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Methods for studying endoplasmic reticulum stress

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Abstract: The endoplasmic reticulum (ER) comprises a network of tubules and vesicles that constitutes the largest organelle of the eukaryotic cell. Being the location where most proteins are synthesized and folded, it is crucial for the upkeep of cellular homeostasis. In addition, it is the largest ionic calcium reservoir in cells, tightly regulating the levels of this second messenger according to cellular necessities. Disturbed ER homeostasis triggers the activation of an intricate and conserved molecular machinery, termed the unfolded protein response (UPR). Given the impact of this signaling network upon an extensive list of cellular processes, ER stress is involved in the onset and progression of multiple diseases, including cancer and neurodegenerative disorders. There is, for this reason, an increasing number of publications focused on characterizing and/or modulating ER stress, which have resulted in a wide array of techniques employed to study ER-related molecular events. This review aims to sum up the tools available design a study of this nature.

Keywords: unfolded protein response, endoplasmic reticulum, PERK, IRE-1, ATF4

1. Introduction

1.1. Molecular basis of endoplasmic reticulum stress

The endoplasmic reticulum (ER) is the largest organelle of the eukaryotic cell, comprising an intricate network of tubules and branches that emerge from the nucleus, including the nuclear envelope, and are distributed throughout the cytoplasm, forming a highly dynamic structure that is continuously rearranging. From a structural point of view, the peripheral ER (i.e. outside the nuclear envelope) includes the rough ER, constituted by sheets, and the smooth ER, constituted by tubules, each structure being related to the type of processes that takes places at a given ER site. The rough ER is easily distinguished from the smooth ER due to the density on ribosomes it presents on its cytosolic surface, while the smooth ER lodges few ribosomes and presents smoother and more curved surfaces [1-3].

Analogously to its volume in the cell, the ER plays a role of the outmost importance in the homeostasis of a wide array of cellular processes, even though it is classically associated to its main function: the *de novo* synthesis and folding of proteins (mainly in the rough ER). It is in the ER that the synthesis of the vast majority of the proteins takes place, mainly secreted and transmembrane proteins, but also some cytosolic ones. In the presence of a signal recognition particle (SRP), the ribosomes containing mRNAs to be translated are recruited to bind the surface of the ER and proceed with their translation [1]. The next step is protein folding, which is the process that encompasses the formation of disulfide bonds between cysteine residues of peptides. Other than this, post-translational modifications, such as *N*-linked glycosylation, also take place [4]. The ER is also in charge of newly-synthesized protein transportation and delivery to their target sites through the secretory pathway, which involves the

rough ER, ER exit sites, the ER-to-Golgi intermediate compartment, the Golgi apparatus itself and post-Golgi carriers that transport the proteins to their final target site [5]. Although on a smaller scale, the synthesis and transportation of phospholipids and steroids also takes place in this organelle, mostly in the smooth ER [1]. However, the maintenance of proteostasis is not the only role that the ER plays in the cellular upkeep. This organelle is the main calcium reservoir of the cell, and it is also in charge of the tight regulation of its levels, a requirement that the cell needs in order to be able to maintain the homeostasis of multiple events, including cell proliferation, differentiation, metabolism, apoptosis and gene expression. For these reasons, it is crucial for the development of an eukaryotic organism [6].

In cases where homeostasis of any of the aforementioned ER-based processes is disturbed, the resulting stress conditions may compromise this organelle's ability to correctly assemble and process peptides. Eventually the amount of newly synthesized proteins surpasses the amount that is exiting the ER, leading to the accumulation and aggregation of misfolded and/or unfolded proteins in the ER lumen. At this point, the ER counters by triggering the unfolded protein response (UPR), a chain of molecular events that has evolved towards attempting to restore homeostatic conditions when stress at the ER is recognized. Briefly, UPR i) decreases the rate of protein synthesis, in order to alleviate protein overload, while promoting the correct processing of synthesized proteins and preventing aggregation in the ER lumen, and ii) promotes endoplasmic reticulum-associated degradation (ERAD) and boosts the expansion of the ER network. Nevertheless, if the stress upon the ER is of such intensity or duration that it cannot be repaired, the UPR signaling switches from survival to pro-death

mechanisms [7]. The major molecular events resulting from UPR activation will be explained hereafter.

As mentioned before, precise calcium regulation is required to maintain homeostatic conditions in the ER. For this reason, it is important to briefly explain the fundamental mechanisms of calcium concentration upkeep, for which the ER requires three types of proteins: pumps to import Ca^{2+} ions from the cytosol, luminal proteins to bind calcium and channels to release these ions according to the cellular context, controlled by an electrochemical gradient. The normal concentration of calcium outside the cell can be as high as 2 mM, while in the cytosol it is estimated to be 100 nM on average [6, 8]. Several other calcium-binding ER proteins are involved on calcium buffering, like the binding immunoglobulin protein (BiP) and other chaperones, such as GRP94 and calreticulin, as well as proteins from the protein disulfide isomerase (PDI) family. Among these, BiP is particularly relevant, since it is a major player of the UPR and calcium is required to bind nascent proteins. One of the better known pumps involved in this process is the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA). This ATPase imports two calcium ions from the cytosol for each ATP molecule hydrolyzed [9]. Concerning the exit of calcium ions from the ER lumen, it is mainly regulated by a second messenger named inositol 1,4,5-trisphosphate (IP_3), sensed by the IP_3- receptor (IP_3R) [10]. Given its role in cellular physiology, temporal and/or spatial imbalances on calcium upkeep may lead to UPR activation.

The role of BiP, however, is broader than its involvement in calcium homeostasis; in fact, it is a master regulator of the UPR. This member of the heat shock protein 70 kDa family (HSP70), also termed glucose regulated protein 78 (GRP78), senses the

accumulation of misfolded polypeptides in the ER by binding their exposed hydrophobic residues to its *C*-terminal domain, thus acting as a chaperone. On the other hand, it possesses an ATP catalytic site at its *N*-terminal domain. When misfolded proteins are sensed on its *C*-terminal domain, ATP is hydrolyzed at the *N*-terminal domain, increasing the affinity of the *C*-terminal domain towards its incorrectly processed substrate, thus keeping it in the ER lumen for a more extended period of time, in order to allow for other mechanism to intervene and correct the mistake. Under homeostatic conditions, BiP localizes on the ER lumen, binding the luminal domain of all of the three major transmembrane proteins that sense ER stress. The classic UPR model indicates that, upon ER stress and increased levels of misfolded proteins, BiP detaches from these sensors, subsequently leading to their activation [11, 12]. However, more recent models propose that the major UPR sensors may be able to bind and recognize unfolded proteins in a BiP-independent manner [13]. These sensor proteins are the protein kinase RNA-like endoplasmic reticulum kinase (PERK), the inositol-requiring enzyme 1 (IRE1) and the activating transcription factor 6 (ATF6). Their mechanisms of action will be detailed below and are schematized in **Figure 1**.

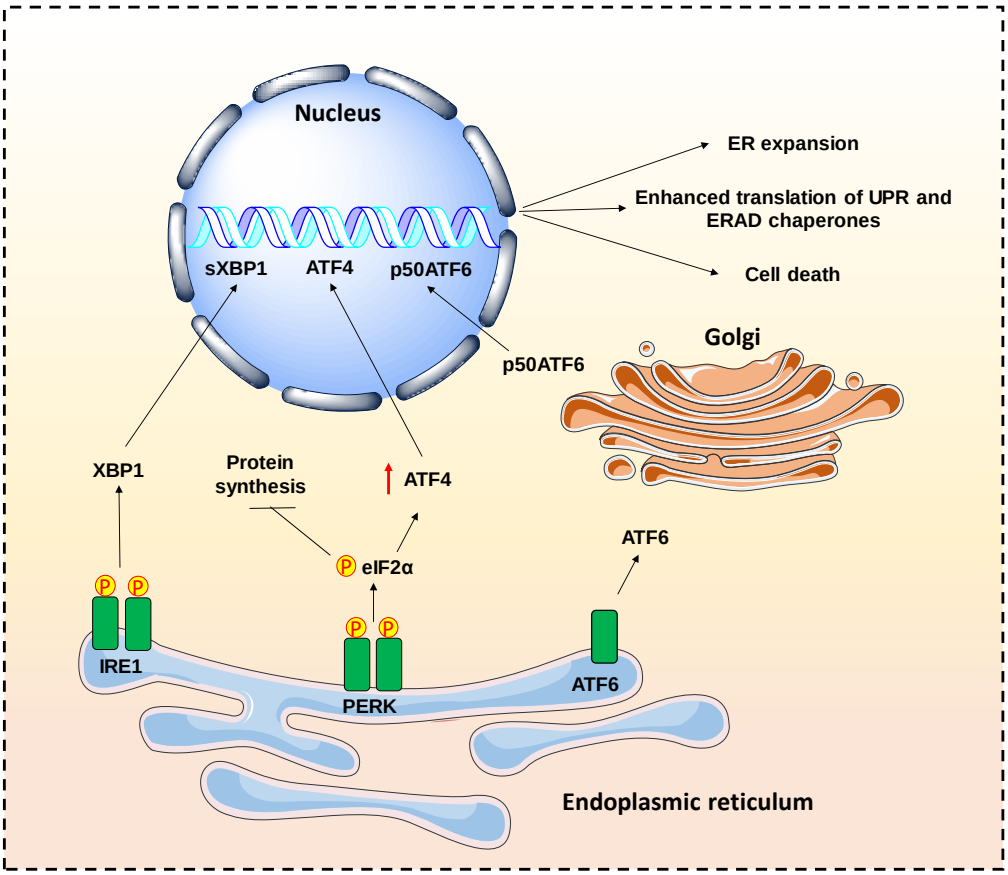

Figure 1. Major molecular machinery involved in the activation of the unfolded protein response (UPR).

1.1.1. PERK → eIF2α → ATF4 → CHOP

The first of the three major branches of the UPR that will be discussed here is initiated by PERK. When BiP dissociates from this enzyme, it is activated by homodimerization and trans-autophosphorylation. Then, it phosphorylates the α subunit of the eukaryotic initiation factor 2 (eIF2), which, in turn, will attenuate protein translation in order to decrease the load of proteins in the ER lumen that await processing. This pro-survival signaling may allow the cell to cope with the stress conditions it is under, and help restore homeostasis. However, if these stress

conditions are too severe or last for too long for this corrective path to rescue the cell, this branch can switch to pro-apoptotic signaling, by activating the activating transcription factor 4 (ATF4). This transcription factor induces the expression of genes like the one encoding for the CCAAT-enhancer-binding protein homologous protein (CHOP), which triggers apoptosis [14]. Although the mechanisms through which CHOP triggers regulated cell death are still not completely understood, it is accepted that there is a correlation between CHOP-induced cell death and downregulation of the pro-survival protein B-cell lymphoma-2 (Bcl-2), concurrently with caspase-3, Bcl-X (Bcl-2 like protein 1), Bax (Bcl-2 associated X-protein), GADD34 (growth arrest and DNA damage-inducible protein), EOR1α and TRB3 upregulation [15, 16]. GADD34 restores protein synthesis rates by stimulating eukaryotic initiation factor 2-alpha (eIF2α) dephosphorylation, which, in turn, will result in proteotoxicity by increased ROS production and ATP depletion [16]. TRB3 is another CHOP-induced gene known to be involved in cell death and to downregulate its own expression, by repressing CHOP activity [17]. Finally, EOR1α sets off the CHOP-EOR1α-IP_3R-calcium-calcium/calmodulin-dependent protein kinase II (CaMKII) pathway, which culminates in mitochondria permeabilization and release of Bax/Bak [15].

Phosphorylation of eIF2α favors the translation of a few genes, designated by integrated stress response (ISR) genes [18]. These genes include ATF4 and CHOP. ATF4 is a basic leucine zipper transcription factor responsible for enhancing the expression of genes related to amino acid metabolism, nutrient uptake, anti-oxidation, protein folding and apoptosis, working along with other transcription factor in the UPR [19, 20]. One of the genes related to apoptosis which expression is enhanced by ATF4 is CHOP, also a transcription factor. As is it associated to triggering stress-induced

apoptosis, its basal levels are low. However, they quickly rise upon ATF4 activation, even though it can also be induced in a PERK-, IRE1- or ATF6-dependent manner. This transcription factor is crucial for ensuing ER stress-induced apoptotic signaling [21]. Molecular events ensuing the activation of the PERK branch of the UPR are schematized on **Figure 2**.

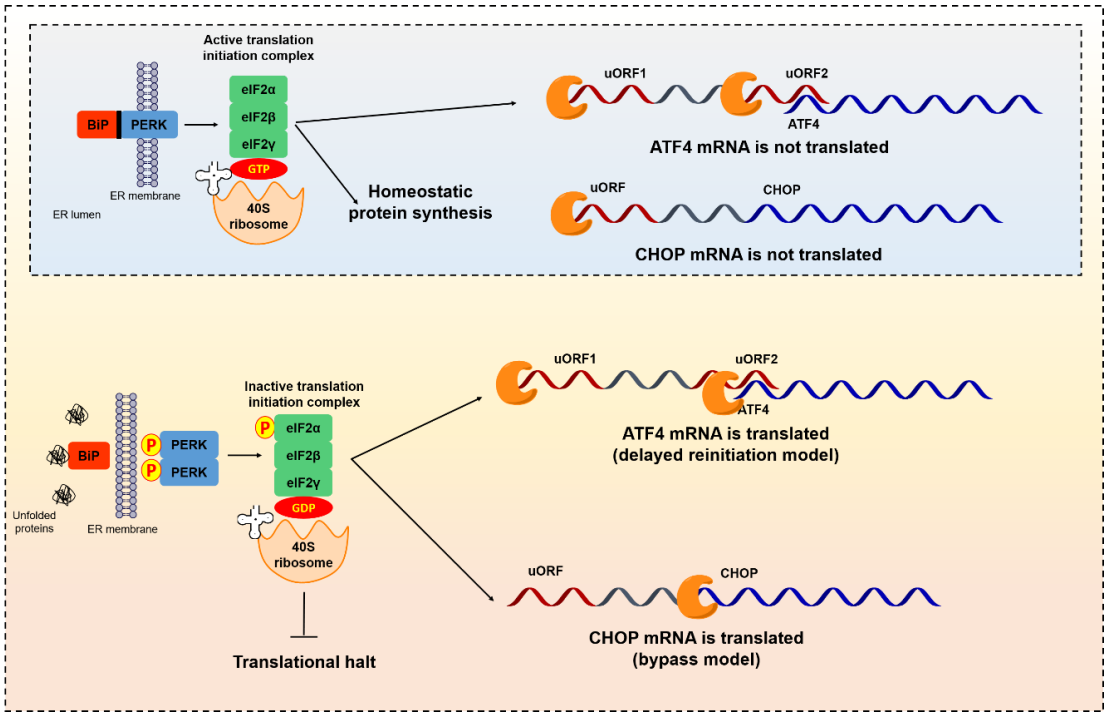

Figure 2. Molecular events ensuing the activation of the PERK branch of the UPR.

1.1.2. IRE1 → XBP1s / IRE1 → RIDD

This branch of the UPR is initiated upon IRE1 activation through autophosphorylation and homodimerization or oligomerization. Of all the UPR transducers, IRE1 is the most conserved [22]. Not unlike PERK, this branch can also promote survival or trigger cell death, as summarized in **Figure 3**. IRE1 has RNAse activity, and it splices the mRNA encoding for X-box binding protein 1 (XBP1), clearing

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its introns and thus leading to the formation of spliced XBP1 (XBP1s) and subsequent translation into a transcription factor. This requires the cooperation of IRE1 units and constitutes its pro-survival action [22-26]. Its pro-cell death action, however, can be carried out by a single unit of the enzyme, and consists of a process termed regulated IRE1-dependent decay (RIDD), ultimately resulting in cell death. RIDD involves the preferential cleavage of ER-associated mRNAs encoding for growth-promoting proteins, the resulting fragments being rapidly degraded by the action of exoribonucleases [22-24]. It is worth mentioning that IRE1 occurs in two different isoforms (IRE1α and –β) and, even though both are activated upon ER stress conditions, IRE1α is the most relevant, since it occurs ubiquitously in the organism, unlike IRE1β, which is restricted to gastrointestinal and respiratory epithelial cells [22, 27, 28].

XBP1s is a potent transcription factor that binds the endoplasmic reticulum stress response element (ERSE) and unfolded protein response element (UPRE) and, consequently, enhances the expression of genes corresponding to the ER machinery, for instance, BiP. Although its cleavage is associated to the IRE1 pathway, its expression can also be induced by ATF6 [29, 30].

1.1.3. ATF6

The transcription factor ATF6 is embedded in the ER membrane, being released upon accumulation of misfolded proteins in the ER lumen. Once activated, it translocates to the Golgi apparatus, where it is cleaved by site-1 and site-2 proteases,

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originating p50ATF6. It then translocates to the nucleus, where it is responsible for inducing the transcription of ER chaperones by binding ERSE, pursuing to restore homeostasis [31-34]. Under optimal conditions, ATF6 is maintained in the ER binding BiP, which inhibits Golgi localization signals [35]. The events resulting from the activation of this transcription factor are depicted on **Figure 3**.

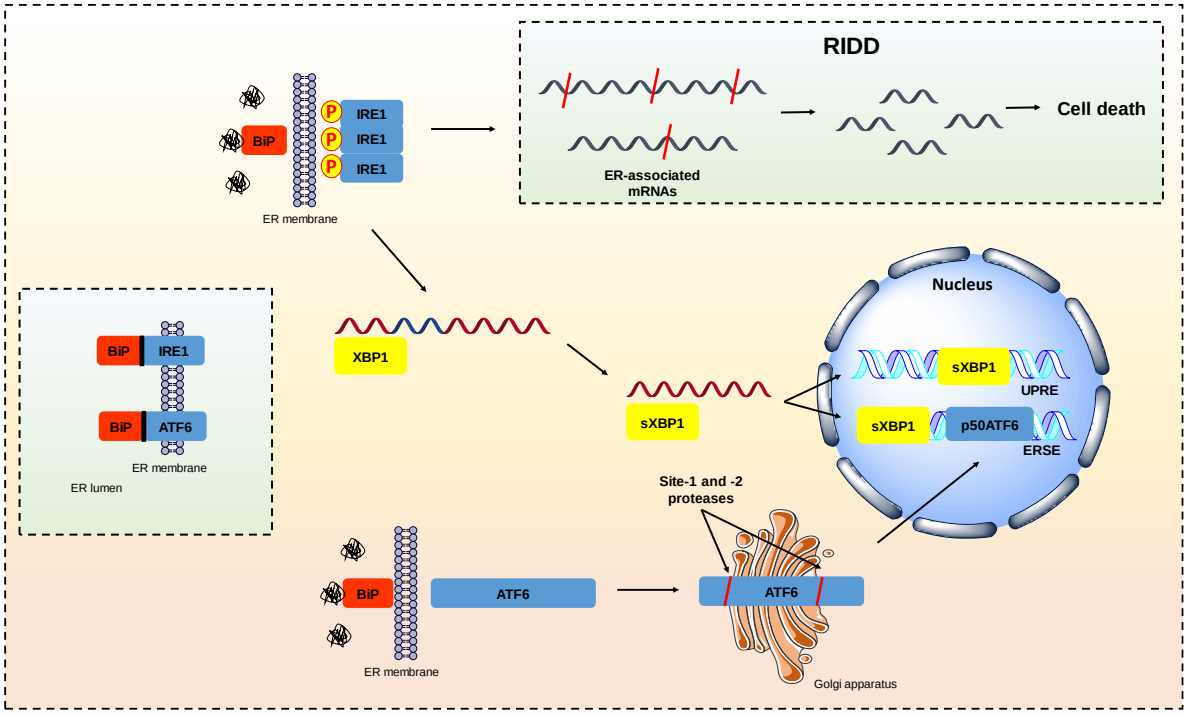

Figure 3. Molecular events ensuing the activation of the IRE1 and ATF6 branches of the UPR.

As mentioned before, prolonged ER stress can lead to the activation of pro-apoptotic proteins from mitochondria. The ER and mitochondria communicate with each other through zones collectively termed mitochondria-associated ER membrane (MAM). Both organelles are dynamic structures that can relocate within the cytoplasm, by moving through the cytoskeleton according to the needs of the cell. For this reason, MAM surface increases under ER stress, particularly on the perinuclear region. MAM

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encloses a significant portion of the outer mitochondrial membrane (OMM), as much as 20%. At these sites, there are direct channels for calcium transfer from the ER lumen into the mitochondria, since chaperone GRP75 directly links IP_3-R on the ER membrane and the voltage-dependent anion channel (VDAC) on the OMM. Under ER stress, this transfer may be overwhelming for the mitochondria, leading to the depolarization of the inner mitochondrial membrane (IMM) and potentially triggering regulated cell death mechanisms, as depicted on **Figure 4** [36].

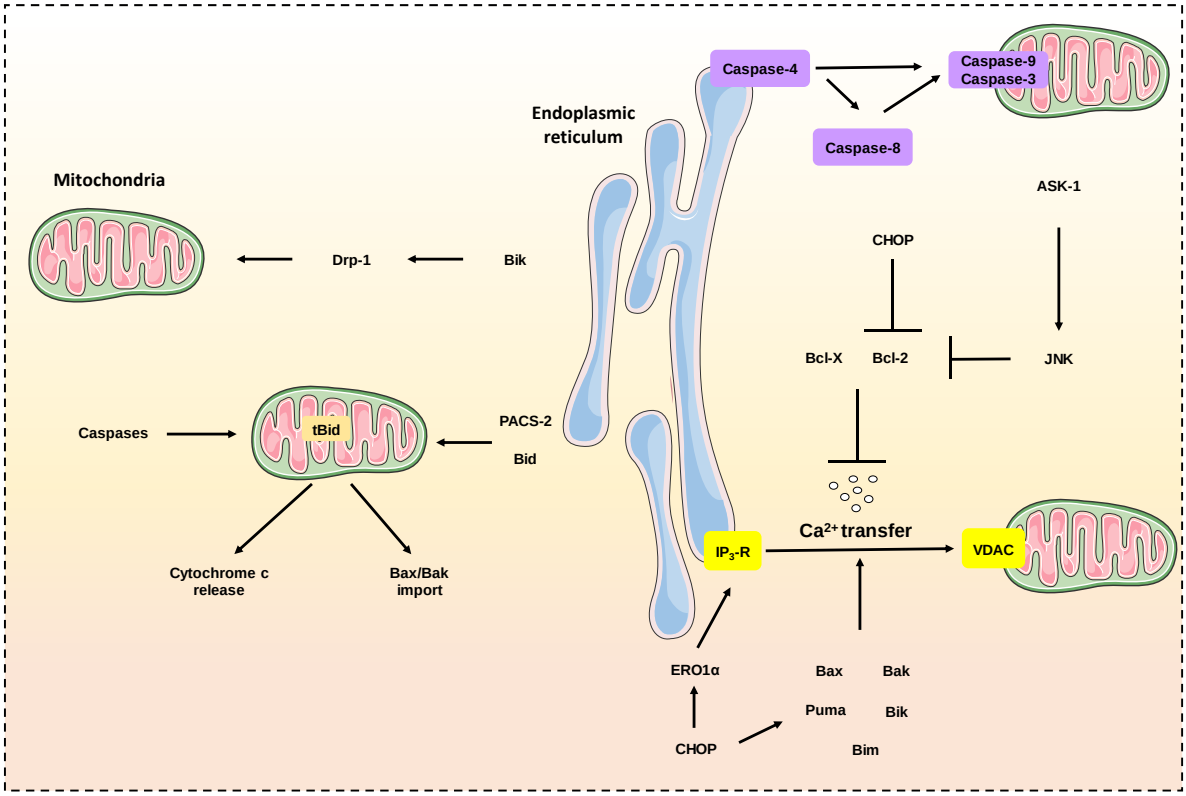

Figure 4. ER-induced mitochondria-dependent regulated cell death. Dynamin-related protein 1 (Drp1) mediates mitochondrial membrane fission. Phosphofurin acidic cluster sorting protein 2 (PACS-2) is necessary for the connection between the ER and mitochondria. Apoptosis signal-regulating kinase 1 (ASK1) is a member of the MAPK

family, as well as dual specificity mitogen-activated protein kinase kinases -4 and -7 (MKK4/7).

Depolarization of IMM disrupts its otherwise low permeability and results in the release of cytochrome *c* and other pro-apoptotic factors. Such proteins include the BH3-only proteins of the Bcl-2 family Bid, Bad, Bim and Puma, which bind other proteins of the Bcl-2 and block their pro-survival action [37, 38]. Apoptotic cell death is classically divided in two pathways: the intrinsic, or mitochondrial, and the extrinsic, or death receptor pathway. Nowadays, however, the ER stress-mediated pathway may be involved on distinct apoptotic models, namely the cell death receptor, mitochondrial or Fas-mediated apoptosis [39]. ER stress-induced regulated cell death involves the activation of the inflammatory caspase-4 in humans (caspase-12 in rodents [40]), which resides in the ER in homeostatic conditions [41, 42]. Shortly, the involvement of the UPR in the process is known to involve changes on PERK and IRE1α signaling, as well as calcium release from the ER lumen, elements of the Bcl-2 family and MAPK-kinases, namely the c-Jun *N*-terminal kinase (JNK) [16, 43].

A brief summary of the functions of the major proteins involved in the UPR can be found on **Table 1**.

Table 1. Description of the activation and mechanisms of action of the major proteins involved in the UPR.

Protein	Class	Mechanism of action	References

ATF4	Transcription factor	Upregulation of pro-apoptotic genes, such as CHOP or activating transcription factor 3 (ATF3) / upregulation of pro-survival genes like BiP	[44]
ATF6	Transcription factor	Upregulation of the UPR machinery and CHOP	[45, 46]
BiP	Chaperone	Binding of unfolded/misfolded proteins, preventing their exit from the ER lumen	[11]
CHOP	Transcription factor	Apoptosis trigger	[21]
eIF2α	Translation initiation factor	Globally impairment of translation initiation, selectively upregulation of a few UPR proteins	[47]
IRE1	Kinase and RNAse	Splicing of the mRNA encoding for the transcription factor XBP1	[23]
PERK	Kinase	Phosphorylation of eIF2α	[48, 49]
XBP1s	Transcription factor	Binding of ERSE and UPRE, upregulating UPR machinery	[30]

Throughout the already performed attempts to understand and manipulate ER stress, several molecules of different origins were established as modulators of one or more of its underlying mechanisms. The molecules that are most commonly employed in research are listed on **Table 2**, along with a brief description of their mechanism of action, in order to provide insights about their use on assays aiming to analyze ER stress. For a deeper insight on the subject of ER stress modulators, the reader is referred to a few available reviews [50-52].

Table 2. ER stress modulators classified according to their respective mechanism of action.

Molecule	Effect	Mechanism of action	References
4-Phenylbutyric acid	Protector	Chemical chaperone.	[53-56]
Brefeldine	Inducer	Inhibition of ER-Golgi transport.	[57-59]
DTT	Inducer	Impairment of protein folding, preventing disulfide bond formation and reducing partially oxidized intermediates.	[60]

Palmitate	Inducer	Induction of caspase-4, ATF4, CHOP and JNK; trigger of autophagy on mature adipocytes.	[61, 62]
Salubrinal	Protector	Inhibition of eIF2α dephosphorylation, leading to decreased protein translation.	[63-67]
Thapsigargin	Inducer	Irreversible SERCA pump inhibition.	[68, 69]
Tunicamycin	Inducer	*N*-linked glycosylation inhibition.	[70]

1.2. Role of endoplasmic reticulum stress in disease

ER stress has been observed throughout the pathogenesis of a number of severe diseases of considerable prevalence worldwide, such as cancer, neurodegenerative diseases, cardiovascular diseases, obesity and diabetes mellitus. Furthermore, the link between ER stress and inflammation has already been established.

Owing to their enhanced metabolic rates, resulting from aberrant proliferation, cancer cells require particularly efficient folding mechanisms and robust pathways to cope with a high protein synthesis rate. Thus, there is a considerable amount of research concerning the establishment of key players of the UPR as molecular targets in chemotherapy. Nonetheless, there is also evidence indicating that cancer cells

susceptibility to ER stress may vary, being that, in some cases, activation of the UPR can be lethal, while in others it can be beneficial for their survival [71]. The latter is the case of multiple myeloma cells, which require IRE1/XBP1s signaling due to their high protein turnover, and are highly sensitive to inhibition of this pathway. On the other hand, IRE1/XBPs signaling may protect against colon cancer by keeping the homeostasis of that organ [72-74]. These facts provide a wide set of proteins as possible molecular targets for the treatment of cancer, with two possible approaches. One would be increasing misfolded protein in the ER to overwhelm the capacity of the cell to restore homeostasis, and the other to inhibit the pro-survival effect of UPR activation, thus increasing malignant cell vulnerability to anticancer therapy, given that ER stress can be significant in drug resistance phenomena [71]. There is evidence that promoting ER stress may sensitize malignant cells to chemotherapy [75]. Inhibiting ER stress-induced autophagy is reported to attain the same effect [76].

Owing to their enhanced metabolic rates, resulting from aberrant proliferation, cancer cells often meet the conditions for the intervention of the UPR, such as accumulation of misfolded/unfolded proteins, hypoxia or hypoglycemia. Thus, there is a considerable amount of research concerning the establishment of key players of the UPR as molecular targets in chemotherapy.

The UPR also plays a dual role in the physiology of the nervous system: while it is important on the maintenance of its homeostatic conditions and synaptic plasticity, it is also involved in neurodegeneration phenomena. Concerning neurodegenerative diseases, the role of protein aggregation into amyloid fibrils is renowned, including huntingtin in Huntington's disease, amyloid-β in Alzheimer's or α-synuclein in Parkinson's. Being proteinopathies, ER stress is a hallmark of the aforementioned

disorders [7]. Alzheimer's and Parkinson's disease are the two neurodegenerative disorders of highest incidence worldwide [77]. Even though the existence of a relationship between ER stress and neurodegenerative disorders is fairly documented in the literature, a lot remains to be described in what seems to be a vicious cycle of cause-effect, in which ER stress triggers and is triggered by the progression of these diseases. Disturbances in the secretory pathway are known to be significant to the onset and progression of PD [78, 79]. Analogously, ER stress increases and is exacerbated by Aβ42, further contributing to the pathogenesis of AD [80].

ER stress has also been implicated in cardiovascular diseases, namely in arrhythmias, ischemic heart disease, cardiac hypertrophy and heart failure. There is evidence suggesting that PERK activation may be involved in the pathogenesis of arrhythmias, a disease associated to disturbed calcium homeostasis. Regarding ischemic hearts, UPR activation results in increased levels of eIF2α, XBP1, ATF6, ATF4 and BiP, and, in severe circumstances, apoptosis is triggered due to JNK activation. In cases of hypertrophy, and subsequent failure, there are described roles for CHOP, XBP1s and BiP, all of them being increased in affected hearts [46]. ER stress has an important role in the pathogenesis of obesity and diabetes mellitus through several pathways. It is implied in promoting inflammation, inducing insulin resistance, interfering with adipokine secretion and lipid metabolism, contributing to the pathogenesis of steatosis, negatively impacting insulin synthesis and inducing apoptosis of pancreatic β-cells [81]. There is evidence that, in human monocytes, ER stress could be related to insulin resistance, a major hallmark of diabetes. Insulin resistance is known to be induced by resistin. Increased levels of this protein correlate with increased levels of BiP, CHOP, PERK and eIF2α [82].

An increasing body of evidence suggests that chronic inflammation is involved in the course of most of the aforementioned diseases [83]. The most broadly reported mechanism resulting in this activation is related to the fact that the UPR both reduces the synthesis and promotes degradation of several short-lived proteins, including the NF-kB inhibitor (IkB). Its eventual depletion, given its relatively short half-life, contributes to the activation of NF-kB [50, 84]. UPR activation leads to the upregulation of several pro-inflammatory proteins. For instance, CHOP induces the expression of IL-23, while ATF6 induces the transcription of acute phase proteins [85]. Furthermore, the IRE1 branch of the UPR mediates, along with TRAF2, JNK phosphorylation, leading to the secretion of multiple pro-inflammatory cytokines, as are IL-1β, IL-6, IL-23, IFN-β, and TNFα [86].

A specific example of the relationship between the UPR and inflammation is its implication in cystic fibrosis, a genetic disease characterized by chronic airway inflammation and infection. This disease induces an adaptive hyperinflammatory response to inflammatory mediators on the airway epithelia, involving UPR activation and ER expansion [87].

On the regard of the relationship between ER stress and disease, the reader is referred to more specific reviews available in the literature [88-93]. ER stress is virtually omnipresent in chronic diseases. There is, however, a lot that remains unknown about its role. For instance, in some cases, it remains unclear whether the UPR is a cause or consequence of the development of a given disease. To clarify such relationship would be the next step towards the establishment of UPR players as biomarkers of said diseases or as targets for drug development.

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2. Methods for studying endoplasmic reticulum stress

2.1. Pharmacological inhibitors

In order to infer about the role of a given protein in an observed effect, selective pharmacological inhibitors can be employed, impairing the activity of a target protein or pathway and allowing to observe the biological outcome under such circumstances. Samples obtained from incubation with pharmacological inhibitors can subsequently be subjected to all types of assays that will be discussed in the remainder of this work. Most of these molecules have been described in the attempt to create drugs acting specifically in the ER, yet their toxicity deemed their use for research only. At the end of this chapter, a summary of the action of all the mentioned inhibitors is schematized on **Figure 5**, while **Figure 6** presents their respective chemical structures.

2.1.1. PERK/eIF2α

GSK2606414 is a selective inhibitor of PERK. In prion-disease *in vivo* models, GSK2606414 restored protein synthesis, resulting in neuroprotection and prevention of the onset of disease. However, the compound presented marked toxicity, inducing weight loss and hyperglycemia [94].

Salubrinal is a pharmacological inhibitor of eIF2α dephosphorylation that acts by blocking the activity of the protein complex growth arrest and DNA-damaged protein 34 (GADD34)/protein phosphatase 1 (PP1) [67]. There is a considerable number of studies indicating that salubrinal is able to protect cells against a set of toxic compounds that act by triggering ER stress. To determine this *in vitro*, several authors have co-incubated cell lines with the target toxic compound in the presence of

salubrinal, and measured cell viability with MTT assays after a chosen time period. The employed concentrations of salubrinal range between 5 and 100 µM, and, in some cases, this molecule is pre-incubated one or two hours before the target toxic compound [65, 67, 95-98]. Salubrinal has also been tested *in vivo*, being injected *via* intracerebral ventricle to study the impact of ER stress in autophagy induced by brain ischemic preconditioning. The concentrations of salubrinal tested in this study are considerably lower (75-150 pM), since the molecule was supplied directly in the brain of the animals [64]. In different reports, however, salubrinal was administrated through intraperitoneal injection at 1 mg/kg [99, 100], in the intra-articular space at 1.5 mg/kg [96] or intravenously at 2.0 mg/kg [98]. Methippara et al. reported that salubrinal may modulate sleep homeostasis in rats [101]. There are also *in vivo* studies that reported unexpectedly high toxicity of the compound, and thus its use was kept to research only [94, 102].

2.1.2. IRE1

4µ8C is one of the pharmacological inhibitors available that acts upon the IRE1 branch of the UPR. It inhibits both the kinase and RNAse activity of IRE1, by binding its kinase domain at K^{599} and its RNAse domain at K^{907} [103, 104]. *In vitro*, the concentration range of 4µ8C found is quite wide [95, 105-107]. There is a report stating 150 nM as its IE_{50} (50% inhibition efficacy) towards XBP1 splicing inhibition in macrophages [108], while concentrations as high as 100 µM can also be found in literature [109]. *In vivo*, it has been used at 10 mg/kg [110].

STF083010, like 4µ8C, acts as an IRE1 inhibitor. However, it inhibits only its RNAse activity of IRE1, thus allowing it to retain its kinase activity, but prevents the

occurrence of XBP1 mRNA splicing and RIDD. The methodology for its use is identical. *In vitro*, it is commonly incubated at 50 or 60 µM [109-112]. *In vivo*, it has been used at 30 mg/kg *via* intraperitoneal injection [110, 113].

2.1.3. ATF6

There are not many examples in the literature of the use of pharmacological inhibitors towards the AT6 branch of the UPR. However, in 2016, Gallagher et al. discovered that a class of pyrazole amides named ceapins can inhibit ATF6 signaling, without affecting other proteins involved in the UPR, and thus allowing for the analysis of the impact of all the three major UPR branches separately. The IC_{50}s of ceapins are 4.9 2.6 for ceapin A1 and 2.6 µM and 0.59 µM for ceapin A6 and ceapin A7, respectively [114]. There have not been noteworthy advances since this, and thus there is still a need for new ATF6 modulators.

2.1.4. Caspase-4

As previously explained, ER stress-induced regulated cell death is widely accepted to rely on the cleavage and activation of the ER-resident procaspase-4. The cell-permeable caspase-4 selective inhibitor Z-LEVD-fmk has shown to prevent ER-stress triggered cell death caused by ER stress-inducing compounds in many cell lines, including the neuroblastoma cell lines SK-N-SH and SH-SY5Y (10 µM) [115], leukemia cells Jurkat T (20 µM) [116], melanoma cell lines Mel-RM, MM200, IgR3, Mel-CV, Me4405, Sk-Mel-28, Mel-FH, and Me1007 (30 µM) [117], A549 and H1299 lung and Seg1 and Bic1 esophageal cancer cell lines (10 µM), although the latter used the variant Ac-LEVD-CHO, which is not an irreversible caspase-4 inhibitor, unlike Z-LEVD-fmk [118]. In the literature, both Z-LEVD-fmk and Ac-LEVD-CHO are commonly

resorted to as caspase-4 inhibitors [119]. The aforementioned caspase-4 inhibitors contain a small peptide, LEVD, which constitutes a caspase-4 substrate. This small peptide may be coupled to a fluorogenic molecule and be used as a fluorogenic substrate to infer about caspase-4 activity on cell lysates. One of such instances is reported by Pallet et al., which incubated 200 µg/mL of cell lysate with 50 µM Ac-LEVD-AFC and the compounds to be tested in 20 mM Tris, 250 mM NaCl at pH 7.4. The fluorescent signal was then read at 410/520 nm [120].

2.1.5. PDI

There are several commercially available irreversible inhibitors of PDI, such as 16F16, thiomuscimol and PACMA31. However, none of them is described as a specific PDI inhibitor [121]. The potential of both 16F16 and thiomuscimol against PDI was first described in 2011 by Hoffstrom et al. in a small molecule screening [122]. Higa and co-workers have employed 16F16 in concentrations up to 20 µM to characterize the role of PDIs on ATF6 signaling on the leukemia cells K562 and LAMA. Their results indicate that drug sensitivity is enhanced by inhibiting PDI activity, being that PDI could be involved in ATF6 activation, and establishing a role for a PDI/ATF6 axis in the development of cancer [123].

Zhao et al. concluded that inhibiting PDI activity with thiomuscimol could prevent ER-stress induced apoptosis [124]. In a different report, thiomuscimol inhibits PDI activity, unlike its inactive analogue muscimol [122]. Similarly, PACMA31 was used to determine that PDI activity is necessary for PERK activation, and resulted in increased cell survival *via* reduction of poly (ADP-ribose) polymerase (PARP) cleavage and

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decreased tumor protein 53 (p53) levels [125]. The importance of PDI on ER homeostasis will be further detailed on section **2.7.**

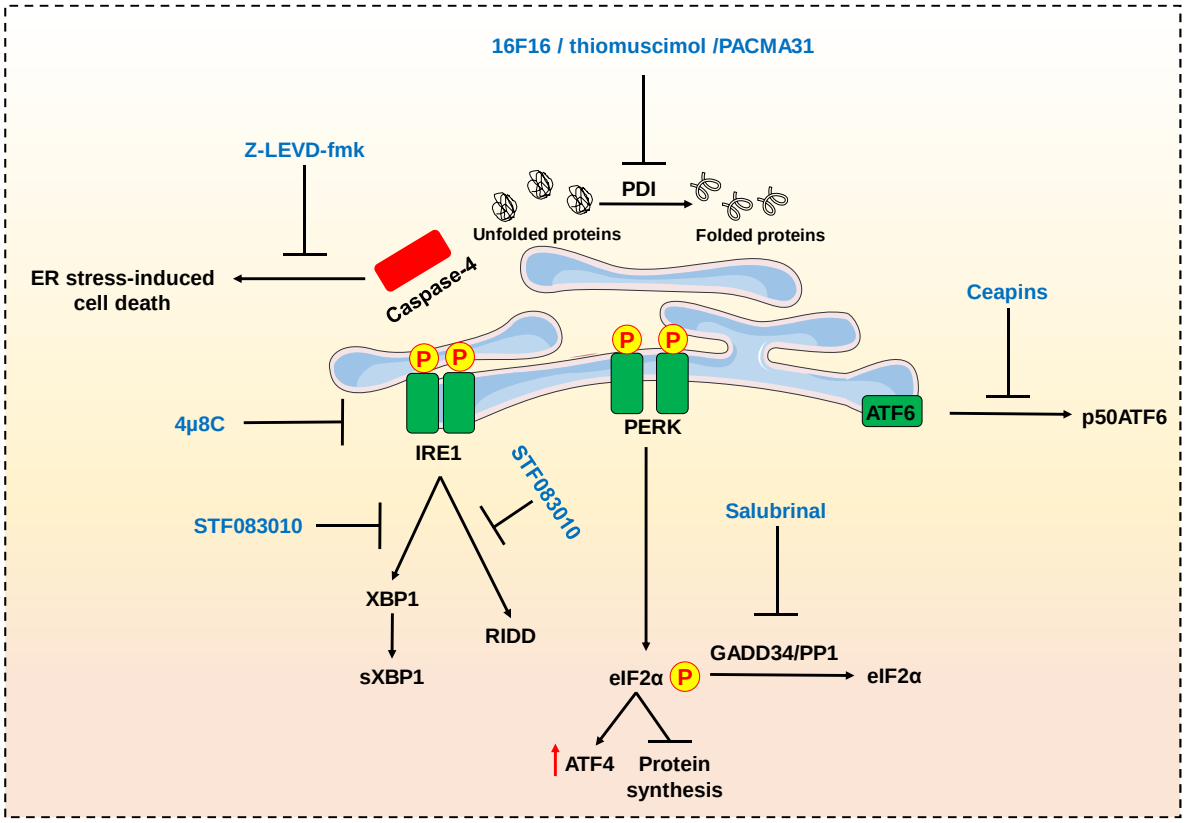

Figure 5. Summary of the mechanisms of action of the pharmacological inhibitors approached throughout this chapter.

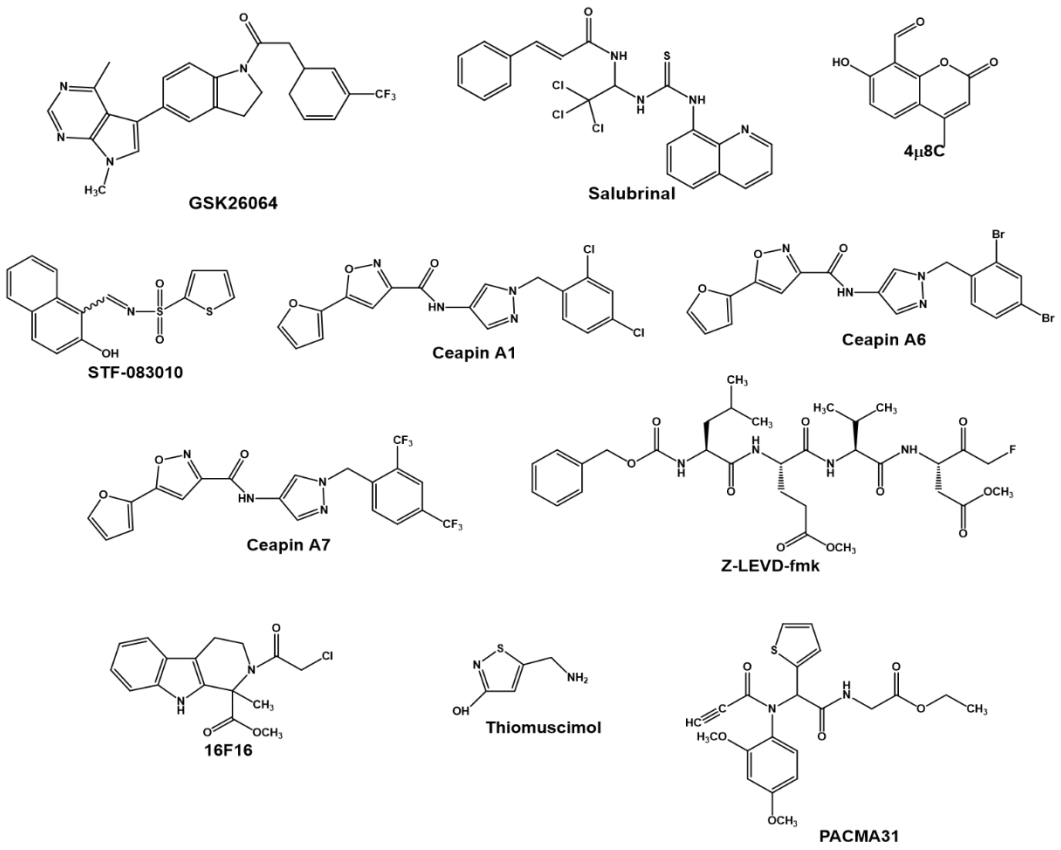

Figure 6. Chemical structures of the pharmacological inhibitors approached throughout this section.

2.2. qPCR

The determination of changes in the mRNA levels of UPR target genes by qPCR is one of the strategies most commonly found in the literature [53, 123, 126-128]. The PCR reaction exponentially replicates a DNA sample, while real-time quantitative PCR or qPCR is the designation given to the detection of PCR amplicons by measuring fluorescence at each cycle of an ongoing exponential reaction [129, 130]. Ended the qPCR reaction, there are several methods available for analyzing the resulting data. The absolute amount of DNA may be determined according to a standard curve or

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through digital PCR. However, the most common way to analyze data is through relative quantification, i.e. determination of fold-changes in gene expression *via* normalization with internal reference genes [131]. It is important that the latter is a gene that is highly expressed and which expression is not altered under the experimental conditions. The selected reference gene to analyze alongside target genes is often GAPDH [98, 132]. However, the latter may be upregulated under hypoxic conditions, reason for which it is not advisable to employ it as the only reference gene on this sort of experiment. Alternatively, β-actin can be used [133].

To name a few instances of the application of this technique in the literature on ER stress-related studies, we can enunciate, for example, that this technique was employed to conclude that increased mRNA levels of GRP78, PERK, eIF2α, CHOP, ATF6, and caspase-12 in placentas are associated to the development of severe pre-eclampsia, while IRE1 and ATF6 mRNA levels remain basal. In this study, as in many others, the results were compared to protein expression analysis, which will be discussed in the following section [134]. To study mechanisms underlying ATF4 translation, researchers used qPCR to analyze ATF4 expression [44]. To confirm the occurrence of ER stress, Bian et al. determined increased BiP expression using RT-PCR [42]. **Table 3** presents important UPR genes, which expression is often evaluated in ER stress-related studies.

Table 3. Characteristics of ER stress-related genes, according to the GenBank database.

Gene/Protein	NCBI Reference Sequence	Chromosomal Location	Expression

ATF4/ATF4	Var1: NM_001675.4 Var2: NM_182810.2	22q13.1	Ubiquitous expression. Broadly expressed in bone marrow.
ATF6/ATF6	NM_007348.4	1q23.3	Ubiquitous expression, particularly high on thyroid, placenta, brain, kidneys and appendix.
DDIT3/CHOP	Var1: NM_001195053.1 Var2: NM_001195054.1 Var3: NM_001195055.1 Var4: NM_001195056.1 Var5: NM_004083.5 Var6: NM_001195057.1	12q13.3	Expressed in virtually every type of tissue, notably on thyroid and bone marrow.

EIF2AK3/PERK	Var1: NM_004836.6 Var2: NM_001313915.1	2p11.2	Higher expression in thyroid, stomach, colon and bone marrow. The Wolcott-Rallison syndrome is associated with mutations on this gene.
EIF2S1/eIF2α	NM_004094.5	14q23.3	Expressed in all tissues, highlighting testis and esophagus.
ERN1/IRE1	NM_001433.5	17q23.3	Ubiquitously expressed. Broadly expressed in adrenal glands and pancreas
HSPA5/BiP	NM_005347.5	9q33.3	Broadly expressed in every tissue, particularly in thyroid, bone marrow and placenta.
XBP1/XBP1	Var1: NM_005080.3	22q12.1; 22q12	Ubiquitous expression, albeit especially high in

Var2: NM_001079539.1	salivary glands, liver and urinary bladder.

In the case of XBP-1, to simply determine total mRNA amounts would not be illustrative of its activity, since its activation as a transcription factor involves mRNA splicing. It is required to compare the amounts of spliced and unspliced XBP-1 mRNA to be able to draw any conclusions [29]. There is a report on a spliced-XBP-1 specific RT-PCR technique, which allows to separate spliced and unspliced mRNA in the human monocytic cell line THP-1. Total RNA reverse transcription and double-stranded cDNA synthesis was required, in order to be able to perform digestion with restriction enzymes (namely *Pst*I). Double-stranded cDNA from unspliced XBP-1 mRNA was digested by the enzyme, and consequently not amplified by the PCR. Oppositely, the spliced form is not digested by the enzyme, since it lacks the enzyme cleavage site [29]. Gene expression levels of IRE1 and ATF6 may also be misleading, since it is more assertive to determine their activation by phosphorylation status for IRE1 and cleavage and localization for ATF6. For this reason, researchers have resorted to the determination of mRNA levels of genes, such as HYOU1 and HERPUD, target genes of ATF6, or ERDJ4, which is ERAD-related gene downstream of XBP-1 [135, 136]. **Figure 7** exemplifies the results we can expect to obtain with this technique with an example performed in our laboratory, namely the effect of thapsigargin upon CHOP, ATF4 and EDEM1 expression on MRC-5 human lung fibroblasts.

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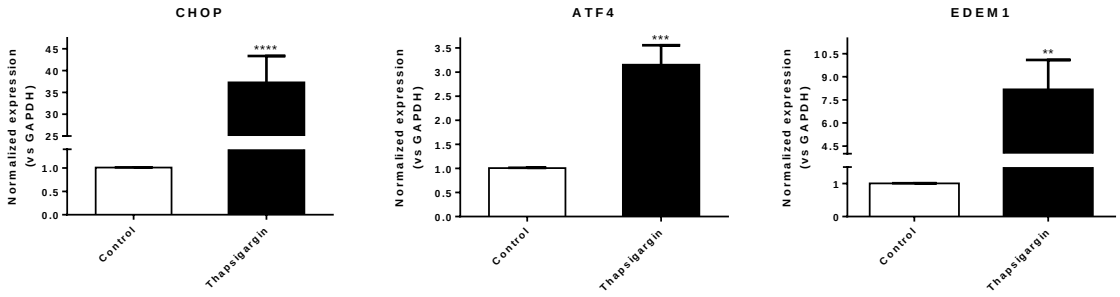

Figure 7. qPCR results representing the increased expression of the UPR markers CHOP, ATF4 and EDEM-1 on MRC-5 human lung fibroblasts after exposure to thapsigargin at 3 µM for 16 h. The expression was normalized against GAPDH. The results are expressed as mean ± SEM of four independent quadruplicates. **p < 0.01; ***p < 0.001; ****p < 0.0001.

2.3. Immunostaining

In the case of several key mediators of the UPR, mRNA expression does not provide sufficient information. Considering IRE1, PERK or eIF2α, the phosphorylation status should also be determined, in order to correctly infer about their activity. Analogously, the gene expression of ATF6 or XBP1 provides little information about its activity as a transcription factor, since it depends on proteolytic cleavage. Thus, it is necessary to distinguish between the native and cleaved forms, rather than just assessing total mRNA amount. For this sort of instances, researchers generally resort to immunoblotting techniques to further confirm the results obtained by qPCR, as will be discussed in this section.

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Along with the development of a reporter gene assay, Badr et al. employed Western Blotting to analyze protein levels of BiP and phosphorylated eIF2α. This is a somewhat laborious technique, since it involves the preparation of cell lysates protein extraction, submission of the protein sample to electrophoresis, transfer of the separated proteins from the SDS-polyacrylamide gels to nitrocellulose membranes and their immersion in a blocking agent, such as nonfat milk or bovine serum albumin, and then in the selected primary antibodies, following incubation in the secondary antibodies, and finally the visualization step ensues with the aid of a substrate, such as a chemiluminescent or a fluorescent [137]. Regarding the aforementioned study designed to analyze mechanisms underlying ATF4 translation, the authors employed this technique to assess ATF4 protein levels and distinguish between phosphorylated and dephosphorylated eIF2α, using a specific antibody for the phosphorylated form and a pan-eIF2α antibody [44]. To determine the role of the ATF6 pathway in glioblastoma development, Dadey and co-workers have observed proteolytic cleavage levels of ATF6 resorting to this technique [136]. ATF6 and BiP levels have been determined this way in a study designed to clarify the role of ER stress in retinal diseases [138]. This technique is widespread throughout the literature regarding ER stress.

Furthermore, the use of immunostaining techniques is not limited to Western Blotting. Immunohistochemistry methods can also be very useful to determine the subcellular localization of ER proteins and the ER itself. The number of selective probes towards the ER is quite scarce, and, for this reason, immunohistochemistry is often used to image the ER. Fluorogenic compounds attached to antibodies towards ER markers, such as BiP, PDI or calnexin, are among the most frequent instances of

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this procedure [139-143]. There are multiple commercially available kits designed to this purpose.

Anti-KDEL staining has been put into practice to visualize increased GRP78/94 protein levels under ER stress, using monoclonal antibodies that bind these ER stress biomarkers. KDEL represents the aminoacid sequence Lys-Asp-Glu-Leu, characteristic of ER-synthesized proteins for the secretory pathway. In this experiment, it was possible to observe evident staining in the perinuclear region of thapsigargin-treated mouse embryonic fibroblasts (MEFs) [144, 145]. There is a vast number of similar instances in the literature, using several ER stress biomarkers [123, 126, 127, 146-148].

2.4. ER labeling

Even though, as mentioned in 2.3., the number of selective probes towards the ER is scarce, there are a few commercial options for direct ER imaging. ER trackers include ER-Tracker™ Blue-White, DPX ER-Tracker™ Green (glibenclamide BODIPY® FL) and ER-Tracker™ Red (glibenclamide BODIPY® TR). The green and red options consist on a dye of the selected color bound to glibenclamide, which binds sulfonylurea receptors of ATP-sensitive K+ channels in the ER and thus impair its normal function. On the other hand, the blue-white option decreased its quantum yield on the presence of highly polar solvents [149-151]. Another option is CellLight® products, that transfect green or red fluorescent protein-based constructs fused to an ER retention signal that, in this case, is calreticulin and KDEL into cells. This sort of product is to be used on live cells only, unlike ER trackers, which allow ER visualization on live or fixed cells [152, 153].

Furthermore, in 2016, McDonald et al. reported the two first fluorescent flavonoids which selectively accumulate in the ER lumen [154]. Meinig et al. synthesized analogues of the flurophore rhodol that display the same properties [155].

2.5. Detection of protein aggregation

Thioflavin T (ThT) is a small molecule that issues a fluorescent response when bound to protein aggregates. Techniques based on this dye can be employed for both imaging and quantitation of misfolded protein accumulation, a hallmark of ER stress, being particularly useful in confirming its occurrence in preliminary assays. Nonetheless, these techniques do not provide insight into the molecular machinery activated during that disturbance. ThT is reported to have a particular affinity towards β-sheets. In a study designed to assess the feasibility of its use to detect ER stress, it was successfully employed in a cell culture system with MEFs, human hepatocarcinoma cells (HepG2), and human aortic endothelial cells (HAEC), and also on ApoE$^{-/-}$ mouse liver sections. ThT at 5 μM resulted in maximum differences of 5.5 fold resulting from cell treated with thapsigargin at 1 μM for 12 h against untreated control cells. It also responded to treatments with other known ER stress inducers, namely DTT, glucosamine and palmitate. ThT fluorescence was determined at Ex. 458 nm excitation and 480–520 nm emission. Increasing thapsigargin concentration (from 0 to 1 μM) revealed a linear response, relatable to the increase in ER stress. This was supported by increased GRP78 and CHOP protein levels, as determined by immunoblotting analysis. Co-incubation with the ER stress inhibitors 4-phenylbutyric acid or tauroursodeoxycholic acid decreased ER stress and ThT fluorescence, which the authors deem as evidence that this is a direct method to measure misfolded protein accumulation in the ER lumen. This study also develops a staining method employing

ThT, which allows visualization of the fluorescent compound in the ER lumen, where misfolded proteins accumulate. This method was completed with the visualization of the ER chaperones GRP78 and GRP94 using anti-KDEL staining [144].

2.6. Reporter gene assays

Given that UPR activation entails differences on the expression of several genes, there are several well-established reporter gene systems to detect these changes. Reporter assays based on secreted alkaline phosphatase (SEAP) have been put into practice for more than two decades to evaluate gene expression [156]. The secretion of SEAP rapidly decreases upon UPR activation. Once this decrease was confirmed to occur in a wide array of engineered *in vitro* and *in vivo* models (employing recombinant SEAP derived from human placental alkaline phosphatase) and in response to several ER stress inducers, SEAP or ER stress-responsive alkaline phosphatase (ESTRAP) was established as a molecular tool for monitoring ER stress in real time. Decreased activity of this enzyme can be associated to increased ER stress and, conversely, to upregulation of ER stress markers [157]. It can be advantageous to base reporter assays on secreted proteins, such as the SEAP, since this can both simplify the experimental procedures (by eliminating the need of protein or RNA extractions) and allow measurements in real time *in vitro* (analyzing culture media samples) or *in vivo* (through analysis of blood samples). The downside of such experiments is that it may be highly unspecific, since changes in its expression are biomarkers to many other physiological events, such as the development of bone diseases [158].

This sort of procedure may be particularly useful in ER stress-related studies. Since it is based on the activity of a secreted enzyme, the outcome will always be affected by ER stress conditions, and that may lead the researcher to misinterpret the results. To determine the impact of this, several cell models were exposed to a range of concentrations of TNF-α and IL-1β in the presence or absence of ER stress conditions. It was observed that the presence of thapsigargin or tunicamycin weakened the dose-dependent secretion of SEAP from the cells in response to the aforementioned cytokines, and thus ER stress as a side effect of a given treatment may exert a significant impact upon the results [159]. In 2007, Badr et al. developed an analogous method using a secreted luciferase, namely a Gaussia luciferase-based method for real-time quantitation and visualization of protein processing, which they claimed to be 20000 times more sensitive than SEAP assay. Briefly, this methodology aims to evaluate the secretory capacity and consists on the delivery to the cells under study, namely human fibroblasts, of a Gaussia luciferase expression cassette and subsequently measuring the inferring the levels of secreted enzyme via determination of its activity [137]. There are several luciferase-based protocols available in the literature [44]. Another example are venus-based constructs, which is a variant of green fluorescent protein. By fusing it to XBP-1, it is possible to analyze its splicing by IRE-1 under ER stress conditions, by detecting the fluorescence of the translated protein [160]. There are also several reports of green fluorescent protein constructs with ATF6 that can be useful in assessing its subcellular localization, and therefore infer about its activation status [161].

2.7. Proteomic studies

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In the last few years, researchers have been employing mass spectrometry in proteomic studies to analyze ER stress, even though proteomic studies on the ER have developed at a slower pace than for other organelles, since isolating the ER proves to be somewhat more complicated. Since this organelle is a continuous membranous structure from the nuclear envelope through the cytoplasm, it is considerably more difficult to obtain pure ER fractions that of other enclosed organelles, such as mitochondria [162].

Recently, the presence of one of the members of the protein disulfide isomerase (PDI) family, namely PDI4, was observed in human placental explants, where it might be regulated by IL11. The determination was carried out resorting to high performance liquid chromatography (HPLC) with tandem mass spectrometry (MS/MS). In order to perform such analysis, placental explants were lysed, the proteins were reduced with DTT, and thiol groups alkylated with iodoacetic acid. Finally, proteins were digested with trypsin, and the resulting solution was lyophilized and fractioned by nanoflow reversed-phase liquid chromatography, coupled to a mass spectrometer with a nanoelectrospray ion source. The authors successfully identified PDI4 out of the nine proteins induced by IL11, and sustain this identification with immunohistochemistry [163].

A similar HPLC-MS/MS method was developed by Cheng et al., on a comprehensive study aiming to map changes at both proteomic and transcriptomic levels on cervical cancer cells HeLa under ER stress caused by the action of DTT (which, as mentioned in **Table 2**, interferes with protein folding). This study concludes that both mRNA and protein maximum levels occur between 2 and 8 h of exposure to stress conditions, and determined the protein levels to be generally more evidently increased than mRNA

levels. After 30 h, mRNA levels were similar to those of control cells (i.e. in homeostatic conditions), and proteins had established a new steady state. Besides employing mass spectrometry for proteomics, the authors used microarrays to analyze the transcriptome and developed a statistical tool to analyze the results, which they named protein expression control analysis (PECA) [164]. Later on, the authors published more extensive results regarding the same study, providing data concerning more proteins than the original publication (2131, against the original 1237 proteins) [165].

2.8. Enzyme activity assays

2.8.1. SERCA pump

Assays to determine SERCA pump activity are not found in the literature quite as often as other less direct approaches to infer about changes on calcium homeostasis. A reason for this may be the technical difficulties posed by direct measurements of its activity.

There are, however, a few reports concerning the determination of SERCA activity. Towards this goal, McMullen and co-workers developed a 96-well plate colorimetric assay. This assay was designed to quantify the amount of inorganic P that is released by the pump following ATP hydrolysis, which then complexes with ammonium molybdate and malachite green. The authors proceeded to isolate microsomes from brain tissue and prepared an inorganic phosphate quantification reagent that binds with inorganic phosphate, whereas the resulting complex issues a green color that can be measured at 660 nm in a spectrophotometer. The aforementioned reagent can be prepared adding 1 part of 10% (w/v) ammonium molybdate and 3 parts 0.2% (w/v)

malachite green, both in 4 M HCl, stirring the mix for 30 min and subjecting it to gravity filtration [8].

Another approach for the analysis of SERCA pump activity *in vitro* is based on the chemiluminescent calcium binding protein aequorin targeted to the ER. This allows for the analysis of the speed of calcium import into the ER lumen, which is related to SERCA activity. The same authors propose a protocol for determining SERCA activity *in vivo*, through isolation of ER vesicles and their analysis of their calcium uptake resorting to the fluorescent calcium dye Fluo-3 [146].

2.8.2. Protein disulfide isomerases

Protein disulfide isomerases (PDIs) have a pivotal role in protein folding. For this reason, cell-free enzymatic assays have been developed to analyze the ability of the molecules to modulate the activity of these enzymes. Since PDIs possess four types of activities, the activity to be measured in a given assay depends on the redox state of the protein chosen as substrate. Isomerase activity is measured when this substrate presents scrambled disulfide bonds. PDI will convert it to its native state, restoring its activity. Oxidase activity occurs when the substrate is completely reduced and PDI performs its oxidative refolding. Reductase activity, on the other hand, occurs when the substrate is oxidized. Finally, chaperone activity can be measured using substrate that does not contain disulfide bonds. This last one can be determined through recovery of substrate activity, thus its subsequent determination is required (such as the reductase or isomerase) and also through changes in substrate aggregation [121, 166].

For analyzing chaperone activity and distinguish from the other types of PDI activity, the substrate must be completely denatured and not need disulfide bonds to fold. Such substrates may be D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), lactate dehydrogenase, rhodanase or citrate synthase or alcohol dehydrogenase. The substrate is incubated in a buffer in the presence of PDI, and then changes in protein aggregation can be inferred by analyzing light scattering or turbidity. Also, to assess effective function repair of the substrate, it can be dissociated from PDI and its activity measured in a subsequent experiment. The substrate used can also be green fluorescent protein, which contains no disulfide bonds and produces fluorescence when its activity is restored. PDI reductase activity is commonly determined on the presence of insulin along with a reducing agent, namely DTT or GSH. Another available approach is a fluorimetric assay, in which PDI will reduce a fluorescent probe (di-(*o*-aminobenzoyl)-GSSG (diabz-GSSG) or dieosinGSSG (Di-E-GSSG)]. Oxidase activity assay protocols available generally use RNAse, bovine pancreatic trypsin inhibitor (BPTI) or lysozyme and determination of the absorbance of the resulting product. Finally, isomerase activity is commonly determined based on restoring the activity of scrambled RNAse or riboflavin-binding protein, which need to be reduced and allowed to reoxidize in a manner that randomizes their disulfide bonds [121, 166].

2.9. Calcium imaging and quantitation

As previously detailed, intracellular calcium levels are tightly regulated within the cell, being that the occurrence of ER stress may lead to its displacement from the ER lumen into the cytosol. For this reason, disturbances in calcium homeostasis are frequently analyzed in ER stress studies.

2.9.1. Fluorescent dyes

Fura-2-acetoxymethyl ester or Fura-2/AM, a membrane-permeable derivative of the original calcium dye Fura-2, is the most commonly employed calcium dye in the literature nowadays. It is a ratiometric dye that is used both for intracellular calcium imaging and quantitation; the fact that it is ratiometric confers it a high reliability, due to decreased errors brought by uneven pipetting, photobleaching, or differences in cell lines. Once inside the cell, cellular esterases will cleave the acetoxymethyl group; then, calcium-bound form will have its excitation wavelength at approximately 340 nm, while the free form has its own excitation maximum around 380 nm, being that the emission is maximum at 510 nm in both cases. Hence, the amount of intracellular calcium is presented as the ratio $F_{340/510}/F_{380/510}$ [167-169].

This fluorescent dye has been used extensively in a wide set of cell lines. One example of a recently published protocol for calcium imaging was performed on the rat hepatoma cell line H4IIEC3, in which the authors loaded the cells with 3 µM Fura-2/AM for 30 min and proceeded to fluorescence imaging under a microscope [170]. A similar protocol has been applied to observe calcium changes on SH-SY5Y cells, consisting in loading of cells with 5 µM Fura-2/AM in HEPES-buffered salt solution (HBSS) [171]. In the literature, we can find a fair amount of suchlike protocols applied to calcium imaging and/or quantitation. Albeit with small differences, all those protocols employ fluorescent dye concentrations around a low µM range, commonly 5 µM [95, 172].

In order to obtain images in which calcium can be observed simultaneously in the cytosol and ER lumen, a technique designated as targeted-esterase induced dye loading has (TED) emerged. This method is based on targeting vector constructs

expressing carboxylesterases and containing a red fluorescent protein. While imaging, the red color will trace the ER structure, and the calcium inside will appear green [173].

There are other fluorescent calcium probes commercially available. One example is Fura-PE3/AM, a variant of Fura-2/AM that is designed to avoid possible compartmentalization in the cell. Suppliers report that hydrolysis of this molecule by esterases will trap it inside the cytosol and make it cell membrane impermeable [174, 175].

2.9.2. Radiolabeled calcium measuring

There are methodologies available for the quantitation of radiolabeled $^{45}Ca^{2+}$ in ER microsomes. One of such instances is performed on 0.45 µm glass fiber type B MAFB micro filter plate, in order to allow the analysis of both microsomes and of the obtained filtrate. The concentration of free calcium is kept at 300 nM. At the time of experiments, several reagents are added to the assay assay buffer in order to form a pre-mixture: 2 mM ATP, 2 mM $MgCl_2$, 1.4 mM DTT, 5 mM PCr (phosphocreatine), and 20 U/mL CPK (creatine phosphokinase). Lastly, radiotracer $^{45}Ca^{2+}$ is added at a final concentration of 0.2 µCi/mL. Then, in the 96-well plate, microsomes are added at 100 µg/mL, the pre-mix, the molecules under study, followed by incubation in the dark at 37 ºC for 60 min. Ended this period, the reaction is finished using filtration with a vacuum plate base. Underneath, a carefully placed clean microplate will gather the filtrate dripping from above, being that microsomes that accumulated $^{45}Ca^{2+}$ are incapable of passing through the filter, due to their charge and size. Finally, filters are washed with a cold buffer (10 mM HEPES-KOH (pH 7.3), 100 mM KCl, 3% (w/v) PEG, 10 mM potassium oxalate, 5 mM MgCl2, and 2 mM EGTA), 50 µL of Ready Value scintillation fluid are

added to the filter wells and $^{45}Ca^{2+}$ accumulation is determined using a Wallac microbeta liquid scintillation counter [8].

3. Concluding remarks

The ER is composed by an intricate network of vesicles and tubules that spread through the cytosol of every eukaryotic cell, continuously changing its architecture according to its requirements. This is related to the variety of cellular processes it regulates or is involved in, even though it is often associated to its predominant role as a protein synthesis, folding and secretion site. The regulation of a wide array of cellular and developmental processes, such as cell proliferation and differentiation, also depends on ER homeostasis, since it is the main calcium reservoir of the cell, the latter being a very important second messenger in such events. The performance of the ER is crucial to the physiology of the cell and to its inbuilt stress response mechanisms. Its importance towards stress recognition and response relies on complex signaling processes that have evolved in eukaryotic organisms. The UPR is a complex molecular response triggered upon the occurrence of disturbances on cellular homeostasis of diversified origin and, for this reason, plays its part in the development of virtually every chronic disease.

These are well established premises in the literature that paved the way for the development of tools to identify ER-related biomarkers of several diseases. Today it is possible to conduct comprehensive studies on models of ER stress or of a determined disease, *in vitro* or *in vivo*, and correctly identify which UPR enzymes and transcription factors are activated, and also whether or not a molecule of choice is able to suppress or ameliorate this situation. Such instances have led to the identification of several ER

modulating molecules, as well as the recognition of their mechanism of action. Unfortunately, several of those molecules have displayed high toxicity and the efforts towards their use in the formulation of new drugs have ceased.

Nonetheless, increasing knowledge of the ER stress molecular network, as well as the establishment of more and more high-throughput techniques that allow testing dozens or even hundreds of molecules at the same time will certainly lead to the identification of novel bioactive molecules on a short term. Furthermore, the field requires more information regarding the impact of the model under study to the stress pathways under way. For example, ER stress responses in 3D and heterotypic systems are lacking. In the near future, new ER-based strategies will hopefully evolve and add to the current pharmacological arsenal for countering ER stress in disease.

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