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# Current Concepts in ER Stress-Induced Apoptosis

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**Abstract**

Tumour development and progression is associated with cancer cell stress, owing to the rapid proliferative rate of cancer cells. During these processes cancer cells encounter severe cytotoxic conditions such as hypoxia, nutrient deprivation, metabolic changes and acidosis. As a consequence cancer cells must possess high adaptive capabilities in order to contend with such stresses. One of the adaptive responses activated in cancer cells is termed the Unfolded Protein Response (UPR), which is triggered by conditions that adversely affect Endoplasmic Reticulum (ER) homeostasis – a condition referred to as ER stress. Activation of the UPR functions to restore ER homeostasis and confer upon cancer cells a survival advantage. If ER stress is prolonged or too severe signalling switches from pro-survival to pro-death and ER stress-induced apoptosis is triggered. In this article we provide an overview of the current concepts concerning ER stress-induced apoptosis, focussing on the role of ER-localized stress sensors and triggering ER stress-induced apoptosis with particular emphasis on the contribution of Bcl-2 family members.

**Keywords:** Apoptosis; Cancer; Endoplasmic reticulum (ER); ER stress; Unfolded Protein Response (UPR)

## Introduction

The Endoplasmic Reticulum (ER) is a membranous network within cells that is important for several cellular functions including translation and folding of secretory and membrane proteins, lipid biogenesis and sequestration of $Ca^{2+}$ [1,2]. It spans from the nuclear envelope to the plasma membrane. The lumen of the ER contains a plethora of proteins involved in the folding and posttranslational modification of newly synthesised proteins, including chaperones, folding enzymes, oxidizing enzymes, and glycosylating enzymes [1]. Many of these proteins are $Ca^{2+}$-dependent and require a highly oxidizing environment. ER homeostasis is very sensitive to perturbations in cellular homeostasis and activates an adaptive response known as the unfolded protein response (UPR) when it senses stressful conditions. This can occur in response to conditions such as hypoxia, disturbed $Ca^{2+}$ homeostasis, accumulation of misfolded proteins, oxidative stress, nutrient deprivation, metabolic changes and acidosis [3].

In this review, we will describe how cells detect ER stress and activate the UPR and how UPR signalling can promote cell recovery or cell death. We also highlight the importance of the intrinsic or mitochondrial apoptotic pathway and Bcl-2 family in the regulation of ER stress-induced cell death. The relevance of these to cancer will be explored briefly.

## ER Stress

To function optimally the ER is dependent on maintaining homeostatic conditions. Any stress which negatively impacts upon energy availability or intracellular $Ca^{2+}$ levels reduces the ability of the ER to function resulting in the accumulation of unfolded proteins within the ER lumen triggering ER stress. Examples of such stresses include hypoxia, oxidative stress and glucose deprivation. Likewise, stresses which place high protein folding demands on the ER such as inflammation or viral infection can also result in the accumulation of unfolded proteins and ER stress. To counteract ER stress, the cell activates the UPR which initially has a pro-survival role. Three ER localised transmembrane receptors, Pancreatic ER Kinase (PKR)-like ER kinase (PERK), Activating Transcription Factor 6 (ATF6) and Inositol Requiring Enzyme 1 (IRE1α) (Figure 1) act as stress sensors and constantly monitor the condition of the ER. Under normal conditions each of these sensors is maintained in an inactive configuration by binding of GRP78 (an ER chaperone protein) to the luminal portion of each receptor (Figure 1). GRP78 has a higher affinity for unfolded proteins and therefore, when they accumulate due to ER stress, GRP78 dissociates from each receptor triggering their activation and induction of the UPR [4].

The primary goal of the UPR is to restore cellular homeostasis by clearing the backlog of unfolded proteins within the ER lumen. UPR signalling shuts down general translation in the cell (to prevent further protein build up), selectively increases the expression of ER chaperone proteins (to aid folding of proteins in the ER lumen) and activates ER-Associated Degradation (ERAD) (which exports misfolded proteins to the cytoplasm where they are degraded by the proteasome). In concert, these processes reduce the load of unfolded proteins and help relieve ER stress. Under circumstances where the level of ER stress is too severe and cannot be resolved, UPR signalling shifts from pro-survival and ER-stress-induced apoptosis ensues [3].

## Key mediators of the Unfolded Protein Response

### PERK

Following dissociation of GRP78, PERK is activated by dimerization followed by autophosphorylation. One important downstream substrate of active PERK is eukaryotic initiation factor 2α (eIF2α) [5] whose phosphorylation on serine 51 inhibits general cap-dependent translation thus reducing further accumulation of proteins within the ER lumen [6]. This block in general translation is important for cell survival as it serves to reduce the continuing build-up of unfolded proteins in the ER thus reducing ER stress. $PERK^{-/-}$ mouse embryonic

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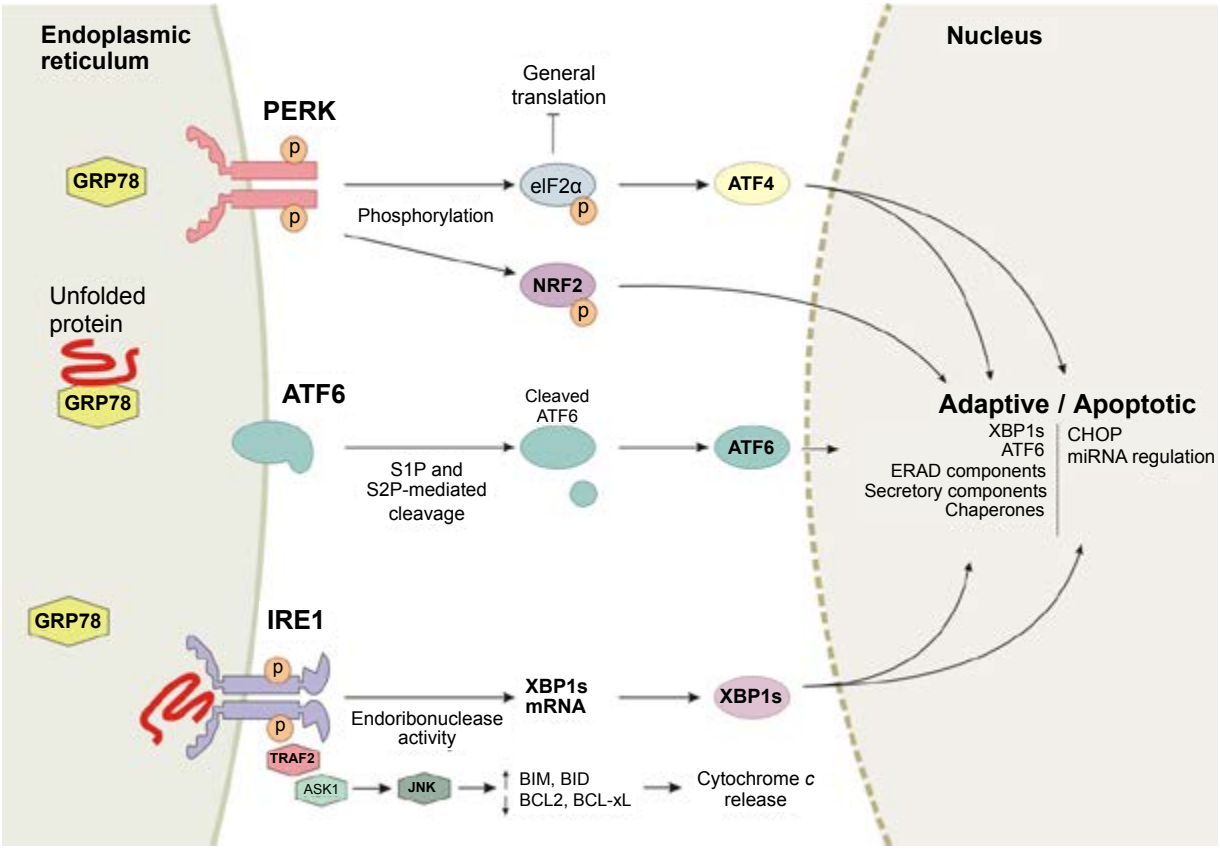


**Figure 1:** Schematic showing activation of the three arms of the UPR. The three ER stress sensors PERK, IRE1 and ATF6 are kept in their inactive state through interaction with the ER chaperone GRP78 (BiP). In response to accumulating misfolded/unfolded proteins in the ER, GRP78 dissociates from the lumilal domain of these sensors, leading to their activation. The concerted action of PERK, IRE1 and ATF6 activates a transcriptional response which can be adaptive or apoptotic and is essential for cell survival during ER stress. The same transcriptional response also contributes to tumour development and progression.

fibroblasts lack this translational block and as a result are hypersensitive to ER stress [6]. Likewise, knock-in non-phosphorylatable eIF2α cells also display increased sensitivity to ER stress agents and cell death [7]. This translational block is not absolute as some genes, such as Activating Transcription Factor 4 (ATF4), have an internal ribosome entry site in their 5' untranslated region enabling their cap-independent translation [8]. ATF4 is a member of the CCAAT/enhancer binding protein family (C/EBP) of transcription factors. Following its translation, ATF4 translocates to the nucleus where it drives expression of ER chaperone proteins (GRP78 and GRP94), genes involved in amino acid biosynthesis, redox reactions, protein secretion and the pro-apoptotic transcription factor CHOP [9].

## ATF6

Due to the presence of an ER-targeted hydrophobic domain ATF6 is an ER tethered protein. Upon detection of ER stress and dissociation of GRP78, ATF6 translocates to the Golgi apparatus. In the Golgi it is cleaved by 2 proteases, Site-1 protease (S1P) and Site-2 protease (S2P) [10]. Active ATF6 is a bZip transcription factor family member which translocates to the nucleus where is drives the expression of chaperone proteins (GRP78, GRP94, Prolyldisulphide Isomerise (PDI)), ER Degradation-Enhancing α-Mannose-Like Protein 1 (EDEM1) and the pro-survival transcription factor X Box-Binding Protein 1 (XBP1) [11,12]. ATF6 signalling appears to be predominantly pro-survival with little evidence linking it to cell death. ATF6 has been associated with the down-regulation of Mcl-1 in apoptotic myoblasts during differentiation, however this effect has not been reported in an ER stress specific context [13].

## IRE1α

In a similar mechanism to ATF6 and PERK, IRE1α is held in an inactivate state through binding to GRP78 and upon dissociation of GRP78, IRE1α is activated by dimerisation and autophosphorylation. IRE1α is a bi-functional molecule that has both a serine-threonine protein kinase domain and an endoribonuclease domain [14]. Following its activation, IRE1α cleaves a 26 nucleotide intron from XBP1 mRNA causing a frameshift enabling translation and generation of a basic leucine zipper family transcription factor, spliced XBP1 (XBP1s) [15]. XBP1s activates the transcription of various proteins involved in the maintenance of ER homeostasis such as ER chaperones (GRP78, ERDj4, HEDJ, and PDI-P5), ER associated degradation (ERAD) components (EDEM, p58$^{IPK}$), components of the secretory pathway (SEC23B, SEC24C, SEC61A, SRP54), as well as transcription factors such as CHOP and XBP1 [16,17]. The majority of XBP1s target genes aim to reduce the ER protein load and restore homeostasis. The pro-survival function of IRE1α signalling is further supported in studies examining the temporal activation of UPR signals and overexpression studies. IRE1α has been identified as one of the first arms switched on during ER stress and to be shut down upon prolonged stress [18]. Furthermore, overexpression of a mutant IRE1α (in which RNase activity can be selectively activated) leads to an enhancement of cell

survival upon induction of ER stress further underscoring the pro-survival functions of IRE1α [18].

Recently, the RNase activity of IRE1α has been linked to a process termed regulated IRE1α-dependent decay of mRNAs (RIDD). While this process was first discovered in *D. melanogaster*, further studies have identified a mammalian counterpart [19,20]. Although IRE1α RNase activity is required for RIDD to occur, this process is distinct from XBP1 splicing, with RIDD activity being reported to target mRNAs encoding secretory proteins [19]. The importance of RIDD activation is not yet fully understood. However, it is expected to decrease the protein load on the ER helping to restore ER homeostasis. On the other hand extensive or prolonged RIDD activation has recently been associated with increased apoptosis [20]. RIDD is a relatively new discovery in ER stress research and therefore much remains unknown as to its role in ER stress-induced cell death or cancer.

## Mechanism of ER Stress-Induced Apoptosis: Role of Bcl-2 Family

The UPR is predominantly a pro-survival response which offers a window of opportunity to resolve ER stress and return to normal functioning. However, in cases where ER stress is severe or prolonged and the UPR cannot restore homeostasis, signalling switches from pro-survival to pro-apoptotic.

Cell death induced by pro-longed or excessive ER stress predominantly occurs via the intrinsic (or mitochondrial) apoptosis pathway. Release of cytochrome *c* from the mitochondrial inter-membrane space is a key event in intrinsic cell death and is associated with opening of the mitochondrial permeability transition pore and loss of mitochondrial transmembrane potential. Once in the cytosol, cytochrome *c* recruits pro-caspase-9 to apoptosis protease activating factor 1 (APAF-1) to form a complex referred to as the apoptosome which enables pro-caspase-9 activation [21,22]. Active caspase-9 cleaves and activates downstream effector caspases such as caspase-3, which in turn cleave cellular substrates leading to the ordered dismantling of the cell [23]. Activation of the intrinsic apoptotic pathway as a result of unresolved ER stress is mediated by members of the B cell lymphoma protein 2 (Bcl-2) family which control the release of cytochrome *c* from the mitochondrial intermembrane space.

The Bcl-2 family of proteins is comprised of pro and anti-apoptotic members with all members containing at least one of the four conserved alpha-helical motifs known as 'Bcl-homology domains' or BH1, BH2, BH3 and BH4. Anti-apoptotic family members possess all four BH domains and include Bcl-xL, Bcl-w, the founder protein Bcl-2, Mcl-1 and A1 [24]. The opposing pro-apoptotic proteins can be divided into subfamilies based on which BH-domains they possess. Some, such as Bak, Bax and Bok, contain BH-domains 1-3, while others have a BH3 domain only and are referred to as BH3-only proteins (Bim, Bad, Bik, Bid, Bmf, Hrk, Puma and Noxa). Anti-apoptotic members of the Bcl-2 family interact with pro-apoptotic members neutralising their function. All anti-apoptotic Bcl-2 family members can target Bax while only Bcl-xL and Mcl-1 have been shown to target Bak. Members of the pro-apoptotic BH3-only family can be subdivided into two distinct groups based on their affinity for multidomain Bcl-2 family proteins. Bid, Bim and Puma interact with and enhance the activity of pro-apoptotic Bcl-2 proteins, while Bad, Bik, Bmf, Hrk and Noxa interact with anti-apoptotic Bcl-2 proteins to displace Bax and Bak. Once "free" Bax and Bak can translocate to the mitochondrial outer membrane where they homooligomerize to form pores and enable cytochrome *c* release [25-28].

## ER Stress Mediated Regulation of Bcl-2 Family Members

Regulation of Bcl-2 family members, especially BH3-only proteins, occurs in response to internal stress signals, including ER stress, and leads to cytochrome *c* release and caspase activation. The significance of this to ER stress-induced death is clearly illustrated in $Bax^{-/-}/Bak^{-/-}$ cells which display resistance to apoptosis [29]. Additionally, overexpression of anti-apoptotic Bcl-2 or Bcl-xL has been demonstrated to protect cells from ER stress-induced cell death underscoring the importance of Bcl-2 proteins and the intrinsic pathway [30]. In recent years, much focus has been placed on understanding the mechanisms regulating expression of Bcl-2 family proteins during ER stress-induced apoptosis. As a result of this we now have an understanding of how different signalling arms of the UPR can modulate expression of pro- and anti-apoptotic Bcl-2 family members.

CHOP is a bZIP-containing transcription factor that is a common point of convergence for all three arms of the UPR, with binding sites for ATF6, ATF4 and XBP1s present within its promoter. CHOP is primarily considered a pro-apoptotic transcription factor that mediates ER stress induced cell death through the regulation of Bcl-2 family members. Upregulation of the BH3-only protein Bim has been demonstrated to occur in a CHOP-dependent manner in tunicamycin-treated MCF7 cells. Further analysis of this pathway revealed Bim expression was modulated by a combination of CHOP-dependent transcriptional upregulation and post-translational modification by protein phosphatase 2a (PP2a) increasing protein stability [31]. CHOP has also been reported to interact with FOXO3a to regulate Puma and Bim expression (in neuronal cells) and with AP1 to modulate Puma expression (in hepatocytes) [32,33]. CHOP mediated downregulation of Bcl-2 has also been reported as a means by which CHOP can tip the balance in favour of pro-apoptotic Bcl-2 proteins and cell death [34,35].

Upon its activation IRE1α recruits TNF receptor associated factor 2 (TRAF2) to its cytosolic domain, thus triggering phosphorylation cascades involving ASK1 and culminating in JNK activation. IRE1α-medaited activation of JNK provides a means by which Bcl-2 family member activity can be regulated. JNK-mediated phosphorylation of Bcl-2/Bcl-xL has been reported to decrease their anti-apoptotic ability while phosphorylation of Bid and Bim by JNK has been demonstrated to increase their pro-apoptotic ability [36-39]. Therefore, IRE1α-mediated JNK activation may represent a mechanism through which IRE1α can manipulate relative levels of pro- and anti-apoptotic Bcl-2 family members thus tipping the balance in favour of apoptosis.

Other transcription factors, not part of the UPR, have also been implicated in ER stress-induced regulation of Bcl-2 family members. For examples, upregulation of the BH3-only proteins Puma and Noxa has been reported to occur during ER stress-induced death in a p53-dependent manner [40]. Furthermore, partial suppression of cell death was observed in $p53^{-/-}$ cells and attributed to the reduced expression of Puma and Noxa [41]. The mechanism triggering p53 signalling during ER stress-induced death is currently unknown although NFκB activation is thought to play an important role [40]. All three arms of the UPR can be linked in various ways to NFκB activation. For example, PERK-mediated repression of translation causes a reduction in IκB levels (since IκB has a short half-life), thereby relieving the repression on NFκB and allowing it to translocate to the nucleus [42]. IRE1α signalling has been implicated in NFκB activation via its recruitment of IKK through TRAF2, thereby permitting nuclear translocation of NFκB [43]. Finally, ATF6 activation has been implicated in NFκB activation

in rat renal proximal tubular cells during shiga toxin treatment [42]. NFκB has hundreds of downstream targets and it is likely that some of these are pro-apoptotic such as BH3-only proteins further committing the cell to death.

ER stress regulation of the levels of certain microRNAs (miRNAs), can also influence expression of Bcl-2 family members and hence affect cell fate. Direct regulation of miRNA expression by ER stress sensors especially PERK has been reported. We recently observed that miRNAs belonging to the miR-17-92 cluster and their paralog cluster miR-106b-25 were downregulated during ER stress, in a PERK-dependent manner. Further analysis revealed that the PERK-regulated transcription factors NRF2 and ATF4 were responsible for this repression. PERK-dependent induction of ATF4 and phosphorylation of NRF2 leads to repression of the miR-106b-25 cluster, thus removing their inhibition of Bim and pushing the cell towards ER stress-induced cell death [44]. Based on the current literature miRNA regulation may help shift the balance between survival and cell death during ER stress-induced apoptosis. Further studies are required to understand the range of Bcl-2 family members regulated by miRNAs during ER stress-induced apoptosis.

## ER Stress and Cancer

Cancer cells are uniquely dependent on UPR signalling to enable their survival within the tumour microenvironment especially in the early stages of tumourigenesis before sufficient vascularisation has occurred. The tumour microenvironment is characterised by conditions such as hypoxia, low nutrients and pH fluctuations, all potent inducers of ER stress. To survive under these conditions cancer cells must initiate adaptive strategies. To this end, elevated expression and dependence on various ER chaperone proteins and arms of the UPR has been observed, and ER stress and the UPR have been linked to the development and progression of cancer. In fact, the UPR is currently emerging as a viable therapeutic target for the treatment of cancer [45].

Numerous studies have shown that GRP78 is overexpressed in many cancers, including prostate, breast and lung cancers [46-53]. Jamora and colleagues have demonstrated cells incapable of inducing GRP78 are incapable of tumour formation [54], thus illustrating the importance of GRP78 in tumour development. Additionally, expression of GRP78 was found to correlate with high levels of proliferation in glioma cells while knockdown decreased the cells' proliferative capacity [55]. Upregulation of GRP78 expression has been linked to protection of dormant tumour cells against drug toxicity, possibly via reduced Bax activation [56]. By upregulating GRP78 expression cancer cells may simply be increasing the protein-folding capacity of the ER, thereby avoiding the induction of ER stress and cell death.

The IRE1α/XBP1s branch of the UPR has also been implicated in cancer cell survival and proliferation. Xenograft models have clearly highlighted the importance of IRE1α/XBP1s to tumour development. Injection of $XBP1^{-/-}$ cells into immune-compromised mice failed to result in tumour formation as compared to their wild type counterparts [57]. Sustained IRE1α signalling and selective XBP1s overexpression has also been linked to increased cell proliferation, while specific knockdown of XBP1s decreases the proliferative capacity of cells. The mechanism of how IRE1α/XBP1s signalling can influence cell proliferation has not yet been fully elucidated although increased expression of cyclin A1 has been reported [58]. Basal overexpression of XBP1s has been reported across a range of cancer types including breast cancer, multiple myeloma and hepatocellular cancer [59-61]. Studies by Carassaco and colleagues reported XBP1s-overexpressing mice induced neoplastic transformation of plasma cells and spontaneously developed multiple myeloma [62]. Numerous studies examining gene regulation in the development of breast cancer have shown increased expression of XBP1 mRNA. Moreover, tissue microarray studies found XBP1s positively correlated with aggressive, highly proliferative, and/or high grade mammary tumours [63] suggesting that XBP1s may be important in the progression of breast cancer.

In contrast, xenograft models using $PERK^{-/-}$ cells demonstrated tumour establishment (unlike $IRE1\alpha^{-/-}$ cells) [64] but reported an attenuated growth rate and increased apoptosis rate compared to wild type cells highlighting a need for PERK signalling in tumour development.

## Targeting the UPR

As previously described the UPR has both pro-survival and pro-death potential. Therefore, targeting this response either by attenuating the adaptive arm or enhancing pro-death signalling may have clinical potential in the treatment of cancer. To date, drug development targeting the UPR has mainly focused on the inhibition of pro-survival IRE1α signals. This has lead to the development of compounds such as STF-083010 and MKC-3646, which act by inhibiting the catalytic core of the RNase domain [65,66]. Directly targeting the endoribonuclease domain of IRE1α blocks its ability to cleave XBP1 mRNA therefore reducing levels of the pro-survival transcription factor XBP1s. Attenuating signalling via this arm of the UPR, is thought to reduce pro-survival responses and push the cell in the direction of death. Indeed, recent work by Mimura and colleagues has highlighted the therapeutic potential of limiting XBP1 cleavage in multiple myeloma (MM) cells [67]. Xenograft mouse models of MM displayed a significant decrease in tumour volume following treatment with MKC-3646 compared to vehicle alone [66]. Increased levels of heat shock proteins is frequently reported in cancer [68]. Recently, Hsp72 demonstrated to interact with IRE1α enhancing its activity and splicing of XBP1 [69], while Hsp90 association is required for IRE1α stability and activation [70]. Therefore, specific targeting of Hsps for example, Hsp72 or Hsp90, by compounds such as MAL3-101, 17-AAG and radicicol may also help counteract IRE1α pro-survival signalling and enhance cell death. In fact, 17-AAG in combination with MKC-3646 had increased cytotoxicity when compared to either treatment alone in RPMI 8226 and INA6 MM cell lines [66].

PERK signalling contributes to pro-survival UPR responses by attenuating cap-dependent translation through the phosphorylation of eIF2α, thus preventing further protein accumulation in the ER. GSK2656157, an ATP competitive inhibitor of PERK catalytic activity, limits the ability of PERK to block translation enhancing cell death. Recent *in vivo* xenograft studies, using GSK2656157, have reported diminished growth in MM and pancreatic-derived tumours, thus highlighting the clinical potential of targeting PERK as an anti-cancer treatment [71].

Alternatively, rather than suppress the adaptive phase of the UPR another approach is to enhance the level of ER stress pushing the cell towards cell death. Proteasome inhibitors, such as Bortezomib, prevent the degradation of damaged/misfolded proteins increasing the level of ER stress and committing the cell to death. Bortezomib has been successfully used as a single agent therapy but has also been combined with an inhibitor of IRE1α endoribonuclease activity providing an effective treatment where ER stress is increased and pro-survival IRE1α signals inhibited. Alternative strategies for increasing ER stress levels

include inhibitors of ERAD pathway. p97ATPase is involved in ERAD retrotranslocation and the ubiquitin fusion degradation pathway, inhibitors such as Eeyarestatin and DBeQ can block p97ATPase activity with the latter reported to induce rapid caspase activation and death in Hela cells [72].

Overall, the UPR presents itself as a clinically relevant and druggable target as it is often the deciding factor between cell survival and cell death. Manipulation of these processes is only starting to be exploited in cancer treatment with initial studies in MM looking promising; however other cancers also need to be explored for drug development to fully exploit this mechanism in cancer therapy.

## Conclusion

ER stress is implicated in many diseases such as cardiovascular disease, metabolic diseases, neurodegenerative diseases including Alzheimer's disease and cancer. The identification of the important players and mediators of these diseases presents promising drug targets and potential therapeutic strategies. The targeting of the UPR and ER stress is a promising area in anti-cancer therapies, however there are some challenges associated with their use as drug targets. A therapeutic window in which it is possible to selectively target and kill the cancer cells with an increased UPR without affecting the normal cells is the most desirable scenario for treatment. Another challenge is the need for the development of suitable UPR biomarkers that can be used to determine the role of the UPR in predicting disease-free survival in patients [73]. In 2008 Davies and colleagues measured XBP1s mRNA levels by Q-PCR in breast cancer patients during treatment with tamoxifen and found that those patients with elevated XBP1s levels had a significantly worse clinical outcome compared to their counterparts with lower XBP1s expression [63]. This presents the exciting possibility of using XBP1s as a potential biomarker in breast cancer. It has been established that the UPR is essential for tumourigenesis and in establishing a suitable tumour microenvironment, but relatively little is known of the UPR's involvement in maintaining already-formed tumours. As cancer patients will usually not present to a clinician until the tumour has been established it would be advantageous to elucidate the UPR's role in already-formed tumours. This is one possible future direction for studies into ER stress, the UPR and their role in cancer.

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### Declaration of conflict of Interest

A Samali is co-founder and director of Aquila Bioscience Limited.

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