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REVIEW

Proteostasis regulation at the endoplasmic reticulum: a new perturbation site for targeted cancer therapy

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To deal with the constant challenge of protein misfolding in the endoplasmic reticulum (ER), eukaryotic cells have evolved an ER protein quality control (ERQC) mechanism that is integrated with an adaptive stress response. The ERQC pathway is comprised of factors residing in the ER lumen that function in the identification and retention of aberrantly folded proteins, factors in the ER membrane for retrotranslocation of misfolded polypeptides, and enzymes in the cytosol that degrade retrotranslocated proteins. The integrated stress response (termed ER stress or unfolded protein response, UPR) contains several signaling branches elicited from the ER membrane, which fine-tune the rate of protein synthesis and entry into the ER to match the ER folding capacity. The fitness of the cell, particularly those bearing a high secretory burden, is critically dependent on functional integrity of the ER, which in turn relies on these stress-attenuating mechanisms to maintain protein homeostasis, or proteostasis. Aberrant proteostasis can trigger cellular apoptosis, making these adaptive stress response systems attractive targets for perturbation in treatment of cell malignancies. Here, we review our current understanding of how the cell preserves ER proteostasis and discuss how we may harness the mechanistic information on this process to develop new cancer therapeutics.

***Keywords*:** retrotranslocation/ERAD/dislocation; ER stress/UPR; proteasome; BH3-only protein; IRE1; PERK; ubiquitin; p97/Cdc48; targeted cancer therapy; bortezomib/Velcade

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Introduction

Secretory and membrane proteins begin their journey in the cell when they are translocated into the lumen of the endoplasmic reticulum (ER) or integrated into the ER membrane via the Sec61 translocon. An elaborate cohort of chaperones in the ER then assist polypeptides in folding and assembly so that they acquire functional, native conformations, as well as proper oligomeric states. Correctly folded and assembled polypeptides are then sorted to ER exit sites where they are loaded into distinct vesicles for transport to different cellular destinations. Successful completion of this long journey is not a trivial task. Polypeptides must pass stringent check points enforced by an evolutionarily conserved ER protein quality control program (ERQC), which efficiently targets and destroys aberrant polypeptides or unassembled protein complexes [1]. The accumulation of aggregation-prone misfolded polypeptides is so detrimental to the fitness of the cell that the ERQC has evolved to destroy almost any protein of questionable quality, such as those that stay in the folding process for a prolonged period of time.

The cell is constantly modulating its protein folding and degradation capacities to avoid accumulation of misfolded proteins and maintain protein homeostasis, or proteostasis. When the production of misfolded proteins exceeds degradation, as often occurs in damaged or aging cells, or in cells exposed to chemical agents that perturb protein folding or the ERQC pathway, the unfolded protein response (UPR) is elicited. The UPR can mobilize several additional mechanisms to restore ER homeostasis [2, 3]. However, if these efforts fail to overcome the folding crisis, persistent ER stress can switch on an apoptotic program, which results in cell elimination [4].

Cells with different specialized functions can bear different secretory burdens, resulting in different levels of intrinsic stress among various cell types. Consequently, cells can have distinct sensitivities to extra stressors that

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perturb ER proteostasis. For example, the plasma B cells are specialized for antibody production. To accommodate this increased secretory burden, the ER in differentiated plasma B cells is drastically expanded to boost the folding capacity. Despite this adaptive change, the massive secretory flux still puts these cells at risk for apoptosis induced by external stressors that might otherwise be inert. Indeed, a recent study demonstrated that plasmacytic differentiation without increased secretory load confers resistance to the proteasome inhibitor bortezomib [5]. Thus, specific therapeutic strategies aimed at targeting ER proteostasis may be employed to selectively treat certain tumors carrying a high secretory burden [6, 7].

ER-associated protein degradation (ERAD)

Elimination of misfolded proteins from the ER by the ERQC program counteracts the production of aberrant proteins from various folding mishaps. This essential housekeeping function operates in the ER of all eukaryotic cells. Misfolded proteins are exported from the ER and subsequently destroyed by the ubiquitin-proteasome system in the cytosol by a process called retrotranslocation or ER-associated protein degradation (ERAD) [8, 9]. In this section, we will discuss the players identified to date in this pathway based on their known functions in order to reveal potential sites of perturbation for targeted cancer therapy.

Substrate recognition and targeting

Misfolding signals can reside in a variety of locations on a polypeptide. Nonetheless, all such signals seem to be associated with the exposure of certain hydrophobic patches that are normally embedded in the interior of a folded polypeptide or properly assembled protein complex. Misfolding signals on polypeptides are usually recognized by chaperones [10, 11]. For secretory and ER luminal proteins, misfolding signals reside entirely within the ER and thus must be recognized as such in the ER lumen. For membrane proteins, depending on their topology and the position of the lesions, substrate recognition may occur in the ER lumen, within the membrane, or in the cytoplasm. Accordingly, studies in yeast have classified ERAD substrates into three categories, called ERAD-L, ERAD-M, and ERAD-C based on the location of the lesions in the lumen, membrane, or cytosol, respectively [12, 13]. Each substrate cohort can also be distinguished by its unique genetic and biochemical requirements for degradation [12, 14]. For example, the degradation of the ERAD-L and ERAD-M substrates is strictly dependent on the ubiquitin ligase Hrd1p (see below), whereas ERAD-C substrates require a different ligase Doa10p for turnover [12, 15]. In mammalian cells, the degradation of different categories of ERAD substrates does not seem to follow the exact same pattern of labor division [16]. Perhaps, the increased complexity of the secretory pathway in these cells calls for more flexibility and redundancy in the ERQC program.

The rules for substrate recognition by ER chaperones can be quite degenerative. In some cases, chaperones act in parallel [17], whereas other times they may work sequentially to 'interrogate' substrates bearing multiple misfolding signals [18, 19]. In general, a short hydrophobic segment exposed on a misfolded protein can be recognized by the Hsp70 family of chaperones such as glucose regulated protein of 78kDa (GRP78/BiP) in mammals and Kar2p in yeast [20, 21], which may be sufficient to initiate retrotranslocation. However, it is worth mentioning that the Hsp70 family of chaperones normally functions as a folding catalyst. BiP/GRP78 also associates with the Sec61 complex and can act as a ratcheting molecule in this context to facilitate the translocation of polypeptides into the ER [22]. Thus, the early steps of the ERQC pathway overlap with ER protein biogenesis. It is currently unclear how chaperones can switch their job from a folding assistant to an ERQC triaging factor. One possibility is that prolonged association of a polypeptide with one or more chaperones without productive folding is sufficient to target the chaperone-substrate complex to a retrotranslocation channel (see below) in the ER membrane, which triggers export and destruction. An analogous timer mechanism has been proposed for the disposal of misfolded glycoproteins (Figure 1).

A large number of proteins entering into the ER carry the consensus sequence Asn-X-Ser/Thr (X designates any residues) that can be modified with an N-linked oligosaccharide (GlcNAc2-Man9-Glu3), which adds an extra layer of complexity for substrate recognition in the ERQC. Glycoproteins are usually folded with the assistance of the lectins calnexin and calreticulin, the glucosidase-1 and -2, and the UDP-glucose:glycoprotein glucosyltransferase (UGGT) (Figure 1) [23, 24]. If a polypeptide remains unfolded after extended association with these lectins, ER-resident mannosidases (Htm1p in yeast or EDEM in mammals) associated with calnexin may trim mannose from the glycan [25-27], generating a signal containing a terminal α1, 6-linked mannose residue [28-30], which is then recognized by the downstream lectin Os9/Yos9p [31, 32]. This results in the extraction of the polypeptide from the folding pathway, which causes its retrotranslocation and degradation. Interestingly, Yos9p not only recognizes the trimmed glycan using its mannose 6-phosphate receptor homology (MRH) motif, but it also has the capacity to recognize unfolded

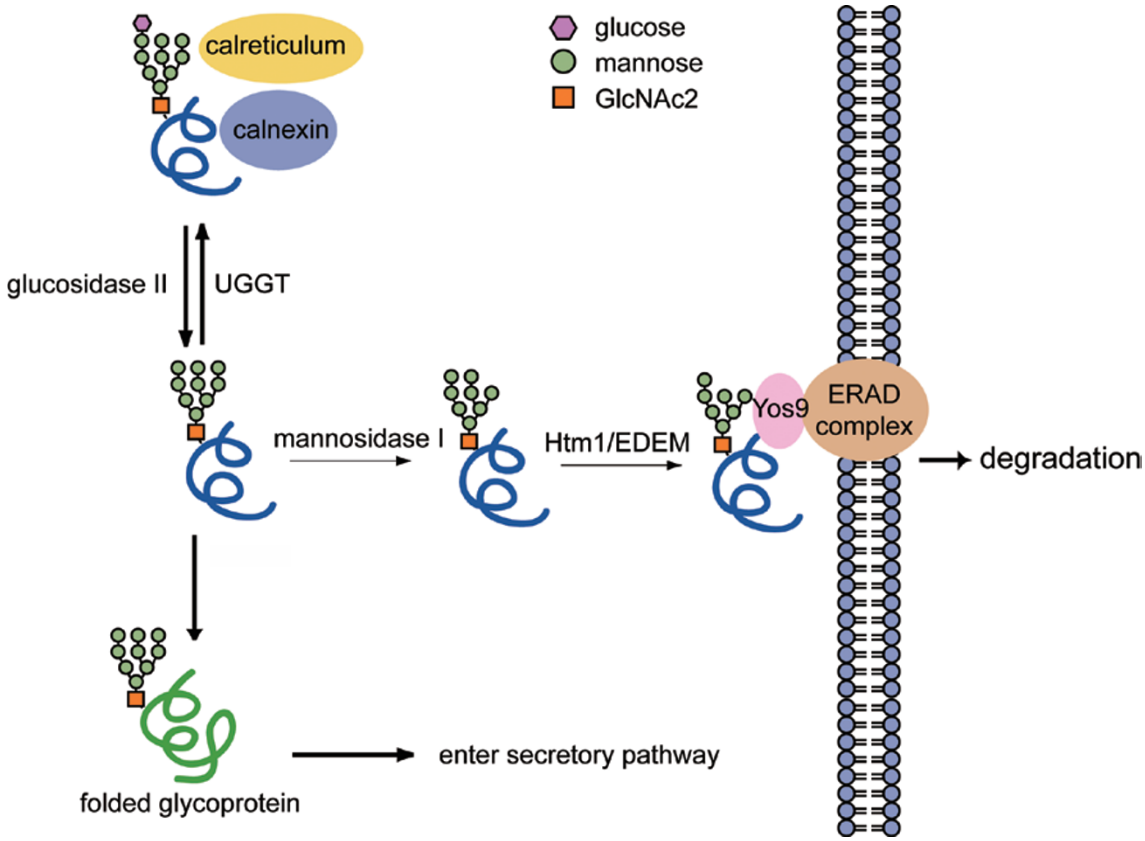

Figure 1 The proposed timer mechanism for degradation of misfolded glycoproteins. Upon entry into the ER, glycoproteins are folded with the assistance of the lectins, Calnexin and Calreticulin. In the so-called Calnexin cycle, the terminal glucose residues on a glycan are removed by glucosidases I and II. In higher eukaryotic cells, a UDP-glucose:glycoprotein glucosyltransferase (UGGT) can subsequently add a glucose residue back if the protein is unfolded. The mono-glucose residue is recognized by Calnexin and Calreticulin, which retains unfolded proteins in the folding cycle. The deglucosylated proteins can exit the Calnexin cycle upon folding, or when the mannose in the N-glycan is cleaved by the ER mannosidase I (Man I). Cleavage of mannose irreversibly extracts glycoproteins out of the folding cycle, which is followed by further mannose trimming by Htm1p/EDEM. Neither mannosidase I nor Htm1p/EDEM displays strong activities *in vitro* (as indicated by the thin arrows). Perhaps, the sluggish action of these enzymes gives newly synthesized glycoproteins sufficient time to fold in the Calnexin cycle. Mannose trimming by ER Man I and Htm1p/EDEM generates a glycan signal that is recognized by the downstream lectin Yos9. Yos9 resides in a large membrane protein complex containing other ERAD factors, which initiates retrotranslocation.

proteins [33, 34], presumably via association with an exposed hydrophobic patch. Accordingly, the number and the position of glycans relative to the misfolding signal in a glycoprotein can influence the rate of substrate recognition and degradation [35].

Protein folding in the ER often involves the formation of disulfide bonds, which is catalyzed by the family of Protein Disulfide Isomerases (PDIs). PDI also has the capacity to break non-native disulfide bonds and reshuffle them to form proper disulfide links. Like other folding catalysts mentioned above, PDI can also partake in the ERQC program by facilitating substrate recognition and partition in the ERAD pathway. Several lines of evidence suggest that the ERAD machinery has limited capacity when handling tightly folded substrates or protein aggregates containing intermolecular disulfide bonds. Bhamidipati and colleagues demonstrated that the fusion of a tightly folded domain to the ERAD-L substrate CPY* impedes its degradation [36]. During retrotranslocation, polypeptides presumably move across the ER membrane in an unfolded monomeric form. In line with this notion, a recent study shows that two basic intramembrane residues in unassembled TCRα chain are required to prevent TCRα oligomerization via inter-chain disulfide bond formation, and this facilitates its degradation [37]. Moreover, Okuda-Shimizu *et al*. found that retrotranslocation of non-secreted κ-LC involves the conversion of a partially oxidized precursor into a reduced monomer [38].

Thus, PDI may promote retrotranslocation by breaking disulfide bonds in oligomerized ERAD substrates, which generates a retrotranslocation competent monomer. Additionally, PDI was also found to interact with certain retrotranslocation complexes in the ER membrane [39, 40], which may define the point of no return at which substrates are irreversibly switched to the degradation-bound track. Ushioda *et al*. recently uncovered another ER reductase ERdj5, which may act analogously to PDI to facilitate retrotranslocation. ERdj5 associates with EDEM, which provides a convenient means to couple protein unfolding with mannose trimming and substrate hand-off to a Yos9p-containing retrotranslocation complex in the ER membrane [41].

Retrotranslocation across the ER membrane

Once segregated from the cohort of properly folded polypeptides, misfolded proteins are then bound to the cytosol for degradation by the proteasome. The transit of polypeptides across the ER membrane most likely occurs via a protein conducting channel(s), although a lipid-mediated dislocation hypothesis has also been proposed [42]. Early evidence indicated that the Sec61 complex, which mediates polypeptide import into the ER, might also act to translocate misfolded polypeptides out of the ER. Although some genetic and biochemical evidence supports this view [43-45], the degradation of many ERAD substrates proceeds normally in the absence of a functional Sec61 complex [46-49]. Thus, it seems that the Sec61 complex is unlikely to serve as a major channel for retrotranslocation.

Recent studies suggest that the multispanning membrane proteins Derlin-1, -2 and -3, which reside in a large protein complex together with the ER- associated E3 ubiquitin ligase Hrd1, define a site of retrotranslocation in the ER membrane [50-53]. With four predicted transmembrane segments, the Derlin proteins may serve as a channel component or scaffold/accessory factors that facilitate channel assembly. Intriguingly, Derlins can associate with luminal ERAD factors such as EDEM as well as with the cytosolic ERAD components including the AAA ATPase p97 and the cytosolic N-glycanase. This may provide a physical link between substrate recognition in the ER and dislocation to the cytosol [53-55]. However, definitive evidence in support of a Derlin-containing proteinous channel is still missing. Likewise, the E3 ligase (see below) Hrd1 can also coordinate actions on both sides of the ER membrane through protein-protein interactions [33, 56]. In yeast, Hrd1p can interact with ER luminal proteins such as the lectin Yos9p through its interacting partner Hrd3p. It also binds p97/Cdc48p (see below) in the cytosol. Moreover, although the N-terminal transmembrane segments of Hrd1p are not required for its ligase activity [57], they are essential for ERAD function [58]. One likely scenario is that the transmembrane domains of Hrd1 may participate in the formation of some kind of membrane pore to translocate ERAD substrates. Consistent with this view, a recent study used an elegant crosslinking approach to demonstrate that a retrotranslocation intermediate is in close proximity to Hrd1p [59].

It is worth mentioning that many ERAD substrates do not require the Derlin-Hrd1 complex for degradation. It is clear from studies in yeast that the multiple-spanning E3 ubiquitin ligase Doa10p acts in parallel to Hrd1p to degrade membrane proteins containing a misfolded cytosolic domain [12, 15]. Like Hrd1p, Doa10p also contains multiple transmembrane segments, which may act analogously to Hrd1p in assisting client transport across the ER membrane. Finally, in mammalian cells, newly synthesized major histocompatibility complex (MHC) class I heavy chain can be dislocated from the ER membrane under the influence of the human cytomegaloviral protein US2 by a novel ERAD mechanism that involves signal peptide peptidase and the multiple membrane-spanning ubiquitin ligase TRC8 [60, 61]. Whether this mechanism is used to degrade naturally occurring misfolded proteins is unclear. Taken all together, the existing evidence suggests the presence of multiple parallel retrotranslocation pathways, each of which channels a subset of misfolded proteins into the cytosol.

The ubiquitination machinery

Once emerging from the ER lumen, polypeptides undergo ubiquitination with one or more chains of ubiquitin molecules covalently linked to either lysine or serine/threonine residues in the substrate [62, 63]. Polyubiquitination of ERAD substrates is not only required for subsequent dislocation from the ER membrane, but also for targeting dislocated polypeptides to the 26S proteasome for degradation [64-66].

Ubiquitination requires the sequential actions of three types of enzymes, an E1 activating enzyme, an E2 conjugating enzyme and an E3 ubiquitin ligase. The human genome contains two E1 enzymes, ~40 E2s and 600-1 000 E3 ligases. In yeast, the relevant ERAD E2s are Ubc7p, Ubc6p, and Ubc1p [57, 64, 67, 68]. Deletion of these genes individually or in combination inhibits ubiquitination and degradation of many misfolded ER proteins. In mammals, the Ube2g and Ube2j subfamilies are homologous to the E2s Ubc7p and Ubc6p, respectively [69]. Members of these E2 families have been implicated in ERAD of many misfolded substrates [70-74]. In yeast, Hrd1p and Doa10p are the two major

ubiquitin ligases dedicated to ERAD [15, 57]. In mammals, orthologs of the yeast Hrd1p and Doa10p, as well as a Hrd1-related E3 named gp78, mediate the degradation of most misfolded ER proteins [73-79]. Mammalian cells also employ several additional E3 ligases including RMA1 [80], the U-box containing ubiquitin ligase CHIP [81], and Parkin, a RING finger E3 linked to the juvenile Parkinson's disease [82]. Two F-box-containing proteins, Fbs1 and Fbs2, each of which is part of a multi-subunit Skp, Cullin, F-box-containing (SCF) ubiquitin ligase, have been shown to recognize carbohydrate chains to facilitate ubiquitination and degradation of retrotranslocated glycoproteins [83, 84]. Finally, a ubiquitin chain-elongating factor named Ufd2p in yeast or E4a and E4b in mammals was shown to extend short ubiquitin chains to enhance the efficiency of substrate targeting to the proteasome [85-87]. The involvement of multiple E3 ligases across species in ERAD is consistent with the notion that ubiquitin ligases confer substrate specificity. However, the number of misfolded ER proteins clearly exceeds the available ligases. It is likely that each retrotranslocation complex contains at least one E3 ubiquitin ligase that modify any substrate emerging from the same translocon.

As expected, many ERAD-specific E2 and E3 enzymes are bound to the ER membrane with their catalytic domains facing the cytosol. Ubc6p and the Ube2j family of E2s contain a short carboxyl terminal transmembrane segment that anchors them to the ER membrane. Hrd1, gp78, RMA1, and Doa10 are multi-spanning membrane proteins carrying a cytosolically localized RING-finger domain. The transmembrane segments of these E3s may not only anchor the ligases in the ER membrane, but may also serve a direct role in the retrotranslocation process (see above). Some E2 and E3s such as Ubc7p, CHIP, Parkin, Fbs1 and Fbs2 are soluble proteins. Nonetheless, in yeast, Ubc7p is recruited to the ER membrane by association with the membrane protein Cue1p [65], and in mammals, Ube2g2 interacts with the multispanning membrane ligase gp78 with a high affinity [88, 89]. It is currently unclear whether or not the other soluble ubiquitin ligases mentioned above are recruited to the site of translocation by interaction with an ER membrane partner.

The dislocation-driving ATPase Cdc48/p97

Misfolded ER proteins undergoing retrotranslocation are dislocated from the ER membrane before being targeted to the proteasome. The precise mechanism by which polypeptides are extracted from the membrane is unclear, but the major player of this reaction has been identified as the AAA (ATPase associated with various cellular activities) ATPase p97 in mammals or its homolog Cdc48p in yeast [90-93].

p97 belongs to the type II AAA ATPase family as it contains two similar Walker type AAA ATPase domains. It forms an evolutionarily conserved hexameric ring with a central pore [94, 95]. It was proposed that Cdc48/p97 might function as a ubiquitin-selective chaperone to segregate ubiquitinated substrates from a large, relatively immobile entity such as the ER membrane [96]. Through its actions on diverse substrates, p97 can influence a variety of cellular processes, including retrotranslocation, the activation of membrane-anchored transcription factors, nuclear envelop formation, spindle disassembly, the homotypic fusion of the ER/Golgi membranes, DNA replication, and transcriptional regulation [96-100]. The substrate specificity probably lies in the diverse p97 cofactors, each of which could in principle link p97 to a distinct set of substrates [97, 101].

The major cofactor that assists p97 in retrotranslocation is a dimer consisting of Ufd1 and Npl4, although there are known exceptions [102, 103]. Different p97 cofactors may each affiliate with a specific type of retrotranslocation complex, allowing p97 to act on almost all ERAD substrates regardless of their translocation route. The p97-Ufd1-Npl4 complex contains several ubiquitin-binding motifs that each can recognize polyubiquitin signals on an ERAD substrate [104-107]. Moreover, p97 itself has an intrinsic affinity to unfolded substrates [108]. Existing evidence suggests that p97/Cdc48 may first bind a non-modified, presumably unfolded substrate emerging from a translocation site. This interaction may serve as a ratcheting mechanism that prevents substrate from slipping back into the ER, and thus allows substrate to be efficiently ubiquitinated. Once ubiquitin chains are conjugated to the substrate, the ubiquitin signal is further recognized by the ubiquitin-binding motifs in the ATPase complex, leading to the extraction of polypeptides from the ER membrane [104].

Substrate delivery to the proteasome

It has been demonstrated that ERAD substrates are exported into the cytosol in an unfolded form, perhaps due to size limitation of the putative membrane retrotranslocons [36]. Once released from the ER membrane, polypeptides need to be rapidly targeted to the proteasome for degradation. Otherwise, the dislocated ERAD products, many of which bear aggregation-prone hydrophobic transmembrane segments, may form toxic aggregates in the cytosol. It has been proposed that certain ubiquitin-binding factors may interact with the ATPase Cdc48/p97 and the proteasome in an alternate mode, leading to substrate hand-off from p97 to the proteasome [85]. Such shuttling molecule candidates include Ufd2 and ataxin-3, which are remarkably distinct in biochemical activity:

Ufd2 is an ubiquitin E4 enzyme that extends short polyubiquitin chains on a substrate [87, 109], whereas ataxin-3 is a member of the Joseph family of deubiquitinating enzymes that can trim or edit ubiquitin chains [110-112]. Nevertheless, both molecules are able to interact with Cdc48/p97 as well as the UBA-UBL domain-bearing proteins Rad23 and Ubiquilin/Dsk2 [113, 114]. Rad23 and Ubiquilin/Dsk2 belong to a family of ubiquitin receptors that are docked on the proteasome to promote substrate-proteasome interactions. In yeast, it seems that several redundant mechanisms exist to channel ubiquitinated proteins to the proteasome. For example, Rad23p can cooperate with the N-glycanase Png1p to facilitate the degradation of glycoproteins and with Ufd2p to turnover non-glycoproteins [115]. Little is known about how these factors shuttle substrates to the proteasome, but the fact that the delivery process can involve ubiquitin modifying enzymes with opposing activities underscores the importance of ubiquitin chain dynamics in this process.

The degradation machinery

The 26S proteasome is the major degradation machinery in the cell for dysfunctional or damaged proteins. Proteasomal degradation also regulates a variety of cellular process such as cell cycle progression. The proteasome is a large multi-subunit enzyme that is comprised of two sub-complexes, the 19S regulatory complex and the 20S proteolytic complex [116]. The 20S sub-complex is made of four stacked rings in a α-β-β-α geometry with each ring containing either 7 α or β subunits. Together, they are assembled into a barrel-like structure that harbors a chymotrypsin-like, a trypsin-like, and a peptidyl-glutamyl peptide-hydrolyzing (PHGH) proteolytic activity inside the barrel. The 19S sub-complex is comprised of at least 19 proteins that can be further divided into two sub-assemblies. Among the 10 proteins in the base, six AAA ATPases are assembled into a hexameric ring that sits on top of the 20S sub-complex. These ATPases can unfold substrates to facilitate their entry into the degradation chamber. They also regulate the gated proteasome opening and provide the driving force that pulls substrates into the degradation chamber. The remaining proteins in the 19S particle form a lid on top of the base. The lid contains ubiquitin-binding sites that collect ubiquitinated proteins, interacts with substrate delivery factors, and harbors deubiquitinating activities that modulate ubiquitin conjugates on substrates en route to the destruction chamber.

Additional factors

Many additional factors have been identified to facilitate ERAD. Some of these factors are conserved from yeast to human, whereas others are specific for higher eukaryotes. In yeast, Usa1p was recently shown to function as a scaffold protein to recruit the Derlin ortholog Der1p protein to the ubiquitin ligase Hrd1p [117, 118]. The mammalian homolog of Usa1p is Herp, which is also required for ERAD, but its precise function is unclear [38, 119]. In yeast, the Cdc48p complex is recruited to the ER membrane via binding to the Ubx2p protein [120, 121], whereas in mammalian cells, several ER-associated membrane proteins contain high affinity binding sites for p97. These include the ubiquitin ligase gp78 [122], a single-spanning membrane protein termed VIMP [53], the Ubx domain-containing protein UbxD8 [123], and Erasin [124]. These factors may act in parallel or sequentially to engage the p97 ATPase in ERAD. In mammals, several substrate-specific ERAD factors have also been identified. For example, a membrane protein complex comprised of SPFH1 and SPFH2 facilitates ubiquitination and degradation of inositol 1, 4, 5-trisphosphate receptors [125]. A second example is Bap31, which promotes the degradation of a mutant variant of the cystic fibrosis transmembrane conductance regulator [126]. The exact functions of these factors in ERAD are currently unclear, but their existence underscores the complexity, and perhaps the underlying specificity, of the ERAD system in higher eukaryotes.

ER stress: an intersection of life and death pathways

In addition to ERQC, eukaryotic cells have also evolved an integrated, adaptive stress response program, also known as the unfolded protein response (UPR) that helps alleviate protein-folding crises in the ER. This topic has been extensively reviewed [2, 127-130]. In brief, the core of the UPR is comprised of several signaling branches mediated by transmembrane proteins such as IRE1, PERK, and ATF6. These proteins sense protein misfolding in the ER lumen and then relay the signals to other parts of the cell to induce adaptive changes (see below and also Figure 2), which include transcriptional upregulation of genes involved in protein folding, degradation, and trafficking, transient inhibition of protein translation and translocation into the ER, decay of ER-localized mRNAs, and induction of autophagy [2, 131].

Although the unfolded protein response is elicited to promote cell viability under stress conditions, persistent ER stress can also switch on an apoptotic program to eliminate stressed cells. Intriguingly, the cyto-protective and the cyto-destructive signals are initiated by the same set of ER stress sensor proteins, raising the question of how a pro-survival or pro-death cell fate is determined

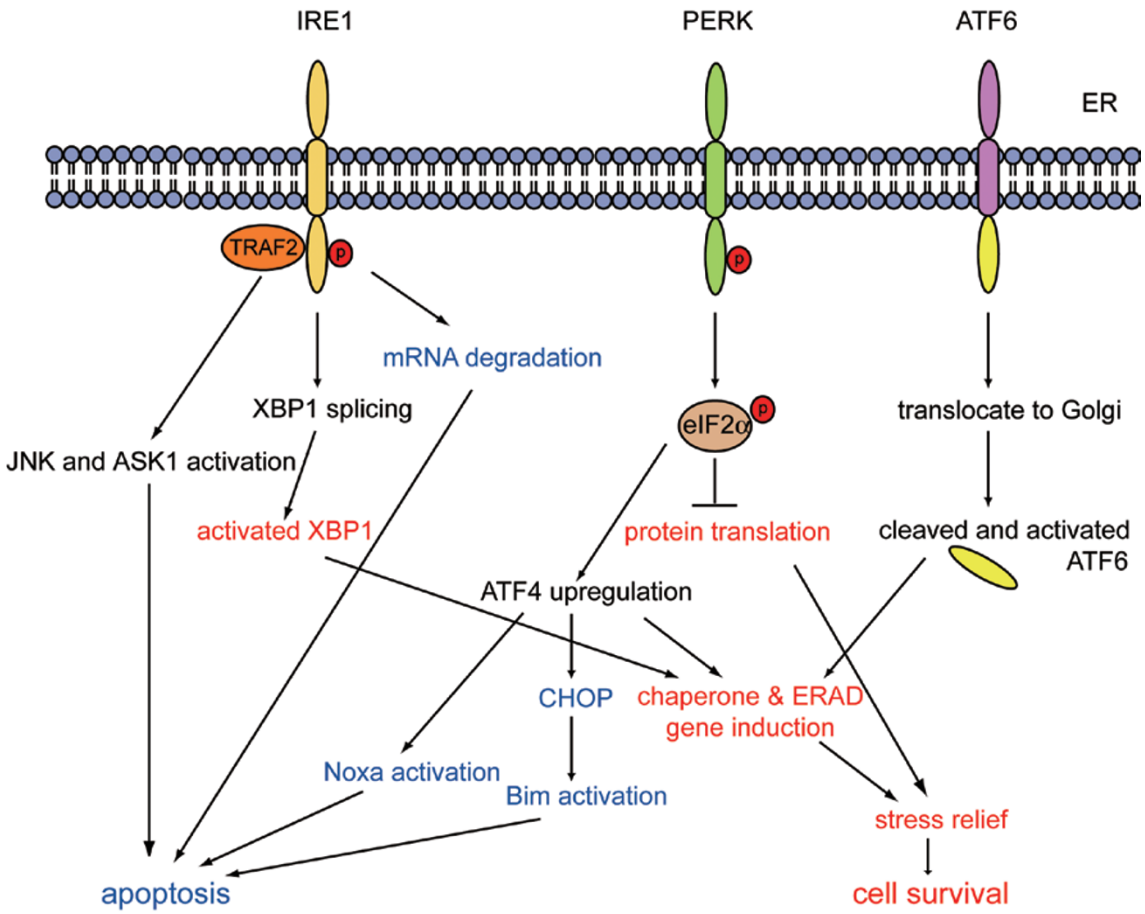

Figure 2 The two faces of the UPR signaling. In the mammalian ER, protein-misfolding stress is sensed by three membrane-associated proteins, ATF6, PERK, and IRE1. ATF6 is a transcription factor whose activation requires the translocation of ATF6 to the Golgi and the subsequent processing of ATF6 by two membrane proteases. PERK is a protein kinase that is autophosphorylated and activated when GRP78 dissociates from its ER luminal domain in response to ER stress. PERK phosphorylates eIF2α, leading to global attenuation of protein synthesis that only a few proteins including the transcription factor ATF4 can escape. ATF4 activates downstream genes that can have either a pro-survival or pro-apoptotic role (pro-death events are marked in blue whereas pro-survival events are marked in red). IRE1 is also a membrane bound protein kinase that is activated when GRP78 dissociates from it. Phosphorylation of IRE1 activates its endonuclease activity, which processes the XBP1 mRNA to produce a potent transcription factor. IRE1 endonuclease activity can also degrade ER-localized mRNA. The cytosolic domain of IRE1 can also bind TRAF2, which modulates the activity of two pro-apoptotic kinases, JNK and ASK1.

under stress conditions.

The IRE1 signaling

Inositol-requiring enzyme 1 (IRE1), a Ser/Thr protein kinase and endoribonuclease, regulates the most evolutionarily conserved branch of the ER stress signaling network [132, 133]. The ER luminal domain of IRE1 can bind BiP/GRP78, which prevents IRE1 oligomerization and maintains it in an inactive state [134-136]. Upon ER stress induction, BiP dissociates from IRE1, allowing it to oligomerize [137]. Moreover, IRE1 also contains a luminal MHC-like domain that may directly interact with misfolded proteins [130, 138]. Recent studies suggest that at least in yeast direct interaction of Ire1p with misfolded proteins is essential for orienting the Ire1 oligomer into a proper configuration to activate its kinase and ribonuclease activities [139]. In mammals, it appears that dissociation of BiP/GRP78 from IRE1 is sufficient to activate it [140]. IRE1 activation results in the unconventional splicing of the mRNAs encoding the transcription factor XBP1 in mammals or Hac1p in yeast [141, 142-144], which results in the production of an activated form of the transcription factor and subsequent upregulation of genes involved in protein folding, degradation and

trafficking. This improves both the ER folding capacity and ERAD efficiency of the cell. Lastly, recent studies reveal that IRE1 activation can activate autophagy [145], which presumably routes a fraction of misfolded ER proteins to the lysosome for degradation. These activities act together to restore ER proteostasis and thereby promote cell vitality under stress conditions.

In addition to its pro-survival actions, IRE1 signaling in mammalian cells also has a pro-apoptotic role. The cytosolic domain of IRE1 was reported to interact with the Ser/Thr kinase, tumor necrosis factor (TNF) receptor-associated factor 2 (TRAF2), which modulates the c-Jun N terminal kinase (JNK) activity to induce cell death [146]. The IRE1-TRAF2 interaction also activates the apoptosis signal-regulating kinase 1 (Ask1) [147], which may contribute to the neuronal cell death induced by polyglutamine repeat-containing proteins. Moreover, a recent study shows that IRE1 autophosphorylation activates its RNAase activity, which not only catalyzes the splicing of XBP1 mRNA, but also induces the decay of many ER-localized mRNAs that encode secretory and membrane proteins [148, 150]. Interestingly, the degradation of ER-localized mRNAs and XBP1 splicing are mechanistically uncoupled events that involve distinct modes of IRE1 RNAase actions. Accordingly, while overexpression of wild type IRE1, which induces both mRNA decay and XBP1 splicing, triggers apoptosis, an IRE1 mutant that only induces XBP1 splicing in the presence of a pseudokinase activator compound fails to induce cell death. The degradation of mRNAs encoding ER chaperones and other secretory proteins may be beneficial to cell survival at the initial phase of ER stress, as it helps reduce protein flux to the ER. However, it may become detrimental if persistent mRNA degradation leads to a depletion of ER chaperones, which would impair the ER folding capacity.

The PERK-eIF2α-ATF4 cascade

In addition to IRE1, the mammalian UPR also activates another ER-localized transmembrane protein kinase termed PERK [protein kinase RNA (PKR)-like ER kinase] [151]. The ER luminal portion of PERK contains a stress-sensing domain that is both structurally and functionally related to that of IRE1 [135]. The cytoplasmic portion of PERK also has a protein kinase domain that is activated when PERK oligomerizes in stressed cells. Activated PERK phosphorylates the α-subunit of the eukaryotic translation initiation factor-2 (eIF2α), resulting in globally attenuated protein translation. This reduces the protein load the ER [152]. In addition to translation inhibition, PERK also contributes to the adaptive stress response by influencing the gene expression landscape. A key player in this process is the cAMP response element-binding (CREB) transcription factor ATF4, which is selectively activated at the translational level despite the global translation inhibition in response to eIF2α phosphorylation. ATF4 upregulates the expression of many pro-survival genes including many ER chaperones, which helps cells adapt to the misfolding stress in the ER [152].

Interestingly, the ATF4 target genes also include some well-known pro-apoptotic factors such as CHOP/GADD153 and Noxa (see below) [153-155]. CHOP/GADD153 is a transcription factor whose induction inhibits cell proliferation and induces apoptosis. CHOP can act as both a transcriptional repressor and activator. For example, it suppresses the expression of the multi-domain anti-apoptotic protein Bcl-2 [156], but upregulates the expression of GADD34, a subunit of a phosphatase complex that dephosphorylates eIF2α [154]. It was proposed that reduced eIF2α phosphorylation by the CHOP-mediated negative feedback loop may increase cell sensitivity to ER stress-induced apoptosis. In support of this model, GADD34-deficient cells have a sustained level of phosphorylated eIF2α and are more resistant to cytotoxicity imposed by ER stress [154]. Moreover, a chemical inhibitor of the eIF2α phosphatase, called salubrinal, also protects cells against ER stress-induced apoptosis [157]. CHOP also regulates the expression of the BH3-only pro-apoptotic protein Bim (see below) [158]. Thus, a number of transcriptional changes are induced as a result of PERK activation that impact the cellular choice between survival and apoptosis.

The mechanism that translates the complex PERK-eIF2α-ATF4 signaling into either a pro-survival or a pro-death cellular action is unclear. It has been proposed that the duration of PERK signaling relative to that of XBP1 activation may influence this critical decision-making process. In response to prolonged ER stress, XBP1 splicing is attenuated, while the PERK activity is maintained [159]. This may shift the cell towards a more destructive fate given the above mentioned connections between prolonged PERK signaling and the activation of pro-apoptotic proteins.

The Bcl-2 protein family

Mitochondria-initiated apoptosis is regulated by the Bcl-2 family proteins, which include Bax and Bak (two pro-apoptotic effectors that participate directly in the permeabilization of the mitochondrial outer membrane), anti-apoptotic factors such as Bcl-2, Bcl-xL, Mcl-1, and Bfl/A1, and factors termed BH3-only proteins. Many of the first two classes of Bcl-2 family proteins belong to the so-called tail-anchored proteins that contain a

carboxyl-terminal transmembrane domain, which localizes these proteins to the mitochondrial outer membrane. In contrast, the BH3-only proteins do not have any transmembrane segment and contain only a Bcl-2 homology domain 3 (BH3). However, they can interact with both pro-apoptotic and pro-survival Bcl-2 family proteins to regulate their activities. Although some BH3-only proteins may promote apoptosis by antagonizing the function of the pro-survival Bcl-2 proteins, recent studies have suggested a direct role for at least some BH3-only proteins in the activation of Bax and Bak [160-162]. Upon the binding of a BH3 helix to Bax, Bax undergoes a conformational change that mobilizes its carboxyl terminal helix for membrane translocation. Importantly, the BH3 domain of Bax is now exposed, which can interact with a yet-to-be activated Bax monomer to propagate the death signal within a Bax homo-oligomeric assembly [163].

Several Bcl-2 family proteins have established connections with the UPR signaling at the ER. It is apparent that many of these BH3 proteins act as downstream executors to initiate cell death in response to an overwhelming ER stress signal. In addition, direct interactions between some Bcl-2 family proteins and ER stress signaling molecules were observed. Specifically, the pro-apoptotic Bcl-2 family proteins Bax and Bak were found to be physically present at the ER membrane in addition to their mitochondrial localization. These ER-associated Bcl-2 family proteins were reported to interact with IRE1 to modulate its signaling properties upon ER stress induction. Accordingly, in Bax and Bak double knockout mice, the IRE1 branch of the UPR signaling was impaired [164]. Along the same line, an ER-localized natural Bak inhibitory polypeptide (BI-1) also forms a complex with IRE1, and this interaction suppresses IRE1 signaling during ER stress [165]. The ER pool of Bax and Bak may also regulate an ER-to-mitochondria calcium flux in response to stress signals, which may also contribute to apoptosis [166, 167].

The expression of many BH3-only proteins including Bim, Puma and Noxa, is induced by ER stress. The mechanisms by which UPR regulates the expression of these genes are variable. Even for a given BH3-only protein, its induction can be mediated by different mechanisms in different cell types or in response to different stress stimuli. For example, Noxa upregulation in mouse embryonic fibroblasts (MEFs) or human melanoma cells treated with the ER stressors thapsigargin or tunicamycin appears to be dependent on the tumor suppressor p53 [168, 169]. However, in neuroblastoma and melanoma cells treated with the chemotherapeutic agents feretinide or in transformed 293T cells exposed to the ERAD inhibitors Eeyarestatin I (EerI) and bortezomib, Noxa induction is independent of p53 [155, 170-172]. Instead, Noxa up-regulation involves the ER stress-regulated transcription factors ATF4 and ATF3 [155, 170] (see below). In addition, the oncogene c-Myc was shown to be required for bortezomib-induced Noxa expression [172]. Likewise, both p53-dependent and -independent mechanisms have been reported for the induction of PUMA during ER stress [168, 169, 173]. For Bim, it has been shown that ER stress elevates Bim protein levels by two independent mechanisms. ER stress can reduce proteasomal degradation of Bim, while at the same time the UPR signal can activate CHOP, which in turn upregulates Bim mRNA expression [158]. Clearly, components of the mitochondria-initiated apoptotic pathway are closely integrated with the UPR program.

Targeting ER proteostasis in cancer therapy

Cells bearing different secretory capacities and basal levels of ER stress can differ significantly in sensitivity to ER stress-induced cell death. Certain cancers such as multiple myeloma are particularly sensitive to ER stress-induced cell death, perhaps because these cells constantly carry a high secretory load due to their specialized role in antibody production. Thus, a therapeutic window exists that allows some ER stress inducers to selectively kill these malignant cells without imposing significant damage to surrounding healthy cells. In this section, we discuss the potential cancer treatment strategies that target ER proteostasis.

The proteasome

Bortezomib (Velcade™, also named PS-341) is a first-in-class proteasome inhibitor that acts at least in part by targeting ER proteostasis to treat cancer. Bortezomib is a peptide boronic acid analog initially designed to inhibit the chymotrypsin activity of the proteasome by mimicking substrate binding. It was shown to be a potent inhibitor of the pro-inflammatory NFκB signaling pathway due to inhibition of IκB degradation [174]. Subsequent studies in the National Cancer Institute (NCI) set of 60 cancer cell lines demonstrated broad spectrum anti-tumor activities for bortezomib [175], which were later confirmed in a mouse xenograft model [176]. Following rigorous clinical testing, the United States Food and Drug Administration (FDA) approved the use of bortezomib for the treatment of multiple myeloma in 2003 and later extended its use to mantle cell lymphoma.

The approval of bortezomib by the FDA as an anti-cancer agent has fueled the interest in understanding the mechanism underlying its anti-cancer activity. Given

the diverse cellular activities of proteasomal substrates, it is not surprising to learn that many cellular pathways contribute to bortezomib-induced cell death. Moreover, the cellular factors involved in bortezomib-induced cell death can vary among different cell types. For example, bortezomib acts through p53 to induce growth arrest and cell death in mammary epithelial cells [177], but in PC-3 prostate cancer cells, the p53 function becomes dispensable for bortezomib-induced cytotoxicity [178]. It was initially thought that inhibition of the pro-survival NFκB signaling pathway might be the predominant cause of bortezomib-induced cell death. However, subsequent studies comparing the activities of bortezomib with an NFκB-specific inhibitor showed that NFκB inhibition by itself cannot fully account for the anti-tumor activity of bortezomib [179].

Recent studies have underscored the importance of the pro-apoptotic protein Noxa in bortezomib-mediated cytotoxicity. In bortezomib-treated cells, Noxa mRNA and protein levels are dramatically increased. Knock down of Noxa in a variety of transformed cell lines renders resistance to bortezomib-induced apoptosis [172, 180-184]. Although Noxa was initially discovered as a transcriptional target of p53 [185], the mechanism that activates Noxa in bortezomib-treated cells appears to be independent of p53. Instead, the UPR plays a critical role. Specifically, bortezomib activates the PERK branch of the UPR, leading to an upregulation of the transcription factors ATF4 and ATF3, which form a hetero-oligomer on the Noxa promoter. In addition, bortezomib attenuates ubiquitination of histone H2A to relieve its inhibitory effect on Noxa transcription [155]. These observations establish an important link between ER stress and the anti-cancer action of bortezomib. Consistent with this idea, several recent studies show that the UPR induction upon proteasome inhibition is essential for bortezomib-induced cytotoxicity [186-189]. Together, these studies highlight an important role for the UPR pathway in the anti-cancer action of bortezomib, and suggest that ER proteostasis can be targeted in anti-cancer therapies.

p97 and associated deubiquitinating enzymes

As discussed above, the ERQC program employs several independent mechanisms for substrate recognition and retrotranslocation from the ER. However, the retrotranslocation of almost all ERAD substrates converges on the p97 ATPase for membrane extraction and for subsequent transfer to the proteasome [90, 97]. Accordingly, inhibition of p97 and the proteasome usually generates a more pronounced effect on ER homeostasis than interference with an upstream ERAD step. Therefore, it is conceivable that p97 may be a potential target for cancer therapy. In support of this hypothesis, recent studies have demonstrated that an ERAD-specific inhibitor, termed Eeyarestatin I (EerI), which targets p97, can induce cell death in hematologic cancer cells via a mechanism similar to that of bortezomib [155, 190]. Like bortezomib, EerI induces ER stress and causes downregulation of histone H2A ubiquitination, which result in Noxa activation and cell death. The anti-tumor profile of EerI in the NCI 60 cancer cell lines is similar to that of bortezomib (Figure 3) [175]. Importantly, EerI can dramatically synergize with bortezomib to induce cancer cell death [155]. These results show that inhibition of p97 can achieve a similar anti-cancer effect as blocking the proteasome. Indeed, interest in searching for more potent inhibitors that block p97 ATPase activity is mounting [191].

EerI is a bi-modular compound that is comprised of two functionally independent domains. An aromatic module targets EerI to the ER membrane, allowing a nitrofuran-containing (NFC) module to directly bind to p97 and to interfere with its membrane-associated functions. As a result, EerI is a much more specific disruptor of ER proteostasis compared to a compound that only has the NFC domain [192]. EerI does not block the nucleotide hydrolysis cycle of p97. Instead, the binding of EerI to p97 induces a conformational change in p97, which may alter its interactions with cofactors [192]. The phenotypic consequence of EerI binding to p97 is complex. In cells exposed to EerI for a prolonged period, p97-mediated retrotranslocation is completely blocked [193]. However, in the initial phase of EerI treatment, a major consequence is the disruption of ubiquitin homeostasis as polyubiquitinated proteins accumulate. This phenotype may be due to an indirect effect of EerI on the action of some deubiquitinating enzymes bound to p97 [190]. These deubiquitinating enzymes can act either upstream (e.g., YOD1) [194] or downstream (e.g., ataxin-3) of the p97 ATPase cycle to modulate the ubiquitin contents on retrotranslocation substrates [110]. EerI's capability to influence deubiquitinating activities associated with p97 is critical for its cytotoxic action, and disruption of ubiquitin homeostasis due to insufficient deubiquitination may be the cause for the loss of ubiquitinated histone H2A. In this regard, chemical inhibitors directly targeting p97-associated deubiquitinating enzymes may achieve similar anti-cancer effects as EerI.

Other anti-cancer targets

Given the essential role of the IRE1 endonuclease activity in promoting cell vitality under ER stress conditions, it has been thought that inhibitors targeting this nuclease activity could have anti-cancer activities [195]. Indeed, Feldman and Koong recently reported the iden-

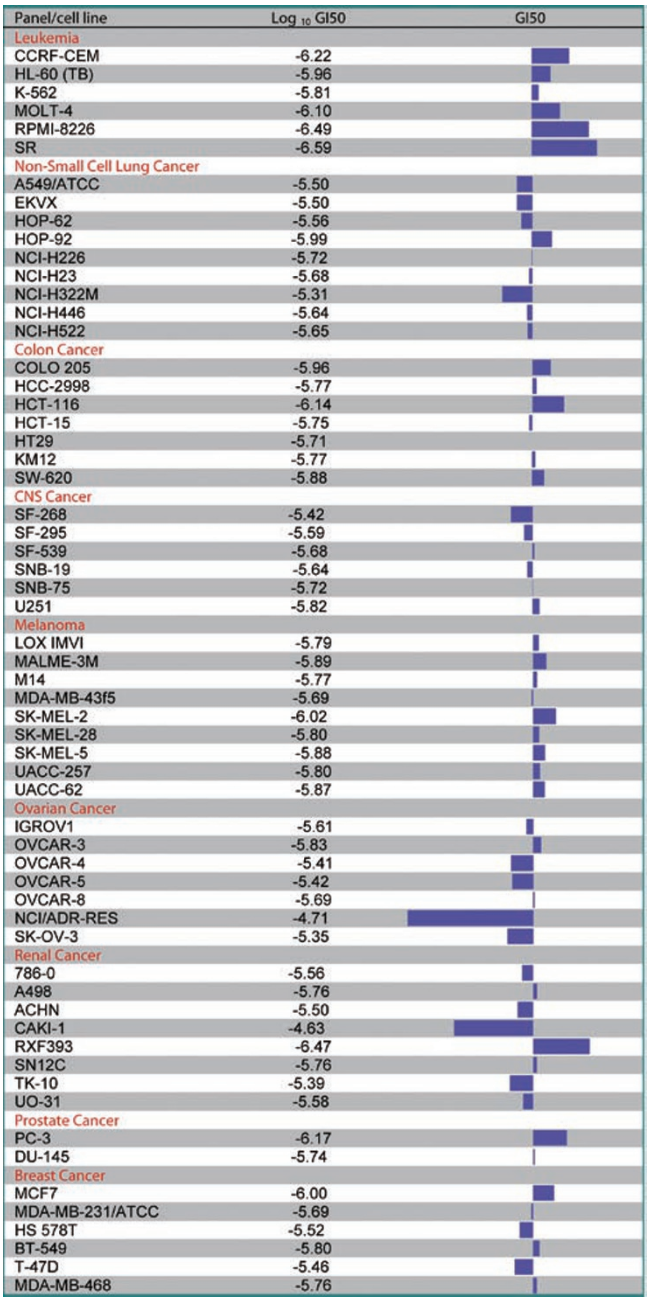

Figure 3 The p97 inhibitor Eerl has broad spectrum anti-cancer activities. The NCI 60 cancer cells were treated with Eerl at 5 doses ranging from 10 nM to 100 μM. Concentration-response curves from two independent experiments were used to calculate the average GI50 (the concentration of drug required to obtain 50% of growth inhibition) for each cell line. The graph shows the GI50 of each cell line relative to the mean value.

tification of the first-in-class IRE1 endonuclease inhibitors, which they named Irestatins. These compounds were shown to be potent cell death inducers, particularly for oxygen-starved cancer cells. One of these compounds was also shown to have anti-cancer activity in a mouse xenograft model [196]. Another potential anti-cancer target in the UPR signaling network is the PERK kinase [197] given the precedent success in developing kinase inhibitors for cancer therapy. However, no potent inhibitor for this enzyme has been reported so far despite some serious efforts from several research groups.

Perspectives

Researchers over the past decade have made tremendous progress towards a better understanding of how eukaryotic cells cope with protein misfolding in the ER and how deregulation of ER proteostasis can cause cell dysfunction and death. The knowledge obtained to date has, no doubt, fueled the development of novel cancer therapeutics concepts, which are now being transformed into potential new medicines. Nonetheless, many fundamental aspects regarding ER proteostasis regulation remain poorly understood. For example, it is still unclear how the cell distinguishes misfolded ER proteins from those in the midst of the folding process. How misfolded proteins are moved across the ER membrane during retrotranslocation is also unknown. In addition, we are almost completely ignorant about the regulation of ERAD capacity in various cell types or in cells facing different stress challenges. In particular, understanding the precise mechanism that governs the life or death decision-making process of stressed cells, such as those stressed by bortezomib, may provide a better guide to developing new treatments that are more effective in battling against cancer.

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