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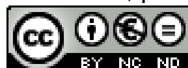

Stress management at the ER: regulators of ER stress-induced apoptosis

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Running title: Regulators of ER stress-induced apoptosis

Abstract

The endoplasmic reticulum (ER) is an elaborate cellular organelle essential for cell function and survival. Conditions that interfere with ER function lead to the accumulation and aggregation of unfolded proteins which are detected by ER transmembrane receptors that initiate the unfolded protein response (UPR) to restore normal ER function. If the ER stress is prolonged, or the adaptive response fails, apoptotic cell death ensues. Many studies have focused on how this failure initiates apoptosis, particularly because ER stress-induced apoptosis is implicated in the pathophysiology of several neurodegenerative and cardiovascular diseases. In this review we aim to shed light on the proteins that are not core components of the UPR signaling pathway but which can influence the course of the ER stress response by regulating the switch from the adaptive phase to apoptosis.

Keywords: Apoptosis, Bcl-2 family, Endoplasmic reticulum (ER) stress, Unfolded protein response (UPR)

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List of abbreviations

ASK1	Apoptosis signal-regulating kinase 1
ATF	Activating transcription factor
BAK	Bcl-2 homologous antagonist/killer
BAX	Bcl-2 associated protein X
BCL-2	B-cell lymphoma 2
BH3	Bcl-2 homology domain 3
BI-1	Bax inhibitor 1
Bim	Bcl-2 interacting mediator of cell death
C/EBP	CAAT/enhancer binding protein
CHOP	C/EBP homologous protein
cIAP	cellular inhibitor of apoptosis protein
eIF2α	Eukaryotic initiation factor 2 α
ER	Endoplasmic reticulum
GADD	Growth arrest and DNA damage inducible gene
GRP	Glucose regulated protein
HSP	Heat shock protein
IAP	Inhibitor of apoptosis protein
IRE1	Inositol-requiring enzyme 1
JNK	c-Jun N-terminal kinase
MEF	Mouse embryonic fibroblast
PERK	Pancreatic ER kinase (PKR) -like kinase
RIDD	Regulated IRE1-dependent decay of mRNA
XBP1s	spliced X box-binding protein 1
TRAF2	Tumor necrosis factor (TNF) receptor-associated factor 2

TRB3	Tribbles homolog 3
UPR	Unfolded protein response
XBP1	X box-binding protein 1
XIAP	X chromosome-linked IAP

1. Introduction

The endoplasmic reticulum (ER) is a continuous membranous network of sacs and tubes in the cell. It includes the regions known as the smooth ER, rough ER and the outer nuclear envelope. It is also in close contact with the mitochondria. It is the primary site for the synthesis and folding of secreted, membrane-bound and some organelle-targeted proteins. Optimum protein folding within the ER lumen requires ATP, Ca^{2+} and an oxidizing environment to allow disulfide bond formation [27]. Conditions that perturb cellular energy levels, the redox state or Ca^{2+} concentration reduce the protein folding capacity of the ER. Such conditions, as well as mutations within proteins that impair their correct folding or otherwise impede their further processing or transport within the ER, result in the accumulation and/or aggregation of unfolded proteins, a condition referred to as ER stress. To overcome the deleterious effects of ER stress, cells have evolved a series of adaptive and protective strategies collectively termed the unfolded protein response (UPR). However, if unresolved, ER stress is lethal to cells via what is known as ER stress-induced apoptosis. Intriguingly, both responses are downstream of UPR and unsurprisingly, there are numerous pathophysiological conditions associated with ER stress including ischemia, neurodegenerative diseases, cancer and diabetes [17, 38].

The UPR is a concerted and complex cellular response that is mediated through three ER transmembrane receptors: pancreatic ER kinase or PKR-like ER kinase (PERK), activating transcription factor-6 (ATF6) and inositol-requiring enzyme 1 (IRE1, also known as ERN 1). In resting cells, all three ER stress receptors are maintained in an inactive state through association with the ER chaperone, 78 kDa glucose regulated protein (GRP78). Under conditions of ER stress, accumulating unfolded proteins leads to GRP78 dissociation and activation of the three ER stress receptors triggering the UPR. The UPR is a pro-survival response aimed at to reducing the backlog of unfolded proteins and restoring normal ER

function [94] (Fig 1). However, if the stress cannot be resolved, this otherwise protective signaling switches to a pro-apoptotic response. This review examines the molecular mechanisms that promote an apoptotic response by influencing UPR-mediated signals during the three distinct phases of ER stress-induced apoptosis: initiation, commitment and, execution [99]. We will discuss regulators of ER stress-induced apoptosis, where we define a regulator as any protein that is not required for transducing the signal in the UPR pathway, but which can modulate the activity of the pathway such that the likelihood of apoptosis occurring is altered.

2. Regulation of the unfolded protein response and initiation phase of ER stress-induced apoptosis

2.1 PERK, ATF6 and IRE1 mediate the initiation phase of the UPR

2.1.1 PERK:

Dissociation of GRP78 from PERK results in its dimerization, autophosphorylation and activation. Active PERK phosphorylates the alpha subunit of the eukaryotic initiation factor 2 (eIF2α), inhibiting general protein translation [34]. Inhibition of protein translation aids cell survival by decreasing the load of nascent proteins in the ER. In fact, PERK$^{-/-}$ mouse embryonic fibroblasts (MEFs), when challenged with ER stress-inducing agents, failed to block protein translation and exhibited increased cell death. Inhibition of translation with cycloheximide reduced ER stress-induced cell death, confirming that blocking the buildup of unfolded nascent proteins is critical for cell survival [36]. This attenuation of translation is, however, not absolute; mRNAs carrying certain regulatory sequences in their 5' untranslated regions can bypass the phospho-eIF2α-mediated translational block and can sometimes be translated at even higher rates [94]. The most studied of such transcripts encodes ATF4, a member of the

CCAAT/enhancer-binding protein (C/EBP) family of transcription factors. ATF4 translation is upregulated upon eIF2α phosphorylation and promotes cell survival by inducing genes involved in amino-acid metabolism, redox reactions, stress response and protein secretion [37]. However, not all the genes induced by ATF4 are associated with cell survival. The transcription factor C/EBP homologous protein (CHOP), whose induction strongly depends on ATF4 [35], is thought to promote apoptosis (Fig 1). In conclusion, activation of PERK is initially protective and critical for survival in the face of mild ER stress. However, activation of PERK also leads to induction of CHOP, which, as detailed later, is an important element in the switch from pro-survival to pro-death signaling.

A second target of PERK is the transcription factor, nuclear factor (erythroid-derived 2)-like 2 (NRF2) whose phosphorylation liberates it from its inhibitor KEAP1, allowing for induction of target genes that are mainly involved in oxidative stress signaling [13].

2.1.2 ATF6:

Dissociation of GRP78 from ATF6 allows its translocation to the Golgi where it is cleaved to its active form by Site-1 and Site-2 proteases (S1P and S2P). Active ATF6 then translocates to the nucleus to induce expression of genes with an ER stress response element (ERSE) in their promoter [94]. The targets of ATF6 include ER chaperone proteins such as GRP78, GRP94, protein disulphide isomerase, and the transcription factors CHOP and X box-binding protein-1 (XBP1) [116]. XBP1 is important in IRE1 signaling and thus links ATF6 with pro-survival signaling emitted through IRE1, as discussed below in more detail (Fig 1). Although ATF6 has long been thought to transduce purely pro-survival signals and counteract ER stress, overexpression of ATF6 can induce CHOP mRNA expression as well, whereas overexpression of a dominant negative ATF6 mutant blocks CHOP induction by ER stress [116]. Recently, ATF6

has been linked to ER stress-induced apoptosis in a myoblast cell line where it was shown to induce apoptosis by indirectly downregulating expression of anti-apoptotic protein MCL-1 [69].

2.1.3 IRE1α:

IRE1α (hereafter referred to as IRE1) is a dual-activity enzyme. In its cytoplasmic part it possesses a serine-threonine kinase domain and a C-terminal endoribonuclease domain. Once activated, the endonuclease activity induces the rapid turnover of mRNAs encoding membrane and secreted proteins, through a pathway referred to as regulated IRE1-dependent decay (RIDD) [42]. However, a more selective function of the endonuclease is removal of a 26-nucleotide intron from the XBP1 mRNA transcript, which is induced by ATF6. The frame-shift splice variant thus generated (XBP1s) codes for a stable, active transcription factor [117]. XBP1s has diverse targets including ER chaperones and the HSP40 family member, $p58^{IPK}$ [50] (Fig 1). $p58^{IPK}$ was initially thought to inhibit PERK by interacting with the cytoplasmic kinase domain, thereby providing a negative feedback loop that relieves the PERK-mediated translational block [113]. Recent data, however, suggest it is located inside the ER lumen where it functions as a co-chaperone of GRP78 [88]. In contrast to its RNase activity, IRE1 kinase function can induce pro-death signaling through binding to TRAF2 which leads to JNK activation [104].

Recently it was shown that experimental prolongation of IRE1 signaling (as determined by XBP1 splicing), independent of ER stress can promote cell survival [53, 54]. During ER stress, IRE1 is switched off earlier than PERK, therefore the cytoprotective function of IRE1 is no longer present while PERK signaling is still enduring [53]. Mechanisms to modulate the duration of IRE1 signaling could therefore influence cell fate in terms of death and survival. The activity of IRE1 is influenced by several interacting proteins (see below) with which it forms a protein complex referred to as the UPRosome [41]. It is noteworthy that of all of the three ER stress

sensors, IRE1 is the most highly regulated, which may reflect a key role in controlling the switch between adaptive responses and initiation of the apoptosis programme.

2.2 HSP family regulation of the three mediators

Heat shock proteins (HSPs), also called stress proteins, are highly conserved proteins whose expression is induced by different kinds of cellular stresses [12, 23]. HSPs have strong cytoprotective effects and behave as molecular chaperones for other cellular proteins. Inappropriate activation of signaling pathways occurs during acute or chronic stress as a result of protein misfolding, protein aggregation or disruption of regulatory complexes. The action of chaperones, through their roles in protein homeostasis, is thought to restore the balance. Mammalian HSPs have been classified into two groups according to their size: high molecular weight HSPs and small molecular weight HSPs. The first group includes three major families: HSP90, HSP70 and HSP60. Some of these are expressed constitutively whereas expression of others is induced by stressful conditions. These proteins can be targeted to different sub-cellular compartments. High molecular weight HSPs are ATP-dependent chaperones and require co-chaperones to modulate their conformation and ATP binding. In contrast, small HSPs, such as HSP27, are ATP-independent chaperones [79]. Among the different HSPs, HSP27 and HSP70 are the most strongly induced after stresses such as anticancer drugs, oxidative stress or irradiation. While expressed at very low levels in non-transformed cells, both HSP27 and HSP70 are abundantly expressed in cancer cells, and therefore have been suggested to be important prognostic factors in malignant diseases [25]. Also within the ER lumen, homologues of HSPs are present that aid in protein folding, e.g., GRP78. HSPs can regulate ER stress signaling from both the cytoplasmic and the ER-luminal side. Conceivably, interactions of ER stress mediators with cytoplasmic HSPs would integrate general cellular stress pathways and

UPR signaling outputs. Regulation of UPR by HSP family proteins is described in the sections below.

2.2.1 GRP78 regulation of the three mediators

The intraluminal ER chaperone GRP78 (also termed BiP) is a member of the HSP70 family of heat shock proteins and is the main regulator of the ER stress mediators PERK, ATF6, and IRE1 which are kept inactive by binding of GRP78 to their luminal domains (reviewed in [46]). Classically it is thought that accumulating unfolded proteins in the ER lumen compete for binding of GRP78 to the ER stress mediators, leading to their activation when GRP78 dissociates. This model proposes that unfolded proteins behave as client proteins of GRP78, involving rapid cycles of substrate binding and release in an ATP-dependent manner. There is, however, evidence that GRP78 might remain stably bound to substrate proteins until these change their conformation. In addition, it is not entirely clear whether GRP78 dissociation from the luminal domains of the three ER stress mediators is fully explained by this competition mechanism, and it has been suggested that co-chaperones might stimulate the release of GRP78 [95]. In this regard it is of note that $p58^{IPK}$ is reported to be localized inside the ER lumen where it acts as co-chaperone of GRP78 [88]. Together with the earlier report showing $p58^{IPK}$ interacts with the kinase domain of PERK [113], this suggests that $p58^{IPK}$ is present in both the cytoplasm and the ER lumen.

How GRP78 dissociation activates the mediators is also not fully understood. The ER-luminal domains interacting with GRP78 are highly conserved between IRE1 and PERK but not so with ATF6. In yeast, IRE1 is activated in a receptor-like manner by direct binding of unfolded proteins to the luminal domain rather than by dissociation of GRP78 [24]. Activation of

mammalian IRE1, as well as of PERK, however seems to be triggered by dissociation of GRP78 as the main mechanism [46].

Expression of GRP78 is upregulated by ER stress through transcription factors ATF6 [115] and ATF4 [58]. The resulting increase in GRP78 protein therefore not only aids folding of proteins inside the ER, but may inactivate PERK, ATF6 and IRE1 by binding to their luminal domains. Thus, GRP78 is component of a feedback mechanism that ensures protein refolding and inactivation of the UPR. As suggested by Rutlowski and Kaufman [87], it is conceivable that differential affinity of the three UPR mediators for GRP78 might differentially regulate the kinetics of their inactivation. However, there is limited evidence to support such a model. During UPR-induced B-cell differentiation, there is activation of the IRE1 pathway [83] in the absence of CHOP induction (which is dependent on ATF6 and PERK pathways) [26]. However, it has recently been reported that this limited ER stress response may be the result of suppression of the PERK signal rather than selective activation of IRE1 [60].

2.2.2 Regulation of IRE1 and PERK by cytoplasmic HSPs

2.2.2.1 HSP90 regulation of PERK and IRE1

HSP90 has been shown to physically interact with the cytoplasmic part of IRE1 and PERK [62]. Disruption of this interaction by HSP90 inhibitors leads to a reduced half-life of IRE1 and PERK protein, resulting in less protein, suggesting stabilization of both proteins by HSP90 in the normal situation. In this study the influence of HSP90 on IRE1 activity and signaling outputs was not addressed, however, it was noted that inhibition of Hsp90 did not impair translational attenuation during ER stress, suggesting that even reduced amounts of PERK were sufficient to block translation.

2.2.2.2 Hsp70 regulation of IRE1

HSP72 is a stress-inducible member of the HSP70 family whose chaperoning activity is dependent on ATP [118]. It also requires co-chaperones for efficient activity. When overexpressed in cells HSP72 was found to be protective against ER stress- induced cell death [29]. Remarkably this cytoprotection was not solely attributable to general effects on the apoptotic machinery but was dependent on production of spliced XBP1. In fact, HSP72 overexpression leads to enhanced and prolonged XBP1 splicing during ER stress, suggesting stimulation of IRE1 activity. Co-immunoprecipitation experiments revealed a physical interaction between the cytoplasmic part of IRE1 and HSP72 that was dependent on the ATPase domain of HSP72 (Gupta et al 2010) and involved the co-chaperone HSP40 (unpublished observations) (Fig. 2). Thus, as a stress-inducible protein HSP72 might serve as a hub connecting other stress pathways with the UPR. It is noteworthy that HSP72 is normally not expressed in unstressed cells but is expressed at high levels in cancer cells. This implies that HSP72 is not an essential component of the UPRosome. However, in cancer cells that express high levels of Hsp72, it forms a complex with IRE1, and by stimulating XBP1 splicing activity, HSP72 contributes to enhanced pro-survival signaling.

2.2.2.3 p58^{IPK} - modulator of PERK or co-chaperone of Grp78

The stress-inducible protein p58^{IPK} is a HSP40 family member, initially identified as an inhibitor of the PERK-related eIF2α kinase PKR, possibly modulating HSP70 activity to refold and inhibit the kinase [67]. Later p58^{IPK} was found localized at the ER and by co-immunoprecipitation experiments shown to physically interact with the cytoplasmic PERK kinase domain, inhibiting PERK activity [113]. Consistent with this finding, p58^{IPK} expression leads to a decrease in eIF2α phosphorylation [105, 113]. Since p58^{IPK} expression is regulated by XBP1s, it is part of a negative feedback regulation limiting PERK activity during ER stress. In

a later study, however, $p58^{IPK}$ was suggested to be part of a protein complex with HSP70 that mediates degradation of proteins during their co-translational import into the ER lumen, thereby lowering protein load of the stressed ER [78]. Importantly, both proposed functions of $p58^{IPK}$ were recently challenged by a study which demonstrated localisation of $p58^{IPK}$ within the ER lumen where it acts as a co-chaperone of GRP78 [88]. While this study could not confirm the aforementioned effects on protein import into the ER, it remains possible that $p58^{IPK}$ indirectly, via GRP78 binding, affects PERK activity.

2.3 Bcl-2 family regulation of IRE1

Members of the BCL-2 family of proteins are major regulators of apoptotic cell death (see [11, 101] for review). They are characterized by up to four conserved BCL-2 homology (BH) domains which are involved in their protein-protein interactions. Multi-domain family members can be subdivided according to their pro-apoptotic (BAX and BAK) and anti-apoptotic effects (such as BCL-2, BCL-X_L, MCL-1). They are located at intracellular membranes, and their best studied mechanism of apoptosis regulation is by controlling the release of cytotoxic molecules from mitochondria. The activity of multi-domain BCL-2 family members is modulated by interaction with BH3-only proteins (such as BIM, BIK, BAD) that contain only the third BH domain and are generally pro-apoptotic. The first reports linking ER stress-induced cell death to the BCL-2 family of proteins showed that overexpression of BCL-2, or deficiency of BAX and BAK, conferred protection against lethal ER stress [16, 112]. These studies assumed that ER stress-induced apoptosis proceeds via the intrinsic pathway, which is controlled by mitochondrially-localized BCL-2 proteins. However, from early on it was noticed that members of the BCL-2 family also reside and are active at the ER membrane [48, 121] and that artificial targeting of BCL-2 to the ER membrane provided protection against ER stress [31].

BCL-2 family proteins where shown to influence ER Ca^{2+} homeostasis and ER-mitochondrial crosstalk and might by this means indirectly affect ER stress-induced cell death [101]. However, they can also interact directly with IRE1, modulating its functioning during ER stress, and thus regulate ER stress-induced cell death at the level of IRE1 signaling [40] (Fig. 3).

BAX and BAK were shown to form a complex with IRE1 in an ER stress dependent manner, and BAX/BAK-deficient cells displayed reduced levels of spliced XBP1 and phosphorylated IRE1 during ER stress [40]. When the BAX/BAK-deficient cells were reconstituted with a BAK mutant that was fused to an ER-localisation motif (BAK-cb5), levels of XBP1s and of JNK phosphorylation were increased [40]. Therefore, the interaction with BAX and BAK appears to be essential for activation of IRE1 and might represent a possible switching mechanism towards pro-apoptotic signaling by IRE1. This was suggested by overexpression of BAK-cb5 in BAX/BAK-deficient cells. In these cells, co-expression of ER-targeted BH3-only proteins Bim or Puma resulted in IRE1-dependent JNK activation in the absence of XBP1 splicing [47]. As JNK activation could be abolished by interfering with either TRAF2 or IRE1, these experiments reveal a specific activation of pro-apoptotic signaling of IRE1 by pro-apoptotic BH3-only proteins acting at the ER membrane. The association of IRE1 with Bax and BAK is influenced by BAX Inhibitor 1 (BI-1), a transmembrane protein localized at the ER and nuclear envelope, that had initially been identified in a genetic screen for suppressors of BAX-induced cell death [84]. BI-1 forms a complex with the cytoplasmic part of IRE1 (Fig. 3). BI-1 deficiency of cells resulted in higher IRE1 activity and enhanced XBP1 splicing, while BI-1 overexpression disrupted the interaction between IRE1 and Bax or Bak [55]. BI-1 itself is regulated by the bifunctional apoptosis regulator (BAR), an ER-localised RING-type E3 ligase, that mediates the proteasomal degradation of BI-1 and thus removes the block of IRE1 activation provided BI-1 [86]. However, BAR becomes downregulated during ER stress, and the

resulting increase in BI-1 might thus contribute to attenuation of IRE1 signaling during prolonged ER stress (Fig. 3).

2.4 Regulation of pro-apoptotic functions of IRE1

If IRE1 has both pro- and anti-apoptotic functions, how are these two opposing functions separated? As mentioned above, IRE1 is part of a large protein complex termed the UPRosome (Fig. 3). Most, but not all, of the components have been shown to modulate both IRE1's kinase and RNase function, in parallel. For example, in pancreatic beta cells glucose was shown to stimulate assembly of a complex between IRE1, the scaffolding protein RACK and protein phosphatase 2A (PP2A), leading to dephosphorylation and inactivation of IRE1. Under ER stress PP2A dissociates, concomitant with IRE1 activation [81]. IRE1 was also found to associate with ASK1 interacting protein (AIP) which stimulates IRE1 dimerization, enhancing JNK activation and XBP1 splicing [57]. In contrast, the specific activation of the IRE1/TRAF2-mediated JNK activation by BH3-only proteins described above offers one example of how the two signaling outputs of IRE1 can be separated by specific interactions within the UPRosome. Another example of this separation is possibly provided by JNK inhibitory kinase (JIK) and Jun activation domain-binding protein-1 (JAB1) which have been identified as IRE1-interacting proteins in yeast two-hybrid screens. The ability of JIK to bind both IRE1 and TRAF2 was also shown, pointing to a potential role for JIK in regulating the recruitment of TRAF2 and thus activation of the JNK pathway. The other IRE1-binding partner, Jun activation domain binding protein 1 (JAB1), was shown to bind to IRE1 in resting conditions. Mild ER stress enhanced this interaction, whereas strong ER stress diminished it. Thus, JAB1 might regulate the choice between the UPR-induced survival and apoptosis by association with or

dissociation from IRE1. Whether JIK or JAB1 provide the switch between the pro-survival and pro-apoptotic IRE1 signaling remains to be further investigated.

Another mechanism of pro-apoptotic signaling by IRE1 is the RNase domain-mediated decay of mRNAs (RIDD, see section 2.1) during prolonged ER stress [42]. As this affects mRNAs encoding ER resident proteins such as chaperones, as well as transmembrane or secreted proteins such as growth factors and their receptors, that are crucial for cell viability, RIDD is considered pro-apoptotic [33]. At present it is completely unknown whether regulators of IRE1 within the UPRosome might switch specificity of the IRE1 RNase domain from XBP1 splicing to mRNA decay and how they might do so. However, there is evidence that XBP1 splicing and RIDD can be induced separately. Certain kinase inhibitors as well as specific IRE1-derived peptides are reported to selectively stimulate XBP1 splicing in absence of RIDD [6, 33]. Since this was paralleled by the absence of IRE1 kinase and JNK activation, it appears that the pro-apoptotic functions of IRE1, RIDD and TRAF2-JNK signaling are coordinately activated, possibly involving a specific conformational or oligomeric status of IRE1.

On the basis of these studies, IRE1 seems to be important for the initiation of pro-apoptotic signals. Interestingly, IRE1 is thought to be the last arm of the UPR to be activated, with PERK being the first, closely followed by ATF6. It is possible that the PERK- and ATF6-mediated pathways attempt to resolve the stress prior to activation of IRE1. Once activated, IRE1 initially aids the UPR by splicing XBP1, but this activity is transient and its termination, either alone or in combination with pro-apoptotic effects of continued RIDD or JNK signaling, and/or sustained PERK signaling, ultimately triggers apoptosis.

3. Commitment phase of ER stress-induced apoptosis

Signaling through PERK, ATF6 and IRE1 can trigger pro-apoptotic signals during prolonged ER stress. They do so indirectly through the activation of downstream molecules such as CHOP or JNK, which regulate the expression and activity of various pro- and anti-apoptotic proteins such as BCL-2 family members and further push the cell down the path of apoptosis. The commitment phase of ER stress-induced apoptosis focuses on how CHOP and JNK relay the pro-apoptotic signal to the final execution phase.

3.1 CHOP

One of the characteristic features of ER stress is increased expression of CHOP which is also known as growth arrest- and DNA damage-inducible gene 153 (GADD153). It is a member of the C/EBP family of transcription factors. Although CHOP was originally identified as part of the DNA damage response pathway, its induction is probably most sensitive to ER stress conditions where it plays a key role in ER stress-induced apoptosis, through mechanisms that are not entirely delineated.

The PERK-eIF2α-ATF4 arm of the UPR is required to induce CHOP protein expression [59]. In addition to being controlled at the level of transcription and translation, CHOP is also regulated through post-translational phosphorylation on serine residues 78 and 81 by p38 MAPK, which increases CHOP activity [109]. In fact, CHOP-mediated myocardiocyte apoptosis is reduced in transgenic mice expressing a dominant negative version of p38α [92]. Notably, p38 is a substrate of apoptosis signal-regulating kinase (ASK1), which is recruited to the IRE1-TRAF2 complex upon ER stress. Thus, during prolonged stress, the PERK and the IRE1 pathways might converge on CHOP, with IRE1-mediated ASK1 activation possibly potentiating CHOP activity

[85]. CHOP activity is also regulated through its dimerization with other basic leucine zipper proteins such as C/EBPα and activating transcription factors (ATFs) [21, 80].

The role of CHOP in ER stress-induced apoptosis has been illustrated using $CHOP^{-/-}$ mice. While born at the expected frequency and with normal phenotypic appearance, these mice display resistance to ER stress-induced apoptosis in a number of disease models such as diabetes, Parkinson's disease, atherosclerosis and cardiac disease [22, 77, 96, 97, 102, 120]. Nonetheless, MEFs derived from $CHOP^{-/-}$ mice show that CHOP deficiency provides only partial resistance to ER stress-induced apoptosis [120].

It is becoming apparent that CHOP mediates cell death primarily through two mechanisms. Firstly, CHOP alters the transcription of genes involved in apoptosis and oxidative stress (including BCL-2 family members, endoplasmic reticulum oxidoreductin 1 (ERO1α), Tribbles-related protein 3 (TRB3) and death receptor 5 (DR5)), and secondly, through a feedback loop, CHOP relieves the inhibition of protein translation imposed by PERK signaling (via induction of GADD34 expression). CHOP transcriptional activity has been reported to shift the balance between pro- and anti-apoptotic BCL-2 family members in favor of pro-apoptotic members. CHOP-induced cell death has been associated with downregulation of BCL-2 levels [66]. This would relieve constraints on BAX allowing it to translocate to the mitochondria and cause cytochrome *c* release resulting in apoptosis. However, in a number of reports no change in BCL-2 expression is detected despite robust induction in CHOP, suggesting that other BCL-2 family members may be more critical for the initiation of ER stress-induced apoptosis in certain cell types [80, 100]. In this regard, BIM has also been identified as a target of CHOP that is required for ER stress-induced apoptosis [80, 100]. ER stress-induced upregulation of *bim* mRNA is mediated by CHOP-C/EBPα heterodimers [80] and although the conventional *bim* promoter does not contain a CHOP binding site, there is a CHOP-C/EBPα

heterodimer binding site (TGCAAT) within the first intron of mouse and human *bim* genes. ERO1α, induced by CHOP, causes oxidation of the ER lumen [61], which promotes disulphide bond formation to help protein folding. However, during ER stress-induced apoptosis a hyperoxidizing environment may be produced leading to cell death. It has also been observed that ERO1-α can activate the inositol-1,4,5-trisphosphate receptor 1 (IP3R1) which stimulates ER calcium release [51]. This calcium release can lead to mitochondrial membrane permeability transition and apoptosis [76]. TRB3 is another target of CHOP, with CHOP and ATF4 cooperating to activate the TRB3 promoter [74]. TRB3 is an intracellular pseudokinase that modulates the activity of several signal transduction cascades [39]. Knockdown of TRB3 expression decreased tunicamycin-induced death in 293 and HeLa cells, with overexpression increasing tunicamycin cell death in these cells [74]. It has been suggested that TRB3 promotes apoptosis by binding to the pro-survival serine/threonine kinase AKT, thereby preventing its phosphorylation and reducing its kinase activity [18, 122]. In MCF-7 breast cancer cells, ER stress caused a transient activation of AKT, and blocking of AKT activity during this response sensitized the cells to ER stress-induced apoptosis [43]. TRB3 is regulated in a number of ways. In a negative feedback loop TRB3 binds to CHOP and represses CHOP/ATF4 transactivation, thus downregulating its own induction [74]. Furthermore, it is an unstable protein and is degraded by the ubiquitin-proteasome system [75]. Together, these would allow the cell to return to normal function following transient ER stress. There has also been a suggestion that TRB3 inhibition of the AKT/mammalian target of rapamycin complex 1 (mTORC1) axis promotes autophagy, thus providing a link between these two pathways [89].

GADD34, a growth arrest and DNA damage gene and another important target of CHOP that is translated during ER stresss, mediates the relief of inhibition of protein translation. GADD34 binds to the α-isoform of protein phosphatase-1 (PP1α), and facilitates PP1-mediated dephosphorylation of eIF2α, thus creating a feedback loop that relieves the translation

repression imposed by PERK-dependent phosphorylation of eIF2α [72]. Phosphorylation of eIF2α and the consequent translational repression are linked to prolonged survival of the cells. Therefore, GADD34 dephosphorylation of eIF2α will have the opposite effect, promoting cell death. In a number of studies, expression of GADD34 has been linked to increased sensitivity to apoptosis [8]. For example, GADD34 can sensitize cells to induction of apoptosis by ionizing radiation [1] and oxidative stress [56]. Notably, GADD34 protein has a short half-life, a factor which may be important in the switch between survival and death in mammalian cells and indeed in ensuring the survival of cells in the face of subsequent bouts of stress. The mechanism by which GADD34 promotes apoptosis is unknown, although it is likely that its effect on eIF2α plays an important role. For example, restoration of protein synthesis by GADD34 may allow the synthesis of pro-apoptotic proteins such as pro-apoptotic BCL-2 family members. Indeed, compared with wild type mice, mice deficient in GADD34-directed eIF2α dephosphorylation display milder renal toxicity by tunicamycin, a drug which causes ER stress through the inhibition of protein glycosylation [61]. Moreover, chemical inhibition of eIF2α phosphatases (with salubrinal) has been reported to reduce ER stress-induced apoptosis suggesting that maintaining eIF2α phosphorylation and thus repressing translation has an overall pro-survival effect [7]. This is supported by the observation that complete loss of PERK-mediated eIF2α phosphorylation sensitizes cells to ER stress-induced death [36, 37, 93].

3.2 JNK regulation of Bcl-2 family

ER stress-induced apoptosis proceeds via the intrinsic pathway of apoptosis which is mediated by BCL-2 family proteins that control release of cytotoxic molecules from mitochondria (Boya et al 2002, Hacki et al 2000). Nonetheless, how expression and activity of BCL-2 family

proteins are regulated by ER stress is less well understood. To date, UPR-mediated activation of CHOP and JNK has been shown to be involved. As described above, CHOP is known to shift the balance of pro- and anti-apoptotic BCL-2 family members in favor of the former. Overexpression of CHOP has been shown to induce apoptosis, which is associated with the activation and mitochondrial translocation of BAX. In this model, overexpression of BCL-2 can block CHOP-induced apoptosis [65, 66]. As discussed in the section on the initial phase, JNK is activated by the IRE1-TRAF2 branch of the UPR. JNK is known to phosphorylate different members of the BCL-2 family, altering their activity and stability. First of all, JNK is able to phosphorylate BCL-2 localized to the ER. This has a knock-on pro-apoptotic effect, as phosphorylated BCL-2 is unable to sequester and inhibit pro-apoptotic BH3-only proteins and cannot control ER Ca^{2+} fluxes (Bassik *et al*, 2004; Fig. 3). JNK can also target the BH3-only proteins, whose induction and/or posttranslational modification has a central role in setting the apoptotic cascade in motion. Of the BH3-only subfamily, p53-upregulated modulator of apoptosis (PUMA), NOXA and BIM have been reported to have a role in ER stress-induced apoptosis. BIM exists in several isoforms, including a short version (BIM_S) and two longer forms: BIM_L and BIM_{EL}, with the latter two being constitutively expressed. In unstressed cells the pro-apoptotic effects of BIM_L and BIM_{EL} are restricted by their binding to the dynein motor complex. Phosphorylation by JNK releases Bim from this inhibitory association allowing it to exert its pro-apoptotic effects (Lei & Davis, 2003; Fig. 3). BIM translocates from the cytoskeleton to the ER in C2C12 cells exposed to tunicamycin [68], whereas others have detected strong induction of BIM in a range of different cell types treated with thapsigargin [80, 100]. Together, these data suggest that JNK activated by ER stress targets BCL-2 proteins, which would permit the activation of BAX and BAK leading to the execution of apoptosis. Interestingly, a reverse interaction between JNK and BAX/BAK was recently reported [40]. In $BAX/BAk^{-/-}$ mice, tunicamycin failed to induce XBP1s and JNK phosphorylation. Moreover,

BAX and BAK were found to interact directly with IRE1 upon ER stress. Reconstitution of BAK expression in the BAX/BAK$^{-/-}$ MEF cells restored tunicamycin-induced JNK phosphorylation, suggesting a direct, although unexpected, connection between the UPR and the apoptotic machinery.

Another two BH3-only proteins have been shown to be regulated by ER stress. Microarray analysis of tunicamycin-treated SH-SY5Y neuroblastoma cells reported an upregulation of PUMA [82]. Likewise, another study examining the expression of BH3-only proteins in response to either thapsigargin or tunicamycin showed upregulation of both PUMA and NOXA in a p53-dependent manner [52]. However, we have no knowledge of how p53 is activated during the UPR. Nevertheless, the induction or the activation of BH3-only proteins during ER stress activates BAX and BAK leading to mitochondrial membrane permeabilization, caspase activation and cell death (Fig. 3).

3.3 Cross-talk between mitochondrial and ER stress signaling at the commitment phase

Although the upstream initiation of apoptosis due to ER stress is distinct from that invoked by other apoptosis initiation pathways it is notable that the commitment phase of ER stress-induced apoptosis is largely dependent on mitochondria. The weight of evidence indicates that, at least in most cell types, ER stress-induced apoptosis leads to caspase activation through the release of cytochrome c from mitochondria. Indeed as noted earlier calcium-mediated mitochondrial permeability transition can occur as a result of IP3R1 regulation by the CHOP target ERO1α [51]. Also at the intersection of the two pathways are Bcl-2 family members which control the release of pro-apoptotic factors from mitochondria.

However, BCL-2 proteins may not be the only proteins providing cross-talk between the ER and mitochondria during apoptosis commitment. A recent paper has identified CRK as a major pro-apoptotic signal required for the execution of ER stress-induced apoptosis [2]. Interestingly, CRK, an adaptor protein with no known catalytic activity, possesses a putative BH3 domain that is important for its apoptotic activity and that sensitizes isolated mitochondria to tBid-induced cytochrome c release [2]. Moreover, proteolytic cleavage of CRK due to an ER stress-activated cysteine protease leads to enhanced pro-apoptotic activity [2]. The identity of the protease, and the generality of the role of CRK in ER stress-induced apoptosis in different cell types remain to be clarified.

4. The execution phase of ER stress-induced apoptosis

4.1 Caspases

All upstream signals in apoptosis pathways, such as the activation of transcription factors, kinase pathways and the regulation of BCL-2 family members, ultimately lead to caspase activation, resulting in the ordered and sequential dismantling of the cell. Caspase activation is a key feature of ER stress-induced apoptosis. To date, the cohort of caspases linked to ER stress-induced apoptosis has not been established conclusively. Processing of caspases-12, -2, -3, -4, -6, -7, -8 and -9 has been observed in different models of ER stress-induced apoptosis. Although caspase activation is required for the apoptotic process, the identity of the apical caspase is of most interest, yet remains subject to debate. Caspase-12 was proposed as a key mediator of ER stress-induced apoptosis [70, 98]. Caspase-12 is expressed in most mammals; but its human homologue has been rendered inactive by several mutations during evolution in most humans except those of African heritage, where caspase-12 has been linked with increased risk of sepsis [90]. Caspase-4 has been proposed to fulfill the function of caspase-12 in humans, but

this is presently under debate [73]. Caspase-12$^{-/-}$ MEF cells have been reported to exhibit partial resistance specifically against ER stress-inducing agents, suggesting an important role for this caspase [70]. However, other work also using caspase-12$^{-/-}$ MEFs (although from a different source) observed no resistance to ER stressors such as thapsigargin [91]. If caspase-12 functions as an initiator caspase during ER stress, as proposed, it should activate downstream caspases. However, little consistent data linking caspase-12 to downstream caspase activation is available. Moreover, no definitive substrates or human orthologue for caspase-12 (except people of African descent) have yet been identified, making it difficult to establish a general role for this caspase as the apical caspase in ER stress-induced apoptosis. There is some evidence emerging pointing to caspase-8 processing/activation at the ER during ER stress-induced apoptosis [10, 45]. However, whether caspase-8 is the apical caspase activated during ER stress is not clear, although at least one study has reported that caspase-8 deficiency reduced ER stress-induced cell death [45]. Alternatively, it seems likely that, through activation of the intrinsic apoptosis pathway, caspase-9 is the apical caspase. Indeed, in several studies, BAX/BAK$^{-/-}$ cells or cells over-expressing Bcl-2 have been found to be resistant to ER stress-induced apoptosis [28, 121]. Furthermore, mouse embryonic fibroblasts (MEFs) lacking caspases-3, 7, or 9 are resistant to ER stress-induced apoptosis [64]. In another study caspase-2 was identified as a key caspase in initiation of ER stress-induced apoptosis in BAX/BAK$^{-/-}$ MEFs, where it was responsible for cleavage of BID, upstream of mitochondrial events [103]. However, in the absence of a systematic shRNA/siRNA knockdown studies of all apical caspases in a single cell line it would be difficult to determine whether there is a single pathway to caspase activation or if parallel pathways activated, and determine the apical caspases.

4.2 PERK regulation of IAPs

It was recently shown that ER stress can regulate the execution phase of apoptosis by causing the transient induction of certain mammalian inhibitor of apoptosis proteins (IAPs) [32, 43, 110]. IAPs are a family of proteins which are involved in the regulation of caspases, [30] and thus their induction results in a delay in caspase activation and the execution of apoptosis. Mammalian X-chromosome-linked IAP (XIAP) can directly bind and inhibit caspase-3, caspase-7 and caspase-9 [19, 20]. In contrast, cellular IAP (cIAP1) and cIAP2 are not direct caspase inhibitors, but are understood to regulate the execution phase of apoptosis by sequestering SMAC away from XIAP, allowing XIAP to remain active and to inhibit caspases [19]. It should be noted that IAPs also act as ubiquitin-E3 ligases regulating nuclear factor kappa B (NF-κB) signaling [30] and the tumor suppressor PTEN that counteracts PI3 kinase activation [106].

Various groups have reported that cIAP1, cIAP2 and XIAP are induced by ER stress, and that this induction is important for cell survival [32, 43, 111]. Recently it was reported that cIAP1 and cIAP2, but not XIAP, are induced by ER stress in a PERK-dependent manner [32]. Others have shown that the induction of IAPs is dependent on phosphatidylinositol 3-kinase (PI3K)-AKT signaling [43]. This may be linked to PERK signaling since PERK can cause a transient activity of PI3K-AKT signaling [32]. ER stress-mediated induction of IAPs occurs through both transcriptional and translational responses, both of which are dependent on PERK. Whereas AKT activation has been implicated in IAP transcription [32, 43], it is not required for their translation [32]. However, it appears that increased transcription may not be necessary for IAP protein induction. IAP mRNA is preferentially translated during ER stress in a PERK and phospho-eIF2α-dependent manner [32], possibly through upstream open reading frames within the 5'-untranslated regions of both cIAP1 and cIAP2 mRNAs or internal ribosome entry sites that have been reported [111]. The specific transcription factors downstream of AKT have not

been identified, although NF-κB is a likely candidate since it is known to be regulated by AKT [5], to be activated by ER stress in a PERK-dependent manner [15, 44] and its target genes include cIAP1 and cIAP2 [5].

Importantly, ER stress-induced IAP expression was found to delay the onset of ER stress-induced caspase cleavage (indicative of activation) and apoptosis [32]. This ability of PERK to induce cIAPs, and also to cause a transient activity of PI3K-Akt signaling demonstrates that PERK not only promotes cellular adaptation to ER stress, but also actively inhibits the ER stress-induced apoptotic program downstream of the commitment phase, allowing a further layer of regulation. Another possible and intriguing role for cIAP1 and cIAP2 in ER stress may lie in the modulation of signaling by death receptors which act as receptors for ligands such as tumor necrosis factor alpha (TNFα). Signaling downstream of TNFα can be either pro-survival or pro-apoptotic, and the E3 ligase activity of cIAPs is crucial for the pro-survival pathway (Wang et al., 1998a). Autocrine TNFα pro-apoptotic signaling can occur during ER stress through IRE1 activity (Hu et al., 2006). However, the role of cIAP1 and cIAP2 in ER stress-induced TNFα signaling has not yet been addressed.

5. Novel regulation of UPR and ER stress-induced apoptosis

Thus far we have described the more well known mechanisms by which the UPR machinery is regulated. However, there are several other mechanisms that are emerging that may prove significant and develop into attractive targets in the future. For example, regulation of the adaptor protein CRK may prove to be an important factor in controlling ER stress-induced apoptosis (this has already been described in Section 3.3).

There are a number of recent reports describing roles of microRNAs in ER stress. Both CHOP and XBP1s have been shown to control expression of certain miRNAs [3, 4]. In human

hepatocellular carcinoma (HCC) cells, ER stress caused downregulation of the microRNA miR-221/222 which contributed to protection against ER stress-induced apoptosis [14]. Another microRNA, miR-122, was shown to negatively regulate the UPR in HCC cells and downregulation of miR-122 enhanced chemoresistance [114]. Thus, miRNAs are possible regulators of the switch from survival to death in cells under ER stress.

An intriguing novel link has been established between innate immunity and UPR signaling by uncovering activation of IRE1 and subsequent XBP1 splicing through Toll-like receptors (TLRs) [63]. In these experiments, IRE1 activation by TLRs contributed to cytokine production and was important for the host defense of mice against an intracellular pathogens. However, the mechanisms of regulation of IRE1 by TLRs have not yet been elucidated.

Enzymes mediating posttranslational modification of XBP1s are emerging as another group of ER stress regulators and include acetyl transferases and deacetylating enzymes, as well as E3-ligases mediating modification by small ubiquitin-like modifiers (SUMO). Whereas acetylation (mediated by p300) was shown to enhance transcriptional activity of XBP1s, deacetylation (by sirtuin 1) had the opposite effects [108]. Furthermore, SUMOylation by PIAS [protein inhibitor of activated STAT (signal transducer and activator of transcription)] was shown to decrease transcriptional activity of XBP1s [9].

There have been some interesting new studies regarding GRP78 and cancer. High levels of cell surface GRP78 have been detected on a variety of cancer cells but not on normal cells [71, 107, 119]. Cell surface GRP78 forms complexes with a growing number of extracellular ligands and membrane-anchored proteins, influencing regulation of pro-survival or pro-apoptotic signalling pathways, such as PI3K/Akt signalling [71]. Thus, cell surface GRP78 could regulate ER stress-induced apoptosis.

6. Conclusion

ER stress conditions have been observed in numerous diseases including Alzheimer's disease, Creutzfeldt-Jakob disease, Huntington's disease, Parkinson's disease, diabetes, cardiovascular diseases, and cancer indicating that ER stress-induced apoptosis is an important factor in pathophysiological conditions. To be able to intervene in such conditions, a firm understanding of the mechanisms mediating ER stress-induced apoptosis is essential. Currently, it appears that many candidate proteins are involved in orchestrating the switch from the protective UPR signaling to pro-apoptotic signaling. Some of these genes, like $P58^{IPK}$, GADD34 and TRB3, are involved in shutting down the PERK-mediated pathway. Ending the protective UPR can understandably be a central element of the switch from adaptation to suicide. These proteins, however, seem to affect sensitivity to ER stress in opposing ways. Besides these proteins with negative-feedback functions, two additional, quite potent, pro-apoptotic molecules are activated during the UPR: CHOP and JNK. Both overexpression and knock down experiments have confirmed the pro-apoptotic role of these proteins in ER stress. Both molecules can target the BCL-2 family and are able to set the death machinery in motion. Furthermore, activation of ASK1/JNK might be regulated by stress-sensitive adaptor proteins like JAB1, offering another possible mechanism to switch from an adaptive response to cell suicide.

It should be remembered that in studying ER stress responses a variety of different inducers are used, the most common being thapsigargin (inhibitor of the sarco/endoplasmic reticulum Ca^{2+} pump), tunicamycin (inhibitor of N-linked glycosylation) and brefeldin A (inhibitor of ER-Golgi transport). Since these have very different targets it is possible there are differences in the precise set of responses induced and therefore caution should be exercised when comparing data from different systems. For example, Sep15, a thioredoxin-like selenoprotein is differentially regulated depending on the underlying mechanism by which ER stress is

induced [49]. Tunicamycin and brefeldin A, which both rely on protein synthesis to exert their ER stress effects led to increased expression of Sep15, while thapsigargin and dithiothreitol, which induce protein folding independently of protein synthesis, stimulated rapid degradation of Sep15 [49]. Finally, it is worth noting that some of the regulators of ER stress-induced apoptosis are specific to ER stress, while others are common to other stress. They therefore could serve as useful targets for therapeutic intervention where ER stress plays a key role. Continued research in this field is necessary in order to tease out the complexities of this cell-death pathway.

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Figure legends

Figure 1. The UPR is mediated by three ER stress sensors. Binding of unfolded proteins to GRP78 within the ER lumen allows activation of PERK, ATF6 and IRE1. PERK dimerises and autophosphorylates. It phosphorylates eIF2α and thus general Cap-dependent translation is inhibited. Cap-independent translation allows the translation of certain proteins such as ATF4 which activates CHOP transcription. One of the genes induced by CHOP is GADD34 which regulates protein phosphatase 1 (PP1), which can dephosphorylate eIF2α. Activation of ATF6 allows its translocation to the Golgi where it undergoes cleavage by S1P and S2P proteases. Cleaved ATF6 activates XBP1 transcription. Active IRE1 is a dual kinase and endonuclease. One of its targets is XBP1 mRNA which undergoes splicing to produce an active transcription factor, XBP1s. One of the targets of XBP1s is $p58^{IPK}$.

Figure 2. Regulators of IRE1 signaling. Under resting conditions GRP78 in the ER lumen keeps IRE1 in an inactive state. On the cytosolic side, protein phosphatase 2A (PP2A) prevents its phosphorylation, probably utilizing RACK as a scaffold protein. BI-1 and JAB1 also interact with IRE1 under resting conditions. HSP90 interacts with IRE1, stabilizing it. Upon ER stress, GRP78 dissociates allowing IRE1 to dimerize. It undergoes autophosphorylation in the kinase domain. Binding of Bax/Bak enhance IRE1-mediated XBP1 splicing and JNK signaling. AIP stimulates IRE1 dimerization. HSP72 interaction with the cytosolic region enhances XBP1 splicing activity. BI-1 becomes ubiquitinated ubiquitination by the E3 ligase BAR and is degraded by the proteasome. JAB1 interaction with IRE1 is enhanced. With prolonged ER stress BAR becomes downregulated, thus allowing stabilization of BI-1 which inhibits Bax/Bak effect on IRE1 signaling. TRAF2 is recruited to the kinase domain, either directly or perhaps via JIK. TRAF2 recruits ASK1 which leads to JNK activation.

Figure 3. Pro-apoptotic signaling by IRE1. JNK is activated downstream of TRAF2-ASK1 interaction with IRE1. Phosphorylation by JNK of Bim causes it to dissociate from the dynein motor complex, which stabilizes it. This allows it to draw anti-apoptotic Bcl-2 family members such as Bcl-2 away from Bax/Bak which are thus free to mediate mitochondrial outer membrane permeabilization and cytochrome *c* release. JNK can also phosphorylate Bcl-2, which inhibits its ability to regulate ER Ca^{2+} homeostasis. Bax and Bak can also interact with IRE1 at the ER membrane. This interaction can be blocked by BI-1. The endonuclease activity of IRE1 mediates Regulated IRE1-dependent decay of mRNA (RIDD), which leads to a decrease in the production of ER chaperone proteins.

Figure 1

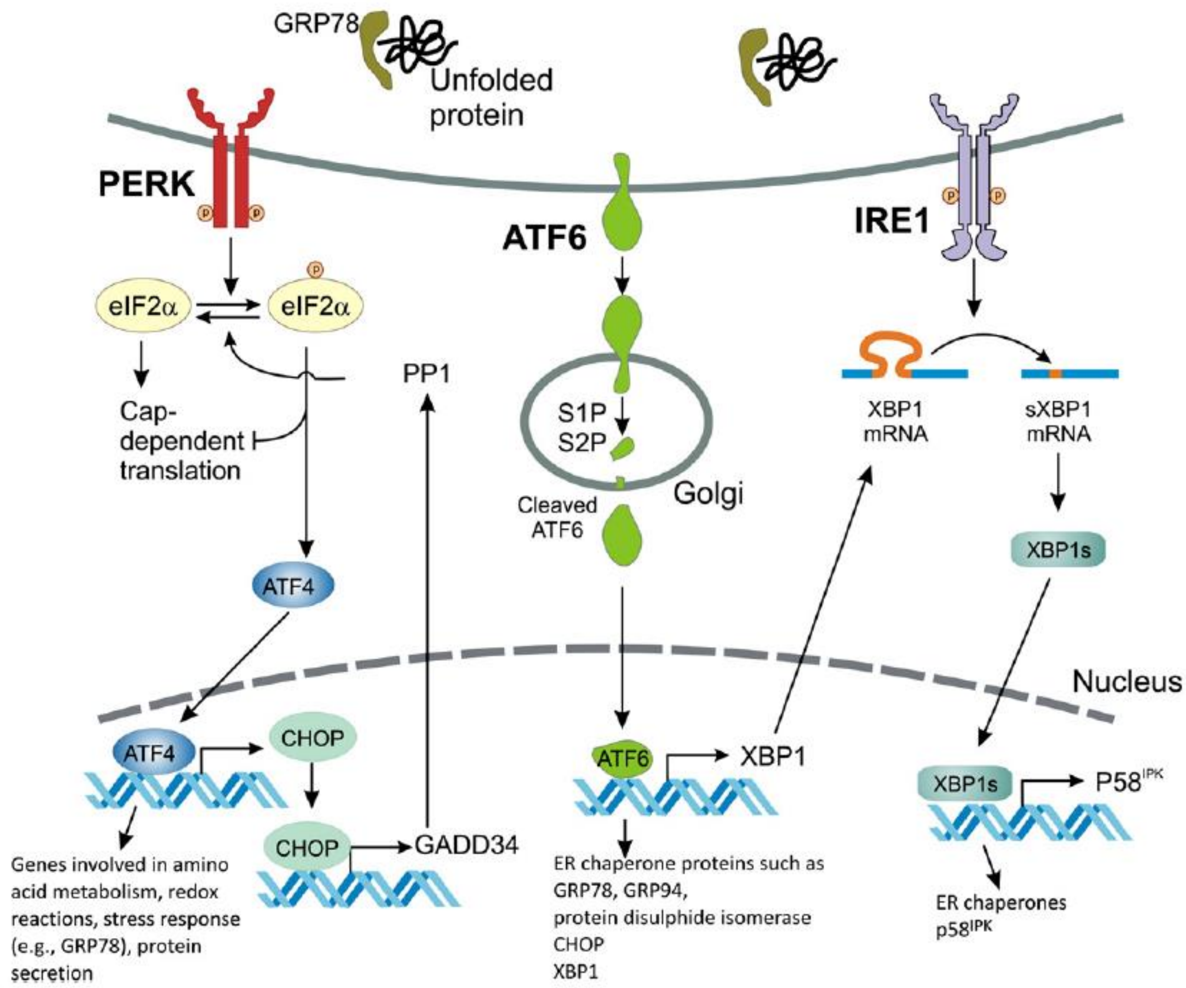

Figure 2

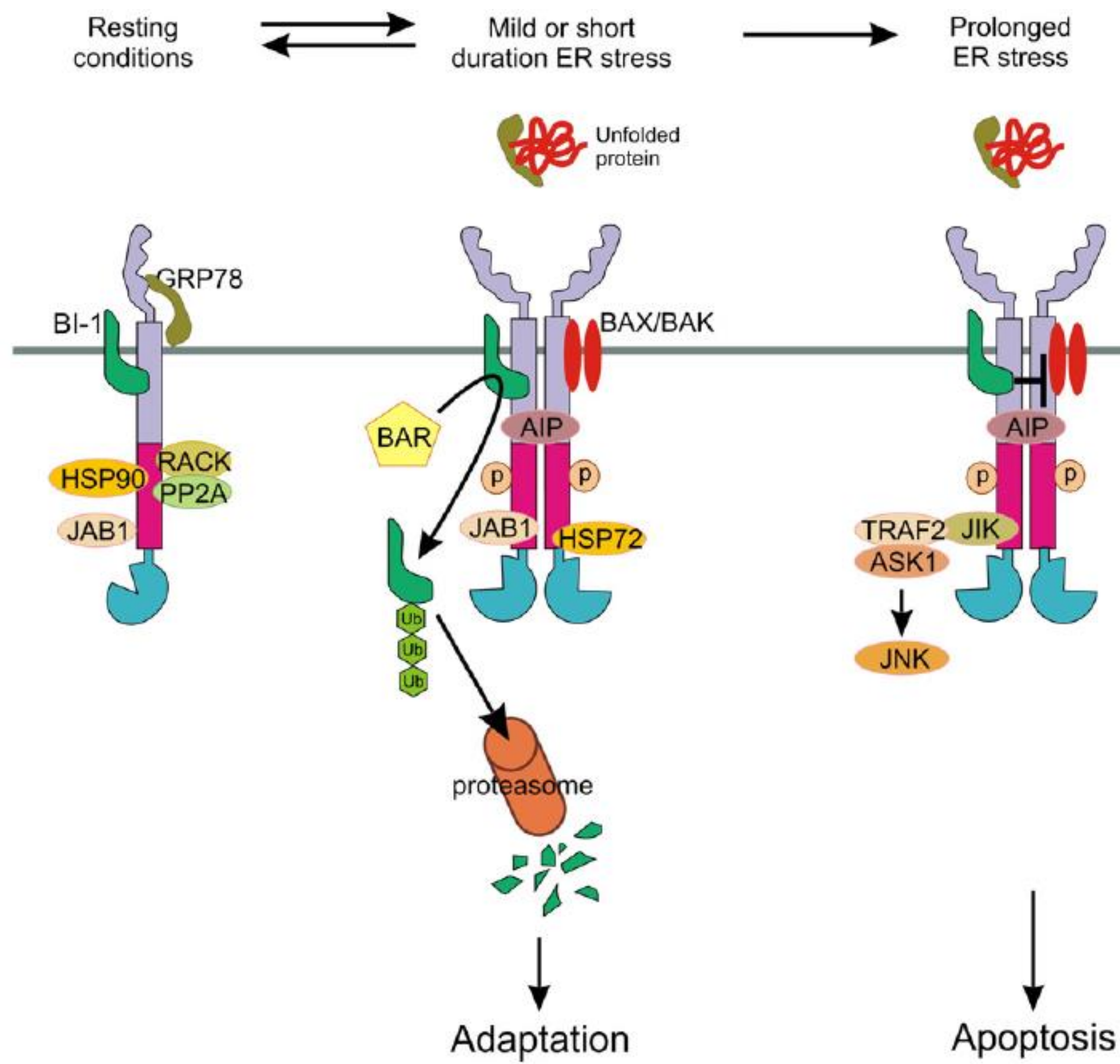

Figure 3