Regulation of calcium homeostasis and flux between the endoplasmic reticulum and the cytosol

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Abstract

The endoplasmic reticulum (ER) is the major store for calcium ions (Ca^{2+}) in the cell. Under physiological conditions, a steep concentration gradient exists between the ER (100 μM up to 1 mM [Ca^{2+}]) and the cytosol (about 100 nM [Ca^{2+}]) (1). Influx of Ca^{2+} into the ER is mainly achieved by the energy-consuming ATPase SERCA2b (sarcoplasmic/endoplasmic reticulum calcium transport ATPase pump, building the Ca^{2+} gradient across the ER membrane required for ATP import. Meanwhile, Ca^{2+} leakage from the ER has been reported to occur via the Sec61 translocon following protein translocation. In this review, we provide an overview of the complex regulation of Ca^{2+} homeostasis, Ca^{2+} flux between subcellular compartments, and the cellular stress response (the unfolded protein response) induced upon dysregulated luminal Ca^{2+} metabolism. We also provide insight into the structure and gating mechanism at the Sec61 translocon and examine the role of ER-resident cochaperones in assisting the central ER-resident chaperone BiP in the control of luminal Ca^{2+} concentrations.

The concentration of Ca^{2+} in the endoplasmic reticulum (ER) is critically important for maintaining its oxidizing environment as well as for maintaining luminal ATP levels required for chaperone activity. Therefore, local luminal Ca^{2+} concentrations and the dynamic Ca^{2+} flux between the different subcellular compartments are tightly controlled. Influx of Ca^{2+} into the ER is enabled by a reductive shift, which opens the sarcoplasmic endoplasmic reticulum calcium transport ATPase pump, building the Ca^{2+} gradient across the ER membrane required for ATP import. Meanwhile, Ca^{2+} leakage from the ER has been reported to occur via the Sec61 translocon following protein translocation. In this review, we provide an overview of the complex regulation of Ca^{2+} homeostasis, Ca^{2+} flux between subcellular compartments, and the cellular stress response (the unfolded protein response) induced upon dysregulated luminal Ca^{2+} metabolism. We also provide insight into the structure and gating mechanism at the Sec61 translocon and examine the role of ER-resident cochaperones in assisting the central ER-resident chaperone BiP in the control of luminal Ca^{2+} concentrations.

Apart from Ca^{2+} flux between the ER and the cytosol, there are also Ca^{2+} flux between the ER and mitochondria. This Ca^{2+} transfer from the ER to mitochondria is closely linked to mitochondrial ATP production and cell survival (8, 9) and requires a close apposition of the ER membrane and the outer mitochondrial membrane thereby forming microdomains. These microdomains are named mitochondria-associated ER membranes (MAMs) (Fig. 1). MAM contacts have a width of 10 to 25 nm and are optimal for Ca^{2+} transfer, so called “Ca^{2+}-hot spots”. In these zones, the concentration of Ca^{2+} is very high reaching levels of more than 10 μM (see review (8)). Efficient Ca^{2+} flux from the ER to mitochondria is mediated via IP3R, located within the ER membrane, the voltage-dependent anion channel 1 (VDAC1), which is located at the outer mitochondrial membrane, and mitochondrial Ca^{2+} uniporter 1, located at the inner mitochondrial membrane (8). Interaction of IP3R with VDAC1 is facilitated by the chaperone GRP75, which is enriched in MAMs and associated with both, IP3R and VDAC1 (9) (Fig. 1). Although mitochondrial Ca^{2+} levels are closely linked to mitochondrial ATP synthesis, Ca^{2+} influx into and prevailing [Ca^{2+}] within mitochondria need to be carefully surveyed, since excessive Ca^{2+} accumulation within mitochondria leads to oxidative stress through production of reactive oxygen species and to subsequent apoptosis by releasing cytochrome c and caspase activation (8, 10).

Replenishment of intracellular Ca^{2+} stores is achieved by a mechanism known as store-operated Ca^{2+} entry (SOCE) (11, 12) (Fig. 1). SOCE is also known to be triggered by Ca^{2+} efflux from the ER to establish a rapid and sustained elevation of Ca^{2+} levels in the cytosol. Elevated Ca^{2+} levels are required to initiate signaling pathways by regulating Ca^{2+}-dependent proteins, such as kinases and phosphatases (13). SOCE depends on two proteins, STIM, located at the ER membrane, and ORAI, at the plasma membrane, which is a structural component of the Ca^{2+}-release activated Ca^{2+} channel (CRAC). Two isoforms STIM1 and STIM2 exist, which differentially sense ER Ca^{2+} levels. Depletion of ER luminal [Ca^{2+}] below its resting levels (of about 400 μM) is sensed by STIM1. While STIM1 senses significant store depletion and activates SOCE, STIM2 senses mild store depletion to maintain basal Ca^{2+} homeostasis (14). Activated STIM1 relocalizes to ER/plasma membrane junctions to bind to ORAI1 at the plasma membrane and to induce opening of CRAC channels.
which triggers Ca\textsuperscript{2+} entry into the cell (15). After cessation of the stimulus, Ca\textsuperscript{2+} is pumped into the ER by SERCA. STIM1 is deactivated and releases ORAI1, which results in closing of the CRAC channels (15). Having depicted the most important Ca\textsuperscript{2+} flux between the ER and the cytosol as well as mitochondria, we will now delineate the interconnection of luminal Ca\textsuperscript{2+} levels, ER redox state, and ATP levels.

**Luminal calcium ion levels affect ER redox equilibrium and luminal ATP levels**

The concentration of Ca\textsuperscript{2+} in the ER is linked to the prevailing redox conditions. Low luminal Ca\textsuperscript{2+} concentrations induce a reductive shift, which is required for reducing disulfide bonds in the SERCA channel thereby enabling Ca\textsuperscript{2+} import into the ER (Fig. 2). When luminal Ca\textsuperscript{2+} levels are restored, oxidation of the respective cysteine residues arrests further Ca\textsuperscript{2+} import and Ca\textsuperscript{2+} levels decrease due to the Ca\textsuperscript{2+} “leakage” at the translocon (16, 17). In order to experimentally study the effects of Ca\textsuperscript{2+}-depletion, the activity of SERCA can be inhibited by the chemical compound thapsigargin (18). Ca\textsuperscript{2+} depletion in thapsigargin-treated cells results in a reductive shift as shown in HEK293 cells transfected with an ‘ER-tuned’ GFP redox-responsive probe (roGFPiE), which enables fluorescent lifetime imaging in the ER lumen (19, 20). This reductive shift was shown to be associated with a decrease in protein disulfide isomerase (PDI) mobility, discussed to be due to complexing of PDI with calreticulin (20) (Fig. 2). However, downregulation of PDI using shRNA only resulted in a slight reduction of the fluorescent lifetime of roGFPiE in HEK293 cells (20) indicating that PDI does not play a major role in providing the reductive shift required for the opening of the SERCA channel. Recently, it was shown that the reductive shift—resulting from Ca\textsuperscript{2+} depletion—is largely independent of the Ca\textsuperscript{2+}-binding calreticulin but associated with increased import of GSH into the ER (21) (Fig. 2). The GSH system with the reduced GSH and the oxidized GSSG redox pair provides an important thiol-disulfide buffer system in the ER (22). Since GSSG cannot be reduced in the ER, maintenance of the ER redox state likely depends on GSH import and GSSG export (23) (Fig. 2). Since thapsigargin-treated cells were shown to have increased GSH levels in the ER lumen, it is now suspected that Ca\textsuperscript{2+}-depletion drives GSH transport through a yet unknown Ca\textsuperscript{2+}-sensitive GSH transporter in the ER membrane (21).

Taken together, the low [Ca\textsuperscript{2+}]\textsubscript{ER} induces a reductive shift in the ER, which leads to the opening of the SERCA pump enabling Ca\textsuperscript{2+} import. In the presence of high luminal [Ca\textsuperscript{2+}], the oxidizing milieu is readjusted, which is required for protein folding, and further [Ca\textsuperscript{2+}] import is inhibited due to the oxidized state of the SERCA pump.

Besides the function of the ER to be the major cellular Ca\textsuperscript{2+} store enabling rapid cellular responses, high luminal Ca\textsuperscript{2+} levels are also mandatory for maintaining a Ca\textsuperscript{2+} gradient across the ER membrane, which is required for ATP import.
As shown above for GSH, ATP cannot be synthesized in the ER but solely stems from glycolysis (in the cytosol) and oxidative phosphorylation (at the inner mitochondrial membrane) (25) and, therefore, also needs to be transported into the ER (Fig. 1). The corresponding ATP/ADP carrier, named AXER (ATP/ADP exchanger in the ER membrane) has been identified only recently by screening databases (26). In thapsigargin-treated HeLa cells, Ca\(^{2+}\) depletion in the ER results in transiently increased ATP levels, an effect that largely decreased when AXER was downregulated (26). This transient elevation in [ATP]ER is followed by a drastic decline in luminal ATP levels (24–26). The reason for the transient increase in luminal ATP upon Ca\(^{2+}\) depletion is not known. It is discussed to be due to downregulation of ATP-consuming activities within the ER and could serve to promote chaperoning activity during the initial phase of ER-stress sensed by accumulating misfolded proteins (as outlined further below). The subsequent downregulation of luminal ATP upon Ca\(^{2+}\) depletion has been shown to be due to corresponding increases in cytosolic Ca\(^{2+}\) levels (24). High cytosolic Ca\(^{2+}\) concentrations (ranging between 500 nM to 2 \(\mu\)M)—sensed by a yet unidentified Ca\(^{2+}\) sensing component—block AXER activity and inhibit ATP import into the ER (Fig. 1). The regulation of ATP import by cytosolic Ca\(^{2+}\) levels has been termed CaATiER (Ca\(^{2+}\) antagonizing transport into ER) by Yong et al. (24) postulate the existence of a Ca\(^{2+}\) sensing component, which attenuates the ATP transport in the presence of high [Ca\(^{2+}\)]\(_{\text{cyt}}\) enabling the transport of ATP into the ER only when the [Ca\(^{2+}\)]\(_{\text{cyt}}\) is at physiological levels.

**Dysregulated calcium homeostasis induces the unfolded protein response**

As discussed above, Ca\(^{2+}\) levels within the ER have to be tightly controlled in order to ensure appropriate luminal ATP levels. Furthermore, Ca\(^{2+}\) and ATP are required for the activity of molecular chaperones and folding enzymes.

Therefore, it is not surprising that depletion of Ca\(^{2+}\) from the ER and subsequent energy deficiency lead to ER-stress due to impaired folding capacity and subsequent accumulation of misfolded or unfolded proteins within the ER lumen (2, 27–29). Likewise, agents that impair the ER redox homeostasis such as DTT or thapsigargin cause ER-stress (30). During ER-stress, a stress response is induced to reestablish ER homeostasis and to minimize cell damage. This complex stress response is named the unfolded protein response (UPR). The UPR succeeds in transiently arresting protein synthesis by inducing transcriptional and translational upregulation of
chaperones in an attempt to refold proteins or at least to keep unfolded and misfolded proteins in a soluble conformation to enable restoration of proteostasis. Simultaneously, irreversibly misfolded proteins are subjected to ER-associated degradation (ERAD). Prolonged ER-stress eventually induces apoptosis (29). During the UPR, ER-stress can mainly be sensed by three ER membrane proteins: protein kinase RNA-like ER kinase (PERK), inositol-requiring enzyme 1 α (IRE1α) and activating transcription factor 6 (ATF6) (29, 31).

With regard to IRE1α, autophosphorylation of its ribonuclease domain activates the endoribonuclease domain of IRE1α, which catalyzes the splicing of prevailing mRNA of X-Box binding protein 1 (Xbp1) resulting in spliced Xbp1 mRNA (Xbp1s) (32). Xbp1s is efficiently translated into XBPI protein, which travels to the nucleus to act as a transcription factor for several genes associated with the ERAD pathway and the folding machinery in the ER lumen (33). Apart from upregulating a subset of genes that are important for restoration of ER homeostasis, the activated endoribonuclease domain of IRE1α degrades a subset of coding and noncoding cytosolic mRNAs to stop ongoing protein synthesis. This mechanism is called regulated IRE1α-dependent decay of mRNA (34). Termination of IRE1α signaling occurs via dephosphorylation of IRE1α and its monomerization (29, 35). If ER homeostasis cannot be restored, apoptosis is induced at late stages of ER-stress. Interestingly, besides its classical function as ER-stress transducer, IRE1α is found to be located at MAM sites (36, 37) (Fig. 1). Experiments with different IRE1α mutants revealed that the function of IRE1α at MAM sites is independent of its enzymatic activities but due to its ability to form a stable complex with IP3R (36). At MAM sites, IRE1α was shown to control Ca2+ transport from the ER to mitochondria i) by serving as a scaffold keeping the optimal distance between ER and mitochondrial membranes and ii) by directing IP3R to MAMs (36).

Autophosphorylation of PERK results in phosphorylation of the transcription factor NRF2 and the eukaryotic initiation factor 2α (eIF2α) (31, 38). Phosphorylation of eIF2α inhibits general protein synthesis in the cytosol but simultaneously stimulates preferential synthesis of specific proteins including the activating transcription factor 4 (ATF4) (29). ATF4 increases transcription of genes involved in transport of amino acids, synthesis of GSH, and maintenance of redox homeostasis (31, 39, 40). Upon prolonged ER-stress, ATF4 induces the transcription of the transcription factor CHOP, which can induce subsequent apoptosis. Furthermore, ATF4 can activate the transcription of GADD34, which dephosphorylates p-eIF2α to restore general protein synthesis (29). Phosphorylation of NRF2, on the other hand, results in dissociation of the protein from its binding partner KEAP1 in the cytosol, which enables trafficking of the transcription factor to the nucleus (31). NRF2 target genes include many detoxifying enzymes as well as cellular transporters, proteins involved in protein folding, as well as genes of proteasomal subunits (40). Besides these classical ER-stress transducer functions, PERK is also found to be enriched at MAMs, where it is required to maintain ER-mitochondrial juxtapositions, a function that is independent of the kinase domain but—similar to the findings for IRE1α (see above)—requires the cytosolic domain (Fig. 1). PERK deficient cells were shown to contain considerable weaker MAMs than WT cells (41), see also the recent review (37). The drastic structural modifications at MAM sites and the reduced ER-stress–induced mitochondria-mediated apoptotic rate in PERK-deficient cells indicate the necessity of PERK for propagation of ER-stress to the mitochondria to induce apoptotic signaling (41). An additional, UPR-independent function could be assigned to PERK by the identification of the actin-binding protein Filamin A (FLNA) as a novel PERK-interaction partner. This interaction seems to be crucial for the formation of juxtapositions of the ER membrane with the plasma membrane, the close vicinity of the membranes is a prerequisite for SOCE-mediated Ca2+ influx (42) (Fig. 1).

The third sensor of ER-stress, activating transcription factor 6 (ATF6), which is also silenced by its binding to BiP and activated upon its dissociation (29), differs in its activation pattern from PERK and IRE1α. When activated, ATF6 travels to the Golgi complex where the cytosolic N-terminus of ATF6 (ATF6N) is cleaved off by regulated intramembrane proteolysis (29). ATF6N travels to the nucleus to function as transcription factor to upregulate transcription of BiP and other chaperones (43). ATF6N has also been reported to dimerize with the transcription factor XBPI to increase upregulation of several ERAD components and co-chaperones (43). There is no evidence to date that ATF6 contributes to the formation, stabilization, or architecture of membrane juxtapositions.

Activation of the UPR is mediated by the ER-resident Hsp70 chaperone BiP, which is the central protein for chaperoning activity in the ER. It has been shown that the binding of BiP to the luminal domains of IRE1α, PERK, and ATF6 keeps them in a silenced state (29, 31, 44, 45). BiP has two distinct domains: a substrate-binding domain (SBD) and a nucleotide-binding domain (NBD). Under control conditions, BiP is bound to luminal domains of the transmembrane proteins IRE1α and PERK by its NBD site (44) (see Fig. 3). When misfolded proteins accumulate in the ER, their binding to the SBD induces the release of BiP from IRE1α and PERK thereby activating them and enabling their oligomerization and autophosphorylation (31, 38, 46, 47) (Fig. 3B). In addition to this long-standing model, an extended model based on structural and mutational analyses in yeast was presented by Credle et al (48). They showed that unfolded proteins can activate and enhance the UPR by directly binding into the highly conserved groove of the Ire1 (45, 48) (Fig. 3C). It is, thus, possible that BiP release is not a requirement for Ire1 activation, but that it might provide a regulatory role under extreme activation conditions when the pool of free BiP becomes severely depleted. Since the Ire1 mutant, defective in binding BiP, is mildly constitutively active, BiP binding to Ire1 might dampen activation of Ire1 under conditions of mild accumulation of unfolded protein, thereby reducing “noise” in UPR signaling (48). Since the luminal domains of IRE1α and PERK are highly homologous, this model can also be extended to the activation mechanism of PERK by unfolded proteins (48) (Fig. 3C).
Apart from its role in silencing the transmembrane proteins IRE1α, PERK, and ATF6 and in transmitting ER-stress via activation of the UPR, BiP has manifold additional functions. BiP mediates proper protein folding and refolding of misfolded proteins, processes which depend on the availability of ATP. Due to its Ca²⁺-binding capacity, BiP is a major Ca²⁺ store in the ER (49) and it also controls [Ca²⁺]ER efflux at the Sec61 translocon and at IP3R. At the Sec61 translocon, BiP is required to keep it in a closed state (50), while at the IP3R (type 1) channel, BiP was shown to activate it and to promote [Ca²⁺] efflux (51). The chaperoning and gating functions of BiP are ATP dependent as shown for IP3R1 (51) and outlined below for its function at the Sec61 translocon. Chaperoning activity of BiP is known to be assisted by cochaperones, which stimulate—due to their highly conserved J-domain—the ATPase activity of BiP. These cochaperones are also known by now to exert diverse functions. In the following section, we will briefly describe the chaperone cycle of BiP and its cochaperones and then consider the role of these individual cochaperones in regulating the Ca²⁺ flux across membranes.

The ATP-consuming chaperoning activity of the Hsp70 protein BiP is based on two conformationally distinct structures of BiP depending on its binding to ADP or ATP at its NBD (Fig. 3D). When bound to ATP, the affinity of BiP-SBD to substrate is low, while in the ADP-bound state, substrate affinity is high. Changes in the affinity of BiP to substrates are mediated by conformational changes following ATP hydrolysis. As soon as folding of a substrate protein has been accomplished, BiP is released from the substrate. This release is mediated by so called nucleotide exchange factors (NEF) resulting in release of the substrate protein (3).

Figure 3. Induction of ER-stress by misfolded proteins. A, BiP has a substrate-binding domain (SBD) and a nucleotide-binding domain (NBD). IRE and PERK are transmembrane proteins with high homologous luminal domains, which are silenced by BiP-NBD binding under normal conditions, when nascent proteins are folded properly (red coiled lines). B, in ER-stressed cells, misfolded proteins accumulate and engage luminal BiP for refolding. By binding of the misfolded proteins to the SBD, BiP dissociates from the luminal domains, enabling the oligomerization and autophosphorylation of the transmembrane proteins thereby activating the UPR. C, according to the extended model, misfolded proteins bind directly to the luminal domains of the transmembrane proteins, enabling their activation and stimulation of the UPR. Under more severe stress conditions, even BiP, which is usually bound to the transmembrane domains, binds unfolded protein and dissociates from the respective luminal domains thereby enforcing the UPR. D, misfolded proteins, bound by BiP, are subjected to a cycle to enable their refolding. This refolding, ATP-consuming chaperoning is assisted by ERdJ proteins and nucleotide exchange factors (NEF). In a first step (1), BiP is shown in its ATP-bound state, in which binding affinity to the substrate (unfolded, native protein or misfolded protein) is low. In a second step (2), ERdJ proteins, as cochaperones, bind to BiP and stimulate ATP hydrolysis. In the ADP-bound state, the affinity of BiP to the substrate is increased. When protein folding has been completed, ADP is exchanged for ATP by the nucleotide exchange factor (NEF) resulting in release of the substrate protein (3).
and required for their function as cochaperones, some JDPs contain additional features such as a glycine/phenylalanine rich region (G/F region) as well as a zinc finger domain. It was proposed that apart from the J-domain, the G/F region is also important for binding of JDPs to Hsp70 chaperones (55). The G/F rich region was further shown to be essential for interaction with target proteins as was shown for the ERdJ4 (56–59). Up to now, eight members of the ER-resident JDPs, ERdJ1 – ERdJ8, are characterized that stimulate ATPase activity of BiP and assist in maintaining ionic homeostasis and proteostasis in the ER (52, 60). Apart from their function in assisting BiP in folding of target proteins, BiP and four of the eight ER-resident cochaperones are also known to be involved in the regulation of Ca\(^{2+}\) homeostasis. Of these, ERdJ3 and ERdJ6 are operating at the Sec61 translocon (Fig. 4), ERdJ1 and ERdJ5 at the plasma membrane or acting as oxidoreductases, respectively (Fig. 2). No evidence is currently available pointing to a contribution of ERdJ2 and ERdJ4 in controlling or regulating Ca\(^{2+}\) homeostasis. Little is yet known about the function of the recently detected proteins ERdJ7 and ERdJ8. Kaz Nagata showed that ERdJ8 (DNAJC16) is an ER-membrane protein with both, the J and a thioredoxin domain, facing the luminal side of the ER. Due to its location in ER membrane subdomains, which are in close vicinity to mitochondrial membranes, ERdJ8 is presumed to be localized at MAMs (61). Yet, knockdown experiments indicate that ERdJ8 regulates autophagosomal degradation of mitochondria and controls the size of newly formed autophagosomes (60). In the following, we will briefly summarize recent understandings of the Sec61 translocon and the impact of BiP in gating and inhibiting Ca\(^{2+}\) efflux at the translocon, before we discuss cochaperone-mediated mechanisms, which regulate the luminal Ca\(^{2+}\) homeostasis.

**BiP and cochaperone-mediated, Sec61-dependent regulation of calcium ion homeostasis**

The Sec61 translocon constitutes the main channel for protein translocation into the ER in mammalian cells (62). Around one third of all cellular proteins have to transit this channel to enter the ER where they undergo maturation, quality control, and—in case of enduring misfolding—their delivery to the ERAD pathway. The processes at the Sec61 translocon are highly demanding since the translocon must allow for passage of nascent proteins while maintaining the permeability barrier between the cytosol and ER lumen. Leakiness of this channel for Ca\(^{2+}\) has been shown in experiments using planar lipid (6), and in Sec61A1-deficient cells, live cell Ca\(^{2+}\) imaging experiments revealed a significant decline in [Ca\(^{2+}\)]\(_{ER}\) efflux in the presence of thapsigargin (6). These results strongly indicated that experimental inhibition of the SERCA pump results in Ca\(^{2+}\) depletion of the ER lumen that is not based on an active IP3R- or ryanodine receptor-mediated Ca\(^{2+}\) burst but based on a Ca\(^{2+}\) efflux along the Ca\(^{2+}\) gradient at the end of protein translocation across the Sec61 channel (63–68) (Fig. 4). The structure of Sec61 pore and the mechanism engaged in gating this channel are depicted in the following section.

The translocon is composed of three subunits (α, β, and γ) (69). The Sec61α subunit is the largest subunit with 10 transmembrane domains that create a pore whereas the smaller β and γ subunits span the ER membrane only once (69) (Fig. 4). A lateral gate enables the insertion of transmembrane domains of translocated proteins. At the cytosolic side, the translocon is sealed by tight ribosome-membrane junctions (70). At the luminal side, a plug-like structure is formed by Sec61α that keeps the translocon closed in the absence of protein translocation and opens the translocon upon initiation of translocation (69, 71, 72).

In addition, BiP was shown to be necessary to seal the translocon during early stages of translocation as well as repetitively during transmembrane domain insertion of emerging proteins (70, 73, 74). BiP was also shown to be required for sealing nontranslocating translocons (70). The sealing of the translocon by BiP occurs in a stochiometric, noncatalytic manner (70). By using hydrophilic collisional quenching agents in isolated microsomes, reconstituted with purified BiP, it was shown that BiP-mediated sealing of the

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**Figure 4. Schematic illustration of the function of ERdJ3 and ERdJ6 in controlling Ca\(^{2+}\) efflux.**

A, protein translocation across the ER membrane is mediated via the Sec61 translocon. B, at the end of the translocation process, the Sec61 channel mediates efflux of Ca\(^{2+}\) from the ER to the cytosol resulting in loss of Ca\(^{2+}\) within the ER. C, translocon closure is achieved by binding of BiP to loop7 of the Sec61α subunit and by binding of ERdJ3 and ERdJ6. Translocon closure results in upregulation of [Ca\(^{2+}\)]\(_{ER}\) levels. The exact mechanism how ERdJ3 and ERdJ6 gate the translocon and whether interaction with BiP is required for this function has still to be elucidated. Inset: The translocon Sec61 is composed of three subunits (α, β, γ). Sec61 β and Sec61 γ have one transmembrane domain. Sec61α has 10 transmembrane domains. Loop7, which is located between the transmembrane domains 7 and 8, has been shown to be required for interaction with BiP. ER, endoplasmic reticulum.
translocon closely resembles the BiP ATPase cycle described above (see also Fig. 3D). BiP associates with the translocon in its ATP-bound state. When ATP is hydrolyzed to ADP, affinity of BiP to the translocon increases so that the translocon is sealed. Exchange of ADP for ATP then induces a conformational change in BiP so that the translocon is opened (74). Even though it was shown that BiP has to be in the ADP-bound state to seal the translocon during protein translocation (74), recently, the necessity of ATP to close the Sec61 channel in order to limit Ca$^{2+}$ efflux was verified by downregulation of the ATP carrier AXER, which resulted in increased Ca$^{2+}$ leakage from the ER (26). Experiments with reconstituted microsomes further revealed that a membrane-bound JDP is required for translocon closure (74). BiP-mediated closure of the pore seems to depend on a functional interaction of BiP with a membrane-bound JDP at or near the translocon (70, 74). Interestingly, since it is the ADP-bound conformation of BiP that is required for sealing the translocon, the interaction of BiP and ERdJ protein is important for something other than stimulation of the ATPase activity of BiP (74). According to these results, a model was proposed in which an ERdJ protein is located within the ER membrane near the translocon, which recruits ADP-BiP to the translocon to close it in a plug-like way. According to their localization near the translocon, the transmembrane proteins ERdj1 and ERdj2 are good candidate J-proteins to fulfill such a required function, which comprises possibly the targeting of ADP-BiP to the translocon. Yet, neither downregulation of ERdj1, ERdj2, nor ERdj7 in HeLa cells did increase Ca$^{2+}$ efflux from the ER (75). This draws the attention to two other cochaperones ERdJ3 and ERdJ6, both of them being known to be involved in the degradation of proteins. ERdJ3 was shown to deliver substrate proteins to BiP to facilitate their retrograde translocation within the ERAD pathway (76–80). Furthermore, secreted ERdj3 was shown to bind to aggregation-prone proteins in the extracellular environment (81). ERdJ3 has different subcellular locations and functions (as reviewed (82)). It is known to mediate protein degradation via a cotranslational degradation pathway (83). Recently, it was shown that ERdJ6 is required for Golgi-independent trafficking of BiP to the cell surface (84). This unconventional traffic of BiP to the cell surface has been reported in lung and colon cancer cell lines (84). Here, ER-derived vesicles translocate to the cell surface through early and recycling endosomes. The loading into the ER-derived vesicles was shown to require ERdj6, which is upregulated by PERK via the PERK–Akt–mTOR axis in response to ER-stress (84). Both, ERdj3 and ERdj6 are known to be soluble proteins in the ER lumen (85, 86) but a membrane-bound pool of both cochaperones has been shown to exist (see recent review (82)). ERdj3 and ERdj6 were both shown to interact with BiP and the Sec61 translocon at the ER membrane (76, 83, 85–87) (Fig. 4). Knockdown experiments revealed that downregulation of either ERdj3 or ERdj6 increases Ca$^{2+}$ efflux from the ER. This effect could be abolished when silencing the expression of Sec61 itself pointing to the ability of both cochaperones to survey Ca$^{2+}$ efflux at the translocon (75). It is of interest to note that downregulation of ERdj6 leads to a compensatory upregulation in ERdj3 protein levels, the increased levels are, however, not able to complement for downregulated ERdj6 levels. Down-regulation of both cochaperones even increased Ca$^{2+}$ leakage from the ER (75), suggesting that both cochaperones cooperate during translocon closure and that they do not have overlapping functions in this respect. An even increased Ca$^{2+}$ efflux from the ER was reported to occur upon simultaneous downregulation of ERdj3 or ERdj6 and BiP (75).

Since knocking down of ERdj3 or ERdj6 could possibly result in a general disturbance of ER proteostasis, which induces ER-stress and subsequent Ca$^{2+}$ efflux (88), it is of interest to note that neither silencing of ERdj3 nor ERdj6 resulted in activation of the UPR (75). These data further manifest that Ca$^{2+}$ efflux in ERdj3/ERdj6 knockdowns is not a consequence of ER-stress but that Ca$^{2+}$ efflux is prevented by interaction of both cochaperones with BiP at the translocon. Nothing is yet known about the binding dynamics of ERdj3 and ERdj6 to BiP with regard to gating the Sec61 translocon. It would be of major interest to investigate the effect of ERdj3/ERdj6 mutants that cannot bind to BiP on Ca$^{2+}$ efflux to investigate whether interaction with BiP or even stimulation of the ATPase activity of BiP by ERdj3 or ERdj6 activity is required for closing the translocon and whether ERdj3 and ERdj6 act simultaneously or sequentially.

It is also not known whether ADP-BiP or the complex ADP-BiP–J-protein serve as a plug for sealing the translocon or whether the underlying mechanism for translocon closure is based on a conformational change induced by the interaction of ADP-BiP or the complex ADP-BiP–J-protein with Sec61. Yet, the binding site of BiP to the translocon could be specified using different Sec61 mutants. The Y344H Sec61 mutant carrying a point mutation within loop7—located between transmembrane domain 7 and 8 of Sec61α—showed impaired binding to BiP and increased Ca$^{2+}$ leakage revealing that loop7 of the Sec61 translocon is central for translocon closure (50). ATP and J-domain dependency for gating the translocon points to the participation of cochaperones in stimulating the ATPase activity of BiP and furthermore clearly shows that the Sec61 translocon is in fact a gated Ca$^{2+}$ channel, regulated by the activity of BiP.

Taken together, ERdj3 and ERdj6 are good candidates in cooperating with BiP at the translocon since a subpopulation of both has been shown to be membrane bound and both assist BiP in sealing the translocon. Since ERdj3 and ERdj6 cannot compensate each other’s function in limiting Ca$^{2+}$ efflux, they affect translocon closure by distinct mechanisms.

Having specific functions in translocon closure to limit Ca$^{2+}$ efflux, ERdj3 and ERdj6 are expected to be upregulated in response to Ca$^{2+}$ depletion within the ER to prevent further Ca$^{2+}$ efflux. This assumption could be confirmed by showing that indeed, thapsigargin treatment, which depletes ER Ca$^{2+}$ stores by blocking SERCA2b, leads to upregulation of ERdj3 (78) and ERdj6 (89, 90).

Considering the experimental data outlined above, a regulatory feedback loop can be postulated linking ATP homeostasis to Ca$^{2+}$ homeostasis in the ER lumen, which enables the cell to regain proteostasis in the ER at the early onset of ER-stress. Since chaperoning and closing the Sec61 translocon

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mediated by BiP require energy (26), changes in cellular microenvironment challenge the maintenance of the ionic and protein homeostasis and more ATP is required to meet the increasing chaperoning demand for proper folding of translocated proteins. Eventually, the ER-localized ATP pool will decline. Since the closure of the Sec61 translocon requires ATP (74), it cannot be kept in a closed state (26), resulting in Ca\(^{2+}\) depletion from the ER via the Sec61 translocon. The initial increase in [ATP]\(_{ER}\) observed in response to Ca\(^{2+}\) depletion (25, 26) could be a mechanism to restore ER Ca\(^{2+}\) homeostasis by closing the Sec61 translocon as well as by enabling BiP to continue its chaperoning function. In case, the temporary rise in ATP is not sufficient to restore ER homeostasis (e.g., due to high levels of ER-stress, and an ATP demand that cannot be met by the rise in ATP), Ca\(^{2+}\) leakage into the cytosol would progress resulting in abrogation of the Ca\(^{2+}\) gradient across the ER membrane and increased cytosolic Ca\(^{2+}\) concentrations, conditions which inhibit AXER and ATP transport into the ER (24). This scenario could be resolved by regaining physiological [Ca\(^{2+}\)]\(_{cyt}\) levels either by pumping Ca\(^{2+}\) across the plasma membrane into the extracellular space or by refilling luminal Ca\(^{2+}\) stores via SOCE and SERCA activity.

This model is in accordance with another hypothetical model presented by Ushioda et al., who discuss that occasional Ca\(^{2+}\) depletion could clear the ER of misfolded proteins (16). This hypothesis is based on the finding that Ca\(^{2+}\) depletion can activate the UPR signaling cascade, which results in an arrest of general mRNA translation and increased degradation of ERAD substrates. Thus, occasional periods of Ca\(^{2+}\) depletion could be exploited to clear the ER of misfolded cargo while preventing degradation of nascent proteins due to translational arrest. Ca\(^{2+}\) homeostasis could then be regained by the presented regulatory feedback mechanism.

**Diabetic phenotype in ERdj6-deficient mice**

Since the dynamics in Ca\(^{2+}\) flux across membranes and the prevailing Ca\(^{2+}\) levels within every subcellular compartment are key positions for regulating the cellular metabolism, disturbances in Ca\(^{2+}\) homeostasis have been shown to be associated with metabolic diseases, such as diabetes mellitus (9). A diabetic phenotype was reported in mice carrying the Y344H mutation in loop7 of the Sec61 translocon, which was found to abolish BiP binding and resulted in an increased Ca\(^{2+}\) efflux from the ER (50, 91). Considering the suggested role of ERdj6 in Sec61 channel closure, it is interesting that a diabetic phenotype was also obtained in ERdj6 KO mice (92). ERdj6 KO mice present with glucosuria, hyperglycaemia, and hypoinsulinemia. Furthermore, there is a reduced amount of pancreatic beta cells in these mice, presumably due to increased proapoptotic signaling (92). Increased apoptotic rates could be due to [Ca\(^{2+}\)]\(_{ER}\) depletion, resulting in general ER-stress and subsequent apoptosis by UPR signaling pathways. Furthermore, [Ca\(^{2+}\)]\(_{ER}\) depletion could prevent Ca\(^{2+}\) flux between the ER and mitochondria. Increased mitochondrial Ca\(^{2+}\) concentrations are important for mitochondrial ATP production, which in turn is required for insulin secretion (9), when cells are stimulated with glucose to produce around one million molecules of proinsulin per minute. In addition, downregulation of ERdj6 might increase PERK signaling and PERK-induced apoptosis due to lack of inhibition through ERdj6 (92).

Hypoinsulinemia in ERdj6-deficient mice could also be based on the resultant reductive shift due to [Ca\(^{2+}\)]\(_{ER}\) depletion (20). For folding and sorting of insulin in the ER, three intramolecular disulfide bonds have to be formed by PDI (9). Accordingly, defective insulin folding and maturation could be based on the lack of PDI, which is sequestered by calreticulin upon [Ca\(^{2+}\)]\(_{ER}\) depletion (20), thereby affecting redox homeostasis (as further outlined in the following section).

While ERdj3 and ERdj6 assist in closing the Sec61 translocon to limit Ca\(^{2+}\) leakage from the ER, ERdj1 and ERdj5 have been found to regulate cellular Ca\(^{2+}\) homeostasis by Sec61-independent mechanisms.

**Cochaperone mediated, Sec61-independent regulation of calcium ion homeostasis**

**ERdj5 activates SERCA2b upon [Ca\(^{2+}\)]\(_{ER}\) depletion and regulates mitochondrial dynamics**

ERdj5 is an ER-resident reductase, which contains a J-domain at its N-terminus and four thioredoxin-like domains with redox-active motifs (Cys-X-X-Cys, CXXC) (93). It is known to cleave disulfide bonds of misfolded proteins to target them to BiP for retrotranslocation via the ERAD pathway (94, 95). Within the ER, ERdj5 maintains luminal Ca\(^{2+}\) stores by controlling Ca\(^{2+}\) influx in response to [Ca\(^{2+}\)]\(_{ER}\) depletion (16) (Fig. 2). In HEK293T cells, it was shown that ERdj5 is normally present in its oxidized form without reductase activity. However, upon decreasing Ca\(^{2+}\) concentrations within the ER, an increasing fraction of ERdj5 becomes reduced (Fig. 2). Thus, upon Ca\(^{2+}\) depletion, the reductive power of ERdj5 increases while the oxidizing PDI1A is sequestered by calreticulin thereby increasing the reductive potential of ERdj5 in the ER (16). This reductive power is required for activating SERCA. ERdj5 can interact with the Ca\(^{2+}\) pump SERCA2b, as shown in HeLa and HEK293T cell, and increases its ATPase activity (16). This effect is lost when all four thioredoxin domains of ERdj5 were mutated (16). Since ATPase activity was shown to positively correlate with the amount of reduced SERCA2b, it is assumed that ERdj5 activates SERCA2b by reduction of an intramolecular disulfide bond under low Ca\(^{2+}\) concentrations (16).

In line with this is the observation that ERdj5 KO MEFs had decreased [Ca\(^{2+}\)]\(_{ER}\) levels, a defect that could be compensated by overexpression of ERdj5 (16). Interestingly, compensation was also obtained by overexpression of an ERdj5 mutant that could not bind to BiP, indicating that the interaction of ERdj5 with BiP is not required for controlling Ca\(^{2+}\) influx. The ability to control Ca\(^{2+}\) influx into the ER thus seems to be solely due to the reductive potential of ERdj5. Taking into account that ERdj5 regulates SERCA activity and Ca\(^{2+}\) influx in response to [Ca\(^{2+}\)]\(_{ER}\) depletion (16), ERdj5 should be upregulated upon Ca\(^{2+}\) depletion. Indeed, transcription of ERdj5 was shown to be upregulated in response to treatment with thapsigargin and...
Ca\(^{2+}\) ionophore, which depletes [Ca\(^{2+}\)]\(_{ER}\) stores by promoting Ca\(^{2+}\) efflux from the ER (96).

Though interaction with BiP is not required for the function of ERdj5 to control Ca\(^{2+}\) influx into the ER, BiP has been shown to modulate ERdj5 motility and its ability to bind to SERCA2b in a [Ca\(^{2+}\)]-dependent way (16). While Ca\(^{2+}\) depletion did not affect ERdj5 mobility, high Ca\(^{2+}\) concentrations promoted oligomeric assembly of ERdj5 within the ER keeping the complex less mobile as shown by coimmunoprecipitation studies using FLAG- and myc-tagged ERdj5 constructs as well as by cellular fractionation experiments (16, 20). Monomerization and mobility of ERdj5 is regained in the presence of BiP as indicated by two independent experiments: While overexpression of BiP facilitated binding of ERdj5 to SERCA2b even under higher Ca\(^{2+}\) concentrations, in the absence of BiP, ERdj5 is found in higher molecular weight fractions having less binding affinity (16). This BiP-controlled availability of ERdj5 points to the presence of an additional mechanism that allows fine-tuning of Ca\(^{2+}\) import. All in all, the data point to an important role of ERdj5 reductase activity in maintaining the physiological Ca\(^{2+}\) influx into the ER (Fig. 2). By means of ERdj5 KO cells, it was recently shown that ERdj5 is not only required for Ca\(^{2+}\) homeostasis in the ER but also for the Ca\(^{2+}\) homeostasis in the cytosol (97). Interestingly, mitochondrial Ca\(^{2+}\) levels were unaffected in ERdj5-deficient cells although fragmentation of mitochondria was observed, and ERdj5-deficient cells proved to be more sensitive to apoptosis (97). The observed mitochondrial fission in ERdj5-deficient cells was shown to be due to aberrant phosphorylation of the cytosolic GTPase DRP1 (dynamin-related protein 1), which is caused by the increase in cytosolic [Ca\(^{2+}\)] (97). The exact mechanism how ERdj5 affects cytosolic [Ca\(^{2+}\)] levels is not fully understood, yet the measured elevation in [Ca\(^{2+}\)]\(_{cyt}\) is very likely due to the inhibition of SERCA2b activity. The impact on DRP1 phosphorylation is very interesting, since the phosphorylation pattern of DRP1 has been shown to be a key driver of metabolic diseases and inhibition of DRP1 phosphorylation, by overexpression of the phosphorylation mutant of S600A, was shown to improve glucose tolerance and to establish the Ca\(^{2+}\) gradient required for AXER activity and ATP transfer into the ER. The rise in ER luminal ATP levels would result in closing the translocon and subsequent restoration of ER homeostasis.

**ERdj1 mediates BiP translocation to the cell surface and regulates intracellular Ca\(^{2+}\) levels**

ERdj1 is a transmembrane protein with its J-domain in the ER lumen and a cytosolic C-terminus (99). The cytosolic C-terminus of ERdj1 can associate with ribosomes (99), thereby arresting protein synthesis, an arrest that is released upon binding of BiP (99–101). ERdj1 and BiP, which are primarily localized in the ER compartment, have also been shown to form a complex at the plasma membrane of macrophages (102–104). At the plasma membrane, BiP functions as a cell surface receptor for various ligands including α2-macroglobulin (104, 105) (Fig. 2). Under normal conditions, this leads to an increase of the intracellular [Ca\(^{2+}\)], probably via G-protein-mediated signaling (102–104). Coimmunoprecipitation experiments in peritoneal macrophages revealed that downregulation of ERdj1 by RNA interference reduced the binding of BiP to the plasma membrane and the binding of α2-macroglobulin to the cell surface. Consequently, downregulation of ERdj1 inhibited the α2-macroglobulin–mediated increase of the intracellular Ca\(^{2+}\) concentrations (103). These results indicate that—in the presence of low cellular Ca\(^{2+}\) levels—ERdj1 plays a crucial role in targeting BiP to the plasma membrane of cells to regulate the Ca\(^{2+}\) influx across the plasma membrane (103), possibly by inducing SOCE, which had been shown to be activated by ER Ca\(^{2+}\) depletion (Fig. 1). As ERdj1 stimulates Ca\(^{2+}\) influx into the cytosol, it is reasonable to assume that ERdj1 is upregulated upon Ca\(^{2+}\) depletion. Yet, presently no data are published with respect to the regulation of ERdj1 upon treatment of cells with thapsigargin or ionophore.

The mechanism how BiP enables Ca\(^{2+}\) influx is yet not fully understood. As a receptor of α2-macroglobulin, BiP could assist and control the formation and opening of the CRAC channel. Studies in 1-LN cells implicated that BiP colocalizes with plasma membrane–located VDAC channels and that binding of ligands to BiP induces a rise in intracellular Ca\(^{2+}\) through VDAC (106).

Another interesting aspect is the question how BiP is transported to the plasma membrane, since BiP contains the KDEL sequence at its C-terminus, which redirects it to the ER compartment. The phenomenon itself, that BiP is localized at the plasma membrane, was observed as early as in 1997, when a “highly homologous protein to BiP” was reported to be localized to the cell surface of lymphoma cells of malignant cutaneous T cell lymphoma (107). One year later, it was shown that cell surface expression of BiP was induced by thapsigargin in human rhabdomyosarcoma cells (108). Also, in NG108-15 glioblastoma cells, cell surface expression of BiP was detected (109). In NG 108-15, it was found that BiP translocation to the plasma membrane was blocked after treatment with brefeldin A, suggesting that BiP translocation is mediated through the Golgi apparatus (109), which was confirmed in liver and...
pancreatic cell lines (84). By now, the underlying mechanism has been elaborated. KDEL receptors at the Golgi apparatus are normally responsible for binding to proteins carrying a KDEL sequence (KDEL proteins) that have translocated from the ER to the Golgi. Upon binding of these proteins to the KDEL receptors, the proteins are retrotranslocated to the ER resulting in the retention of KDEL proteins in the ER (110).

The translocation of KDEL receptors from the Golgi apparatus to the ER is due to the activation of tyrosine kinase SRC, which is localized at Golgi membranes (111). In ER-stressed HeLa cells, it was shown that IRE1α binds to SRC and induces a dispersion of KDEL receptors in the Golgi apparatus, enabling BiP to escape from ER retention and to traffic to the plasma membrane (112). The exact mechanism on how ERdj1 is involved in mediating BiP transport to the plasma membrane is yet not known.

**Conclusion**

Protein and ionic homeostasis in ER are essential for cellular metabolism and survival. Disturbance of this finely balanced homeostasis results in metabolic diseases such as diabetes. Within the ER, the Hsp70 chaperone BiP and its ER-resident cochaperones have manifold functions. Besides their role in controlling cotranslational and posttranslational translocation of proteins, in assisting in protein folding and promoting the degradation of mutated or misfolded proteins, four of the eight ER-resident cochaperones are known to be involved in regulating Ca^{2+} flux along cellular membranes. ERdj3 and ERdj6 control Ca^{2+} leakage from the ER by closing the Sec61 channel at the end of protein translocation. Two other ER-resident cochaperones, ERdj1 and ERdj5, control Ca^{2+} homeostasis by Sec61-independent mechanisms. ERdj1 plays a central role in targeting BiP to the plasma membrane. Here, the ERdj1–BiP complex seems to control Ca^{2+} influx with BiP acting as a receptor for alpha2-macroglobulin. ERdj5 has a central role in controlling Ca^{2+} influx into the ER. Due to its reductive potential, it has the ability to reduce and open the SERCA to enable Ca^{2+} influx and to maintain luminal Ca^{2+} stores. In this review, we present the current knowledge on the impact of these ERdj proteins in controlling the cellular Ca^{2+} metabolism, yet the elaboration of the underlying mechanisms is at its very onset.

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