The schematic shows the activation of the three ER transmembrane sensors to transduce UPR signaling, following the sequestration of BiP by an increase in misfolded proteins in the ER. The transcriptional pathways are activated by IRE1 dimerization and autophosphorylation by the IRE1 kinase. This activates the nuclease activity associated with IRE1 to promote the splicing of the Xbp1 mRNA and association of Xbp1s transcription factor to UPRE sequences in the promoters of ERAD genes to enhance their transcription. ATF6 is transported from ER to golgi (not shown), where it is proteolysed (depicted by scissors) to release the mature ATF6 transcription factor. ATF6 translocates to the nucleus to activate chaperone genes containing ERSE sequences. The translational repression pathway is activated by PERK dimerization and autophosphorylation. PERK phosphorylates eIF2α to inhibit global protein translation. The phosphorylated eIF2α enhances the translation of ATF4, a transcription factor that promotes the expression of CHOP and GADD34, which feeds back to recruit PP1α (not shown) and enhance the dephosphorylation of eIF2α.

The primary goal of translation repression is to reduce the protein load in the ER and buy cells time to deal with the acquired burden of misfolded proteins. However, in cells experiencing severe or chronic ER stress, CHOP/GADD153, a transcription factor, activates the transcription of proapoptotic genes, such as Bim (and caspase-12), to trigger programmed cell death and thereby eliminate the irreparably damaged cells.[37] CHOP also activates the transcription of ERO1 (ER oxidoreductin I), which catalyzes the thiol–disulfide exchange with protein disufide isomerase to promote protein disufide

bridges.[38] This increases oxidative stress, which can reach lethal levels in the face of excessive protein misfolding. However, under conditions of acute or reversible ER stress, ATF4, either acting alone[39] or in conjunction with CHOP,[38] activates the transcription of GADD34. GADD34 is an ER-associated phosphatase regulatory subunit, which binds the alpha isoform of protein phosphatase-1 (PP1) to generate an active eIF2α phosphatase. Thus, GADD34 functions in a feedback loop to restore general protein synthesis,[39,40] which is essential for the continued cell growth and viability.

The eIF2α phosphatase places a strong brake on the phosphorylation of eIF2α by PERK (and other eIF2α kinases), impairing their ability to repress translation in response to a subsequent stress. Moreover, the persistent or prolonged expression of GADD34 by enhancing general protein translation has the potential to further exacerbate protein misfolding. Mathematical modeling of the PERK pathway activated by acute, reversible, or chronic terminal ER stress suggested that the instability or rapid turnover of the GADD34 protein plays a critical role in the cell's decision to die or survive.[41] This was supported by studies that highlighted the hypersensitivity of GADD34-overexpressing cells to cell death induced by oxidative stress[42] and irradiation.[43] Thus, mammalian cells have developed elaborate mechanisms, including the trafficking of GADD34 protein from cytosol to ER[44] and polyubiquitination of the α-amino group at the N-terminus of GADD34,[45] to actively degrade the protein using the 26S proteasome. The GADD34 protein is also highly phosphorylated, but the physiological role of these covalent modifications has not been defined. We speculate that GADD34 phosphorylation may also control its turnover and/or function to determine cell fate. It is worth noting that stress-activated BiP expression was markedly reduced in the GADD34-null mouse embryo fibroblasts,[46] suggesting that GADD34 may be required to complete the later transcriptional phase of UPR signaling that results in the increased expression of BiP and protection of cells from apoptosis in response to protein misfolding.

Emerging studies have indicated a critical role for PERK in the activation of autophagy,[47] which may also promote cell survival following ER stress. The precise mechanistic link between eIF2α phosphorylation and the expression of ATF4, CHOP, and GADD34 in the activation of autophagy proteins remains to be investigated.

B. Enhancing Protein Folding Capacity

The ER transmembrane protein ATF6 (activating transcription factor 6) is another UPR sensor, which when dissociated from BiP is trafficked from ER to golgi, where it is successively cleaved by site-1 and site-2 proteases, which are better known for their role in processing SREBPs,[48] transcription factors that control fatty acid and cholesterol biosynthesis.[49] The cleaved ATF6 migrates to

the nucleus and activates the transcription of genes encoding BiP and other chaperones.[50] The multiple steps involved in trafficking of ATF6 from ER to golgi and, following its proteolytic processing, entry into the nucleus mean that the ATF6 pathway is slower to activate than the PERK pathway. Moreover, BiP is a longer lived protein than ATF4, CHOP, or GADD34. Thus, the activation of the ATF6 pathway and expression of BiP precondition cells[41] and allow them to adapt better to subsequent bouts of protein misfolding, thereby ensuring their survival. High levels of BiP are often seen in diseased tissues and may reflect the presence of a chronic ER stress.

Two isoforms, ATF6α and ATF6β, are both induced by stress, with ATF6α being the predominant isoform in most cells. While targeted gene disruption in mice has suggested that ATF6α and ATF6β display redundant function,[51] other studies have shown that ATF6β expression is slightly delayed compared to ATF6α and that ATF6β attenuates ATF6α function.[52] This hints at yet another feedback loop that regulates the duration of ER stress. Studies of the ATF6α-null mice have suggested that GADD34 (and CHOP) levels are higher in tissues of the mutant mice following ER stress compared to wild-type mice, hinting that ATF6α may repress GADD34 gene transcription.[53] This also suggests that the ATF6α may sustain eIF2α phosphorylation and translational repression while increasing the cellular protein folding capacity through expression of BiP and other chaperones to ensure cell survival. Under circumstances in which the elevated levels of BiP and other chaperones fail to clear the buildup of misfolded proteins, their futile efforts at refolding proteins will likely generate an oxidative stress to damage mitochondria and trigger apoptosis.

C. Increased Degradation of Misfolded Proteins

The third ER stress sensor, IRE1, also contains a luminal domain that binds BiP. In the presence of unfolded proteins, as described above for PERK and ATF6, BiP is competed away from IRE1. IRE1 is a dual-function enzyme, containing both kinase and endonuclease activity. Upon the release of BiP, IRE1 dimerizes and autophosphorylates.[27] This results in its increased endonuclease activity. Two isoforms, IRE1α and IRE1β, are expressed in many cells, with IRE1α being the predominant isoform. IRE1α degrades ER-associated mRNAs and, thus, may also contribute to translational repression.[54] However, the major function of IRE1 is to catalyze the splicing of the mRNA encoding the transcription factor XBP1, which is the product of an ATF6-induced gene.[31] This allows the mature XBP1 protein to be translated and subsequently to activate the transcription of genes encoding components of the ER-associated protein degradation (ERAD) machinery. ERAD is associated with the translocation of misfolded proteins from the ER lumen to cytosol for degradation by the 26S proteasome.[55]

One gene activated by XBP1 is p58IPK, a member of the Dnaj family of proteins.[56] p58IPK was first identified as a cellular factor utilized by influenza virus to antagonize PKR and promote viral replication.[57,58] Subsequent studies showed that p58IPK targets both PKR and PERK to inhibit eIF2α phosphorylation.[59,60] In addition, p58IPK contains a signal peptide sequence that targets it to the ER lumen, where it binds BiP and functions as a cochaperone to enhance protein folding.[61] Thus, in addition to the activation of ERAD, the IRE1 signaling pathway may also terminate eIF2α phosphorylation and activate BiP function, highlighting the crosstalk and coordination of the three UPR signaling pathways.

In summary, mammalian cells use a three-pronged approach to reduce protein load, increase protein folding, and degrade misfolded proteins to ensure their survival. Emerging data suggest that the crosstalk between the three UPR signaling pathways is critical for cells to effectively manage the stress. In particular, distinct components of the three pathways work to dictate the extent and duration of eIF2α phosphorylation and translational repression required for cell recovery.[31] It is also clear that the three pathways allow different cell types to respond differently to stress. For example, the ATF6 pathway is preferentially activated by ischemia.[62] This diversity also plays a role in the susceptibility of tissues to different protein misfolding disorders.

VI. UPR and Human Disease

Numerous human diseases arise from problems in protein folding. While many mechanisms contribute to protein misfolding, these disorders can be clustered into three major groups (Table I). These include diseases caused by cells experiencing a protein overload which overwhelms the ER protein quality control machinery that normally manages the day-to-day protein folding. Mutations in proteins that compromise their ability to be folded and errors in the cellular protein processing machinery also create a chronic UPR that results in disease. Such mutations when introduced into mice can be used to model the human disease, and these mouse models highlight the dramatic activation of ER stress in diseased tissues. However, these experimental models have not provided clear information about the stress signaling pathways most relevant to the disease process, nor any hints on whether pharmacological targeting of any one pathway can achieve the desired therapeutic benefit without toxicity. For example, mice expressing the various mutant human superoxidase dismutases (SOD1) associated with human amylotrophic lateral sclerosis (also known as Lou Gehrig's disease) acquire motor neuron disease similar to that seen in humans.[63] Likewise, the transgenic expression of mutant human Huntingtin[64] or α-synuclein[65] have yielded useful mouse models of Huntington's and Parkinson's disease, respectively. All these models highlight

TABLE I
HUMAN DISEASES ASSOCIATED WITH PROTEIN MISFOLDING

Protein overload	Alzheimer's disease
	Parkinson's disease
	Hereditary hyperthyroidism
	Creutzfeld–Jacob disease (prion disease)
	Type-2 diabetes (hyperinsulinemia)
	Familial amyelotrophic lateral sclerosis
Mutant proteins	Cystic fibrosis (mutant CFTR)
	Nephrogenic diabetes insipidus (mutant aquaporin)
	Hereditary hypercholesterolemia (mutant LDLR)
	Wallcott–Rallison syndrome (mutant PERK)
	Osteogenic imperfecta (mutant collagen)
	Retinitis pigementosa (mutant rhodopsin)
Protein processing	Albinism (tyrosine deficiency)
	Obesity (prohormone convertase-1 deficiency)
	α1-Antitrypsin deficiency
	Thyroglobulin deficiency
	Lipoprotein lipase deficiency

Three major contributors to protein misfolding include protein overload, mutations resulting in folding-defective proteins, and errors in protein processing. The diseases listed exemplify human disorders associated with protein misfolding. While diseases such as Parkinson's are linked to gene mutations, these account for only a small fraction of the disease population, and the mechanism underlying in accumulation of Lewy bodies in most patients may be indirect, such mitochondrial damage and high levels of reactive oxygen species. Yet others, such as retinitis pigmentosa, result from many mutations, and for simplicity, only one example is shown. Finally, errors in UPR, such as mutations in PERK, can also contribute to protein misfolding.

the remarkable increase in ER stress that accompanies the expression of mutant proteins in the target tissues.[66–68] However, it has still been difficult to decipher the precise role of ER stress in the disease process, and, most importantly, these models have provided only limited insights into the risks and benefits of pharmacologically targeting individual UPR signaling pathways. With this in mind, our discussion will focus on gene disruption or knockout studies in mice that may help us to evaluate the contribution of individual UPR signaling components in the physiology and pathophysiology of different cells and organs (Table II). It is anticipated that, when combined with the aforementioned mouse models of human disease, these animal models will yield invaluable information on the most appropriate therapeutic strategy for the treatment of diseases associated with chronic or severe ER stress.

A. Genetic Targeting of Transcriptional Pathways Activated by UPR

Following the detection of misfolded proteins in the ER, ATF6 and IRE1 activate parallel transcriptional pathways that are crucial for the long-term adaptation of cells to ER stress. As both pathways enhance the expression of

TABLE II
DISRUPTION OF GENES ENCODING MOUSE UPR SIGNALING COMPONENTS

Component	Function	Outcome of mouse mutations
eIF2α	Eukaryotic translation intitation factor	Homozygous eIF2α (S51A) mice died 1 day after birth.[69]
GCN2	eIF2α kinase	GCN2-null mice were viable but showed sensitivity to lack of dietary amino acids.[5,6]
HRI	eIF2α kinase	HRI-null mice were viable but had low red blood cells and erythroid progenitors.[70]
PKR	eIF2α kinase	PKR-null mice were viable and showed increased susceptibility to virus infections.[71,72]
PERK	ER-transmembrane eIF2α kinase	PERK-null mice were viable but displayed early onset diabetes and mild skeletal defects.[11,73]
GADD34	Stress-induced ER-associated subunit of eIF2α phosphatase	GADD34-null mice were viable and had no overt phenotype. One strain showed aberrant red cell count and morphology.[74]
CReP	ER-associated subunit of a constitutive eIF2α phosphatase	CReP-null mice were pale (reduced red blood count), stunted and died within 1 day of birth.[75]
ATF4	Transcription factor	ATF4-null mice were viable but blind due to lens defects.[76,77]
CHOP	Transcription factor	CHOP-null mice were viable and with no overt phenotype.[38]
ERO1	ER luminal oxidoreductin	ERO1-α-null mice had mild perturbations in cardiac calcium homeostasis.[78] ERO1-β mutant mice had mild diabetes.[79]
Caspase-12	ER-associated caspase	Caspase-12 mutant mice were viable and displayed no overt phenotype.[80]
ATF6	ER-transmembrae protein and transcription factor	ATF6α and ATF6β mutant mice were both normal, but the double knockouts were embryonic lethal.[52,53]
BiP	ATF6-induced ER luminal chaperone	BiP-null mice were embryonic lethal.[81]
IRE1	ER-transmembrane kinase/nuclease	IRE1α-null mice were embryonic lethal with defects in B cell lymphopoesis while IRE1β-null mice were viable with no obvious phenotype.[82,83]
Xbp1	ATF6-induced transcription factor processed by IRE1	Xbp1-null mice were embryonic lethal with defects in liver and exocrine gland development.[84–86]

A number of UPR components have been mutated in mice and provided insights into the tissues whose physiology is most reliant on these components. Yet others highlight the risk associated with impairment of their function. These mutant mice provide an excellent experimental platform to test the physiological impact of specific disease genes and alterations in diet and other factors implicated in human protein misfolding disorders.

chaperones and components of the ERAD machinery, together they enhance the cell's tolerance for misfolded proteins and protect cells from death induced by chronic stress.

ATF6α is the major isoform expressed in most mammalian cells but the disruption of the mouse ATF6α gene has no discernable effect on mouse development.[51,53] When presented with a transient pharmacological stress that induces liver dysfunction and steatosis (fatty liver) in mice, however, the ATF6α-null mice showed significant difficulty in recovering from this insult compared to wild-type mice.[87] In contrast to ATF6α, which is a short-lived protein and a strong activator of ERSE (ER stress response element) containing genes, ATF6β is a more stable protein and a weaker transcriptional activator.[52,88] The loss of function of the mouse ATFβ gene was also without any notable phenotype.[51] The disruption of both ATF6 genes, however, resulted in embryonic lethality.[51] This suggested a critical role for the combined actions of both ATF6 isoforms in mammalian development but provided no hint of their role in human disease. Analysis of UPR signaling in the ATF6α-null mouse embryo fibroblasts (MEFs) highlighted a severe deficit in BiP induction in response to various pharmacological stresses, which was consistent with ATF6α's known role in activating BiP transcription.[51,53] By contrast, ATF6β MEFs were indistinguishable from wild-type MEFs in their response to ER stress.[51] These data questioned the proposed role for ATF6β as a repressor of ATF6α function[52] but, instead, suggested a nonredundant role for the two ATF6 isoforms in transducing UPR signals. The function of ATF6β in UPR signaling remains unknown.

Interestingly, ATF6α-null MEFs also showed an attenuated induction of CHOP and p58IPK by ER stress.[51,53] These data were consistent with the notion that ATF6α cooperates with ATF4 to induce CHOP expression and raised the possibility that ATF6α may also collaborate with Xbp1, a known transcriptional activator of p58IPK.[56] Indeed, ATF6α formed a heterodimer with Xbp1 to bind UPRE elements in promoters of ERAD genes. Moreover, the induction of ERAD genes by ER stress was either strongly attenuated or completely lost in the ATF6α-null MEFs.[51] Another surprise was that GADD34 induction was greatly enhanced in the ATF6α-null MEFs,[53] which was inconsistent with GADD34 simply being an ATF4-[40] or CHOP-induced gene[38]; the transcriptional regulation of the GADD34 gene is still poorly understood. On the other hand, GADD34 mRNA levels were unchanged in yet other ATF6α-null MEFS, suggesting that the mode of gene disruption, deletion of exon 4[53] or exons 8 and 9,[51] or the particular mouse strain influenced these results.

As with the embryonic lethality seen in the ATF6α/β double knockout, disruption of the mouse BiP gene, a major target of ATF6α, was also embryonic lethal.[81] Loss-of-function mutations in the mouse IRE1α[82,83] and Xbp1[84] genes also resulted in early embryonic lethality. These studies suggested a critical role

for the UPR-activated transcriptional pathways, specifically ATF6/BiP and IRE1α/Xbp1, in mouse development. Most notably, Xbp1 was required for the differentiation of hepatocytes[85] and the lethality of the Xbp1-null mice was rescued by the liver-specific reexpression of Xbp1.[86] However, the role of UPR in tissue differentiation and development is still poorly understood. Remarkably, gene expression analyses in the knockout MEFs suggested that ATF6α and Xbp1 served overlapping or redundant functions, activating a subset of UPRE (UPR element) containing genes.[56,89] Similarly, there was significant overlap in the genes whose expression required functional ATF6α and PERK pathways.[89] Genome-wide analysis of Xbp1 binding sites highlighted genes associated in protein synthesis, folding, and trafficking as expected, but also identified DNA damage and repair genes and genes associated with neurodegenerative and myodegenerative disorders.[89] Most importantly, these studies pointed to genes that were expressed in a tissue- and condition-specific manner and suggested that Xbp1 regulated the differentiation of muscle, pancreas, and salivary glands.

In contrast to BiP knockout mice, homozygous "knockin" mice expressing a mutant BiP and lacking the KDEL ER-targeting sequence, survived several hours after birth. These mutant animals died of respiratory failure due to chronic ER stress induced by the impaired synthesis of pulmonary surfactants.[90] By comparison, the heterozygous mutant BiP (KDEL) mice developed age-dependent renal lesions that were exacerbated by protein overload.[91] These data suggested that BiP played a critical role in protecting the kidney from chronic disease or injury resulting from an excessively high protein diet.

B. Gene Targeting of Translational Pathway Activated by UPR

Phosphorylation of eIF2α is an early response to misfolded proteins and is activated by osmotic stress,[92] glucose deficiency,[93] and a wide range of other stresses. To define the physiological importance of eIF2α phosphorylation at serine-51, Scheuner et al.[69] generated a "knockin" mouse in which serine-51 was substituted by alanine to abolish eIF2α phosphorylation. The homozygous eIF2α (S51A) mutant mice died within the first day of life, as a result of a severe loss in pancreatic β-cells and hyperglycemia. While the heterozygous eIF2α (S51A) mutant mice appeared normal on a chow diet, these animals also developed severe β-cell dysfunction when placed on a high-fat diet.[94] These data hinted at a critical role for eIF2α phosphorylation in the development and functioning of pancreatic β-cells, in which the elevated basal translation or proinsulin induced by eIF2α (S51A) likely elicited a chronic ER stress[94] and subsequent apoptosis.

As discussed earlier, four distinct kinases catalyze eIF2α phosphorylation at serine-51 in mammalian cells (Chapter 8). GCN2 is the primary sensor of nutrient availability.[95] It promotes eIF2α phosphorylation in liver of mice fed a diet lacking leucine.[96] While homozygous GCN2-null mice were viable and fertile, when presented with a diet deficient in amino acids, many stillborn pups were delivered.[6,96] Injection of threoninol, a drug that blocks threonyl-tRNA charging in wild-type mice, altered their preference for a threonine-rich diet over a threonine-depleted diet. By contrast, threoninol had no impact on the dietary preference of the GCN2-null mice.[97] Finally, amino acid deprivation failed to increase eIF2α phosphorylation in tissues of the GCN2 knockout mice,[97,98] providing strong support for a pivotal role of GCN2 in nutrient sensing.[95] Indeed, GCN2 promoted the survival of cancer cells derived from solid tumors that were poorly perfused and therefore deprived of nutrients.[99]

HRI is the major eIF2α kinase in erythroid cells and inhibits the synthesis of α and β globins in response to iron deficiency.[70] Consistent with this observation, HRI-null mice have an abnormal blood count and, when stressed, erythrocytes from the mutant mice overproduced globins and experienced hemolysis.[70] Thus, HRI appears primarily essential for the viability of red cell precursors.

PKR has broader substrate specificity than other eIF2α kinases, and its substrates include many transcription factors and other proteins. Thus, PKR not only controls cell growth and differentiation but also activates apoptosis.[8] Deletion of the double-stranded RNA-binding domain in PKR mutant mice resulted in no apparent phenotype other than a modest increase in their susceptibility to viral infection.[71,72] Deletion of the PKR kinase domain also yields mice with no overt phenotype,[100] but analysis of these PKR-null MEFs identified a pathway by which fatty acids activated PKR, which then phosphorylated IRS1 (insulin receptor substrate-1) to inhibit insulin signaling. Consistent with these findings, the PKR-null mice are significantly protected from the development of insulin resistance when placed on a high-fat diet.[93] These data suggest an intriguing link between obesity and viral infections, both of which activate PKR, and the development of diabetes.[93] These studies also highlighted the challenges of generating genetically modified mice in which different functional domains are selected for gene disruption, yielding different phenotypes.

PERK is the major eIF2α kinase in most tissues, particularly secretory tissues, and the only UPR sensor known to be mutated in humans. The Wolcott–Rallison syndrome (WRS) is a rare genetic disorder associated with missense mutation in the human PERK gene that prevents the expression of a functional kinase.[101] WRS is characterized by early onset diabetes with a severe loss of β-cell mass, aberrant skeletal development, mental retardation, cardiovascular abnormalities, and other defects.[101] Interestingly, SNPs (single

nucleotide polymorphisms) in the human PERK gene[102] are also linked to an increased risk for type-2 diabetes. Homozygous PERK-null mice were born with a normal pancreas but soon lost β-cells to develop early diabetes.[11,103] These animals also developed skeletal dysplasia associated with defective bone mineralization similar to WRS patients. Yet other studies of PERK-null MEFs highlighted the severely attenuated eIF2α phosphorylation in response to a variety of stresses, although compensatory increases in IRE1α activation and reduced cell survival when exposed to ER stress were also observed.[11] Interestingly, cells and tissues from the PERK-null mice displayed altered ER morphology and defective calcium mobilization.[104] These data suggest that PERK activity is required for calcium signaling and insulin secretion and that the loss of PERK function impacts multiple cellular processes to elicit diabetes. Finally, PERK was required for the induction of autophagy, a pathway that contributes to cell survival under conditions of severe nutrient starvation.[47] More recent studies suggest that PERK (and ATF4) is necessary for ATF6 transcription and translation[105] and, therefore, for downstream events such as expression of BiP and other chaperones. These data argue for a protective role for PERK in chronic ER stress and the development of human disease, particularly type-2 diabetes.

Additional evidence supporting a role for UPR signaling in β-cell viability and human diabetes comes from the analysis of Wolfram syndrome, an autosomal recessive disorder associated with juvenile-onset diabetes. This disease results from loss-of-function mutations in the WFS1 gene encoding Wolframin, a β-cell ER membrane protein.[106,107] ER-stress-induced Wolframin expression and the levels of Wolframin were greatly reduced in both PERK-null and IRE1α-null MEFs following ER stress. This suggested that both these UPR signaling pathways contributed to WFS1 gene activation. The WFS1 mutations increased ER stress in β-cells and suggested a chaperone role for Wolframin in proinsulin folding.[108] Polymorphisms in WFS1 gene were also linked to type-2 diabetes.[109,110] Homozygous WFS1-null mice are small and male sterile, making it difficult to accurately model Wolfram syndrome in mice.[111] The conditional deletion of WFS1 in pancreatic β-cells yielded mice with overt diabetes resulting from increased ER stress and apoptosis in β-cells.[112]

The Xbp1-induced gene p58IPK encodes an ER-associated Hsp40 family protein induced by ER stress and inhibits eIF2α phosphorylation by PKR and PERK.[59,60] Deletion of the mouse p58IPK gene had no impact on the pancreatic development but the mutant animals displayed a progressive loss of β-cells, with concomitant reduction in insulin production and diabetes.[113] As p58IPK serves many functions in the ER (e.g., cochaperone for BiP), it is difficult to attribute these findings solely to changes in PERK activity. Nevertheless, the above data support a key role for PERK and eIF2α phosphorylation in insulin synthesis and β-cell survival.

Phosphorylation of eIF2α in mammalian cells is opposed by a signaling complex composed of protein phosphatase-1α bound to either GADD34 (protein encoded by the PPP1R15A gene[114]) or CReP (the product of the PPP1R15B gene[115]) (Fig. 2). Initially discovered as an mRNA transcript induced in Chinese hamster ovary (CHO) cells by ultraviolet irradiation,[116] GADD34 was postulated to be a proapoptotic gene and, consistent with this, GADD34 overexpression sensitized cells to apoptosis induced by ionizing radiation.[43,117] GADD34 was also identified as Myd116, a gene required for myeloid differentiation.[118,119] Later studies established GADD34 as a component of a stress-activated eIF2α phosphatase.[12,46] Three distinct GADD34 knockout mice have been generated,[46,74,120] and in one of these (deleting

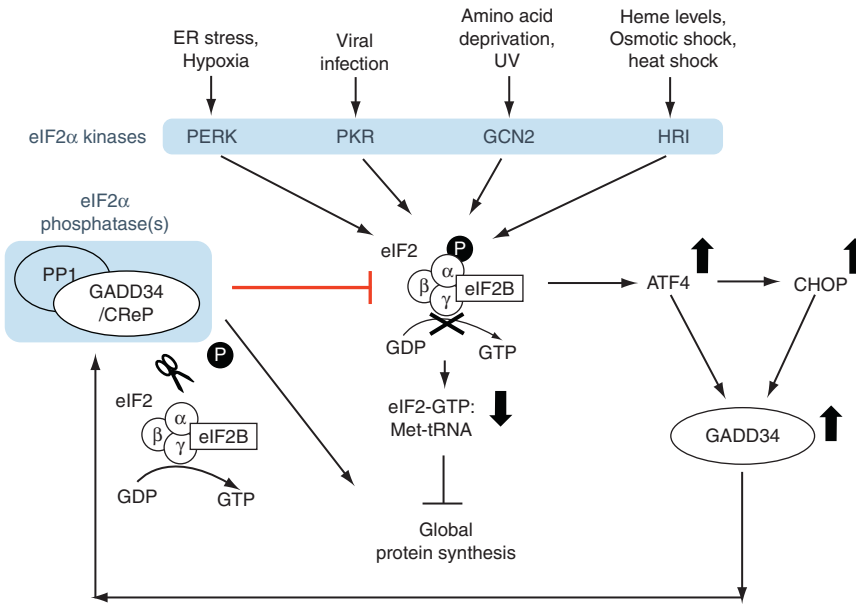

FIG. 2. Cellular control of eIF2α phosphorylation. Four eIF2α kinases, PERK, PKR, GCN2, and HRI, are activated by distinct stresses to phosphorylate eIF2α at serine 51 and inhibit the recycling or exchange of GDP for GTP from the heterotrimeric eIF2 complex. This prevents the assembly of the eIF2 complex as well as initiation of translation at other mRNA start sites and attenuates general protein synthesis. The phosphorylated eIF2α, however, permits the translation of ATF4 a well as its downstream targets CHOP and GADD34. GADD34 gene expression may utilize both ATF4 and CHOP. Once expressed, GADD34 binds the PP1α catalytic subunit. The PP1 complexes containing GADD34 and its homolog CreP catalyze the dephosphorylation of eIF2α to promote GDP/GTP exchange on the eIF2 complex and reinitiate protein synthesis.

exons 2 and 3) a thalassemia-like phenotype with hypersplenism and decreased hemoglobin content in erythrocytes were observed, suggesting a key role for GADD34 in hemoglobin synthesis.[74] This phenotype was remarkably similar to that of the HRI-null mouse,[70] suggesting a requirement for both kinase and phosphatase to control eIF2α phosphorylation and protein translation in red blood cells. In two other GADD34 mutant mice, one deleting exon 1[120] and the other exon 3,[46] the blood count was normal. ER-stress-induced eIF2α phosphorylation was prolonged in all three GADD34-null MEFs, supporting GADD34's role as a key component of an eIF2α phosphatase. Intraperitoneal injection of tunicamycin in wild-type mice-induced ER stress and renal lesions resembling human acute tubular necrosis, while the kidneys of GADD34-null mice were remarkably spared under similar circumstances.[46] GADD34-null mice also showed a remarkably enhanced capacity for wound healing,[121] suggesting that GADD34 attenuates cell migration by inhibiting myosin IIA expression; but the underlying mechanism remains unclear. GADD34 overexpression also activated autophagy and protected cells expressing mutant Huntingtin from ER-stress-induced programmed cell death.[122] These studies hinted at a cytoprotective role for GADD34 in ER stress, linking changes in eIF2α phosphorylation to mTOR signaling.[123] Transgenic mice expressing an activated form of GADD34 in the liver maintained insulin sensitivity and diminished hepatic steatosis induced by a high-fat diet,[124] demonstrating an additional protective role for GADD34 in the liver. On the other hand, these mutant mice showed reduced insulin sensitivity in skeletal muscle and fat tissue, possibly resulting from elevated circulating IGFBP-3 (insulin-like growth factor binding protein 3).[125] These data suggest that changes in eIF2α phosphorylation in liver are communicated via plasma IGFBP-3 to other tissues to regulate their insulin sensitivity. Thus, elevated GADD34 levels either directly or indirectly alter the physiology of other tissues to modulate insulin resistance, hyperinsulinemia, and eventually type-2 diabetes. In this regard, it is noteworthy that new studies point to the ability of stressed cells to transmit ER stress to other as yet unstressed neighboring cells.[126]

The cellular content of GADD34 protein is regulated by multiple mechanisms, including the stress-induced transcription of the GADD34 gene possibly via the combined actions of ATF4 and CHOP.[38,39] The GADD34 mRNA is highly labile,[36,41] though the molecular basis for its instability is still unclear. ER-stress-induced phosphorylation of eIF2α promotes the translation of GADD34 mRNA, which contains two uORFs[36] as noted for CHOP[35] and ATF4.[34] Finally, the GADD34 protein is actively degraded by a mechanism involving its translocation to ER,[44] polyubiquitination of its N-terminal α-amino group,[45] and degradation by the proteasome. Mutations that stabilized GADD34 resulted in the accumulation of intracellular protein aggregates[45] and cell death (Brush and Shenolikar, unpublished data).

CReP, a less well-studied homolog of GADD34, assembles a constitutive eIF2α phosphatase that is unresponsive to ER stress.[115] Homozygous CReP-null mice showed a reduced red cell count and were pale and stunted at birth. These mutant animals failed to nurse and died within 1 day of birth.[75] Interestingly, the double knockout of GADD34 and CReP genes in mice resulted in a more severe phenotype, with embryonic lethality occurring prior to the implantation stage.[75] This latter phenotype was largely rescued by crossing the GADD34/CReP double knockout mice with the homozygous eIF2α S51A mice. These data demonstrated that the function of GADD34 and CReP as eIF2α phosphatases was not shared by other cellular phosphatases. The role of eIF2α phosphatases in mammalian development was transmitted primarily by the dephosphorylation at a single phosphoserine residue, serine-51, in a single phosphoprotein substrate, namely eIF2α.[75]

Examining some of the downstream targets of eIF2α phosphorylation provides further insights into the physiological role of the translation pathway activated by UPR. For example, eIF2α phosphorylation results in the enhanced translation of ATF4 (activating transcription factor 4), which in turn activates the transcription of CHOP[127] and GADD34.[40] Homozygous ATF4 knockout mice are born at less than expected Mendelian ratios and have defects in eye lens development.[76,77] Moreover, ATF4-null MEFs failed to induce GADD34 in response to stress, thereby showing a prolonged eIF2α phosphorylation and delayed translation recovery.[40] Interestingly, ATF4−/− MEFs, like the GADD34−/− MEFs, showed significantly reduced BiP expression following ER stress, emphasizing the role for GADD34 in the translation (or transcription) of BiP. CHOP has been implicated in the expression of GADD34[38] as well as proapoptotic genes, such as Bim and caspase-12.[37] Consistent with these observations, the disruption of mouse CHOP gene protected both cells and animals against protein aggregation and cell death induced by ER stress.[38]

Remarkably, CHOP activates the transcription of genes implicated in both cell survival, such as the ER-associated oxidoreductin 1 (ERO1), and cell death, such as the ER-localized caspase-12. The mice lacking ERO1-α, which is expressed in most tissues, were phenotypically normal, although calcium homeostasis in cardiomyocytes of the mutant animals was perturbed.[78] By contrast, mice lacking ERO1-β, selectively expressed in pancreatic β-cells, showed a mild diabetic phenotype with modest fasting hyperglycemia.[79] Surprisingly, ERO1-α was also present in β-cells but did not compensate for the loss of ERO1-β, suggesting nonredundant roles for the two ERO1 isoforms. Cellular studies showed that the loss of function of ERO1-β enhanced the ability of β-cells to cope with ER stress induced by the expression of a folding-defective mutant insulin *Akita Ins2*(C96Y). However, this benefit was not realized in mice, where the loss of a single ERO1-β allele exacerbated the

diabetic phenotype associated with the *Akita Ins2*(C96Y) mutation.[79] These results highlight the necessity to utilize animal models rather than cell lines to reliably assess the role of UPR signaling in disease.

The caspase-12-null mice were resistant to ER-stress-induced apoptosis, namely renal toxicity induced by tunicamycin injection,[80] and the cortical neurons from these mutant mice were also protected from neurotoxicity and death induced by amyloid-β. However, caspase-12-null mice were not protected from infectious prion disease,[128] indicating that caspase-12 is not essential for the pathogenesis of all neurological protein misfolding disorders.

Together, the above studies revealed a complex interplay between the translational and transcriptional pathways that mediate UPR and control the function and viability of different tissues. The data also highlighted that cellular studies, using either overexpression or knockdown, suggested roles for UPR signaling components that frequently did not replicate in animals, Nevertheless, the broad conclusions of the gene disruption studies in mice were that suppression of the transcriptional pathways that impaired chaperone expression or ERAD might incur severe toxicity or side effects. By comparison, modulation of the translational pathway, specifically eIF2α phosphorylation, provided a safer outcome from the pharmacological targeting of UPR. Even here, targeting specific isoforms, for example, GADD34 versus CReP, may be important to ensure greater safety.

VII. Modulation of eIF2α Phosphorylation: Potential for Treating Human Disease

Although ER stress or protein misfolding has been implicated in a wide range of disorders, the strategy for targeting UPR signaling may differ depending on whether the therapeutic aim is to protect cells, such as β-cells, neurons, and cardiomyocytes, from cell death in the face of chronic ER stress, or to evoke terminal ER stress and apoptosis in cancers that are difficult to treat by current therapies. For simplicity, we will limit this discussion to potential cytoprotective strategies, but even here the strategy may differ depending on whether ER stress is incurred by genetic mutations that result in folding-defective proteins or one wishes to target diseases resulting from protein overload or deficits in protein processing (Table II). We speculate that chemical chaperones, drugs that elevate cellular expression of heat shock proteins or antioxidants that reduce oxidative stress caused by futile protein folding, may be better suited for the treatment of diseases arising from expression of folding-defective proteins. By comparison, protein overload associated with viral infections or hyperinsulinemia associated with insulin resistance that

contributes to β-cell exhaustion and type-2 diabetes might be better managed by attenuating protein translation. In the latter situation, the modulation of eIF2α phosphorylation may offer an attractive opportunity for the development of novel antidiabetic (or antiviral) drugs.

Salubrinal was discovered while screening for small molecules that protected rat PC12 cells from death caused by a pharmacological ER stress.[129] Further analyses established that salubrinal, which elevated eIF2α phosphorylation, inhibited one or more cellular eIF2α phosphatase(s). Indeed, salubrinal inhibited eIF2α phosphatases containing GADD34, CReP (Fig. 3), or ICP34.5, the HSV-1 protein that overcomes PKR activation.[129,130] These data suggested that salubrinal targeted the conserved PP1-binding domain in these three proteins and disrupted their ability to scaffold PP1α, and possibly eIF2α,[131] and assemble a functional eIF2α phosphatase.

Salubrinal showed remarkable protective effects in a number of experimental models of neurodegenerative disease. For example, amyloid β-protein (Aβ) aggregates, seen as plaques in postmortem brain of individuals with Alzheimer's disease, induce ER stress in cells.[132] In this regard, salubrinal increased eIF2α phosphorylation and attenuated apoptosis induced by Aβ in human neuroblastoma cells.[133] In cells expressing mutant Huntingtin, the primary cause of protein aggregates and neuronal death in Huntington's disease,[134] salubrinal abrogated apoptotic signaling and increased cell survival.[135] Salubrinal also protected PC12 cells from cell death induced by α-synuclein,[135] a component of Lewy bodies seen in Parkinson's disease. In animals, salubrinal protected against neuronal death caused by ischemia and excitotoxicity in the rat brain.[136] In the transgenic mouse models of familial amyotrophic lateral sclerosis (fALS), salubrinal reduced the buildup of polyubiquitinated proteins in motor neurons, delayed the progression of motoneuron disease, improved muscle strength, and extended the lifespan of the mutant mice.[137]

Interestingly, salubrinal inhibited the replication of HSV1,[129,130] dengue,[138] and other viruses in cultured cells, indicating the potential of eIF2α phosphatase inhibitors as pan antiviral agents. Salubrinal also overcame the apparent resistance of multiple myeloma cells to bortezomib (also known as velcade), a proteasomal inhibitor clinically approved for these cancers. Bortezomib elevates GADD34 in target cells, antagonizing eIF2α phosphorylation and leading to cell cycle arrest.[139] Salubrinal, by inhibiting GADD34 function, synergized with bortezomib to enhance the death of multiple myeloma[139] and leukemic cells.[140]

One concern associated with the use of salubrinal and possibly other eIF2a phosphatase inhibitors is that, by increasing eIF2a phosphorylation, these drugs will likely increase cellular levels of their target GADD34 and thereby limit the duration of their action. While this could make it difficult to develop

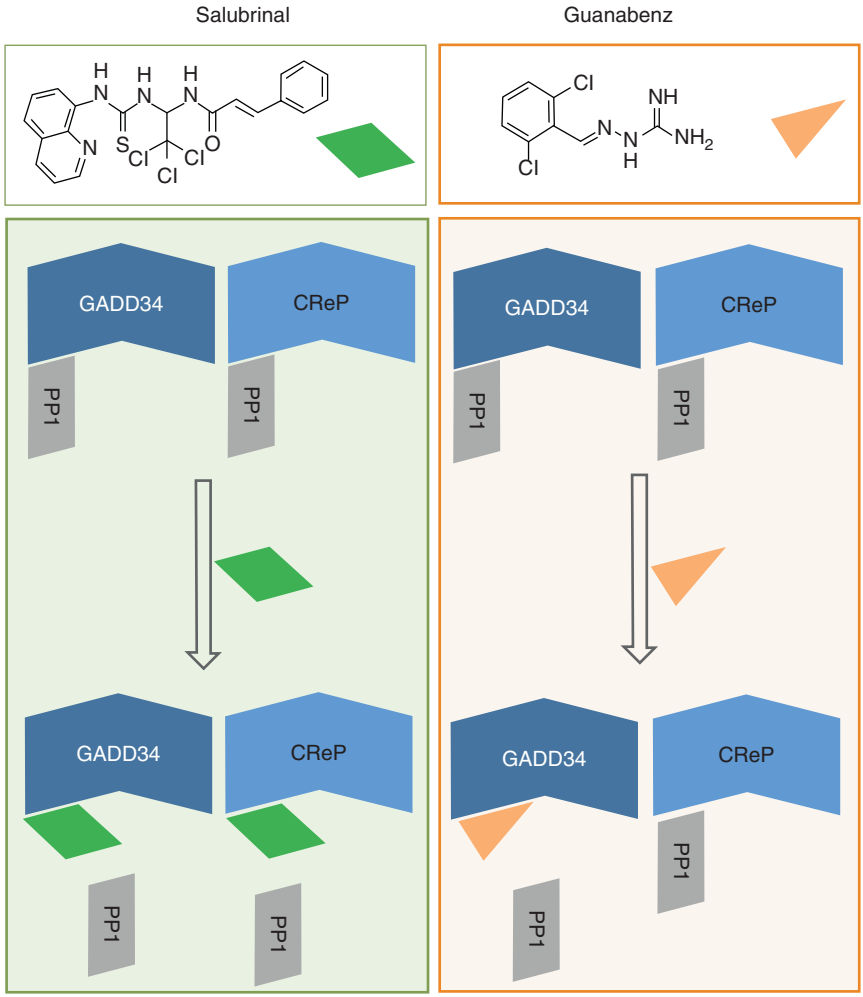

FIG. 3. Mode of action of Salubrinal and Guanabenz as inhibitors of cellular eIF2α phosphatases. Salubrinal (green diamond) and Guanabenz (orange triangle) are structurally unrelated compounds. Recent studies show that they both target cellular eIF2α phosphatases comprised of PP1 bound to either GADD34 or CreP. The prevailing evidence suggests that salubrinal disrupts the PP1 complex containing both GADD34 and CReP (left panel), while guanabenz selectively binds to GADD34 and disrupts its association with PP1 catalytic subunit. In this manner, both drugs elevate the phosphorylation of eIF2α, particularly in response to ER stress.

an effective dosing regimen, these same properties would reduce the possibility of overdosing, making these drugs safer. Salubrinal also has low solubility and bioavailability[137] and needs to be modified to improve its pharmacokinetic properties and druggability. Initial attempts at synthesizing more efficacious salubrinal analogs were unsuccessful.[141]

Guanabenz (also known as Wytensin), a clinically approved α2-adrenergic agonist used to treat hypertension,[142] was shown to possess an antiprion activity[143] that was independent of the α2-adrenergic receptor. Bertolotti and colleagues recently established that Guanabenz bound GADD34, but not CReP, and disrupted PP1α binding.[144] Guanabenz protected β-cells from death induced by expression of the mutant insulin, *Akita Ins2*(C96Y).[144] This novel property of Guanabenz raises exciting possibilities, as the drug has been in use in patients for several decades without notable side effects, suggesting a significant margin of safety. By comparison, gene disruption studies hinted that inhibiting both GADD34 and CReP may have much greater liability. Nevertheless, salubrinal and guanabenz represent an exciting opportunity for drug development aimed at enhancing eIF2α phosphorylation to treat human protein misfolding disorders. Indeed, this has encouraged new small-molecule screens to identify novel modulators of ER stress.[145,146]

In conclusion, UPR signaling pathways are critical for cells to manage protein folding associated with everyday protein synthesis. Extensive work over the last two decades have identified components of this complex translational and transcriptional regulatory network and analyzed their role in cells and animals. While transient translational repression allows cells time to more effectively cope with protein folding, the expression of numerous protein chaperones and activation of ERAD further aid this process. The latter have the added advantage of preconditioning cells to tolerate repeated bouts of protein misfolding. Our understanding of how transient, reversible ER stress differs from chronic terminal ER stress that contributes to cell death and disease is still incomplete, but the use of genetically modified mice have provided new insights into the potential beneficial effects of modulating UPR components as well as highlighting the hazards of targeting others. The discovery of small molecules that disrupt the PP1/GADD34 complex to enhance eIF2α phosphorylation may, for the first time, allow us to mimic physiological perturbations, such as hibernation and ischemia,[147,148, 149] as well as endogenous mechanisms such as the activation of GLP1 receptors,[73] which disrupt this eIF2α phosphatase complex. Thus, these experimental tools offer the opportunity to analyze the physiological impact of acute, reversible modulation of UPR signaling. Finally, Salubrinal and Guanabenz provide two distinct chemical templates from which to design novel drugs to treat a variety of human diseases associated with ER stress.

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