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Review:

Molecular signal networks and regulating mechanisms of the unfolded protein response*

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Abstract: Within the cell, several mechanisms exist to maintain homeostasis of the endoplasmic reticulum (ER). One of the primary mechanisms is the unfolded protein response (UPR). In this review, we primarily focus on the latest signal webs and regulation mechanisms of the UPR. The relationships among ER stress, apoptosis, and cancer are also discussed. Under the normal state, binding immunoglobulin protein (BiP) interacts with the three sensors (protein kinase RNA-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1α (IRE1α)). Under ER stress, misfolded proteins interact with BiP, resulting in the release of BiP from the sensors. Subsequently, the three sensors dimerize and autophosphorylate to promote the signal cascades of ER stress. ER stress includes a series of positive and negative feedback signals, such as those regulating the stabilization of the sensors/BiP complex, activating and inactivating the sensors by autophosphorylation and dephosphorylation, activating specific transcription factors to enable selective transcription, and augmenting the ability to refold and export. Apart from the three basic pathways, vascular endothelial growth factor (VEGF)-VEGF receptor (VEGFR)-phospholipase C-γ (PLCγ)-mammalian target of rapamycin complex 1 (mTORC1) pathway, induced only in solid tumors, can also activate ATF6 and PERK signal cascades, and IRE1α also can be activated by activated RAC-alpha serine/threonine-protein kinase (AKT). A moderate UPR functions as a pro-survival signal to return the cell to its state of homeostasis. However, persistent ER stress will induce cells to undergo apoptosis in response to increasing reactive oxygen species (ROS), Ca^{2+} in the cytoplasmic matrix, and other apoptosis signal cascades, such as c-Jun N-terminal kinase (JNK), signal transducer and activator of transcription 3 (STAT3), and P38, when cellular damage exceeds the capacity of this adaptive response.

Key words: Unfolded protein response; Endoplasmic reticulum (ER) stress; Mechanism; Signal networks; Homeostasis
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1 Introduction

In human and animal cells, many stringent quality control systems are employed to ensure the normal metabolism of cells. The endoplasmic reticulum (ER) is responsible for the folding and maturation of newly synthesized transmembrane and secretory proteins in the Golgi compartment. Alterations in ER homeostasis cause accumulation of misfolded/unfolded proteins in the ER, which activates signaling

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pathways to orchestrate adaptive cellular responses. However, if homeostasis fails to be restored, the ER will initiate cell death signaling pathways, chronic inflammation, or tumorigenesis. The mechanisms that couple protein translation with protein folding in the ER have been reviewed (Kaufman, 2004) as well as the basic stress signal pathway of the ER (Shen *et al.*, 2004; Rutkowski and Kaufman, 2007). Some papers have discussed the function of unfolded protein response (UPR) signaling pathways in disease, and concluded that persistent ER stress, as well as inflammation, triggers the basic mechanisms involved in the development or pathology of metabolic disease, neurodegenerative disease, and inflammatory disease (Wang and Kaufman, 2012; Chaudhari *et al.*, 2014; Zhu *et al.*, 2014). Most studies have focused on the relation between UPR and cancer, and concluded that ER stress and UPR activation in both tumor cells and endothelial cells stimulate tumor angiogenesis, and the activity of different UPRs involved in tumorigenesis has been described (Nagelkerke *et al.*, 2014; Wang and Kaufman, 2014; Wang *et al.*, 2014; Yadav *et al.*, 2014). In this review, we primarily focus on our understanding of the network of ER stress signaling pathways and its regulatory mechanisms revealed in recent years. The latest signal webs of ER stress are summarized and their regulatory mechanisms discussed. These webs and mechanisms form the basis for a more thorough understanding and further investigation of the functions of the UPR, as well as its relationship with other cellular signaling cascades that could modulate its output.

2 Endogenous homeostasis and the surveillance mechanism

2.1 ER functions in protein production

The functions of the ER in protein processing are sensitive to environmental conditions and cellular changes. Protein production rates are primarily regulated by the mammalian target of rapamycin (mTOR) pathway and the UPR. As environmental conditions change, mTOR signaling coordinately regulates the rate of global translation initiation and ribosome biogenesis to maintain homeostasis. However, when misfolded or unfolded proteins accumulate in the ER, eukaryotic translation initiation factor 2 α (eIF2α), a downstream component of the UPR, is phosphorylated and inhibits protein expression (Kaufman, 2004; DuRose *et al.*, 2009). This is referred to as ER stress.

2.2 ER functions in protein modification and misfolded/unfolded protein degradation

In addition to protein processing, the ER functions as a sensitive surveillance system that attempts to monitor and prevent the accumulation of misfolded or unfolded proteins. This system prevents these misfolded/unfolded proteins from entering the secretory pathway and directs persistently misfolded proteins towards a degradative pathway (Cao *et al.*, 2013).

Translation of secretory proteins is initiated in the cytoplasm followed by translocation to the ER membrane via signal-recognition particle (SRP) receptor interactions. These nascent polypeptide chains enter the ER lumen with the help of the SEC61 translocon after their signal peptide is cleaved by a signal peptidase on the ER membrane. Once in the ER lumen, $(glucose)_3(mannose)_9$-*N*-$(acetylglucosamine)_2$ ($Glc_3Man_9GlcNAc_2$) is added to a Asn-X-Ser/Thr consensus sequence, where X is any amino acid except proline (Fig. 1: A1–A3). As part of the protein modification and folding process, the two terminal glucoses are cleaved by glucosidase I and glucosidase II, and then calnexin (CNX) and calreticulin (CRT) recognize and bind with glucose-(1,3)-mannose glycosidic bond, which is present on high mannose-containing asparagine-linked oligosaccharides. This promotes proper folding and disulfide bond formation with the help of protein disulfide isomerase (PDI) and other molecular chaperones (Shen *et al.*, 2004; Chaudhari *et al.*, 2014).

Proteins that are not properly assembled are subjected to reglycosylation by uridine diphosphate (UDP)-glucose: glycoprotein glucosyltransferase (UGT) and rebind CNX/CRT, until they attain the correct conformation for secretion (Fig. 1: A1-1–A1-3). The retained misfolded proteins are tagged by ER degradation-enhancing α-mannosidase-like protein (EDEM) for ER-associated degradation (ERAD) via the ubiquitin proteasome degradation system or autophagy (Chaudhari *et al.*, 2014) (Fig. 1: A1-4–A1-6). Correctly folded/assembled proteins are transported to the *cis*-Golgi compartment by coat protein complex II (COPII)-coated vesicles that form at ribosome-free

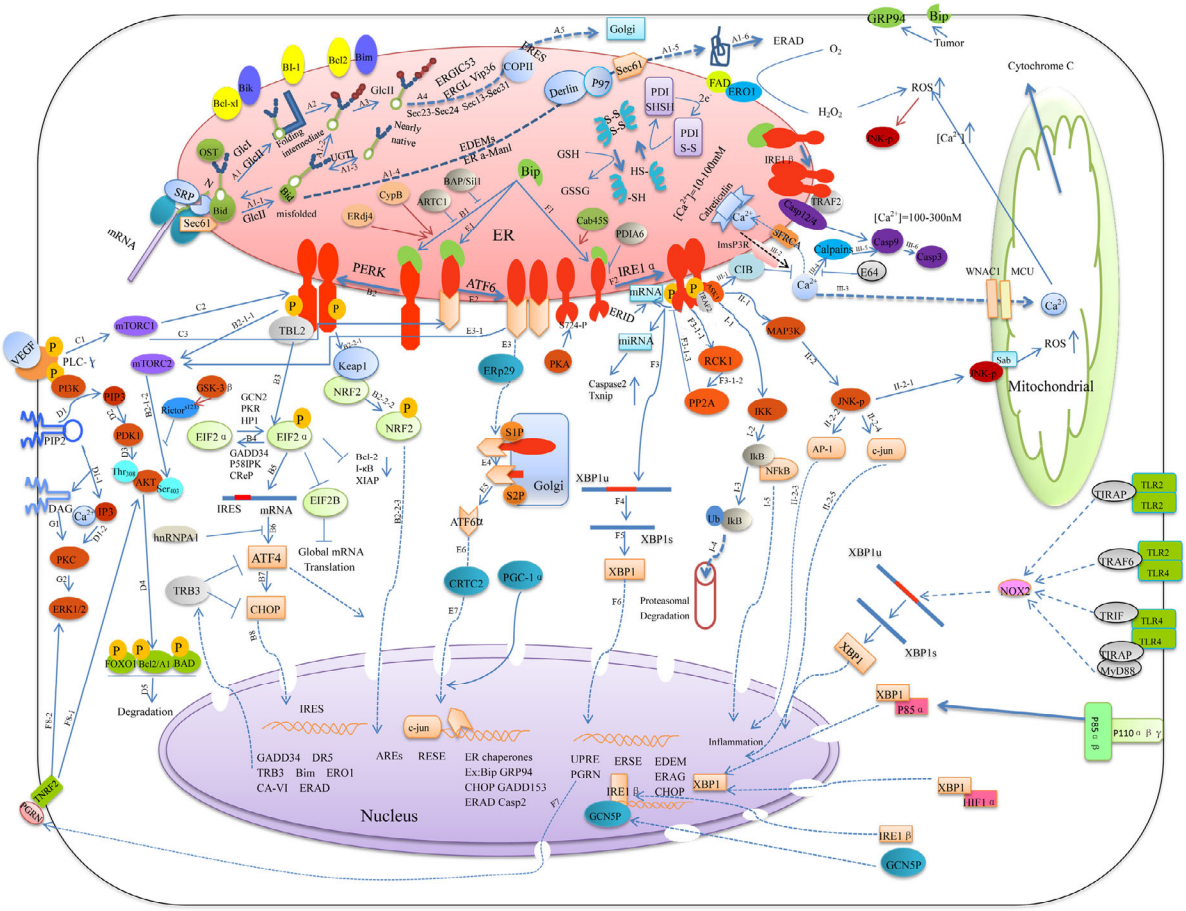

Fig. 1 Signal network and regulating mechanism of UPR

ER exit sites (ERESs) (Shen *et al.*, 2004; Braakman and Bulleid, 2011). COPII assembled at ERES is initiated by the activation of Sar1, a small GTPase, followed by the successive recruitment of the Sec23-Sec24 dimer and Sec13-Sec31 tetrameric complex to the scaffold protein Sec16. These vesicles undergo fusion with the ER membrane and then fission as a 60–80 nm diameter vesicle and bud off (Farhan *et al.*, 2008). These chaperones and oxidoreductases with Lys-Asp-Glu-Leu (KDEL) sequences in the budding of COPII vesicles are continually recycled back to the ER by interacting with the KDEL receptor in the ER membrane (Malhotra and Kaufman, 2007). Under the UPR, ER export ability is augmented by the merger, within minutes, of ERES and the formation of additional ERES after a period of hours to days (Farhan *et al.*, 2008).

However, in a solid tumor, chaperones and oxidoreductases, such as binding immunoglobulin protein (BiP), also known as 78 kDa glucose-regulated protein (GRP78), PDI, ER oxidoreductin 1α (ERO1α), and GRP94, are not partially recycled back to the ER, but instead are translocated to the plasma membrane. The localization of these chaperones and oxidoreductases in the plasma membrane can in some cases serve in tumor immunorecognition or promote tumor proliferation (Malhotra and Kaufman, 2007). For example, BiP/GRP78 overexpression has been found to correlate with both the rate of patient survival and the depth of tumor invasion (Yadav *et al.*, 2014).

2.3 ER functions in mRNA quality control and RIDD and rRNA synthesis

Another buffering system is nonsense-mediated mRNA decay (NMD) (Mühlemann and Lykke-Andersen, 2010). Nonsense mRNAs contain an intact open reading frame (ORF) with a premature stop codon that would generate a misfolded polypeptide. NMD is important in preventing the generation of misfolded proteins by capturing the nonsense mRNA and repressing initiation of its translation. It differs from ERAD which primarily functions in the degradation

of misfolded proteins. The mechanism of NMD includes the capture of substrate mRNA by a decay-inducing complex, its subsequent release from the ribosome and eukaryotic release factors (eRFs), nonsense mediated mRNA decay associated PI3K related kinase 1 (SMG1)-mediated RNA helicase and ATPase 1 (UPF1) phosphorylation and then SMG6-dependent dephosphorylation of UPF1 by protein phosphatase 2A. Finally, SMG6-mediated cleavage of mRNA occurs at a premature termination codon (PTC)-proximal site in the cytoplasm followed by exosomal degradation (Eberle *et al.*, 2009; Hwang *et al.*, 2010). Though ER stress does not affect UPF1 phosphorylation and NMD complex assembly, it increases exosome endoribonuclease and 3'-5' exoribonuclease 3 (DIS3) and exosome component 3 (EXOSC3), two exosomal subunits, and SMG6 expressions. Also, during ER stress, the regulated inositol-requiring enzyme 1 (IRE1)-dependent decay (RIDD) pathway promotes the degradation of ER membrane-localized mRNAs, which limits the synthesis of secretory proteins on the ER membrane (Sakaki *et al.*, 2012; Maurel *et al.*, 2014). Apart from inducing NMD and ERID for mRNA degradation, the UPR also down-regulates the synthesis of rRNA by inactivation of the RNA polymerase I basal transcription factor RNA polymerase I transcription factor (RRN3/TIF-IA) (DuRose *et al.*, 2009).

3 BiP/GRP78-dependent or -independent UPR activation mechanisms

3.1 BiP/GRP78-dependent UPR activation mechanisms

When ER homeostasis is perturbed by intraluminal calcium, improper glycosylation, nutrient deprivation, pathogen infection, accumulation of misfolded proteins, or changes in redox status, the UPR is activated in an attempt to maintain protein fidelity and minimize unfolded protein accumulation (Malhotra and Kaufman, 2007; Sakaki *et al.*, 2012). The UPR comprises three basic pathways, which are controlled by three respective ER sensors: protein kinase RNA-like ER kinase (PERK), activating transcription factor 6 (ATF6), and IRE1α. The three sensors form stable complexes with immunoglobulin proteins (BiP/GRP78) and prevent the UPR under normal cellular conditions. When misfolded proteins accumulate, the BiP/GRP78s are released from the three sensors. Consequently, the three sensors homodimerize and subsequently autophosphorylate and initiate UPR signaling cascades as a transcription factor (Nagasawa *et al.*, 2007).

There are three kinds of basic elements involved in the transcription of ER chaperone genes: one is the *cis*-acting ER stress response element (*ERSE*). *ERSE* contains a CCAAT-N9-CCACG sequence, which can be bound by ATF6α and X-box binding protein 1 (XBP1) via its CCACG conserved sequence. This is followed by the binding of the general transcription factor, nuclear factor Y (NF-Y), to the CCAAT part of the *ERSE*, leading to the activation of ER chaperone gene transcription (Yamamoto *et al.*, 2004). The second element is the UPR element (UPRE), which contains a TGACGTGG/A sequence and is preferentially bound by XBP1 compared with ATF6. Activation of transcription from this element does not require the assistance of NF-Y (Yamamoto *et al.*, 2004). The third element, *ERSE-II*, is very similar to *ERSE* except that it is separated by a single nucleotide spacer and the CCAAT and CCACG sites are in the opposite orientation, resulting in the sequence ATTGG-N-CCACG. ERSE-II binds ATF6 in an NF-Y-dependent fashion, while XBP1 is bound in an NF-Y-independent fashion (Yamamoto *et al.*, 2004). ERSE regulates the expressions of ER-localized molecular chaperones such as BiP in order to refold unfolded proteins in the ER. UPRE may primarily regulate the expressions of components of the ERAD system in order to degrade unfolded proteins in the ER. However, except for *Herp*, the target genes mediated by ERSE-II remain unclear (Yamamoto *et al.*, 2004; Renna *et al.*, 2007).

3.2 BiP/GRP78-dependent UPR regulation mechanisms

The interaction between BiP/GRP78 and the three UPR sensors is also regulated by several proteins. The first is ER-localized cyclophilin B (CypB), which interacts with BiP and stabilizes the BiP/UPR sensors complex via its peptidyl-prolyl *cis-trans* isomerase (PPIase) activity (Kim *et al.*, 2008). The second is ERdj4, which stabilizes the BiP/UPR sensor complex by inhibiting the connection between the nucleotide-binding domain (NBD) and the unfolded/misfolded protein substrate (Shen *et al.*, 2002; Awad *et al.*, 2008; Chen *et al.*, 2014a). Cab45S, the third

regulative protein, stabilizes BiP/IRE1 interactions, inhibiting ER stress-induced IRE1-c-Jun N-terminal kinase (JNK) signaling by specifically interacting with the NBD of BiP/GRP78 (Chen *et al.*, 2014a). Finally, BAP/Sil1 dissociates the BiP/three sensors complex by directly interacting with GRP78/BiP (Chung *et al.*, 2002; Chen *et al.*, 2014a). At the same time, the activity of GRP78/BiP is also regulated by mono-ADP-ribosylation by arginine-specific ecto-enzymes (ARTCs) on two arginine residues (R470 and R492) in UPR, which interferes with BiP to bind with the three sensors and augment the UPR signals (Fabrizio *et al.*, 2014). The expressions of all of these interacting proteins are induced through UPR signaling pathways to regulate cooperatively and balance protein processing with the demands of protein synthesis.

3.3 BiP/GRP78-independent UPR activation mechanisms

In addition to ER stress, ATF6 and PERK are also activated by vascular endothelial growth factor (VEGF)-VEGF receptor (VEGFR)-phospholipase C-γ (PLCγ)-mTOR complex 1 (mTORC1) signaling in tumors (Fig. 1: C1–C3). Solid tumors exist in hypoxic environments and rely on adaptive signaling pathways such as hypoxia-inducible factor 1α (HIF-1α), UPR, and macroautophagy to maintain proteostasis and energy balance. VEGF, induced by HIF-1α, XBP1, ATF6, and ATF4, is the most important proangiogenic driver secreted by an autocrine (tumor cell) and paracrine (endothelial cell) effect. VEGFR interacting with VEGF induces PLCγ activation and subsequent mTORC1 phosphorylation, which activates ATF6 and PERK, but not IRE1, as a result, further activating UPR and mTORC2 (Fig. 1: B2-1-1, E3-1). The dissociation of BiP from UPR sensors is not necessary for the activation of VEGF-VEGFR-PLCγ-mTORC1 pathway in tumors (Urra and Hetz, 2014). VEGF interacting with its receptor also induces phosphoinositide-dependent kinase 1 (PDK1)-dependent RAC-alpha serine/threonine-protein kinase (AKT) phosphorylation at Thr308 and mTORC2-dependent phosphorylation at Ser473, which functionally impacts endothelial cell survival and angiogenesis (Urra and Hetz, 2014). However, ER stress leads to Rictor phosphorylation at S1235 via glycogen synthase kinase 3β (GSK-3β), which interferes with AKT/mTORC2 binding and subsequent AKT Ser473 phosphorylation (Karali *et al.*, 2014) (Fig. 1: D1–D3).

Furthermore, in the absence of an ER stress response, glucagon markedly increases hepatic IRE1α phosphorylation (Ser724) via protein kinase A (PKA) activation leading to the full activation of AKT (Ser473) and other downstream effectors, such as forkhead box O1 (FOXO1), but not tuberous sclerosis 2 (TSC2) (Karali *et al.*, 2014).

4 PERK, ATF6, and IRE1α signaling and regulatory mechanisms

4.1 PERK signaling and regulatory mechanisms

PERK dimerization and *trans*-autophosphorylation lead to the activation of eIF2α by phosphorylation at Ser51 (first 3 h). As a result, the phosphorylated eIF2α (eIF2α(P)) prevents the recycling of the eIF2-bound guanosine diphosphate (GDP) to guanosine triphosphate (GTP) and the subsequent formation of the eIF2-GTP-tRNA-Met ternary complex (Sonenberg and Hinnebusch, 2009). eIF2α phosphorylation consequently turns off the synthesis of a large proportion of proteins, but selectively increases the expressions of other proteins that are involved in transcription regulation, oxidative stress, amino acid synthesis, protein folding, and differentiation apoptosis. These mRNAs have an internal ribosome entry site (IRES) sequence in the 5'-untranslated region (Muaddi *et al.*, 2010; Malhotra and Kaufman, 2011; Chaudhari *et al.*, 2014) (Fig. 1: B1–B5).

In addition to PERK, there are three other eIF2α kinases, including double-stranded RNA-activated protein kinase (PKR), general control non-derepressible kinase 2 (GCN2), and heme-regulated inhibitor kinase (HRI), that also phosphorylate eIF2α at Ser51. PKR is activated by ER stress thereby initiating inflammatory response signaling. Along with PERK, it cytoprotectively functions through the activating transcription factor 4 (ATF4)-C/EBP homologous protein (CHOP) pathway in response to a glucose deficiency (Fig. 1: B6–B8). HRI is activated by an iron or heme deficiency as well as oxidative stress. GCN2 is activated by uncharged tRNA caused by an amino acid deficiency and functions proapoptotically through the induction of expression of the X-linked inhibitor of apoptosis protein (XIAP) (Muaddi *et al.*, 2010). However, GADD34, P58IPK, and CReP can promote eIF2α dephosphorylation and function as a

negative feedback mechanism of eIF2α phosphorylation (Harding *et al.*, 2009).

The PERK signal cascade includes several primary protein components, such as eIF2α, transducin (β)-like 2 (TBL2), nuclear factor erythroid-derived 2-like 2 (Nrf2), ATF4, CHOP, and kelch-like ECH-associated protein 1 (KEAP1) (Fig. 1: B2-2-1–B2-2-3). TBL2 is an ER-localized type-I transmembrane protein that preferentially binds to *p*-PERK, but not GCN2 or IRE1. TBL2 serves as a potential regulator of the PERK pathway by interacting with *p*-PERK via its N-terminus proximal region and with eIF2α via its WD40 domain, which is necessary for phosphorylating eIF2α and ER stress-induced ATF4 expression (Tsukumo *et al.*, 2014). KEAP1 functions as a cytoskeletal anchor to retain Nrf2 in the cytosol of unstressed cells. When PERK is activated, Nrf2/KEAP1 complexes dissociate following Nrf2 phosphorylation by PERK, thereby allowing Nrf2 nuclear translocation and the expressions of antioxidant and detoxification enzymes (Cullinan *et al.*, 2003; Chaudhari *et al.*, 2014). Moreover, ATF4 and Nrf2 have been reported to dimerize and regulate oxidative stress responses (Nagelkerke *et al.*, 2014). Heterogeneous nuclear ribonucleoprotein (hnRNP) A1, induced by ER stress, attenuates IRES-mediated translation of anti-apoptotic mRNAs, including Bcl-2-like protein 1 (Bcl-xL) (Bevilacqua *et al.*, 2010). However, overexpressed Bcl-2-associated athanogene 5 (Bag5) in prostate cancer inhibits ER stress-induced apoptosis by decreasing CHOP and BCL2-associated X (BAX) while increasing Bcl-2 (Li *et al.*, 2014b).

CHOP consists of two functional domains, an N-terminal transcriptional activation domain and a C-terminal basic-leucine zipper (bZIP) domain composed of a basic amino acid-rich DNA-binding region followed by a leucine zipper dimerization motif (Li *et al.*, 2014b). CHOP induces the transcription of many genes, some of which are involved in negative feedback. For example, GADD34 functions in eIF2α dephosphorylation, tribbles homologue 3 (TRB3) represses ATF and CHOP transcriptional activity, histone deacetylase 4 (HDAC4) directly inhibits ATF4 transcriptional activity, and Atg5 is vital for autophagy (Kouroku *et al.*, 2007; Majumder *et al.*, 2012; Li *et al.*, 2014b; Zhang *et al.*, 2014). Some genes, such as stanniocalcin 2 (*STC2*), which mediates invasion, are up-regulated by hypoxia-inducible factor 1 (*HIF1*) which is a downstream gene of ATF4/CHOP in tumors (Nagelkerke *et al.*, 2014). Some of these genes are also involved in apoptosis and inflammation when ER stress and UPR signal activation are prolonged. For example, Bax-Bak mediates mitochondrial membrane permeabilization, the ERO1α-IP3R-calcium-calcium/calmodulin-dependent protein kinase II (CaMKII) pathway, caspase 3 pathway, and c-Jun pathway (Li *et al.*, 2014b; Nagelkerke *et al.*, 2014). Bim, essential for apoptotic induction, is usually phosphorylated by ERK/Rsk1/2, followed by ubiquitylation through the E3-ligase bTRCP. CHOP increases Bim transcription. At the same time, Bim is dephosphorylated by activated protein phosphatase 2A (PP2A), which inhibits Bim-ubiquitylation and proteosomal degradation during ER stress (Häcker, 2014). ER stress can also induce the expressions of interleukin 6 (IL-6) and several chemokines by the activation of the Janus kinase 1 (JAK1)/signal transducer and activator of transcription 3 (STAT3) axis (Meares *et al.*, 2014).

Apoptosis is further promoted by BCL-2 transcriptional suppression and the induction of expression of death receptor 5 (DR5) by eIF2α phosphorylation (Karali *et al.*, 2014). Furthermore, PERK down-regulates X-Linked inhibitor of apoptosis (XIAP) synthesis through p-eIF2α and promotes XIAP degradation through ATF4 (Hiramatsu *et al.*, 2014). eIF2α phosphorylation is both necessary and sufficient to activate NF-κB DNA binding and an NF-κB reporter gene. eIF2α phosphorylation-dependent NF-κB activation correlates with decreased levels of inhibitor of κBα (IκBα), an inhibitory protein, by repression and not by increased phosphorylation or decreased stability. This differs from canonical signaling pathways that promote IκBα phosphorylation and degradation (Deng *et al.*, 2004).

4.2 ATF6 signal cascade and regulatory mechanisms

ATF6 is a type II ER transmembrane protein that contains a bZIP domain in the cytosol and a stress-sensing domain in the ER lumen. During ER stress, ATF6 is released from BiP and translocates to the Golgi compartment where it is sequentially cleaved by site-1 and site-2 proteases (Fig. 1: E1–E4). This

causes the release of an N-terminal fragment that translocates to the nucleus as a transcription factor to increase XBP1-, BiP/GRP78-, CHOP-, PDI-, and ERAD-associated protein transcriptions (Zhang *et al.*, 2006; Nagelkerke *et al.*, 2014). The activation of ATF6α can also drive lipid biosynthesis and ER expansion (Bommiasamy *et al.*, 2009).

Endoplasmic reticulum protein 29 (ERp29), belonging to a redox-inactive PDI-Db-subfamily of PDI proteins, acts as an escort factor for ATF6 (Hirsch *et al.*, 2014). Various auxiliary proteins also play an important role in ATF6α nuclear translocation and its tissue specificity function. For example, ATF6α interacting with CRTC2 antagonizes the ability of cyclic adenosine 3,5-monophosphate response element-binding protein (CREB) to activate gluconeogenesis in the liver (Malhotra and Kaufman, 2011), and ATF6α associated with the transcriptional coactivator PGC-1α enables ATF6α to regulate several exercise-associated aspects of skeletal muscle function. PGC-1α is also necessary for efficient recovery from acute exercise (Wu *et al.*, 2011) (Fig. 1: E6, E7).

4.3 IRE1α signal cascade and regulatory mechanisms

4.3.1 Different activation mechanisms of IRE1α

The amino terminus of IRE1α resides in the ER lumen to sense the protein folding status, while the carboxyl terminus resides in the cytosol to initiate a unique signaling through its AKT and endoribonuclease (RNase) activities to induce an unconventional splicing reaction. IRE1α activation is mediated by oligomerization and *trans*-autophosphorylation leading to a conformational change that fully elicits its RNase activity (Poothong *et al.*, 2010). In addition to IRE1α being activated by disassociation from BiP during ER stress, hepatic IRE1α phosphorylation (Ser724) is markedly increased via PKA activation in the presence of glucagon (Mao and Shao, 2011). Furthermore, Toll-like receptor (TLR) 2/4 mediates IRE1/XBP1 activation through the adaptor proteins TIRAP and MyD88 (TLR2), MyD88 or TRIF (TLR4), and TRAF6 (TLR2/4). Intriguingly, this activation appears to depend on the activity of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase NOX2, thus providing further insight into the functional significance of XBP1 in innate immunity (Kaufman and Cao, 2010).

4.3.2 Synthesis and translocation mechanisms of XBP1

Activated IRE1α initiates the splicing of several kinds of mRNA. The unspliced mRNA (XBP1u) of the transcription factor XBP1 is a primary target, from which 26 nucleotides are excised to form the spliced XBP1 mRNA (XBP1s). The protein encoded by the XBP1u is rapidly degraded, while the protein XBP1 from the spliced variant XBP1s induces ER stress-related gene expression (Majumder *et al.*, 2012). XBP1, whose expression is induced by both XBP1 itself and activated ATF6, is an unstable protein with a half-life of 22 min (Majumder *et al.*, 2012) (Fig. 1: F1–F6). Also, phosphorylation of eIF2α and translational repression during early UPR augment the stabilization of the XBP1s (Majumder *et al.*, 2012). All of these increase the expression of XBP1 during UPR.

There have been few recent insights into the mechanisms of XBP1 nuclear translocation under traditional UPR. However, following growth factor stimulation, XBP1 nuclear translocation is facilitated by p85α, but not p85α-p85β heterodimers, with the increased translocation occurring independently of phosphoinositide-3 kinase (PI3K) catalytic activity (Kaufman and Cao, 2010). In addition to p85α, HIF1α increases XBP1 nuclear translocation by interacting with its N-terminal bZIP domain, leading to augmented VEGF-A, PDK1, glucose transporter type 1 (GLUT1), and DNA damage inducible transcript 4 (DDIT4) expressions under normoxic and hypoxic conditions (Chen *et al.*, 2014b).

4.3.3 Regulatory webs of the IRE1α/XBP1 signal cascade

As in the PERK pathways, several proteins function in limiting IRE1α/XBP1 signaling and maintaining it within a physiologically appropriate range. In the luminal domain of IRE1α, IRE1α activity is limited by the direct binding of PDIA6, a resident ER protein disulfide isomerase, at Cys148, which is oxidized when IRE1α is activated. Furthermore, in the cytoplasmic domains of IRE1α, IRE1α activity is limited cytoplasmically by interaction with BCL-2-related proteins and Bax inhibitor-1 (BI-1), BCL2 binding component 3 (PUMA), or Bcl-2-interacting mediator of cell death (BIM) (Eletto *et al.*, 2014). The RNase activity of IRE1α can be

modulated by unique amino acid sequences in subdomains V and VIA within the adenosine diphosphate (ADP)/adenosine triphosphate (ATP) binding domain (Poothong *et al.*, 2010). IRE1α activity can also be regulated by the scaffold protein receptor for activated C-kinase 1 (RCK1), which binds to IRE1α and PP2A and forms the glucose-inducible ternary IRE1α-RCK1-PP2A complex (Fig. 1: F3-1-1–F3-1-3). This association forms the ternary IRE1α-RCK1-PP2A complex, promotes the dephosphorylation of IRE1α by PP2A, and attenuates IRE1α-dependent increases in insulin production (Qiu *et al.*, 2010).

4.3.4 Downstream targets and their functions regulated by the IRE1α/XBP1 signal cascade

The primary function of IRE1α/XBP1 is to promote cell survival by up-regulating genes encoding proteins involved in protein folding, quality control, and ERAD by both transcription factor activity of XBP1 and RNase activity of IRE1α. The cleaved IRE1α also has been reported to function as a transcription factor in mammals when increased presenilin-1 under UPR controls IRE1α proteolysis (Niwa *et al.*, 1999). IRE1β can interact with the transcriptional coactivator, Gcn5p, and recruit a transcription coactivator complex to a specific chromosomal locus to mediate localized histone acetylation, thus making specific gene sequences accessible for transcription (Welihinda *et al.*, 1997).

The IRE1α/XBP1 pathway simultaneously induces the expression of all four mammalian translocon-associated protein (TRAP) complex subunits, which accelerates misfolded protein ERAD by distinguishing them from correctly folded proteins (Nagasawa *et al.*, 2007). Furthermore, the actions of IRE1α can vary depending on the tissue and cell type. In chondrocytes, IRE1α expression is induced by the granulin-epithelin precursor (GEP), a growth factor known to stimulate chondrogenesis. It inhibits GEP-mediated differentiation by reducing collagen II (ColII), sex determining region Y-box 9 (Sox9), collagen X (ColX), matrix metalloproteinase 13 (MMP-13), Indian hedgehog (IHH), and Runx2 expressions, while enhancing parathyroid hormone-related peptide (PTHrP) expression (Guo *et al.*, 2014a). IRE1α-XBP1-PDI signaling also regulates hepatic triglyceride export and the assembly of lipid-rich very low-density lipoprotein (VLDL) particles (Wang *et al.*, 2012). Moreover, in solid tumors, IRE1α up-regulates the expression of VEGF-A (Drogat *et al.*, 2007) and the expression of valosin containing protein (VCP). VCP is involved in protein folding, cell cycle control, and apoptosis as part of ERAD, and regulates ubiquitin-mediated degradation of misfolded proteins (Wang *et al.*, 2014). More significantly, the IRE1 target XBP1 acts as a negative regulator of apoptosis in osteoarthritis by affecting caspase 3, caspase 9, caspase 12, p-JNK1, and CHOP (Guo *et al.*, 2014b).

Apart from cleavage of XBP1 mRNA, some microRNAs (miRs) that repress translation are also cleaved by IRE1α and release translational blocks. For example, they are involved in regulating the expression of caspase 2 (Hassler *et al.*, 2012).

Furthermore, some genes are regulated synergistically by several pathways. For example, PERK signaling induces ATF5 to activate pro-oxidant thioredoxin-interacting protein (Txnip) transcription while IRE1α cleaves miR-17 to stabilize Txnip mRNA (Hassler *et al.*, 2012; Oslowski *et al.*, 2012). A specificity protein 1 (Sp1)-binding site within a GC-rich region of the cationic amino acid transporter-1 (*Cat-1*) gene controls its basal expression, while ATF4 and XBP1 are required for sustained transcriptional induction of the *Cat-1* gene during the UPR (Huang *et al.*, 2010). All these positive and negative feedback mechanisms provide survival signals and inhibit cell death.

4.3.5 Other signal pathways related to IRE1α and IRE1β

In addition to the traditional IRE1α/XBP1 signal cascade, several other cascades can be activated by IRE1α oligomerization, including: (1) ER stress-induced IRE1α phosphorylation leading to tumor necrosis factor receptor-associated factor 2 (TRAF2) and apoptosis signal-regulating kinase 1 (ASK1) recruitment to the cytosolic leaflet of the ER membrane. ASK1 then stimulates JNK phosphorylation, subsequently activating c-Jun and inducing apoptosis (Chen *et al.*, 2014a; Nagelkerke *et al.*, 2014) (Fig. 1: II-1, II-2, II-2-4). (2) Activated JNK interacts with Sab, a mitochondrial JNK binding protein, and consequently impairs respiration, which increases mitochondrial reactive oxygen species (ROS) culminating in apoptosis (Win *et al.*, 2014) (Fig. 1: II-2-1). (3) The activated JNK also phosphorylates the transcription

factor activator protein 1 (AP-1) and induces the expressions of inflammatory genes (Chaudhari *et al.*, 2014) (Fig. 1: II-2-2). (4) The TRAF2 associates with IκB kinase (IKK) and activates NF-κB by promoting degradation of IκBα, resulting in NF-κB nuclear translocation (Chaudhari *et al.*, 2014) (Fig. 1: I-1–I-5). (5) IRE1β-mediated ASK1/JNK activation leads to the cleavage and activation of procaspase 12 (in mouse) or procaspase 4 (in humans) to promote cell death (Fabrizio *et al.*, 2014; Wang *et al.*, 2014); furthermore, the IRE1α-TRAF2-ASK1 complex inhibits the Ca^{2+}-binding inositol 1,4,5-triphosphate receptor (InsP3R) from binding its inhibitor CIB1, thus inhibiting ER-to-cytosolic efflux of Ca^{2+}. The severe UPR induces the dissociation of ASK1 from the IRE1α-TRAF2-ASK1 complex and increases the assembly of ASK1/calcium and integrin binding 1 (CIB1) complex. CIB1 dissociates from CIB1/InsP3R and increases the cytosolic concentration of Ca^{2+}, which induces mitochondrial production of ROS and cell apoptosis (Son *et al.*, 2014) (Fig. 1: III-1–III-6). A region of mitochondria-associated membranes (MAMs) connects the ER membrane and mitochondrial membrane by some proteins interacting within the two organelle membranes. Interaction of the sigma-1 receptor with IRE1α and BiP allows the receptors to efficiently modulate cell survival signals from the ER to the mitochondria. The sigma-1 receptor interacts with and stabilizes IRE1α to enhance a prolonged activation of the IRE1α-XBP1 pathway, thus promoting cell survival. When ER calcium is depleted, BiP dissociates from the sigma-1 receptor, enabling it to stabilize InsP3Rs at the MAMs and facilitate calcium signaling to the mitochondria during ER stress conditions (Wang *et al.*, 2014). Progranulin (PGRN) induced by XBP1 binds directly to the 2nd and 3rd cysteine-rich domains (CRDs) in the extracellular portion of TNFR2 and activates ERK1/2 as well as the AKT signaling pathway (Li *et al.*, 2014a).

5 Concluding remarks

5.1 ER stress and apoptosis

Cellular homeostasis acts in opposition to both internal and external stimuli to maintain optimal metabolic and proliferative levels via a series of positive and negative feedback networks. Despite recent improvements in our knowledge of the UPR, many of its underlying mechanisms remain poorly understood. In addition to the conventional UPR activation mechanism involving BiP/ER stress sensors, PERK and ATF6 can also be activated by VEGF-PLCγ-mTORC1 pathway in tumors (Urra and Hetz, 2014), and IRE1α can be activated by activated AKT (Karali *et al.*, 2014). In normal cells, moderate ER stress, functioning in re-establishing cellular homeostasis, is strictly regulated and favors survival, while prolonged UPR activation induces apoptosis (Rutkowski *et al.*, 2006). CHOP increases folding ability as well as the burden of misfolding in the ER by re-establishing protein folding (Malhotra and Kaufman, 2011; Wang *et al.*, 2014). IRE1α also functions as a double-edged sword: on the one hand, protein synthesis is decreased by RIDD and successively protects cells by reducing the protein-folding burden, but on the other hand, the mRNAs that encode pro-survival proteins are also degraded (Hassler *et al.*, 2012).

Apoptosis induced by ER stress is achieved through the expressions of pro-apoptotic proteins (Hassler *et al.*, 2012; Chen *et al.*, 2014a; Lu *et al.*, 2014; Nagelkerke *et al.*, 2014; Wang *et al.*, 2014) and the stabilities of these proteins and their mRNAs, as previously discussed (Häcker, 2014). An additional apoptotic mechanism includes decreasing the translational efficiency of anti-apoptotic proteins, such as hypertonicity-induced eIF2α phosphorylation inducing cytoplasmic hnRNP A1 accumulation, which attenuates IRES-mediated translation of anti-apoptotic mRNAs, including Bcl-xL (Bevilacqua *et al.*, 2010). Another apoptotic mechanism involves increasing the expression of tumor necrosis factor α (TNF-α) via IRE1α- and NF-κB-dependent activation, which is mediated through TNF receptor 1/caspase 8 (Hu *et al.*, 2006). Lastly, apoptosis can occur by augmenting the expression or proteolytic cleavage of caspases. In force-induced apoptosis, PERK, not PKR, GCN2, eIF2α, CHOP, IRE1, or ATF6, activates caspase 3 in a caspase 9-dependent and mitochondria-independent fashion, implying a novel function of PERK that occurs in addition to its canonical UPR role (Mak *et al.*, 2008). Expressions of caspase 2 and subsequent Bid proteolytic cleavage increase, contributing to apoptotic cell death (Hassler *et al.*, 2012). Furthermore, IRE1-mediated ASK1/JNK activation leads to

the cleavage and activation of procaspase 12 (in mouse) or procaspase 4 (in humans) to promote cell death (Fabrizio *et al.*, 2014; Wang *et al.*, 2014).

A systemic inflammatory response is another important mechanism resulting in cell death apart from the activation of AP-1 and NF-κB, as described previously (Malhotra and Kaufman, 2011; Chaudhari *et al.*, 2014; Chen *et al.*, 2014a; Nagelkerke *et al.*, 2014). ER stress-induced activation of the JAK1/STAT3 axis leads to the expressions of IL-6 and several chemokines to drive inflammation (Meares *et al.*, 2014). Txnip, induced by ER stress through the PERK and IRE1 pathways, induces IL-1β mRNA transcription, activates IL-1β production by the NLRP3 inflammasome, and mediates ER stress-mediated β cell death (Hassler *et al.*, 2012; Oslowski *et al.*, 2012).

ROS and Ca^{2+} are two important apoptosis executors. ROS, harmful at high concentrations while beneficial at moderate/low concentration, increase in response to ER stresses (Chaudhari *et al.*, 2014). The formation of disulfide in the ER is believed to contribute to 25% of the ROS generated by the cell (Chaudhari *et al.*, 2014). Furthermore, mitochondrial ROS are increased by JNK/Sab complex activation when ER stress activates the JNK (Win *et al.*, 2014). CHOP induces endoplasmic reticulum oxidoreductase 1α (ERO1α), which hyperoxidizes the ER environment and further commits the cell to apoptosis (Wang *et al.*, 2014).

The ER, mitochondria and nucleus are the main intracellular Ca^{2+} stores, with the ER being able to store up to 10–100 mmol/L Ca^{2+} (versus 100–300 nmol/L in the cytoplasm). InsP3Rs and ryanodine receptors on the ER membrane facilitate the transfer of Ca^{2+} from the lumen of the ER to the cytoplasm, and Ca^{2+} is taken up again by the sarco-endoplasmic reticulum Ca^{2+}-ATPases (SERCAs) also located in the ER membrane. However, Ca^{2+} uptake into the mitochondria is controlled by the voltage-dependent anion channel (VDAC) residing at the outer mitochondrial membrane interface and the Ca^{2+} uniporter residing at the inner mitochondrial membrane interface, while mitochondrial Ca^{2+} is expelled by antiporters in an exchange process for either Na^+ or H^+. Thus, the antiporter and the exchanger maintain mitochondrial membrane potential and optimal Ca^{2+} concentrations in the mitochondria (Malhotra and Kaufman, 2011). Furthermore, InsP3Rs are primarily clustered in the MAM regions and serve as primary subcellular microdomains of Ca^{2+} transfer from the ER to the mitochondria (Son *et al.*, 2014). ER stress promotes the association of the CIB1/InsP3R complex, which contributes to the increase in cytosolic Ca^{2+} concentration (Hu *et al.*, 2006; Son *et al.*, 2014). Bak and Bax, which localize to both the ER and mitochondria, have been shown to be associated with ER calcium release concomitant with increased mitochondrial calcium (Malhotra and Kaufman, 2011). ER stress also up-regulates FBXO6, which inhibits cadmium-induced ER stress and JNK1 activation (Du *et al.*, 2014). Pathological conditions accompanied by a prolonged increase in calcium levels can overwhelm the calcium regulatory network, making it difficult for cells to recuperate. A disruption in Ca^{2+} homeostasis can trigger apoptosis through the activation of ER resident caspases or mitochondrial dysfunction due to Ca^{2+} overload (Son *et al.*, 2014).

5.2 Persistent ER stress and cancer

To re-establish cellular homeostasis, UPRs are activated at different levels. They also serve as apoptotic inducers that denote normal cells destined for death during persistent ER stress. However, the precise regulatory mechanisms remain unclear. Surprisingly, cancer cells have evolved ways to adapt to unfavorable microenvironments consisting of low pH, oxygen, glucose, or other nutrient levels, in which a persistent UPR is necessary for tumor survival. These cell fate differences between normal cells and cancer cells in response to a persistent UPR can be attributed to differing UPR molecular mechanisms in tumor versus normal cells. For example, in normal hypoxic/ischemic retinal ganglion neurons, activated IRE1α degrades the classical guidance cue netrin-1, subsequently hindering vascular regeneration (Binet *et al.*, 2013), while in paralleled tumors IRE1α induces HIF-1α expression, which induces VEGF expression and vascular regeneration (Urra and Hetz, 2014). Several reviews have concluded that ER stress and UPR activation stimulate tumor angiogenesis (Nagelkerke *et al.*, 2014; Wang and Kaufman, 2014; Wang *et al.*, 2014; Yadav *et al.*, 2014). The relation between tumorigenesis and persistent ER stress still remains unclear. However, it can be reasonably hypothesized that cells have a higher probability of cancerization due to abnormal activation of signal

cascades, mutations in vital signaling proteins, a persistent inflammation response, or altered gene expression under persistent ER stress. For instance, chaperones and oxidoreductases promote tumorigenesis when they are abnormally translocated on the plasma membrane during a persistent stress response (Malhotra and Kaufman, 2007). As a consequence of disrupted homeostasis, the probability of cancerization and disease is increased.

Compliance with ethics guidelines

Jing GONG, Xing-zhi WANG, Tao WANG, Jiao-jiao CHEN, Xiao-yuan XIE, Hui HU, Fang YU, Hui-lin LIU, Xing-yan JIANG, and Han-dong FAN declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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中文概要

题　目：内质网应激的信号通路以及调控机制

概　要：文章阐述了内质网应激信号通路及其调控机制；补充了最新的调控通路；探讨了内质网应激与肿瘤发生和细胞凋亡的关系。同时将内质网信号通路全面概括到一张图中，综合阐述了未折叠蛋白反应（UPR）的调控机制，并把内质网应激与细胞凋亡、肿瘤发生关联到一起，方便读者更好地了解与学习内质网应激。

关键词：未折叠蛋白；内质网应激；调控机制；信号通路；稳态