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REVIEW ARTICLE

# Negative feedback and modern anti-cancer strategies targeting the ER stress response

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**Endoplasmic reticulum (ER) stress is a cell state in which misfolded or unfolded proteins are aberrantly accumulated in the ER. ER stress induces an evolutionarily conserved adaptive response, named the ER stress response, that deploys a self-regulated machinery to maintain cellular proteostasis. However, compared to its well-established canonical activation mechanism, the negative feedback mechanisms regulating the ER stress response remain unclear and no accepted methods or markers have been established. Several studies have documented that both endogenous and exogenous insults can induce ER stress in cancer. Based on this evidence, small molecule inhibitors targeting ER stress response have been designed to kill cancer cells, with some of them showing excellent curative effects. Here, we review recent advances in our understanding of negative feedback of the ER stress response and compare the markers used to date. We also summarize therapeutic inhibitors targeting ER stress response and highlight the promises and challenges ahead.**

**Keywords:** anticancer strategy; ER stress response; ERAD; ER-phagy; feedback regulation; UPR

The endoplasmic reticulum (ER) is an essential protein folding organelle of eukaryotic cells and monitors approximately 40% of all cellular protein biosynthesis, folding and trafficking [1].

Endoplasmic reticulum stress is a state in which misfolded or unfolded proteins accumulate in the ER and can arise as a result of numerous types of internal or external insults; for example, mutant proteins, nutrient status and pathogen stimuli. An adaptive mechanism, the so-called ER stress response, becomes activated to counter this problem and to maintain protein homeostasis [2]. Because of increased protein synthesis rates, more gene mutations and the stressful microenvironment, it is not surprising that ER stress could be triggered in cancer cells. Indeed, numerous studies have demonstrated that ER stress is present in many types

**Abbreviations**
ASK1, apoptosis signal-regulating kinase-1; ATF4, activating transcription factor 4; ATF6α, activating transcription factor 6α; ATL3, atlastin-3; Bcl-2, B cell lymphoma 2; BiP, immunoglobulin heavy-chain binding protein; BTZ, bortezomib; CCPG1, cell cycle progression-1; CHOP, C/EBP homologous protein; csBiP, cells surface BiP; DUBs, deubiquitinases; EerI, eeyarestatin I; eIF2α, eukaryotic translation initiation factor 2α; EMT, epithelial-to-mesenchymal transition; ERAD, ER-associated protein degradation; ERLAD, ER-to-lysosome-associated degradation; eroGFP, ER-targeted redox-sensitive GFP; FAM134B, sequence similarity 134 member B; FGF, fibroblast growth factor; GADD34, DNA-damage-inducible protein 34; GPT, UDP-*N*-acetylglucosamine:dolichyl-phosphate *N*-acetylglucosamine-phosphotransferase; HSR, heat shock response; IL, imterleukin; IRE1α, inositol-requiring protein 1α; ISR, integrated stress response; JNK, Jun-N-terminal kinase; MM, multiple myeloma; MRH, mannose 6-phosphate receptor homology; PDI, protein disulfide isomerase; PDIA6, protein disulfide isomerase family A member 6; PERK, PRKR-like ER kinase; RIDD, regulated IRE1-dependent decay; RTN3, reticulon-3; UPR, unfolded protein response; VEGFA, vascular endothelial growth factor-A; XBP1, X-box binding protein 1.

of human cancers and have also shown that the ER stress response plays a vital role in the initiation and development of cancer [3].

In this review, we start with a concise exposition of mechanisms underlying the adaptive response upon ER stress. Next, we describe the feedback regulation of the ER stress response and then compare and contrast the ER stress response and heat shock response (HSR). Finally, the role of ER stress in cancer and the available anticancer therapeutics targeting ER stress are analyzed.

## Canonical mechanisms of unfolded protein response

### Three signal branches of the UPR

The UPR comprises two steps: first, misfolded or unfolded proteins compete and bind to chaperone immunoglobulin heavy-chain binding protein (BiP) from three UPR sensors [4]. Second, once activated, the sensors further activate downstream signaling pathways. The three UPR sensors and their corresponding downstream pathway are PRKR-like ER kinase (PERK)-eukaryotic translation initiation factor 2α (eIF2α) [5], inositol-requiring protein 1α (IRE1α)-X-box binding protein 1 (XBP1) [6,7] and activating transcription factor 6α (ATF6α) [8].

### IRE1α

IRE1α is the most evolutionarily conserved ER stress sensor among the three UPR signaling branches. It is a type I transmembrane protein, containing a luminal sensor domain, a cytosolic serine/threonine kinase and an RNase domain [9]. Under nonstress conditions, the luminal domain of IRE1α is associated with the ER chaperone BiP to prevent dimerization. Upon mild ER stress, unfolded and misfolded proteins compete for BiP from IRE1α. IRE1α homodimerizes, autophosphorylates and subsequently activates its endoribonuclease activities [10]. Activated IRE1α cleaves a 26-base intron from XBP1u (uncleaved form mRNA), generating a translational frame-shift mRNA, XBP1s (active form) [11]. XBP1 (transcription factor) enters the nucleus and regulates a set of UPR-related genes to enhance protein folding, secretion and ER-associated protein degradation (ERAD) to cope with the accumulation of misfolded protein in ER [12]. Additionally, XBP1 can inhibit C/EBP homologous protein (CHOP), thereby promoting cell survival [13]. On the other hand, under irremediable ER stress, unfolded proteins may bind to IRE1α to induce oligomer [14] and trigger a promiscuous cleave at an XBP1-like site of ER-related mRNAs, ribosomal RNA and miRNAs [15], known as regulated IRE1-dependent decay (RIDD), which can either preserve ER homeostasis or induce cell death [2].The cytoplasmic part of activated IRE1α also binds tumor necrosis factor receptor-associated factor 2, an adaptor protein, which activates apoptosis signal-regulating kinase 1 (ASK1) and JUN-N-terminal kinase (JNK), leading to ER stress-induced apoptosis by activation of BIM and inactivation of B cell lymphoma 2 (Bcl-2) [16].

### PERK

PERK is a type I transmembrane protein with a cytosolic serine/threonine kinase domain and it is the most rapidly activated pathway among the three branches [17]. Similar to IRE1α, PERK is homodimerizated and autophosphorylated when BiP is recruited to misfolded proteins, under conditions of acute ER stress [18]. Activated PERK is one of the integrated stress response (ISR) kinases that can suppress global protein synthesis by phosphorylation-mediated inactivation of eIF2α at Ser51 [19,20]. As a subunit in the eIF2–GTP–tRNAmet ternary, phosphorylation of eIF2α greatly inhibits the exchange of eIF2 bound GDP for GTP, which is essential for translation initiation [21]. As a result, global protein translation is suppressed and causes the cell cycle to arrest in the G1 phase [22]. This process helps ameliorate the ER protein burden and recover from ER stress.

Although shutting down global mRNA translation, p-eIF2α paradoxically increases the translation of activating transcription factor 4 (ATF4) because of its short inhibitory upstream open reading frames in 5'-UTR [23]. ATF4 enters the nucleus to activate cascades of gene expression constituting the ISR, which is responsible for remediating stress, restoring homeostasis and promoting cell survival [24]. ATF4 also stimulates the expression of CHOP (also known as DDIT3 and GADD153), which is responsible for the initiation of the apoptotic cascade [23]. Ample evidence has suggested expression of CHOP is strictly involved in cell apoptosis; however, ChIP-sequencing studies did not confirm that CHOP occupies pro-apoptotic family genes. Instead, ATF4 and CHOP formed heterodimers directly induce genes encoding protein synthesis, but not apoptosis. The increased protein synthesis causes ATP depletion and oxidative stress, which is an essential signal for cell death [25]. These results indicate that cell apoptosis induced by CHOP may result from increased protein synthesis and associated oxidative stress [26]. The heterodimer also upregulates the transcription of growth arrest and DNA-

damage-inducible protein 34 (GADD34), which promotes eIF2α dephosphorylation in a negative feedback manner and recovery from global mRNA translational inhibition [27].

During the early stages of ER stress, ATF4 induces miR-211 expression, which represses CHOP transcription by enhancing histone methylation at the CHOP promoter [25]. Moreover, mRNAs and proteins of CHOP are degraded rapidly because its short half-life results in their downregulated expression, allowing cells to adapt. However, upon intense and persistent stress, constitutive PERK-eIF2α activation increases CHOP stability and induces apoptosis [28]. Therefore, activation of the PERK–eIF2α pathway can promote both survival or apoptosis depending on the severity of the stress.

### ATF6

ATF6 is a leucine zipper-containing transcription factor and a type II transmembrane protein that has two homologues, ATF6α and ATF6β. Under basal conditions, ATF6α is retained on the ER membrane by interacting with BiP. Upon accumulation of misfolded protein in ER, ATF6α is released from BiP and transported to the Golgi apparatus, where it is cleaved sequentially by membrane bound transcription factor peptidase, site 1, and membrane bound transcription factor peptidase, site 2, to generate an active transcription factor (cleaved ATF6α) [29]. Cleaved ATF6α enters the nucleus to increase transcription of XBP1 and a set of genes that increase ER protein folding capacity and ERAD [30,31]. Unlike ATF6α, ATF6β may act as a translational repressor of ATF6α-mediated transcription and function [32].

## The UPR increases capacity for protein clearance

The activation of the UPR results in a transient attenuation of protein synthesis, as well as an increased capacity for protein folding. Moreover, the UPR also upregulates the activity of protein degradative pathways such as the ERAD and selective ER-phagy to decrease ER stress.

### Main components and processes of ERAD

Unlike the classical UPR pathway, novel components of the ERAD system are still being identified. The main known mammalian components include E2s (UBE2G2, UBE2J1, UBE2J2), E3s (HRD1, GP78, TRC8, TEB4, RNF5, RNF170, RNF103, RFP2, Fbx2, Fbx6, Parkin, CHIP, UBE4a, TMEM129 [33], RNF185 [34]) and deubiquitinases (DUBs) (YOD1, USP13, USP25, Ataxin-3, VCIP135, OTUB1) [35].

The ER does not contain degradative devices and misfolded proteins need to be dislocated across the ER membrane for degradation by the cytosolic 26S proteasome. The main processes of ERAD include substrate recognition, retrotranslocation, polyubiquitination, substrate extraction and proteasomal degradation [36].

#### *Substrate recognition*

Substrate recognition is a vital process in ERAD because inefficient recognition causes the accumulation of misfolded protein and overactive recognition may have costs. However, how the ERAD components recognize the misfolded protein is largely unclear. Many studies have focused on the recognition of luminal *N*-glycosylated ERAD substrates in yeast. A large number of ER proteins are modified at asparagine residues within the canonical *N*-glycosylation sites (NxS/T) with a well-defined, branched glycan chain Glc3Man9GlcNAc2 (Glc, glucose; Man, mannose; GlcNAc, *N*-acetylglucosamine) [37]. A prolonged ER residency, usually originating from the protein folding problem, results in the trimming of terminal mannoses by the mannosidase Htm1 (EDEM in mammals) and the formation of an α-1,6-linked mannose residue [38,39]. The mannose 6-phosphate receptor homology (MRH) domain of ER-resident lectins Yos9 (OS-9/XTP3-B in mammalian) binds to this biochemical mark [40] and directs the mannose-trimmed misfolded proteins to the groove of Hrd3 (SEL1L in mammals), which can bind the misfolded polypeptide segment around the glycan [41,42]. Because the affinity between substrates and Hrd3 or MRH domain is low, the chaperone Kar2 (BiP in mammals) may help in this process [43]. The mechanisms of nonglycosylated protein recognition are less clear. A study focusing on the degradation of three ERAD nonglycosylated proteins suggested that glycosylated and nonglycosylated proteins shared a similar recognition complex comprising of calnexin, EDEM1, OS-9, XTP3-B and HRD1 [44]. However, in another study, nonglycoproteins were found to be captured by BiP and then transferred to the ER disulfide reductase ERdj5 for reduction of disulfide bonds without going through the calnexin/EDEM1 pathway [45].

#### *Retrotranslocation and polyubiquitination*

According to the location of the misfolded protein domain (the ER lumen, the ER membrane, or the cytosolic side of the ER membrane), misfolded ER proteins can be retrotranslocated in three pathways

(ERAD-L, ERAD-M and ERAD-C) even though ERAD-L and ERAD-M share the similar components [46]. After being selected, ERAD-L substrates are retrotranslocated across the ER membrane back into the cytoplasm by the complex consisted of the RING-finger ligase Hrd1p and other cofactors (Hrd3, Usa1 and Der1) [47]. Hrd1p allows the misfolded luminal domain of a substrate to move across the membrane by autoubiquitination on several lysines within its RING-finger domain [48]. Hrd1p contains eight transmembrane segments and can only form a 'half-channel'. The whole channel across the membrane comprises two 'half-channels' corresponding to the cavities of Hrd1p and the rhomboid-like Der1 protein, linked by Usa1. The two proteins make the membrane between them much thinner by distorting it, which weakens the energy barrier to accommodate a polypeptide chain. The ERAD-L substrate inserts into the retrotranslocon as a hairpin, one side interacting with Der1 and the other with Hrd1p. Then, polyubiquitination occurs at a suitable lysine residue that emerges in the cytosol, which could prevent backsliding of the polypeptide into the ER lumen [42]. Hrd1p overexpression can mediate the retrotranslocation in the absence of its cofactors Hrd3, Usa1 and Der1 [49], even though the selectivity for misfolded proteins is lost [47]. This indicates Hrd1 plays a central role in this retrotranslocation. This comprises the most conserved ERAD mechanism in mammals. ERAD-M substrates also use Hrd1p and Hrd3, but this is independent of Usa1 and Der1. ERAD-C substrates are directly targeted to the Doa10 [46]. Doa10 also recognizes some substrates with the misfolded domain within their membrane-spanning region [50]. Recently, the fourth ERAD pathway to dispose of inner nuclear membrane proteins has been identified. It relies on an Asi RING-finger ligase complex that comprises three integral membrane proteins: Asi1, Asi2 and Asi3 [51,52].

### *Substrate extraction and degradation*

Almost all ERAD polyubiquitinated substrates are pulled out of the ER membrane by a common machine, the ubiquitin-interacting p97 ATPase complex [53]. It consists of an AAA + ATPase Cdc48 (called p97 or VCP in mammals) hexamer that forms a double-ring construction and associates with two cofactors, Ufd1/Npl4, which can bind the polyubiquitin chain of the substrate [54]. Some nonubiquitinated substrates such as cholera toxin can be translocated into the cytosol in a p97 independent manner, which is consistent with the requirement for polyubiquitination in p97 function [55]. The misfolded protein is pulled through the central pore of the double-ring ATPase, causing its unfolding by hydrolyzing ATP. When the substrate is completely translocated, the release from the p97 ATPase complex requires a DUB, Otu1, because the Ufd1/Npl4 is still associated with the polyubiquitin chain. A minority of substrates lose all ubiquitins, whereas most of them retain an oligoubiquitins chain [56]. How the trimmed substrates from Cdc48 are transferred to the proteasome is still unclear. They are probably transferred directly to the proteasome through the oligoubiquitin chain or the polyubiquitin chain extended by Ufd2 [57]. The unfolded substrates may be kept soluble by cytosolic chaperones such as the BAG6 [58] complex and transferred to the proteasome by the shuttling factors Rad23 and Dsk2 because they have both ubiquitin- and proteasome-binding domains [59]. Finally, the ERAD substrates are degraded by the proteasome.

## Aggregated proteins and ER components are cleared by selective ER-phagy

Autophagy, a self-degradative process, is another adaptive mechanism upon ER stress. Activation of ER stress and autophagy was demonstrated by detecting the protein markers and it was found that inhibited ER stress could reduce the level of autophagy [60]. Autophagy triggered by ER stress mainly includes the ER stress-mediated autophagy, selective ER-phagy and ER-to-lysosome-associated degradation (ERLAD).

The ER stress-mediated autophagy is characterized by the generation of autophagosomes that include aggregated proteins, worn-out proteins and damaged subcellular organelles under ER stress conditions. $Ca^{2+}$ and the three UPR branches are necessary for the activation of ER stress-mediated autophagy [60,61]. ER contains a high concentration of $Ca^{2+}$. The escalation of intracellular $Ca^{2+}$ release into the cytoplasm from ER upon ER stress stimulates a CamKK/AMPK dependent pathway that relieves mTOR inhibition on the ULK1 complex and induces autophagy [62]. $Ca^{2+}$ also activates death-associated protein kinase, which phosphorylates Beclin1 on T119 and promotes Beclin1 dissociating from Bcl-2, inducing autophagy [63]. As mentioned above, p-IRE1α could activate JNK, and JNK1, but not JNK2, mediates Bcl-2 phosphorylation. Phosphorylation at T69, S70 and S87 promotes the disruption of the Beclin-1/Bcl-2 complex and autophagy activation [64]. XBP1s, another product from p-IRE1α, triggers autophagy through transcriptional activation of Beclin-1 [65]. Additionally, the PERK/eIF2α pathway is essential for stress-induced autophagy because overexpression of ATF4 results from eIF2α phosphorylation,

and the ATF4-mediated CHOP activation was shown to transcriptionally induces a set of autophagy-related genes such as LC3, ATG5, ATG3, ATG7, ATG10, beclin1 and p62 [66]. Furthermore, the ATF6 pathway indirectly regulates autophagy via the transcriptional activation of CHOP [67].

The ER-phagy might be a new branch of ER stress-mediated autophagy, in which ER fragments are sequestered within double membrane autophagosomes and transported to the lysosomes for degradation with the assistance of ER-phagy receptors. ER-phagy receptors are the ER-resident Atg8-binding proteins that decorate specific ER subdomains for capture by autophagosomes [68]. To date, six mammalian ER-phagy receptors have been identified: family with sequence similarity 134 member B (FAM134B) [69], reticulon-3 (RTN3) [70], Sec62 [71], cell cycle progression-1 (CCPG1) [72], atlastin-3 (ATL3) [73] and TEX264 [74]. FAM134B is an ER membrane protein that is mainly located at the edges of the ER sheets and is the first of the ER-phagy receptors to be described and characterized. Downregulation of FAM134B protein causes an expansion of the ER, whereas FAM134B overexpression results in ER fragmentation [69]. RTN3 and ATL3 are located at ER tubules and are involved in the turnover of ER subdomains on nutrient deprivation [70,73]. Recently, TEX264 was identified as a single-pass transmembrane protein involved in starvation-induced ER-phagy [74]. CCPG1 is a type II, single-pass transmembrane protein. By contrast to FAM134B and RTN3, CCPG1 carries a luminal domain, which recognizes and binds to lumenal protein aggregates and sequesters them into autophagosomes. CCPG1 will respond to an overloaded ER with misfolded or aggregated proteins [72]. Sec62 mainly plays a role in RecovER-phagy, which is discussed below.

ERLAD was specially defined in 2018 to distinguish it from ER-phagy, even though the phenomenon that nonautophagic clearance from the ER of aggregated proteins by direct conversion of ER into lysosomes was discovered several decades ago [75]. In ERLAD, ER-derived vesicles containing material to be removed from cells are not captured by autophagosomes, but can fuse with lysosomes [76].

## Negative feedback loop in the ER stress response

### Negative feedback regulation of the HSR in prokaryotes

Negative feedback regulation in the stress response is highly conserved with respect to the evolution of eukaryotes and prokaryotes. HSR plays a major role in sustaining protein homeostasis in prokaryotes. When *Escherichia coli* cells are suddenly exposed to high temperature, transcription factor σ32 is rapidly translated by rpoH mRNA for which the 5' region is required for thermal regulation, repression at low temperature or nonstress conditions [77]. σ32 rapidly increases the synthesis of chaperone HSP (e.g. DnaKJ) and protease (e.g. FtsH) to refold or degrade the denaturation proteins resulting from the high temperature. As a manner of feedback regulation, free chaperones can directly bind and inactivate σ32 [78], which is extremely unstable and rapidly degraded by the membrane-localized FtsH protease in a chaperone-dependent manner. Signal recognition particle-dependent targeting of σ32 to the membrane is an essential step in this progress [79,80].

The HSR in bacteria may be evolutionarily related to an ER stress response such as the UPR in eukaryotic organisms [80]. The breakthrough in prokaryotes research may provide inspiration for UPR investigations. The similarities between the HSR in bacteria and the UPR in eukaryotic organisms are compared in Table 1.

Actually, ER stress response should be a fine-tuning regulation machinery like the HSR in prokaryotes. The overactivated or defective responses that do not match the stress level will harm cells. Excess UPR products also need to be disposed during stress recovery (Fig. 1). By contrast to the classical UPR pathways that have been constantly proposed, the feedback regulation of the ER stress response largely remains mysterious.

### BiP and its J-domain co-chaperones (ERdj4) restrain excessive IRE1α activity

BiP and ERdj4 are key regulators for restraining the UPR in the feedback regulation machinery. ERdj4 has two functional domains: the targeting domain and the J-domain. The targeting domain can associate with the IRE1α luminal domain and recruit BiP. The J-domain stimulates the ATPase activity of BiP. Thus, it promotes BiP binding to IRE1α. Subsequently, ERdj4 is ejected and a repressive BiP-IRE1α complex is formed. During ER stress, accumulated unfolded proteins compete for BiP and ERdj4, and IRE1α dimerizes and activates downstream pathways as described above. Once the stress is relieved or as a result of UPR activation, redundant ERdj4 and BiP will disrupt the active IRE1α luminal domain dimmer, forcibly monomerize IRE1α and repress the UPR [81]. Previous studies have reported that XBP1s, the product of IRE1α activation, induces the expression of ERdj4

**Table 1.** Similarities between the HSR in prokaryotes and the ER stress response in eukaryotes.

| | HSR | ER stress response |
|---|---|---|
| Inducer | Heat | Misfolded protein |
| Sensors | 5′ region of rpoH mRNA | PERK, IRE1α, ATF6 |
| Transcript factors | σ32 | ATF4, XBP1, cleaved ATF6 |
| Consequences | | |
| | • Chaperones: DnaK, GroEL, GroES | • Attenuate global protein translation: p-eIF2α<br>• Increase protein folding capability: chaperones |
| | • Proteases: HslVU, FtsH | • Promote protein degradation: ERAD, ERLAD<br>• Induce apoptosis |
| Feedback regulation | | |
| | • Chaperones inhibit σ32 activity | • Chaperones inhibit IRE1α activity<br>• GADD34 promotes eIF2α dephosphorylation<br>• XBP1u degrades ATF6 and XBP1s |
| | • Proteases degrade σ32 | • HRD1-SEL1L complex degrades IRE1α<br>• RecovER-phagy clears excess ER components |

and BiP [82]. As a result, the 'ERdj4-BiP-IRE1α' axis may represent a form of negative feedback for fine-tuning the IRE1α pathway.

## Protein disulfide isomerase (PDI) limits excessive UPR signaling

PDI is a multifunctional protein catalyzes the formation, breakage and rearrangement of disulfide bonds [83] or functions as a chaperone at high concentrations [84]. Recently, S357 of PDI was found to be phosphorylated by Fam20C and induce an open conformation of PDI that can bind to the lumenal domain of IRE1α and attenuate excessive IRE1α activity [85].

Protein disulfide isomerase family A member 6 (PDIA6), a resident ER protein, attenuates the IRE1α signaling by directly binding to C148 in the IRE1α luminal domain, which is oxidized when IRE1α is activated [86]. However, PDIA6 was demonstrated to interact with IRE1α and enhance IRE1α activity in another study [87]. In addition to IRE1α, PDIA6 was also found to interact with PERK and inhibit its signaling [86]. By contrast to PERK and IRE1, PDIA6 cannot affect ATF6 signaling. However, another two protein disulfide isomerases, PDIA5 and ERp18, were found to monitor ATF6 ER quality control [88,89]. Moreover, the production of XBP1u, a 261aa protein, functions as a negative regulator of ATF6 and XBP1s by direct association and promotes rapid degradation of the complex by the proteasome [90]. This evidence provides a mechanism for maintaining UPR activation within a physiologically appropriate range.

## IRE1α is an endogenous substrate of ERAD

ERAD was described and named in 1996 [91] and it has long been thought to be an integral part of the UPR, as previously discussed. However, a recent study revealed that the UPR sensor could be degraded by ERAD to downregulate the ER stress response. In a quantitative liquid chomatography-tandem mass spectrometry analysis of purified microsomal/ER fractions from SEL1L induced knockout and wild-type mouse embryonic fibroblasts, IRE1α was found to be an endogenous substrate for the SEL1L-HRD1 ERAD complex without a significant change in the mRNA abundance of IRE1α [92].

First, as a membrane protein, the recognition of IRE1α for ERAD may depend on BiP and OS-9, as well as three intramembrane hydrophilic residues of IRE1α. Under basal conditions, IRE1α is sequestered by redundant BiP, selected and transferred to the SEL1L-HRD1 complex and then degraded. BiP can not only act as an IRE1α blocker, but also an IRE1α-ERAD trigger. Under ER stress, BiP is competed by misfolded proteins leading to IRE1α dimer-/oligomerization, which is resistant to the SEL1L-HRD1 complex and activation, as discussed above. BiP may play a key role in the inactivation of IRE1α. IRE1α dissociates from ERAD and is stable when BiP is depleted and degrades faster when BiP is overexpressed. As further evidence, D123P mutant IRE1α has a shorter half-life than wild-type IRE1α because mutant IRE1α associates with BiP constitutively even under stress conditions [92]. Thus, this unique feedback mechanism

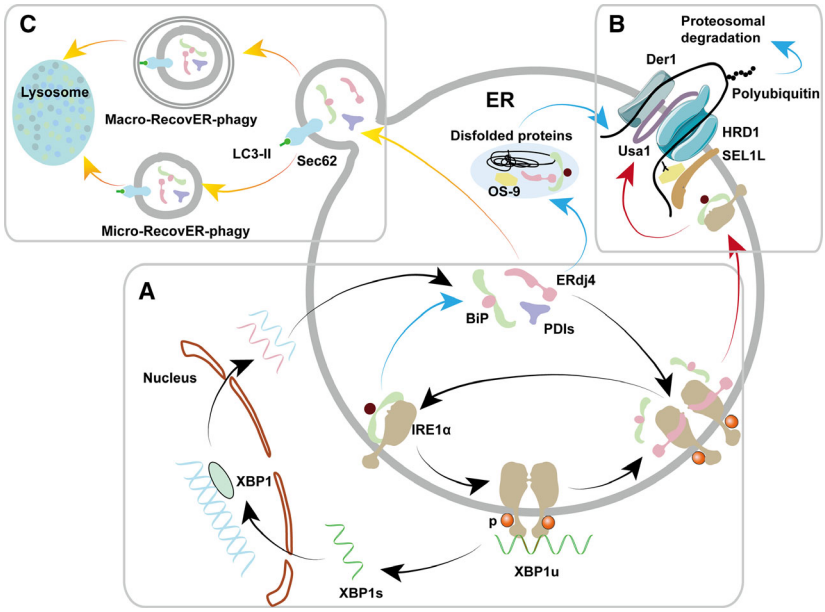


**Fig. 1.** Feedback regulation of the ER stress response. (A) Upon ER stress, misfolded proteins compete for and recruite BiP from monomeric IRE1α. Then, IRE1α homodimerizes, autophosphorylates and subsequently induces activation of transcription factor XBP1, which regulates a set of protein folding genes such as BiP and ERdj4. Although ER stress is ameliorated, redundant ERdj4 and BiP forcibly disrupt the IRE1α dimer into a monomer to repress the UPR. PDIs, such as p-PDI and PDIA6, can also bind to IRE1α and limit excessive IRE1α activity. (B) Under basal conditions, monomeric IRE1α can be transferred to the SEL1L-HRD1 complex, where it is degraded with the assistance of OS-9 and BiP. This is a bridge between the UPR and ERAD. (C) When cells recover from ER stress, excess ER subdomains decorated with SEC62 containing molecular chaperones and folding enzymes will be delivered to the lysosome for clearance. SEC62 serves as an LC3-II binding protein. Chaperone BiP plays a central role in ameliorating ER stress and negative feedback of the ER stress response (p, phosphorylation; XBP1s, transcriptionally active XBP1; XBP1u, unspliced XBP1).

between the UPR and ERAD fills an important gap in the ingenious self-regulatory mechanism of the ER stress response.

### RecovER-phagy help ER return to prestress condition

Another feedback mechanism in the ER stress response is RecovER-phagy [71], which is triggered, or substantially enhanced, when recovery from ER stress takes place. Upon resolution of ER stress, excess ER components must be removed so that both the volume and the content of the ER can return to prestress conditions. This process is assisted by selective autophagic programs. Excess ER subdomains decorated with Sec62 containing molecular chaperones and folding enzymes, although excluding ERAD factors, will be delivered to the autolysosomal system for clearance. Sec62 is a channel protein of the translocon complex, being responsible for importing proteins into the ER and acting as an ER-resident autophagy receptor. Sec62 contains a conserved LC3-II-interacting region in the C-terminal cytosolic domain, promoting the delivery of select ER domains to autolysosomes for clearance [71].

In sum, ER stress response is a conserved self-regulatory process that can maintain homeostasis via a multiple feedback loop. However, some aspects of the process have not be clarified; for example, how ERAD components are downregulated when the stress is relieved and whether the ATF6 pathway also has a feedback loop. Accordingly, additional studies are worthwhile, which may use findings in HSR in prokaryotes for reference.

## The adaptive ER stress response in human cancers

### UPR signal proteins are used as ER stress markers

To confirm whether ER stress exists in cancer cells, markers of ER stress should first be determined. This is an equivocal area because ER stress is characterized by misfolded or unfolded proteins in the ER, and any

direct measurement remains challenging even though two methods attempt to bypass this difficulty.

The ER lumen is remarkably enlarged, as can be observed by electron microscopy during ER stress. This method has been used to monitor ER stress in pancreatic β cells [93]. Another method is the use of fluorescent protein reporter ER-targeted redox-sensitive GFP (eroGFP) to dynamically measure ER redox status [94]. The ER should maintain an oxidizing environment because oxygen is essential for disulfide bond formation during post-translational protein folding, as discussed above [95]. As a result, redox changes should accompany the unfolded protein concentration. Under oxidizing conditions, eroGFP becomes disulfide-linked because it has an engineered cysteine pair on adjacent surface-exposed β-strands, with increased excitation from 400 nm and decreased excitation from 490 nm. The ratio of the fluorescence measured from these two wavelengths reports the ER redox status in ER and represents the unfolded protein concentration.

Such methods are direct and powerful, although they are inconvenient with respect to use in complex research such as clinical studies. Indded, the cells are described as experiencing 'ER stress' if the downstream UPR components are positively detected. The UPR is an ingenious self-regulatory mechanism that makes it highly coordinated to ER stress levels in cells, as discussed above. Activation of UPR signal proteins may represent ER stress levels in cells. Positive detection of partial components of the UPR has been used as a marker for ER stress, including under physiological or pathological conditions [96,97]. Nevertheless, the UPR is a complex and dynamic process and the idea that any UPR protein can be used as a golden marker remains uncertain.

A review comprising 17 primary studies summarized ER stress markers in mammalian oocytes and pre-implantation embryos, identifying BiP as the most popular (11/17) marker for indicating ER stress. Other markers, such as ATF4, ATF6, XBP1, CHOP, PERK, IRE1α, ASK1 and GRP94, are also used in related studies [98]. Another review analyzed 14 types of human cancer, revealing that almost all studies (23/27) take BiP as evidence for ER stress and approximately half of them (11/27) take BiP as the only marker to indicate ER stress. Other molecules are used, such as CHOP, GRP94, XBP1, GRP75, HSP60, calreticulin, HSP90, ATF6, HSP27, HSP70, IRE1α and GADD34 [3]. BiP is not only a central regulator of the three ER stress sensors, but also the common consequence of the three parallel signaling branches of the UPR with respect to increasing protein folding capability. However, whether BiP is suitable as a golden marker to diagnose ER stress in cells still requires verification. Additionally, there is a fatal logical fallacy if UPR proteins are used as ER stress predictor. Cells lacking UPR components are likely to be more stressed because they are unable to evoke an effective adaptive response to cope with the accumulated misfolded protein. This indicates that the development of a direct and convenient method or marker would be worthwhile.

## Numerous exogenous and endogenous stimuli make it presumable that higher ER stress exists in cancer cells

Internal insults such as aberrant proteins resulting from gene mutations and high rates of protein translation may induce ER stress in cancer cells. Human cancer cells possess dozens of nonsynonymous mutations, on average 100–120 mutations [99], with certain cancers such as melanoma and lung cancers harboring more than 200 mutations [100]. It is well-known that increased protein synthesis and translocation into the ER, as a result of hyperactivation of proto oncogenes or loss-of-function mutations in a tumor suppressor, is one of the hallmarks of cancer. This comprises a source of excess misfolded or unfolded protein, which could overwhelm ER protein folding capacity and induce ER stress.

Cancer cells usually have more stringent microenvironment conditions, such as nutrient deprivation (hypoxia or glucose deprivation), pathogens or inflammatory stimuli, which may perturb protein folding and trigger ER stress. The rapid expansion of the tumor mass and less efficient vasculature lead to the generation of hypoxia and a lack of glucose regions in solid tumors. Disulfide bond formation is a main post-translational modification during protein synthesis in the ER. It is evident that, during post-translational protein folding or isomerization, oxygen is essential for disulfide bond formation, even though it is formed rapidly during protein synthesis without oxygen [95]. Glucose metabolism supplies tumor cells with ATP and functions as a donor for glycosylation. Glucose deprivation can lead to the efflux of $Ca^{2+}$ from the ER because of inhibition of the sarcoplasmic/ER $Ca^{2+}$-ATPase pump [101] and improper protein glycosylation of misfolded proteins. As a result, cancer cells are presumed to harbor a higher ER stress level.

The ER stress adaptive response has been documented in many types of human cancer. Understandably, cancer cells are highly secretory and prone to constitutive UPR activation. Some types of cancer, including multiple myeloma (MM), can express high

levels of immunoglobulins. Additionally, many solid cancers, such as pancreatic, lung, breast, ovarian and colon cancers, also show evidence of increased expression of mucin [102,103]. However, ER stress responses have also been documented in other types of cancer, such as skin, prostate, brain, gastric, liver and kidney carcinoma [3]. This indicates that ER stress responses may play an essential role in cancer at multiple stages.

## Role of the ER stress adaptive response in cancer cells

The ER stress adaptive response is an indispensable process for cancer development and cancer cells facilitate multiple mechanisms to achieve this goal, including activating the pro-survival branch of the UPR during neoplastic transformation, bolstering viability under the harsh microenvironment, stimulating angiogenesis and promoting metastasis by supporting epithelial-to-mesenchymal transition (EMT) or dormancy.

### Cancer cell survival

The ER stress adaptive response promotes cancer cell survival in normal cells in a similar manner. Neoplastic transformation is initiated by overactivation of oncogenes (H-Ras and c-MYC) or loss of tumor suppressors (p53) driving unrestricted rapid cell division and protein synthesis, which overwhelms the protein folding capacity of ER and results in ER stress. This triggers the ER stress adaptive response to ameliorate stress and protects cancer cells from apoptosis.

Oncogenic H-Ras during cancer development is a canonical example. Following the proliferative response just after H-Ras transduction, primary mouse keratinocytes undergo premature senescence after 7–10 days. As expected, inhibiting IRE1α or XBP1 using pharmacological or genetic approaches reduces the proliferation of H-Ras keratinocytes. However, there is an odd phenomenon where prolonged treatment of H-Ras keratinocytes with 4-phenyl butyric acid, a molecular chaperone that can decrease unfolded proteins [104], not only reduces the UPR activation, but also inhibits proliferation. Further study reveals that the proliferation of cancer cells requires XBP1 splicing to cope with rapid protein synthesis, whereas cleavage of helix–loop–helix transcription factor ID1, one of the RIDD mRNA targets, drives senescence upon prolonged ER stress [105].

### Cancer angiogenesis

Neovascularization is a limiting factor for solid cancers that are unable to grow beyond 1–2 mm without angiogenesis. The UPR has been shown to play a vital role in cancer angiogenesis.

Silencing PERK, IRE1α and ATF6 can strongly suppress cancer angiogenesis induced by hypoxia or nutrient deprivation [106–108]. This may result from the XBP1, ATF4 and cleaved ATF6, which can directly bind to the promoter or enhancer sites of the vascular endothelial growth factor-A (VEGFA), the predominant mediator of angiogenesis, and transcriptionally upregulate its expression [109]. In addition to VEGFA, other pro-angiogenic factors such as interleukin (IL)-6 and fibroblast growth factor (FGF)2 are also suppressed when knocking down PERK or ATF4 in an orthotopic squamous cell carcinoma upon glucose deprivation treatment [107]. Similar to PERK, underexpression of IRE1α or XBP1 is also correlated with the downregulation of prevalent pro-angiogenic cytokines such as FGF2, IL-1β, IL-6 and IL-8, which likely contributes to the reduction of angiogenesis in malignant glioma and triple-negative breast cancer [106,108]. However, in contrast to IRE1α-XBP1 activity, improved IRE1α-RIDD activity via mutation in glioblastoma multiform suppresses cancer angiogenesis. This highlights the dual RNase function of IRE1α [110].

### Cancer metastasis

EMT is a cell-biological program, where polarized epithelial cells lose their adhesion property and obtain mesenchymal cell phenotypes. It not only is involved in embryogenesis, but also plays an important role in cancer invasion and metastasis [111]. Abnormal activation of EMT during cancer metastasis loses contacts between cells and upregulates extracellular matrix proteins, thus facilitating the migration and invasion of cancer cells that are confirmed to be related to activation of PERK. Cancer cells that are hypersecretory undergo EMT and it is reasonable to oberve constitutive activation of PERK–eIF2α–ATF4 signaling upon EMT [112]. Furthermore, pretreatment of metastatic 4T1 cells with a PERK inhibitor diminishes their metastatic capacity, as assessed by lung tumor burden after tail-vein injection. Additionally, ATF4 was reported to protect EMT cells from anoikis, a type of apoptosis following matrix detachment, via inducing the expression of the major antioxidant enzyme heme oxygenase 1 and promoting metastasis [113].

The function of IRE1α-XBP1 in EMT of cancer cells is complex. Expression of EMT genes and ATF4 target genes is strongly correlated in breast, colon, gastric, lung cancers and metastatic cancers of various

origins. Nevertheless, no significant correlation was observed between the expression of EMT and other branches of the UPR, such as the IRE1α-XBP1 pathway [112]. Another study induced EMT via overexpression of lysyl oxidase-like 2 (LOXL2) in MDA-MB-231 and Hs578T basal-like carcinoma cell lines, indicating the crucial roles of IRE1α- XBP1 in EMT. The accumulation of LOXL2 in ER sequesters BiP and activates IRE1α-XBP1, which transcribes EMT-TFs such as SNAI1, SNAI2, ZEB2 and TCF3 directly. Furthermore, inhibition of XBP1 with 4μ8c, a blocker targeting IRE1α RNase activity, can reduce LOXL2-mediated loss of E-cadherin, a cell adhesion marker [114].

Even if cancer cells invade stromal cell layers and enter the circulatory system successfully, their growth may be restrained because of the inhospitable tissue microenvironments. They can remain in this quiescent state for years, or even several decades, until microenvironmental conditions improve [115]. This process is referred to as tumor dormancy and overexpression of the UPR marker, BiP, has been reported in dormant cancer cells [116]. Increased p38 activity activates a pro-survival mechanism in dormant HEp3 squamous carcinoma cells via upregulating BiP and activating PERK. This allows dormant cells to resist chemotherapy inducing apoptosis by preventing Bax activation [117]. Constitutive ATF6 activation is also documented in dormant HEp3 cells rather tumorigenic HEp3 squamous carcinoma cells, which is related to cell survival via the Rheb and mTOR pathway [118].

### Other roles of the adaptive ER stress response in cancer cells

Similar to its dual role in normal cells, the ER quality control system can promote cancer cell survival or death in a context-dependent manner. Nevertheless, for better development, some special strategies may be taken by cancer cells to suppress UPR-induced apoptosis and promote survival.

Similar to normal cells, acute ER stress activates the three UPR branches (IRE1α, PERK and ATF6) to promote cancer cell survival via reducing misfolded protein levels. IRE1α and ATF6 activities are rapidly attenuated upon chronic ER stress, whereas PERK signaling, including the expression of pro-apoptotic transcription regulator CHOP, was maintained, which triggers apoptosis in normal cells [119]. However, in melanoma cells, IRE1α and ATF6 are reported to sustain activation by increasing activation of the MEK/ERK pathway and protecting cancer cells from ER stress-induced apoptosis [120].

Under mild/acute ER stress, IRE1α self-associates into dimmers and activates XBP1 to ameliorate ER stress. Under severe/chronic ER stress, IRE1α surpasses an oligomerization threshold to induce RIDD and induce apoptosis. Those cell processes occur in normal cells. However, in some human cancers, the activity of IRE1α with respect to inducing RIDD is inhibited by IRE1α mutants that can still splice XBP1 mRNA [121]. Hence, cancer cells escape from ER stress-induced apoptosis at the same time as sustaining their pro-survival function.

Another cancer-supporting role of the UPR is to help cancer cells escape from the immune defense by decreasing the MHC I-peptide presentation. Decreased surface expression of MHC I was found upon ER stress even though intracellular MHC I proteins were not changed. An impaired MHC I-peptide presentation is more related to global translation attenuation regulated by p-eIF2α, reducing the overall peptide pool for MHC I loading [122].

The role of ERAD and ER-phagy in cancer development and progression remains largely unknown. SEL1L is upregulated in colorectal cancer [123], whereas ectopic SEL1L induction in pancreatic cancer cells leads to G1 phase cell cycle arrest via the induction of a phosphatase and tensin homolog. Moreover, high levels of SEL1L in pancreatic cancer cells also lead to a reduction in invasiveness, possibly by modulating genes related to cell–matrix interactions [124]. Another well-studied ERAD component in cancer cells is OS-9, which was proposed as an important link between hypoxia regulation and cancer progression by mediating the degradation of HIF-1α [125]. The roles of ER-phagy receptors, FAM134B and Sec62, in cancer cells have been reported. Mutations in FAM134B have been observed in various malignancies and may play a dual role. FAM134B loss may promote colorectal cancer cell tumorigenicity [126], whereas it acts as an oncogene and promotes cancer development in esophageal squamous cell carcinoma [127]. The Sec62 gene is also amplified in several cancers, including lung adenocarcinomas, prostate, thyroid, and head and neck squamous cell carcinoma [128,129]. However, its molecular role in cancer remains undefined.

## Therapeutics targeting ER stress response: current strategies and undesired side effects

Cancer cells may require an appropriate UPR activation level to protect from stress and promote survival. Either increasing ER stress or decreasing the UPR activation level should tip the balance and induce

apoptosis. Hence, there are two main anticancer therapeutics exploiting the UPR. The first approach comprises ER stress inducers, which raise ER stress to a critical level and trigger cell apoptosis. The second approach involves protective pathway antagonists, which block the adaptive response acting as a pro-survival mechanism. Small molecule inhibitors are designed based on those strategies, as discussed in many reviews [130,131]. Some of them not only inhibit the UPR, but also simultaneously increase ER stress. In this review, some representative ones are taken as examples (Fig. 2).

## ER stressors

As a result of intrinsic and extrinsic insults, cancer cells usually stay in a chronic or severe ER stress state and are pushed near the apoptotic threshold. Other stimuli such as ER stress inducers act as the straw that breaks the camel's back. Tunicamycin is a canonical ER stress inducer via inhibition of UDP-*N*-acetylglucosamine:dolichyl-phosphate *N*-acetylglucosamine-phosphotransferase (GPT), which can transfer GlcNAc to dolichol phosphate in ER and disrupt protein *N*-glycosylation. Based on this mechanism, tunicamycin has been identified as a potential anticancer drug. It

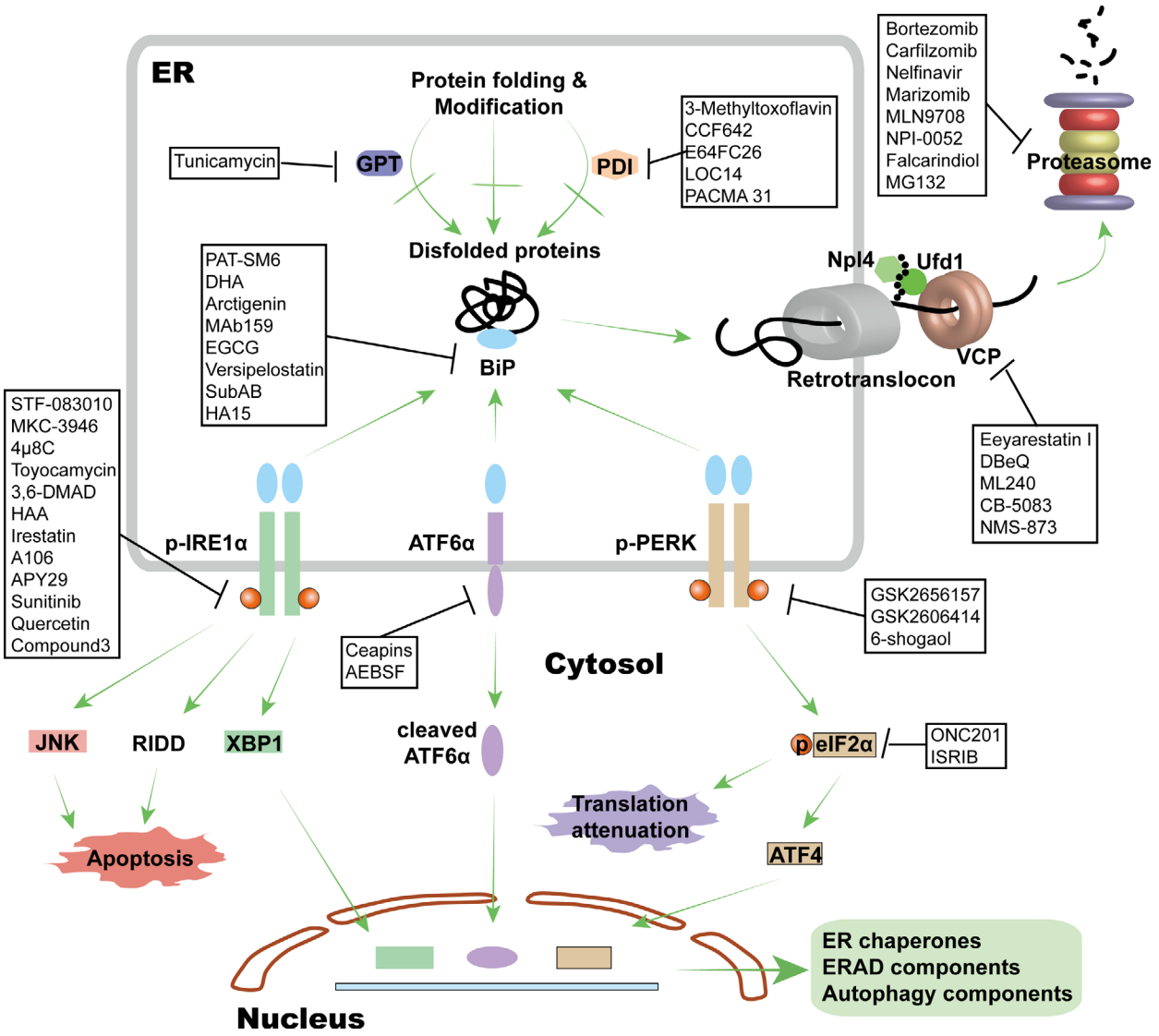


**Fig. 2.** Pharmacological targeting of the ER stress response. EGCG, epigallocatechin gallate; 3,6-DMAD, *N*9-(3-(dimethylamino) propyl)-*N*3, *N*3,*N*6,*N*6-tetramethylacridine-3,6,9-triamine; HAA (hydroxy-aryl-aldehydes); DHA, docosahexaenoic acid; AEBSF, 4-(2-aminoethyl) benzenesulfonyl fluoride; GPT, UDP-*N*-acetylglucosamine:dolichyl-phosphate *N*-acetylglucosamine-phosphotransferase.

has been reported that tunicamycin treatment alone could promote autophagy and apoptosis in several cancers. Moreover, tunicamycin increases chemotherapy and radiation therapy-induced apoptosis by increasing basal ER stress in cancer cells [132,133]. Other ER stress inducers include PDI inhibitors (3-methyltoxoflavin, CCF642, E64FC26, LOC14, PACMA31) [131], ER $Ca^{2+}$ATPase noncompetitive inhibitor thapsigargin [134] and brefeldin A, which is a very common inducer of ER stress by inhibiting protein transport from the ER to the Golgi apparatus [135]. Celecoxib, a COX-2 selective nonsteroidal anti-inflammatory drug approved by the US Food and Drug Administration for treating the pain and inflammation, could lead cancer cell to death through inducing ER stress [136].

### Rescuer blockers

XBP1 mRNA splicing is the main activation product of IRE1α and promotes cell survival, as discussed above. STF083010 is an IRE1α inhibitor that blocks the IRE1α RNase domain without affecting its kinase activity and oligomerization. It can form a covalent bond with IRE1α K907 in the RNase catalytic site and disrupt its endoribonuclease activity for XBP1 mRNA and RIDD. Treatment of human MM cells with STF-083010 alone showed cytotoxic activity in various *in vitro* and *in vivo* models [137]. MM cells may under inherent ER stress. STF-083010 blocks XBP1 and hampers the protective function, making cells intolerant to the existing stress and tipping the balance toward apoptosis. However, a recent study reported that pretreating gastric cancer cells with STF-083010 considerably relieved ER stress-induced autophagy and apoptosis induced by melatonin [138]. Other rescuer blockers include IRE1α inhibitors (MKC-3946, 4μ8C, toyocamycin, 3,6-DMAD, HAA, irestatin, A106, APY29, sunitinib, quercetin, compound 3) [139], ATF6 inhibitors (ceapins, AEBSF) [140], PERK inhibitors (GSK2656157, GSK2606414, 6-shogaol) and p-eIF2α inhibitors (ONC201, ISRIB) [141].

### Multifunctional inhibitors

Small molecule inhibitors targeting ERAD usually result in the accumulation of misfolded proteins and cause ER stress-related apoptosis. Eeyarestatin I (EerI) is an ERAD inhibitor targeting p97. It has two functional domains. One is an aromatic domain responsible for localizing EerI to the ER membrane and improving its target specificity. Another is a nitrofuran-containing group responsible for binding to the p97 ATPase [142]. EerI has been shown to have antitumor activity in malignant myeloma cells and HER2 positive breast cancer cells. However, p97 protein expression is decreased in almost all primary lung cancers. Moreover, EerI treatment induces ER stress and potently activates an EMT-like state in cells, which contributes to chemoresistance and poor survival [143]. EerI not only acts as an ERAD inhibitor hampering misfolded protein degradation activity, but also results in increased accumulation of misfolded protein in the ER, making the ER stress tenser. Bortezomib (BTZ) is similar to EerI in this respect. It not only targets to ERAD, but also is an ER stress inducer approved by the US Food and Drug Administration for treating MM and mantle cell lymphoma. The sensitivity of BTZ is related to the amount of immunoglobulin retained within MM cells. Preplasmablasts do not synthesize high levels of immunoglobulin and are resistant to BTZ [144]. Other multifunction inhibitors include p97 inhibitors (DBeQ, ML240, CB-5083, NMS-873) [145] and proteasome inhibitors (carfilzomib, nelfinavir, marizomib, MLN9708, NPI-0052, falcarindiol, MG132) [146].

### Therapeutics targeting cell-surface BiP (csBiP) in cancer cells

Another therapeutic targeting ER stress is BiP, which can be overexpressed on the surface of cancer cells but not normal cells [147]. Multiple csBiP ligands, such as prostate apoptosis response-4, isthmin1, plasminogen Kringle5, secreted BiP, synthetic peptides and monoclonal antibodies, have been identified. They play multiple roles, including pro-proliferation, pro-survival and pro-apoptosis, even though the transduction mechanism is unclear [148]. PAT-SM6, a human monoclonal IgM antibody isolated from a gastric cancer patient, has shown promising results in early-stage clinical trials of relapsed or refractory MM [149,150]. The antibody can induce cancer cell death by complement-dependent cytotoxicity and antibody-dependent cellular cytotoxicity by selectively targeting csBiP of MM cells [151]. Moreover, csBiP auto-antibodies decrease the metastatic properties of cancer cells in ovarian cancer patients [152]. Other BiP blockers include DHA, arctigenin, MAb159, EGCG, versipelostatin, SubAB and HA15 [153].

### Limitations and side effects of therapeutic approaches targeting the ER stress response

The ER stress adaptive response is an essential mechanism for cancer cell survival. As a result, it is a

promising target for cancer therapy. However, some special characteristics should be considered.

Different cancer types have different ER stress levels and, even in the same cancer tissue, different cancer cells could have different ER stress levels. ER stress inducers or UPR antagonists only selectively kill cells with a lower threshold. As noted above, several small molecule inhibitors targeting ER stress function as an anticancer drug in some cancer types, whereas they have anti-apoptosis effects or induce chemoresistance in others [138,143].

When ER stress inducers or UPR antagonists push cancer cells toward death thresholds, normal cells that are dependient on the protective mechanism of the UPR may suffer the same stress insults, especially secretory organs such as the pancreas. GSK2656157 is an ATP mimetic PERK kinase inhibitor with respect to blocking PERK autophosphorylation and phosphorylation of eIF2α. GSK2656157 exhibits significant antitumor efficacy in multiple human tumor xenograft growths in mice. However, the pharmacological inhibition of PERK in adult mice by GSK2656157 causes damage to pancreatic exocrine acinar cells and islet cells, which limits its application in cancer therapy [154].

## Conclusions and perspectives

In recent decades, great progress has been made in the identification of key components of the ER stress response, as well as the underlying mechanisms. However, important aspects such as the feedback regulation of the ER stress response remain incompletely understood. As a fundamental adaptive response, tumor cells hijack the ER stress response to protect themselves from various insults occurring during cancer initiation and progression. The ER stress response represents a promising target for anticancer therapies and dozens of molecules targeting every branch of the ER stress response have been developed. However, most of them have not reached the clinic because of insufficient efficacy or obvious negative side effects, with the latter likely arising as a result of the compensatory functions of the ER stress response. Thus, new and more specific targets that can distinguish normal cells from malignant neoplastic cells are urgently needed. We still have a long way to go before targeting of the ER stress response can become an effective anticancer therapy.

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