IRE1–XBP1 pathway regulates oxidative proinsulin folding in pancreatic β cells

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In mammalian pancreatic β cells, the IRE1α–XBP1 pathway is constitutively and highly activated under physiological conditions. To elucidate the precise role of this pathway, we constructed β cell–specific Ire1α conditional knockout (CKO) mice and established insulinoma cell lines in which ire1α was deleted using the Cre–loxP system. ire1α CKO mice showed the typical diabetic phenotype including impaired glycemic control and defects in insulin biosynthesis postnatally at 4–20 weeks. Ire1α deletion in pancreatic β cells in mice and insulinoma cells resulted in decreased insulin secretion, decreased insulin and proinsulin contents in cells, and decreased oxidative folding of proinsulin along with decreased expression of five protein disulfide isomerase (PDIs): PDI, PDIR, P5, ERp44, and ERp46. Reconstitution of the IRE1α–XBP1 pathway restored the proinsulin and insulin contents, insulin secretion, and expression of the five PDIs, indicating that IRE1α functions as a key regulator of the induction of catalysts for the oxidative folding of proinsulin in pancreatic β cells.

Introduction

ER stress and the unfolded protein response (UPR) have important implications for cellular functions and are linked to various human diseases including diabetes; accordingly, a detailed knowledge of these processes is critical. In metazoans, three principal ER stress sensors activate the UPR, i.e., PKR-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol requiring 1 (IRE1; Rutkowski and Hegde, 2010; Kimata and Kohno, 2011). Among them, IRE1 is the mostly highly conserved from yeast to humans (Mori, 2009). Mammalian genomes encode two IRE1 paralogs, IRE1α and IRE1β. Whereas IRE1β is specifically expressed in digestive tissues such as the intestine and stomach, IRE1α is ubiquitously expressed (Bertolotti et al., 2001; Tsuru et al., 2013). Upon ER stress, IRE1α forms a dimer/oligomer for the trans-autophosphorylation and activation of its RNase domain (Kimata et al., 2007; Li et al., 2010). The activated IRE1α RNase domain then cleaves the unspliced form of X-box–binding protein 1 (XBP1u) mRNA to initiate the splicing of XBP1u mRNA on the ER membrane, leading to formation of the spliced form of XBP1 (XBP1s; Yoshida et al., 2001; Calfon et al., 2002; Yanagitani et al., 2011). This splicing reaction creates a translational frameshift to produce functional XBP1s and a transcriptional activator that up-regulates genes encoding ER-associated degradation machinery proteins, ER chaperones, lipid synthesis enzymes, glycosylation enzymes, and secretory machinery proteins (Yoshida et al., 2003; Ron and Walter, 2007).

Pancreatic β cells synthesize and secrete a large amount of insulin in response to high–blood glucose concentrations. Accumulating evidence suggests that insulin production causes physiological ER stress and the activation of the UPR in pancreatic β cells (Hassler et al., 2015; Sharma et al., 2015; Szabat et al., 2016). Because some mutations in PERK are related to Wolcott–Rallison syndrome, the role of PERK in pancreatic β cells is well characterized (Harding et al., 2001, 2012). However, the role of IRE1α in pancreatic β cells, especially in the biosynthesis of insulin, is not fully understood (Lipson et al., 2006; Han et al., 2009).

Insulin is secreted from pancreatic β cells by regulated exocytosis in high–blood glucose conditions, and it is synthesized as preproinsulin from Ins genes. Preproinsulin is targeted to the ER membrane and, upon translocation, is processed to proinsulin. Proinsulin is folded via three disulfide bonds into its native structure (Weiss, 2009). Disulfide bond formation in the ER is catalyzed by protein disulfide isomerase (PDI) family proteins.
Mammals have at least 20 PDI family proteins (hereafter referred to as PDIs; Braakman and Bulleid, 2011; Okumura et al., 2015). The knockdown of PDI family genes results in decreased secretion of specific secretory proteins (Wang et al., 2007, 2015). However, it is not clear which PDI family proteins facilitate proinsulin folding.

To elucidate the physiological significance of the constitutive activation of the IRE1α–XBP1 pathway in pancreatic β cells, we established pancreatic β cell–specific Ire1α conditional knockout (CKO; Ire1αβ(fl/ΔR)) mice and insulinoma cells, MIN6 (Ire1αβ(fl)), harboring floxed Ire1α derived from Ire1αβ(fl); IT-6 mice. Using these experimental models, we uncovered a new role of the Ire1α–XBP1 pathway in pancreatic β cells.

Results

Physiological activation of the Ire1α–XBP1 pathway in pancreatic β cells

Accumulating evidence suggests that the Ire1α–XBP1 pathway plays important roles in the pancreas at the neonatal and postnatal stages (Iwawaki et al., 2004; Lee et al., 2005, 2011). To directly observe the activation of the Ire1α–XBP1 pathway in adult mouse tissues, we used RT-PCR to examine the ratio of the spliced form of Xbp1 mRNA to total Xbp1 mRNA. In this analysis, Xbp1 mRNA splicing was slightly higher in the pancreas than in other mouse tissues (Fig. 1A). Consistent with the level of Xbp1 mRNA splicing, both Ire1α and ER resident proteins harboring the KDEL motif (e.g., ER folding enzymes such as immunoglobulin heavy chain binding protein [BiP], GRP94, and PDI) exhibited higher expression in the pancreas of mice than chaperones localized in other cell compartments, including HSP90 in the cytosol and HSP60 in the mitochondria (Fig. 1B).

The pancreas has two major types of secretory cells: exocrine acinar cells and endocrine islet cells. We observed moderate Xbp1 mRNA splicing in pancreatic acinar cells but extremely high splicing in pancreatic islets (Fig. 1A). β-Cells occupy ~70% of pancreatic islets in mice (Pechhold et al., 2009) and humans (Wang et al., 2013). In addition, Xbp1s protein is highly expressed in pancreatic islets in mice and humans (Engin et al., 2013, 2014). Collectively, these findings suggest that the Ire1α–XBP1 pathway is constitutively and highly activated under physiological conditions in pancreatic β cells in mammals.

Impaired glycemic control and defective insulin biosynthesis in Ire1αβ(fl/ΔR) mice

To elucidate the role of the Ire1α–XBP1 pathway in pancreatic β cells, we generated pancreatic β cell–specific Ire1α CKO (Ire1αβ(fl/ΔR)) mice by crossing Ins-Cre mice (Herrera, 2000) with Ire1αβ(fl) mice (Iwawaki et al., 2009, 2010). The Ire1αβ(fl/ΔR) mice exhibited steady increases in blood glucose beginning at 4 wk, whereas glucose levels in control (Ire1αβ(fl/ΔR)) mice did not increase (Fig. 2A). In addition, the Ire1αβ(fl/ΔR) mice showed more significant decreases in glucose tolerance than control Ire1αβ(fl/ΔR) mice at 12 wk (Fig. 2B). To understand the mechanism underlying the diabetic phenotype in Ire1αβ(fl/ΔR) mice, we examined serum insulin levels. The Ire1αβ(fl/ΔR) mice exhibited significantly lower serum insulin levels at 20 wk of age than Ire1αβ(fl/ΔR) mice (Fig. 2C). Consistent with this result, the intracellular contents of proinsulin and insulin were significantly decreased in the islets of Ire1αβ(fl/ΔR) mice (Fig. 2, D and E), which explains the diabetic phenotype of Ire1αβ(fl/ΔR) mice. However, the diabetic phenotype of Ire1αβ(fl/ΔR) mice was not associated with changes in the size or number of pancreatic islets within 20 wk (Fig. S1, A–C). These findings indicate that Ire1αβ(fl/ΔR) mice exhibited declined insulin secretion owing to a decreased capacity for insulin production and not a decreased number of islets or size of pancreatic β cells. Thus, these results suggest that the Ire1α–XBP1 pathway plays an important role in insulin production.

Establishment of a model cell line to study the role of Ire1α in pancreatic β cells

Ire1αβ(fl/ΔR) mice showed a typical diabetic phenotype with hypoinsulinemia and hyperglycemia (Fig. 2). We examined the mechanism underlying defective insulin biosynthesis in Ire1αβ(fl/ΔR) mice using a MIN6 cell line carrying a floxed Ire1α-RNase domain from a mouse obtained by crossing an IT-6 mouse expressing SV40 large T antigen under the control of the INS promoter (Miyazaki et al., 1990, 2010) with an Ire1αβ(fl) mouse. This cell line was named MIN6 (Ire1αβ(fl)) (Fig. S2 A). We easily deleted the Ire1α-RNase domain from this model pancreatic β cell line by infecting the cells with adenovirus harboring Cre recombinase (Ad-Cre) referred to as Ad-Cre, MIN6 (Ire1αβ(fl/ΔR)) (Fig. S2 B). After infection, cells were subjected to genotyping PCR and immunoblotting with an antibody against Ire1α. The results clearly showed that the Ire1α-RNase domain was almost completely deleted from MIN6
(Ire1α<sup>fl/fl</sup>) cells after infection with Ad-Cre (Figs. 3 A and S2 C). Importantly, Xbp1 mRNA splicing was completely inhibited in Ad-Cre, MIN6 (Ire1α<sup>ΔR/ΔR</sup>) cells (Fig. 3, A and B).

Consistent with the results obtained for the islets of Ire1α<sup>B(+/ΔR)</sup> mice, the intracellular contents of proinsulin and insulin in MIN6 (Ire1α<sup>ΔR/ΔR</sup>) cells were remarkably decreased compared with those of control cells (Con, MIN6 (Ire1α<sup>ΔR/ΔR</sup>) cells; adenovirus harboring GFP [Ad-GFP], MIN6 (Ire1α<sup>ΔR/ΔR</sup>) cells; see Materials and methods; Fig. 3, A, C and D). Furthermore, insulin secretion from MIN6 (Ire1α<sup>ΔR/ΔR</sup>) cells was dramatically decreased compared with that from MIN6 (Ire1α<sup>ΔR/ΔR</sup>) cells (Fig. 3 E). Thus, MIN6 (Ire1α<sup>ΔR/ΔR</sup>) cells retain the basic characteristics of the islets of Ire1α<sup>B(+/ΔR)</sup> mice. Based on these findings, we used MIN6 (Ire1α<sup>fl/fl</sup>) and MIN6 (Ire1α<sup>ΔR/ΔR</sup>) cells as model cell lines to study the role of IRE1α in pancreatic β cells.

**Morphology of insulin granules and the ER in MIN6 (Ire1α<sup>ΔR/ΔR</sup>) cells**

The insulin granule is the site of insulin maturation and storage before secretion from β cells. Because intracellular proinsulin and insulin contents were reduced in MIN6 (Ire1α<sup>ΔR/ΔR</sup>) cells compared with control cells (Fig. 3, A, C, and D), we examined the number and size of insulin granules in MIN6 (Ire1α<sup>ΔR/ΔR</sup>) cells by electron microscopy. The number of insulin granules was 27% lower in MIN6 (Ire1α<sup>ΔR/ΔR</sup>) cells than in MIN6 (Ire1α<sup>ΔR/ΔR</sup>) cells (Fig. 3, F–H). Furthermore, the size of insulin granules was significantly decreased in MIN6 (Ire1α<sup>ΔR/ΔR</sup>) cells (Fig. 3, F, G, and I).

These results are consistent with the decreased insulin content in MIN6 (Ire1α<sup>ΔR/ΔR</sup>) cells.

Owing to their roles in ER homeostasis, deletion of ER stress sensors often results in abnormal ER morphology (Harding et al., 2001; Tsuru et al., 2013; Hassler et al., 2015). Thus, we expected that the deletion of one of the three ER stress sensors in secretory cells might result in increased ER stress and activation of other ER stress sensors such as PERK and ATF6. To test this possibility, we examined whether PERK and ATF6 were more highly activated in MIN6 (Ire1α<sup>ΔR/ΔR</sup>) cells. Upon ER stress, PERK phosphorylates IRE1α and elf2α (Harding et al., 2001). Thus, to examine the activation of PERK, we quantified the phosphorylation of these proteins using antibodies that detect the phosphorylated forms. Levels of phosphorylated PERK and elf2α were significantly higher in MIN6 (Ire1α<sup>ΔR/ΔR</sup>) cells than in control cells (Fig. S3, A–C), indicating that PERK is more highly activated in MIN6 (Ire1α<sup>ΔR/ΔR</sup>) cells. Mammals have
two paralogs of ATF6: ATF6α and ATF6β. In response to ER stress, ATF6α and ATF6β are transported to the Golgi apparatus, where they are cleaved to release their cytosolic domain (Yamamoto et al., 2007). The level of cleaved ATF6α was slightly increased in MIN6 (Ire1αΔR/ΔR) cells (Fig. S3, D and E), indicating that ATF6α was slightly more activated in MIN6 (Ire1αΔR/ΔR) cells than in control cells. In contrast, the level of cleaved ATF6β was not significantly elevated in MIN6 (Ire1αΔR/ΔR) cells (Fig. S3, D and F). These results indicate that the deletion of the RNase domain of Ire1α causes the activation of PERK and ATF6α in MIN6 (Ire1αΔR/ΔR) cells. It has been reported that the constitutive activation of PERK and ATF6α causes CCAAT enhancer–binding protein (CHOP) induction (Arensdorf et al., 2013). Chop mRNA expression was slightly higher in MIN6 (Ire1αΔR/ΔR) cells than in control cells (Fig. S3 G). In pancreatic β cells, increased CHOP activity causes cell toxicity, e.g., sustained ER stress, oxidative stress, and apoptosis (Tabas and Ron, 2011). We examined whether the activation of PERK and ATF6α causes the decreased insulin secretion in MIN6 (Ire1αΔR/ΔR) cells. Treatment with a PERK kinase inhibitor, GSK 2606414 (Harding et al., 2012), and an ATF6α inhibitor, 4-(2-aminoethyl) benzylsulfonyl fluoride (AEBSF; Okada et al., 2003), did not restore insulin secretion in MIN6 (Ire1αΔR/ΔR) cells (Fig. S3 H). Thus, the activation of PERK and ATF6α is not the main factor explaining the decreased insulin secretion in MIN6 (Ire1αΔR/ΔR) cells.

Insulin mRNA levels are not altered in MIN6 (Ire1αΔR/ΔR) cells

The level of insulin secretion and cellular contents of proinsulin and insulin were substantially lower in Ad-Cre, MIN6 (Ire1αΔR/ΔR) cells than in control cells (Fig. 3, A and C–E). However, these decreases were not caused by activation of PERK and ATF6 (Fig. S3 H), suggesting that efficient insulin biosynthesis requires the Ire1α-XBP1 pathway. To understand the role of Ire1α in the biosynthesis of insulin, we first examined the effect of the deletion of the Ire1α-RNase domain on the transcription of Ins genes. Mice have two Ins paralogs: Ins1 and Ins2. Ins genes are up-regulated
by various transcription factors such as Pdx1, MafA, and Beta2 (Han et al., 2011). To determine whether IRE1α is involved in Ins gene transcription in pancreatic β cells, we examined the effect of IRE1α–XBP1 pathway on the expression levels of Ins genes and related transcription factors in MIN6 cells. The expression levels of Ins1 and Ins2 were not decreased in MIN6 (Ire1αΔR/ΔR) cells (Fig. 4A). Moreover, the expression levels of genes encoding transcription factors that regulate Ins genes were not decreased in MIN6 (Ire1αΔR/ΔR) cells (Fig. 4A). These findings indicate that the IRE1α–XBP1 pathway is not involved in the regulation of Ins gene transcription, at least under the tested conditions, in pancreatic β cells.

**Translation and translocation of preproinsulin in MIN6 (Ire1αΔR/ΔR) cells**

Next, we examined the role of the IRE1α–XBP1 pathway in the translation and translocation of insulin in pancreatic β cells. To examine the translation of Ins mRNA, MIN6 cells were labeled with [35S]Met/Cys for 30 min and subjected to immunoprecipitation (Fig. 4B). Preproinsulin and proinsulin levels did not differ between MIN6 (Ire1αfl/fl) cells and MIN6 (Ire1αΔR/ΔR) cells (Fig. 4B), indicating that the IRE1α–XBP1 pathway is not required for efficient translation of Ins mRNAs. To examine the translocation of preproinsulin into the ER, cells were pulse-labeled for 1 min, chased, and subjected to immunoprecipitation. During or immediately after translocation into the ER, the signal sequence of preproinsulin is processed to form proinsulin. As expected, the majority of preproinsulin was converted to proinsulin during the chase period. The processing rate of the signal sequence of preproinsulin did not differ between MIN6 (Ire1αΔR/ΔR) cells and MIN6 (Ire1αfl/fl) cells (Fig. 4C). Thus, the IRE1α–XBP1 pathway is not required for the efficient translocation of preproinsulin into the ER.

**Detection of mixed-disulfide complexes involving proinsulin**

The formation of disulfide bridges between the correct pairs of cysteines, which is presumably mediated by PDI family members, is vital for the folding of most secretory proteins with disulfide bonds. The formation of disulfide bridges between the correct pairs of cysteines, which is presumably mediated by PDI family members, is vital for the folding of most secretory proteins with disulfide bonds.
in MIN6 (Ire1αΔR/ΔR) cells. From these findings, we concluded that oxidative folding of proinsulin was significantly decreased in MIN6 (Ire1αΔR/ΔR) cells and that oxidative folding of proinsulin was significantly decreased in MIN6 (Ire1αΔR/ΔR) cells (Fig. S4 D). In contrast, among ER chaperones, decreased expression levels were observed only for Erdj4 and Orp150 (Fig. S4 C). We further examined the protein expression levels of ER chaperones and PDIs by immunoblotting. ORP150 expression was significantly lower in MIN6 (Ire1αΔR/ΔR) cells than in MIN6 (Ire1αΔR/ΔR) cells, and the levels of PDI/PDIA1, PDIR/PDIA5, P5/PDIA6, Erp44/PDIA10, and Erp46/PDIA15 were substantially lower in MIN6 (Ire1αΔR/ΔR) cells (Fig. 5, A and B). Erdj4 and PDip/PDIA2 were not detectable by immunoblotting in either MIN6 (Ire1αΔR) cells or MIN6 (Ire1αΔR/ΔR) cells (not depicted). Next, we examined the expression levels of the five PDIs and ORP150 in islets of Ire1αΔR/ΔR mice by immunoblotting. Consistent with the results in MIN6 (Ire1αΔR/ΔR) cells, the levels of PDI, PDIR, P5, Erp44, Erp46, and ORP150 were significantly and specifically lower in the pancreatic islets of Ire1αΔR/ΔR mice than in those of Ire1αΔ(+/ΔR) mice (Fig. 5, C and D). From these findings, we concluded that the Ire1α–XBP1 pathway is required for efficient induction of PDIs, PDIR, P5, Erp44, Erp46, and ORP150.

Insulin secretion and insulin content were restored by reconstitution of the Ire1α–XBP1 pathway in MIN6 (Ire1αΔR/ΔR) cells

To examine whether insulin secretion and insulin content could be restored by reconstituting the Ire1α–XBP1 pathway in MIN6 (Ire1αΔR/ΔR) cells, we stably expressed WT Ire1α or Xbp1s in the mutant MIN6 cells. As expected, the splicing of Xbp1s was restored by the expression of WT Ire1α or Xbp1s in MIN6 (Ire1αΔR/ΔR) cells (Fig. 6, A and B). Importantly, the stable expression of one of these genes also fully restored the intracellular contents of proinsulin and insulin (Fig. 6, A, C, and D) and moderately restored insulin secretion in high-glucose conditions (Fig. 6 E) in MIN6 (Ire1αΔR/ΔR) cells. Moreover, the expression levels of the five PDIs, i.e., PDI, PDIR, P5, Erp44, and Erp46, were also restored in MIN6 (Ire1αΔR/ΔR) cells stably expressing WT Ire1α or Xbp1s (Fig. 6, F and G). These findings clearly support the idea that the Ire1α–XBP1 pathway is responsible for the oxidative folding of proinsulin and the elevated expression of the five PDIs in pancreatic β cells.

XBP1s directly binds to the promoter regions of the five PDI family genes

XBP1s binds to the ACGT core sequence of the UPR element in the promoter regions of downstream target genes such as Hspa5 (BIP; Acosta-Alvear et al., 2007). The five PDI family genes also have ACGT core sequences in their promoter regions. To examine whether XBP1s directly binds to the promoter regions of the five PDI family genes, we performed a chromatin immunoprecipitation (chIP) assay using a nuclear extract from WT MIN6 cells. The PCR amplification of the chIP revealed that XBP1s directly binds to the promoter regions of the five PDI family genes (Fig. 7).

Detection of covalent interactions between PDIs and proinsulin

Based on the decreased expression of the five PDIs in MIN6 (Ire1αΔR/ΔR) cells and pancreatic islets of Ire1αΔR/ΔR mice, we speculated that some are involved in the proinsulin folding process. During oxidative folding, PDI family members form bonds. PDI family members catalyze the formation, isomerization, and reduction of disulfide bonds. During the reactions, PDI family members form disulfide-linked intermediates with their substrates. Despite their transience, the intermediates can be stabilized by treating cells directly with TCA to prevent post-harvest oxidation of cysteines and then alkylating the free cysteines with N-ethylmaleimide (NEM). In this experiment, we combined this method with pulse-chase experiments and used a high-titer antibody against newly synthesized proinsulin to follow the oxidative folding of proinsulin (Kadokura and Beckwith, 2009; Kadokura et al., 2013). WT MIN6 cells (Miyazaki et al., 1990, 2010) were labeled with [35S]Met/Cys for 5 min, chased for up to 60 min, and treated with TCA and subjected to immunoprecipitation with an antiproinsulin antibody. Immediately after the pulse, a small fraction of proinsulin was observed at the oxidized position (5.5 kD; Fig. S4 A, red arrowhead). In addition to this band, several bands with different molecular masses were observed from 15–250 kD (Fig. S4 A, black arrowheads). After a 15-min chase, appreciable portions of bands were converted to oxidized proinsulin (Fig. S4 A). To examine whether the bands observed from 15–250 kD indeed contained proinsulin, after pulse labeling, samples were analyzed by 2D gel electrophoresis in which the first dimension was nonreducing and the second was reducing. Appreciable portions of the bands observed from 15–250 kD in the first dimension (Fig. S4 B, black arrowheads) were shifted to the position corresponding with the reduced form of proinsulin (9 kD) in the second dimension (Fig. S4 B, white arrowhead). These bands represented mixed-disulfide complexes involving proinsulin. Some nonspecific bands showed resistance to β-mercaptoethanol treatment.

**Inefficient folding of proinsulin in MIN6 (Ire1αΔR/ΔR) cells**

To study the role of Ire1α in the oxidative folding of proinsulin, we monitored the folding status of proinsulin in MIN6 (Ire1αΔR/ΔR) cells by calculating the ratio of oxidized proinsulin (Fig. 4 D, Nonreducing, red arrowhead) to total proinsulin (Fig. 4 D, Reducing, white arrowhead; and Fig. 4 E). In MIN6 (Ire1αΔR/ΔR) cells, the ratio of folded proinsulin to total proinsulin increased during the chase. Importantly, in MIN6 (Ire1αΔR/ΔR) cells, the ratio of folded proinsulin to total proinsulin was significantly lower than that of control MIN6 cells (Fig. 4 E). From these findings, we concluded that oxidative folding of proinsulin was significantly decreased in MIN6 (Ire1αΔR/ΔR) cells.

**Expression levels of PDIs and ER chaperones in MIN6 (Ire1αΔR/ΔR) cells and islets of Ire1αΔ(+/ΔR) mice**

Oxidative folding of proinsulin takes place in the ER and is assisted by PDI family members and ER chaperones. However, the precise mechanisms of proinsulin folding, including the enzymes involved in this process, remain unclear. Because the oxidative folding of proinsulin was decreased in MIN6 (Ire1αΔR/ΔR) cells, we speculated that Ire1α up-regulates some enzymes and PDIs involved in proinsulin folding in pancreatic β cells. To identify such enzymes and chaperones, we compared the expression levels of ER chaperones and PDIs in MIN6 cells by quantitative real-time PCR (qRT-PCR; Fig. S4, C and D). Among PDI family proteins, the expression levels of Pdi, Pdir, Pdip, P5, Erp44, Erp46, and Erdj5/pdii were significantly lower in MIN6 (Ire1αΔR/ΔR) cells than in MIN6 (Ire1αΔR/ΔR) cells (Fig. S4 D).
transient disulfide-linked intermediates with their substrates (Kadokura et al., 2013). To test whether it is possible to detect covalent interactions between PDI family members and proinsulin, MIN6 cells were transfected with FLAG-tagged PDI, P5, ERp44, ERp46, and ERp72 (Araki et al., 2013). We detected specific bands with greater molecular masses than that of monomer proinsulin by nonreducing electrophoresis (Fig. 8 A and S4, A and B, black arrowheads). These bands represent disulfide-linked complexes formed between proinsulin and one of the PDI family members (PDI, P5, ERp44, or ERp46) as indicated by their conversion to the reduced form of monomer proinsulin after treatment with a reductant before electrophoresis (Fig. 8 A, white arrowhead). These findings indicate that PDI, P5, ERp44, ERp46, and ERp72 indeed interact with proinsulin via a disulfide bond at some point during the biosynthesis of proinsulin. Among the PDI family proteins tested in this experiment, PDI, P5, ERp44, and ERp46, which are downstream targets of the IRE1α-XBP1 pathway, are involved in disulfide formation of proinsulin. Compared with PDI, P5, ERp44, and ERp46, PDIR had relatively minor contribution to disulfide formation of proinsulin, at least in this experiment (Fig. 8 A). Next, we examined whether reconstitution of the five PDIs, i.e., PDI, PDIR, P5, ERp44, and ERp46, can restore insulin secretion in MIN6 (Ire1αΔR/ΔR) cells. In this experiment, we used Cys-dead (CM) PDI mutants (CM PDIs) as a negative control. When we transiently expressed the WT five PDIs in MIN6 (Ire1αΔR/ΔR) cells as shown in Fig. 8 B, insulin secretion was restored to a similar extent (∼50% compared with MIN6 (Ire1αfl/fl)) as the results obtained by the reconstitution of Ire1α in MIN6 (Ire1αΔR/ΔR) cells (∼55% compared with MIN6 (Ire1αfl/fl) cells in Fig. 6 E). It is important to note that overexpression of the WT five PDIs in MIN6 (Ire1αfl/fl) cells resulted in a 19% increase in insulin secretion, whereas overexpression of the only PDI/PDIA1 in MIN6 (Ire1αfl/fl) cells revealed a 13% decrease (Fig. 8 C). This result strongly supports our idea that the five PDIs participate in the proinsulin folding process in the ER.

**Discussion**

In this study, we presented several lines of evidence that the Ire1α-XBP1 pathway plays an important role in the efficient folding of proinsulin and transcriptional induction of the five PDI family proteins, which are critical for correct disulfide bond formation of proinsulin, in pancreatic β cells. We tried to detect both oxidized monomer proinsulin and mixed-disulfide complexes between proinsulin and PDI family proteins to monitor the accurate speed of oxidative folding of proinsulin by an improved method of pulse-chase experiments (see Materials and methods;
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IRE1α induces five PDIs for proinsulin folding 

Figure 6. Reconstitution of the IRE1α–XBP1 pathway in MIN6 (Ire1α ΔR/ΔR) cells. (A–G) To examine whether the phenotype of MIN6 (Ire1α ΔR/ΔR) cells is restored by reconstituting the IRE1α–XBP1 pathway, WT Ire1α and Xbp1s were stably expressed by retroviruses harboring WT Ire1α and Xbp1s in MIN6 (Ire1α ΔR/ΔR) cells, respectively. In B, C, D, and G, black bars indicate MIN6 (Ire1α ΔR/ΔR) cells infected with retrovirus harboring an empty vector (Vec). Red bars indicate MIN6 (Ire1α ΔR/ΔR) cells infected with retrovirus harboring WT Ire1α. Yellow bars indicate MIN6 (Ire1α ΔR/ΔR) cells infected with retrovirus harboring Xbp1s. (A) Expression levels of Ire1α, proinsulin, insulin, and GAPDH were analyzed by immunoblotting in the indicated MIN6 cells. RT-PCR for Xbp1 mRNA of total RNA isolated from the indicated MIN6 cells. (B) The ratio of Xbp1 mRNA splicing in A was quantified. Xbp1 splicing (%) = Xbp1s/total Xbp1 × 100. (C) Expression levels of proinsulin relative to those of GAPDH in A were quantified and normalized as described in C. (D) Expression levels of insulin relative to those of GAPDH in A were quantified and normalized as described in C. (E) Insulin secretion in response to glucose concentration in the indicated MIN6 cells for 4 h was measured by ELISA. (F) Protein expression levels of PDI family proteins and ER chaperones in the indicated MIN6 cells were analyzed by immunoblotting. (G) Protein expression levels of PDI family proteins and ER chaperones relative to those of GAPDH in F were quantified and normalized as described in C. Error bars represent means and SD. n = 3. *, P < 0.05; **, P < 0.01. 

Figure 7. XBP1s directly binds to the promoter regions of the five PDI family genes. (A) ChIP assay using normal IgG and anti-XBP1s IgG. PCR of the WT MIN6 cell extracts were performed using a set of primers outside of the promoter regions containing XBP1s binding sites. (B) ChIP assay followed by quantitative PCR to quantify the binding of XBP1s to proximal promoter regions of the indicated genes. Error bars indicate means and SD. n = 3.
Liu et al., 2005; Kadokura and Beckwith, 2009; Zito et al., 2010; Kadokura et al., 2013). We then detected and distinguished oxidized monomer proinsulin and mixed-disulfide complexes involving proinsulin and showed that IRE1α is required for the efficient folding of proinsulin in pancreatic β cells (Fig. 4, D and E; and Fig. S4, A and B).

What is the precise role of IRE1α in the promotion of proinsulin folding? We found that the expression levels of five PDI family proteins, PDI, PDIR, P5, ERp44, and ERp46, were significantly decreased in MIN6 (Ire1αΔR/ΔR) cells (Fig. 5, A and B; and Fig. S4 D) and in pancreatic islets of IRE1α B(-/ΔR) mice (Fig. 5, C and D). In addition, the expression levels of the five PDIs were restored by reconstituting the IRE1α–XBP1 pathway in MIN6 (Ire1αΔR/ΔR) cells (Fig. 6, F and G). ChIP assays of the five candidate PDIs confirmed that XBP1s protein directly binds to the promoter regions of the five PDI family genes (Fig. 7). Thus, IRE1α is required for the increased expression of PDI, P5, PDIR, ERp44, and ERp46 in pancreatic β cells. Furthermore, proinsulin and insulin contents were also fully restored by reconstituting the IRE1α–XBP1 pathway in MIN6 (Ire1α“/“) cells (Fig. 6, A, C, and D) because the decrease in proinsulin folding was associated with decreases in the expression of the five PDIs, which are downstream targets of the IRE1α–XBP1 pathway. In addition, PDI, P5, ERp44, and ERp46 formed mixed-disulfide complexes with newly synthesized proinsulin (Fig. 8 A). We therefore propose that IRE1α functions as a key regulator of oxidative folding of enzymes involved in proinsulin folding (Fig. 9).

Insulin is a representative secretory protein folded by disulfide bond formation in the ER, but it is not fully understood how proinsulin is folded in the ER. Recently, it was reported that the deletion of ERO1β, an ER disulfide oxidase that is highly expressed in pancreatic β cells, causes a decrease in insulin secretion and oxidative folding of proinsulin in mice (Zito et al., 2010). ERO1 maintains PDIs in their active oxidized forms. However, the PDIs involved in proinsulin folding are not well characterized. In this study, we clearly showed that at least PDI, P5, ERp44, ERp46, and ERp72 form mixed-disulfide complexes with newly synthesized proinsulin (Fig. 8 A). In addition, decreased...
expression of the five PDIs (PDI, P5, PDIR, ERp44, and ERp46) is correlated with decreased proinsulin folding (Figs. 4, 5, and 6), and reconstitution of the five PDIs could moderately restore insulin secretion to a similar extent as reconstitution of Irela (Figs. 6 E and 8 B). Furthermore, when we overexpressed the mixture of five PDIs in MIN6 (IrelaΔR/ΔR) cells, insulin secretion was increased. In contrast, the only PDI/PDIA1 overexpression in Ire1αΔR/ΔR preproinsulin targeting to the ER, and we did not detect such a difference. Thus, the differences among studies may be explained primarily by the differences among cell lines.

In this study, we used two independent experimental models, Ire1αΔR/ΔR mice and MIN6 (IrelaΔR/ΔR) cells, and showed that constitutively activated Ire1α in pancreatic β cells induces specific PDI family proteins that are important for the correct folding of proinsulin in the ER. This combination technique is useful to clarify the novel roles of Ire1α in pancreatic β cells.

**Materials and methods**

**Generation of Ire1αΔR/ΔR mice**

The Ire1αΔR/ΔR (deleted transmembrane domain) and Ire1αΔR/ΔR mice (floxed RNase domain) were generated as previously described (Iwawaki et al., 2009). To generate Ire1αΔR/ΔR mice, Ire1αΔR/ΔR mice were crossed with Ire1αΔR/ΔR mice. Ire1αΔR/ΔR mice were made by crossing these mice with the Ins-Cre transgenic mice Tg(Ins-Cre)23Herr, which were a generous gift from P. Herrera via Y. Kido (Kobe University, Kobe, Japan; Herrera, 2000). All experimental protocols involving animals were approved by the Committee on Animal Research at Nara Institute of Science and Technology (NAIST) and were performed in accordance with the institutional guidelines of NAIST.

**Measurement of blood glucose and serum insulin**

The blood glucose level was measured using LabAssay glucose (Wako). The insulin concentration in serum was determined using a mouse insulin ELISA kit (AKRIN-011T; Shibayagi).

**Glucose tolerance tests**

Glucose tolerance tests were performed on 12-wk-old male Ire1αΔR/ΔR mice and Ire1αΔR/ΔR mice after fasting for 16 h. Mice were administered glucose at 2 g/kg body weight by i.p. injection. Blood glucose levels were measured at 0, 15, 30, 60, and 120 min after the injection.

**Islet morphology analysis and immunohistochemistry**

The pancreas was fixed in 4% paraformaldehyde in PBS buffer at 4°C overnight and then immersed in 30% sucrose in PBS buffer. The pancreas was embedded in optimal cutting temperature compound. Immunohistochemistry was performed on 8-µm tissue sections prepared using a cryostat (HM550; MIC Inter- national) following standard methods. Primary antibodies were used at the following dilutions: guinea pig antiinsulin (Dako) at a 1:500 dilution and rabbit antiglucagon (Dako) at a 1:500 dilution. Images of stained sections were observed using a fluorescence microscope (BZ-X710; Keyence) with a CFI Plan Apochromat 20× 0.5 NA objective lens (Nikon) at room temperature. The acquired images were processed with Imaging Joint BZ-H3XD and analyzed by BZ-H3A and BZ-H3C software (Keyence).

**Islet isolation**

Pancreatic islets were prepared from the mouse pancreas. HBSS (0.8% NaCl, 5 mM KCl, 0.17 mM Na2HPO4, 0.4 mM KH2PO4, 0.1% glucose, 1.2 mM CaCl2, 0.8 mM MgSO4, 7.5 mM NaHCO3, and 0.01% phenol red) was used for perfusion and washing of the pancreas. Collagenase P (Roche) was dissolved in HBSS to a final concentration of 0.01% phenol red. Collagenase P (Roche) was dissolved in HBSS to a final concentration of 0.01% phenol red.

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**Figure 9. Schematic diagram illustrating the function of Ire1α as a key regulator of oxidative proinsulin folding and PDI family levels in pancreatic β cells.**
were obtained (mice. One MIN6 (Ire1αfl/fl) cell line (original name; MINS#12) with alleles of both IRE1α or XBP1s was ligated into the pMXsIP-based retroviral vector (a gift from T. Kitamura, University of Tokyo, Tokyo, Japan) to construct Ire1α_pMXsIP and Xbp1s_pMXsIP. Plat-GP (RV-103; Cell Biolabs) cells were transfected with VSV-G_pMLV.2 and Ire1α_pMXsIP or Xbp1s_pMXsIP using Fugene HD (Promega) following the manufacturer's protocol to generate the retrovirus. VSV-G expressed by VSV-G_pMLV.2 promotes the formation and release into the culture medium of the virus particle. MIN6 (Ire1αΔR/ΔR) cells were infected with retroviruses and cultured for 1 wk in the presence of 5 µg/ml puromycin (Wako) to obtain MIN6 (Ire1αΔR/ΔR) cells stably expressing WT Ire1α and Xbp1s.

Establishment of mouse insulinoma cells harboring the floxed Ire1α-RNase domain

Ire1αΔR mice (Iwawaki et al., 2009) were crossed with IT-6 mice (Miyazaki et al., 1990, 2010), which express SV40 large T antigen under the control of the human insulin promoter. Progeny with the IT-6 transgene and homozygous floxed Ire1α alleles were obtained (Ire1αΔR/ΔR; IT-6 mice), and these mice developed insulinomas at 10 wk of age. Four MIN6 (Ire1αΔR) cell lines were established from insulinomas that developed in independent one MIN6 (Ire1αfl/fl; IT-6 mice), and these mice developed insulinomas at 10 wk of age. Four MIN6 (Ire1αfl/fl) cell lines were established from insulinomas that developed in independent one MIN6 (Ire1αfl/fl) cell line (original name; MINS#12) with high insulin secretion in high-glucose and high-insulin content was then selected (Sato et al., 2017). In the adenovirus infection experiments, two experimental controls were used. In one control, referred to as Con, MIN6 (Ire1αΔR/ΔR) cells, the MIN6 (Ire1αΔR) cells were subjected to the infection procedure in the absence of adenovirus. In the other control, named Ad-GFP, MIN6 (Ire1αΔR) cells, the cells were infected with adenovirus carrying GFP (Fig. S2 B).

Propagation and purification of adenovirus for the infection of MIN6 (Ire1αΔR) cells

Adenovirus harboring either GFP or Cre has been described previously (Miyazaki et al., 2012). To propagate adenoviruses, HEK293 cells were infected with the appropriate adenovirus and grown for 3–5 d. To concentrate the adenovirus, the infected HEK293 cells were collected in a 50-ml tube and subjected to seven freeze–thaw cycles. After centrifugation at 3,500 g at 4°C for 30 min, the supernatants were collected and filtered to purify the adenovirus. The titer of the adenovirus was determined by the infectious titer method using HEK293 cells. The following secondary antibodies were used for immunoblotting: anti-IGG (H+L chain; rabbit) pAb-Alexa Fluor 488 (706-545-148; Jackson ImmunoResearch Laboratories, Inc.) at a 1:500 dilution and anti-ribbit Igg (H+L chain) pAb–Alexa Fluor 594 (A-21207; Thermo Fisher Scientific) at a 1:500 dilution.

Antibodies for immunoblotting

The following primary antibodies were used for immunoblotting: antinsiulin at a 1:500 dilution and antiglucagon at a 1:500 dilution.

Immunoblotting

WT MIN6 cells were plated on six-well plates at 4.0 × 10^6 cells/well, cultured for 4 d, and washed twice with PBS. They were subjected to alkylation with NEM and immunoblotting as previously described (Kadokura et al., 2013). The following primary antibodies were used for immunoblotting: anti-insulin (8138S; Cell Signaling Technology) at a 1:1,000 dilution, anti-IRE1α (3294S; Cell Signaling Technology) at a 1:500 dilution, anti-PERK (3192S; Cell Signaling Technology) at a 1:500 dilution, anti–PERK-P (3179S; Cell Signaling Technology) at a 1:500 dilution, anti-ATF6α and anti-ATF6β (ab155800; Abcam) at a 1:1,000 dilution, and anti-ERp72 (5174S; Cell Signaling Technology) at a 1:1,000 dilution, anti-PDI (5121275; GeneTex) at a 1:1,000 dilution, anti-PERK (3192S; Cell Signaling Technology) at a 1:1,000 dilution, anti-ERp44 (3798S; Abcam) at a 1:1,000 dilution, anti-ERp46 (a gift from K. Inaba, Tohoku University, Sendai, Japan) at a 1:2,000 dilution, anti–eIF2α-P (3398S; Cell Signaling Technology) at a 1:1,000 dilution, anti–PERK-P (3179S; Cell Signaling Technology) at a 1:1,000 dilution, anti–PDI (390862; Santa Cruz Biotechnology, Inc.) at a 1:200 dilution, anti–ERp44 (3798S; Cell Signaling Technology) at a 1:1,000 dilution, anti–ATF6α and anti–ATF6β (Iwawaki et al., 2009) at a 1:1,000 dilution. The following secondary antibodies were used for immunoblotting: anti-IgG (H+L chain; rabbit) pAb–HRP goat Igg/Fab (458; MBL) at a 1:2,500 dilution and anti-mouse IgG-HRP (115-035-003; Jackson ImmunoResearch Laboratories, Inc.) at a 1:2,500 dilution.

Immunofluorescence

MIN6 (Ire1αΔR) or MIN6 (Ire1αfl/fl) or MIN6 cells were plated on six-well plates at 4.0 × 10^6 cells/well, cultured for 4 d, and washed twice with PBS. They were subjected to alkylation with NEM and immunoblotting as previously described (Kadokura et al., 2013).

Comununprecipitation experiment

WT MIN6 cells were transfected with FLAG-tagged PDI, PDIF, P5, ERp44, ERp46, ERp57, ERp72, and ERd5 (a gift from K. Nagata, Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan) at a 1:2,500 dilution.
Kyoto Sangyo University, Kyoto, Japan; Araki et al., 2013) and subjected to a pulse-chase experiment. Then, samples were subjected to alkylation with NEM and immunoprecipitation with an anti-FLAG monoclonal M2 antibody (F1804-5MG; Sigma-Aldrich). The resulting immunoprecipitates were then subjected to a second immunoprecipitation using anti-proinsulin monoclonal antibody and separated by 4–12% NuPAGE (WG1402A; Thermo Fisher Scientific).

Quantification of secreted insulin by ELISA
MIN6 (Ire1α0/0 or Ire1α+/−) cells were plated on six-well plates at 4.0 × 10⁶ cells/well, cultured for 4 d with DMEM, and preincubated for 2 h with Hepes–Krebs–Ringer bicarbonate Hepes (KRKH) buffer (10 mM Hepes, 115 mM NaCl, 5.9 mM KCl, 1.2 mM MgCl₂, 6H₂O, 1.2 mM NaH₂PO₄, 1.2 mM Na₃SO₄, 2.5 mM CaCl₂, 25 mM NaNH₂O₃, and 2 mg/ml BSA) containing 1.67 mM glucose. Insulin secreting cells were then grown for 4 h under 1.67 mM glucose/Hepes-KRKH buffer and visualized after staining with ethidium bromide. Intensities of bands were quantified by sandwich ELISA. The amount of secreted insulin was normalized against the total cellular protein.

Genotyping PCR
For genotyping PCR, tails of mice or MIN6 (Ire1α0/0 or Ire1α+/−) cells were collected in tubes and dissolved in tail lysis buffer (50 mM Tris-HCl, pH 7.5, 20 mM EDTA, pH 8.0, 100 mM NaCl, and 1% SDS) containing proteinase K (1:100; Takara Bio Inc.) at 65°C overnight. The dissolved samples were subjected to PCR amplification with primers (see below) and Ex-Taq DNA polymerase (Takara Bio Inc.). Mice and MIN6 (Ire1α0/0 or Ire1α+/−) cells were genotyped by PCR with primers that flank the loxP sites (floxed) of the RNase domain of Ire1α, a transmembrane region of Ire1α, and rat insulin promoter-Cre region (see below). CreFlpCheck forward, 5′-CCG AAGCCATGAAACACAGG-3′; CreFlpCheck reverse, 5′-CCCTGCGAGGTGTCATGG-3′; 3Rcheck forward, 5′-CAAGTGAGGAGGTATGCCTCTTCGT-3′; Ire1α-ex6-F1, 5′-GCTTCATCATGACACCTCTTCAG-3′; pgk-neo-R1, 5′-CGG TGTGGTGAGATGTGCAAACT-3′; Cre-F001, 5′-ATGTCATATTCTCAGG-3′; and Cre-R001, 5′-CGCCGATAACCAGTGAAAC-3′. The PCR products were separated on a 4% (floxed and transmembrane region of Ire1α) or 0.8% (Ins-Cre) agarose gel in TAE buffer and visualized after staining with ethidium bromide. Intensities of bands were then measured and used to determine the extent of Xbp1 splicing.

RNA extraction
Total RNA was isolated from mouse tissues and MIN6 cells using RNAiso PLUS (Takara Bio Inc.). cDNA was generated from total RNA using Oligo(dT) 18mer and Moloney murine leukemia virus reverse transcriptase (M-MLV; Promega) following the manufacturer’s protocol.

Estimation of the extent of Xbp1 mRNA splicing using RT-PCR
To estimate the extent of Xbp1 mRNA splicing, cDNA prepared from total RNA as described above was subjected to PCR amplification with a pair of primers, i.e., mouse Xbp1 forward primer, 5′-GAGAACCAGGATTTAAGACACG-3′; and mouse Xbp1 reverse primer, 5′-GAAGATGGTAGGGAGGTGAC-3′; and KAPA Taq extra DNA polymerase (KAPA Bioscience). To evaluate the spliced and unspliced forms of Xbp1 mRNA, the PCR products were separated on a 7.5% acrylamide gel with TAE buffer and visualized after staining with ethidium bromide. Intensities of bands were then measured and used to determine the extent of Xbp1 splicing.

qRT-PCR
To estimate the RNA expression of genes, cDNA prepared from total RNA as described above was subjected to qRT-PCR using SYBR green II (Takara Bio Inc.) with a pair of primers (Table S1) on a Light Cycler 480 system (Roche). Primers for RT-PCR were designed using Probe Finder (Roche).

ChIP assay
MIN6 cells were plated on 15-cm dishes and cultured for 4 d and then fixed in 1% paraformaldehyde/PBS for 10 min at room temperature. Chromatin shearing and immunoprecipitation were performed using the ChIP-IT Express ChIP kit (53008; Active Motif) and Bioruptor UCW-310 (Sonic Bio) according to the manufacturer’s protocol. The immunoprecipitated DNA fragments were used as templates for PCR amplification (see below; the number +1 indicates the transcription start site of each gene).

Transmission electron microscopy
MIN6 (Ire1α0/0 or Ire1α+/−) cells were seeded onto Thermomax plastic coverslips (Thermo Fisher Scientific). Cells were fixed in 0.1 M sodium cacodylate buffer, pH 7.4, containing 2.5% glutaraldehyde overnight at 4°C, and subsequently fixed in 0.1 M sodium cacodylate buffer, pH 7.4, containing 1% osmium tetroxide for 1 h at 4°C. Fixed cells were stained en bloc with 0.5% uranyl acetate and uranyl acetate, and examined with an H7100 transmission electron microscope (Hitachi) at an acceleration voltage of 75 kV.
IRE1αB(+/ΔR) and IRE1α B(-/ΔR)

Fig. S1 shows the immunostaining of pancreas sections of Online supplemental material was assumed to be normal, but this was not formally tested.

ends. Error bars in the figures represent SD. Data distribution p-values (*, P < 0.05; **, P < 0.01) are indicated in the figure legends. Statistical significance was calculated by unpaired two-tailed t test unless otherwise indicated. All experiments were repeated at least three times unless otherwise described. The number of independent experiments is described as n, and p-values (*, P < 0.05; **, P < 0.01) are indicated in the figure legends. Error bars in the figures represent SD. Data distribution was assumed to be normal, but this was not formally tested.

Online supplemental material

Fig. S1 shows the immunostaining of pancreas sections of IRE1αB(+/ΔR) or IRE1αB(-/ΔR) or WT MIN6 cells were pulse-labeled with 30 µl of EasyTag express [35S] protein labeling mix (NEG772; PerkinElmer) in 16.7 mM glucose/Hepes-KRBH for the indicated times and then were chased by growing the cells in 16.7 mM glucose/Hepes-KRBH containing 0.13 mg/liter cysteine 2H2O and 0.06 mg/ml methionine for the total duration. After these steps, free cysteines in the cells were subjected to alkylation with NEM as described above. The NEM-treated samples were then diluted ten-fold with ice-cold radioimmunoprecipitation assay buffer without SDS (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, and 50 mM Tris-HCl, pH 8.0) and incubated with an antiproinsulin monoclonal antibody at 4°C for 16 h. The immune complexes were collected using Dynabeads protein G (10003D; Thermo Fisher Scientific). The eluted immune complexes were separated by 4–12% NuPAGE gel (WG1402A; Thermo Fisher Scientific), and the gel was exposed to an imaging plate for 2–10 d. The radioactive bands were detected using a BAS1000 system (Fujix).

Statistical analyses

Statistical significance was calculated by unpaired two-tailed Student’s t test unless otherwise indicated. All experiments were repeated at least three times unless otherwise described. The number of independent experiments is described as n, and p-values (*, P < 0.05; **, P < 0.01) are indicated in the figure legends. Error bars in the figures represent SD. Data distribution was assumed to be normal, but this was not formally tested.

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Author contributions: Y. Tsuchiya established MIN6 (IRE1αB(+/ΔR)) cell lines and performed all experiments using cultured cells. M. Saito generated mouse strains and performed all experiments related to the mouse studies. J.-i. Miyazaki and F. Tashiro provided IT-6 mice and prepared adenovirus. T. Iwawaki provided IRE1α KO, CKO mice, and anti-ATF6 antibody. H. Kadokura designed the analysis of oxidative proinsulin folding. Y. Imagawa performed electron micrograph analyses. Y. Tsuchiya, M. Saito, H. Kadokura, and K. Kohn supervised the research, analyzed the data, and wrote the manuscript. K. Kohn supervised all studies. All authors discussed the results and commented on the manuscript.

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