Reduced IRE1α mediates apoptotic cell death by disrupting calcium homeostasis via the InsP3 receptor

SM Son¹, J Byun¹, S-E Roh², SJ Kim² and I Mook-Jung*¹

The endoplasmic reticulum (ER) is not only a home for folding and posttranslational modifications of secretory proteins but also a reservoir for intracellular Ca²⁺. Perturbation of ER homeostasis contributes to the pathogenesis of various neurodegenerative diseases, such as Alzheimer’s and Parkinson diseases. One key regulator that underlies cell survival and Ca²⁺ homeostasis during ER stress responses is inositol-requiring enzyme 1α (IRE1α). Despite extensive studies on this ER membrane-associated protein, little is known about the molecular mechanisms by which excessive ER stress triggers cell death and Ca²⁺ dysregulation via the IRE1α-dependent signaling pathway. In this study, we show that inactivation of IRE1α by RNA interference increases cytosolic Ca²⁺ concentration in SH-SYSY cells, leading to cell death. This dysregulation is caused by an accelerated ER-to-cytosolic efflux of Ca²⁺ through the InsP3 receptor (InsP3R). The Ca²⁺ efflux in IRE1α-deficient cells correlates with dissociation of the Ca²⁺-binding InsP3R inhibitor CIB1 and increased complex formation of CIB1 with the pro-apoptotic kinase ASK1, which otherwise remains inactivated in the IRE1α–TRAF2–ASK1 complex. The increased cytosolic concentration of Ca²⁺ induces mitochondrial production of reactive oxygen species (ROS), in particular superoxide, resulting in severe mitochondrial abnormalities, such as fragmentation and depolarization of membrane potential. These Ca²⁺ dysregulation-induced mitochondrial abnormalities and cell death in IRE1α-deficient cells can be blocked by depleting ROS or inhibiting Ca²⁺ influx into the mitochondria. These results demonstrate the importance of IRE1α in Ca²⁺ homeostasis and cell survival during ER stress and reveal a previously unknown Ca²⁺-mediated cell death signaling between the IRE1α–InsP3R pathway in the ER and the redox-dependent apoptotic pathway in the mitochondrion.

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The endoplasmic reticulum (ER) is an intracellular organelle not only responsible for protein synthesis and quality control but also serves as a Ca²⁺ store to maintain intracellular Ca²⁺ levels.¹,² Most integral membrane proteins and secretory proteins are synthesized at the ER, where they fold and, when necessary, become covalently modified and assembled into high-quality, functional proteins. As the maintenance of ER homeostasis is essential to cell survival, the cells have an ER stress-sensing system, termed the ‘unfolded protein response’ (UPR).³,⁴ The ER dysfunctions such as glucose deprivation, aberrant Ca²⁺ regulation, or accumulation of misfolded proteins, leads to UPR activation and initiates intracellular signaling pathways for cell protection. The UPR is governed by ER stress sensors, including inositol-requiring enzyme 1α (IRE1α), double-stranded RNA-activated protein kinase (PKR)-like ER kinase (PERK), and activating transcription factor 6 (ATF6), in the ER lumen.⁵ IRE1α, a major ER stress transducer, is a serine/threonine protein kinase/endoribonuclease that, upon activation, initiates the splicing of X-box binding protein 1 (Xbp1) mRNA.⁶,⁷ Spliced Xbp1 mRNA encodes a transcriptional activator that induces the transcription of chaperone protein-encoding genes, whose products have a role in ER protein folding.⁷ Under prolonged stress, IRE1α also interacts with TNF receptor-associated factor 2 (TRAF2) and apoptosis signal-regulating kinase 1 (ASK1) or activates caspase-12, an ER resident caspase, to cause cell death in neuronal cells.⁸,⁹ PERK is a transmembrane kinase that phosphorylates eukaryotic translation initiation factor 2 (eIF2α) during ER stress responses and reveal a previously unknown Ca²⁺-mediated cell death signaling between the IRE1α–InsP3R pathway in the ER and the redox-dependent apoptotic pathway in the mitochondrion. Reduced IRE1α mediates apoptotic cell death by disrupting calcium homeostasis via the InsP3 receptor

1Department of Biochemistry and Biomedical Sciences, Seoul National University College of Medicine, Seoul, Korea and 2Department of Physiology, Seoul National University College of Medicine, Seoul, Korea

*Corresponding author: I Mook-Jung, Department of Biochemistry and Biomedical Sciences, Seoul National University College of Medicine, 28 Yungun-dong, Jongro-gu, Seoul 110-799, Korea. Tel: +82 2 740 8249; Fax: +82 2 3672 7352; E-mail: ihhe@snu.ac.kr

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Abbreviations: ER, endoplasmic reticulum; ASK1, apoptosis signal-regulating kinase 1; IRE1α, inositol-requiring enzyme 1α; InsP3R, inositol-1,4,5-triphosphate (InsP3) receptor; CIB1, calcium- and integrin-binding protein 1; ROS, reactive oxygen species; UPR, unfolded protein response; PERK, double-stranded RNA-activated protein kinase (PKR)-like ER kinase; ATF6, activating transcription factor 6; TRAF2, TNF receptor-associated factor 2; eIF2α, eukaryotic translation initiation factor 2 subunit alpha; RyR, ryanodine receptor; SERCA, Ca²⁺-reuptake pumps consisting of sarco-endoplasmic reticulum Ca²⁺-ATPase; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; TUDCA, tauroursodeoxycholate; TMRM, tetramethyl rhodamine methyl ester; MCU, mitochondrial uniporter; CsA, cyclosporin A; Fura-2 AM, fura-2 acetoxymethyl ester; Xbp-1, X-box binding protein 1

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misfolding, the ATF6 cytoplasmic domain is liberated from its membrane anchor by regulated proteolysis.12

The intracellular Ca2⁺ ion level (\([\text{Ca}^{2+}]_i\)) regulates cellular processes, such as exocytosis, transcription, proliferation, and apoptosis.13 The Ca2⁺ concentration is tightly regulated by multiple Ca2⁺-channels, pumps, and binding proteins; [Ca2⁺]i is increased by Ca2⁺ influx across the plasma membrane and Ca2⁺ release from intracellular stores. The ER, mitochondria, and nucleus are main intracellular Ca2⁺ stores; the ER is the most important, as it can store up to 10–100 mM Ca2⁺ (versus 100–300 nM in the cytoplasm).14 Ca2⁺ movements across the ER membrane are facilitated by Ca2⁺-release channels, including inositol-1,4,5-trisphosphate (InsP3) receptors (InsP3Rs) and ryanodine receptors (RyRs); and Ca2⁺ reuptake pumps consisting of sarco-endoplasmic reticulum Ca2⁺-ATPases (SERCAs) residing in the ER.15-17

The pumps, channels, and buffering proteins finely regulate the spatiotemporal pattern of cytosolic Ca2⁺ levels ([Ca2⁺]cytosol (c)). However, despite tight regulation of Ca2⁺ release from the ER, the depletion of ER Ca2⁺ and the overload of cytosolic Ca2⁺ can be induced by several stimuli. The alterations in [Ca2⁺]c disrupt Ca2⁺ homeostasis, and unchecked increases in [Ca2⁺]c, can trigger apoptosis through the activation of processes in the cytoplasm (e.g., abnormal activation of calpain or phosphatase calcineurin), activation of ER resident caspases, or mitochondrial dysfunction due to Ca2⁺ overload.18-20

As ER stress is intimately associated with cell death, proper manipulation of ER stress is essential for cell survival.21 In this study, we investigated the role of ER stress transducers in cell death. By using IRE1x-, PERK-, or ATF6-specific siRNA, we demonstrated that knockdown (KD) of IRE1x, but not PERK or ATF6, induced ER stress and altered morphology (ER expansion). In SH-SY5Y cells, IRE1x KD caused cell death, not due to unfolded protein accumulation but due to accelerated Ca2⁺ release from the ER. In addition, IRE1x-KD-induced [Ca2⁺]c alterations were mediated by InsP3R. We speculate that IRE1x may regulate InsP3R-mediated Ca2⁺ release by interacting with ASK1 and calcium- and integrin-binding protein 1 (CIB1), the latter of which regulates opening of InsP3R. In IRE1x-KD cells, InsP3R-induced increases of ER Ca2⁺ release resulted in cell death due to prolonged mitochondrial Ca2⁺ accumulation and alterations in morphology (swelling and fragmentation) and function.

Results

Reduced IRE1x levels induce ER stress and alter ER morphology in human neuroblastoma SH-SY5Y cells. Previous studies have shown that ER stress causes cell death through accumulation of unfolded or abnormal proteins in the ER and subsequent activation of ER stress-induced caspases.20,23 ER stress transducers modulate ER-specific stress;7,10,24 therefore, we investigated whether the main ER stress transducer IRE1x regulates ER stress-mediated cell death. After SH-SY5Y cells were transfected with IRE1x-specific siRNA for 48 h, total IRE1x levels were reduced by 40–60% versus control siRNA-transfected cells, without changes in β-actin expression (Figures 1a–c and Supplementary Figures S1a and b). We used western blots to determine whether downregulation of IRE1x expression induces ER stress and observed marked induction of CHOP, an ER stress-related marker protein, as well as GRP78, an ER chaperone25 (Figure 1b). Next, we knocked down other ER stress transducers, PERK and ATF6x, to test their ability to regulate ER stress. PERK- and ATF6x-specific siRNA reduced their respective protein levels by 60–80%, without any change in β-actin expression (Figure 1a). We found, however, unlike IRE1x KD, reduction of PERK or ATF6x did not induce ER stress (Figure 1c), suggesting that only IRE1x regulates ER stress under basal conditions. As IRE1x is localized in the ER membrane26 and the ER structure undergoes dramatic changes upon cellular damage,27,28 we examined ER morphology under IRE1x KD. Western blotting revealed no difference in the expression of ER membrane proteins, such as calreticulin or calciumnexin (Figure 1d), Immunofluorescence experiments using anti-calreticulin antibody as an ER indicator showed that ER morphology was slightly altered in IRE1x-KD cells (data not shown). We used transmission electron microscopy to analyze IRE1x-KD-induced changes in ER morphology. The electron micrographs of IRE1x-KD cells showed ER enlargement and distension (ER expansion) (Figure 1e). Thus, IRE1x KD induced ER stress and caused ER expansion.

Knockdown of IRE1x induces cell death by disrupting intracellular Ca2⁺ homeostasis. ER stress induces cell death,21 therefore, we tested the effect of IRE1x KD on cell viability. The results of MTT and calcein-AM assays showed that reduction of IRE1x induced cell death (Figures 2a and b). To confirm the increase of apoptotic cell death under IRE1x-KD conditions, we performed the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, a method for detecting DNA fragmentation. Consistently, TUNEL staining indicated increased apoptosis in IRE1x-KD cells (Figure 2c). To determine whether this cell death was mediated by ER stress, the IRE1x-KD cells were treated with the chemical chaperone tauoxursoxycholate (TUDCA) to protect the cells from ER stress. Indeed, TUDCA alleviated ER stress induction by IRE1x-KD (Figure 2e) but did not rescue IRE1x-KD-induced cell death (Figure 2d), suggesting that IRE1x-KD-induced cell death could not be rescued by inhibiting ER stress alone. Tunicamycin, an inhibitor of protein glycosylation, causes ER stress-induced apoptosis by accumulating unfolded or misfolded proteins in the ER.30 In this study, tunicamycin not only induced cell death in the SH-SY5Y cells but also enhanced cell death in IRE1x-KD versus control siRNA-transfected cells (Figure 2d). These data suggest that IRE1x-KD-induced cell death is mediated by mechanisms other than ER stress caused by accumulation of abnormal proteins in the ER. Previous studies have shown that dysregulation of intracellular Ca2⁺ levels ([Ca2⁺]c) induces cell death.13 As IRE1x is a type I transmembrane protein localized in the Ca2⁺-storing ER, we examined the effect of reduced IRE1x levels on intracellular Ca2⁺ levels. Using the Fluo-4 calcium assay, we observed that IRE1x reduction triggered [Ca2⁺]c upregulation (Figure 2f). To determine whether dysregulated [Ca2⁺]c induced by IRE1x KD causes cell death, the cells were treated with 1,2-bis(o-aminophenoxo)ethane-N,N,N,N-tetraacetic

by regulating CHOP, an ER stress-related marker protein, as well as GRP78, an ER chaperone25 (Figure 1b). Next, we knocked down other ER stress transducers, PERK and ATF6x, to test their ability to regulate ER stress. PERK- and ATF6x-specific siRNA reduced their respective protein levels by 60–80%, without any change in β-actin expression (Figure 1a). We found, however, unlike IRE1x KD, reduction of PERK or ATF6x did not induce ER stress (Figure 1c), suggesting that only IRE1x regulates ER stress under basal conditions. As IRE1x is localized in the ER membrane26 and the ER structure undergoes dramatic changes upon cellular damage,27,28 we examined ER morphology under IRE1x KD. Western blotting revealed no difference in the expression of ER membrane proteins, such as calreticulin or calciumnexin (Figure 1d), Immunofluorescence experiments using anti-calreticulin antibody as an ER indicator showed that ER morphology was slightly altered in IRE1x-KD cells (data not shown). We used transmission electron microscopy to analyze IRE1x-KD-induced changes in ER morphology. The electron micrographs of IRE1x-KD cells showed ER enlargement and distension (ER expansion) (Figure 1e). Thus, IRE1x KD induced ER stress and caused ER expansion.

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acid-acetoxymethyl ester (BAPTA-AM), a Ca\(^{2+}\) chelator. BAPTA-AM treatment prevented IRE1\(\alpha\)-KD-induced cell death (Figure 2g), suggesting that dysregulation of \([\text{Ca}^{2+}]_{i}\) has a role in IRE1\(\alpha\)-KD-induced cell death. Neither PERK nor ATF6\(\alpha\) induced ER stress (Figure 1c); we explored their role in cell death by MTT and calcein-AM assays and found that knockdown of these regulators did not induce apoptosis (Figures 2h and i). Consistently, reduction of PERK or ATF6\(\alpha\)
had no effect on \([\text{Ca}^{2+}]\), (Figure 2j). These results suggest that only IRE1\(\alpha\) regulates cell survival by maintaining \(\text{Ca}^{2+}\) homeostasis under basal conditions.

**Rescue of IRE1\(\alpha\) restores disrupted intracellular \(\text{Ca}^{2+}\) homeostasis and then inhibits cell death in IRE1\(\alpha\)-KD cells.** To avoid off-target effects during siRNA treatment, we reintroduced IRE1\(\alpha\) into IRE1\(\alpha\)-KD cells. After transfection with control or IRE1\(\alpha\) siRNA for 24 h, an exogenous IRE1\(\alpha\) construct was transfected into the cells for 24 h (Supplementary Figure S2). IRE1\(\alpha\) expression was increased in these IRE1\(\alpha\)-overexpressing (IRE1 o/e) cells (Supplementary Figure S2a). IRE1\(\alpha\) re-introduction rescued calcine-AM signal loss in IRE1\(\alpha\)-KD cells (Supplementary Figure S2b), indicating that altered IRE1\(\alpha\) levels regulated cell death. Figures 2e and f show that IRE1\(\alpha\) regulated cell survival by maintaining \(\text{Ca}^{2+}\) homeostasis under basal conditions. IRE1\(\alpha\) re-introduction also restored \(\text{Ca}^{2+}\) in...
IRE1<sup>±</sup>-KD cells, as demonstrated by Fluo-4 calcium assay (Supplementary Figure S2c). These data indicate that IRE1<sup>±</sup> regulates cell survival by maintaining Ca<sup>2+</sup> homeostasis under basal conditions.

**IRE1<sup>±</sup> KD induces [Ca<sup>2+</sup>]<sub>i</sub> upregulation by accelerating ER Ca<sup>2+</sup> release.** As IRE1<sup>±</sup> is an ER membrane protein<sup>30</sup> and the ER is a major Ca<sup>2+</sup>-storing organelle,<sup>1</sup> we investigated whether IRE1<sup>±</sup>-KD-induced [Ca<sup>2+</sup>]<sub>i</sub> increases are caused by ER Ca<sup>2+</sup> release. Thapsigargin, an inhibitor of ER Ca<sup>2+</sup>-ATPase (SERCA),<sup>31</sup> was used to determine the concentration of free Ca<sup>2+</sup> within the ER lumen ([Ca<sup>2+</sup>]<sub>ER</sub>) by selectively depleting ER Ca<sup>2+</sup>-stores, whereas BAPTA-AM was used to deplete cytoplasmic Ca<sup>2+</sup>. The Fluo-4 assay showed that the IRE1<sup>±</sup>-KD cells showed few Ca<sup>2+</sup>-level changes from ER, compared with control siRNA-transfected cells (Figure 3a), suggesting that increased [Ca<sup>2+</sup>]<sub>i</sub> in the IRE1<sup>±</sup>-KD cells was caused by ER Ca<sup>2+</sup> release. To confirm this result, we directly measured the effects of IRE1<sup>±</sup> downregulation by Ca<sup>2+</sup>-imaging with the fluorescent dye Fura-2-AM. Although the IRE1<sup>±</sup>-KD cells showed increases in the basal [Ca<sup>2+</sup>]<sub>i</sub>, the levels of [Ca<sup>2+</sup>]<sub>ER</sub> increases in the IRE1<sup>±</sup>-KD cells after challenge with thapsigargin (a measure of [Ca<sup>2+</sup>]<sub>ER</sub> stores) was lower than that in the control siRNA-transfected cells (Figure 3b). The experiment was repeated three times; the average increase after thapsigargin challenge was 48% of the control level (average baseline level). These data indicate that reduced IRE1<sup>±</sup> levels caused [Ca<sup>2+</sup>]<sub>i</sub> upregulation by accelerating Ca<sup>2+</sup> release from the ER.

InsP<sub>3</sub> mediates IRE1<sup>±</sup>-KD-induced [Ca<sup>2+</sup>]<sub>i</sub> alterations and increases ER Ca<sup>2+</sup> release resulting in cell death. The ER is the most important intracellular Ca<sup>2+</sup>-store. Regulation of intracellular Ca<sup>2+</sup> by the ER is mainly mediated by Ca<sup>2+</sup> uptake into the ER through SERCA Ca<sup>2+</sup>-pumps and Ca<sup>2+</sup> release through Ca<sup>2+</sup>-channels, such as InsP<sub>3</sub>Rs or RyRs<sup>33–35</sup>. To determine whether [Ca<sup>2+</sup>]<sub>i</sub> increases in IRE1<sup>±</sup>-KD cells are caused by ER Ca<sup>2+</sup>-related channels, we treated the cells with control siRNA- and channel blockers dantrolene and 2-aminoethoxydiphenyl borate (2-APB) to inhibit RyRs and InsP<sub>3</sub>R, respectively. As shown in Figure 4a, 2-APB treatment blocked the increase of [Ca<sup>2+</sup>]<sub>i</sub> in the IRE1<sup>±</sup>-KD cells, whereas dantrolene did not. Notably, western blotting indicates no difference in RyRs and InsP<sub>3</sub>R expression between IRE1<sup>±</sup>-KD and control siRNA-transfected cells (Figure 4b), suggesting that upregulation of [Ca<sup>2+</sup>]<sub>i</sub> in the IRE1<sup>±</sup>-KD cells is associated with InsP<sub>3</sub>R but does not alter the expression of ER Ca<sup>2+</sup>-related channels. Next, to determine whether InsP<sub>3</sub>R-mediated Ca<sup>2+</sup>-release in IRE1<sup>±</sup>-KD cells influences cell death, the viability of IRE1<sup>±</sup>-KD cells treated with vehicle, dantrolene, or 2-APB was determined by calcein-AM assay. Inhibition of InsP<sub>3</sub>R rescued cell death in the IRE1<sup>±</sup>-KD cells, whereas the blockers of RyRs (dantrolene) did not (Figure 4c).

To confirm this result, caspase-3 and -9 activities were measured. Essential for apoptosis,<sup>36,37</sup> caspases exist as inactivezymogens and require proteolytic processing for activation. Under apoptotic conditions, the activation of upstream caspasess (caspase-8 and/or -9) proteolytically activates downstream caspasess, such as caspase-3.<sup>38</sup> In comparison to control siRNA-transfected cells, the IRE1<sup>±</sup>-KD cells showed increased levels of cleaved caspase-3 and -9 (Figure 4d); these effects were rescued by 2-APB, but not dantrolene. To confirm these results, the cells were treated with xestospongin C (XeC), an InsP<sub>3</sub>R-specific antagonist.<sup>39</sup> XeC reversed [Ca<sup>2+</sup>]<sub>i</sub> increases and IRE1<sup>±</sup>-KD-induced cell death in the IRE1<sup>±</sup>-KD cells (Figures 4e and f). These data suggest that, upon IRE1<sup>±</sup> downregulation, InsP<sub>3</sub>R-mediated Ca<sup>2+</sup>-release induces apoptotic cell death.

IRE1<sup>±</sup> regulates InsP<sub>3</sub>R-mediated Ca<sup>2+</sup>-release through the ASK1-CIB1 interaction. To explore the mechanisms through which IRE1<sup>±</sup> KD promoted InsP<sub>3</sub>R-mediated Ca<sup>2+</sup>-release, we tested whether IRE1<sup>±</sup> interacts directly with InsP<sub>3</sub>R. By co-immunoprecipitation (co-IP), no interaction was detected between IRE1<sup>±</sup> and InsP<sub>3</sub>R (Figure 5a). We next investigated whether IRE1<sup>±</sup> downstream signaling is associated with InsP<sub>3</sub>R-mediated Ca<sup>2+</sup>-release in the IRE1<sup>±</sup>-KD cells. When the IRE1<sup>±</sup> kinase activity was inhibited by addition of the ATP-competitive inhibitor 1NM-PPT<sup>40</sup> to the SH-SY5Y cells, [Ca<sup>2+</sup>]<sub>i</sub> was not affected (Supplementary Figure S3), suggesting that the IRE1<sup>±</sup> kinase activity and its downstream signaling pathway are not associated with the opening of InsP<sub>3</sub>R. Previous studies have demonstrated that CIB1 binding to InsP<sub>3</sub>R led to an inhibition of Ca<sup>2+</sup>-release from InsP<sub>3</sub>R.<sup>22</sup> CIB1 was recently suggested to function as a Ca<sup>2+</sup>-sensitive modulator by interacting directly with ASK1.<sup>41</sup> Based on these findings, we tested the association between the regulation of the opening of InsP<sub>3</sub>R and...
changes in binding partners. The co-IP analysis shows that the extent of the CIB1-ASK1 interaction was increased in the IRE1\textsubscript{x}-KD cells, compared with the control siRNA-transfected cells. In addition, the IRE1\textsubscript{x}-KD cells also showed increased CIB1-ASK1 interaction and decreased InsP3R-CIB1 interaction, indicating that IRE1\textsubscript{x} downregulation reduced recruitment of TRAF2-ASK1 to IRE1\textsubscript{x}, thereby resulting in increases in free ASK1-CIB1 binding and decreases in the CIB1-InsP3R interaction (Figure 5b). Decreased CIB1-InsP3R interaction induces Ca\textsuperscript{2+} release from InsP3R,\textsuperscript{22} thus IRE1\textsubscript{x}-KD-induced Ca\textsuperscript{2+} release from InsP3R may result from changes in CIB1-InsP3R binding. To visualize the ASK1-CIB1 and CIB1-InsP3R interactions under IRE1\textsubscript{x}-KD conditions, we performed the proximity ligation assay. In this novel assay, close proximity of the target proteins generates punctate signals. The IRE1\textsubscript{x}-KD cells produced more signals in the presence of ASK1 and CIB1 antibodies and fewer signals in the presence of CIB1 and InsP3R antibodies (Figure 5c), suggesting that reduced IRE1\textsubscript{x} levels enhanced ASK1-CIB1 interaction and inhibited CIB1-InsP3R interaction. To determine whether decreased CIB1-InsP3R binding upregulates [Ca\textsuperscript{2+}], to trigger cell death, SH-SY5Y cells were transfected with CIB1-specific siRNA (CIB1-KD cells) for 48 h. Reduced CIB1 levels led to [Ca\textsuperscript{2+}] increases and cell death; treatment of CIB1-KD cells with 2-APB, but not dantrolene, reversed these effects (Figures 5d and e), suggesting that reduction of CIB1 may upregulate Ca\textsuperscript{2+} release from InsP3R, in turn enhancing cell death.

**IRE1\textsubscript{x}-KD induces mitochondrial dysfunction and reactive oxygen species (ROS) generation, leading to cell death.** To investigate the mechanism through which IRE1\textsubscript{x}-KD-induced [Ca\textsuperscript{2+}] increases mediate cell death, we focused on mitochondrial functions because of their role as modulators of the apoptotic process. Previous studies have shown that enhanced [Ca\textsuperscript{2+}] mediated by InsP3R and RyRs increased sequestration of vast amounts of Ca\textsuperscript{2+} in mitochondria ([Ca\textsuperscript{2+}]\textsubscript{im}), which subsequently triggered mitochondrial membrane permeabilization and led to apoptotic cell death.\textsuperscript{42} This pathway also depends on Ca\textsuperscript{2+}-induced opening of the permeability transition pore (PTP).\textsuperscript{43}

As the IRE1\textsubscript{x}-KD cells showed increased mitochondrial fission (Figure 6a), stable Mito-DsRed-expressing cells were used to investigate the effect of IRE1\textsubscript{x} KD on mitochondrial morphology. Electron microscopy (EM) showed increased mitochondrial fragmentation in the IRE1\textsubscript{x}-KD cells (Figure 6b). We also determined the effect of IRE1\textsubscript{x} KD on
**Figure 4** InsP3R mediates IRE1α-KD-induced [Ca²⁺]i alterations and increase ER Ca²⁺ release, leading to cell death. (a) Treatment with InsP3R blocker (2-APB), not RyRs blocker (Dant; dantrolene), blocked the increase of [Ca²⁺]i in the IRE1α-KD cells. Changes in [Ca²⁺]i were determined by the Fluo-4 assay. After siRNA transfection for 48 h, 5 μM of Fluo-4 AM in DMEM was added at 37 °C for 60 min. After washing, dantrolene (20 μM), 2-APB (10 μM), and BAPTA-AM (5 μM) were added for 6 h, and 0.5 μM of thapsigargin was treated for 30 min, and then fluorescent signals were captured using a fluorescence microscope and analyzed in ImageJ (N = 3 experiments). Data are shown as the mean percentage ± S.E.M. IK, IRE1α-KD cells; Tg, thapsigargin (positive control for Fluo-4 assay); BAPTA, BAPTA-AM (negative control for Fluo-4 assay). **P < 0.01 and *** P < 0.001 versus vehicle (DMSO)-treated control siRNA-transfected cells; ### P < 0.001 versus vehicle-treated IRE1α-KD cells. NS indicates no significant difference. Scale bar = 40 μm. (b) RyRs and InsP3R expression in control and IRE1α-siRNA-transfected cells was determined by western blotting, with β-actin as a loading control. (c) The viability of the IRE1α-KD cells treated with dantrolene (20 μM), 2-APB (10 μM), and BAPTA-AM (5 μM) was determined by calcein-AM assay. Data shown are the mean percentage ± S.E.M. **P < 0.01 versus vehicle-treated control siRNA-transfected cells; # P < 0.05 versus vehicle-treated IRE1α-KD cells. Data were obtained from at least five replicates per group (N = 5 experiments). (d) IRE1α-KD cells showed activation of caspase-3 and -9, which was inhibited by 2-APB treatment. fl, full-length form; cle, cleaved (activated) form. β-Actin is a loading control. (e) Xestospongin C, one of InsP3R-specific antagonists, reversed increased [Ca²⁺]i in IRE1α-KD cells. In all, 2 μM of xestospongin C was treated with Fluo-4 loaded cells for 6 h, and changes in [Ca²⁺]i were determined by the Fluo-4 assay. XeC, xestospongin C. Representative images are shown. Scale bar = 40 μm. (f) Cell viability was analyzed by calcein-AM assay. 2-APB (10 μM) or xestospongin C (2 μM) were treated with IRE1α-KD cells, and then calcein-AM assay was performed. Data are shown as the mean percentage ± S.E.M. * P < 0.05 versus vehicle-treated control siRNA-transfected cells; * P < 0.05 versus vehicle-treated IRE1α-KD cells. Data were obtained from at least five replicates per group (N = 5 experiments).
IRE1α knockdown-induced cell death

**Figure 5.** IRE1α regulates InsP3R-mediated Ca^{2+} release through the ASK1-CIB1 interaction. (a) Co-immunoprecipitation (IP) with InsP3R- and IRE1α-specific antibodies revealed no interaction between InsP3R and IRE1α. The lower panel (‘Lysate’) shows a western blot of IRE1α, using the β-actin as a loading marker. Representative images are shown; IP3R indicates InsP3R. (b) Co-IP with CIB1-, ASK1-, InsP3R-, and IRE1α-specific antibodies. The left and middle panel show increased CIB1-ASK1 interaction in IRE1α-KD cells. The right panel shows decreased CIB1-InsP3R interaction in IRE1α-KD cells. The lower panel (‘Lysate’) shows a western blot of ASK1, CIB1, IRE1α, TRAF2, and InsP3R, with β-actin as a loading control. Representative images are shown. (c) Proximity ligation assay showed reduced that IRE1α levels induced ASK1-CIB1 interaction and decreased CIB1-InsP3R interaction. DAPI (blue) was used to stain nuclei. Red dot-like signals indicate close proximity of two specific proteins (anti-ASK1 (rabbit polyclonal), anti-CIB1 (mouse monoclonal), and anti-InsP3R (rabbit polyclonal) antibodies). Scale bar = 25 μm. *P < 0.05 and ***P < 0.001 versus control siRNA-transfected cells (Con). (d) Alteration in [Ca^{2+}]_{i} in CIB1-KD cells was measured by Fluo-4 assay. Data were analyzed by ImageJ program. **P < 0.01 versus control siRNA-transfected cells; #P < 0.05 versus CIB1-KD cells. Data were obtained from at least five replicates per group (N = 5 experiments).
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Figure 6  IRE1α KD induces mitochondrial dysfunction and ROS generation leading to cell death. (a) Changes in mitochondrial morphology in IRE1α-KD cells. Mitochondria in IRE1α-KD cells were shortened (represented by the aspect ratio) and had a more circular shape (represented by the form factor) in comparison with control siRNA-transfected cells. Scale bar = 20 μm. (b) EM images of IRE1α-KD cells showed mitochondrial fission. The lower panels show enlarged figures, and the arrowheads show expanded ER. Scale bar = 2 μm. (c) The mitochondrial membrane potential was measured by TMRM assay. Representative images are shown. Data presented are the mean ± S.E.M of three experiments. *P < 0.05 versus control siRNA-transfected cells. Scale bar = 50 μm. (d) ROS generation in IRE1α-KD cells was determined by DCFDA staining. Arrow indicates DCF fluorescence (ROS generation). Treatment with InsP3R blocker (2-APB), not RyRs blocker (Dant; dantrolene), blocked the increase of ROS levels in the IRE1α-KD cells. After siRNA transfection for 36 h, dantrolene (20 μM) or 2-APB (10 μM) were added for 12 h. After washing, 1 μM DCFDA in OPTI-MEM was added at 37°C for 60 min, and fluorescent signals were captured by fluorescence microscopy. Representative images are shown. (N = 3 experiments); Scale bar = 50 μm. (e) Generation of mitochondrial superoxide in IRE1α-KD cells was measured by MitoSOX staining. Red fluorescence indicates the existence of mitochondrial superoxide. IRE1α-KD cells showed increased mitochondrial superoxide levels. Scale bar = 50 μm. (f) Treatment with ROS scavenger (NAC) or mitochondrial calcium uptake blocker (Ru360) blocked increased cell death in the IRE1α-KD cells. Cell viability was measured by calcine-AM assay. After siRNA transfection for 24 h, NAC (1 mM), CsA (200 nM), Ru360 (10 μM), and CCCP (2.5 μM) in DMEM were added for 24 h. After washing, the calcine-AM assay was performed. Data shown are the mean percentage ± S.E.M of four experiments. *P < 0.01 versus control siRNA-transfected cells; †P < 0.05 ‡P < 0.01 versus vehicle (DMSO)-treated IRE1α-KD cells. NS indicates no significant difference.
mitochondrial membrane potential. The tetramethyl rhodamine methyl ester (TMRM) assay showed depolarization of mitochondrial membrane potential in IRE1α-KD cells (Figure 6c). Depolarized mitochondrial membrane potential induces ROS generation. Dichlorofluorescin diacetate (DCFDA) staining showed increased DCF fluorescence, representing increased ROS levels in IRE1α-KD cells (Figure 6d). To detect mitochondrial superoxide accumulation, we performed MitoSOX Red staining and found that the IRE1α-KD cells showed significantly higher levels of MitoSOX Red fluorescence in mitochondria (Figure 6e). To determine whether alterations in mitochondrial homeostasis induce cell death under IRE1α-KD conditions, we performed the cell death assay with several blockers. Treatment with NAC, a well-known ROS scavenger, or Ru360, a blocker of the mitochondrial uniporter (MCU), rescued cell death caused by IRE1α KD (Figure 6f), indicating that IRE1α-KD-induced [Ca2+]c increases enhanced Ca2+ load in the mitochondria, thereby leading to mitochondrial dysfunction and cell death. Notably, treatment with carbonyl cyanide m-chlorophenyl hydrazine (CCCP), the mitochondrial uncoupler, did not induce additional cell death in comparison with IRE1α KD alone (Figure 6f), suggesting that IRE1α KD induced cell death through mitochondrial dysfunction. Cyclosporin A (CsA), which inhibits mitochondrial permeability transition, partially rescued the IRE1α-KD cells from cell death (Figure 6f). These results suggest that IRE1α KD induces mitochondrial dysfunction and cell death through increased ROS generation.

Discussion

Accumulating studies have shown that ER stress is closely associated with cell death. As the ER mediates protein synthesis, folding, and Ca2+ maintenance, the ER disruption causes cell death through several mechanisms. In response to ER stress, the cells activate the ER stress-specific defense system. It is well known that IRE1α acts as the main ER stress transducer; however, its role in cell death is not yet fully understood. In this study, we chose SH-SYSY cells based on previous reports on the roles of IRE1α in ER stress and mitochondria-ER crosstalk. Our results demonstrate that cell death was induced in IRE1α knocked down SH-SYSY cells (IRE1α-KD cells) compared with control siRNA-transfected cells (Con). As the ER is a Ca2+-storing organelle, [Ca2+]c increased when IRE1α was knocked down, and treatment with a Ca2+-chelating agent rescued cell death induced by IRE1α KD. We explored the underlying mechanism of IRE1α-induced [Ca2+]c increases and found that IRE1α-KD-induced [Ca2+]c upregulation resulted from ER Ca2+ release. Our results also indicate that the ER Ca2+-related channel InsP3R mediated IRE1α-KD-induced [Ca2+]c increases, thereby leading to cell death. Previously, abnormal Ca2+ release from InsP3R has been suggested to

Figure 7 Proposed model of cell death in IRE1α-KD cells. Reduced IREx appeared to induce cell death through an accelerated ER-to-cytosolic efflux of calcium through InsP3R, followed by mitochondrial dysfunction and calpain-activated pathway.
act as an important apoptotic signal. Here, we found that treatment with InsP3R blockers inhibited [Ca2+]i, increases and cell death in the IRE1a-KD cells. In addition, treatment of SH-SY5Y cells with the InsP3R agonist adenosphin A caused significant cell death, whereas co-treatment with BPAT-A decreased cell death (Supplementary Figures S4a and b). These results suggest that enhanced InsP3R-mediated Ca2+ release may induce cell death in a Ca2+-dependent manner.

We explored the underlying mechanism of IRE1a-KD-induced InsP3R activation by co-IP and found that IRE1a did not interact with InsP3R directly. In previous studies of InsP3R’s binding partners, CIB1 binding to InsP3R inhibited Ca2+ release from InsP3R. CIB1 is thought to function as a Ca2+-sensitive modulator by interacting directly with ASK1. We tested the association between opening of InsP3R and the CIB1-ASK1 interaction under IRE1a-KD conditions and found that IRE1a KD increased the CIB1-ASK1 interaction but reduced the CIB1-InsP3R interaction, which in turn resulted in increased Ca2+ release from InsP3R. There are previous studies that IRE1a-ASK1 pathway mediates cell death under pathological conditions. In contrast, we focused on the role of IRE1a itself under normal condition. We compared with control siRNA-transfected cells and IRE1a siRNA-transfected cells without any stimuli. We found first that IRE1a regulates Ca2+ homeostasis in the ER by trapping ASK1. The downregulation of IRE1a increased the increased ASK1-CIB1 interaction through the decreased IRE1a-ASK1 interaction, resulting in the reduction of inhibitory roles of CIB1 in Ca2+ release through IP3R. Consistently, CIB1 KD increased [Ca2+]i, likely through reduced interaction between CIB1 and InsP3R and thus induced cell death (Figures 5d and e).

Mitochondria are the intracellular organelles associated with Ca2+ handling. Mitochondrial Ca2+ uptake regulates intracellular Ca2+ signaling and cell survival by buffering cytosolic Ca2+ levels. Previous studies have shown that Ca2+ accumulation in mitochondria induced apoptotic cell death through Ca2+-induced MPT opening. As IRE1a KD induced [Ca2+]i increases and cell death, we focused on mitochondrial alterations, including abnormal mitochondrial fission and reduced mitochondrial functions in the IRE1a-KD cells. In addition, IRE1a KD increased the levels of ROS, a well-known cell death-inducing factor. Ca2+ accumulation in mitochondria occurs via the MCU across a steep electrochemical gradient. Treatment of IRE1a-KD cells with MCU blockers inhibited cell death, indicating that Ca2+ accumulation in mitochondria may act as a main apoptotic factor in the IRE1a-KD cells. We also found that ROS scavengers reduced cell death in the IRE1a-KD cells. Based on the finding that treatment of the IRE1a-KD cells with 2-APB reduced ROS generation (Figure 6d), we suggest that IRE1a-KD-induced [Ca2+]i,inito accumulation caused increased ROS generation and eventually induced apoptotic cell death. Notably, IRE1a-KD-induced cell death was also mediated, at least in part, by calpain activation (Supplementary Figures S5a–d and Figure 7). Treatment of the IRE1a-KD cells with a calpain inhibitor blocked cell death induced by [Ca2+]i upregulation. These results are consistent with previous studies showing that excessive Ca2+ binds and activates Ca2+-dependent enzymes, such as calpain, thereby activating caspase-12 and triggering the apoptotic pathway.

Surprisingly, unlike IRE1a, knockdown of the other two ER stress transducers, PERK and ATF6a, did not lead to [Ca2+]i increases or cell death. In conclusion, reduced expression of the ER stress transducer IRE1a induced ER stress and caused cell death by accelerating ER Ca2+ release via InsP3R. InsP3R-induced ER Ca2+ release in the IRE1a-KD cells caused cell death via prolonged mitochondrial Ca2+ accumulation and alterations in ER morphology and function (Figure 7).

Materials and Methods

Cell cultures, transfection, and drug treatment. Human neuroblastoma SH-SY5Y cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; HyClone, Irvine, CA, USA) supplemented with 10% fetal bovine serum (HyClone) and an antibiotic mixture of penicillin (100 U/ml) and streptomycin (100 μg/ml). The control siRNA (sc-37007) and siRNA against IRE1a (sc-40705 and 1171247), PERK (1046373), ATF6a (1009444), and CIB1 (sc-43271) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and/or Bioneer Inc. (Daejeon, Korea). Cy3-tagged IRE1a siRNA was made by Bioneer, Inc. and IRE1a cDNA was purchased from Addgene (ID:20744; Cambridge, MA, USA). Cells were cultured for 48 h after transfection with Lipofectamine for cDNA and siRNA for mRNA according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA) and treated with vehicle or appropriate concentrations of TUDCA (T0266), tunicamycin (T7785), taspigen (T9033), BPA-MA (A1078), CCRP (C2759), dantrolene (D9175), 2-APB (D9754), N-acetyl-l-cysteine (NAC) (A7250), L-NG-nitroarginine methyl ester (N5751), CsA (30024), and Calpain Inhibitor I (A6185) from Sigma-Aldrich (St. Louis, MO, USA); Xestospongin C (sc-201505) and Ru362 (sc-222265) from Santa Cruz Biotechnology; DEVK-in (S30378) from BD Biosciences (Franklin Lakes, NJ, USA); I-ter-buthyl-5-naphthalen-1-ylmethyle-1H-pyrazolo[1,5-d]-pyrimidin-4-ylmine (1MU-PPI) (13330) from Cayman Chemical (Ann Arbor, MI, USA); and adenosphin A (115500) from Calbiochem (San Diego, CA, USA). Sequences were as follows: siRNA against IRE1a (1171247) 5'-CUCGCUAUAUGACGCUAC-3' (sense), 5'-GUAGCUAGAUAGACGAC-3' (antisense); siRNA against PERK (104637) 5'-GAGAACAAAGAGAAGACGAC-3' (antisense), 5'-GUAGCUUCUGUGUCACGUGUA-3' (antisense); and siRNA against ATF-6a (1009444) 5'-CAGAGAAGACGCUACGAC-3' (sense), 5'-AGUACCUAGAGACGUGCU-3' (antisense).

Antibodies. Cell pellets were prepared as described and western blotted with the following antibodies: anti-IRE1a (ab287073; 1:1500) from Abcam (Cambridge, MA, USA); anti-Jun-Jun (A1578; 1:5000) from Sigma-Aldrich; anti-PERK (sc-13073; 1:1000), anti-ATF6a (sc-22799; 1:1000), and anti-GAD513 (sc-575; 1:1000), anti-ASK1 (sc-7931 and sc-5294; 1:1000 for WB, 1:100 for IP and PLA), anti-caspase-12 (sc-70227; 1:1000), and anti-GRP78 (sc-1050; 1:1000), anti-TRAF2 (sc-7346; 1:1000), and anti-calpain (sc-7530; 1:1000) from Santa Cruz Biotechnology; anti-ryanodine receptor (MAb196; 1:1000); Thermo Scientific, Hudson, NH, USA); anti-InsP3R (07-1210; 1:2000 for WB, 1:300 for IP, 1:100 for PLA and anti-CIB1 (MAb2601; 1:1500 for WB, 1:500 for IP, 1:100 for PLA) (Millipore, Schwabach, Germany); and anti-calreticulin (2891; 1:2000), anti-calsequestrin (2433; 1:2000), anti-cleaved caspase-9 (9501; 1:2000), anti-caspase-9 (9502; 1:2000), and anti-caspase-3 (9662; 1:2000) (Cell Signaling Technology, Beverly, MA, USA). Immune reactive bands were photographed and quantified on LAS-3000 with MultiGauge (Fuji Film Inc., Tokyo, Japan).

Live and dead cell assay. To measure cell viability, calcein-AM, MTT, and TUNEL assays were performed. The calcein-AM assay was performed according to the manufacturer’s instructions (C3099, LIVE/DEAD Viability and Cytotoxicity Kit; Molecular Probes, Invitrogen, Carlsbad, CA, USA). Briefly, 5 x 104 cells were incubated for 24 h after seeding in 96-well plate, and then transfected with 20-50 μl siRNA for 24–48 h. Treatments were administered after transfection at optimal dose (see figure legends). Calcein-AM reagent in phenol red-free media (1:1000) was added, incubated for 1 h at 37 °C, and washed three times with PBS. Fluorescence was measured at excitation and emission wavelengths (ex/em) of 485 nm/530 nm on a fluorescence plate reader (Infinite M200 Pro; TECAN, Männedorf, Switzerland). The MTT assay was performed as described. Briefly, after transfection and drug treatment, 2.5 mg/ml MTT (M2003, Sigma-Aldrich) in phenol red-free medium was
added and incubated for 2 h at 37 °C, followed by aspiration of the MT solution, addition of isopropanol to dissolve the formazan crystals, and incubation at 37 °C for 1 h. Absorbance was measured at 540 nm. Experiments were independently repeated at least three times, and data were expressed as a percentage of the control (control siRNA-transfected or vehicle-treated cells). The TUNEL assay (G7361; Promega, Madison, WI, USA) was performed according to the manufacturer’s protocol. Cells (1 × 10^5) were incubated for 24 h after seeding in 96-well plates and transsected with control or IRE1α siRNA for 4 h. TUNEL-positive cells were counted under a fluorescence microscope (Olympus, Tokyo, Japan) and expressed as the percentage of apoptotic cells relative to counted cells (n = 500) in 96 wells.

ROS measurement. Hydrogen peroxide levels were determined using DCFDA (C6827; Invitrogen). In brief, treated cells were incubated with 1 μM DCFDA for 30 min and washed with PBS. Fluorescent signals were captured using a fluorescence microscope. Changes in mitochondrial oxidation product were measured using MitoSOX Red staining (5 μM for 15 min at 37 °C; M36008; Invitrogen), according to the manufacturer’s instructions.

Mitochondrial membrane potential measurement. In depolarized cells, mitochondrial labeling with potential-indicating probes like TMRM disappears; therefore, red fluorescence serves as an indicator of mitochondrial membrane potential. The medium was replaced with phenol red-free medium containing 500 mM TMRM (100 μM well; T-668; Invitrogen). Plates were incubated for 1 h at 37 °C and washed three times with PBS (50 μM well). Fluorescent signals were captured using a fluorescence microscope (Olympus), and analyzed in >500 cells per group.

Morphology of mitochondria. Mitochondria were visualized after the expression of MiTo-DeRed (DeRed2 fused to the mitochondrial targeting sequence from subunit VIII of human cytochrome c oxidase). Images were captured under a confocal laser scanning microscope (FV10-I-w; Olympus), and analyzed in 100 cells per group with the ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Fluo-4 fluorescence imaging for [Ca^{2+}]_i measurement. For intracellular Ca^{2+} imaging, cells were loaded with the Ca^{2+}-sensitive dye fluo-4-acetoxyethyl ester (Fluo-4 AM; 5 μM; F10471; Invitrogen) at 37 °C for 60 min and washed with PBS to remove extracellular Fluor-4 AM. After drug treatment, fluorescent signals were captured using a fluorescence microscope. The fluorescence intensity reflected [Ca^{2+}]_i. Images for 1000 cells per group were analyzed with the ImageJ software.

Fura-2 intracellular calcium imaging. Cytosolic calcium levels ([Ca^{2+}]_c) were assessed by ratiometric analysis using fura-2 acetoxyethyl ester (Fura-2 AM; F1221; Molecular Probes). Fura-2 AM was applied in the perfusion system throughout the imaging process. SH-SY5Y cells plated on polystyrene-coated coverslips were loaded with Fura-2 AM (2 μM) for 30 min in Normal Tyrode’s solution (140 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM glucose, and 10 mM HEPES, pH 7.35), supplemented with 0.01% pluronic acid. Imaging was performed using an inverted microscope (Nikon Ti, Tokyo, Japan) with a ×40 UV objective lens (Nikon, Tokyo, Japan). Fura-2 AM was excited by sequential illuminations at 340 and 380 nm from a Lambda DG-4 illumination system (Sutter, Novato, CA, USA). Image processing was controlled by the Axon Imaging Workbench software 6.0 (AIW; Union City, CA, USA). Emission was detected at a wavelength of 510 nm. Fura-2 emission ratios following excitation at 340 and 380 nm were processed by AIW. Video images were obtained using an intensified CCD Workbench software 6.0 (AIW; Union City, CA, USA). Image processing was controlled by the Axon Imaging Workbench software 6.0 (AIW; Union City, CA, USA).

Conflict of Interest
The authors declare no conflict of interest.

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Author contributions
SMS wrote the manuscript and researched data. JB researched data. S-ER, SJK researched Ca^{2+} data. IM-J supervised the study and reviewed and edited the manuscript.

1. Meldolesi J, Pozzan T. The endoplasmic reticulum Ca^{2+} store: a view from the lumen. Trends Biochem Sci 1998; 23: 10–14.
2. Ma Y, Hendershot LM. The unfolding tale of the unfolded protein response. Cell 2001; 107: 827–830.
3. Kaufman RJ. Orchestrating the unfolded protein response in health and disease. J Clin Invest 2002; 110: 1389–1398.
4. Ron D, Walter P. Signal integration in the endoplasmic reticulum unfolded protein response. Nat Rev Mol Cell Bio 2007; 8: 519–529.
5. Schroder M, Kaufman RJ. The mammalian unfolded protein response. Annu Rev Biochem 2005; 74: 739–789.
6. Shamu CE, Walter P. Oligomerization and phosphorylation of the Ire1p kinase during intracellular signaling from the endoplasmic reticulum to the nucleus. EMBO J 1996; 15: 3028–3039.
7. Yoshida H, Matsui T, Yamamoto T, Okada T, Mori K. XBPl mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. Cell 2001; 107: 681–691.
8. Urano F, Wang X, Bertolotti A, Zhang Y, Chung P, Harding HP et al. Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. Science 2000; 287: 664–666.

9. Nishitoh H, Matsuzawa A, Tobiume K, Saegusa K, Takeda K, Inoue K et al. ASK1 is essential for endoplasmic reticulum stress-induced neuronal cell death triggered by expanded polyglutamine repeats. Genes Dev 2002; 16: 1345–1355.

10. Harding HP, Zhang Y, Ron D. Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase. Nature 1999; 397: 271–274.

11. Rützlski DT, Kaufman RJ. All roads lead to ATF4. Dev Cell 2003; 4: 442–444.

12. Ye J, Rawson RB, Komuro R, Chen X, Dave UP, Prywes R et al. ER stress induces cleavage of membrane-bound ATF6 by the same proteases that process SREBPs. Mol Cell 2000; 6: 1355–1364.

13. Carafoli E. Calcium signaling: a tale for all seasons. Proc Natl Acad Sci USA 2002; 99: 1115–1222.

14. Gorlach A, Klapa P, Kietzmann T. The endoplasmic reticulum: folding, calcium homeostasis, signaling, and redox control. Antioxid Redox Signal 2006; 8: 1391–1418.

15. Beprzo-przyni I. The inositol 1,4,5-trisphosphate receptors. Cell Calcium 2005; 38: 261–272.

16. Rossi D, Sorrentino V. Molecular genetics of ryanodine receptors Ca\(^2\)+ release channels. Cell Calcium 2002; 32: 307–313.

17. East JM. Sarco(endoplasmic reticulum calcium pumps: recent advances in our understanding of structure/function and biology (review). Mol Membr Biol 2000; 17: 189–200.

18. Squer MJ, Sehnert AJ, Sellins CS, Malkinson AM, Taekore E, Cohen JJ. Calpain and Ca2\(+\) homeostasis, signaling, and redox control. Antioxid Redox Signal 2006; 8: 1391–1418.

19. Wang HJ, Pathan N, Eihel M, Krajewska S, Yamaguchi Y, Shibasaki F et al. Ca2\(+\)-induced apoptosis through calcinurin dephosphorylation of BAD. Science 1999; 284: 339–343.

20. Nakagawa T, Zhu H, Morishima N, Li E, Xu J, Yankner BA et al. Tauroursodeoxycholic acid on endoplasmic reticulum stress-induced caspase-12 activation. J Neurochem 2008; 107: 1738–1749.

21. Kim, KU, Xu W, Reed JC. Cell death and endoplasmic reticulum stress: disease relevance and therapeutic opportunities. Nat Rev Drug Discov 2007; 6: 1013–1030.

22. Hennings JK, Burhner N, Stahler F, WINNIG M, BARTZ M, MELKERT W et al. Sweet taste receptor activation induces mitochondrial apoptosis. J Cell Physiol 1999; 178: 311–319.

23. Yao M, Desai S, Garcia-Perez C, Saotome M, Sinha Roy S et al. Mitochondrial calcium signalling and cell death: approaches for assessing the role of mitochondrial Ca2\(+\) uptake in apoptosis. Cell Calcium 2006; 40: 553–560.

24. D. Lee, J. BENDER, E. Kadenbach B. Control of mitochondrial membrane potential and ROS formation by reversible phosphorylation of cytochrome c oxidase. Mol Cell Biochem 2003; 213–223.

25. Hansson MJ, Mansson R, Mattisson G, Ohlsson J, Karlsson J, Keep MF et al. Brain-derived relaxing factor, a vasodilator, suppresses microglial oxidative stress. J Neurochem 2004; 89: 715–729.

26. Lee H, Noh JH, Oh Y, Kim Y, Chung JW, CHUNG CW et al. IRE1 plays an essential role in ER stress-mediated aggregation of mutant huntingtin via the inhibition of autophagy flux. Hum Mol Genet 2012; 21: 101–114.

27. De SIMONI, L. LINARD, D. HERMANNS, E. KOOPS, B. GOEMARES, J. M. MITCHELL, P. PEROXISOMAL-5 as potential modulator of mitochondrial calcium-ER crosstalk in MPP(-) induced cell death. J Neurochem 2012; 125: 473–485.

28. Jayaraman T, Marks AR. T cells deficient in inositol 1,4,5-trisphosphate receptor are resistant to apoptosis. Mol Cell Biol 1997; 17: 3005–3012.

29. Jayaraman T, Marks AR. Calcinurin is downstream of the inositol 1,4,5-trisphosphate receptor in the apoptotic and cell growth pathways. J Biol Chem 2000; 275: 6037–6042.

30. Rizzuto R, De Stefani D, Raffaello A, Mammucari C. Mitochondria as sensors and effectors of calcium waves. J Cell Sci 2001; 114: 2249–2252.

31. Tampon W, Weihlinda AA, Kaufman RJ. An stress response pathway from the endoplasmic reticulum to the nucleus requires a novel bifunctional protein kinase for endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. Nature 2000; 403: 98–103.

32. Kim, XU, WU, REED JC. Cell death and endoplasmic reticulum stress: disease relevance and therapeutic opportunities. Nat Rev Drug Discov 2007; 6: 1013–1030.

33. Supattapone S, Worley PF, Baraban JM, Ferguson JM, Sholapurkar, purification, and characterization of an inositol triphosphate receptor. J Biol Chem 1988; 263: 1530–1534.

34. Ellisman MH, Debnick T, Ouyang Y, BECK CF, TANKSELY SJ, WALTON PD et al. Identification and localization of ryanodine binding proteins in the avian central nervous system. Neuron 1990; 5: 135–145.

35. Guntinas-Lrim AM, Grace J, SHELL GE. A novel Ca2\(+\) pump expressed in brain, kidney, and stomach is encoded by an alternative transcript of the slow-twitch muscle sarcoplasmic reticulum Ca2\(+\) ATPase gene. Identification of cDNAs encoding Ca2\(+\) and other contract-transporting ATPases using an oligonucleotide probe derived from the ATP-binding site. J Biol Chem 1998; 263: 15032–15040.