Redox-assisted regulation of Ca^{2+} homeostasis in the endoplasmic reticulum by disulfide reductase ERdj5

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Calcium ion (Ca^{2+}) is an important second messenger that regulates numerous cellular functions. Intracellular Ca^{2+} concentration ([Ca^{2+}]_i) is strictly controlled by Ca^{2+} channels and pumps on the endoplasmic reticulum (ER) and plasma membranes. The ER calcium pump, sarco/endoplasmic reticulum calcium ATPase (SERCA), imports Ca^{2+} from the cytosol into the ER in an ATPase activity-dependent manner. The activity of SERCA2b, the ubiquitous isoform of SERCA, is negatively regulated by disulfide bond formation between two luminal cysteines. Here, we show that ERdj5, a mammalian ER disulfide reductase, which we reported to be involved in the ER-associated degradation of misfolded proteins, activates the pump function of SERCA2b by reducing its luminal disulfide bond. Notably, ERdj5 activated SERCA2b at a lower ER luminal [Ca^{2+}]_i (Ca^{2+}ER), whereas a higher Ca^{2+}ER induced ERdj5 to form oligomers that were no longer able to interact with the pump, suggesting [Ca^{2+}]_ER-dependent regulation. Binding Ig protein, an ER-resident molecular chaperone, exerted a regulatory role in the oligomerization by binding to the J domain of ERdj5. These results identify ERdj5 as one of the master regulators of ER calcium homeostasis and thus shed light on the importance of cross talk among redox, Ca^{2+}, and protein homeostasis in the ER.

ERdj5 | SERCA2 | endoplasmic reticulum | calcium homeostasis | redox regulation

Intracellular Ca^{2+} acts as one of the most important signaling molecules in the cytosol and regulates numerous cellular functions including muscle contraction, cellular motility, and vesicular transport through the function of calcium-binding proteins including calmodulin in the cytosol (1, 2). Thus, maintenance of the intracellular Ca^{2+} concentration ([Ca^{2+}]_i) is critical for cellular signaling. Calcium homeostasis in the cytosol is maintained by release and influx of Ca^{2+} through calcium channels and pumps, respectively, in the plasma and endoplasmic reticulum (ER) membranes (1, 2). Calcium release from the ER into the cytosol is mediated by the inositol 1,4,5-trisphosphate receptor channel (3) and ryanodine receptor (4, 5) localized on the ER membrane. On the other hand, intracellular Ca^{2+} is taken up by the ER via the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) in an ATPase activity-dependent manner (2, 6, 7).

Subtypes of SERCAs (SERCA1s–3) are variously expressed in eukaryotic cells. SERCA1a and -1b are expressed in fast skeletal muscles, whereas SERCA2a is expressed in cardiac, smooth, and slow skeletal muscles. There are five isoforms of SERCA3, which are expressed in various mammalian tissues in a cell-type-dependent manner. Among subtypes of SERCAs, SERCA2b is a housekeeping isoform that is ubiquitously and abundantly expressed in nonmammalian cells and smooth muscles. SERCA2b consists of 11 transmembrane domains, and its activity is negatively regulated by the oxidation of two cysteines in its ER-luminal domain (8). In the resting state of SERCA2b under the high ER luminal [Ca^{2+}]_ER condition, the N domain of calreticulin, a molecular chaperone in the ER, interacts with the C-terminal sequence of SERCA2b and recruits Eps8l, a ubiquitous ER thiol-dependent oxidoreductase that promotes the formation of disulfide bonds, to target the intraluminal loop 4 of SERCA2b (8). Intramolecular disulfide bond formation between two cysteines in loop 4 inhibits the pump activity of SERCA2b. When [Ca^{2+}]_ER decreases to lower than 50 μM, Eps8l dissociates from SERCA2b to reactivate the pump function (8). Considering the oxidative condition in the ER lumen, the reduction of this disulfide bond should require a molecule(s) with reductase activity, which has not been identified.

Newly synthesized secretory proteins are cotranslationally translocated into the ER, where they are correctly folded with the aid of various molecular chaperones and enzymes. The major chaperones in the ER, including calnexin, calreticulin, BiP, and some protein disulfide isomerases (PDIs), require Ca^{2+} for their functions (6, 9). Inhibition of Ca^{2+} uptake into the ER by the SERCA inhibitor thapsigargin causes ER stress due to the accumulation of misfolded proteins. Consequently, maintenance of calcium homeostasis in the ER by the SERCA calcium pump is critically important for the functional integrity of the ER.

Proteins that harbor genetic mutations or are terminally misfolded must be eliminated to prevent formation of toxic aggregates. Terminally misfolded glycoproteins in the ER are transferred from calnexin/calreticulin to ER degradation-enhancing α-mannosidase-like protein 1 (EDEM1) in an N-glycan trimming-dependent manner (10, 11) and are subsequently transferred to ERdj5, the first disulfide

Significance

Ca^{2+} is one of the most important second messengers regulating numerous cellular functions; therefore, the regulation of Ca^{2+} release from and its uptake into the endoplasmic reticulum (ER) are both critical for calcium signaling. The activity of sarco/ endoplasmic reticulum Ca^{2+}-ATPase isoform 2b (SERCA2b), a calcium pump on the ER membrane, was reported to be negatively regulated by the oxidation of two cysteines in its ER-luminal portion, and it is expected to be activated by its reduction. However, no molecules responsible for this reduction have been identified. Here, we showed for the first time that ERdj5, the reductase in the ER of mammalian cells, activates SERCA2b by reducing its disulfide bonds in a [Ca^{2+}]_ER-dependent manner.


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reductase identified in the mammalian ER, which contains a J domain at its N terminus and four thioredoxin-like domains with redox-active motifs (Cys-X-X-Cys, CXXC) (12). ERdj5 cleaves disulfide bonds within the misfolded proteins to facilitate their passage through the retro-translocation channel in the ER membrane. Misfolded substrates are transferred from ERdj5 to Binding Ig Protein (BiP), a major molecular chaperone in the ER, which binds to ERdj5 through the HPD (Hys-Pro-Asp) motif in the J domain. BiP recruits the substrates to the retro-translocation channel to promote their associated degradation (ERAD) pathway (13, 14).

ERdj5 is the first identified ER reductase; therefore, we postulated and examined whether ERdj5 regulates SERCA2b activity by cleaving the disulfide bond in its intraluminal loop. Here, we show that ERdj5 activates the pump function of SERCA2b by reducing its luminal disulfide bond. Remarkably, we also found that ERdj5 activates SERCA2b in a [Ca\(^{2+}\)]\(_{\text{ER}}\)-dependent manner. These results have established that ERdj5, in addition to ERp57, works as one of the master regulators of SERCA2b and thus for ER calcium homeostasis. ERdj5 is a PDI family member involved in redox homeostasis in the ER; therefore, our findings shed light on the importance of cross talk among redox, Ca\(^{2+}\), and protein homeostasis in the ER.

Results

ER-Resident Reductase ERdj5 Interacts with SERCA2b. Although dissociation of ERp57 from the intraluminal loop 4 of SERCA2b was reported to reactivate its ATPase activity under the lower [Ca\(^{2+}\)]\(_{\text{ER}}\) condition, the fate of the disulfide bond after release of ERp57 was not examined. The redox condition in the ER lumen is oxidizing; therefore, an oxidoreductase(s) with reducing activity is postulated to be required for reduction of this disulfide bond. We hypothesized and examined whether ERdj5 can activate SERCA2b by cleaving the disulfide bond.

Immunoprecipitation and immunoblot analysis clearly showed that endogenous ERdj5 interacted with endogenous SERCA2b. The specificity of this interaction was confirmed by small interfering RNA (siRNA) knockdown of SERCA2b (Fig. 1A). Calnexin, a SERCA2b-binding protein (15), was also coprecipitated in this complex. In nonreducing gels, the band containing ERdj5 was shifted to around 200 kDa because cysteines of CXXC motifs of ERdj5 make mixed disulfide bonds with SERCA2b (Fig. 1B). When all CXXC motifs were converted to Ala-X-X-Ala (AXXA) (Fig. 1C), this ERdj5/AA mutant lost its reducing activity (12, 13). The ERdj5/AA mutant barely bound to SERCA2b (Fig. 1D). These results suggest that this complex forms via the mixed disulfide bond(s) between ERdj5 and SERCA2b. Consistent with this, the ERdj5/CA mutant, in which all CXXC motifs were converted to CXXXA (Fig. 1C), bound much more strongly to SERCA2b because the CA mutant acts as a trapping mutant of redox proteins (Fig. 1E). Using a set of ERdj5 mutants in which only one CXXC motif remained intact, we found that SERCA2b preferentially bound to ERdj5 via Trx3 and Trx4 (Fig. 1D), which have significant reductase activity and are hence involved in ERAD (13). This was more directly shown using the ERdj5/CA4 mutant, in which the CXXC motif in the Trx4 domain was mutated to CXXXA and other CXXCs to AXAXA. The complex of ERdj5/CA4 mutant and SERCA2b was shown as an around 200-kDa band in the presence of divinylsulfone (DVSF), which is a chemical cross-linker for disulfide bridge (Fig. S1).

A mutation in the J domain of ERdj5, in which histidine 63 in the HPD motif was replaced with alanine (ERdj5/H63A), weakened the interaction with SERCA2b (Fig. 1D). This suggests that BiP contributes to efficient formation of the SERCA2b-ERdj5 complex.

ERdj5 Regulates SERCA2b Pump Function Through Its Reductase Activity. Next, we examined the effect of ERdj5 binding to SERCA2b on Ca\(^{2+}\) uptake into the ER from the cytosol using ERdj5-disrupted mouse embryonic fibroblasts (MEFs) semipermeabilized with digitonin. Very little Ca\(^{2+}\) was imported into the ER of ERdj5−/− cells (blue line), whereas it was imported rapidly into the ER of wild-type ERdj5+/-/− cells (black line) (Fig. 2A and B). Ca\(^{2+}\) uptake into the ER of ERdj5−/− cells was rescued by overexpression of wild-type ERdj5 (Fig. 2C).

Fig. 1. Interaction between ERdj5 and SERCA2b. (A and B) Forty-eight hours after transfection of nonspecific (NS) or SERCA2-specific siRNA into HeLa cells, cell lysates were prepared for immunoprecipitation with an anti-SERCA2 antibody. Immunoprecipitates were subjected to (A) reducing or (B) nonreducing (Left) and reducing (Right) SDS/PAGE for the analysis by immunoblotting with the indicated antibodies. (C) The series of ERdj5 mutants constructed in this work. (D and E) Twenty-four hours after cotransfection of (D) HA-tagged or (E) PA-tagged SERCA2b and FLAG-tagged ERdj5/WT or the indicated ERdj5 mutants into HEK293 cells, cell lysates were prepared for immunoprecipitation with (D) anti-HA or (E) anti-PA antibodies. (D and E) All immunoprecipitates were subjected to reducing SDS/PAGE and analyzed by immunoblotting with the indicated antibodies. The asterisk in D indicates an NS band. CNX, calnexin.
ERdj5 (red line), whereas the ERdj5/AA mutant (purple line) had no effect. Overexpression of the ERdj5/H63A mutant (green line) had almost the same effect on Ca\(^{2+}\) uptake as ERdj5/WT in semipermeabilized cells. In these experiments, the expression levels of SERCA2b were confirmed not to be changed among these cells (Fig. S2). These results clearly indicate that redox-active ERdj5 is required for the efficient uptake of Ca\(^{2+}\) into the ER.

We next investigated calcium storage in the ER by treating cells with thapsigargin, an inhibitor of SERCA ATPase activity (Fig. 2 C and D). Ca\(^{2+}\) release from the ER under inhibition of SERCA2b was lower in ERdj5 (−/−) MEFs than in ERdj5 (+/−) MEFs, which suggests that the inactive state of SERCA2b in the absence of ERdj5 caused low calcium storage in the ER. Overexpression of ERdj5/WT and the ERdj5/H63A mutant recovered Ca\(^{2+}\) storage in the ER, but the ERdj5/AA mutant failed to rescue Ca\(^{2+}\) storage in the ER, which is consistent with the results shown in Fig. 2B. Taken together, these observations strongly suggest that ERdj5 stimulates SERCA2b activity and that, in the absence of ERdj5, SERCA2b is maintained in an inactive state. Indeed, ERdj5 knockdown decreased cellular tolerance to ER stress induced by A23187, a Ca\(^{2+}\) ionophore that decreases [Ca\(^{2+}\)]_ER (Fig. S3).

Fig. 2. Deficiency of ERdj5 suppresses Ca\(^{2+}\) uptake into the ER through SERCA2b. (A and B) Cells were loaded with Mag-Fura-2 to estimate [Ca\(^{2+}\)]_ER. Semipermeabilized MEFs were treated with EGTA for 20 min to remove Ca\(^{2+}\). After depletion of Ca\(^{2+}\), ATP and Ca\(^{2+}\) were added to stimulate SERCA pump functions. Mag-Fura-2 fluorescence was measured as [Ca\(^{2+}\)]_ER. The quantifications of each Ca\(^{2+}\) uptake are as shown in B. (C and D) After 1 \(\mu\)M thapsigargin treatment, [Ca\(^{2+}\)]_i was measured using Mag-Fura-2. Peak amplitudes are shown as a bar graph in D.
The interaction of ERdj5 with SERCA2b was strengthened when cells were treated with thapsigargin or ionomycin, both of which decrease $[\text{Ca}^{2+}]_{\text{ER}}$ (Fig. 4B). When $[\text{Ca}^{2+}]_{\text{ER}}$ was titrated in the presence of 1 mM ethylene glycol tetraacetic acid (EGTA), the ERdj5/SERCA2b interaction was maximal at submillimolar $[\text{Ca}^{2+}]_{\text{ER}}$ (Fig. 4C).

**ERdj5 Forms Oligomers at High $[\text{Ca}^{2+}]_{\text{ER}}$.** To address why ERdj5 is incapable of binding to SERCA2b at high $[\text{Ca}^{2+}]_{\text{ER}}$, we referred to previous studies of calsequestrin, a regulator of the ryanodine receptor (18, 19). Calsequestrin consists of three tandem repeats of thiooxidoredoxin-like domains, which are similar to a part of ERdj5, and was reported to convert its molecular conformation from a monomer to an oligomer depending on $[\text{Ca}^{2+}]_{\text{ER}}$, and this conversion affects the interaction with the ryanodine receptor (20, 21). Thus, we analyzed the size distribution and hydrodynamic diameter of ERdj5 in solution by dynamic light scattering (DLS) measurement of recombinant ERdj5 at various $[\text{Ca}^{2+}]_{\text{ER}}$. Upon addition of submillimolar $[\text{Ca}^{2+}]_{\text{ER}}$, the scattering intensity derived from the monomer species rapidly decreased (Fig. 5A), whereas the average diameter rapidly increased (Fig. 5B). These observations indicate that ERdj5 is converted to homo-oligomers at high $[\text{Ca}^{2+}]_{\text{ER}}$, in sharp contrast to the behavior of PDI.

**[Ca$^{2+}]_{\text{ER}}$ Influences the Interaction of ERdj5 with SERCA2b.** Next, we sought to determine whether the activation of SERCA2b by ERdj5 is constitutive or regulative. To maintain Ca$^{2+}$ homeostasis in the ER, ERdj5 should activate SERCA2b in a $[\text{Ca}^{2+}]_{\text{ER}}$-dependent manner. Although ERdj5 was present in its oxidized form in normal cells, treatment with thapsigargin converted it to the reduced form, which is capable of reducing SERCA2b (Fig. 4A). The redox state of another ER-resident oxidoreductase, PDI, was not changed by thapsigargin treatment, suggesting that Ca$^{2+}$ deficiency in the ER specifically converted ERdj5 to the reduced form. This was not simply due to ER stress because tunicamycin, an inhibitor of N-glycosylation, had no effect on the redox state of ERdj5 (Fig. 4A, Right).
Finally, we examined the effect of BiP binding on ERdj5 oligomerization using the ERdj5/H63A mutant, which is incapable of BiP binding, and the BiP/T37G mutant, which lacks ATPase activity (22). First, to examine the effect of BiP on the interaction between ERdj5 and SERCA2b, BiP was cotransfected with ERdj5 and SERCA2b. Lysates of cells transfected with FLAG-SERCA2b were treated with Tg or ionomycin for the indicated amount of time. The per-cent binding of ERdj5 was normalized to that in untreated cells. (C) FLAG-SERCA2b was captured with FLAG-conjugated beads in lysates of cells transfected with FLAG-SERCA2b. Lysates of cells transfected with Myc-tagged ERdj5 were adjusted to contain the indicated concentration of CaCl2. The adjusted cell lysates were treated with Tg or ionomycin for the indicated amount of time. The percentage binding of ERdj5 was normalized to that in untreated cells. (C) FLAG-SERCA2b was captured with FLAG-conjugated beads in lysates of cells transfected with FLAG-SERCA2b. Lysates of cells transfected with Myc-tagged ERdj5 were adjusted to contain the indicated concentration of CaCl2. The adjusted cell lysates were incubated with FLAG-SERCA2b-bound beads for the pull-down assay. ERdj5-Myc bound to SERCA2b was detected by immunoblotting with an anti-Myc antibody. Binding of ERdj5 with SERCA2b was quantified and shown below the panel.

**Discussion**

In this report, we showed that ERdj5 activated the pump function of SERCA2b by reducing the intraluminal disulfide bond of SERCA2b. This activation by ERdj5 was [Ca2+]ER-dependent, and at a higher [Ca2+]ER, ERdj5 was converted to an oligomer form, which was no longer effective for the activation of SERCA2b.

A possible regulatory mechanism is shown in Fig. 6D. The oxidized (low-activity state) form of SERCA2b can be converted into the high-active form by reduction of the disulfide bond in the ER-luminal portion, which activates Ca2+ uptake into the ER. When [Ca2+]ER becomes higher than 1 mM, it causes oligomerization of ERdj5, which loses the ability to bind to SERCA2b. This feedback regulation by oligomerization of ERdj5 serves to maintain Ca2+ homeostasis in the ER lumen. When [Ca2+]ER is lowered by treatment of cells with thapsigargin, the decrease in [Ca2+]ER shifts the lumen to a reducing state (23), providing favorable conditions for the reducing activity of ERdj5 and activation of SERCA2b.

Treatment with thapsigargin also causes inactivation of PDI, a major oxidoreductase in the ER, and contributes to the shift of the ER redox condition to a reducing state (24). The decrease in [Ca2+]ER induces ER stress, resulting in higher expression levels of ER molecular chaperones such as BiP, which prevents the oligomerization of ERdj5 to counteract the decrease in [Ca2+]ER by activating SERCA2b. Molecular chaperones induced by ER stresses including BiP require Ca2+, which is provided by activation of SERCA2b (6). Thus, regulation of SERCA2b by ERdj5 and BiP provides a calcium-mediated regulatory mechanism that maintains organelle homeostasis of the ER.

One important question is how SERCA2b activity is regulated by oxidation/reduction of the Cys875-Cys887 pair located in L7-L8. To obtain insights into this question, homology models of SERCA2 in several catalytic and redox states were built based on the previously reported crystal structures of SERCA1 (25-27) (Fig. 7A-D and Fig. S5). In these homology models, L7-L8 of SERCA2 is predicted to consist of two short α-helices with loops. Cys875 and Cys887 are located in each of the two α-helices, respectively (Fig. 7A and B). In the oxidized state, Cys875 and Cys887 form a disulfide bridge, stabilizing the L7-L8 conformation (Fig. 7C). Comparison of the oxidized and reduced states suggests that reduction of the Cys875-Cys887 disulfide bond alters the backbone structure and orientation of the side chains of several residues, leading to significant conformational change of L7-L8. On the other hand, the C-terminal tail characteristic of SERCA2b is predicted to interact with the cleft between L7-L8 and L5-L6 and stabilize the Ca2+-bound E1 state (Fig. 7C). Therefore, reduction of the Cys875-Cys887 disulfide bond will change the L7-L8 conformation and affect the interaction between the loop and the C-terminal tail, thus destabilizing the E1 state. Consequently, Ca2+ release is likely facilitated in the reduced form of SERCA2b.

In addition to the redox-dependent conformational changes of L7-L8, homology models in the E1-2Ca-ATP state suggest that the cystolic N domain is also involved in the regulation of the catalytic cycle in a redox-dependent manner. The relative position of the N domain differs significantly between the reduced (green) and oxidized (red) forms of SERCA2 in the E1-2Ca-ATP state (Fig. 7D). Thus, compared with the reduced state, the larger movement of the N domain is expected in the oxidized state during the transition from the E1-2Ca to the E1-2Ca-ATP state. These observations suggest that the reduced state requires lower activation energy than the oxidized state for the transition from the E1-2Ca to the E1-2Ca-ATP states, resulting in acceleration of the catalytic cycle.

In addition to the reducing activity of ERdj5, the interaction of ERdj5 with BiP through its J domain is also noted. The interaction of BiP with ERdj5 is not necessary for the activation of Ca2+ uptake under the low [Ca2+] condition because the ERdj5/H63A mutant and ERdj5/WT had an equivalent activity in the semi-permeabilized assay system (Fig. 2A and B). On the contrary, BiP binding to ERdj5 was necessary to prevent oligomerization of ERdj5 and thus for the interaction of ERdj5 with SERCA2b.
under high \([\text{Ca}^{2+}]\) (Fig. 6 A–C). The involvement of BiP in the activation of SERCA2b by ERdj5 may be particularly relevant to the maintenance of \([\text{Ca}^{2+}]\) homeostasis in the ER under the stress conditions as mentioned above.

We previously showed that the interplay of ERdj5 with BiP is indispensable for the elimination of misfolded proteins from the ER through ERAD. Glycoproteins misfolded in the ER are recognized by EDEM1 and are recruited to ERdj5, which reduces the intramolecular or intermolecular disulfide bonds of misfolded proteins to facilitate retro-translocation through the dislocon channel. When nonglycoproteins are misfolded in the ER or when EDEM1 is overwhelmed with misfolded glycoproteins, BiP recruits these misfolded proteins to ERdj5 (14). After reduction of disulfide bonds, substrates to be degraded are

![Diagrams](image-url)

**Fig. 5.** ERdj5 regulates \([\text{Ca}^{2+}]_{\text{ER}}\) by oligomer formation. (A) Relative scattering intensity derived from the monomeric component of ERdj5/SS or PDI at various calcium concentrations. (B) The Z-average, the mean diameter of ensemble particles in solution, of ERdj5/SS or PDI under various calcium concentrations. Values are the means ± 2 SD of five independent experiments. (C) ERdj5FLAG was captured with FLAG-conjugated beads in lysates of cells transfected with ERdj5-FLAG. Lysates of cells cotransfected with Myc-tagged ERdj5 and BiP/WT or the T37G mutant were adjusted to contain the indicated concentration of CaCl\(_2\). The cell lysates were incubated with ERdj5-FLAG-bound beads for the pull-down assay. After pull down with ERdj5-FLAG-bound beads, Myc-tagged ERdj5 bound to FLAG-tagged ERdj5 was detected by immunoblotting with an anti-Myc antibody. (D) Twenty-four hours after transfection of the indicated constructs into HEK293 cells, cell lysates containing 1 mM EGTA and titrated CaCl\(_2\) were prepared. Cell lysates were applied to a 10–40% sucrose density gradient and centrifuged. Each fraction was separated by SDS/PAGE for immunoblotting with the indicated antibodies.

![Diagrams](image-url)

**Fig. 6.** BiP binding to ERdj5 prevents ERdj5 oligomer formation. (A) Twenty-four hours after cotransfection of FLAG-tagged SERCA2b, FLAG-tagged ERdj5/WT, and BiP into HEK293 cells, cell lysates were prepared for immunoprecipitation with an anti-FLAG antibody. Myc-tagged ERdj5 bound to FLAG-tagged SERCA2b was detected by immunoblotting with an anti-Myc antibody. (B) Twenty-four hours after cotransfection of BiP and ERdj5/WT or the H63A mutant into HEK293 cells, cell lysates containing 1 mM EGTA and 5 mM CaCl\(_2\) were prepared. Cell lysates were applied to a 10–40% sucrose density gradient and centrifuged. Each fraction was separated by SDS/PAGE for immunoblotting with an anti-Myc antibody. (C and D) SERCA2b activation mechanism via the reducing activity of ERdj5 depending on \([\text{Ca}^{2+}]_{\text{ER}}\). ERdj5 activates SERCA2b only at a lower \([\text{Ca}^{2+}]_{\text{ER}}\), whereas a higher \([\text{Ca}^{2+}]_{\text{ER}}\) induces ERdj5 to form oligomers that are no longer able to interact with the pump. BiP exerts a regulatory role in the oligomerization of ERdj5 by binding to its J domain.
Materials and Methods

Cell Culture and Transfections. HEK293T cells, HeLa cells, and MEFs were cultured in Dulbecco's Modified Eagle's Medium (Gibco 11995) supplemented with 10% (vol/vol) inactivated FCS. Plasmids were transfected using Lipofectamine 2000 (Life Technologies) for HEK293T or HeLa cells and Lipofectamine 2000 (Life Technologies) (5 μg) for MEFs. To measure [Ca\textsuperscript{2+}]i, transfected MEFs were loaded with Fura-2 at 00 AM (Life Technologies) and incubated for 20–30 h of transfection. Transfected MEFs were loaded with Mag-Fura-200 AM (Life Technologies) (5 μM) for 60 min at room temperature and perfused with cytosolic-like medium (CLM) containing 125 mM KCl, 20 mM NaCl, 10 mM CaCl\textsubscript{2}, and 10 mM MgCl\textsubscript{2}. MEFs were transfected with pIRE52-NSL-EGFP or ERdj5s/pIRE52-NSL-EGFP using TransIT-LT1 (Mirus Bio). To measure [Ca\textsuperscript{2+}]i, after 20–30 h of transfection, transfected MEFs were loaded with Mag-Fura-200 AM (Life Technologies) (5 μM) for 60 min at room temperature and perfused with cytosolic-like medium (CLM) containing 125 mM KCl, 20 mM NaCl, 10 mM CaCl\textsubscript{2}, and 10 mM MgCl\textsubscript{2}. MEFs were transfected with pIRE52-NSL-EGFP or ERdj5s/pIRE52-NSL-EGFP using TransIT-LT1 (Mirus Bio). To measure [Ca\textsuperscript{2+}]i, after 20–30 h of transfection, transfected MEFs were loaded with Mag-Fura-200 AM (Life Technologies) (5 μM) for 60 min at room temperature and perfused with cytosolic-like medium (CLM) containing 125 mM KCl, 20 mM NaCl, 10 mM CaCl\textsubscript{2}, and 10 mM MgCl\textsubscript{2}. MEFs were transfected with pIRE52-NSL-EGFP or ERdj5s/pIRE52-NSL-EGFP using TransIT-LT1 (Mirus Bio).

Imaging. ERdj5 (ΔN) and ERdj5 (ΔC) MEFs were transfected with pIRE52-NSL-EGFP or ERdj5s/pIRE52-NSL-EGFP using TransIT-LT1 (Mirus Bio). To measure [Ca\textsuperscript{2+}]i, after 20–30 h of transfection, transfected MEFs were loaded with Mag-Fura-200 AM (Life Technologies) (5 μM) for 60 min at room temperature and perfused with cytosolic-like medium (CLM) containing 125 mM KCl, 20 mM NaCl, 10 mM CaCl\textsubscript{2}, and 10 mM MgCl\textsubscript{2}. MEFs were transfected with pIRE52-NSL-EGFP or ERdj5s/pIRE52-NSL-EGFP using TransIT-LT1 (Mirus Bio). To measure [Ca\textsuperscript{2+}]i, after 20–30 h of transfection, transfected MEFs were loaded with Mag-Fura-200 AM (Life Technologies) (5 μM) for 60 min at room temperature and perfused with cytosolic-like medium (CLM) containing 125 mM KCl, 20 mM NaCl, 10 mM CaCl\textsubscript{2}, and 10 mM MgCl\textsubscript{2}. MEFs were transfected with pIRE52-NSL-EGFP or ERdj5s/pIRE52-NSL-EGFP using TransIT-LT1 (Mirus Bio). To measure [Ca\textsuperscript{2+}]i, after 20–30 h of transfection, transfected MEFs were loaded with Mag-Fura-200 AM (Life Technologies) (5 μM) for 60 min at room temperature and perfused with cytosolic-like medium (CLM) containing 125 mM KCl, 20 mM NaCl, 10 mM CaCl\textsubscript{2}, and 10 mM MgCl\textsubscript{2}. MEFs were transfected with pIRE52-NSL-EGFP or ERdj5s/pIRE52-NSL-EGFP using TransIT-LT1 (Mirus Bio).

Cell Lysis and Immunoprecipitation. Cells were washed with PBS [−], incubated on ice for 20 min in lysis buffer (50 mM Tris HCl [pH 7.4], 150 mM NaCl, 10 mM N-ethylmaleimide, and 1% Nonidet P-40 or 1% CHAPS) supplemented with protease inhibitors, and then immunoprecipitated with specific antibodies. Immunoprecipitations were separated by SDS-PAGE. Immunoblotting was conducted under reducing or nonreducing conditions with the antibodies indicated in the text (Figs. 1 and 3–6). To detect the mixed disulfide complex (Fig. S1), cells were treated with 100 μM DVSF for 1 h. After the cell lysis, the cell lysates are incubated with 1% SDS and diluted with lysis buffer by 10 times for immunoprecipitation.

BiP and ERdj5 are regulated to induce either ERAD or BiP-recycling. However, our findings shed light on coordinated cross talk among protein, redox, and calcium homeostasis, which are three major types of homeostasis in the ER.

release from the ER, cells were stimulated with 1 μM thapsigargin (Sigma-Aldrich) in BS3 lacking CaCl₂. Imaging data analysis was performed using MetaMorph (Molecular Devices) and Igor Pro (Wave Metrics) software.

Expression and Purification of Recombinant SERCA2b and ERdj5. The PA-SERCA2b vector was transfected into HEK293T cells to establish a cell line stably expressing PA-SERCA2b. High expression of PA-SERCA2b was induced with cumate, 50 ng/mL phorbol 12-myristate 13-acetate (PMA), and 1 mM sodium butyrate (36, 37). The cells were solubilized with 1% n-dodecyl-β-D-maltoside (DDM), 50 mM Hepes (pH 7.0), 100 mM NaCl, 20% (vol/vol) glycerol, 1 mM CaCl₂, and 1 mM MgCl₂. PA-SERCA2b was bound to anti-PA tag N2-1 Sepharose and eluted with 0.2 mM PA14 peptide, as described previously (34). The eluted PA-SERCA2b sample was treated with 1 mM diamide or 1 mM DTT for 1 h at 4 °C for full oxidation or reduction of the Cys875-Cys887 pair, respectively, and then loaded onto a Superose6 10/300 GL column (GE Healthcare) pre-equilibrated with 50 mM Hepes (pH 7.0), 100 mM NaCl, 20% (vol/vol) glycerol, 1 mM CaCl₂, and 1 mM MgCl₂, and 0.1% DDM.

C-terminally FLAG-tagged ERdj5 and its cysteine mutant (ERdj5/SSS; 12, 13) were inserted into the PiggyBac Captchim Switch Inducible Vector (System Biosciences). The resultant vector was transfected into HEK293T cells to generate cell lines stably overexpressing ERdj5/FLAG or ERdj5/FLAGSSS. The cells were induced with 10× cumate and 50 ng/mL PMA, and the harvested cells were solubilized with 1% (wt/vol) (DDM), 50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 10% (vol/vol) glycerol, 1 mM EDTA, 1 mM DTT, and 1 mM PMSF. ERdj5/FLAG was purified with anti-FLAG agarose (Medical & Biological Laboratories). The eluted sample was concentrated in the presence of 1 M Non-Detergents Sulfobetaines-201 (NDSB-201) and then loaded onto a Superdex200 10/300 GL column (GE Healthcare) pre-equilibrated with 20 mM Hepes (pH 8.0), 0.05% Tween-20, 10% (vol/vol) glycerol, and 1 M NDSB. The purity of the resultant samples (reduced and oxidized PA-SERCA2b, ERdj5/FLAG, and ERdj5/FLAGSSS) was assessed by reducing SDS/PAGE (Fig. S4).

ATPase Activity Measurement of SERCA2b With/Without ERdj5. The purified PA-SERCA2b samples were incubated in buffer containing 1 mM MgCl₂, 10 μM CaCl₂, and 0.1% DDM for 10 min at 37 °C. After incubation, 1 mM ATP was added to initiate the SERCA2b ATPase cycle. The rereduced SERCA2b was precipitated with 10% (vol/vol) NDSB-201 and 1 mM DTT for 1 h at 4 °C. Reduced forms of ERdj5/FLAG and ERdj5/FLAGSSS were prepared by treatment with 1 mM DTT for 1 h at 4 °C. Oxidized PA-SERCA2b (50 nM) was incubated with 12.5, 25, 50, and 125 mM of the reduced form of ERdj5/FLAG or ERdj5/FLAGSSS for 10 min at 37 °C. After incubation, 1 mM ATP was added to initiate the SERCA2b ATPase cycle. The phosphate group released from the ER, cells were stimulated with 1 μM thapsigargin (Sigma-Aldrich) in BS3 lacking CaCl₂. Imaging data analysis was performed using MetaMorph (Molecular Devices) and Igor Pro (Wave Metrics) software.

Detection of the Redox States of SERCA2b and ERdj5. Cells in suspension were either untreated or treated with 10 mM DTT or 1 mM dipityridyl disulfide and then precipitated with 10% (vol/vol) TCA and washed with 70% ethanol and dimethyl ether, as described above. To detect the PA-tagged SERCA2b, oxidized pellets and rereduced pellets were treated with lyophilization and eluted with PA antibody-conjugated beads. The immunoprecipitants were incubated overnight at 37 °C in buffer containing Acr-Arg protease (Promega). Samples were adjusted to contain 1× Laemmli buffer for SDS/PAGE and subjected to immunoblotting with the anti-PA tag antibody N2-1.

Pull-Down Assay. FLAG-tagged SERCA2b or ERdj5 transiently expressed in HEK293T cells was immunoprecipitated with Dynabeads Protein G (Thermo Fisher) conjugated with the anti-FLAG M2 antibody. Lysates of cells transfected with Myc-tagged ERdj5 were incubated with titrated CaCl₂ and 1 mM EGTA for 1 h at 4 °C. The conjugated beads were mixed with cell lysates and washed with rotation for 10 min at room temperature. Coprecipitated ERdj5-Myc was resolved by SDS/PAGE and immunoblotted with an anti-Myc antibody.

Sucrose Density Gradient Centrifugation. Cell lysates were applied to a 10–40% sucrose density gradient created using a Gradient Master (BioComp). Lysates were centrifuged at 36,000 × g for 10 min. Each fraction of 250 μL collected from the top was adjusted to contain 1× Laemmli buffer for separation by SDS/PAGE.

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Supporting Information

Ushioda et al. 10.1073/pnas.1605818113

**Fig. S1.** ERdj5/CA4 interacts with SERCA2 through the disulfide bond. (A) Schematic representation of ERdj5/CA4. (B) Twenty-four hours after cotransfection of PA-tagged SERCA2b and FLAG-tagged ERdj5/AA or ERdj5/CA4 mutant into HEK293 cells, cells were treated with DVSF, which is the cross-linker specific for disulfide bond. The lysates were added with 1% SDS to dissociate the noncovalent interaction. After the dilution of SDS to 0.1%, the cell lysates were immunoprecipitated with anti-PA antibodies and analyzed by SDS/PAGE under reducing condition.

**Fig. S2.** The expression levels of SERCA2b were unaffected by the overexpression of ERdj5s. Twenty-four hours after transfection of ERdj5 WT, AA mutant, or H63A mutant into ERdj5 (−/−) MEF cells, cell lysates were prepared for immunoblotting with anti-SERCA antibody (Upper) or anti-β-actin antibody (Lower).

**Fig. S3.** ERdj5-knockdown cells are sensitive to ER stress induced by the Ca²⁺ ionophore A23187. Unfolded protein response induction was examined by detecting the spliced form of XBP1 mRNA. Total RNA was prepared from HEK293T cells 48 h after transfection. Cells treated with 100 nM A23187 for the indicated amounts of time were used as positive controls. Unspliced (U) and spliced (S) forms of hXBP1 mRNA amplified by RT-PCR are shown.
Fig. S4. Preparation of recombinant PA-SERCA2b, ERdj5/WT-FLAG, and ERdj5/SS-FLAG. Purified PA-SERCA2b, ERdj5/WT-FLAG, and ERdj5/SS-FLAG were subjected to reducing SDS/PAGE and stained with Coomassie brilliant blue (CBB).

Fig. S5. Simplified reaction scheme during the Ca^{2+} pump cycle of SERCA.