ANTICANCER RESEARCH *33*: 4683-4694 (2013)

Review

The Paradox of the Unfolded Protein Response in Cancer

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Abstract. *The endoplasmic reticulum (ER) is an elaborate organelle that is essential for cellular function and survival. Conditions that interfere with ER functioning can lead to the accumulation of unfolded proteins, which are detected by transmembrane sensors that then initiate the unfolded protein response (UPR) to restore ER proteostasis. If 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 diseases, including cancer. Whether the UPR inhibits tumour growth or protects tumour cells by facilitating their adaptation to stressful conditions within the tumour microenvironment is unknown, and dissection of the UPR network will likely provide answers to this question. In this review, we aim to elucidate the paradoxical role of the UPR in apoptosis and cancer.*

The endoplasmic reticulum (ER) consists of a membranous network that extends throughout the cytosol; here, proteins are synthesized, post-translationally modified and folded into correct conformations. Unlike the cytosol, the ER luminal environment is sufficiently oxidised to permit for cysteine oxidation and subsequent formation of the disulfide bonds that are critical to the correct conformations of many mature proteins (1). The ER contains stringent quality control systems that selectively export correctly-folded proteins and extract terminally-misfolded proteins for ubiquitin-dependent proteolytic degradation, a process known as ER-associated protein degradation (2) (Figure 1). However, if degradation is insufficient, misfolded proteins can accumulate. This phenomenon is called ER stress, and it activates the unfolded protein response (UPR). The UPR is generally considered to be the transcriptional induction of molecular chaperones in response to ER stress (3). However, gene expression profiling has demonstrated that, parallel to the chaperones, the UPR regulates genes involved in protein entry into the ER, calcium and redox homeostasis, ER quality control, autophagy, lipid biogenesis, and vesicular trafficking. Additionally, ER stress attenuates global protein synthesis, a process that subsequently reduces the protein load to help re-establish equilibrium and is associated with cell-cycle arrest and tumour dormancy. Three ER stress transducers have been identified: protein kinase RNA-like endoplasmic reticulum kinase (PERK), inositol-requiring enzyme-1 (IRE1), and activating transcription factor-6 (ATF6; Figure 2) (4, 5). Most targets are co-regulated by IRE1, PERK, and ATF6 to ensure the redundancy and robustness of this adaptive response (6).

Following initiation of malignancy, rapid tumour growth and inadequate vascularization result in microenvironmental stress. This condition activates a range of stress response pathways, including the UPR, which meticulously coordinate adaptive and apoptotic responses to ER stress. During tumourigenesis, the UPR enhances the ER protein-folding capacity and maintains ER protein homeostasis (or proteostasis), thereby counteracting apoptosis. The UPR, when coupled with induced tumour dormancy, dually protects neoplastic cells from apoptosis and permits recurrence once favourable growth conditions have been restored (7, 8). However, if ER stress is prolonged and the

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Key Words: Cancer, endoplasmic reticulum, stress, unfolded protein response, molecular chaperones, activating transcription factor 6, PERK kinase, inositol requiring enzyme-1, review.

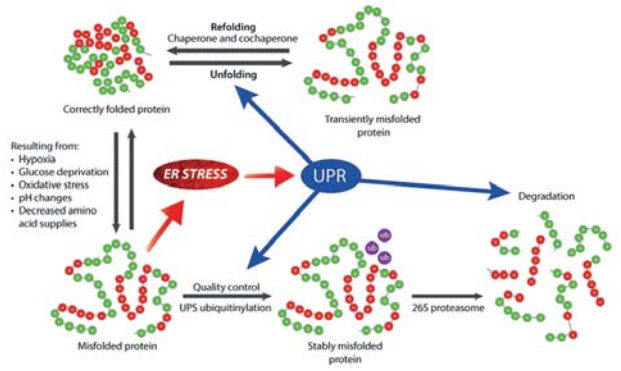

Figure 1. *Cellular stress as the cause of protein misfolding. Molecular chaperones stabilise and (un)fold newly-synthesised proteins into their proper conformations. During tumour formation, continuous endoplasmic reticulum (ER) stress eventually causes damage that the chaperones cannot correct. These proteins might then be recognised and degraded by the ubiquitin-proteasome system (UPS). However, if this process is insufficient to counter the accumulation of misfolded proteins, the unfolded protein response (UPR) is activated to induce chaperones, protein quality control and degradation.*

UPR fails to restore ER proteostasis, tumour cell apoptosis ensues. This review addresses this paradoxical role in cancer.

Extrinsic and Intrinsic Stressors that Activate the UPR During Tumourigenesis

Although tumours secrete angiogenic factors to promote angiogenesis, this is often insufficient to meet the elevated tumour metabolic requirements. Therefore, in addition to hypoxia (9), cells in developing tumours are subject to glucose deprivation, lactic acidosis, oxidative stress, and decreased amino acid supplies (Figure 1). In addition to these extrinsic stressors, tumour-intrinsic stressors, such as errors in glycoprotein and lipid biosynthesis that result from an increased mutation rate, might also contribute to the induction of ER stress (10).

Hypoxia-mediated UPR activation is essential for tumour cell survival. The major UPR-inducing pathway in tumours is mediated by hypoxia. Human fibrosarcoma and lung carcinoma cells up-regulate 78-kDa glucose-regulated protein (GRP78) and X-box-binding protein 1 (XBP1) splicing under hypoxic conditions *in vitro*, whereas in human colon cancer cells, hypoxia induces the PERK-dependent phosphorylation of eukaryotic initiation factor-2α (eIF2α) and the translation of activating transcription factor-4 (ATF4; Figure 2) (8). A strong positive correlation was demonstrated between spliced XBP1 (XBP1s)-induced bioluminescence and tumour hypoxia in transgenic mice that developed spontaneous mammary carcinomas and exhibited luciferase reporter-coupled XBP1 splicing (11). Additionally, the exposure of transformed mouse embryonic fibroblasts (MEFs) to hypoxia led to increased GRP78 and XBP1 expression, as well as increased ATF4 and C/EBP homologous protein transcription factor (CHOP) expression. A potential UPR trigger in hypoxic conditions is ER oxidase 1α (ERO1α), an oxidoreductase that catalyses disulfide bond formation in nascent proteins in an oxygen-dependent manner. Although hypoxia transcriptionally induces ERO1α, reduced oxygen tension impairs ERO1α activity and subsequent protein folding. Another UPR-inducing mechanism is the up-regulation of glycogen synthase kinase 3B, which activates the PERK branch (12).

The UPR is required for tumour cell growth under hypoxic conditions (13). Cells are sensitised to hypoxia *in vitro* by antisense-mediated *GRP78* inhibition (14). PERK inactivation due to the generation of mutations in its kinase domain impairs cell survival under extreme hypoxia (15). PERK promotes cancer cell proliferation by limiting oxidative DNA damage through ATF4 (16).

Additionally, XBP1-deficient tumour cell survival was reduced during severe hypoxia *in vitro*, and these cells were unable to grow as tumours *in vivo*. Spliced XBP1 expression restored tumour growth, suggesting that the IRE1 branch is also required for tumour cell survival during hypoxia (17).

Thus, tumour formation with aberrant microcirculation leads to hypoxia, which induces the UPR. In turn, the UPR increases cellular survival and proliferation, which further enlarges the tumour and thereby increases hypoxia in the tumour core (Figure 3).

Activation of the UPR by glucose deprivation and subsequent acidosis. Tumour cells adapt to low glucose levels by switching to a high rate of aerobic glycolysis, which is known as the Warburg effect (18). The resulting lactic acid production reduces the pH, leading to aggravated local distress. Acidosis is a prominent feature of the tumour microenvironment that surprisingly promotes tumour survival and progression by regulating several B-cell leukemia/lymphoma-2 (BCL-2) family members and CHOP (see below) (19). The glucose-regulated protein family, which includes the master UPR regulator GRP78, was originally discovered due to the up-regulation of its members in response to glucose deprivation (20). In the XBP1s reporter mouse model, which develops spontaneous mammary tumours, XBP1 splicing was found to increase upon exposure to a non-metabolizable glucose analog that simulates glucose deprivation (11).

CHOP deletion in a mouse model of Kirsten rat sarcoma viral oncogene homolog-induced lung cancer increases tumour incidence and thus supports the notion that ER stress serves as a barrier to malignancy. UPR activation and the subsequent $p58^{IPK}$ expression control the fates of malignant

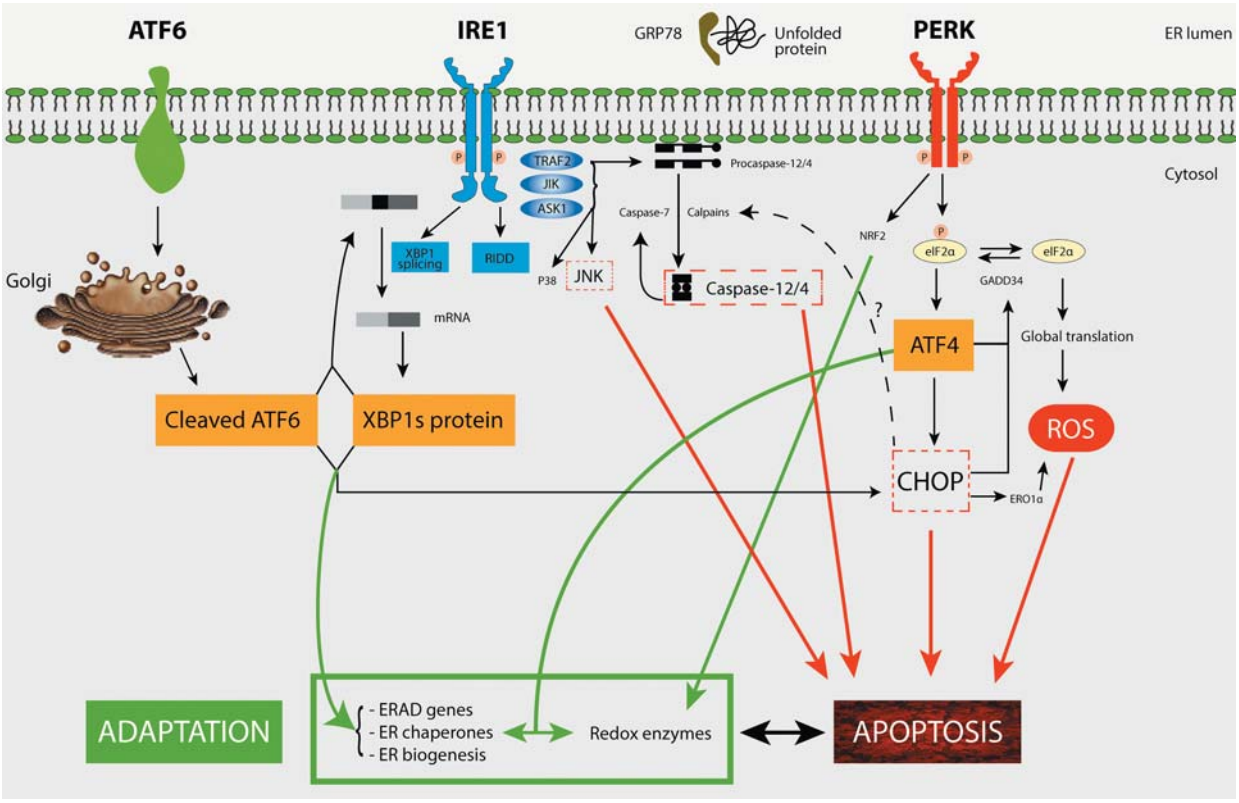

Figure 2. *Endoplasmic reticulum (ER) stress induces the unfolded protein response (UPR) through a triple transcription factor system. Misfolded proteins sequestrate 78-kDa glucose-regulated protein (GRP78), thus allowing the activation of three ER membrane-associated proteins. Activating transcription factor-6 (ATF6) translocates to the Golgi for cleavage, and the cleaved fragment subsequently regulates UPR gene expression. Inositol-requiring enzyme 1 (IRE1) cleaves X-box-binding protein 1 (XBP1) mRNA to a spliced form (XBP1s) that is translated to a strong transcription factor. Along with selective XBP1 mRNA splicing, other mRNAs are degraded by the IRE1 RNase activity (RIDD). IRE1 promotes c-Jun N-terminal kinase (JNK) and p38 phosphorylation through direct interactions. Caspase-12 (murine) or -4 (human) activation is ER stress-dependent. Protein kinase RNA-like endoplasmic reticulum kinase (PERK) phosphorylates eukaryotic initiation factor 2α (eIF2α) to attenuate global translation. Phosphorylated eIF2α favours activating transcription factor 4 (ATF4) translation. The latter induces growth arrest and DNA damage-inducible protein (GADD34), which dephosphorylates eIF2α. PERK also phosphorylates nuclear factor erythroid 2-related factor 2 (NRF2), which induces an anti-oxidative response. ASK1: apoptosis signal-regulating kinase; CHOP: C/EBP homologous protein transcription factor; ERAD: ER-associated protein degradation; ERO1α: ER oxidase 1α; JIK: jun kinase-inhibitory kinase; ROS: reactive oxygen species; TRAF2: tumor necrosis factor receptor-associated factor-2.*

cells that face glucose deprivation. Overcoming this barrier requires for selective attenuation of the PERK-CHOP branch by $p58^{IPK}$. Furthermore, this $p58^{IPK}$-mediated fine-tuning enables cells to benefit from the protective features of chronic UPR (21).

Dual Role of GRP78 in and on Surface of Tumour and Endothelial Cells

GRP78 is a key player in tumourigenesis and is involved in the three major hallmarks of cancer, namely the enhancement of cell proliferation, protection against apoptosis and promotion of tumour angiogenesis (22). The phosphoinositide-3 kinase (PI3K)/phosphatase and tensin homolog (PTEN)/protein kinase B (PKB) pathways play central roles in these hallmark processes. In mice, PKB activation in *PTEN*-null prostate epithelium was potently suppressed in a *GRP78*-knockout model, and a similar suppression of PKB activation was observed in human prostate cancer cells that had been transfected with small-interfering RNA (siRNA) targeted against *GRP78* (23, 24). As *PTEN* mutations and PKB activation are major drivers of tumourigenesis, GRP78 inactivation might represent a novel approach to reducing tumourigenesis that results from loss of PTEN tumour suppression or oncogenic PKB activation (1). Apart from its abundant expression in the ER, GRP78 can localise at the cell surface, within the cytoplasm, in the mitochondria and in the nucleus, as well as in secretions from tumour and endothelial cells, and this protein is implicated in processes beyond protein folding.

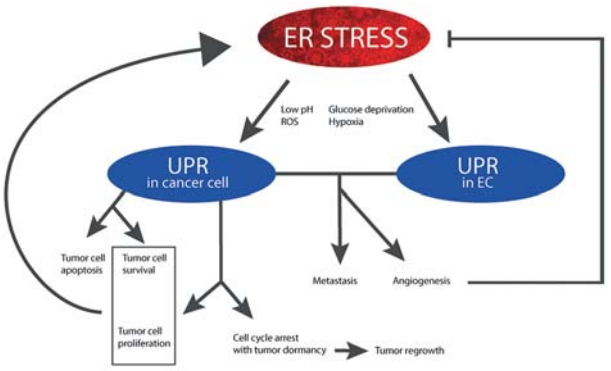

Figure 3. *The paradoxical role of the unfolded protein response (UPR) in cancer. During tumourigenesis, specific stressors activate the UPR in both cancer and endothelial cells (EC). In cancer cells, both apoptosis and survival can be induced by UPR components. Furthermore, cell-cycle progression or arrest (e.g. by reduced cyclin D1 translation) can occur in response to protein kinase RNA-like endoplasmic reticulum kinase (PERK) activation. This arrest can be temporary during stressful conditions such as chemotherapy. After the induced dormancy, tumour re-growth can occur upon the restoration of more favourable conditions. A positive feedback loop increases ER stress via cellular adaptation during tumour formation. Due to its effects on endothelial and cancer cell survival and function, the UPR also modulates metastasis and angiogenesis, which, if functional, reduces ER stress. ROS: Reactive oxygen species.*

GRP78 in tumour cells. The first causal correlation between GRP78 and *in vivo* carcinogenesis was reported in fibrosarcoma cells. *GRP78* silencing in these cells inhibited their ability to form tumours upon xenografting into mice (25). The essential role of GRP78 was confirmed in a transgenic mouse mammary tumour model. Mice that lacked one *GRP78* allele exhibited decreased breast adenocarcinoma growth and angiogenesis as well and showed survival compared to wild-type mice (26). Likewise, in glioma cells, high GRP78 levels were found to correlate with increased proliferation, and siRNA-mediated *GRP78* suppression reduced the cell proliferation rate (27).

GRP78 levels are known to be increased in various solid tumour types, including prostate, head and neck, melanoma, breast, lung, brain, gastric, colon, and hepatocellular carcinoma (HCC) (14, 28). Furthermore, elevated GRP78 levels correlate with gastric, breast, and liver cancer metastasis (7). In contrast, a recent report suggested that *GRP78* is down-regulated in mouse prostate cancer models (29). Thus, although GRP78 and malignancy appear to be positively-correlated, exceptions might occur. However, these unexpected results might be due to time-dependent alterations. Additionally, GRP78 plays a dual role in tumour cells. GRP78 controls early tumour development through tumour suppressive mechanisms such as the induction of dormancy (30). On the other hand, at more advanced stages of progression, during which tumours are exposed to more severe stress, GRP78 has been shown to promote tumour progression through its pro-survival (26) and pro-metastatic functions (7).

GRP78 on the tumour cell surface. Severe ER stress promotes GRP78 cell surface localization in various types of neoplastic and endothelial cells (14). The cell surface form of GRP78 affects cell membrane signalling pathways that regulate proliferation, apoptosis and tumour immunity (31). A growing number of cell surface GRP78-binding partners have been identified (1, 14). In prostate cancer cells, cell surface GRP78 binds the activated form of the proteinase inhibitor α_2-macroglobulin. This interaction promotes cell proliferation by activating p38 and PI3K (32). In addition to α_2-macroglobulin, cell surface GRP78 can interact with Cripto, a small tumour cell surface protein that regulates tumour progression by blocking the growth-inhibitory transforming growth factor β and activating c-SRC and PKB. Interestingly, antibody-mediated blockade of this interaction with cell surface GRP78 is sufficient to inhibit its oncogenic signalling (14, 33). Finally, neovascularization, together with the formation of cell surface GRP78/T-cadherin complexes, was accelerated by ER stress (34), whereas cell surface GRP78 might also serve as a receptor for the angiogenesis inhibitor Kringle 5; the binding of Kringle 5 to GRP78 is required to exert its anti-angiogenic and pro-apoptotic activities in stressed tumour and endothelial cells (14). Thus, the dual effects of cell surface GRP78 signalling depend on the availability of binding partners in the tumour microenvironment.

GRP78 in endothelial cells. The importance of GRP78 in tumour angiogenesis is reflected by its constitutively high expression within the glioblastoma vasculature, which is suggestive of the sustained stress experienced by tumour-associated endothelial cells (31). In a mammary tumour model, conditional heterozygous *GRP78* knockout in endothelial cells led to a dramatic reduction in tumour angiogenesis and metastatic growth, with minimal effects on normal tissue microvascular densities. *GRP78* knockdown in immortalised human endothelial cells revealed that *GRP78* regulated endothelial cell proliferation, survival and migration (7). Vascular endothelial growth factor (VEGF) is a major driver of endothelial proliferation, and all three UPR pathways directly regulate VEGF expression (35). However, the downstream target GRP78 also plays an active role in VEGF regulation. *GRP78*-knockdown suppresses VEGF receptor-2, as well as VEGF-induced endothelial cell proliferation (14).

Three Proximal UPR Sensors in Cancer: An Integrated View

After the sequestration of GRP78 by unfolded proteins, ATF6, IRE1, and PERK are activated to transduce the ER stress signal to the cytosol and nucleus (Figure 2).

ATF6: Fine-tuning of the UPR. Although the ATF6 branch in cancer is the least investigated, its potential as an effector of clinical outcomes should not be underestimated. Activated ATF6 translocates to the Golgi, where proteases cleave it and release a fragment into the cytosol. Indeed, enhanced nuclear translocation of the ATF6 fragment is observed in various types of cancer, including HCC (28) and Hodgkin's lymphoma (36), and its expression has been linked to metastasis and relapse (37). Additionally, whereas XBP1s is required for organismal development, the functional roles of ATF6 in ER proteostasis remodelling are adaptive and can adjust the ER capacity to match demand. Therefore, ATF6 modulation might sensitively tune proteostasis without globally influencing proteome folding, trafficking, or degradation (38).

In contrast to PERK and IRE1, ATF6 activation has no obvious paradoxical outcomes. The latter primarily induces cytoprotective responses, such as ER biogenesis, chaperone up-regulation and protein degradation (38, 39). Moreover, ATF6 induces transcription of *XBP1* mRNA, the major splicing target of the IRE1 endonuclease. Recently, ATF6 was identified as a survival factor for quiescent, but not proliferative, squamous carcinoma cells and as essential for the adaptation of dormant tumour cells to chemotherapy, a process that is mediated by Ras homolog enriched in brain (RHEB) and mammalian target of rapamycin (mTOR) activation (37). ATF6 or RHEB down-regulation was able to reverse dormant cell resistance *in vivo*. Therefore, targeting survival signalling in dormant tumour cells after chemotherapy by abrogating the adaptive ATF6-RHEB-mTOR pathway might reduce the metastatic cancer relapse rate.

IRE1: The conserved core branch. After oligomerisation, IRE1 has at least three established outputs: *XBP1* mRNA splicing, regulation of IRE1-dependent decay (RIDD) of other mRNAs and direct interactions with downstream mediators (40) (Figure 2).

Increased XBP1 splicing has been demonstrated in numerous haematological and solid types of cancer and has been associated with more malignant phenotypes and poor survival (41-43). IRE1 has been shown to promote cell proliferation by regulating cyclin A1 expression through XBP1 splicing in prostate cancer cell lines (44). Notably, XBP1s enhances catalase expression, and the loss of XBP1s sensitizes cells to oxidative stress-induced apoptosis. Indeed, XBP1-deficient cells produce less catalase, which is associated with reactive oxygen species (ROS) generation and p38 activation (45). Moreover, XBP1 splicing itself might directly lead to tumourigenesis, as was evidenced by the observation that the maintenance of elevated XBP1s levels in B and plasma cells could drive multiple myeloma pathogenesis and promote hallmark myeloma characteristics, including bone lytic lesions and sub-endothelial immunoglobulin deposition (46). Moreover, a putative inhibitor of IRE1 RNase exhibited anti-myeloma activity in xenograft mice, suggesting that the IRE1-XBP1 pathway is an appealing target for anticancer therapies (47).

Xenograft glioma cells that expressed dominant-negative *IRE1* exhibited reduced proliferation. In this model, wild-type gliomas were characterised by an angiogenic/massive phenotype, whereas tumours that expressed dominant-negative *IRE1* exhibited an avascular/diffuse phenotype, suggesting that IRE1 is required for angiogenesis and functions as a switch between angiogenesis and invasion (48). The requirement for IRE1 in tumour angiogenesis during stress conditions *in vitro* could be attributed to its role in VEGF expression regulation (49). Additionally, the loss of XBP1 was shown to inhibit both tumour growth and blood vessel formation. However, these effects appeared to be VEGF-independent, indicating that the IRE1-XBP1s-VEGF axis only partially regulates the angiogenic functions of IRE1 (50). On the other hand, VEGF induces internalization of the VEGF receptor, which subsequently interacts with IRE1 to enhance XBP1 splicing (51).

The role of RIDD and the interactions of IRE1 with several downstream mediators during tumour growth and angiogenesis are not currently understood. Prolonged RIDD activation has been reported to increase apoptosis (40). Activated IRE1 recruits the adaptor protein tumor necrosis factor receptor-associated factor-2 (TRAF2) to the ER membrane, which has been reported to further activate c-Jun *N*-terminal kinase (JNK) (see below), resulting in caspase-12 activation and apoptosis in a mouse model (52). The JNK pathway is a member of the mitogen-activated protein kinase superfamily, which also includes p38 (53), and this activated pathway is involved in ER stress-mediated apoptotic cascades.

XBP1s overexpression in breast cancer cells increased BCL-2 levels after antiestrogen stimulation, thereby suppressing apoptosis (54); however, JNK phosphorylates and paradoxically inhibits BCL-2. Thus, the effects of IRE1 on the BCL-2 family vary according to the output, which is anti-apoptotic when mediated by XBP1 splicing *versus* pro-apoptotic when mediated by JNK.

PERK and protein translation in cancer. PERK phosphorylates eIF2α, leading to a translation blockade and cap-independent ATF4 translation, as well as nuclear factor erythroid 2-related factor-2 (NRF2), leading to the up-regulation of antioxidative enzymes (6) (Figure 2). PERK has been implicated in tumour progression and angiogenesis. PERK inactivation in mouse fibroblasts and human colon cancer cells, using targeted mutagenesis or a dominant-negative *PERK*, resulted in smaller tumours that demonstrated impaired angiogenic abilities upon grafting into immunodeficient mice (13, 55). *PERK* deletion in a mammary tumour mouse model was found to modestly increase tumour latency while profoundly inhibiting metastatic spread (16).

Similar observations were made in a colorectal carcinoma xenograft model that expressed a dominant-negative *PERK*. *PERK*-knockdown in human esophageal and breast carcinomas resulted in cell-cycle arrest at the G_2/M phase (16). This G_2/M arrest could likely be attributed to reduced NRF2 activity in these PERK-deficient cells, resulting in ROS accumulation that causes oxidative DNA damage and subsequently triggers cell-cycle arrest *via* the DNA double strand-break checkpoint (31). Similar to IRE1 deficiency, PERK-deficient tumours exhibited reduced viability and impaired angiogenic ability during hypoxia; these effects were attributed to the losses of phosphorylated eIF2α and ATF4. The requirement for PERK in tumour angiogenesis was further confirmed with a mouse $PERK^{-/-}$ insulinoma model in which $PERK^{-/-}$ tumours exhibited reduced vascularity (56). Thus, both downstream transcription factors of PERK, namely ATF4 and NRF2, contribute to cellular adaptation and tumour promotion.

In contrast to the previous results, p38-induced dormancy in squamous cell carcinoma cells was associated with increased PERK activation. Accordingly, pharmacologically-activated PERK was found to induce growth arrest *in vitro* and to suppress tumour growth *in vivo*, indicating an additional role for PERK in tumour growth suppression (57). Indeed, eIF2α phosphorylation-induced translational arrest down-regulates cell-cycle regulators such as cyclin D1, resulting in cell-cycle arrest in the G_1 phase. Accordingly, a non-phosphorylatable eIF2α mutant was sufficient to drive the malignant transformation of human kidney cells or fibroblasts, and conditional *PERK* deletion was found to de-regulate mammary acinar morphogenesis and to cause hyperplastic growth *in vivo* (58, 59).

Taken together, activation of the PERK axis induces tumour suppression (by G_1/S arrest) and dormancy, whereas inactivation appears to induce paradoxical effects on specific hallmarks of carcinogenesis (22), such as tumourigenesis, angiogenesis and metastasis.

Recently, a context-dependent impact of PERK on cell fate has been indicated. Downstream of PERK, CHOP directly transactivates the growth arrest and DNA damage-inducible protein (GADD34). The latter promotes eIF2α de-phosphorylation, thereby creating a negative feedback loop that leads to translational recovery (60). Additionally, both ATF4 and CHOP induce protein synthesis (61). This finding could explain the time-dependent balance in protein synthesis. After acute ER stress, protein synthesis is inhibited by eIF2α phosphorylation. However, downstream induction of *ATF4, CHOP*, and *GADD34* leads to protein synthesis recovery. If acute ER stress is addressed, survival is promoted by the restoration of translation. Conversely, if chronic ER stress continues or the acute ER stress was too severe to be addressed during a transient reduction of translation, protein synthesis leads to ROS formation and ultimately triggers apoptosis. Accordingly, salubrinal, an eIF2α dephosphorylation inhibitor, protects cells from ER stress-associated apoptosis (62).

The UPR and Apoptosis: Adaptation or Suicide – A Double-edged Sword

During ER stress, cells either survive by inducing adaptation mechanisms or commit suicide by apoptosis. The intrinsic apoptosis pathway is closely related to factors anchored on the mitochondria. The membrane insertion of pro-apoptotic proteins changes mitochondrial membrane permeability, resulting in cytochrome *c* release and caspase activation (53, 63).

CHOP: A key mediator of ER stress-induced apoptosis. Notably, *CHOP* induction strongly correlates with the onset of ER stress-associated apoptosis, and CHOP silencing protects cells (53). However, mouse embryonic fibroblasts (MEFs) derived from *CHOP*-knockout mice exhibit only partial resistance to ER stress-driven apoptosis, indicating that CHOP is not the only death pathway in this context (64). Precisely how CHOP mediates ER stress-induced apoptosis remains controversial because CHOP regulates numerous genes, the majority of which are involved in hallmarks of cancer, such as cell migration, proliferation, and survival (22, 65).

The down-regulation of anti-apoptotic *BCL-2* and the induction of the proapoptotic BCL-2 interacting mediator of cell death (BIM), p53 up-regulated modulator of apoptosis (*PUMA*) and BCL-2-associated X protein (*BAX*) are believed to contribute to CHOP-mediated apoptosis (63). *In vivo* data from breast carcinoma-derived cells corroborate these findings (66).

CHOP transcriptionally induces ERO1α (see above), which promotes disulfide bond formation but also generates hydrogen peroxide leakage into the cytoplasm (60). *In vivo*, partial *ERO1α* silencing was shown to protect against ER stress-induced death, and CHOP deficiency suppressed pancreatic β-cell apoptosis, which was associated with decreased ERO1α expression and oxidative stress markers. ERO1α activates the ER calcium channel inositol-1,4,5-trisphosphate receptor 1 (IP3R1) (67). Upon release from the ER, calcium triggers apoptosis by activating calcium/calmodulin-dependent kinase II (CaMKII), which subsequently induces four apoptotic pathways. Firstly, CaMKII triggers JNK-mediated Fas antigen induction. Secondly, CaMKII promotes mitochondrial calcium uptake, thereby activating intrinsic apoptosis. Thirdly, CaMKII activates signal transducer and activator of transcription-1 (STAT1), a pro-apoptotic signal transducer (68). Finally, the CHOP-ERO1α-IP3R1-CaMKII axis induces NADPH oxidase subunit 2 and generates ROS to possibly amplify CaMKII activation as part of a positive feedback loop because ROS induces CHOP expression. Surprisingly,

NADPH oxidase-induced ROS are also part of a second positive feedback loop that activates dsRNA-dependent protein kinase to subsequently phosphorylate eIF2α, thereby amplifying *CHOP* expression. For example, CHOP-induced hepatocyte death in a mouse protein-misfolding model was associated with oxidative stress and was relieved by an antioxidant. Because CHOP-induced apoptosis can be blocked by buffering cytosolic calcium, the ERO1α-IP3R1 pathway appears to comprise its main signalling axis (69).

Furthermore, CHOP activity is increased in response to phosphorylation by p38. p38 is a substrate of apoptosis signal-regulating kinase (ASK1, see below), which is recruited to the IRE1-TRAF2 complex upon ER stress. Thus, during prolonged stress, the PERK and IRE1 pathways might converge on CHOP, with IRE1-mediated ASK1 activation potentiating CHOP activity (4).

The JNK pathway in ER stress-mediated apoptosis. Several studies indicate a pivotal role for JNK in the mediation of ER stress-induced apoptosis (70). JNK recruitment by IRE1 is regulated by c-Jun NH_2-terminal inhibitory kinase (JIK), which has been reported to interact with both IRE1 and TRAF2. The IRE1-TRAF2 complex then recruits ASK1, causing ASK1 activation and regulating the JNK pathway that leads to cell death. In a mouse HCC model, ASK1 deficiency promoted HCC, whereas the reintroduction of ASK1 suppressed tumour development (71). Furthermore, cells from *ASK1*-knockout mice were found to be resistant to ER stress-associated apoptosis and exhibited reduced JNK and p38 activity (72). JIK overexpression was shown to promote the interaction between IRE1 and TRAF2 and JNK activation in response to ER stress, whereas the overexpression of an inactive JIK mutant inhibited JNK activation (52). Thus, the IRE1-TRAF2-JIK-ASK1-JNK pathway exerts an opposite effect on cell survival than that of the cytoprotective IRE1-XBP1s pathway. The regulation of these paradoxical IRE1 outputs requires further investigation. As described previously, JNK is a downstream effector of the CHOP-CaMKII pathway. Therefore, in conditions where both proapoptotic IRE1 activation and CHOP expression are prolonged, additive JNK activation might play a crucial role in apoptosis regulation.

Downstream apoptosis-related targets of JNK include antiapoptotic BCL-2, B-cell lymphoma-extra large (BCL-XL) and myeloid cell leukaemia sequence 1 (MCL-1), all of which are inhibited by JNK, and proapoptotic BID and BIM, which are activated by JNK-mediated phosphorylation (63, 73). During non-stress conditions, BIM is sequestered by dynein motor complexes. ER stress increases BIM levels by reducing BIM degradation and by CHOP-mediated gene induction. Phosphorylation by JNK releases BIM from its inhibitory association with the motor complexes, thus permitting its translocation to the mitochondrial outer membrane where it promotes cytochrome *c* release and caspase activation. Interestingly, a positive feedback loop exists between BIM and caspase-3. Phosphorylated BIM is a caspase-3 target and, once cleaved, becomes a more potent inducer of cytochrome c release (74, 75).

The mitochondria and the BCL-2 family. The proapoptotic BCL-2 family members trigger mitochondrial dysfunction and are sub-divided into the multi-BCL-2 homology (BH) domain proteins, such as BAX and BAK, and the BH3-only proteins, such as BAD. Of the 11 BH3-only protein subfamily members, PUMA, NOXA, BID, HARAKIRI and BIM have been reported to mediate ER stress-induced apoptosis (74, 75).

Additionally, the BCL-2 family also regulates ER stress through physical interactions with certain UPR components. For example, BAX and BAK directly interact with the IRE1 cytosolic domain upon ER stress; this interaction is essential for IRE1 activation (76). In cells that exclusively express ER-localised BAK, BIM and PUMA selectively activate the TRAF2-JNK arm of IRE1 in the absence of XBP1 splicing (77). In *BAX*/*BAK* double-knockout mice, ER stress failed to induce XBP1s, IRE1 or JNK. Moreover, *BAX*/*BAK* double-knockout MEFs are resistant to apoptosis mediated by various ER stressors, and the reconstitution of BAK expression in these MEFs restored JNK phosphorylation, suggesting a direct connection between the UPR and the apoptotic machinery (76). Thus, BAX and BAK are required for IRE1 signalling, although both are also involved in ER stress-induced apoptosis. This response could represent a switch toward pro-apoptotic signalling by IRE1. The association of IRE1 with BAX and BAK is influenced by BAX inhibitor-1 (BI-1), an ER transmembrane protein. BI-1 directly interacts with the IRE1 cytosolic domain to inhibit its endoribonuclease activity. BI-1-deficient cells were found to exhibit enhanced IRE1 activity and sustained XBP1 splicing, whereas BI-1 overexpression disrupted the interaction between IRE1 and BAX or BAK (78). Similar to ER-localised BAX/BAK oligomers (see below), BI-1 also modulates ER calcium homeostasis by forming a calcium-permeable channel pore (79).

BCL-2, BAX and BAK associate with both mitochondrial and ER membranes. During ER stress, ER-targeted BAX and BAK undergo conformational changes and oligomerisation, which leads to calcium release from the ER to the cytosol to activate m-calpain and, subsequently, procaspase-12 (see below) (81, 82). In contrast, mitochondria-targeted BAK enhances caspase-7 cleavage to create parallel pathways of caspase activation by BAX and BAK (80).

Each branch acts on different levels to tightly modulate the BCL-2 family. During hypoxia, ATF4 induces the BH3-only proteins HARAKIRI, PUMA and NOXA following PERK activation (75). Additionally, CHOP transactivates

BIM and down-regulates *BCL-2* and *MCL-1*. The less-studied ATF6 has been linked to ER stress-induced apoptosis in a myoblast cell line through the indirect inhibition of MCL-1 expression (83). The IRE1 branch can affect BH3-only proteins such as PUMA or BID. Functional integration likely occurs because BAX/BAK acts through mitochondrial permeabilisation, a key pro-apoptotic effect of the CHOP-ERO1α-CaMKII pathway.

Caspases. The processing of caspases-2 to -9 and caspase-12 has been observed in various models of ER stress-induced apoptosis (84, 85). In mouse models, caspase-12 was proposed as a key mediator of ER stress-induced apoptosis. Caspase-12-knockout MEFs exhibited partial resistance, specifically against ER stressors. However, another study that used different caspase-12-knockout MEFs did not show any resistance to apoptosis (86). Procaspase-12 is localised on the cytosolic ER surface and is activated by ER stress *via* IRE1-TRAF2-dependent mechanisms. TRAF2 promotes procaspase-12 clustering at the ER membrane (52). The interaction between TRAF2 and procaspase-12 is inhibited by ER stress, and IRE1 overexpression. Therefore, caspase-12 activation might require for the dissociation of procaspase-12 from TRAF2, which is subsequently recruited to IRE1. Calpains, a family of calcium-dependent proteases, also play a role in caspase-12 activation, and calpain-deficient MEFs exhibit reduced ER stress-mediated caspase-12 activation and apoptosis (85). Therefore, it is plausible that a CHOP-ERO1α-IP3R-calcium-calpain pathway contributes to caspase-12 activation. Surprisingly, human caspase-12 has no similar function because its gene has been disrupted by a frame shift. Instead, human caspase-4 is specifically cleaved under ER stress, suggesting that it might be a functional mouse caspase-12 ortholog. Transmembrane protein 214 (TMEM214) mediates stress-induced apoptosis by acting as an anchor for the ER recruitment and subsequent activation of procaspase-4 (87).

Caspase-7, which also translocates from the cytosol to the cytosolic ER surface in response to ER stress, cleaves procaspase-12. A dominant-negative catalytic caspase-7 mutant was shown to inhibit caspase-12 activation. Caspase-7 is also a downstream executioner of caspase-12, a fact that suggests an amplification loop in the ER stress-induced apoptotic cascade (53).

Future Perspectives and Remaining Conundrums

Recently, ER stress research has received unprecedented attention. A basic PubMed search revealed that more than 830 ER stress investigational studies have so far been published in 2013. However, to integrate these links with hypoxia-inducible factor 1(HIF1)-VEGF or inflammatory pathways in a comprehensive network, the focus should be placed on elucidating the downstream mediators and crosstalk for all three UPR pathways. The observed paradox of the UPR in cancer (Figure 3) is likely due to functional redundancy and time-dependent outcomes of the UPR, although there are also some methodological issues.

ER stress-induced apoptosis is not completely suppressed when a single UPR effector is experimentally silenced. The fact that *CHOP* is a transcriptional target of both PERK and IRE, and even ATF6 provides an obvious link among all three branches. One caveat is that the IRE1 and ATF6 branches have weaker activities, compared to the PERK-CHOP branch, during prolonged ER stress (63). Most targets can be regulated separately by each pathway; moreover, each pathway possesses its own transcriptional activity for a certain target that determines its effect on cell fate. Some targets even require the concomitant activation of two pathways, for example, $p58^{IPK}$ requires ATF6/IRE1 cooperation (38). Additionally, a single downstream effector can exhibit different mechanisms of action. For example, the anti-apoptotic mechanisms of GRP78 include the prevention of UPR sensor activation, and the preservation of ER calcium homeostasis and its chaperone activity by limiting misfolded protein aggregation (1, 26).

The majority of studies measured the expression of only two or three ER stress markers such as GRP78 or CHOP; only a minority included target genes from each branch. Future therapeutic targeting of the UPR will likely affect one branch. The pleiotropic effects and acute toxicities of global UPR inducers, including the most commonly used thapsigargin (an ER calcium pump inhibitor) and tunicamycin (an N-linked glycosylation inhibitor), complicate studies that focus on an understanding of how the UPR remodels ER proteostasis in the absence of acute ER stress or how partitioning between ER client protein folding and trafficking *versus* degradation can be influenced by arm-selective UPR modulation. Moreover, UPR research that includes a variety of acute ER stress inducers introduces difficulties when comparing data from different studies. The recent development of targeted inhibition [*e.g.* PERK inhibitors (88)] or activation [*e.g.* PERK activators (89)] approaches, as opposed to the concomitant activation of all three branches, will lead to a new era in UPR research.

The duration and severity of ER stress determine the survival/apoptosis switch. The three branches provide opposing signals, and the timing of their induction shifts the balance between cytoprotection and apoptosis in response to unmitigated ER stress. For example, IRE1 signalling is an early event that is attenuated upon prolonged ER stress (90), and likewise, PERK induces its own de-activation *via* the up-regulation of GADD34 (Figure 2). Both pathways thus contain intrinsic 'timers' that likely contribute to cellular life-or-death decisions. Because *CHOP* mRNA and protein half-lives were found to be short, compared to those of pro-survival UPR outputs such as GRP78, sustained PERK activity (which is primarily responsible for *CHOP* up-

regulation) might therefore be necessary to accumulate CHOP levels sufficiently to stimulate the pro-apoptotic BCL-2 family proteins. Additionally, despite ATF4, CHOP, and GADD34 being able to restore protein synthesis, sustained PERK activity results in a protracted translational block that is incompatible with cell survival (38, 61). Similarly, sustained IRE1-mediated mRNA degradation might deplete ER cargo and protein-folding activities (40). Currently, it is unclear how tumour cells adapt to chronic ER stress *in vivo*. Although the UPR components are clearly activated in several types of tumours, the long-term evolution of this activity is unknown. Therefore, the use of experimental models with which to monitor temporal dynamics is required. For example, under hypoxia, there is a bi-phasic response to eIF2α phosphorylation. Phosphorylation is increased after 8 h but reduced after 24 h (possibly by PERK-ATF4-CHOP-GADD34) and is again enhanced after 48 h. Thus, following the initial attenuation in protein translation, there might be a transient period in which additional protein synthesis is permitted before a more permanent reduction occurs (15). Consequently, the effects of future drug interventions might be time-dependent, and whether cancer incidence might be reduced through the enhancement of protein-folding capacities during carcinogen exposure remains unknown. For example, the development of molecules that protect the liver by reducing alcohol-induced ER stress might dramatically reduce the incidence of HCC because chronic alcohol use increases HCC risk (91).

Deciphering this paradox could permit for the development of novel therapeutic modalities. In cancer, the UPR could be targeted to promote apoptosis by inhibiting UPR components and thus abrogating cellular adaptation (*e.g.* the use of versipelostatin, a repressor of GRP78 expression) or overloading the UPR (*e.g.* the use of proteasomal inhibitors). Overall, an ideal approach would integrate both targets without any toxicity.

In principle, UPR inhibitors should specifically target the tumour tissue. However, certain normal cell types place high demands on ER function, such as antibody-producing B-cells or insulin-secreting β-cells, and the potential toxicities against these cell types would require close monitoring during future drug discovery efforts (92). Notably, tissue-specific UPR patterns might help to differentiate target tissues. However, current molecular insights into the adaptation/apoptosis switch during ER stress are insufficient, and UPR drugs might block ER stress-mediated apoptosis and might unintentionally promote tumour progression. In general, protein kinases such as PERK represent favourable targets for the development of small-molecule inhibitors. However, as described above, PERK exhibits both pro- and anti-tumour properties. PERK-targeted therapies might facilitate the proliferation of dormant tumour cells or might drive cancer cells into dormancy, thereby protecting them from chemotherapy. Additionally, the inhibition of one branch might result in altered signalling through the other branches. Indeed, HEK293 cells that overexpressed a kinase-dead PERK mutant were found to exhibit increased XBP1 splicing and ATF6 activation in response to ER stress. Despite delayed dynamics, these cells still induced CHOP expression, which partially accounts for the increased susceptibility of these cells to ER stress-induced apoptosis (93).

In conclusion, the ability of the UPR to regulate cell fate has been highlighted as a primary pathophysiology research focus and represents a potential cancer therapeutic axis. However, its paradoxical effects on survival and proliferation of neoplastic and endothelial cells complicate the clinical applications of UPR modulators. This paradox is primarily due to our incomplete understanding of redundancy, the opposing effects of the separate outputs of each pathway, the interplay between the UPR and other pathways and the temporal UPR dynamics in cancer, as well as other confounding factors, including the absence of a standardized definition of ER stress, and a lack of branch-specific research.

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Received August 6, 2013
Revised September 27, 2013
Accepted September 30, 2013