

As shown in Fig. 1D, a shorter form of XBP1 mRNA was increased. This form is produced by 26-nucleotide splicing from primary XBP1 mRNA by the ribonuclease activity of IRE1, increasing active XBP1 protein levels in mutant islets (Fig. 1E). HRD1, a ubiquitin ligase involved in ERAD, is one of the XBP1 target genes (20). In *wfs1*-deficient islets, levels of HRD1 protein were markedly increased (Fig. 1E). In addition, mRNA levels of EDEM (ER-associated degradation-enhancing  $\alpha$ -mannosidase-like protein) (23), another target of XBP1, were significantly increased in mutant islets ( $100 \pm 5$  arbitrary units (wild type) vs.  $136 \pm 18$  (mutant),  $n = 6$ ,  $p < 0.05$ ). These data indicate that the IRE1-initiated subpathway of the UPR is also activated in *wfs1*-deficient islets.

#### **Establishment of MIN6 $\beta$ -cell lines deficient in WFS1**

To examine the influence of WFS1-deficiency specifically in a homogenous  $\beta$ -cell population,  $\beta$ -cell lines were established by crossing *wfs1*<sup>+/-</sup> and *wfs1*<sup>-/-</sup> mice (17) with IT6 mice expressing simian virus 40 (SV40) large T antigen under the insulin promoter (24) and were designated MIN6wfs1<sup>+/-</sup> and MIN6wfs1<sup>-/-</sup>, respectively (see Materials and Methods). IT6 mice were previously reported to develop insulinoma, from which the MIN6 cell line (24), one of the most highly differentiated  $\beta$ -cell lines, was generated. We established two cell lines each for the *wfs1*<sup>+/-</sup> and *wfs1*<sup>-/-</sup> genotypes. As shown in Fig. 2A, the two cell lines with the *wfs1*<sup>-/-</sup> genotype (MIN6wfs1<sup>-/-</sup>-1 and 2) show similar UPR characteristics. Similarly, characteristics of two cell lines with the *wfs1*<sup>+/-</sup> genotype (MIN6wfs1<sup>+/-</sup>) were indistinguishable (data not shown). Therefore, only one line of each genotype was used for subsequent analyses. We compared MIN6wfs1<sup>-/-</sup> with MIN6wfs1<sup>+/-</sup> at the same passage numbers (passages 5 to 8), but not with the original

MIN6 cells. This is because we were concerned that a difference in passage number between the original MIN6 and MIN6wfs1<sup>-/-</sup> cells, irrespective of WFS1-deficiency, might affect the protein expression profile, rendering the former an inappropriate control for the latter. After completion of a series of experiments, MIN6wfs1<sup>+/-</sup> cells reached passages 15 - 20, the same passage of original MIN6 cells we have. The function and survival of MIN6wfs1<sup>+/-</sup> cells are similar to those of wild-type MIN6 cells at similar passage numbers (data not shown).

### **Effects of WFS1-deficiency on UPR in $\beta$ -cell lines**

As shown in Fig. 2A, altered expressions of UPR-related proteins observed in *wfs1*-deficient islets were reproduced in MIN6wfs1<sup>-/-</sup> cells; PERK phosphorylation, as well as expressions of active XBP1 and HRD1, were increased in *wfs1*-deficient MIN6 cells. ATF4 levels were also shown to be increased in these cells. Furthermore, although GRP78 and GRP94 protein levels were similar (Fig. 2B, upper panel), the activity of the GRP78 promoter containing three ER stress response elements was greater in MIN6wfs1<sup>-/-</sup> cells than in MIN6wfs1<sup>+/-</sup> cells (Fig. 2B), strongly suggesting activation of the ATF6 subpathway of the UPR in MIN6wfs1<sup>-/-</sup> cells. To confirm that alterations in UPR-related proteins are due to WFS1-deficiency, wild-type human WFS1 protein was expressed in MIN6wfs1<sup>-/-</sup> cells. We took advantage of the tetracycline-inducible expression system. MIN6wfs1<sup>-/-</sup> cells were infected with the Tet-repressor expressing virus (AdCAG-TR) together with a recombinant adenovirus bearing wild-type human WFS1 cDNA under the CMV promoter containing the Tet-operator (AdCTO-WFS1). The cells were then treated with doxycycline (2  $\mu$ g/ml). As shown in Fig. 2C, when WFS1 expression was restored to levels comparable to those of the original MIN6 cells,

the increase in PERK phosphorylation was prevented. In addition, overexpression of GRP78, a master regulator of the ER stress response, also resulted in normalization of PERK phosphorylation levels (Fig. 2D), clearly indicating the observed alteration in UPR-related proteins to be due to exacerbation of ER stress caused by WFS1-deficiency.

#### **No UPR induction in heart, skeletal muscle or brown adipose tissues from *wfs1*-deficient mice**

WFS1 protein is expressed in a variety of non-pancreatic tissues, though less abundantly than in islets (Fig. 3A). Therefore, we also examined expressions of UPR genes in tissues other than pancreatic islets. Cardiac function is reportedly not impaired in subjects with Wolfram syndrome (12) or in *wfs1*-deficient mice (17). Skeletal muscle and brown adipose tissue also appear essentially normal in mutant mice (data not shown). In contrast to islets, no UPR alterations were observed in these tissues from *wfs1*-deficient mice (Fig. 3B-D). Thus, UPR activation is tissue-specific in WFS1-deficiency.

#### **Increased $\beta$ -cell apoptotic response in *wfs1*-deficient islets**

ER stress induces apoptosis through activation of various signaling molecules including JNK and pro-apoptotic proteins, such as CHOP (5-7). CHOP expression was increased at both the mRNA (Fig. 4A) and the protein level (Fig. 4B), in mutant as compared to wild-type islets. In contrast, JNK expression levels and phosphorylation states were not altered in *wfs1*-deficient islets (Fig. 4B). We also found increased levels of cleaved caspase-3, a hallmark of apoptosis, in mutant islets (Fig. 4B). CHOP expression and

cleaved caspase-3 levels were also increased in *wfs1*-deficient MIN6 cells (Fig. 4C), while no such changes were observed in heart, skeletal muscle or adipose tissue (data not shown).

We also measured apoptosis in MIN6*wfs1*<sup>-/-</sup> and MIN6*wfs1*<sup>+/-</sup> cells by counting adherent cells positive for annexin V staining under fluorescent microscope. We found 1 - 2% cells to be annexin V positive for both the *wfs1*<sup>-/-</sup> and the *wfs1*<sup>+/-</sup> genotype cultured under standard conditions, i.e. no differences between MIN6*wfs1*<sup>-/-</sup> and MIN6*wfs1*<sup>+/-</sup> cells. An increase in the number of apoptotic cells was observed when MIN6*wfs1*<sup>-/-</sup> cells were challenged with 0.5 μM thapsigargin for 24 hours, as compared to MIN6*wfs1*<sup>+/-</sup> cells under the same conditions (2.7 ± 1.0% (MIN6*wfs1*<sup>+/-</sup>) vs. 6.2 ± 1.1% (MIN6*wfs1*<sup>-/-</sup>), n = 3, p < 0.05). Therefore, MIN6*wfs1*<sup>-/-</sup> cells exhibited increased apoptosis susceptibility. These data, together, indicate that an ER stress mediated -apoptotic process is activated in *wfs1*-deficient β-cells.

### **Impaired β-cell proliferation in *wfs1*-deficient islets**

In addition to increased apoptosis, decreased proliferation may contribute to loss of β-cell mass in *wfs1*-deficient mice. When β-cell proliferation activity was assayed by 5-bromodeoxyuridine (BrdU) incorporation in pancreases from wild-type and mutant mice, BrdU incorporation was found to be significantly reduced in *wfs1*-deficient β-cells (Fig. 5A and B). This observation suggested impaired proliferation, along with increased apoptosis, to contribute to β-cell loss in *wfs1*-deficient islets.

We next explored possible causes of the decreased β-cell proliferation in *wfs1*-deficient islets. The link between the UPR and cell cycle arrest was previously reported to be mediated by down-regulation of cyclin D1 due to general translational

suppression via eIF2 $\alpha$  phosphorylation (25). However, neither expression of cyclin D1 nor that of cyclin D2, major isoforms of the D type cyclins in  $\beta$ -cells (26,27), was changed in mutant islets (data not shown). CHOP has also been recognized as causing cell cycle arrest and apoptosis (28,29). Since GADD34 is reportedly a target of CHOP (30) and is involved in cell growth and survival (31), GADD34 expression was examined. GADD34 transcript levels were found to be increased in *wfs1*-deficient islets ( $100 \pm 11$  vs.  $151 \pm 14$ ,  $p < 0.05$ ). Recent studies have demonstrated that cell cycle regulation is critical for maintenance of  $\beta$ -cell mass (25,26). Since GADD34 reportedly induces p53 phosphorylation and enhances expression of the cell cycle inhibitor p21<sup>CIP1</sup> (32), p53 and p21<sup>CIP1</sup> expressions were assessed. We found phosphorylation of p53 to be increased, though total p53 was not elevated (Fig. 5C). In addition, increased expressions of p21<sup>CIP1</sup> mRNA ( $100 \pm 11$  vs.  $413 \pm 32$ ,  $p < 0.01$ ) and p21<sup>CIP1</sup> protein (Fig. 5C) were observed in *wfs1*-deficient islets. We also examined the expression of another cell cycle inhibitor, p27<sup>KIP1</sup>, and found no difference between wild-type and mutant islets (Fig. 5C). Increased expression of p21<sup>CIP1</sup> protein was also observed in *wfs1*-deficient MIN6 cells, SV40 large T antigen-transformed cells in which p53 activity was considered to be suppressed (Fig. 5D). Expression of p21<sup>CIP1</sup> protein was not increased in heart, skeletal muscle or brown adipose tissues from *wfs1*-deficient mice (data not shown).

In order to determine whether increased expression of p21<sup>CIP1</sup> is attributable to ER stress, wild-type islets were treated with thapsigargin (0.5  $\mu$ M) for 12 hours. As shown in Fig. 5E, expression of p21<sup>CIP1</sup> was significantly increased. In addition, expression of p21<sup>CIP1</sup> was markedly increased in MIN6 cells treated with thapsigargin (Fig. 5F) or tunicamycin (data not shown). These data suggest p21<sup>CIP1</sup> expression to be

induced by ER stress in  $\beta$ -cells.

Finally to assess the effects of p21<sup>CIP1</sup> expression on  $\beta$ -cell proliferation, p21<sup>CIP1</sup> was expressed in wild-type MIN6 cells in a tetracycline-inducible manner (Fig. 6A). Overexpression of p21<sup>CIP1</sup> suppressed a MIN6 cell number increase (Fig. 6B), suggesting that increased p21<sup>CIP1</sup> expression contributes to the reduced  $\beta$ -cell mass in *wfs1*-deficient islets.

## DISCUSSION

We systematically investigated UPR in *wfs1*-deficient islets and MIN6  $\beta$ -cells as well as heart, skeletal muscle and brown adipose tissues from the mutant mice in this study. Enhanced UPR was specifically observed in  $\beta$ -cells but not in other tissues examined. These findings indicate that diabetes in Wolfram syndrome is caused by increased ER stress in  $\beta$ -cells and establish Wolfram syndrome as an ER stress-based disease, as is the case in Wolcott-Rallison syndrome with PERK-deficiency (9). Furthermore, we found enhanced UPR to be associated with not only activation of the apoptotic pathway but also impaired cell cycle progression in  $\beta$ -cells. These observations provide evidence of novel mechanisms underlying ER stress-mediated  $\beta$ -cell loss.

We demonstrated activation of the PERK and IRE1 subpathways of the UPR. Increased activation of the GRP78 promoter indicates the ATF6 subpathway to be induced as well. GRP78 expression was also reportedly increased by knockdown of WFS1 expression in INS1 insulinoma  $\beta$ -cells (20). Collectively, these data indicate that all three UPR subpathways are activated by WFS1-deficiency in  $\beta$ -cells. The UPR is activated when ER homeostasis is perturbed by defective ER calcium homeostasis, mutations in ER resident proteins and/or abnormalities of the ERAD system. Disturbed ER homeostasis is also induced by defect(s) in components of the UPR system, as is the case in Wolcott-Rallison syndrome with PERK-deficiency. The present data suggest that impaired ER homeostasis does not result from defect(s) in a specific pathway(s) of the UPR. Our previous study demonstrated an abnormal cytosolic  $\text{Ca}^{2+}$  response in *wfs1*-deficient  $\beta$ -cells (17), suggesting that impaired ER  $\text{Ca}^{2+}$  homeostasis is a possible cause of ER stress associated with WFS1-deficiency.

We found that WFS1 protein is highly expressed in heart, skeletal muscle and brown adipose tissues. However, there is no UPR activation in these tissues from mutant mice. Thus, the UPR is tissue-specific in *wfs1*-deficient mice. One possible explanation of this tissue specificity is that a protein(s), compensating for loss of WFS1 protein function, is present in these tissues but not in  $\beta$ -cells. This interesting possibility merits further investigation and elucidation of WFS1 function is necessary to resolve the tissue-specific effects of WFS1-deficiency.

Our results demonstrate, in addition to the augmented apoptotic process evidenced by increased caspase-3 cleavage, that  $\beta$ -cell proliferation is decreased in *wfs1*-deficient mice. Impaired proliferation was also reported in BRIN-BD11 cells expressing the human WFS1 antisense transcript (33). Our observation is in contrast to that by Riggs et al., who detected no changes in the numbers of BrdU-positive cells in islets from  $\beta$ -cell specific *wfs1* knockout mice (21). The reason for this discrepancy is currently unclear, but may reflect differences in the ages of the mice studied: 6-week-old mice were used in the present versus 12- or 24-week-old animals in their study (21). Cell cycle dysregulation in *wfs1*-deficient islets was associated with increased expression of p21<sup>CIP1</sup>, a cell cycle regulator. p21<sup>CIP1</sup> can serve, depending on which tissues or cells it is activated in, as both an inhibitor and an agonist of cell cycle progression (34). Our observation that forced expression of p21<sup>CIP1</sup> suppressed MIN6  $\beta$ -cell proliferation suggests that p21<sup>CIP1</sup> operates as a cell cycle inhibitor in  $\beta$ -cells, although our results must be interpreted cautiously since forced overexpression of p21<sup>CIP1</sup> may produce effects different from those occurring in mutant  $\beta$ -cells with increased p21<sup>CIP1</sup> levels. A very recent study, demonstrating that p21<sup>CIP1</sup> acts as a molecular brake on mitogenic stimuli in  $\beta$ -cells (35), supports the notion of p21<sup>CIP1</sup>



functioning as a cell cycle inhibitor in  $\beta$ -cells. ER stress inducers were recently reported to cause p21<sup>CIP1</sup> expression and cell cycle arrest in chondrocytes (36) and prostatic cancer cells (37), suggesting that cell cycle arrest associated with increased p21<sup>CIP1</sup> expression is a common feature in cells under ER stress. Furthermore, reduced proliferation associated with increased expression of p21<sup>CIP1</sup>, in *wfs1*-deficient  $\beta$ -cells (the present study) and  $\beta$ -cells transgenic for hepatocyte growth factor and/or placental lactogen (35), highlights an important role for p21<sup>CIP1</sup> in regulation of  $\beta$ -cell mass in addition to the roles of p27<sup>KIP1</sup> recently reported (38).

CHOP induces GADD34 expression (32), which then activates p53 phosphorylation and p21<sup>CIP1</sup> transcription (36). Therefore, the CHOP --> GADD34 --> p53 pathway is a candidate for ER stress-mediated p21<sup>CIP1</sup> expression. Indeed, an increase in p21<sup>CIP1</sup> expression was associated with increased GADD34 expression and p53 phosphorylation in *wfs1*-deficient  $\beta$ -cells. However, induction of p21<sup>CIP1</sup> expression by thapsigargin was observed in MIN6 cells transformed with SV40 large T antigen, a well-known suppressor of p53. In addition, an ER stress-induced increase in p21<sup>CIP1</sup> expression was observed in p53-deficient prostatic cancer cells (37). Thus, ER stress appears to induce p21<sup>CIP1</sup> expression through both p53-dependent and -independent mechanisms.

Since  $\beta$ -cells are apparently much more sensitive to ER stress than other types of cells and tissues (39), ER stress might be a more common cause of  $\beta$ -cell failure than previously thought, especially in terms of the increased insulin demands of modern lifestyles. Our data indicate that both increased apoptosis and impaired proliferation, in  $\beta$ -cells, are mechanisms leading to  $\beta$ -cell loss in *wfs1*-deficient islets, a model of ER-stress mediated  $\beta$ -cell failure. Further studies designed to elucidate the molecular

mechanisms of  $\beta$ -cell loss under chronic ER stress are anticipated to contribute to future treatments for type 2 diabetes.

## MATERIALS AND METHODS

### Antibodies

The monoclonal antibody against P58<sup>IPK</sup> was a generous gift from Prof. M.G. Katze (University of Washington). Other antibodies were purchased from the indicated sources: anti-GRP94, anti-KDEL (Stressgen Biotechnologies), anti-GRP78, anti-XBP1, anti-p21<sup>CIP1</sup>, anti-CHOP, anti-p53, anti-phosphorylated p53, and anti-ATF4 (Santa Cruz Biotechnology), anti-HRD1 (Abgent), anti-phosphorylated PERK, anti-JNK, anti-phosphorylated JNK, anti-eIF2 $\alpha$ , anti-phosphorylated eIF2 $\alpha$ , and anti-cleaved caspase-3 (Cell Signaling), and anti-p27<sup>KIP1</sup> (BD Transduction Laboratories).

### Mouse islet isolation, real-time RT-PCR and Western blot

The *wfs1*-deficient mice used had a C57Bl/6 background and were described previously (17). All animal experiments were approved by the Tohoku University Institutional Animal Care and Use Committee (#15-45). Islets were isolated by collagenase infusion through the common bile duct and harvested by hand. Total RNA was prepared immediately after islet isolation using an RNAeasy kit (Qiagen). For real-time RT-PCR analysis, cDNA was synthesized by reverse transcription using the oligo d(T)<sub>16</sub> primer and subjected to PCR amplification with gene-specific primers (Table 1) using a SYBR Green 1 kit (Roche). Data are presented as relative values to actin mRNA. For detection of the spliced form of XBP1 mRNA, the primers were: 5'-TGAGAACCAGGAGTTAAGAAACGC-3' and 5'-TTCTGGGTAGACCTCTGGGAGTTCC-3'. For immunoblotting, islets from 3 to 4 mice were pooled, dissolved immediately after isolation in a lysis buffer (approximately 100 islets/15  $\mu$ l) and

subjected to SDS-polyacrylamide gel electrophoresis. In several experiments, isolated islets were cultured overnight and treated with 0.5  $\mu$ M thapsigargin for 12 h. All Western blot experiments were repeated at least three times, with different sets of samples, throughout this study. Immunoblot band intensities were analyzed using Scion image software (Scion Corporation) and normalized with those of actin.

#### **Establishment of MIN6*wfs1*<sup>-/-</sup> and MIN6*wfs1*<sup>+/-</sup> cell lines**

The *wfs1*<sup>-/-</sup> mice (17) were bred with IT6 mice expressing SV40 large T antigen under the human insulin promoter (24) and the resulting *wfs1*<sup>+/-</sup>:SV40Tag/+ mice were further bred with *wfs1*<sup>-/-</sup> mice. Tumors from pancreases of 10- to 12-week-old *wfs1*<sup>+/-</sup>:SV40Tag/+ and *wfs1*<sup>-/-</sup>:SV40Tag/+ mice were carefully excised and placed in Dulbecco's Modified Eagle's Medium containing penicillin and streptomycin. Cells were expanded and frozen at passages 3 and 4. We used these cells at 5 to 8 passages in this study. For study of apoptosis, MIN6 cells were infected with AdRIPeGFP expressing enhanced green fluorescent protein under the insulin promoter to facilitate detection of cells under fluorescent microscope. Apoptosis was examined by staining with annexin V using the Annexin V-Cy3 apoptosis detection kit (Medical and Biological Laboratories). At least 1,000 cells per sample were counted for annexin V positive cells.

#### **GRP78 promoter assay**

The pGL3-promoter, pTK-RLuc and pGL3-basic plasmids were purchased from Promega. The mouse GRP78 promoter fragment spanning -172 to -21 (positions relative to the transcription start site) was amplified by PCR using oligonucleotides

5'-GACTCGAGGCCGCTTCGAATCGGCAG-3' and 5'-TCAAGCTTGGCCAGTATC GAGCGCGC-3'. This fragment contains three ER stress response elements (40) and the corresponding regions of human (40) and rat (41) GRP78 genes were shown to respond to ATF6 activation. A GRP78 promoter-driving luciferase reporter plasmid (designated pmGRP78pro(-172)-Luc) was constructed by subcloning this fragment into the *Xho*I and *Hind*III sites of the pGL3-basic vector. MIN6wfs1<sup>+/-</sup> or MIN6wfs1<sup>-/-</sup> cells were co-transfected with pGL3-promoter or pGRP78pro(-172)-Luc together with pTK-RLuc using the LipofectAMINE reagent (Invitrogen). Luciferase activities were assayed with Dual-Luciferase reporter system (Promega) using a Lumat LB9507 luminometer (Berthold).

#### **BrdU incorporation assay**

BrdU (100 mg/kg) was injected into the mice intraperitoneally. Six hours later, the mice were sacrificed and their pancreases were fixed with 4% paraformaldehyde. Immunohistochemical analyses were performed with a Cell Proliferation Assay kit (BD Pharmingen). Sections were also stained with anti-insulin. BrdU-positive  $\beta$ -cells were counted in at least 50 sections per mouse.

#### **Recombinant adenovirus experiments**

Human *GRP78* cDNA was purchased from Open Biosystems. Human *WFS1* cDNA was a generous gift from Prof. Y. Tanizawa (Yamaguchi University). The CMV promoter containing two Tet-operator sequences (designated CTO) was excised from pcDNA5/TO (Invitrogen) and ligated to these cDNAs. The Tet-repressor cDNA was excised from pcDNA6/TR (Invitrogen) and ligated to the CAG promoter unit (39).

These expression units were used to generate recombinant adenoviruses by a previously described method (40). The resulting viruses were designated AdCAG-TR for the Tet-repressor expressing virus and AdCTO-GRP78 for the GRP78 expressing virus under the CTO promoter, and so on. MIN6 and its derivative cells were infected with AdCAG-TR at a multiplicity of infection (m.o.i.) of 30 together with viruses with the CTO promoter at an m.o.i. of 100. One day after infection, cells were reseeded and divided into two groups. Two days thereafter, the cells were fed media with or without doxycycline (2 µg/ml). We have observed no adverse effects of infection of a control recombinant adenovirus expressing green fluorescence protein at an m.o.i. of less than 250 on MIN6 cell function in terms of cell proliferation and glucose-stimulated insulin secretion (data not shown). For the cell number assessment, MIN6 cells infected with AdCAG-TR and AdCTO-p21<sup>CIP1</sup> were seeded in 6-well plates at  $2 \times 10^5$  per well, cultured in media with or without doxycycline (2 µg/ml) and harvested after the indicated intervals. Cells were then stained with trypan blue and counted.

### **Statistical analysis**

Data are presented as means  $\pm$  S.E. Differences between groups were assessed by Student's *t-test*.

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## FIGURE LEGENDS

**Figure 1.** Activation of three subpathways of the UPR in *wfs1*-deficient islets.

**A)** Activation of the PERK/eIF2 $\alpha$  pathway. Islets isolated from wild-type and *wfs1*-deficient mice were subjected to SDS-PAGE and probed with the indicated antibodies: P-PERK, phosphorylated-PERK; P-eIF2 $\alpha$ , phosphorylated-eIF2 $\alpha$ ; T-eIF2 $\alpha$ , total eIF2 $\alpha$ .

**B)** Real-time RT-PCR analysis of GRP94, GRP78 and P58<sup>IPK</sup> gene expressions in wild-type (open columns) and *wfs1*-deficient (closed columns) mice. Relative mRNA levels were obtained after normalization to actin mRNA. \*p < 0.05. n = 6.

**C)** Expressions of chaperone proteins in *wfs1*-deficient islets. Lysates of isolated islets were probed with the indicated antibodies.

**D)** Increased XBP1 mRNA splicing in *wfs1*-deficient islets. Amplification of XBP1 mRNA from islet total RNA with specific primers yields spliced (303 bp) and non-spliced (329 bp) XBP1 transcripts.

**E)** Activation of the IRE1/XBP1 pathway. Lysates of isolated islets were probed with the indicated antibodies.

Western blot data shown are representative of at least three experiments with different sets of samples.

**Figure 2.** Increased UPR and its reversal by expression of WFS1 or GRP78 in an SV40 transformed *wfs1*-deficient  $\beta$ -cell line (MIN6wfs1<sup>-/-</sup>).

**A)** Expression of UPR-related proteins in various MIN6 cell lines. MIN6, MIN6wfs1<sup>+/-</sup>-1, MIN6wfs1<sup>-/-</sup>-1 and MIN6wfs1<sup>-/-</sup>-2 cells were lysed and probed with the