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Cell Metabolism
Short Article

ATF4-Mediated Induction of 4E-BP1 Contributes to Pancreatic β Cell Survival under Endoplasmic Reticulum Stress

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SUMMARY

Endoplasmic reticulum (ER) stress-mediated apoptosis may play a crucial role in loss of pancreatic β cell mass, contributing to the development of diabetes. Here we show that induction of 4E-BP1, the suppressor of the mRNA 5′ cap-binding protein eukaryotic initiation factor 4E (eIF4E), is involved in β cell survival under ER stress. 4E-BP1 expression was increased in islets under ER stress in several mouse models of diabetes. The *Eif4ebp1* gene encoding 4E-BP1 was revealed to be a direct target of the transcription factor ATF4. Deletion of the *Eif4ebp1* gene increased susceptibility to ER stress-mediated apoptosis in MIN6 β cells and mouse islets, which was accompanied by deregulated translational control. Furthermore, *Eif4ebp1* deletion accelerated β cell loss and exacerbated hyperglycemia in mouse models of diabetes. Thus, 4E-BP1 induction contributes to the maintenance of β cell homeostasis during ER stress and is a potential therapeutic target for diabetes.

INTRODUCTION

Recent studies have shown decreased pancreatic β cell mass to be a common feature of subjects with type 2 diabetes mellitus (Butler et al., 2003). Susceptibility to stress-induced apoptosis may underlie β cell loss. Translational regulation is an essential strategy by which cells cope with stress conditions (Clemens, 2001). Translation of eukaryotic mRNA is regulated primarily at the level of initiation. Translational initiation begins with formation of a ternary complex composed of the methionine-charged initiator tRNA, eukaryotic initiation factor 2 (eIF2), and GTP (Holcik and Sonenberg, 2005). The ternary complex then binds to the 40S ribosomal subunit and several other initiation factors, generating the 43S preinitiation complex. The mRNA 5′ cap-binding protein eIF4E associates with eIF4A and eIF4G to form the eIF4F complex and interacts with the 5′ cap structure of the mRNA. The eIF4F complex then recruits the 43S preinitiation complex to the mRNA, allowing the complex to scan toward the initiator AUG codon. The two best characterized regulatory steps in this translational control are formation of the ternary complex and assembly of the eIF4F complex. Phosphorylation of the α subunit of eIF2 (eIF2α) prevents ternary complex formation and thereby suppresses global translation. In addition, eIF4E-binding proteins (4E-BPs) inhibit eIF4F assembly by competitively displacing eIF4G from eIF4E. Global translational suppression through eIF2α phosphorylation is a mechanism shared among different stress-response pathways. Depending on the nature of the stress stimulus, eIF2α can be phosphorylated by four different kinases (Holcik and Sonenberg, 2005). Global attenuation of protein biosynthesis then paradoxically increases expression of several proteins, including the transcription factor ATF4 (Harding et al., 2000).

Because of their high insulin secretory activity, β cells are vulnerable to endoplasmic reticulum (ER) stress, a condition of disrupted ER homeostasis due to accumulation of misfolded proteins (Schroder and Kaufman, 2005). Cells respond to ER stress by activating an adaptive cellular response known as the unfolded protein response (UPR). Under ER stress conditions, global translation is suppressed through eIF2α phosphorylation by an ER-resident kinase, PERK. The importance of PERK-mediated translational suppression has been demonstrated in infancy-onset diabetes and skeletal defects caused by loss of PERK in humans (Delepine et al., 2000) and mice (Harding et al., 2001; Zhang et al., 2002). However, the roles of translational control through inhibition of eIF4F assembly by 4E-BPs under stress conditions, including ER stress, have yet to be fully clarified. Herein, we have studied roles of 4E-BP1,

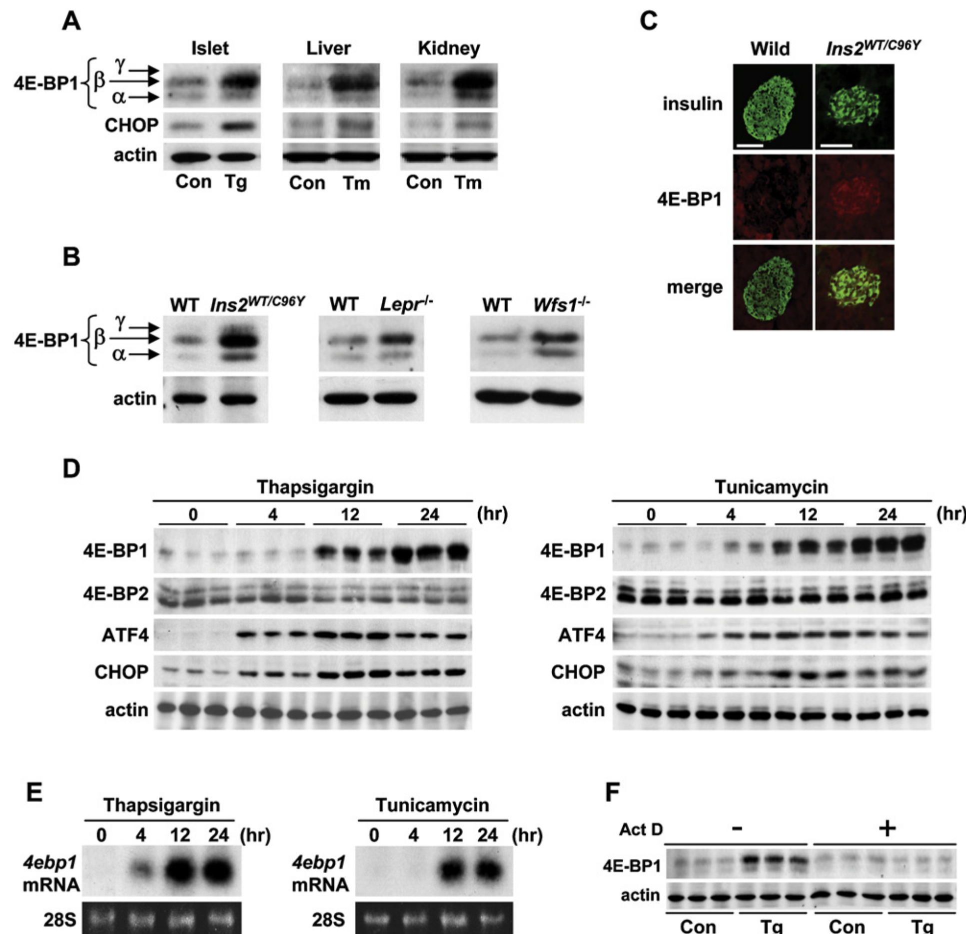

Figure 1. ER Stress Induces 4E-BP1 Expression

(A) Expression of 4E-BP1 protein in isolated islets treated with vehicle (0.05% DMSO) control (Con) or 0.5 μM thapsigargin (Tg) for 12 hr. 4E-BP1 expression was also examined in the livers and kidneys of mice that had received intraperitoneal injections of tunicamycin (Tm) 96 hr previously.

(B) Expression of 4E-BP1 protein in islets from wild-type (WT), *Ins2*$^{WT/C96Y}$, *Lepr*$^{-/-}$, and *Wfs1*$^{-/-}$ mice.

(C) Immunostaining of pancreatic sections from WT and *Ins2*$^{WT/C96Y}$ mice using anti-insulin and anti-4E-BP1 antibodies. Scale bars = 50 μm.

(D and E) Time courses of 4E-BP1, 4E-BP2, ATF4, and CHOP expression (D) and *4ebp1* mRNA expression (E) in MIN6 cells treated with thapsigargin (left panel) or tunicamycin (right).

(F) Inhibition of 4E-BP1 induction by actinomycin D (1 μg/ml) in MIN6 cells treated with thapsigargin for 12 hr.

one of three isoforms of the 4E-BP family, in β cells under ER stress.

RESULTS

ER Stress Induces 4E-BP1

4E-BP1 protein is present in three forms with different phosphorylation states. The hypophosphorylated α and β forms are active and the hyperphosphorylated γ form is inactive in terms of eIF4E binding. Expression of 4E-BP1 protein, especially the hypophosphorylated forms, was markedly induced, with an increase in CHOP, a stress marker protein, in isolated islets treated with thapsigargin (an ER Ca^{2+} pump inhibitor causing ER stress) (Figure 1A). 4E-BP1 induction was also observed in liver and kidneys of mice administered tunicamycin (a protein glycosylation inhibitor), another ER stress inducer (Figure 1A).

Furthermore, 4E-BP1 protein expression was markedly increased in *Ins2*$^{WT/C96Y}$ islets (Figures 1B and 1C), in which misfolded insulin molecules with a C96Y mutation cause ER stress (Wang et al., 1999). Islets from leptin receptor null (*Lepr*$^{-/-}$) mice, which have been shown to suffer from ER stress (Laybutt et al., 2007), also exhibited increased 4E-BP1 expression (Figure 1B). The *Wfs1*$^{-/-}$ mouse (Ishihara et al., 2004) is a model of Wolfram syndrome, which is characterized by juvenile-onset diabetes mellitus and optic atrophy and is caused by *WFS1* mutations (Inoue et al., 1998; Strom et al., 1998). WFS1-deficient islets are affected by chronic ER stress (Ishihara et al., 2004; Riggs et al., 2005). Again, 4E-BP1 protein was increased in *Wfs1*$^{-/-}$ islets (Figure 1B).

Induction of 4E-BP1 by ER stress was also observed in insulinoma MIN6 cells (Miyazaki et al., 1990) (Figure 1D). Expression of 4E-BP2, another member of the 4E-BP family, remained unchanged. While expression of ATF4 and CHOP peaked at 12 hr after treatment with thapsigargin or tunicamycin, 4E-BP1 protein was further increased at 24 hr posttreatment (Figure 1D). 4E-BP1 protein induction appeared to result from transcriptional

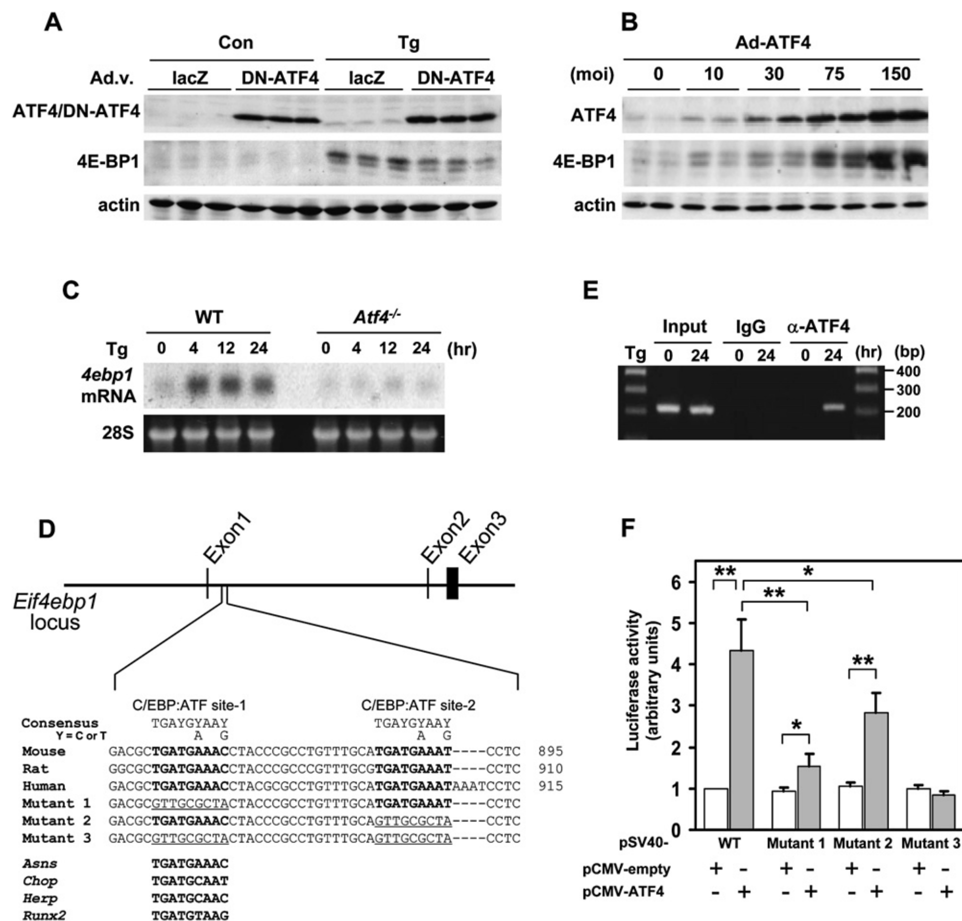

Figure 2. *Eif4ebp1* Is a Direct Target of ATF4

(A) Suppression of thapsigargin (Tg, 0.5 μM)-induced 4E-BP1 expression by dominant-negative ATF4 (DN-ATF4). MIN6 cells were infected with an adenovirus expressing either lacZ or DN-ATF4. Two days later, the cells were treated with vehicle (0.05% DMSO) control (Con) or Tg for 12 hr.

(B) 4E-BP1 expression in MIN6 cells infected with an adenovirus expressing wild-type ATF4 at the indicated multiplicity of infection (moi).

(C) *4ebp1* mRNA levels in wild-type and $Atf4^{-/-}$ MEFs treated with thapsigargin.

(D) C/EBP:ATF composite sites in intron 1 of the *Eif4ebp1* gene. Mouse, rat, and human *Eif4ebp1* gene segments are aligned with ATF4 binding sequences in several genes. Numbers are positions relative to A of the initial ATG codon. *Asns*, asparagine synthetase; *Herp*, homocysteine-induced ER protein; *Runx2*, runt-related transcription factor 2.

(E) Chromatin immunoprecipitation assay of MIN6 cells treated with thapsigargin. DNAs precipitated with nonspecific or anti-ATF4 IgG were amplified using primers for the *Eif4ebp1* intron 1 region.

(F) ATF4 induction of luciferase reporters with the *SV40* promoter and an *Eif4ebp1* gene segment with C/EBP:ATF composite sites or their mutants shown in (D). MIN6 cells were transfected with luciferase reporters together with either pCMV-empty or pCMV-ATF4. Error bars represent SEM. n = 4; *p < 0.05, **p < 0.01.

activation since *4ebp1* mRNA levels were also increased by these ER stress inducers (Figure 1E) and the transcriptional inhibitor actinomycin D completely blocked 4E-BP1 induction by thapsigargin (Figure 1F).

ATF4 Directly Activates the *Eif4ebp1* Gene

MIN6 cells were infected with recombinant adenoviruses expressing dominant-negative (DN) forms of transcription factors involved in the UPR. Expression of DN-ATF4 (He et al., 2001) (Figure 2A), but not DN-ATF6 or DN-XBP1 (see Figure S1 available online), suppressed 4E-BP1 induction by thapsigargin. Conversely, expression of wild-type ATF4 dramatically induced 4E-BP1 expression (Figure 2B). Furthermore, *4ebp1* mRNA levels were not increased by thapsigargin in $Atf4^{-/-}$ murine embryonic fibroblasts (MEFs) (Harding et al., 2003) (Figure 2C).

A survey of the mouse *Eif4ebp1* gene using a luciferase assay identified a segment in intron 1 that conferred thapsigargin sensitivity to a luciferase reporter (Figure S2). Indeed, we found two potential ATF4 binding sequences (C/EBP:ATF composite sites) in this segment (Figure 2D). Chromatin immunoprecipitation (ChIP) assays revealed that ATF4 binds this segment (Figure 2E). Furthermore, cotransfection of a luciferase reporter containing the C/EBP:ATF sites with an ATF4-expressing plasmid increased luciferase activity by 4.3-fold (Figure 2F). Disruption of the upstream C/EBP:ATF site (mutant 1) or the downstream site (mutant 2) decreased the ATF4-mediated increase in luciferase activity by 83% or 47%, respectively, and disruption of both (mutant 3) completely abolished the increase (Figure 2F).

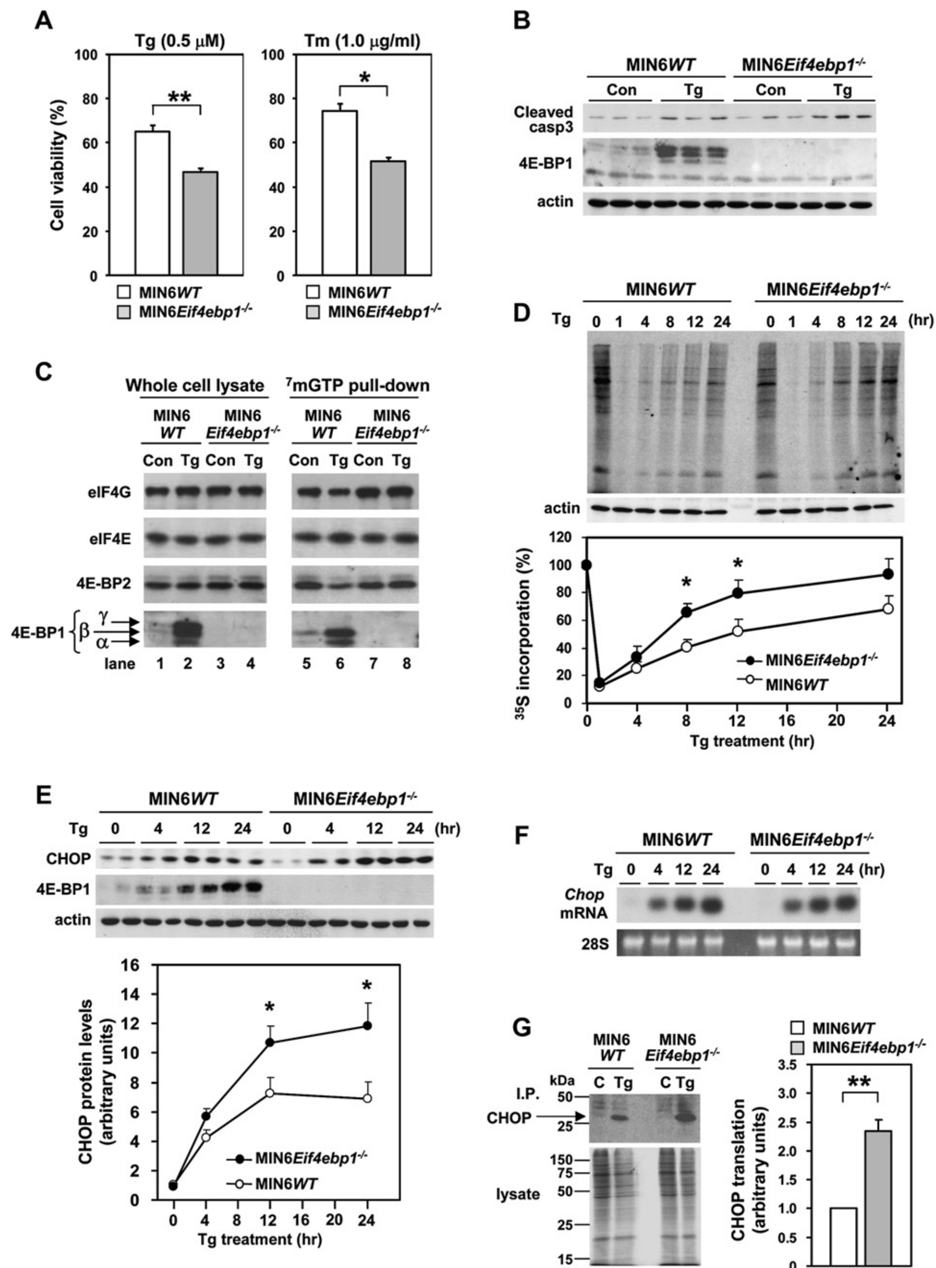

Figure 3. 4E-BP1-Deficient Cells Exhibit Increased Apoptosis Susceptibility with Deregulated Translational Control

(A) Viability of MIN6*WT* and MIN6*Eif4ebp1*$^{-/-}$ cells treated with 0.5 μM thapsigargin (Tg) or 1.0 μg/ml tunicamycin (Tm) for 36 hr, normalized to MIN6*WT* cells treated with vehicle (0.05% DMSO). n = 3–4.

(B) Immunoblot of cleaved caspase-3 in MIN6*WT* and MIN6*Eif4ebp1*$^{-/-}$ cells treated with vehicle control (Con) or thapsigargin for 24 hr.

(C) Immunoblot analysis of 4E-BP1, 4E-BP2, eIF4E, and eIF4G in whole-cell lysates (left) or in a complex associated with ^{7}mGTP-Sepharose (right) in cells treated with thapsigargin for 24 hr.

(D) [^{35}S]methionine/cysteine incorporation during a 15 min pulse labeling in MIN6*WT* and MIN6*Eif4ebp1*$^{-/-}$ cells pretreated with thapsigargin for the indicated periods. Ten percent of the lysates were also probed with an anti-actin antibody. A representative autoradiogram is shown in the upper panel; data from three experiments are summarized in the lower panel.

(E) Increased CHOP induction in MIN6*Eif4ebp1*$^{-/-}$ cells treated with thapsigargin. Representative blots are shown in the upper panel; data from four experiments are summarized in the lower panel.

(F) *Chop* mRNA levels in MIN6*WT* and MIN6*Eif4ebp1*$^{-/-}$ cells treated with thapsigargin.

(G) Greater *Chop* translation in MIN6*Eif4ebp1*$^{-/-}$ cells treated with thapsigargin. MIN6*WT* and MIN6*Eif4ebp1*$^{-/-}$ cells treated with vehicle (C) or thapsigargin (Tg) for 12 hr were labeled with [^{35}S]methionine/cysteine. Lysates were either directly subjected to SDS-PAGE or immunoprecipitated with anti-CHOP antibody. Representative autoradiograms are shown in the left panel; data from four experiments are summarized in the right panel.

Error bars represent SEM. *p < 0.05, **p < 0.01.

4E-BP1-Deficient β Cells Are More Vulnerable to ER Stress

A 4E-BP1-deficient β cell line, MIN6*Eif4ebp1*$^{-/-}$, was established by crossing *Eif4ebp1*$^{-/-}$ mice (Tsukiyama-Kohara et al., 2001) with IT6 mice expressing SV40 large T antigen in β cells (Miyazaki et al., 1990). MIN6 cells with wild-type *Eif4ebp1* alleles, established in parallel, were designated MIN6*WT* cells. MIN6*Eif4ebp1*$^{-/-}$ cells were more vulnerable to ER stress inducers than MIN6*WT* cells (Figure 3A). 4E-BP1 re-expression restored this diminished viability of MIN6*Eif4ebp1*$^{-/-}$ cells to control levels (Figure S3A). The increased susceptibility to ER stress-induced cell death was accompanied by enhanced caspase-3 cleavage (Figure 3B), indicating that the reduced viability of MIN6*Eif4ebp1*$^{-/-}$ cells was due at least in part to increased apoptosis. In addition, DNA fragmentation under ER stress was greater in *Eif4ebp1*$^{-/-}$ islets than in wild-type islets (Figure S3B). These results suggest that 4E-BP1 induction contributes to β cell survival under ER stress.

We then examined the impact of 4E-BP1 deficiency on the integrity of the eIF4F translational initiation complex. Pull-down assays of eIF4E and its binding partners with a cap analog, 7-methyl-GTP, revealed that thapsigargin-induced 4E-BP1 expression resulted in marked increases in the amounts of hypophosphorylated 4E-BP1 α and β forms bound to eIF4E, displacing eIF4G from eIF4E in MIN6*WT* cells (Figure 3C, compare lane 5 with lane 6). The amount of eIF4G bound to eIF4E was reduced to 63% ± 3% ($n = 4$, $p < 0.05$) of that in vehicle-treated MIN6WT cells. In contrast, levels of eIF4G bound to eIF4E were not decreased by thapsigargin in MIN6*Eif4ebp1*$^{-/-}$ cells (Figure 3C, compare lane 7 with lane 8). Thus, eIF4E availability for translational initiation was greater in MIN6*Eif4ebp1*$^{-/-}$ cells than in MIN6*WT* cells under ER stress. Measurement of the global translation rate revealed that recovery from translational suppression by thapsigargin was more rapid in 4E-BP1-deficient cells (Figure 3D).

Translation of newly synthesized mRNA molecules is reportedly much more dependent on eIF4E availability than that of preexisting mRNAs (Novoa and Carrasco, 1999). Expression of CHOP, a mediator of ER stress-induced apoptosis, was thus studied in MIN6*Eif4ebp1*$^{-/-}$ cells since *Chop* mRNA is one of the transcripts most abundantly synthesized during ER stress (Pirot et al., 2007). *Eif4ebp1* deletion caused greater CHOP protein induction by thapsigargin in MIN6 cells (Figure 3E), with unaltered *Chop* mRNA accumulation (Figure 3F). Pulse-labeling experiments demonstrated enhanced CHOP translation (Figure 3G). Thus, CHOP expression during ER stress was augmented via increased translation in 4E-BP1 deficiency.

Eif4ebp1 Deletion Accelerates β Cell Loss in Mouse Diabetes Models

To examine the roles of 4E-BP1 under ER stress in vivo, *Eif4ebp1*$^{-/-}$ mice on the 129S6 background were fed a high-fat diet (HFD), which is thought to produce ER stress in β cells through peripheral insulin resistance (Scheuner et al., 2005). *Eif4ebp1*$^{-/-}$ mice developed glucose intolerance (Figures S4A and S4B), which was associated with blunted insulin secretion (Figure S4C) and reduced pancreatic insulin content (Figure S4D) as compared to HFD-fed wild-type mice. These data suggest that *Eif4ebp1*$^{-/-}$ mice have a β cell defect. However, HFD-fed *Eif4ebp1*$^{-/-}$ mice gained more weight and were more insulin resistant than HFD-fed wild-type mice (Figures S4E and S4F). Therefore, the possibility remains that β cell failure in HFD-fed *Eif4ebp1*$^{-/-}$ mice resulted from greater ER stress rather than from a defect in β cells lacking 4E-BP1.

We next crossed *Eif4ebp1*$^{-/-}$ mice with two genetic models of diabetes in which β cells are under ER stress, *Ins2*$^{WT/C96Y}$ and *Wfs1*$^{-/-}$ mice on the 129S6 background. 4E-BP1 deficiency did not alter body weight (Figures S5A and S5B) or insulin sensitivity (Figures S5C and S5D) but worsened hyperglycemia in *Ins2*$^{WT/C96Y}$ (Figure 4A) and *Wfs1*$^{-/-}$ (Figure 4B) mice. In *Eif4ebp1*$^{-/-}$ *Ins2*$^{WT/C96Y}$ mice, pancreatic insulin content was less than half of that in *Ins2*$^{WT/C96Y}$ mice at 5 weeks of age (Figure 4C), and the majority of islets in *Eif4ebp1*$^{-/-}$ *Ins2*$^{WT/C96Y}$ mice were smaller as compared to those in *Ins2*$^{WT/C96Y}$ mice (Figure 4D). We also observed a 38% decrease in pancreatic insulin content in *Eif4ebp1*$^{-/-}$ *Wfs1*$^{-/-}$ mice as compared to *Wfs1*$^{-/-}$ mice (Figure 4E). Importantly, the insulin-positive area was smaller in pancreatic sections from *Eif4ebp1*$^{-/-}$ *Wfs1*$^{-/-}$ mice than in pancreatic sections from *Wfs1*$^{-/-}$ mice at 27–30 weeks of age (Figure 4F), indicating that ER stress-mediated β cell loss is exacerbated by 4E-BP1 deficiency in vivo.

Global protein synthesis was studied in these mouse islets. A tendency toward decreased protein synthesis was observed in both *Ins2*$^{WT/C96Y}$ (Figure 4G, hatched bar; $p = 0.074$) and *Wfs1*$^{-/-}$ islets (Figure 4H, hatched bar; $p = 0.079$) as compared to wild-type islets. *Eif4ebp1* deletion ablated this regulation and resulted in significantly increased protein synthesis in *Eif4ebp1*$^{-/-}$ *Ins2*$^{WT/C96Y}$ ($p = 0.013$) and *Eif4ebp1*$^{-/-}$ *Wfs1*$^{-/-}$ ($p = 0.045$) islets as compared to that in corresponding single mutants (compared hatched with filled bars in Figures 4G and 4H). These data suggest that accelerated β cell loss under ER stress is due to deregulated translational control.

DISCUSSION

Our results implicate 4E-BP1, identified as a component of the UPR, in β cell survival under ER stress. Important roles of 4E-BPs under various stress conditions have been recently demonstrated in yeast (Ibrahimo et al., 2006) and *Drosophila* (Teleman et al., 2005; Tettweiler et al., 2005). These data suggest that translational suppression by 4E-BPs is an evolutionarily conserved strategy against stress conditions. Although we focused on β cells, ER stress-mediated induction of 4E-BP1 was also observed in the liver and kidneys, suggesting the general importance of the present findings.

Our results suggest that, in addition to translational regulation by eIF2α phosphorylation due to PERK activation, another mode of translational control mediated by 4E-BP1 plays a role in the maintenance of β cell homeostasis under ER stress. Since translational suppression by eIF2α phosphorylation is transient owing to feedback dephosphorylation by GADD34 (Novoa et al., 2001), prolonged translational suppression by 4E-BP1 might be needed in the later stages of the UPR. However, in contrast to PERK, 4E-BP1 deficiency alone does not cause diabetes in mice under normal conditions, suggesting that 4E-BP1 protein is not a key regulator but rather functions with other molecules to maintain β cell homeostasis under ER stress. The preferential role of 4E-BP1 in the later stages of the UPR might be puzzling since expression of

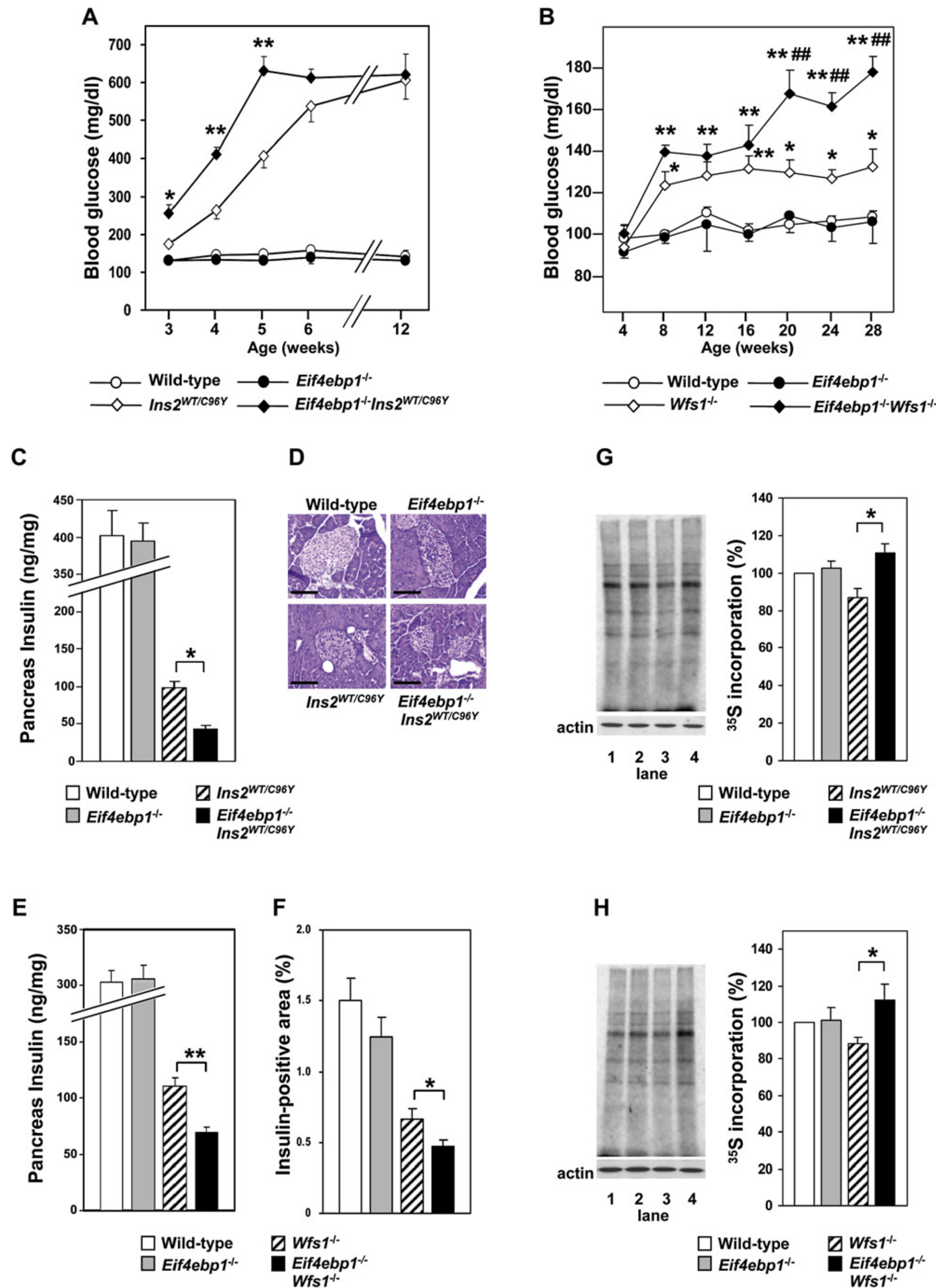

Figure 4. β Cell Loss Is Exacerbated by 4E-BP1 Deficiency in Mouse Diabetes Models

(A) Fed blood glucose levels of wild-type (n = 6), *Eif4ebp1*$^{-/-}$ (n = 5), *Ins2*$^{WT/C96Y}$ (n = 9), and *Eif4ebp1*$^{-/-}$ *Ins2*$^{WT/C96Y}$ (n = 11) mice. Data from three cohorts are combined. *p < 0.05, **p < 0.01 versus *Ins2*$^{WT/C96Y}$ mice.

(B) Fed blood glucose levels of wild-type (n = 12), *Eif4ebp1*$^{-/-}$ (n = 8), *Wfs1*$^{-/-}$ (n = 15), and *Eif4ebp1*$^{-/-}$ *Wfs1*$^{-/-}$ (n = 10) mice. Data from three cohorts are combined. *p < 0.05, **p < 0.01 versus wild-type mice; ##p < 0.01 versus *Wfs1*$^{-/-}$ mice.

(C) Pancreatic insulin content of mice of the indicated genotypes at 5 weeks of age. n = 3 for each genotype. *p < 0.05.

(D) Hematoxylin and eosin staining of sections showing representative islets from mice of the indicated genotypes at 5 weeks of age. Scale bars = 50 μm.

(E) Pancreatic insulin content of wild-type (n = 8), *Eif4ebp1*$^{-/-}$ (n = 4), *Wfs1*$^{-/-}$ (n = 15), and *Eif4ebp1*$^{-/-}$ *Wfs1*$^{-/-}$ (n = 12) mice at 27–30 weeks of age. **p < 0.01.

(F) Insulin-positive area in pancreatic sections of wild-type (n = 3), *Eif4ebp1*$^{-/-}$ (n = 3), *Wfs1*$^{-/-}$ (n = 4), and *Eif4ebp1*$^{-/-}$ *Wfs1*$^{-/-}$ (n = 5) mice at 27–30 weeks of age. *p < 0.05.

(G) [^{35}S]methionine/cysteine incorporation in islets of the indicated genotypes at 5–6 weeks of age. Ten percent of the lysates were also probed with an anti-actin antibody. A representative autoradiogram is shown in the left panel. Lane 1, wild-type; lane 2, *Eif4ebp1*$^{-/-}$; lane 3, *Ins2*$^{WT/C96Y}$; lane 4, *Eif4ebp1*$^{-/-}$ *Ins2*$^{WT/C96Y}$. Data from four experiments are summarized in the right panel. *p < 0.05.

ATF4, the primary inducer of *Eif4ebp1* under ER stress, is activated by translational suppression by eIF2α phosphorylation during the acute phase. We found that 4E-BP1 protein is stable with a half-life of approximately 20 hr (Figure S6). Thus, 4E-BP1 protein seems to continue to be expressed abundantly during the later stages of the UPR. This is consistent with the recent observation that several prosurvival proteins involved in the UPR are stable, while proapoptotic proteins are not (Rutkowski et al., 2006). We found that global protein synthesis was higher in 4E-BP1-deficient β cells than in wild-type cells under ER stress conditions. In particular, expression of CHOP was augmented in 4E-BP1 deficiency. Enhanced CHOP expression in 4E-BP1-deficient cells suggests that a reduction in eIF4E availability due to 4E-BP1 induction suppresses CHOP translation during ER stress in wild-type cells, possibly accounting for one of the mechanisms by which 4E-BP1 plays a role in adaptation to ER stress. Important roles of translational control via eIF4E availability have also been suggested in prolonged hypoxia (Koritzinsky et al., 2006). However, the signaling mechanisms for translational control are different: ER stress increases 4E-BP1 protein levels via ATF4 in β cells, while hypoxia enhances 4E-BP1 activity via dephosphorylation and also causes eIF4E nuclear localization in HeLa cells.

The present results also suggest that variations in genes regulating eIF4E availability and/or eIF4F formation may have an impact on susceptibility to diabetes. In this context, a recent report demonstrating that a gene encoding eIF4A2, a component of eIF4F, is possibly linked to type 2 diabetes in French families (Cheyssac et al., 2006) is of great interest. Furthermore, our findings raise the possibility that 4E-BP1 may be a potential target for diabetes mellitus treatment.

EXPERIMENTAL PROCEDURES

Animal Experiments

All animal experiments were approved by the Tohoku University Institutional Animal Care and Use Committee. *Wfs1*$^{-/-}$ mice were backcrossed to a 129S6 (Taconic) background for six generations. *Ins2*$^{WT/C96Y}$ mice (Charles River Laboratories) were backcrossed to a 129S6 background for five generations. *Eif4ebp1*$^{-/-}$ mice were maintained on a 129S6 background. Only male mice were used. For the in vivo studies shown in Figures 4A, 4C, and 4D, littermates from crosses of male *Ins2*$^{WT/C96Y}$ *Eif4ebp1*$^{+/-}$ and female *Ins2*$^{WT/WT}$ *Eif4ebp1*$^{+/-}$ mice were used. For Figures 4B, 4E, and 4F, littermates from intercrosses of *Eif4ebp1*$^{+/-}$ *Wfs1*$^{+/+}$ mice and littermates from intercrosses of *Eif4ebp1*$^{+/-}$ *Wfs1*$^{-/-}$ mice were used. For isolated islet experiments (Figures 4G and 4H), age-matched nonlittermate mice were used. To induce ER stress in vivo, mice were given a 0.5 μg/g body weight intraperitoneal injection of tunicamycin. After 96 hr, kidneys and livers were removed. Tissue sample processing, immunostaining of pancreatic sections, and determination of β cell area and pancreatic insulin content were performed as described previously (Ishihara et al., 2004).

Cell Culture and Cell Viability Assay

Pancreatic tumors in *Eif4ebp1*$^{-/-}$:SV40Tag mice on a mixed background were excised, yielding MIN6*Eif4ebp1*$^{-/-}$cells, which were used at 5–10 passages in this study. MIN6 cells were cultured in DMEM supplemented with 15% FCS. *Atf4*$^{-/-}$ MEFs were cultured in DMEM supplemented with a nonessential amino acid mixture and 10% FCS. Cells seeded in 24-well plates 2 days previously were treated with thapsigargin or tunicamycin and used for western blotting or cell viability assay. Cell viability was determined with a cell proliferation assay kit (Promega). Construction of adenoviruses and infection of MIN6 cells were performed as described previously (Ishihara et al., 2004).

Northern and Western Blotting and Cap-Binding Affinity Assay

Total RNA extracted using ISOGEN (Nippon Gene) was probed with ^{32}P-labeled cDNAs. Tissue homogenates and cell lysates were subjected to SDS-PAGE and probed with primary antibodies against 4E-BP1, 4E-BP2, eIF4E, eIF4G, cleaved caspase-3 (Cell Signaling), ATF4, CHOP (Santa Cruz), and actin (Sigma). Cell lysates were incubated with 7-methyl-GTP (^{7}mGTP)-Sepharose (Amersham) overnight at 4°C. The ^{7}mGTP-Sepharose was then pelleted and boiled. Experiments were performed at least three times. Band intensity was quantified using Scion Image software.

Metabolic Labeling

Due to the low islet yields from *Ins2*$^{WT/C96Y}$, *Ins2*$^{WT/C96Y}$ *Eif4ebp1*$^{-/-}$, *Wfs1*$^{-/-}$, and *Eif4ebp1*$^{-/-}$ *Wfs1*$^{-/-}$ mice, islets with these genotypes were pooled from two or three mice. Fifty to eighty islets were cultured for 3 days in RPMI supplemented with 10% FCS. Islets washed with methionine/cysteine-free RPMI containing 10% dialyzed FCS were labeled with a protein labeling mix (PerkinElmer) (1.0 MBq/tube) for 15 min and then resolved in sample buffer (1.0 μl/islet for wild-type and *Eif4ebp1*$^{-/-}$ islets and 0.75 μl/islet for other genotypes). The level of protein synthesis was quantified from autoradiograms. For measurement of *Chop* translation, 4×10^6 cells treated with thapsigargin for 12 hr were washed with methionine/cysteine-free DMEM containing 15% dialyzed FCS and labeled with [^{35}S]methionine/cysteine (20 MBq/bottle) for 2 hr. Cells were then resolved in lysis buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 2 mM $MgCl_2$, 0.1% Triton X-100, and protease inhibitors [Roche]). Lysates were precleared with Protein A Sepharose Fast Flow (Amersham) and incubated with anti-CHOP antibody (R-20, Santa Cruz) overnight.

Firefly Luciferase Reporter Assay

Oligonucleotides containing ATF4 binding sites were annealed and subcloned into the pGL3-Promoter vector (BamHI-SalI, Promega). MIN6 cells were transfected with luciferase reporters using Lipofectamine (Invitrogen). Luciferase activity was assayed with a dual-luciferase system (Promega) using a luminometer (Berthold).

Chromatin Immunoprecipitation Assay

Proteins bound to DNA were crosslinked with 1% formaldehyde at 4°C for 20 min. After sonication, the protein-DNA complexes were immunoprecipitated using an anti-ATF4 antibody (C-20, Santa Cruz). After reversal of the crosslinks at 65°C for 6 hr, DNA was purified on a DNA purification column (QIAGEN). PCR was performed with the primers 5′-GATGAGGAAGAGGAAGCTGAGTTG-3′ and 5′-AGTTGTAAGAGGAGTAGTTGGGGG-3′.

Statistical Analysis

Data are presented as means ± SEM. Differences between groups were assessed by Student's t test. $p < 0.05$ was considered significant.

SUPPLEMENTAL DATA

Supplemental Data include six figures and Supplemental References and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/7/3/269/DC1/.

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(H) [^{35}S]methionine/cysteine labeling as in (G) in islets of the indicated genotypes at 6–8 weeks of age. Lane 1, wild-type; lane 2, *Eif4ebp1*$^{-/-}$; lane 3, *Wfs1*$^{-/-}$; lane 4, *Eif4ebp1*$^{-/-}$ *Wfs1*$^{-/-}$. Data from three experiments are summarized in the right panel. *$p < 0.05$.
Error bars represent SEM.

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