ER Stress and UPR Through Dysregulated ER Ca^{2+} Homeostasis and Signaling

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Abstract

The endoplasmic reticulum (ER) has two important biological functions: protein folding and storing intracellular Ca^{2+}. Importantly, adequate ER Ca^{2+}-store filling is critical for proper protein folding. In many occasions, ER stress is tightly linked to disruption of ER Ca^{2+} homeostasis, causing the activation of an integrated signaling pathway, the unfolded protein response (UPR). In this book chapter, we will review the ER as a dynamic intracellular Ca^{2+}-storage organelle
in a constant state of Ca\(^{2+}\) flux that is in close proximity to the mitochondria, thereby controlling cell survival, adaptive responses to stress and apoptosis. Next, we will discuss how altered [Ca\(^{2+}\)]\(_{ER}\) homeostasis leads to ER stress, and how ER stress and their sensors alters Ca\(^{2+}\) flux. Recent studies provided novel insights in the molecular mechanisms underlying these processes, including a dynamic regulation of ER Ca\(^{2+}\)-uptake and –release mechanisms by ER chaperones and the main controller of the ER-stress sensors, GRP78/BiP. Furthermore, recently identified Ca\(^{2+}\)-transport systems also seem to target ER-stress proteins. Overall, it is clear that altered Ca\(^{2+}\) signaling and UPR during ER stress are closely related through dynamic physical interactions between their key players.

**Keywords**

Ca\(^{2+}\) · GRP78/Bip · ER intracellular Ca\(^{2+}\) release channels · IP\(_3\)Rs · ER-stress · Unfolded protein response · Apoptosis · Ca\(^{2+}\) homeostasis · ERO44 · ERO1\(\alpha\) · SERCA · BI-1 · PERK · IRE1\(\alpha\) · Sigma1 receptors · Calcium binding proteins

**Abbreviations**

BI1  Bax inhibitor-1  
BiP  Immunoglobulin heavy chain binding protein  
CaMKII  Calmodulin-dependent protein kinase II  
cyt c  Cytochrome c  
eIF2\(\alpha\)  Eukaryotic initiation factor 2\(\alpha\)  
ER  Endoplasmic reticulum  
ERAD  ER-associated degradation  
ERO1\(\alpha\)  ER oxidoreductin 1\(\alpha\)  
FAD  Familial Alzheimers disease  
GRP  Glucose-regulated protein  
HO-1  Heme oxygenase 1  
IMM  Inner mitochondrial membrane  
IP\(_3\)  Inositol 1,4,5-trisphosphate  
IP\(_3\)Rs  Inositol 1,4,5-trisphosphate receptors  
JNK  C-Jun N-terminal kinase  
MAMs  mitochondria associated membranes  
MCU  Mitochondrial Ca\(^{2+}\) uniporter  
mTOR  Mammalian target of rapamycin  
NPR  NADPH-P450 reductase  
OMM  Outer mitochondrial membrane  
P450  Cytochrome P450  
PDI  Protein disulfide isomerase  
PLC  Phospholipase C  
PML  Promyelocytic leukemia  
PTP  Permeability transition pore  
ROS  Reactive oxygen species  
RyRs  Ryanodine receptors  

SERCA Sarco- and endoplasmic reticulum Ca\(^{2+}\) ATPase
SERCA1 T Truncated sarco- and endoplasmic reticulum Ca\(^{2+}\) ATPase 1
SPCA1 Secretory pathway Ca\(^{2+}\) ATPase 1
STIM Stromal interaction molecule
TRP Transient receptor potential
TRPC6 Canonical transient receptor potential-6
UPR Unfolded protein response
VDAC Voltage dependant anion channel

1 Introduction

Intracellular Ca\(^{2+}\) signals regulate a plethora of cell biological and physiological functions like proliferation, differentiation, secretion, muscle cell contraction, metabolism, trafficking, gene transcription and apoptosis [1]. During recent years, it has become clear that intracellular Ca\(^{2+}\) plays a central role in regulating and sensing key cellular processes, including autophagy and the unfolded protein response (UPR) [2, 3]. Thus, changes in Ca\(^{2+}\)-flux patterns caused by mutations in Ca\(^{2+}\)-permeable channels, Ca\(^{2+}\)-uptake and -release mechanisms, Ca\(^{2+}\)-binding proteins and Ca\(^{2+}\)-pumps underlie many pathological conditions. In Ca\(^{2+}\) signaling, the endoplasmic reticulum (ER) plays a very critical role, since this organelle is the main intracellular Ca\(^{2+}\) store and is in close proximity with the mitochondria. Furthermore, the function of the ER in the synthesis, modification, folding and export strongly depends on proper Ca\(^{2+}\) storage, since many ER-resident chaperones bind Ca\(^{2+}\) and/or regulate Ca\(^{2+}\)-uptake or release mechanisms [4]. Thus, imbalance in the steady-state ER Ca\(^{2+}\) levels will lead to ER stress responses like UPR. Furthermore, ER-stress proteins modulate Ca\(^{2+}\)-signaling processes. Finally, ER Ca\(^{2+}\)-uptake and release mechanisms also directly control ER stress and UPR. In this chapter, we will review the current state-of-the-art on the relationship between Ca\(^{2+}\) signaling and ER stress. It is important to note that the regulation of ER stress by (perturbed) Ca\(^{2+}\) signaling is an emerging field, representing a novel and important concept in biology. It clearly underlies a variety of pathological conditions, but also offers novel therapeutic opportunities to target malignant cells. This is underpinned by the rising number of items published per year and citations to these items per year dealing with Ca\(^{2+}\)/calcium and ER stress (Fig. 1).

2 The ER as an Intracellular Ca\(^{2+}\) Store

2.1 ER Ca\(^{2+}\) Homeostasis

Proper ER function depends on adequate filling of the ER with Ca\(^{2+}\) [4]. Depleting or overfilling ER Ca\(^{2+}\) stores exerts detrimental effects on cellular health and survival. This is achieved by a balance between Ca\(^{2+}\)-uptake mechanisms and Ca\(^{2+}\)-
Ca$^{2+}$ signaling in ER stress only recently emerged in the scientific literature. The results of a Web of Knowledge search in “ALL DATABASES” (http://apps.webofknowledge.com) performed on 14 November 2011 using the search term ((Ca$^{2+}$ or calcium) and “ER Stress”) are presented. The results indicate that the role of Ca$^{2+}$ signaling in controlling ER stress and during ER-stress mechanisms has only recently emerged in literature. However, the role of Ca$^{2+}$ signaling in ER stress is now a rapidly evolving field with an increasing number of papers published per year (black; ■) and an increasing number of citations per year referring to papers (gray; ●) covering these topics. Some key papers elucidating the molecular mechanisms underlying the interrelation between ER stress and Ca$^{2+}$-transport systems, like SERCA2b (red), SERCA1T (purple), IP$_3$Rs (blue), BI-1 (green) and PERK (black).

leak or –release mechanisms of the ER [6]. Ca$^{2+}$ storage in the lumen of the ER is mediated by a variety of Ca$^{2+}$-binding proteins [7] (Fig. 2).

### 2.1.1 ER Ca$^{2+}$-buffering Proteins

The ER functions as the main intracellular Ca$^{2+}$ store, containing 2 mM of total [Ca$^{2+}$] and about 500 μM of free [Ca$^{2+}$], which is at least 50 fold higher than the free [Ca$^{2+}$] in the cytosol which is about 100 nM [8, 9]. While calsequestrin is the major Ca$^{2+}$-buffering protein in the SR of skeletal and cardiac muscle cells [10], other cells express a variety of Ca$^{2+}$-binding proteins that also function as molecular chaperones, such as calreticulin, calnexin, 78-kDa glucose-regulated protein/immunoglobulin heavy chain binding protein (GRP78/BiP), GRP94, and various other protein disulfide isomerases (PDI) [11]. The majority of the ER Ca$^{2+}$ is bound by the ER-resident 46-kDa calreticulin [12]. This chaperone is organized in three functional
domains [9, 13]. The N-domain is involved in polypeptide and oligosaccharide binding [14, 15]. The P-domain contains a flexible arm containing a central Pro-rich stretch involved in oligosaccharide binding and complex formation with ERp57, an oxidoreductase folding enzyme of the ER [16–19]. The N- and P-domains together are critical for the chaperone function of calreticulin [13–15]. Furthermore, the P-domain displays high-affinity (Kd ~ 10 μM) low-capacity (1 mol of Ca²⁺ per 1 mol of protein) Ca²⁺ binding [20]. The C-terminal C-domain, which is enriched in large clusters of acidic amino acids (Asp and Glu) and separated by basic amino acids (Lys and Arg), is involved in low-affinity (Kd ~ 2 M) high-capacity (25 mol of Ca²⁺ per mol of protein) Ca²⁺ binding [13, 20]. GRP78/BiP is another important low capacity Ca²⁺-buffering protein, responsible for ~25% of the Ca²⁺-binding capacity of the ER [21]. GRP94 is one of the most abundant ER Ca²⁺-buffering proteins, which displays a high number of low-capacity, high-affinity Ca²⁺ binding sites as well as high-capacity, low-affinity Ca²⁺-binding sites [22]. In addition, ER oxidoreductases display high-capacity weak-affinity Ca²⁺ binding, participating in buffering ER Ca²⁺ [9].

2.1.2 ER Ca²⁺-uptake Mechanisms

The uptake of Ca²⁺ in the ER is achieved by the sarco- and endoplasmic reticulum Ca²⁺ ATPases (SERCA), which are encoded by three different genes and are expressed
as various splice variants [23]. SERCA2b is the house-keeping isoform widely expressed in a variety of tissues and cell types, and displays the highest Ca\(^{2+}\) affinity [24, 25]. Other SERCA isoforms have a more restricted expression: SERCA2a in the cardiac muscle, while SERCA1 in the skeletal muscle [26]. The Ca\(^{2+}\)-uptake activity of SERCA is controlled by small accessory proteins, like phospholamban, which is expressed in muscle cells [27]. A potent, irreversible inhibitor of SERCA Ca\(^{2+}\) pumps is thapsigargin, which binds to the M3-transmembrane helix with very high affinity (nanomolar range) [28]. In this respect, thapsigargin is commonly used to induce ER stress through blocking ER Ca\(^{2+}\) uptake and depleting ER Ca\(^{2+}\) stores [29].

### 2.1.3 ER Ca\(^{2+}\)-leak and –release Mechanisms

The ER Ca\(^{2+}\)-release mechanisms are very pleiotropic and utilize a tight interplay between them, often through physical interactions [30]. Furthermore, there are mechanisms that exert a constant passive Ca\(^{2+}\) leak from the ER, even in the absence of stimuli (passive Ca\(^{2+}\)-leak channels) and that mediate Ca\(^{2+}\) release in response to cellular stimulation (intracellular Ca\(^{2+}\)-release channels) [6]. Two families of ER-located intracellular Ca\(^{2+}\)-release channels exist: inositol 1,4,5-trisphosphate (IP\(_3\)) receptors (IP\(_3\)Rs) [31–33] and ryanodine receptors (RyRs) [34, 35]. Both families are encoded by three different genes and are expressed as large tetrameric complexes: 1.2 MDa for IP\(_3\)Rs and 2.2 MDa for RyRs. While IP\(_3\)Rs are ubiquitously expressed among almost all tissues, RyRs are highly abundant in skeletal and cardiac muscle. However, RyRs are also expressed at lower levels in other cell types, including neurons [36]. In spite of their low cellular expression, RyRs are critical for Ca\(^{2+}\)-signaling events, since the flux of Ca\(^{2+}\) ions per opening of RyRs is about 20-fold higher than that of IP\(_3\)Rs [34].

The mechanism of IP\(_3\)R activation involves the production of IP\(_3\) by phospholipase C (PLC) \(\beta/\gamma\) in response to extracellular stimuli, like hormones, growth factors and antibodies [37]. IP\(_3\) binds to the N-terminal IP\(_3\)-binding domain, which triggers conformational changes that are sensed by the C-terminal channel domain, thereby opening the Ca\(^{2+}\)-channel pore [32, 38]. Importantly, IP\(_3\)R activity is regulated by cytosolic Ca\(^{2+}\) in a bell-shaped dependent manner: low cytosolic [Ca\(^{2+}\)] enhances IP\(_3\)R opening, while high cytosolic [Ca\(^{2+}\)] promotes IP\(_3\)R closing [39]. In addition, ER luminal Ca\(^{2+}\) too controls IP\(_3\),R-mediated Ca\(^{2+}\) release, since partially depleted ER Ca\(^{2+}\) stores are less sensitive to IP\(_3\) and release less Ca\(^{2+}\) [40, 41]. In general, all three RyR isoforms respond to Ca\(^{2+}\)-induced Ca\(^{2+}\) release [42]. However, in a physiological context, skeletal muscle-type RyR (RyR1) is activated through its direct interaction with the intracellular loop of the dihydropyridine receptor, an L-type voltage-dependent Ca\(^{2+}\) channel, triggering skeletal muscle contraction [42]. RyR2, which is highly expressed in cardiac muscle cells, is activated by Ca\(^{2+}\)-influx through voltage-dependent Ca\(^{2+}\) channels, triggering cardiac muscle contraction [43]. An important concept of physiological RyR function is its “coupled gating” through FK506-binding proteins, which facilitates the coordinated “all-or-none” opening of RyR clusters [44]. Due to the Ca\(^{2+}\)-induced Ca\(^{2+}\)-release properties of RyRs, these channels likely contribute or amplify IP\(_3\)R-originating Ca\(^{2+}\) signals. In addition, the Ca\(^{2+}\)-flux properties of both channel types are tightly regulated by a range of cellular factors and processes, including Ca\(^{2+}\), ATP, phosphorylation, dephosphorylation and accessory proteins [31, 35, 42, 45, 46]. Over the last decade,
it has also become clear that IP₃Rs are critical targets of proteins involved in cell survival, cell death and cellular homeostasis, including anti-apoptotic proteins, autophagy proteins and proteins involved in ER stress [47–49].

Next, it is important to note that other intracellular compartments also may contribute to ER Ca²⁺-signaling events, including the Golgi network [50] and the lysosomes [51]. For a long time, the Golgi network was considered as a homogenous Ca²⁺-signaling compartment [52–54]. Now, recent evidence indicates that a high degree of heterogeneity of the Golgi network exists towards its Ca²⁺-transport mechanisms [55]. While the secretory pathway Ca²⁺ ATPase 1 (SPCA1) Ca²⁺ pumps mediate Ca²⁺ uptake in the cis-Golgi network, this compartment also displays some ER-like features like SERCA-mediated Ca²⁺ uptake and IP₃R-mediated Ca²⁺ release [25]. In contrast, Ca²⁺ uptake into the trans-Golgi network exclusively depends on the activity of SPCA1 Ca²⁺ pumps and through IP₃R activation, while Ca²⁺ mobilization is mediated through RyRs [56]. Ca²⁺ release from acidic Ca²⁺ stores is mediated by the two-pore channels, a recently identified protein family [57–60]. These channels release Ca²⁺ in response to nicotinic acid adenine dinucleotide phosphate [61, 62]. Importantly, while the overall capacity of these Ca²⁺ stores is relatively small in comparison to the ER Ca²⁺ stores, local Ca²⁺ release from lysosomal Ca²⁺ stores can be amplified by Ca²⁺ release from the ER Ca²⁺ stores through Ca²⁺-induced Ca²⁺ release [63, 64].

Besides physiological Ca²⁺-signaling events in response to stimuli, it is clear that the ER contains a high number of mechanisms that mediate basal Ca²⁺ leak from the ER Ca²⁺ stores [6]. A large number of proteins have been proposed to function as basal Ca²⁺-leak channels. Collectively, these channels are responsible for the loss of about 20–200 μM of Ca²⁺ per minute, thereby controlling steady state [Ca²⁺]ER levels under normal conditions [65]. Recently, we have reviewed the contribution of the different channels that mediate passive Ca²⁺ leak [6]. First, translocons, the protein-conducting channels on the surface of the rough ER, mediate Ca²⁺ leak from the ER in response to puromycin, an antibiotic that purges translocons from nascent polypeptide chains [66–68]. Nevertheless, under normal conditions, the translocon complex likely remains closed and thus its contribution to physiological Ca²⁺ leak remains questionable [69]. Second, a number of isoforms of the transient receptor potential (TRP) channel family are expressed at ER membranes and have been implicated in the ER Ca²⁺ leak [70], including TRPC1 [71], TRPV1 [72–75], TRPM8 [76] and TRPP2 (polycystin 2) [77–79]. The latter is a Ca²⁺ permeable channel largely located at the ER membranes [80], where it interacts with both IP₃Rs [81] and RyRs [82], thereby potentiating intracellular Ca²⁺ signals [79, 83]. In addition, polycystin 2 may function as a passive Ca²⁺-leak channel lowering steady state [Ca²⁺]ER [84]. Third, presenilin channels have been proposed to function as Ca²⁺-permeable channels [85]. In lipid bilayer experiments, wild-type presenilin, but not mutant presenilin forms low-conductance divalent cation-permeable ion channels linked to familial Alzheimer disease. Fifth, Bax Inhibitor-1 (BI-1), Lifeguard and hGAAP, a family of conserved anti-apoptotic ER-located proteins containing 6 or 7 transmembrane domains, have been shown to display Ca²⁺-channel properties [86, 87]. In many cases, the exact mechanism by which these proteins lower [Ca²⁺]ER have not been elucidated. BI-1 has been proposed to function as a
Ca\(^{2+}\)/H\(^{+}\) antiporter, whose oligomerization and activity is enhanced by lowering of cytosolic pH [88–91]. We found that BI-1 contains four conserved Asp residues in its C-terminal domain, which function as the Ca\(^{2+}\) pore of BI-1 [92]. In addition to this, it remains to be elucidated whether BI-1 directly regulates intracellular Ca\(^{2+}\)-release channels, like IP\(_{3}\)Rs. Sixth, pannexin channels, which belong to the family of connexin/pannexin proteins involved in building head-to-head docked gap junctional channels and unopposed “free” channels present in ER membranes forming Ca\(^{2+}\)-permeable channels [93, 94]. Pannexin 1 overexpression decreased the ER Ca\(^{2+}\) content. Seventh, alternative splice variants of SERCA1 have been described, which lead to the expression of C-terminally truncated proteins (SERCA1\(_{T}\)) that are deficient in pumping Ca\(^{2+}\) in the ER [95, 96]. SERCA1\(_{T}\) is expressed in a variety of tissues, but is absent in skeletal and cardiac muscle. SERCA1\(_{T}\) forms homodimers and has been proposed to form a Ca\(^{2+}\)-leak channel and/or enhance IP\(_{3}\)R-mediated Ca\(^{2+}\) leak. In any case, overexpression of SERCA1\(_{T}\) reduces [Ca\(^{2+}\)]\(_{ER}\), thereby counteracting full-length SERCA.

Finally, it is important to note that [Ca\(^{2+}\)]\(_{ER}\) also controls Ca\(^{2+}\)-influx pathways through store-operated Ca\(^{2+}\) channels, \textit{i.e.} ER Ca\(^{2+}\)-store depletion will trigger Ca\(^{2+}\) influx across the plasma membrane from the extracellular environment [97]. The molecular mechanisms underlying store-operated Ca\(^{2+}\) influx involve the ER Ca\(^{2+}\) sensors, stromal interaction molecule (STIM) 1 and 2 and plasmalemmal Ca\(^{2+}\)-influx channels, Orai 1, 2 and 3 [98]. Upon ER Ca\(^{2+}\)-store depletion, STIM1 aggregates in ER patches that are in close proximity of the plasma membrane, thereby recruiting and activating Orai channels [99]. Importantly, STIM2 activity seems to be tightly controlled by basal ER Ca\(^{2+}\) levels, thereby functioning as homeostatic regulator of ER Ca\(^{2+}\) homeostasis and keeping cytosolic and ER Ca\(^{2+}\) levels within tight limits [100].

Thus, it is clear that ER Ca\(^{2+}\) levels are in constant state of dynamic flux. Altering one of these three parameters, the level of Ca\(^{2+}\)-binding proteins, the overall rate of Ca\(^{2+}\) release or the overall rate of Ca\(^{2+}\) uptake, has an immediate effect on the steady state ER Ca\(^{2+}\) levels. This is critical for survival and apoptosis, since the ER is in close proximity with the mitochondria [101–105]. Furthermore, dysregulation of these Ca\(^{2+}\)-buffering and –transport mechanisms will affect steady state Ca\(^{2+}\) levels in the ER. Severe and/or chronic decreases in [Ca\(^{2+}\)]\(_{ER}\) will trigger ER stress and apoptosis.

### 2.2 The IP\(_{3}\)R at the Mitochondria Associated Membranes (MAMs)

The molecular and functional properties of ER-associated MAMs have been discussed elsewhere [3, 106]. In addition, an overview of the most important MAM associated proteins is given in the previous chapter: “ER-mitochondria connections, calcium cross-talk and cell fate: a closer inspection”. Here, we want to highlight the role of the IP\(_{3}\)R at the MAMs.

IP\(_{3}\)Rs are present in the MAMs, allowing the localized release of Ca\(^{2+}\) from the ER in hotspots that are in close apposition to mitochondrial Ca\(^{2+}\)-uptake mechanisms [104]. The amount and functional properties of IP\(_{3}\)Rs present in the MAMs ought to be carefully regulated. For instance, GM1 ganglioside accumulates in the
MAMs in GM1-gangliosidosis and interacts with phosphorylated IP$_3$Rs, enhancing IP$_3$R clustering and IP$_3$R-mediated Ca$^{2+}$ flux into the mitochondria [107]. In addition, it is important to take into account that different IP$_3$R isoforms display different functional properties, including differences in sensitivity towards IP$_3$ [108, 109]. In the MAMs, IP$_3$R3 isoforms seem to be enriched [110]. This is elegantly shown in CHO cells in which IP$_3$R3 is the least abundant IP$_3$R isoform; yet, silencing IP$_3$R3 in CHO cells has the most profound effect on the mitochondrial Ca$^{2+}$ accumulation and apoptosis in these cells [110]. These observations are underpinned by the observation that IP$_3$R3 is the isoform that displays the highest degree of co-localization with the mitochondria [104].

Furthermore, many proteins critically control the Ca$^{2+}$-flux properties of IP$_3$Rs. Bcl-2-family members target IP$_3$Rs and affect their functional properties [47]. Bcl-2 was shown to suppress IP$_3$R-mediated Ca$^{2+}$ signals, in particular the large pro-apoptotic Ca$^{2+}$ transients [111–113]. In contrast, Bcl-XL seems to enhance IP$_3$R-mediated Ca$^{2+}$ signals, in particular the oscillatory pro-survival Ca$^{2+}$ spikes [114, 115]. The differences in IP$_3$R regulation by Bcl-2 versus Bcl-XL likely underlie the selective interaction with different protein domains [116, 117]. The C-terminal part of the IP$_3$R containing the channel pore seems to interact with all anti-apoptotic Bcl-2-family members through their hydrophobic cleft involved in scaffolding the BH3 domain of pro-apoptotic Bcl-2-family members [118]. In contrast, the central, modulatory domain of the IP$_3$R responsible for transferring N-terminal IP$_3$ binding to C-terminal channel opening only binds the BH4 domain of Bcl-2, but not that of Bcl-XL [116]. In this respect, Bcl-2 may bind to two IP$_3$R domains, thereby limiting channel opening, whereas Bcl-XL may bind to the C-terminal channel domain, facilitating channel opening. Alternatively, Bcl-2-family members may affect the sensitivity of IP$_3$Rs towards basal IP$_3$ levels by enhancing PKA-dependent phosphorylation of the IP$_3$R channel, thereby increasing passive Ca$^{2+}$ leak through hypersensitive IP$_3$Rs [119]. This will result in decreased steady-state [Ca$^{2+}$]$_{\text{ER}}$ levels, limiting IP$_3$R-mediated Ca$^{2+}$ transfer into the mitochondria. In addition, Bcl-2-family members may regulate IP$_3$R-expression levels [120].

Beyond Bcl-2 proteins, other pro-survival and/or pro-cell death proteins regulate IP$_3$Rs. Recently, extranuclear promyelocytic leukemia protein (PML) has been identified as another key regulator of IP$_3$R activity in the MAMs [121–123]. At these microdomains, the tumor suppressor PML promotes the Ca$^{2+}$-flux properties of the IP$_3$R by recruiting PP2A, which counteracts PKB/Akt activity and thus suppresses PKB/Akt-mediated phosphorylation of IP$_3$Rs. This causes increased IP$_3$R-mediated Ca$^{2+}$ transfer into the mitochondria and thus OMM permeabilization.

In addition, apoptogenic factors released from mitochondria can also regulate IP$_3$R activity and provide feedback towards ER Ca$^{2+}$ signaling. For instance, cytochrome c (cyt c) is released from the mitochondria upon mitochondrial outer membrane permeabilization and binds to the IP$_3$Rs [124, 125]. As a result, the bell-shaped dependent regulation of IP$_3$R opening by cytosolic [Ca$^{2+}$] is perturbed and IP$_3$Rs fail to close in response to high cytosolic [Ca$^{2+}$]. Thus, upon apoptosis induction, cyt c will provide a positive feedback loop in which IP$_3$R-mediated Ca$^{2+}$ release is further promoted and mitochondrial Ca$^{2+}$ overload is inevitable. In other words, cyt
binding to IP$_3$Rs seems to be important for adequate apoptosis induction [126, 127]. In addition to this, IP$_3$Rs are targets of downstream apoptosis executioners, like caspase 3, which is activated upon cyt c release and apoptosome formation [128, 129]. Proteolytic cleavage of IP$_3$Rs leads to a 95-kDa C-terminal channel fragment that provokes uncontrolled Ca$^{2+}$ leak from the ER during apoptosis, further amplifying the cellular and mitochondrial Ca$^{2+}$ overload. Finally, during agonist-induced stimulation, activated IP$_3$Rs seem to be rapidly and selectively removed through ER-associated degradation (ERAD) in a variety of cellular systems [130–133]. Thus, down-regulation of the number of IP$_3$R channels responding to IP$_3$ may act as a homeostatic process for adaptation towards persistent IP$_3$ signaling. This will protect cells from the deleterious effects of aberrant IP$_3$R-mediated Ca$^{2+}$ signaling. IP$_3$R degradation involves its tagging with ubiquitin and targeting to the ubiquitin-proteasome pathway [131].

Given the fact that IP$_3$R activity critically controls mitochondrial apoptosis and that anti- and pro-apoptotic proteins target and modulate IP$_3$Rs, it is not surprising that cancer cells have pursued these mechanisms to promote their survival. For instance, chronic lymphocytic leukemia cells, characterized by a chromosomal rearrangement of the Bcl-2 gene resulting in elevated Bcl-2-protein levels, seem to be protected from pro-apoptotic Ca$^{2+}$ signals through the inhibitory effect of Bcl-2 on IP$_3$R activity [134]. Abolishing Bcl-2’s inhibitory properties on IP$_3$R function using cell-permeable peptides that cause dissociation of Bcl-2 from IP$_3$Rs causes exaggerated spontaneous Ca$^{2+}$ elevations, resulting in apoptotic cell death. Glioblastoma cells that display hyperactive PKB/Akt, have dampened IP$_3$R signals in the MAMs, decreasing their susceptibility towards apoptosis [135]. Likewise, mutations in PML affect the PKB/Akt signaling towards IP$_3$Rs and contribute to tumor development [123].

### 2.3 ER Ca$^{2+}$ Controls Survival, Apoptosis and Autophagy

ER Ca$^{2+}$ homeostasis is critical for cellular health [136]. Changes in ER Ca$^{2+}$ content and ER Ca$^{2+}$-release properties will affect cellular survival through a complex network of cellular responses, including autophagy, ER stress and apoptosis [3, 4].

Ca$^{2+}$ oscillations are typically associated with increased mitochondrial bioenergetics, including ATP and NADH synthesis and thus cell survival and proliferation [137–139]. Recently, spontaneous IP$_3$R-mediated Ca$^{2+}$ transfers into the mitochondria have been shown to be critical in this process [140]. Impaired IP$_3$R activity perturbed mitochondrial bioenergetics in response to lowered ATP/AMP ratio and caused the activation of autophagy through an increase in AMP kinase activity.

Excessive Ca$^{2+}$-release events and Ca$^{2+}$ transients are known to trigger mitochondrial Ca$^{2+}$ overload and opening of the PTP, resulting in apoptosis [141–143]. In this respect, a modest lowering in the steady-state ER Ca$^{2+}$ levels, e.g. by increasing the ER Ca$^{2+}$ leak through IP$_3$Rs or by anti-apoptotic proteins, act as a protective mechanism that enhances cellular survival [119, 144–146]. However, severe and/or chronic ER Ca$^{2+}$ depletion will act as an ER stress triggering UPR [4]. The regulation and mechanisms underlying these responses are discussed below.
3 [Ca^{2+}]_{ER} Controls ER-Stress Responses: Altered Steady-State [Ca^{2+}]_{ER} Levels Cause ER Stress

3.1 Disturbed ER Ca^{2+} Homeostasis Triggers ER Stress

ER-resident chaperones like calreticulin, GRP78/BiP, and GRP94 need a high [Ca^{2+}]_{ER} for their activity with Ca^{2+} binding to paired anionic amino acids [9, 147, 148]. In addition, several of the ER chaperones also act as Ca^{2+} buffers [7, 21], allowing ER Ca^{2+} accumulation up to millimolar levels. While a slight decrease in ER Ca^{2+} content may be beneficial for cell survival, severe depletion of ER Ca^{2+} by treating cells with a Ca^{2+} ionophore or by inhibiting SERCA ER-Ca^{2+}-uptake activity with thapsigargin, leads to inappropriate secretion, aggregation, and degradation of unassembled proteins [147]. Hence, the [Ca^{2+}]_{ER} must be maintained in an environment of continuous intracellular Ca^{2+} signaling. Furthermore, there is evidence that depletion of ER Ca^{2+} stores by itself is sufficient to trigger ER stress and apoptosis [149]. Failure of this homeostatic mechanism, either by toxic chemical agents or the altered expression of proteins disturbing ER Ca^{2+} homeostasis, triggers UPR to either re-establish normal ER function or to eliminate the cell [136]. These ER-stress responses may be triggered by aberrant IP_{3},R- and RyR-mediated Ca^{2+} release, the expression of ER Ca^{2+}-leak channels like CALHM1 and BI-1, inhibition of SERCA activity, deficiencies in Ca^{2+} ATPases in the Golgi and the expression of truncated isoforms of SERCA [4]. Furthermore, it is also important to note that the extent of ER Ca^{2+} depletion may control the outcome of ER stress, pro-survival or pro-death. A physiologically relevant example of this principle is the partial depletion of ER Ca^{2+} stores in β-cells upon glucose deprivation [150]. During fasting, the ATP/energy status of β-cells is decreased, which results in a decrease in SERCA Ca^{2+}-uptake activity and a decrease in the steady-state [Ca^{2+}]_{ER}. The latter causes the activation of PERK, but not of IRE1α (inositol-requiring enzyme 1), thereby protecting β-cells against oxidative stress and apoptotic ER stress [150]. The activation of PERK and subsequent eukaryotic initiation factor 2α (eIF2α) phosphorylation is known to be physiologically relevant for β-cell function and survival, including the repression of insulin expression. Thus, this PERK pathway seems to be enhanced by ER Ca^{2+} depletion during low glucose levels.

The adaptive mechanisms initiated by the UPR involve reduced translation of proteins, enhanced translation of ER chaperones to increase the folding capacity of the ER, and the degradation of misfolded proteins through ERAD [151–153]. Global mRNA translation is inhibited for a few hours to reduce the influx of new proteins into the ER, whereas alarm signals involving the activation of mitogen activated protein kinases are induced [154]. A chronic decrease in [Ca^{2+}]_{ER}, perturbing the function of chaperones, provokes the accumulation of unfolded proteins [5]. These act as a sink for luminal GRP78/BiP [155]. As a result, GRP78/ BiP shuffles from the ER-stress sensors (PERK, IRE1α and ATF6) to the unfolded proteins, thereby assisting their proper folding and preventing protein aggregation (Fig. 3). ER-stress sensors become de-repressed/activated, yielding early adaptive responses promoting survival or late responses promoting apoptosis under conditions of severe or on-going ER stress. Furthermore, cells coping with (mild) ER
The UPR in response to ER stress caused by (partial) ER Ca\(^{2+}\)-store depletion. At normal [Ca\(^{2+}\)]\(_{\text{ER}}\) the ER-stress sensors are scaffolded and inactivated by GRP78/BiP. Protein trafficking and quality-control mechanisms work normally. Polypeptides are translocated through Sec61 and become glycosylated. This transport is facilitated by the molecular chaperone GRP78/BiP. Glucosidases then prepare the glycoprotein for binding to the ER lectins, calreticulin and calnexin, whereas oxidoreductases catalyze disulfide-bond formation. ER-resident chaperones facilitate the proper folding of the nascent protein and prevent its aggregation. Further deglucosidation releases the ER lectins and once the protein is correctly folded and processed, the protein leaves the ER via the coat protein (COPII)-coated vesicles to the secretory pathway. Misfolded proteins, in contrast, associate with various chaperones, including GRP78/BiP, and are removed from the ER through ERAD. Under these conditions, IP,R1 activity is maintained by binding of GRP78/BiP, which facilitates its assembly. GRP78/BiP also scaffolds the chaperone Sigma-1 receptor, which is released from GRP78/BiP during physiological Ca\(^{2+}\) signaling and targets IP3R3, stabilizing its role at the MAMs and protecting its degradation. In contrast, when the [Ca\(^{2+}\)]\(_{\text{ER}}\) is chronically decreased, the function of chaperones becomes disturbed and unfolded proteins accumulate and act as sponge for luminal GRP78/BiP. As a consequence, ER-stress sensors are devoid of GRP78/BiP and become activated, yielding early adaptive responses promoting survival (indicated in green) or late responses promoting apoptosis under conditions of severe or ongoing ER stress (indicated in red). IRE1α undergoes dimerization and activation of its kinase and endoribonuclease activity, thereby splicing XBP1 mRNA and yielding a potent transcriptional activator that induces the expression of genes involved in ERAD, protein folding (like GRP78/BiP) and lipid synthesis. ATF6 goes to the Golgi compartment, where it is proteolytically cleaved to yield a cytosolic fragment (p50) that migrates to the nucleus and activates the transcription of UPR genes, like GRP78/BiP and CHOP. PERK dimerizes, autophosphorylates and phosphorylates eIF2α, thereby suppressing its activity and reducing the rate of translation initiation, while increasing the rate of translation of ATF4, a potent transcription factor that augments the expression of genes involved in anti-oxidative stress, amino-acid metabolism, and protein chaperoning. During ongoing ER stress...
stress will up-regulate their GRP78/BiP levels, preventing cells from undergoing apoptosis. In addition, increasing the level of ER Ca2+-binding proteins like calreticulin rendered cells more resistant towards toxic environmental agents known to up-regulate GRP78/BiP and to induce ER stress by preventing cytosolic Ca2+ rises, oxidative stress and thus cell death [156]. Collectively, it is clear that up-regulation of GRP78/BiP levels is beneficial to prevent cell death. This principle has been used in a compound screen for chemical GRP78/BiP inducers [157]. A novel compound, BiP inducer X was shown to activate ATF6 signaling and elevate BiP levels, thereby protecting neuronal cells against ER-stress-induced apoptosis in vitro and in vivo. Beyond GRP78/BiP-mediated depressing of ER-stress sensors (PERK, IRE1α and ATF6), cytosolic signaling molecules may also modulate the activity of these proteins through direct binding as endogenous ligands [158]. Finally, during recent years, it has become clear that IP3Rs too are (direct and indirect) targets of GRP78/BiP [159, 160].

3.2 Cytoplasmic Ca2+ Rise Triggers ER-stress Responses

Some reports indicate that the depletion of the ER Ca2+ stores by itself is the major factor for triggering ER stress and apoptosis without any involvement of the capacitative Ca2+ entry or a sustained elevation of intracellular Ca2+ concentrations [149]. However, it is clear that not only the decrease in [Ca2+] in the ER underlies ER-stress responses, but also the concomitant [Ca2+] elevations in the cytosol control the activation of pro-apoptotic ER-stress events. The mechanism involves the activation of calmodulin dependant kinase II (CaMKII), which enables apoptosis through c-Jun N-terminal kinase (JNK)-mediated Fas induction and promotion of mitochondrial Ca2+ uptake, followed by mitochondrial membrane permeabilization and release of cyt c [161, 162]. Recently, calcineurin has been implicated in ER stress, causing its (transient) activation upon cytosolic Ca2+ rises and positive feedback on PERK signaling through direct interaction, thereby stimulating its autophosphorylation and kinase activity [210]. Furthermore, RCAN1, a regulator of calcineurin 1, is a downstream target of ATF6, thereby coordinating cell growth and ER stress signaling [211]. In addition, calcineurin hyperactivation has been or irreparable ER damage, apoptotic pathways are activated. IRE1α phosphorylates JNK, leading to inhibition of Bcl-2 activity and activation of Bim, and recruits, releases and activates pro-caspases in the cytosol. Induction of CHOP via XBP1, ATF6 or ATF4, down-regulates pro-survival Bcl-2-family members, increases pro-death proteins (like Bim) and ROS species, and decreases the levels of glutathione, a ROS scavenger. In the presence of ROS species, Ca2+ transfer to the mitochondria leads to the release of cyt c. The balance between pro- and anti-apoptotic Bcl-2-family members is disturbed, activating the intrinsic apoptotic pathway. During ER stress, IP3,R function is completely remodeled. GRP78/BiP will be released from IP3,R1, causing a decline in IP3,R activity through decreased subunit assembly. On the other hand, GRP78/BiP will release the Sigma-I receptor and increase its chaperone function, causing an enhanced IP3,R3-mediated Ca2+ flux into the mitochondria. The outcome of these processes may promote survival or cell death. This figure has been adapted from [4]. © Cold Spring Harbor Laboratory Press
associated with neurodegeneration in response to misfolded proteins and ER stress [212]. In any case, the co-operation of decreased $[\text{Ca}^{2+}]_{\text{ER}}$ and the increase in cytosolic $[\text{Ca}^{2+}]$ is underpinned by the observation that oxidative stress-induced ER stress and cell death is enhanced by depleting the ER $\text{Ca}^{2+}$ stores using thapsigargin [163]. Similar findings were obtained in neurons exposed to hyperactivation of ionotropic glutamate receptors, e.g. by using NMDA. The latter causes a $\text{Ca}^{2+}$ overload into the cytoplasm that leads neurons to excitotoxic death. However, aberrant activation of intracellular $\text{Ca}^{2+}$-release channels, like RyRs and IP$_3$Rs, contribute to this $\text{Ca}^{2+}$ overload and the disruption of ER $\text{Ca}^{2+}$ homeostasis, leading to ER-stress responses, like eIF2α phosphorylation or GRP78/Bip elevation. Importantly, NMDA-induced ER-stress events were counteracted using inhibitors of IP$_3$R and RyR channels [164].

On the other hand, ER-stress events may elicit $\text{Ca}^{2+}$ influx. For instance, the accumulation of unfolded proteins and ER stress triggered cytosolic $\text{Ca}^{2+}$ elevations through the stimulation of $\text{Ca}^{2+}$-influx pathways, resulting in calcineurin activation and in long-term survival of cells undergoing ER stress [165]. This pathway seems to be pursued by pathogenic fungi to promote resistance towards azole antifungal drugs.

Finally, it is important to note that there is emerging evidence that ER stress also activates autophagy, a conserved lysosomal degradation pathway turning over cellular macromolecules like protein aggregates, and damaged or dysfunctional organelles [166] (see further Chap. 7). Activation of PERK and IRE1α in response to ER stress has been linked with the stimulation of autophagy [167]. A third mechanism involves the rise in cytosolic $\text{Ca}^{2+}$ that accompanies (partial) ER $\text{Ca}^{2+}$ depletion, thereby activating CAM-kinase kinase β [168]. The latter activates AMPK, a negative regulator of mammalian target of rapamycin (mTOR). mTOR signaling negatively regulates autophagy. Thus, AMPK activation will lead to an increased autophagic flux. A recent study provided novel insights in the regulation of autophagy induction by AMPK, indicating its regulation of ULK1 phosphorylation and kinase activity [169]. In normal conditions, ULK1 forms a complex with mTORC1. During mTORC1 inactivation (e.g. starvation), mTORC1 dissociates from ULK1, allowing AMPK recruitment, ULK1 phosphorylation and activation by autophosphorylation. As a result, mATG13 is phosphorylated, forming a stable complex with ATG101 and FIP200 and causing autophagy induction [170, 171]. Independent of AMPK, cytosolic $\text{Ca}^{2+}$ increases may also directly affect other autophagy proteins, like the recruitment of Phosphatidylinositol 3-phosphate -binding protein WIPI-1 (Atg18) to autophagosomal membranes [172]. In any case, the rise in cytosolic $\text{Ca}^{2+}$ seems an important factor for autophagy induction [48], and thus may also be a critical factor for ER-stress-induced autophagy and its cytoprotective effects. The activation of autophagy may be beneficial to remove cytosolic protein aggregates, which induce ER stress by a global reduction in proteasome activity [173]. This is particularly important in neurodegenerative diseases and in prion-based diseases, which are associated with mutant proteins and aggregates of protein [167, 174]. However, it should be noted that continually activated autophagy will result in cell death and thus may also contribute to cell death in response to (severe) ER stress [175].
4 ER Stress and Altered Ca\textsuperscript{2+} Signaling Are Tightly Connected

4.1 Altered ER Ca\textsuperscript{2+} Fluxes During ER Stress

4.1.1 Altered IP\textsubscript{3}R Signaling During ER Stress
IP\textsubscript{3}R activity has been shown to be regulated during ER stress [4, 159, 160, 176]. However, heterogeneity among IP\textsubscript{3}R isoform function during ER stress was observed since IP\textsubscript{3}R1, but neither IP\textsubscript{3}R2 nor IP\textsubscript{3}R3, function was affected during ER stress [160]. IP\textsubscript{3}R1 channels are targets of GRP78/BiP, a necessary chaperone for their assembly into functional tetrameric units (see also 4.2.1). During ER stress, GRP78/BiP is released from IP\textsubscript{3}R1 and ERp44 now binds to the same site on IP\textsubscript{3}R1, thereby suppressing its Ca\textsuperscript{2+}-flux properties [177]. Moreover, ER stress may also disrupt Ca\textsuperscript{2+}-signaling complexes, thereby hampering efficient physiological IP\textsubscript{3} signaling, as observed in PERK-deficient cells [178]. In addition, prolonged ER stress that recruits CHOP activation may lead to the binding of ER oxidase 1\alpha (ERO1\alpha) to IP\textsubscript{3}Rs, thereby potentiating Ca\textsuperscript{2+} flux through IP\textsubscript{3}R channels and triggering excessive Ca\textsuperscript{2+}-signaling events that induce cell death [176].

4.1.2 Expression of Defective SERCA Pumps During ER Stress
Second, during ER stress, the specialized SERCA1 T isoforms is expressed. Indeed, ER-stress inducers cause the accumulation of SERCA1 T, but not of full-length SERCA1 [96]. The induction of SERCA1 T occurs through PERK/eIF2\alpha phosphorylation/ATF4 activation and amplifies the ER-stress responses, since siRNA against SERCA1 T inhibited the up-regulation of ER-stress markers. The mechanism could be attributed to its role in enhancing ER Ca\textsuperscript{2+} leak and depleting ER Ca\textsuperscript{2+} stores. ER-stress inducers caused a dramatic decrease in steady-state ER [Ca\textsuperscript{2+}], which was severely reduced upon knock down of endogenous SERCA1 T. Furthermore, SERCA1 T is preferentially expressed at ER/mitochondrial contact sites, increasing the docking of mitochondria to the ER and reducing the distance between ER and mitochondria. As a consequence, SERCA1 T enhances IP\textsubscript{3}R-mediated Ca\textsuperscript{2+} leak into the mitochondria while leaving global IP\textsubscript{3}R-mediated Ca\textsuperscript{2+} transients in the cytosol unaffected, causing Ca\textsuperscript{2+}-dependent apoptosis through mitochondria.

4.2 ER-stress Proteins Control Ca\textsuperscript{2+} Signaling from the ER

4.2.1 GRP78/BiP
Brostrom et al. were among the first to demonstrate that GRP78/BiP levels were tightly controlled by ER Ca\textsuperscript{2+} levels. Indeed, low concentrations of the Ca\textsuperscript{2+} ionophore ionomycin, causing ER Ca\textsuperscript{2+} mobilization, were sufficient to up-regulate GRP78/BiP-protein levels, while the phosphorylation of the alpha-subunit of eIF-2 responsible for the rapid suppression of translational initiation required higher concentrations of ionomycin [179]. Furthermore, up-regulation of GRP78/BiP levels by treating cells with thapsigargin rendered cells more tolerant to environmental
stress [156]. GRP78/BiP also acts as a Ca\(^{2+}\)-buffering protein in the ER, increasing the ER Ca\(^{2+}\)-store capacity [21]. In this respect, GRP78/BiP not only acts as a molecular chaperone, but also indirectly contributes to the function of Ca\(^{2+}\)-dependent ER chaperones and folding proteins by increasing the ER Ca\(^{2+}\)-store capacity during ER stress. The role of GRP78/BiP in stabilizing ER Ca\(^{2+}\) homeostasis by GRP78/BiP were provided by the laboratory of Miki-shiba [160] (Fig. 4). IP\(_3\)R1 knock down was shown to enhance the susceptibility of neuronal cells towards ER-stress inducers, leading to a decrease in mitochondrial potential and increased apoptosis. Conversely, cells treated with ER-stress inducers significantly impaired IP\(_3\)-R-channel activity in intact cells and microsomal preparations. Importantly, these results were independent from effects on ER Ca\(^{2+}\)-store content. The molecular mechanism involved a direct binding of GRP78/BiP to IP\(_3\)R1. GRP78/BiP selectively bound to IP\(_3\)R1, but did not bind to the other IP\(_3\)-R isoforms. The GRP78/BiP-interaction site of IP\(_3\)R1 corresponds to the divergent region of the largest intraluminal loop of IP\(_3\)R1 located between the fifth and sixth transmembrane domain. Importantly, this divergent region precedes a region that is conserved among all IP\(_3\)-R isoforms, corresponding to the channel-pore-forming region. This GRP78/BiP-binding site on IP\(_3\)-Rs overlaps with the site previously identified for IP\(_3\)-R interaction with the ER chaperone ERp44. As a consequence, GRP78 competes with ERp44 for IP\(_3\)R1 binding and dissociates ERp44 from IP\(_3\)-Rs. Importantly, during ER stress conditions, GRP78 dissociates from IP\(_3\)R1. This decrease in GRP78/BiP binding to IP\(_3\)R1 during ER stress seems to underlie the impaired IP\(_3\)R1-channel activity, since GRP78/BiP knock down impaired Ca\(^{2+}\) release through IP\(_3\)R1, but not through IP\(_3\)R2 or IP\(_3\)R3. This indicates that GRP78/BiP binding to IP\(_3\)R1 is essential for IP\(_3\)R1-channel activity. The mechanism involves a critical role for GRP78/BiP in the assembly of IP\(_3\)R1 monomers into functional tetrameric channel complexes. Importantly, GRP78/BiP knockdown as well as ER-stress induction reduces the amount of IP\(_3\)R1 subunit assembly into tetramers, while ATP seems to enhance IP\(_3\)R1/GRP78-complex formation. Furthermore, a mutant IP\(_3\)R1 channel lacking the GRP78/BiP-binding site displayed a prominent decrease in tetrameric subunit assembly. Further mechanistic insights were provided by the use of a GRP78/BiP mutant which is able to bind ATP but lacks ATP-induced conformational change. In contrast to wild-type GRP78/BiP, this ATPase-deficient GRP78/BiP mutant inhibits IP\(_3\)R1-subunit assembly and does not enhance IP\(_3\)R1-mediated Ca\(^{2+}\) signaling. Hence, these results suggest that IP\(_3\)R1 may function as the fourth ER-stress sensor, besides PERK, IRE1\(\alpha\) and ATF6. Indeed, during (prolonged) ER stress IP\(_3\)R1 activity will decline, leading to a decrease in mitochondrial potential and thus apoptosis. This may be relevant under conditions of severe ER stress. During mild ER stress, the decline in IP\(_3\)R1 activity may result in the activation of autophagy in response to a decrease in ATP/AMP ratio and activation of AMP kinase [140]. Indeed, IP\(_3\)-R knock down or inhibition using xestospongin B has been shown to induce autophagy as a compensatory survival pathway in response to a decline in mitochondrial bioenergetics.
Finally, it is important to note that GRP78 not only directly targets IP₃R₁, but also indirectly influences IP₃R₃-channel activity by scaffolding Sigma-1 receptors, which seem to stabilize functional IP₃R₃-channel complexes at the MAMs not only during physiological signaling but also during the induction of ER stress [159]. During physiological signaling, Sigma-1 receptors seem to protect IP₃R₃ from degradation. During ER stress, Sigma-1 receptors are released from GRP78/BiP. This increases Sigma 1 receptor’s chaperone activity and their binding to IP₃R₃ localized at the MAM, thereby sustaining Ca²⁺ transfer during the adaptive phase of ER stress.
4.2.2 PERK

While it is clear that PERK activation is a hallmark of ER Ca\(^{2+}\) depletion, there is also recent evidence that PERK may control intracellular Ca\(^{2+}\)-signaling events in response to agonist stimulation [178]. Indeed, cells deficient in PERK displayed increased ER stress associated with a distended and fragmented ER that was disconnected from contacts with the plasma membrane. Since ER/plasma-membrane contacts are important for efficient IP\(_3\)-induced Ca\(^{2+}\) release upon the binding of agonist to receptors that activate PLC\(\beta/\gamma\), PERK deficient cells displayed decreased rates of agonist-induced Ca\(^{2+}\) release. Hence, due to increased distance between the ER and the plasma membrane, diffusion of IP\(_3\) from the site of production (the plasma membrane) to its target site (the ER) will be very inefficient and rate limiting. Furthermore, in intact cells, IP\(_3\) is rapidly turned over, further hampering IP\(_3\) signaling. Thus, PERK seems to be important for proper ER integrity and thus contributing to efficient IP\(_3\)/Ca\(^{2+}\) signaling in plasma membrane/ ER microdomains [178]. PERK does not seem to directly regulate IP\(_3\)Rs, since Ca\(^{2+}\) release triggered by exogenous IP\(_3\) application in a permeabilized cell system was not affected.

Recently, PERK was shown to interact with the Ca\(^{2+}\)/calmodulin-dependent phosphatase calcineurin during ER stress [181] (Fig. 5). ER stress activated and up-regulated calcineurin binds to PERK and indirectly increases PERK autophosphorylation and activity, enhancing eIF2\(\alpha\) phosphorylation and shutting down protein translation. In addition, when Ca\(^{2+}\) is restored, PERK will also phosphorylate calcineurin and reduce its activity and expression. As a consequence, PERK signaling will be turned off and in combination with restored ER Ca\(^{2+}\) filling, normal protein translation and ER homeostasis will re-establish. Hence, calcineurin/PERK seems to function as an important rheostat linking Ca\(^{2+}\) changes during ER stress to control of the activity of ER-stress sensors.

4.2.3 ER-redox Enzymes

ER chaperones, like calreticulin, clearly regulate ER Ca\(^{2+}\) signaling by affecting SERCA Ca\(^{2+}\)-uptake activity and IP\(_3\)-induced Ca\(^{2+}\) signaling [182–184]. Furthermore, calreticulin and calnexin can interact with proteins like protein disulfide isomerase and ERP57, an ubiquitous ER thiol-dependent oxidoreductase that promotes the formation of intra- or intermolecular disulfide bonds during glycoprotein folding [14, 19, 185]. It is clear that these complexes dynamically regulate Ca\(^{2+}\)-transport mechanisms. During high [Ca\(^{2+}\)]\(_{\text{ER}}\), calreticulin through its N domain directly targets glycosylated residues in the C-terminal tail of SERCA2b and through its P domain recruits ERP57 to SERCA2b, thereby promoting disulfide bond formation between thiol groups in the fourth intraluminal loop domain [186]. Furthermore, under these conditions, calnexin is phosphorylated and interacts with SERCA2b [184]. These mechanisms reduce SERCA Ca\(^{2+}\)-uptake activity. During low [Ca\(^{2+}\)]\(_{\text{ER}}\), ERP57 dissociates from SERCA2b, resulting in increased oxidation of the intraluminal thiol groups and an increased SERCA Ca\(^{2+}\)-uptake activity [186]. Furthermore, the increase in cytosolic Ca\(^{2+}\) may result in calnexin dephosphorylation, causing its dissociation from SERCA2b [184]. These mechanisms increase SERCA Ca\(^{2+}\)-uptake activity. Recently, calcineurin
was shown to be implicated in calnexin dephosphorylation, alleviating its inhibitory effect on SERCA in response to ER-stress-induced Ca$^{2+}$ rises and attempting to restore normal ER Ca$^{2+}$ filling [181]. During this process, calcineurin activity will be decreased through phosphorylation by PERK. This in turn will turn off PERK autophosphorylation and activity and mediate a return to normal ER homeostasis (Fig. 5).

Another thioredoxin-family member, ERp44, directly targets the variable region of the third intraluminal loop domain of IP$_3$R1, which is divergent from IP$_3$R2 and IP$_3$R3 [177]. The binding of ERp44 to IP$_3$R1 was dependent on steady-state ER [Ca$^{2+}$], since depletion of ER Ca$^{2+}$ stores enhanced the IP$_3$R1/ERp44-complex formation. Also, the redox state of the ER influenced ERp44’s binding to IP$_3$R1, since
mutating Cys residues in the third intraluminal loop of IP₃R1 decreased ERp44 binding, indicating that free thiol groups are required for ERp44 binding. In addition, ERp44 levels determine Ca²⁺ release through IP₃R1 channels, since overexpression of ERp44 inhibited IP₃R1-mediated Ca²⁺ signaling, while knock down of ERp44 enhanced these events. Consistent with the binding characteristics of ERp44 to IP₃R1, free thiol groups are required for the inhibition of IP₃R1 by ERp44, since mutating two specific Cys residues in the variable region of the third intraluminal loop (Cys2496 and Cys2504 of IP₃R1) make IP₃R1 channels resistant to inhibition by ERp44.

Taking together SERCA2b and IP₃R1 regulation by redox enzymes, it is clear that the redox state of the cells works in a coordinated manner to regulate [Ca²⁺]ER homeostasis (Fig. 6). Under normal physiological conditions, including filled ER Ca²⁺ stores, the ER is an oxidizing environment [187, 188] and chaperones, which depend on adequate ER Ca²⁺ levels will function properly [9]. To prevent ER Ca²⁺ overload, SERCA2b Ca²⁺-uptake activity is suppressed by ERp57 binding to disulfide bonds in the ER lumen and by binding of phosphorylated calnexin [184, 186], while IP₃R1 activity is enhanced by low levels of ERp44 binding and high levels of GRP78/BiP binding [160, 177]. Under reducing conditions in the ER or decreased [Ca²⁺]ER, SERCA2b and IP₃R1 will expose thiol-free groups in their luminal domains. As a result, SERCA2b activity will be stimulated through release of ERp57 and calnexin dephosphorylation by calcineurin [181, 184, 186], while IP₃R1 activity will be inhibited through loss of GRP78 binding and increased binding of ERp44 [160, 177]. This will lead to an increase in steady-state [Ca²⁺]ER levels. During this return, calcineurin activity is proposed to decline and restore dormant PERK [181]. Likely, these conditions will favor the function of Ca²⁺-dependent ER chaperones and oxidoreductases. These dynamic mechanisms are important to regulate Ca²⁺-transport mechanisms in an adequate manner to restore proper filling of the ER Ca²⁺ stores. Failure in these homeostatic mechanisms to restore adequate ER Ca²⁺-store filling likely will result in chronic ER stress and cell death.

The redox regulation of these Ca²⁺-transport mechanisms are highly relevant during ER stress, since the redox state of the ER is altered during ER stress [189, 190]. One mechanism involves the induction of ERO1α downstream CHOP activation, leading to hyperoxidation of the ER lumen [176]. The latter may completely remove ERp44 from IP₃Rs, causing IP₃R hypersensitivity (Fig. 4). Importantly, in vitro ER-stress models (e.g. in lipoprotein-cholesterol models), ERO1α is up-regulated in a CHOP-dependent manner in macrophages and protected against ER-stress induced apoptosis, but not against non-ER-stress-induced apoptosis (like staurosporine). Importantly, ER-stress inducers (like tunicamycin) strongly activated IP₃R-mediated Ca²⁺ signaling when ERO1α was expressed, but not when ERO1α expression was knocked down or lacking in the macrophages. Furthermore, both IP₃R1 and ERO1α seemed necessary for ER-stress-induced apoptosis. The mechanism likely involves IP₃R1 hyperoxidation, since the antioxidant N-acetyl-Cys inhibited both the enhanced IP₃R activity and increase in apoptosis in macrophages undergoing ER stress. It also requires the presence of CHOP, indicating that systemic ER stress that leads to CHOP activation will activate IP₃R1-mediated Ca²⁺ signaling through
ERO1α-mediated hyperoxidation of the ER lumen. The latter seems to affect IP₃R1/Erp44-complex formation in the macrophages by shifting the equilibrium towards disulfide bridge formation in the third intraluminal loop of IP₃R1. Thus, chronic ER stress through ERO1α activity will abolish the low level binding of Erp44 to IP₃R1 and result in hyperactive IP₃R1 channels and apoptosis. Importantly, similar observations were made in the macrophages of insulin-resistant obese mice, suggesting a relevance of this mechanism in pathological conditions, like diabetes.

Importantly, during these oxidative stress conditions, other Ca²⁺-transport mechanisms might be affected, including the luminal ER Ca²⁺-sensor Stim1. Recently, it has been shown that Stim1 is S-glutathionylated at position Cys56, thereby decreasing its affinity for Ca²⁺ and facilitating its aggregation and activation of Orai1 Ca²⁺-influx channels [191]. This will contribute to a constitutive Ca²⁺ influx and increase in basal cytosolic Ca²⁺ levels. Thus, increased store-operated Ca²⁺ influx through partial ER Ca²⁺ depletion and hyperactivation of Stim1/Orai1 signaling through redox modifications at the level of the Stim1 protein, may cooperate to cellular Ca²⁺ overload to eliminate cells in response to oxidative stress, e.g. such as during prolonged ER stress.

It is important to note that other Ca²⁺-transport proteins may help to fight the accumulation of reactive oxygen species (ROS) during ER stress. A well-documented example is BI-1 [192].

### 4.3 Ca²⁺-transport Mechanisms Directly Regulate ER-stress Sensors with Complex Outcomes: the Example of BI-1

Work from John C. Reed’s lab and many others showed that BI-1, a conserved ER protein that suppresses cell death in yeast, plants and animals, protects against ER-stress-mediated cell death, but not against mitochondrial or Fas/death-receptor pathways [87, 88, 193, 194]. BI-1-deficient cells were hypersensitive to ER-stress inducers, while BI-1 overexpression protects against apoptosis induced by ER stress [88]. These cytoprotective properties of BI-1 correlate with its ability to reduce the steady-state ER Ca²⁺ levels, presumably via its Ca²⁺/H⁺-antiporter activity [89, 90]. In addition, BI-1’s protective role against ER stress seems to involve the regulation of vacuolar H⁺-ATPase activity in the lysosomes, thereby maintaining proper lysosomal function during ER stress [195]. In vivo, BI-1-deficient mice are hypersensitive to ER-stress induction and display increased elevated tissue damage e.g. after ischemic reperfusion injury [196]. In this respect, BI-1 overexpression in neurons protects against cell death in response to low glucose concentrations through decreasing steady-state ER Ca²⁺ levels [197, 198]. Hence, BI-1 likely plays an important role to preserve tissue integrity in major organs, like liver, kidney and brain in order to promote cell survival and homeostasis after ER stress and/or injuries [196]. This is supported by the fact that kidney and liver tissue from BI-1-deficient mice displayed a prominent increase in ER-stress markers, including induction of CHOP through the activation of IRE1α. This event leads to splicing of XBP-1 and phosphorylation of JNK and the cleavage of the transcription factor ATF6, whose
cytosolic domain travels to the nucleus. Importantly, PERK signaling, eIF2α phosphorylation and ATF4 processing remained unaffected by the lack of BI-1.

Further studies revealed important insights in the mechanisms by which BI-1 regulates ER-stress responses. BI-1 prevents the accumulation of ROS that arises during ER-stress induction. The mechanism involves the up-regulation and increased activity of heme oxygenase 1 (HO-1), the rate-limiting enzyme in the degradation of heme into biliverdin, CO, and free divalent iron (Fe^{2+}) [199]. HO-1 regulation during ER stress strongly correlated with the presence of BI-1. Furthermore, HO-1 was critical for BI-1-mediated cytoprotection against ER stress, since HO-1-deficient cells overexpressing BI-1 were not protected against ER-stress inducers. BI-1 regulation of HO-1 expression seemed dependent on Nrf2, a transcription factor that regulates ARE-driven gene expression. Nrf2 has been shown to induce the expression of phase 2 detoxifying and antioxidant enzymes and other stress-inducible genes, including GSH S-transferase, quinine reductase (NQQ1), and HO-1 (30). In addition to HO-1, BI-1 targets cytochrome P450 (P450), a component of the microsomal monooxygenase system, composed of P450 2E1, NADPH-P450 reductase (NPR) and phospholipids [200]. The relatively low degree or efficiency in electron coupling between NPR and P450 is thought to be a major source for ROS production. During ER stress, ROS production is enhanced in part due to up-regulation of P450 2E1 resulting in lipid peroxidation of the ER membranes and ER-stress-mediated cell death. Cells lacking P450 2E1 activity are protected against these events due to decreased ROS accumulation. Importantly, the increase in P450 2E1 during ER stress is counteracted by BI-1 overexpression, while cells lacking BI-1 displayed increased ROS accumulation and P450 expression. BI-1 also modulated electron flow between NPR and P450 2E1 by reducing the interaction between NPR and P450 2E1 and the catalytic activity of P450 2E1 enzyme. To summarize, these data indicate that BI-1 fights ROS production and accumulation by down-regulating P450 2E1 activity and up-regulating HO-1, thereby limiting the accumulation of misfolding proteins due to oxidative dysregulation and thus reducing unfolded protein responses.

In addition to these mechanisms, the Hetz laboratory revealed that BI-1 also targets the ER-stress sensor IRE1α [201]. Signaling through IRE1α has been shown to be regulated by physical interaction with the pro-apoptotic executioner Bel-2-family members, Bax and Bak [202]. Now, BI-1 negatively regulates IRE1α activity and thus suppresses XBP-1 splicing, while BI-1-deficient cells display increased XBP-1 splicing and the expression of its downstream targets, i.e. ER-stress-induced genes that promote folding, degradation of misfolded ER proteins through the ERAD pathway and genes involved in the translocation of proteins into the ER [201]. BI-1 seemed to be involved in the inactivation of the IRE1α complex and the decrease in XBP-1 splicing resulting in recovery of full-length XBP-1. This was observed during conditions of mild but prolonged ER stress (chronic) and conditions of transient but strong ER stress induction (acute). Furthermore, inhibition of spliced XBP-1 expression selectively increased the susceptibility of BI-1-knockout cells, but not of wild-type cells, to chronic ER-stress. However, XBP-1 seemed to protect both BI-1-knockout and wild-type cells in response to acute ER-stress. The molecular mechanism involved the binding of BI-1 to the C-terminal domain of IRE1α, which
contains its kinase and endoribonuclease domains. A constitutive binding of BI-1 to IRE1α was observed during basal conditions as well as during ER stress. BI-1 binding to IRE1α was shown to potently block its endoribonuclease activity, since XBP-1 splicing by recombinantly expressed and purified IRE1α was inhibited by the presence of BI-1. The BI-1 domain responsible for IRE1α binding is its C-terminal part, which contains the pH sensor and which is essential for BI-1’s Ca2+-leak properties. Hence, these data indicate that BI-1 negatively regulates IRE1α/XBP-1 signaling, which is involved in adaptive responses and protection against ER stress. This seems to contrast with its known anti-apoptotic function against (severe) ER stress. However, low doses of ER stress may reflect the in vivo condition, in which cells are able to cope with injury and activate distinct pathways that do not involve apoptosis induction [203]. In this respect, BI-1 may negatively regulate adaptive responses. This seems to be supported by a very recent study by Hetz and co-workers, showing that BI-1 represses autophagy through JNK and IRE1α expression [204]. Furthermore, BI-1-deficient cells display up-regulated autophagic flux in comparison to wild-type cells. As a consequence, survival of BI-1-deficient cells was enhanced during nutrient deprivation. Similar findings were found in flies lacking BI-1 expression. Thus, BI-1 seems to function as a stress integrator controlling a variety of homeostatic processes, including adaptive ER-stress response, ER-stress dependent apoptosis and pro-survival signaling through autophagy.

4.4 Ca2+ Signaling Contributes to Survival, Adaptation and Death Decision upon ER Stress

Finally, it is important to present an integrated overview of the different mechanisms that control Ca2+ signaling determining the outcome of ER-stress (Fig. 6). It is important to note that Ca2+ signaling in microdomains may be totally different than global cytosolic Ca2+ levels, thereby affecting different targets. During mild or transient ER stress, adaptation and/or survival mechanisms may be activated through elevated cytosolic Ca2+ levels like the activation of calcineurin, which helps PERK activation and CaMKIIβ, which in turn activates autophagy through dampened IP3R1-mediated Ca2+ transfer from ER into mitochondria in the MAM microdomain. This could occur through AMPK activation, a positive regulator of autophagy that inhibits mTOR and enhances ULK activity or through maintaining IP3R3 activity in the MAMs and thus increasing mitochondrial bioenergetics. On the other hand, irreparable ER stress may lead to cell elimination through apoptosis. This can be triggered by a severe reduction in mitochondrial potential due to dampened Ca2+ transfer into the mitochondria or by mitochondrial Ca2+ overload. The latter is due to the activation of ERO1α-dependent aberrant IP3R1 signaling and the activation of CaMKII signaling, which leads to Fas induction and enhanced mitochondrial Ca2+ uptake. This toxic Ca2+ signaling may be aggravated by aberrant activation of store-operated Ca2+ entry through glutathionylation of STIM1. It is clear that the cocktail of the Ca2+ signals are primary detectors of the cellular health at the level of the ER, critically controlling the balance between pro-survival and pro-death pathways.
In this book chapter, we provided an overview of the tight connection between ER Ca²⁺ homeostasis and its role in protein folding. It is clear that many mechanisms contribute to adequate ER Ca²⁺ filling, a requisite for the proper function of many ER chaperones and oxidoreductases. On the one hand, chronic or severe dysregulation of ER Ca²⁺ levels is known to underlie a variety of diseases and results in ER stress due to accumulation of misfolded proteins, redox dysregulation and the
activation of the UPR. This quality control system provides an integrated signaling pathway, resulting in a variety of adaptive responses to cope with mild ER stress, or pro-apoptotic events to remove cells suffering from irreparable damage during severe ER stress. Furthermore, the close proximity of the mitochondria senses these ER-stress responses due to altered Ca^{2+} fluxes from the ER into the mitochondrial matrix. It also seems clear that the ER Ca^{2+}-signalosome is transformed during ER stress with different regulation of IP_{3}Rs and SERCAs, but also by the expression of alternative isoforms (e.g. SERCA1 T). Part of this altered regulation of Ca^{2+} transports relates to the direct binding of ER-stress proteins, chaperones and redox enzymes to selective SERCA and IP_{3}R isoforms, thereby directly regulating Ca^{2+} fluxes from the ER and controlling cell fate (survival or apoptosis). There is now also accumulating evidence that Ca^{2+} transporters like BI-1 form physical complexes with ER-stress sensors such as IRE1α, thereby strictly regulating the extent and amplitude of their activity and thus their function in UPR upon ER stress. These insights are important in order to develop novel therapeutic strategies for diseases caused by chronic ER Ca^{2+} depletion and ER stress or to trigger ER stress through depleting ER Ca^{2+} stores in malignant cells.

Acknowledgments

The authors wish to thank the Research Council of the K.U. Leuven and the Research Foundation – Flanders (F.W.O.) for their support of the research work performed in their lab. We also wish to apologize to those whose research papers in this field were not included in this book chapter. We also wish to thank our national and international collaborators in this field for fruitful discussions.

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