

# Potential for therapeutic manipulation of the UPR in disease

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**Abstract** Increased endoplasmic reticulum (ER) stress and the activated unfolded protein response (UPR) signaling associated with it play key roles in physiological processes as well as under pathological conditions. The UPR normally protects cells and re-establishes cellular homeostasis, but prolonged UPR activation can lead to the development of various pathologies. These features make the UPR signaling pathway an attractive target for the treatment of diseases whose pathogenesis is characterized by chronic activation of this pathway. Here, we focus on the molecular signaling pathways of the UPR and suggest possible ways to target this response for therapeutic purposes.

**Keywords** Endoplasmic reticulum (ER) stress · Chemical chaperone · XBP1 · Insulin resistance · Type 2 diabetes

## Abbreviations

ER	Endoplasmic reticulum
UPR	Unfolded protein response
ERAD	ER-associated degradation
PERK	Protein kinase RNA (PKR)-like ER kinase
IRE1	Inositol requiring enzyme-1
ATF6	Activating transcription factor-6
GRP78	Glucose regulating protein 78
Hsp70	Heat shock protein 70
eIF2 $\alpha$	Eukaryotic initiation factor 2
ATF4	Activating transcription factor 4
uORFs	Upstream open reading frames

CHOP	C/EBP homologous protein
GADD34	Growth arrest and DNA damage-inducible 34
PP1c	The catalytic subunit of protein phosphatase
C/EBP	CCAAT/enhancer-binding protein
PPAR $\gamma$	Peroxisome proliferator-activated receptor $\gamma$
Nrf2	Nuclear factor erythroid2-related factor 2
Keap1	Kelch-like erythroid-cell-driven protein with cap'n'collar homology-associating protein 1
cLD	Core of yeast IRE1 ER-luminal domain
XBP1	X-box binding protein-1
XBP1s	Spliced form of XBP1
CREB/ATF	cAMP response element binding/activating transcription factor
XBP1u	Unspliced form of XBP1
bZIP	The basic-leucine zipper motif
GLS	Golgi localization signals
SP1	Serine protease site 1 protease
SP2	Metalloprotease site 2 protease
CRE	ATF/cAMP response element
ERSE	ER stress response elements
HFD	High-fat diet
STZ	Streptozotocin
LPS	Lipopolysaccharide
IL-4	Interleukin-4
FFA	Free fatty acids
mTOR	Mammalian target of rapamycin
mTORC1 and mTORC2	mTOR complex 1 and 2
IGF1	Insulin-like growth factor
TSC	Tuberous sclerosis complex
IRS1 and IRS2	Insulin receptor substrate 1 and 2
PI3K	Phosphatidylinositol 3-kinase
SERCA	Sarco(endo)plasmic reticulum Ca <sup>2+</sup> -ATPase

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p38 MAPK	p38 Mitogen-activated protein kinase
TNF- $\alpha$	Tumor necrosis factor $\alpha$
WR	Wolcott-Rallison
$\alpha$ 1-AT	$\alpha$ 1-antitrypsin
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
NBD	Nucleotide-binding domain
PD	Parkinson's disease
HD	Huntington's disease
AD	Alzheimer's disease
AR-JP	Autosomal recessive juvenile parkinsonism
6-OHDA	6-Hydroxydopamine
Herp	Homocystein-induced ER protein
JNK	c-Jun N-terminal kinase
FoxO1	Forkhead box protein O1
A $\beta$	Amyloid $\beta$ peptide
APP	Amyloid precursor protein
PS	Presenilin
FAD	Autosomal dominant familial AD
MEFs	Mouse embryonic fibroblasts
MM	Multiple myeloma
NF $\kappa$ B	Nuclear factor $\kappa$ B
PBA	4-Phenylbutyric acid
TUDCA	Tauroursodeoxycholic acid
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine

### The endoplasmic reticulum and ER stress

The endoplasmic reticulum (ER) is an organelle that comprises a continuous membranous structure consisting of tubules, flattened sacs, and the lumen [1]. The entire ER network is interconnected, and the membranes serve to separate molecules that reside in the lumen of the ER from those in the cytoplasm [2, 3]. The ER is highly sensitive to the nutritional and energy states of the cell, and it dramatically adjusts its machinery depending on the demand created in the cell [4, 5]. The ER folds and modifies newly synthesized peptides into their tertiary, lowest energy conformation [6], following which proteins are translocated to the Golgi apparatus for packaging and secretion, or are transported to membranes [5, 7, 8] (Fig. 1). Proteins that enter the ER are subjected to post-translational modifications such as N-linked glycosylation, disulfide bond formation, and proline hydroxylation [9, 10].

The ER lumen has characteristics of the extracellular environment [10], and it is possible that the ER is evolved

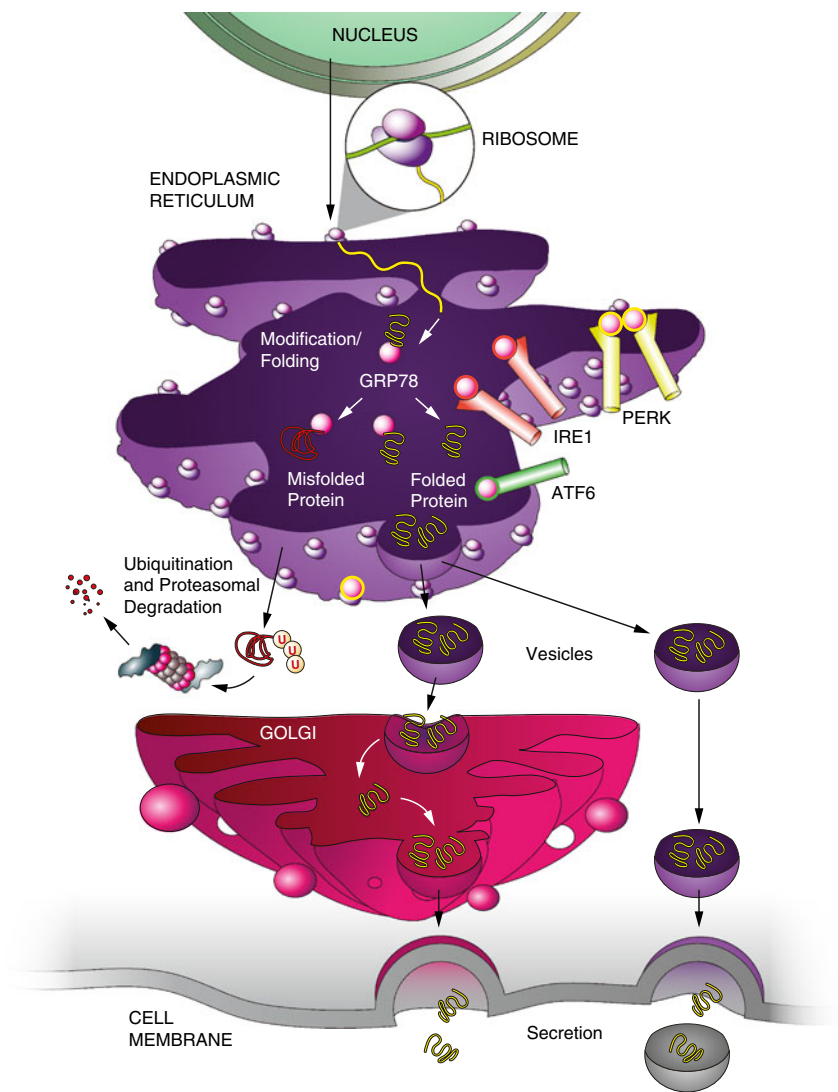
from an invagination of the plasma membrane [10]. This is indeed a viable possibility and is supported by the observation that secretory proteins are produced in the ER in an environment similar to that of the extracellular conditions, enabling proteins to protect their conformation when they are outside the cell. Similarly, the calcium concentration within the ER is the same as that of the extracellular environment [11]. As all secretory and membrane proteins pass through the ER before exiting the cell to face the extracellular environment, it is likely the ER provides a final control point to ensure the fidelity of proteins before exporting them out of the cell. In addition to proteins, sterols, phospholipids, and assembled lipoproteins are also synthesized in the lumen of ER [12, 13]. Finally, the ER serves as a major calcium reservoir: maintenance of a high calcium concentration within the ER is essential for ER function, and for optimizing the activity of enzymes within the ER [14–16].

The so-called misfolded proteins, which are not properly folded into their functionally active structures, are retained in the lumen of ER until they attain their proper conformations [5, 17, 18]. If this final tertiary structure cannot be achieved, misfolded proteins are then transported back to the cytosol and subjected to ubiquitination and proteasome-dependent degradation, a process referred to as ER-associated degradation (ERAD) [17–19] (Fig. 1). Under conditions wherein misfolded proteins accumulate within the lumen of the ER, or when ER capacity cannot meet the demand for protein synthesis, the organelle enters into a state called “ER stress” [5, 17, 18]. This condition is triggered by processes such as increased protein synthesis, genetic mutations that cause defects in folding, alteration in calcium homeostasis, and nutrient starvation such as glucose deprivation [5, 15, 17]. As a cellular recovery and survival mechanism, the ER responds to ER stress by activating a series of complex coordinated signaling pathways, collectively called the unfolded protein response (UPR) [4, 5, 17, 18, 20–22] (Fig. 2). The UPR is a physiological response when it occurs in acute settings, but it can lead to pathologies, or even cell death (reviewed below) under conditions of chronic, unresolved ER stress.

### The unfolded protein response

The UPR is initiated by activation of three major transducers: protein kinase RNA (PKR)-like ER kinase (PERK), inositol requiring enzyme-1 (IRE1), and activating transcription factor-6 (ATF6) [5, 23] (Fig. 2). The primary role of the UPR is to maintain and re-establish ER homeostasis [4, 23]. Under physiological conditions, the UPR is activated as a cellular survival program that protects the cell from ER stress and helps it recover from damage or increased work overloads. In essence, the UPR functions to reset the ER, from the

**Fig. 1** Normal state. Proteins that enter the ER are folded and transported to the Golgi apparatus or other destination. GRP78 is bound to the luminal domains of PERK, IRE1, and ATF6



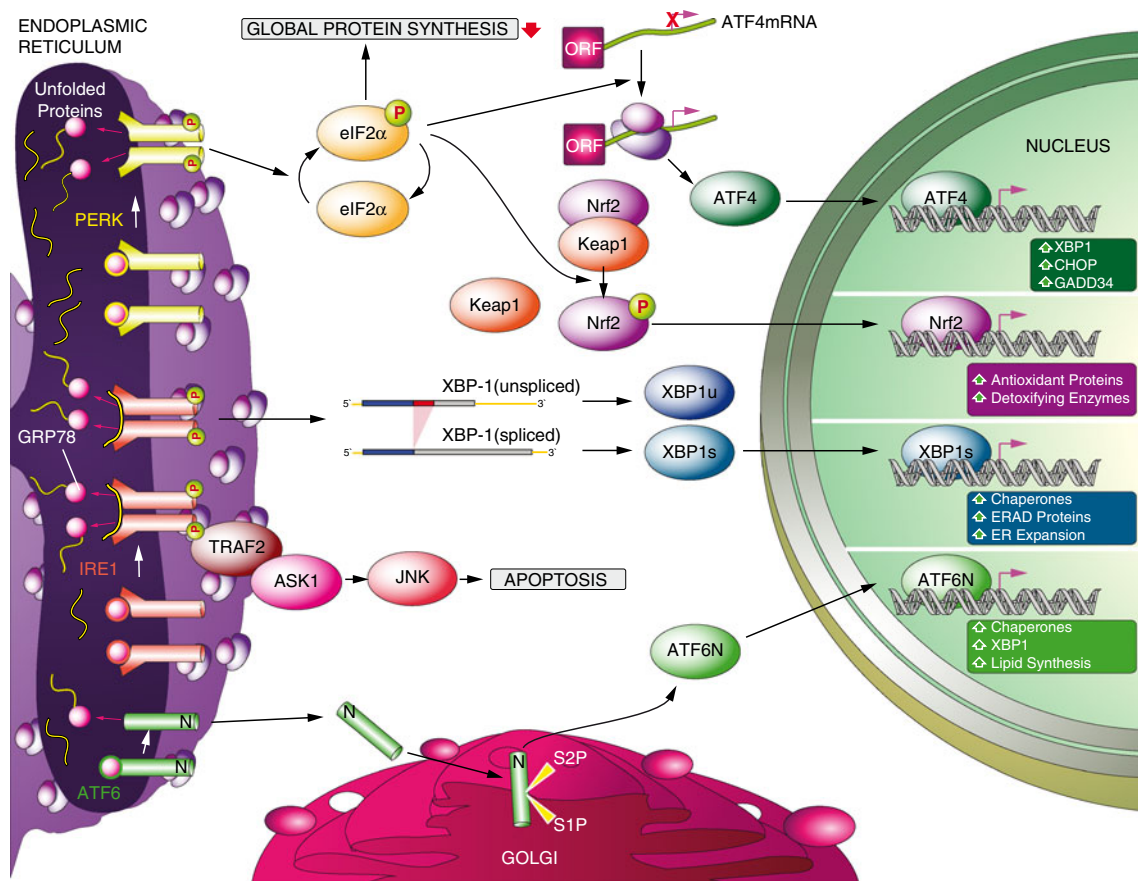
“stress” state back to its normal condition [4, 17]. However, sustained ER stress and prolonged UPR activation can also trigger the apoptotic machinery, and ultimately lead to cell death [4, 18]. Despite the beneficial role of the UPR in the maintenance of cellular homeostasis, prolonged ER stress often leads to pathological conditions [21, 24, 25]. The functional significance of the UPR is not yet fully understood, but it is known to contribute to the pathogenesis of many diseases including diabetes, cancer, atherosclerosis, neurologic diseases, and inflammatory bowel disease [4, 22, 26–28].

Under ER stress conditions, a chaperone called the glucose regulating protein 78 (GRP78) initially cues the three transducers of the UPR for activation of downstream signaling cascades [5, 29]. GRP78, also known as BiP, is a member of the Hsp70 family of chaperones, and it negatively regulates the UPR signaling pathway by physically interacting with the three UPR transducers [5]. In the basal state (no external stimulus or stress condition), GRP78 is

bound to the luminal domains of PERK, IRE1, and ATF6, and the three UPR transducers remain in a state of low activity [5]. Under stress conditions (accumulation of unfolded and misfolded proteins in the ER), GRP78 is released from all three transducers and binds to unfolded and misfolded proteins in the lumen of ER [30]. Exactly how the transducers sense ER stress is still under investigation [23]. Several models have been proposed for activation of the UPR and suggest that each branch of the UPR is separately regulated [17, 18]. Below, we briefly outline how the UPR signaling elements are regulated in order to understand potential therapeutic approaches.

#### Protein kinase RNA (PKR)-like ER kinase

PERK is a type I transmembrane kinase that resides in the ER. Activation of PERK during ER stress reduces protein synthesis globally [29, 31], thereby decreasing the speed of



**Fig. 2** ER stress state. During ER stress, GRP78 dissociates from PERK, IRE1, and ATF6. PERK and IRE1 oligomerize, forming a dimeric structure with a deep groove where peptide can bind. Upon oligomerization, PERK and IRE1 are auto-phosphorylated. PERK phosphorylates eIF2 $\alpha$ , leading to attenuation in global protein synthesis. Phosphorylated eIF2 $\alpha$

leads to translation and nuclear translocation of ATF4 and Nrf2. Activated IRE1 mediates unconventional mRNA splicing of XBP1 to generate XBP1s. IRE1 also recruits TRAF2 and ASK1 and leads to activation of JNK. ATF6 translocates to the Golgi apparatus and the cytoplasmic tail of ATF6 acts as a transcription factor to regulate UPR target genes

entry of new polypeptides into the ER. This mechanism facilitates the process of homeostasis, by enabling the existing unfolded proteins in the lumen to attain their folded conformation. While PERK plays a central role in the regulation of the UPR, we do not fully understand the mechanism by which PERK senses ER stress, although several models have been suggested [18]. For example, the binding of GRP78 to PERK may maintain PERK in an inactive state, as supported by reports that GRP78 and PERK form a complex under normal conditions [30], and that PERK is activated by reduction in the expression of GRP78 and is inhibited when GRP78 is overexpressed [30]. Thus, one model posits that as a result of an increase in the amount of unfolded proteins in the lumen of ER, GRP78 is titrated away from PERK by the misfolded proteins, and this dissociation leads to oligomerization and activation of PERK [17, 18]. Another model proposes that accumulation of unfolded proteins leads to a dissociation of GRP78 from the unfolded proteins that it is bound to in the lumen. This leads to direct binding of the unfolded proteins to the luminal domain of PERK, which in turn results in oligomerization and

autophosphorylation of PERK [18]. The deep groove formed by the dimeric structure of PERK can support the direct binding of a peptide [17, 18]. Additional detailed studies are needed to unravel the mechanisms of PERK activation.

The kinase activity of PERK leads to phosphorylation of Serine51 of its main downstream effector, the eukaryotic initiation factor 2 (eIF2 $\alpha$ ), which then leads to global attenuation of protein synthesis [29, 31]. However, proteins such as activating transcription factor 4 (ATF4) escape from this global inhibition of protein synthesis [32]. ATF4 contains inhibitory upstream open reading frames (uORFs) in its mRNA that normally suppress the initiation of translation in the absence of ER stress [33]. However, when ER stress is elevated, phosphorylated eIF2 $\alpha$  leads to ribosomal skipping of the uORFs and active translation of ATF4 mRNA [33]. ATF4 acts as a transcription factor for genes that contribute to ER function and apoptosis. An example of an immediate ATF4 target gene is the transcription factor C/EBP homologous protein (CHOP) [34, 35], which is believed to participate in the initiation of apoptotic pathways by upregulating

the transcription of genes when accumulation of unfolded/misfolded proteins goes beyond the capacity of the ER [36]. Another gene induced by ATF4 is growth arrest and DNA damage-inducible 34 (GADD34) [37, 38]. GADD34 interacts with the catalytic subunit of protein phosphatase (PP1c) [37] and serves as a negative feedback loop to deactivate PERK action by dephosphorylating eIF2 $\alpha$ , by which it resets the UPR to the basal state once ER stress is resolved [37, 38].

eIF2 $\alpha$  phosphorylation also increases translation of CCAAT/enhancer-binding protein (C/EBP) in *in vitro* models [39]. Likewise, reduced phosphorylation of eIF2 $\alpha$ , achieved by overexpression of GADD34 in the liver, leads to decreased expression of C/EBP $\alpha$  and C/EBP $\beta$ , and of their downstream effector PPAR $\gamma$  [39]. Accordingly, GADD34 transgenic mice display lower blood glucose levels, improved glucose tolerance, lower levels of liver glycogen, and diminished hepatosteatosis [39].

Another effector protein that is phosphorylated by PERK is nuclear factor erythroid2-related factor 2 (Nrf2) [40], which forms a complex with Kelch-like erythroid-cell-derived protein with cap'n'collar homology-associating protein 1 (Keap1). The Nrf2/Keap1 complex is maintained in the cytoplasm under normal conditions (without ER stress) and is subjected to degradation by ubiquitin–proteasome pathway [40]. Upon ER stress, PERK phosphorylates Nrf2 and results in dissociation of the Nrf2/Keap1 complex. The released free Nrf2 stably translocates to the nucleus, and acts as a transcription factor for genes that encode antioxidant proteins and detoxifying enzymes [40].

#### Inositol requiring enzyme 1

The second arm of the UPR is IRE1. Like PERK, IRE1 is also a type I transmembrane kinase, and the two transmembrane proteins share similar structures in their luminal domains [18, 41]. IRE1 is highly conserved from yeast to humans [42, 43]. Two homologues of IRE1 have been identified: IRE1 $\alpha$  and IRE1 $\beta$  [41]. IRE1 $\alpha$  is expressed in all cells throughout the body, while expression of IRE1 $\beta$  is restricted to the intestinal epithelium and lung [44, 45]. IRE1 has endoribonuclease as well as kinase activity [46–48]. During ER stress, IRE1-bound GRP78 is released from IRE1 [49]. Studies in yeast document that, once this release occurs, the structure of IRE1 allows it to bind to unfolded proteins, providing insight into the mechanism of the IRE1 activation that follows ER stress [17, 30, 49]. IRE1 $\alpha$  has a conserved cLD, which contains interface 1 and interface 2 [50]. Interface 1 forms a deep groove where peptides can bind, while interface 2 induces further oligomerization [50]. As IRE1 senses ER stress and oligomerizes, its kinase and endoribonuclease activities are activated [51], and it is auto-phosphorylated, which increases its

kinase activity, initiating a signaling cascade that can activate c-Jun amino terminal kinase (JNK) [52]. The endoribonuclease domain of IRE1 cleaves the mRNA of a transcription factor called X-box binding protein-1 (XBP1) [53–55], and leads to the translation of a higher molecular weight protein, which is the spliced form of XBP1 (XBP1s) [43, 56–58].

XBP1 is a member of the CREB/ATF basic region-leucine zipper family of transcription factors, and is ubiquitously expressed in adult tissues [59]. The full-length XBP1 mRNA is referred to as unspliced XBP1 (XBP1u) [53, 54]. IRE1 cleaves the mRNA of XBP1u and initiates the excision of 26 nucleotides from the mRNA [53], which in turn results in a frame shift and ultimately in the generation of the spliced form XBP1s [53–55]. While XBP1u is extremely unstable, and is subjected to proteasome-dependent and -independent degradation soon after translation [60], XBP1s is a highly active transcription factor and a master regulator of ER capacity [4, 5, 18, 57, 58, 61]. XBP1s upregulates expression of ER chaperones [57, 62] and the components of ER-associated degradation [57], and also plays a key role in ER expansion [58, 63]. While XBP1s target genes are fairly well known [57], the exact role of XBP1u is debated. XBP1u was initially believed to negatively regulate the transcriptional activity of XBP1s by directly interacting with the spliced form, forming a complex, and directing XBP1s to proteasome-mediated degradation [64, 65]. However, a subsequent report suggested that XBP1u is capable of inducing the UPR and increasing expression of XBP1s target genes as well as other non-target genes, but only if XBP1u is stabilized and its degradation is blocked as implicated by a XBP1u mutant with improved stability [60]. It has been documented that the half-life of XBP1u is extremely short and the protein is usually subjected to rapid proteasomal degradation soon after synthesis, at a rate that is almost the same as its level of synthesis [60]. The same study also concluded that the short half-life of XBP1u abrogates a physiologically significant role for the unspliced protein and proposed that the rapid degradation of XBP1u is required to prevent uncontrolled activation of the UPR [60]. We also agree that XBP1u does not have any physiological importance as we have been unable to detect the unspliced protein in any tissue or cells that we have examined to date (unpublished observations).

#### Activating transcription factor 6

The third arm of the UPR is ATF6, which is categorized as a type II transmembrane protein with two homologs, ATF6 $\alpha$  and ATF6 $\beta$  [66]. Structurally, ATF6 contains a DNA-binding domain with the bZIP and a transcriptional activation domain in the cytoplasmic portion [66]. In its N-terminus, ATF6 carries two Golgi localization signals (GLS), referred to as

GLS1 and GLS2 [67, 68]. Under normal conditions without ER stress, GRP78 binds to GLS1, and this interaction retains ATF6 in an inactive state in the ER membrane [67, 68]. Once activated by ER stress, GRP78 is released from ATF6, and triggers the translocation of ATF6 to the Golgi apparatus, a process that requires the GLS2 domain [67]. The transported ATF6 is subjected to intramembrane proteolysis, whereby it is cleaved by SP1 and SP2 [69–71], resulting in the generation of a 50-kDa DNA-binding domain of ATF6 derived from its cytoplasmic tail, referred to as ATF6F or ATF6N [68]. The cleaved fragment translocates to the nucleus and acts as a transcription factor that activates UPR target genes. ATF6 regulates the expression of a variety of genes that contain that CRE and ERSE [62, 72]. While little is known about the negative regulation or deactivation of ATF6, XBP1u also reportedly binds ATF6 $\alpha$  and leads to proteasomal degradation of ATF6 $\alpha$  [65].

### The UPR elements in pathophysiology

The exact functions of the UPR under physiological and pathological conditions, and its role in disease pathogenesis, are not fully understood yet. However, what we do know is that the UPR is involved in various processes, including development, differentiation, maintenance of homeostasis, and apoptosis [17, 25]. The physiological role of the UPR is well defined from studies with animal models. In this section, we review the knockout models that have been developed for elucidating the role of the major UPR signaling molecules in pathophysiology, focusing mainly on metabolic diseases.

#### PERK in pathophysiology

An important characteristic of the *Perk*<sup>-/-</sup> mouse is that it develops diabetes, due to the destruction of pancreatic  $\beta$  cells [73]. Under normal conditions, insulin is synthesized in response to increased glucose levels in the blood, and is secreted into the circulation to keep glucose levels within a very tight range [74]. Insulin biosynthesis takes place in the ER of pancreatic  $\beta$  cells, and is dynamically controlled by ER capacity and stress conditions. When the demand for insulin synthesis exceeds the capacity of the ER, it triggers ER stress, and activates PERK [4] and its downstream effector eIF2 $\alpha$ , to reduce the workload. In *Perk*<sup>-/-</sup> mice, however, protein synthesis cannot be controlled by PERK even under conditions of ER stress [73]. In fact, when *Perk*<sup>-/-</sup> mice are challenged with glucose, uncontrolled synthesis of insulin further triggers ER stress [31, 73]. The prolonged unresolved ER stress initiates the apoptotic pathway, culminating in the destruction of  $\beta$  cells and the development of diabetes [73].

While the study of whole body knockout mouse models of PERK points out an important role this kinase activity in

pancreatic  $\beta$  cell biology,  $\beta$  cell-specific PERK-deficient mice surprisingly do not develop diabetes [75]; rather they have a normal number of  $\beta$  cells and display normal glucose tolerance with low of blood glucose level [75]. Further investigation is needed to clarify the relationship between PERK, insulin biosynthesis, and its regulation in  $\beta$  cells.

#### eIF2 $\alpha$ in pathophysiology

Mice in which Serine51 of eIF2 $\alpha$  is mutated to Alanine (eIF2 $\alpha$ <sup>S51A</sup> mice) have a complete defect in eIF2 $\alpha$  phosphorylation at Serine51. They display defects in pancreatic  $\beta$  cells during late embryonic development, and develop severe hypoglycemia and a failure to survive for more than 18 h after birth [76]. Multiple factors are believed to be responsible for the hypoglycemia in eIF2 $\alpha$ <sup>S51A</sup> mice, including lower levels of gluconeogenic enzymes and diminished glycogen storage in the liver [76]. Heterozygous eIF2 $\alpha$ <sup>S51A</sup> mice, on the contrary, have a functionally active pancreas, plus normal basal glucose and insulin levels [77]. They display normal glucose tolerance levels and insulin sensitivity under normal diet feeding conditions. However, when heterozygous eIF2 $\alpha$ <sup>S51A</sup> mice are challenged with a high-fat diet (HFD), they develop a higher level of obesity compared to control animals [77]. A combination of an eIF2 $\alpha$  mutation and HFD leads to abnormal ER function, glucose intolerance, and reduced insulin sensitivity [77]. Note that transgenic mice, in which the function of eIF2 $\alpha$  is impaired, exhibit features that are similar to those observed in humans with type 2 diabetes. The absence of eIF2 $\alpha$  in mice results in increased ER stress and malfunction of the pancreas, resulting from  $\beta$  cell destruction [77].

#### CHOP in pathophysiology

Depletion of CHOP, a factor that promotes programmed cell death, improves  $\beta$  cell function and cell survival in mice [78]. The type 2 diabetes that is induced by a high fat diet (HFD) and treatment with a moderate dose of streptozotocin (STZ) is reversed by deletion of the CHOP gene [78]. Deficiency in CHOP maintains insulin secretion and prevents hyperglycemia in the HFD/STZ mouse model [78]. These features are also observed in the leptin receptor-deficient obese *db/db* mouse model [78]. CHOP deletion alone leads to an increase in body weight with augmented adiposity, but without any disturbance in glucose metabolism [78–80]. The contributions of CHOP function to other organs have also been reported [81, 82]. In particular, the induction of apoptosis in lung tissue following intraperitoneal treatment with LPS is suppressed in CHOP knockout mice [82], as is ischemia-associated apoptosis of neurons in the brain [81].

Another example of the pathological effects of a chronic UPR is seen in the Akita<sup>Ins2</sup> mouse model [83]. This mouse

has a missense mutation in the proinsulin 2 (*Ins2*) gene (Cysteine96 residue to Tyrosine) [84, 85], which disrupts disulfide bonds between chains of insulin and leads to improper folding of insulin. The resulting unfolded insulin is retained in the ER of pancreatic  $\beta$  cells, induces ER stress, and activates UPR signaling [83]. Phenotypic features of the Akita<sup>Ins2</sup> mouse include hypoinsulinemia and hyperglycemia [83]. These features of type I diabetes are believed to be primarily due to chronic activation of the UPR in  $\beta$  cells, triggered by the accumulation of unfolded insulin protein [83, 86]. This notion is substantiated in studies of Akita<sup>Ins2</sup> mice that are crossed with *Chop*<sup>-/-</sup> mice. Prolonged ER stress in the pancreatic  $\beta$  cells is known to induce expression of CHOP, an ER stress-associated apoptosis factor [87]; thus, increased expression of CHOP, as in the Akita<sup>Ins2</sup> mouse, causes programmed cell death of pancreatic  $\beta$  cells and the development of progressive hypoinsulinemia and diabetes [83]. However, when the Akita<sup>Ins2</sup> mouse is bred with *Chop*<sup>-/-</sup> mice, the offspring Akita<sup>Ins2</sup>*Chop*<sup>-/-</sup> mice exhibit decreased apoptosis of pancreatic  $\beta$  cells and a resulting delay in the progression of diabetes [83].

In parallel, deletion of CHOP prevents UPR-induced apoptosis and improves glucose homeostasis in HFD-fed eIF2 $\alpha$ <sup>S/A</sup> mutant mice that are obese and diabetic [78]. CHOP deletion improves  $\beta$  cell function and preserves pancreatic  $\beta$  cell mass [78]. These phenotypic features are also observed in STZ-treated mice and leptin receptor-deficient *db/db* mouse model wherein CHOP deletion improves glucose tolerance and prevents hyperglycemia in the fasting state [78].

#### ATF6 $\alpha$ in pathophysiology

ATF6 $\alpha$  knockout mice do not display significant differences in the expression of ER chaperones relative to controls [88]. mRNA expression profiling of *Atf6 $\alpha$* <sup>-/-</sup> cells demonstrates no changes except in expression of the *Atf6 $\alpha$*  gene. Moreover, mice in which ATF6 $\alpha$  is deleted do not display any developmental defects, leading to the conclusion that ATF6 $\alpha$  does not have an important role in embryonic and postnatal development [88]. However, in vitro as well as in vivo studies show that ATF6 $\alpha$  is needed for folding, secretion, and degradation during ER stress condition [88], suggesting that ATF6 $\alpha$  is involved in mediating adaptation to chronic ER stress. *Atf6 $\alpha$* <sup>-/-</sup> mice also display persistent ER stress in the liver and kidneys upon induction of ER stress with chemicals [89], and unresolved ER stress in the liver of these mice results in a loss of lipid homeostasis and microvesicular steatosis [89].

#### XBP1 in pathophysiology

A physiological role for XBP1 was first identified for the differentiation of plasma cells [90]. XBP1 expression was found

to be upregulated by IL-4 when plasma cells were induced to differentiate [91]. Accordingly, XBP1 overexpression in B cells induces terminal differentiation of these cells into antibody-producing plasma cells [90]. Likewise, XBP1 depletion results in fewer numbers of plasma cells, while the B cells themselves display a defect in immunoglobulin production in vitro [90]. The importance of XBP1 in physiological conditions is substantiated by reports that germline deletion of XBP1 is lethal [92, 93]. These initial studies on XBP1 were performed prior to the recognition that XBP1 is the mammalian homolog of Hac1p, and that it plays a key role in UPR signaling. Germline *Xbp1*<sup>-/-</sup> mice die from severe liver hypoplasia [92] or necrosis of cardiac myocytes [93]. On the other hand, heterozygous *Xbp1*<sup>+/-</sup> mice are viable and appear to possess no phenotype until they are challenged with a HFD (discussed further below under “Obesity and type 2 diabetes” section) [26].

#### The UPR in disease

As outlined above, elements of the UPR participate in various physiological conditions. Although precise genetic and mechanistic association of the UPR with human disease has not been fully investigated yet, considerable ongoing basic research is focused on uncovering how the UPR contributes to human disease, and results of such studies are rapidly being translated to clinical settings. Malfunction of the ER and failure in localization of proteins to their correct cellular destinations are widely believed to cause a number of diseases in humans [4, 5, 25, 94, 95]. Some of these examples, which are related to metabolic, neurodegenerative, and oncologic disorders, are discussed briefly below and of potential therapeutic relevance to UPR-directed strategies are outlined below.

#### Obesity and type 2 diabetes

Obesity is a complex metabolic disorder that contributes to the development of many other life-threatening diseases, including heart disease, type 2 diabetes, and cancer [96–98]. Type 2 diabetes is a highly debilitating condition that arises in obesity [96, 99]. Over the last decade, increased ER stress signaling has been implicated in the development of insulin resistance and type 2 diabetes [26, 27, 100, 101]: initial observations indicated that ER stress parameters, such as phosphorylation of PERK and IRE1, are increased in the liver and adipose tissues of obese and type 2 diabetic mice [26, 100, 102–105]. Increased ER stress signaling, through activation of IRE1, leads to inhibition of insulin receptor signaling, which in turn results in insulin resistance and type 2 diabetes [26]. Furthermore, when *Xbp1*<sup>+/-</sup> mice, which are on a background that is completely resistant to the development of obesity, insulin resistance,

and type 2 diabetes, are fed a HFD, these mice in fact develop obesity, severe insulin resistance, and type 2 diabetes [26]. These observations provide the first evidence that ER stress has a key role in the pathology of type 2 diabetes. Since then, other groups have also reported that ER stress is increased in obesity, and have established a causal relationship between the protein folding capacity of the ER and insulin sensitivity in obesity [103, 105].

Intense efforts are currently aimed at understanding why ER stress develops in obesity. It was reported that free fatty acids (FFA), which are implicated in the development of insulin resistance in obesity, cause ER stress and activate the UPR [106–108]. For example, the saturated FFA palmitate creates ER stress in pancreatic  $\beta$  cells, hepatocytes, and cardiomyoblasts. Excess palmitate causes perturbations in the ER system, and activates the UPR by altering the integrity of the ER membrane [109–112]. Another possible link between obesity and the development of ER stress is the mammalian target of rapamycin (mTOR) signaling pathway [113]. mTOR is involved in regulating a wide range of cellular events, such as growth, proliferation, metabolism, autophagy, and apoptosis [114, 115]. mTOR functions in two different complexes called mTOR complex 1 and 2 (mTORC1 and mTORC2) [116, 117]. mTORC1 comprises mTOR, raptor, mLST8, Deptor, and PRAS40; and mTORC2 is composed of mTOR, rictor, Deptor, Protor, mSIN1, and mLST8 [117]. Increased activation of the mTORC1 pathway blocks insulin and IGF1 signaling pathways. In tuberous sclerosis complex (TSC)-deficient cells, the insulin-induced tyrosine phosphorylation of insulin receptor substrate 1 and 2 (IRS1 and IRS2) is blocked, and consequently, activation of phosphatidylinositol 3-kinase (PI3K) and its downstream Akt is inhibited [118, 119]. A deficiency of the TSC1 or TSC2 genes leads to constitutive activation of the mTORC1 complex [120, 121]. ER stress levels in these TSC-deficient cells are elevated in an mTORC1-dependent manner, and activation of the UPR contributes to mTORC1-mediated inhibition of insulin signaling via degradation of IRS1 [113]. Considering the fact that obesity is characterized by increased mTORC1 activity, it is possible that this pathway contributes to the development of ER stress in obesity.

As discussed above, even heterozygous deficiency of XBP1s is sufficient to create severe ER stress, insulin resistance, and type 2 diabetes in mice fed a HFD [26]. Our recent observations led us to identify an interesting pathology that plays a central role in the development of ER stress in obesity. We have shown that p85 $\alpha$  and p85 $\beta$ , the regulatory subunits of PI3K, interact with XBP1s, and that this interaction plays an important role in nuclear translocation of XBP1s [122] (Fig. 3). This interaction is also extremely important for driving the nuclear translocation of XBP1s during postprandial states to inhibit gluconeogenesis, and

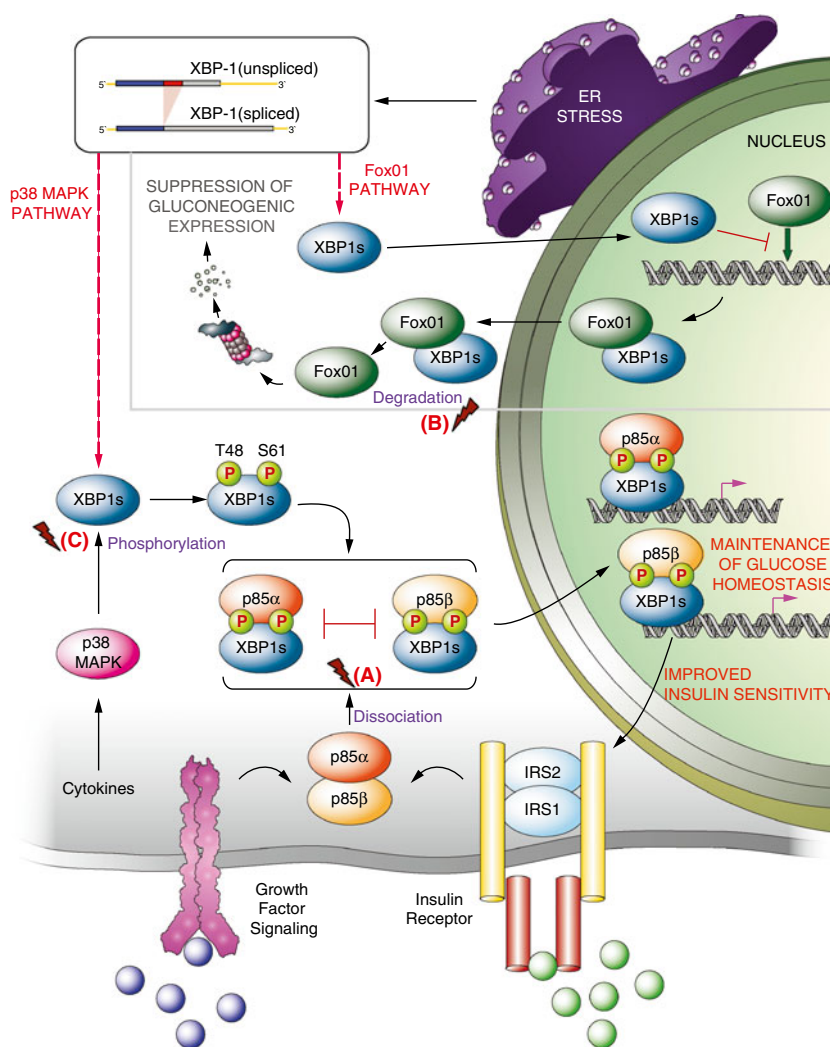
also to reduce ER stress as a result of nutrient fluxes. However, the interaction between p85s and XBP1s is disrupted in obesity, and there is a major reduction in nuclear translocation of XBP1s and in the upregulation of chaperones [122]. These results indicate that obesity is characterized by loss of XBP1s activity or by an XBP1s-deficient state. Given these observations, it is possible to postulate that loss of XBP1s activity contributes significantly to the development of ER stress in obesity, and consequently to insulin resistance and type 2 diabetes. Indeed, reestablishing the activity of XBP1s in the liver of obese and diabetic mice greatly enhances glucose tolerance, increases insulin sensitivity, and reduces blood glucose levels to euglycemia [123].

Calcium levels in the ER also contribute to the development of obesity-related ER stress. Normally, the ER stores free calcium, and the high calcium levels in its lumen are essential for ER functions and ER homeostasis [14, 16]. Perturbation of luminal calcium concentrations in the ER creates severe ER stress as it interferes with the activities of enzymes and chaperones [14, 124]. The Ca<sup>2+</sup> pump sarco(endo)plasmic reticulum Ca<sup>2+</sup>-ATPase (SERCA) resides in the ER membrane and functions to reuptake cytosolic calcium into the lumen of the ER [16]. Inhibition of SERCA activity by thapsigargin blocks calcium reuptake from the cytoplasm into the ER lumen, bringing about severe ER stress and triggering activation of the UPR [16, 124]. Notably, SERCA2b, the main isoform of SERCA2 in the liver [124], is significantly reduced in the liver of obese and diabetic mice, and overexpression of this isoform in the liver of obese mice markedly reduces ER stress and blood glucose levels, resulting in improved glucose tolerance and insulin sensitivity [15]. This role of SERCA2b is likely mediated via increased chaperone activity as a result of restoration of calcium levels in the ER by SERCA2b [15].

A recent surprising finding was that the inflammatory kinase p38 mitogen-activated protein kinase (p38 MAPK) interacts with XBP1s [125] (Fig. 3). In general, inflammatory pathways are believed to be detrimental for metabolic homeostasis [126–128]. Indeed, recent reports indicate that ER stress in obesity could be induced by inflammation and inflammatory signaling cascades [129–134]. However, activation of inflammatory signaling cascades by TNF- $\alpha$  during ER stress conditions has a completely different effect, and in fact may lead to a reduction in ER stress [125]. This outcome is mediated through activation of p38 MAPK. Despite being one of the main inflammatory nodules in the cell, p38 MAPK greatly enhances nuclear translocation and activation of XBP1s by phosphorylating it on Threonine48 and Serine61 [125]. Furthermore, p38 MAPK is normally activated in the liver after refeeding, but this signaling mechanism is blunted in obesity. Thus, phosphorylation of XBP1s is also diminished in obesity and its activity is reduced [125]. Gain-of-function experiments in the liver of



**Fig. 3** ER stress in obesity and type 2 diabetes. **a** The regulatory subunits of PI3K, p85 $\alpha$  and p85 $\beta$ , form a heterodimer, which dissociate from each other during insulin receptor signaling. Binding of p85 $\alpha$  or  $\beta$  to XBP1s leads to nuclear translocation of XBP1s. In obesity conditions, interaction of p85 $\alpha$  or  $\beta$  with XBP1s is disrupted, and it results in defective XBP1s nuclear translocation. **b** XBP1s interacts with FoxO1 and leads to proteasome-mediated degradation of FoxO1. Defective XBP1s nuclear translocation in obesity conditions results in accumulation of FoxO1 in the nucleus. **c** p38 MAPK phosphorylates XBP1s on Thr48 and Ser61 residues, and these phosphorylations are required for XBP1s nuclear translocation, which is defective in obesity conditions



obese and diabetic mice support the finding that activation of p38 MAPK enhances XBP1s activity, relieves ER stress, greatly reduces glucose intolerance and insulin resistance, and ultimately normalizes the blood glucose levels [125]. Collectively, these observations raise the important issue of the various roles played by inflammatory signaling in the development of metabolic diseases, and indicate that inflammation itself might even be beneficial for ER stress states and for metabolic homeostasis under certain circumstances.

Increased ER stress in hypothalamic neurons of obese mice significantly contributes to the development of leptin resistance and obesity [27]. In this context, brain-specific XBP1 depletion in mice leads to the development of obesity and severe leptin resistance when the animals are challenged with a HFD [27].

Taken together, current evidence that links ER stress to obesity and obesity-associated diseases suggests that reducing ER stress in obesity would be an attractive strategy for the treatment of obesity and type 2 diabetes. How can this be achieved by targeting elements of the UPR signaling

pathways? Inhibition of IRE1 kinase activity without effecting its endoribonuclease activity could be a possible therapeutic modality aimed at reducing ER stress-mediated insulin resistance. Increasing the activity of XBP1 also holds great promise for increasing ER capacity and reducing ER stress in obesity. Indeed, as discussed above, XBP1s activity is also reduced in obesity, and re-establishment of this activity has robust anti-diabetic effects [123]. A major challenge in this area of research, however, is that inhibition of a single molecule or arm of the UPR signaling network might be compensated by other mechanisms. However, the strategy of targeting the ER as a whole, and increasing its capacity and efficiency, may yield new therapeutic approaches for treatment of type 2 diabetes and obesity.

#### Wolcott-Rallison syndrome

The possible contribution of UPR elements in the development of human disease has direct relevance for the pathogenesis of Wolcott-Rallison (WR) syndrome, a disorder

characterized by early-onset diabetes and multiple epiphyseal dysplasias [135, 136]. WR syndrome is an autosomal recessive disease caused by mutations in the *EIF2AK3* gene, which encodes PERK in humans [135, 136]. The mutations in WR syndrome impair the ability of PERK to phosphorylate eIF2 $\alpha$  [136]. Patients with this syndrome exhibit features that are similar to those reported for PERK-deficient mice, which is now an accepted model for studying the pathologies associated with this disorder [73, 135]. Patients with WR syndrome develop hypoinsulinemia and hyperglycemia, due to a progressive destruction of pancreatic  $\beta$  cells [135, 137]. Strikingly, however, WR patients often display acute severe hypoglycemia, which is not reported for *Perk*<sup>-/-</sup> mice [138]. WR syndrome has not been investigated in depth at a molecular level, but the fact that PERK is a major contributor to the development of type 1 diabetes mellitus in this syndrome underscores the significance of the UPR in the pathophysiology of WR-related diabetes.

#### $\alpha$ 1-Antitrypsin deficiency

Deficiency of  $\alpha$ 1-antitrypsin ( $\alpha$ 1-AT) is an example of a human disease caused by protein misfolding in the ER.  $\alpha$ 1-AT is a protease inhibitor that is synthesized in the liver, and functions primarily to protect cells from neutrophil elastase activity, which enzymatically destroys tissues, especially in lung alveoli.  $\alpha$ 1-AT deficiency is a genetic disorder caused by a mutation in the  *$\alpha$ 1-AT* gene [139]. The mutant  $\alpha$ 1-ATZ molecule is one of the most common deficiency variants [139, 140], arising from a point mutation at the Glutamate342 which is substituted with a Lysine residue [140]. The substitution disrupts the structure of  $\alpha$ 1-AT such that mutant  $\alpha$ 1-ATZ molecules favor the formation of a dimer [140].  $\alpha$ 1-ATZ is functionally active even after it has been secreted [141–143]. However, the ER machinery tends to retain the misfolded  $\alpha$ 1-ATZ proteins in the lumen until such time as it may be properly folded [142].  $\alpha$ 1-ATZ is degraded by the proteasome-dependent pathway [144], but also by autophagy [145]. The  $\alpha$ 1-ATZ that is trapped in the ER of liver cells triggers elevated ER stress and inflammation [146].

Levels of ER stress and activation of the UPR have not yet been extensively studied within the context of  $\alpha$ 1-AT deficiency in humans. However, increased levels of XBP1s and ATF4 are reported in monocytes from humans with  $\alpha$ 1-AT deficiency [147]. Overexpression of active ATF6 in cells promotes the disposal of  $\alpha$ 1-ATZ by ER-associated degradation pathways [148], directing to a strategy that could limit hepatic damage caused by  $\alpha$ 1-ATZ that is trapped in the ER. However, the lack of functional  $\alpha$ 1-AT in patients by this approach would present an obstacle. Methods that induce proper folding of  $\alpha$ 1-ATZ, or improve release of  $\alpha$ 1-ATZ from the ER, might be more viable in terms of therapeutic applications because the mutated form would be active in this case.

#### Cystic fibrosis

Cystic fibrosis (CF) is caused by a mutation in the gene that encodes the cystic fibrosis transmembrane conductance regulator (CFTR) protein [149], an ion channel that regulates the transport of chloride and sodium ions across epithelial membranes. A mutation in CFTR results in the abnormal movement of ions, and severely affects organs such as the lung, liver, and pancreas [149]. As for other transmembrane proteins, CFTR is synthesized in the ER [150]. The most common CF mutation is the deletion of the phenylalanine508 residue ( $\Delta$ F508-CFTR) [151, 152]. While most of the misfolded  $\Delta$ F508-CFTR protein is subjected to proteasome-mediated degradation [150, 153], some of it is retained in the ER and ER-Golgi intermediate compartment [153, 154]. Newly synthesized CFTR is glycosylated at Asparagine897 and 900 residues and transported to the Golgi apparatus for further modification before the transport to the plasma membrane [154]. The 508 aa resides in one of the two NBD, which is found in the cytoplasmic region of the CFTR protein. The  $\Delta$ F508 mutation results in an overall conformational defect due to alterations in domain–domain interactions within the protein. While the detailed mechanism by which mutant CFTR is retained in the ER is not fully understood, it is suggested that there are diarginine (RXR) ER retention/retrieval signal motifs in the cytoplasmic domain of CFTR. These motifs reside inside of the properly folded CFTR protein. However,  $\Delta$ F508 mutation leads to exposure of these motifs to the ER lumen, and blocks complete folding of the protein, which ultimately leads to retention of CFTR protein in the ER and subsequent degradation [155]. In support, replacing the arginine residues with a lysine restores the trafficking and function of CFTR [156].

CF is often accompanied by chronic airway infection and inflammation. Bronchial epithelia in humans with CF display increased levels of XBP1s and ATF4 [157, 158], and in vitro studies show that ATF6 levels are increased in  $\Delta$ F508-CFTR-expressing cells [159]. Several studies suggest that activation of the UPR in CF protects airway epithelia by increasing the concentration of stored calcium [157, 158]. Activation of the UPR protects from the amino acid loss and oxidative stress caused by inflammation in CF [157, 158]. Meanwhile, CF exhibits mechanisms to overcome increased ER stress and inhibit further activation of the UPR. Mutant CFTR proteins that are trapped in the ER are directly subjected to ubiquitin-dependent proteasomal degradation [160]. In addition, CFTR is transcriptionally repressed during ER stress and activation of the UPR [161, 162]. Calreticulin, an ER stress-responsive molecular chaperone found in the ER, decreases the expression and membrane localization of CFTR [163, 164]. Accordingly, downregulation of calreticulin increases CFTR expression in the membrane, both in in vitro and in vivo settings [163, 164]. Thus, calreticulin likely traps mutant CFTR in the ER lumen. Use of siRNA to downregulate ATF6 also leads to increased

membrane CFTR and better ion flux through the CFTR [159]. The contribution of elements of the UPR to the regulation of CF suggests that the modulation of UPR might yield opportunities for developing novel CF therapeutics. As with  $\alpha$ 1-AT deficiency, because the mutant CFTR molecule still retains some functional capacity [165], a rewarding approach might be to increase the membrane trafficking of CFTR with use of chemical chaperones that facilitate its release from the ER.

#### Neurodegenerative diseases

ER stress and the UPR signaling are closely linked with many neurodegenerative diseases, including Parkinson's (PD), Huntington's (HD), and Alzheimer's (AD) diseases.

##### *Parkinson's disease*

PD is characterized by impairment of movement due to the loss of dopaminergic neurons in the brain. Genetic studies reveal that a familial form of PD, known as autosomal recessive juvenile parkinsonism (AR-JP), results from defects in the *Parkin* gene that encodes the ubiquitin protein ligase E3 [166–168], which tags proteins for degradation. It has been suggested that the development of AR-JP, caused by a mutation of the *Parkin* gene, is related to regulation of the UPR. In this case, defective E3 activity in AR-JP leads to a failure in the tagging of Parkin substrates for degradation, their accumulation in the ER, and the triggering of ER stress in neurons [169]. Prolonged ER stress ultimately leads to neuronal cell death and the development of PD [169–171].

In vitro studies document that Parkin protein actively responds to the UPR. Parkin is upregulated during ER stress to induce protein degradation [166, 167], and overexpression of Parkin in dopaminergic neuroblastoma cells reduces ER stress and suppresses neuronal cell apoptosis induced by the UPR [166, 167]. Several studies indicate that CHOP mediates cell death of dopaminergic neurons in PD [81, 172, 173]. For example, the levels of CHOP are increased following administration of 6-hydroxydopamine (6-OHDA), a neurotoxin that induces apoptosis of dopaminergic neurons [174]. In parallel, *Chop* knockout mice are resistant to 6-OHDA treatment-induced apoptosis of dopaminergic neurons [174]. Thus, within the context of apoptosis and the development of neurodegenerative diseases, CHOP may emerge as an attractive target for a therapeutic modality. However, because current data were obtained from whole body *Chop* knockout mice, further research is essential to confirm the role of CHOP in specific populations of neurons.

##### *Huntington's disease*

HD is also a genetic neurodegenerative disorder caused by an increase in the number of CAG trinucleotide

repeats in the *huntingtin* (*HTT*) gene [175, 176]. Mutations result in abnormally long huntingtin protein [177]. Fragments of long huntingtin protein bind with each other and accumulate in regions of the cytoplasm and in the perinuclear space [178, 179]. In most cases, these accumulations form nuclear inclusions in neurons and disrupt the function of the brain region that mediates movement, thinking, and emotions [180, 181]. Aggregated huntingtin proteins impair the proteasome degradation system, which leads to further accumulation of other misfolded proteins [182, 183] and contributes to the development of ER stress [177].

An in vitro study with the use of siRNA initially suggested that the ER is involved in the development of HD. Specifically, deletion of the *HTT* gene by siRNA disrupts the structure and networks of the ER [184]. The finding that HTT proteins associate with microtubules [185] led to the suggestion that HTT interferes with the ER network by disrupting the configuration of the cytoskeleton [184].

More direct evidence for the correlation of HD and ER function is derived from postmortem brain samples of HD patients, which display elevated expression levels of UPR target genes such as CHOP, GRP78, and Herp [186]. Expression of these ER stress-related genes is augmented in an HD mouse model [186]. In vitro studies by other investigators also confirm that ER stress and expression of UPR target genes are increased in HD [187, 188]. Specifically, expression of CHOP, GRP78, and PDI is higher (relative to control) in a striatal cell line that was established from an *HTT* knock-in mouse model [187]. Another group also reports on elevated JNK activity in cells that overexpress expanded poly(Q) peptides, which form aggregates resembling those of HTT protein in HD [188]. JNK activation is followed by caspase-12 activation and apoptosis [188].

Mutant HTT also disturbs ER calcium homeostasis [189]. Perturbation of high intraluminal calcium concentrations in the ER interferes with the activity of chaperones, and creates severe ER stress [14, 124]. A recent study showed that XBP1 deletion decreases accumulation of HTT protein, and protects against the development of HD symptoms in the YAC128 mouse model, which carries the human *HTT* gene with 128 CAG repeats [190]. The mechanism that links reduced HTT protein with XBP1 deficiency is enhanced autophagy, which is regulated by increased expression of Forkhead box protein O1 (FoxO1) [190, 191]. This observation is in parallel with the notion that XBP1s mediates proteasome-mediated degradation of FoxO1 [123] and by the observation that XBP1 deficiency results in increased levels of FoxO1 [123]. Taken together, the above observations that ER homeostasis is disrupted in HD could be incorporated into future studies aimed at developing novel treatments for HD.

### Alzheimer's disease

Elevated ER stress is a feature of AD, a disorder characterized by formation of insoluble fibrous protein aggregates in the brain. A major component of such aggregates is the amyloid  $\beta$  ( $A\beta$ ) peptide [192, 193].  $A\beta$  is generated via cleavage of the transmembrane glycoprotein amyloid precursor protein (APP) by presenilin (PS), which is a component of the  $\gamma$ -secretase complex, a membrane-resident protease [192].

The length of  $A\beta$  varies from 36 to 43 amino acid residues, depending on the site where APP is cleaved. The most common peptides are  $A\beta_{40}$  and  $A\beta_{42}$ , which arise from cleavage after residues 40 and 42, respectively [194]. In neurons, generation of  $A\beta_{40}$  occurs in the Golgi apparatus, while  $A\beta_{42}$  is generated in the ER [194]. Generation of  $A\beta_{42}$  in the ER may be an initial event in the development of AD. Thus, inhibition of  $A\beta_{42}$  production may arrest the development or progression of disease [194]. Accumulation of unfolded proteins and activation of the UPR is also seen in patients with AD [195], and the levels of GRP78 and phosphorylated PERK are elevated in the temporal cortex and hippocampus of these patients at different stages of AD [195]. Moreover, the UPR is activated in pre-tangle hippocampal neurons of AD patients, as shown by the report of increased levels of phosphorylated PERK, eIF2 $\alpha$ , and IRE1 [196].

Genetic studies reveal more than 100 mutations in the PS gene that are associated with an autosomal dominant familial AD (FAD) [197, 198]. Recent evidence indicates that PS forms an ion channel for calcium trafficking [199] and mutations of PS that are associated with FAD is important for ER calcium homeostasis [199]. Therefore, mounting evidence points to dysregulation of ER function and ER stress signaling as having a key role in AD pathology.

### Cancer

The link between the UPR and cancers has been amply established as the UPR is highly activated in a number of cancers. The expression of GRP78 and other glucose-regulated proteins that are induced during tumor growth [200] was documented in the 1990s. Since then, increased GRP78 levels have been reported in several cancers, including malignant human breast cancer [201], lung cancer [202], colon cancer [203], and ovarian cancer [204, 205]. In agreement with this notion, suppression of GRP78 inhibits cancer cell growth [206, 207]. Increased levels of chaperones could be protective for tumor cells and enable them to grow faster and have a more solid ER homeostasis.

A role for IRE1 in cancer biology has also been highlighted by investigations of malignant gliomas [208], which are deadly, highly proliferative brain tumors. Angiogenesis is a particular hallmark of gliomas. Inhibition of IRE1 signaling results

in decreased angiogenesis, slower tumor growth rates, and reduced invasiveness of the glioma cells [208]. Similarly, depletion of PERK or ATF4 in human tumor cells also reduces tumor growth rate and angiogenesis [209]. Furthermore, PERK induces the translation of pro-angiogenic genes, and PERK deletion in MEFs impairs vasculogenesis and decreases cancer cell proliferation [210]. In addition, ER stress leads to degradation of p53, a tumor suppressor gene [211]. Taken together, many tumors depend on an intact UPR for survival.

Another example of the association between the UPR and cancer is seen in multiple myeloma (MM), which is characterized by excess production of monoclonal proteins in bone marrow plasma cells. MM cells display increased ER stress and elevated levels of XBP1 [212]. Earlier reports identified a requirement of XBP1 in plasma cell differentiation [90], which makes it plausible that XBP1 is involved in the development of MM. In support of this notion, transgenic mice with overexpression of XBP1s in B cells and plasma cells develop pathology, including subendothelial immunoglobulin deposition, similar to that reported for human MM [213]. Furthermore, these mice exhibit aberrant expression of genes that are also dysregulated in human MM [213]. In an effort to treat MM by manipulating the UPR, treatment of MM cell lines with a small molecule IRE1 $\alpha$  endoribonuclease inhibitor called MKC-3946 [214] resulted in reduced ER stress and a reduced rate of tumor cell growth [214].

Bortezomib, a 26S proteasome inhibitor that is used in the treatment of MM [215, 216], has an anti-cancer activity that prevents degradation of pro-apoptotic factors such as I $\kappa$ B, and suppresses production of anti-apoptotic proteins such as Bcl-2 [217, 218]. Degradation of I $\kappa$ B by the proteasome promotes the translocation of nuclear factor  $\kappa$ B (NF $\kappa$ B) to the nucleus, and increases the expression of genes involved in cell growth and cell survival. NF $\kappa$ B activity is reportedly elevated in MM patients [219]. Another mechanism responsible for bortezomib-induced apoptosis of MM cancer cells involves ER stress [220, 221]. Prolonged ER stress created by bortezomib-induced proteasome inhibition disturbs calcium homeostasis, and results in the release of calcium from the ER. Uptake of this calcium by mitochondria is followed by the release of cytochrome c, which activates caspases and induces apoptosis [220]. Bortezomib is known to trigger apoptosis in MM cells by activation of caspase-2 [221]. Bortezomib promotes ER stress-induced apoptosis in pancreatic cancer cells through activation of JNK [222]. Many other reagents, including MKC-3946, enhance the cytotoxic effects of bortezomib in MM [214]. MKC-3946 blocks the splicing of XBP1, induced by bortezomib by inhibiting endoribonuclease activity of IRE1 [214], thereby creating further ER stress. It is not clear how exactly MKC-3946 blocks XBP1 splicing without affecting phosphorylation of IRE1. However, through this mechanism, IRE1 still has apoptotic effect via activation of JNK in the presence of MKC-3946.

Interestingly, low levels of total XBP1 mRNA are correlated with resistance to bortezomib treatment, whereas high XBP1 mRNA levels increase sensitivity to bortezomib [223]. This suggests that the total XBP1 mRNA levels prior to bortezomib treatment are important for the response to therapy [223].

## Chemical chaperones

What are chemical chaperones?

Chemical or pharmaceutical chaperones comprise a group of low molecular weight compounds that are known to stabilize protein conformation against thermally and chemically induced denaturation [224, 225]. Agents that have chemical chaperone activity include polyols, amines, glycerol, trimethylamine *N*-oxide, and dimethyl sulfoxide [225–228], plus compounds such as 4-phenylbutyric acid (PBA) and tauroursodeoxycholic acid (TUDCA) [104, 229].

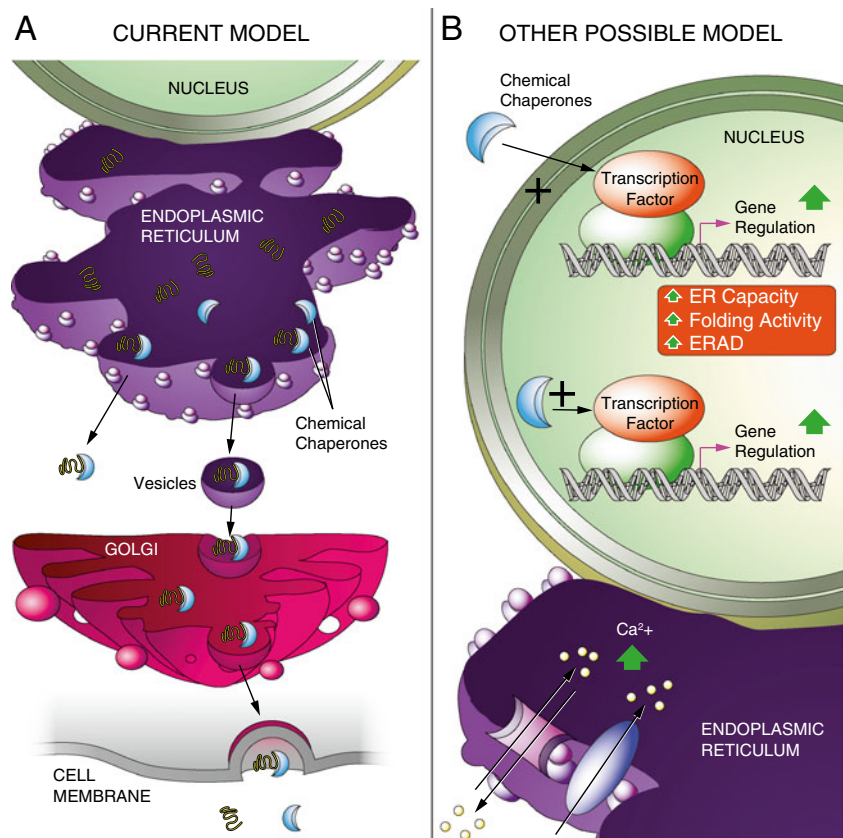
The mechanism underlying chemical chaperone functioning

The mechanisms that mediate the functioning of chemical chaperones in protein folding are not fully understood. We discuss here two categories of action (Fig. 4): (1) compounds

such as DMSO or glycerol have the ability to coat proteins in the ER, and mask hydrophobic patches on unfolded proteins, thereby increasing the secretion of the proteins [230]. These compounds do not increase the folding capacity of the ER; rather they create a detergent effect, which increases the release of unfolded proteins from the ER by allowing them to escape quality control mechanisms of the ER. As discussed below, one example of such a mechanism of action is the release of mutated CFTR protein from the ER before it is completely folded (see below in section on “Cystic fibrosis”) [224, 231]; (2) compounds indirectly affect ER folding capacity. For example, molecules that activate transcriptional programs leading to increased expression of chaperones in the ER can also act as chemical chaperones. We believe it is unlikely that chemical chaperones increase the folding of proteins in the ER directly, thereby increasing their folding. To the best of our knowledge, no examples of such effects of direct binding exist in the literature. The suggestion of direct binding having an influence on protein folding probably is derived from the observations that some chemicals can increase the secretion of the unfolded proteins, which led to the belief that these compounds (chemical chaperones), like the known molecular chaperones, assist protein folding.

In addition to these two possibilities, agents that regulate ER calcium homeostasis could also serve as chemical

**Fig. 4** The action of chemical chaperones. **a** Current model. Chemical chaperones nonspecifically coat the surface of newly synthesized proteins and enhance their process of secretion. **b** Other possible model. Chemical chaperones enhance the transcription of genes that are involved in ER capacity, ER folding activity, and ERAD, by leading to activation of transcription factors or binding to transcription machinery. Chemical chaperones may act on calcium homeostasis by manipulating calcium influx into the ER lumen



chaperones. Recent observations indicate that increased SERCA2b function could be beneficial for ER homeostasis [15]. This approach, without changing the expressions of chaperones, may increase the activity of molecular chaperones or folding enzymes in the ER, thereby increasing ER folding capacity.

### Possible therapeutic implications of chemical chaperones for disease

#### Type 2 diabetes and insulin resistance

The potential use of chemical chaperones for the treatment of type 2 diabetes was first demonstrated in the study where 4-PBA and TUDCA were shown to reduce ER stress and improve insulin sensitivity [104]. Administration of 4-PBA and TUDCA, which have distinct structures but share similar chemical chaperone activity, decreased PERK and IRE1 phosphorylation in the liver of obese and diabetic mice, and greatly enhanced glucose tolerance and the diabetic phenotype [104]. While this study was a proof of principle, 4-PBA and TUDCA are both weak chemical chaperones and must be administered at high doses to reduce ER stress. Our current working hypothesis with regard to the action of these chemical chaperones is that they upregulate a complex transcriptional program that increases ER capacity, and ultimately reduces ER stress. Indeed, 4-PBA activates PPAR $\alpha$  [232], and a more recent report documents that PPAR $\gamma$  regulates ER function [233]. Furthermore, TUDCA also affects other transcription factors [234, 235]. Further and more detailed work is required to understand the mechanism of action of these compounds in regulating ER homeostasis.

The effects of TUDCA on ER stress and insulin sensitivity have been studied in humans [236]. TUDCA was administered orally to obese and insulin-resistant human subjects for 4 weeks, to determine whether it could effectively treat insulin resistance in obese individuals by improving ER capacity. However, our previous observations have shown that TUDCA does not have a good oral availability in mice, and high doses of the compound are required to reduce ER stress in obese mice even when delivered via intraperitoneal injection. Because it would be a challenge to achieve a working dose of TUDCA through oral administration, the experimental design was not optimally suited for exploring possible beneficial effects of TUDCA in terms of reducing ER stress in obese humans. Indeed, administration of 1,750 mg/day of TUDCA was not enough to reduce ER stress in obese humans [236]. Nonetheless, significant increases in insulin sensitivity were recorded following TUDCA administration in this report [236]. The fact that TUDCA caused increased insulin sensitivity in the absence of effects on ER stress may indicate that the reduction in ER stress was

too low to detect by the techniques used or that TUDCA exerted ER stress-dependent as well as -independent effects on the insulin signaling pathway.

#### Leptin resistance

Increasing leptin sensitivity in obesity could provide a unique strategy for treating this debilitating disease. Nevertheless, despite extensive research efforts, no effective leptin sensitizers have been described. Recent reports indicate that reducing ER stress in the hypothalamus of obese mice via the use of chemical chaperones can provide a novel approach for increasing leptin sensitivity and influencing the treatment of obesity [27]. However, the same issues discussed above with regard to a requirement for very high dose administration of 4-PBA and TUDCA also apply in the case of leptin sensitization. A critical next step will be to create more potent chemical chaperones with better pharmacological availability to enable translation of these studies from mice to humans.

#### $\alpha$ 1-Antitrypsin deficiency

The first attempts at using chemical chaperones to stabilize  $\alpha$ 1-ATZ and increase its secretion were with the use of glycerol [143]. The simple addition of glycerol enhanced the fidelity of protein folding, and resulted in better secretion of  $\alpha$ 1-ATZ in *in vitro* models [143]. PBA treatment also significantly increases the secretion of ER-trapped mutant  $\alpha$ 1-ATZ. Moreover, this effect of PBA was demonstrated in transgenic mice that carry the human  $\alpha$ 1-ATZ gene. Oral administration of PBA significantly increased the release of human  $\alpha$ 1-ATZ protein into the circulation [143, 237]. However, only the end result, namely whether or not the chemical chaperone enhances secretion of  $\alpha$ 1-ATZ, was monitored in these experiments. To understand the mechanistic underpinnings of how chemical chaperones act, and to translate this approach to the clinical setting, it is also important to verify whether the levels of ER stress and activation of the UPR are affected.

#### Cystic fibrosis

As discussed above, the mutant  $\Delta$ F508-CFTR protein in CF is retained in the ER [150, 160]. Earlier work showed that glycerol facilitates the folding of mutant CFTR protein, and increases the localization of CFTR to the membrane [238]; this work also examined the effect of PBA on CF in humans [151]. Patients with the  $\Delta$ F508-CFTR mutation, who received 1 week of PBA therapy, exhibited partial improvements of CFTR activity in nasal epithelia [151]. Nasal potential difference responses, used to examine basal chloride transport as an indication of the channel's activity, were also improved following PBA treatment. However, the test scores for sweat chloride concentration, which serves as an

index of CFTR channel function, were unaffected by the treatment [151]. With regard to this seeming discrepancy in the results, it has been suggested that sweat chloride concentration does not necessarily predict the severity of lung disease in CF patients [239]. The use of another compound called benzo(c)quinolizinium has also been proposed for use in increasing  $\Delta F508$ -CFTR expression or for increasing apical membrane trafficking of  $\Delta F508$ -CFTR in the membrane [240, 241]. The detailed mechanism of action of the compound is not fully understood.

#### Neurodegenerative diseases

There is intense interest in how ER stress contributes to the development of neurodegenerative changes, and in identifying potential therapeutics that can target this process. A number of studies have explored the effect of chemical chaperones on neurodegenerative diseases. For example, administration of PBA in in vitro and in in vivo models of AD leads to a reduction in amyloid plaques in the brain and improves phenotypic behaviors [242]. In vitro assays demonstrated that PBA prevents apoptosis in neuronal cells [243]. Similarly, amyloid plaques in the brain are also decreased with use of TUDCA [244], and apoptosis is reduced in cell lines [245, 246]. Administration of TUDCA in a rat model of HD improves the phenotypic features of HD [247]. TUDCA also has neuroprotective effects against MPTP, a neurotoxin used to generate the rodent model of PD [248].

#### Cancer

Deficiency of tuberous sclerosis complex (TSC) genes constitutively activates mTOR signaling [120, 121] and contributes to the development of tumors [219, 249]. It was shown that loss of TSC activity elevates ER stress levels and promotes susceptibility to ER stress-induced apoptosis [113]. In this study, more apoptosis was observed in kidney adenomas from thapsigargin-treated *Tsc2*<sup>+/-</sup> mice when compared to normal kidney tissues; in other words, TSC-deficient tumor cells respond better to ER stress-induced apoptosis. This observation provides an alternate strategy for targeting tumors with TSC deficiency and dysregulated mTOR signaling, and highlight that acute ER stress-induced apoptosis may be stimulated with ER stress-inducing agents in certain circumstances [113].

On the other hand, PBA treatment can induce apoptosis in cancer cells, including those from colon cancer, prostate cancer, and gastric cancer [250–252]. In fact, PBA has been already tested in clinical trials for the treatment of malignant glioma [253] and hematological malignancies [254]. However, the requirement for very high doses of PBA for successful treatment remains as a challenge for this approach. Nonetheless, these studies suggest that in certain tumors, the UPR provides anti-tumor or tumor suppressor functions.

#### Conclusions

The UPR is a sophisticated and highly sensitive signaling pathway that influences a broad range of activities within cells. Its primary roles are to protect cells from undergoing ER stress and to maintain ER homeostasis. However, prolonged activation of the UPR can lead to apoptosis and tissue damage. While significant progress has been made in understanding how the UPR is regulated at the molecular level, more detailed mechanistic knowledge is necessary for effective manipulation of UPR elements in order to develop strategies for the treatment of diseases. Reducing the effects of ER stress in disease will probably require a variety of approaches, depending on the disease. Increasing the folding capacity of the ER could be beneficial for some diseases (e.g., obesity), as well as those that benefit the escape or secretion of mutated proteins from the ER (e.g., CF). In other circumstances, activating the UPR could be more appropriate (e.g., TSC disease). It must be emphasized that there is no universal solution that is relevant for developing strategies to manipulate ER stress in different diseases. Effective solutions will have to be individualized for each condition.

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