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

Suguru Yamaguchi[1,3], Hisamitsu Ishihara[1,*], Takahiro Yamada[1], Akira Tamura[1], Masahiro Usui[1], Ryu Tominaga[1], Yuichiro Munakata[1], Chihiro Satake[1], Hideki Katagiri[2], Fumi Tashiro[4], Hiroyuki Aburatani[5], Kyoko Tsukiyama-Kohara[6], Jun-ichi Miyazaki[4], Nahum Sonenberg[7] and Yoshitomo Oka[1]

[1]Division of Molecular Metabolism and Diabetes,
[2]Division of Advanced Therapeutics for Metabolic Diseases, Center for Translational and Advanced Animal Research,
Tohoku University Graduate School of Medicine, Sendai, Miyagi 980-8575, Japan
[3]Institute for International Advanced Research and Education, Tohoku University, Sendai, Miyagi 980-8578, Japan
[4]Division of Stem Cell Regulation Research, Osaka University Graduate School of Medicine, Suita, Osaka 565-0871, Japan
[5]Research Center for Advanced Science and Technology, University of Tokyo, Tokyo 153-8904, Japan
[6]Department of Experimental Phylaxiology, Faculty of Medical and Pharmaceutical Sciences, Kumamoto University, Kumamoto 860-8556, Japan
[7]Department of Biochemistry and McGill Cancer Center, McGill University, Montreal, Quebec H3G 1Y6, Canada

*Correspondence: hisamitsu-ishihara@mail.tains.tohoku.ac.jp

Running Title: 4E-BP1 in β cell survival under ER stress

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 diabetes models. 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, 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 pre-initiation complex. The mRNA 5' cap-binding protein, eukaryotic initiation factor 4E (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 pre-initiation complex to the mRNA, allowing the complex to scan toward the initiator AUG codon. The two well-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 stimuli, eIF2α can be phosphorylated by four different kinases (Holcik and Sonenberg, 2005). Global attenuation of protein biosynthesis then paradoxically increases expressions 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, 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 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 (ER Ca^{2+} pump inhibitor causing ER stress) (Figure 1A). 4E-BP1 induction was also observed in liver and kidneys of mice administered tunicamycin (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 Cys96Tyr mutation cause ER stress (Wang et al., 1999). Islets from leptin receptor null (*Lepr*$^{-/-}$) mice, which were 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* gene 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 expressions of ATF4 and CHOP peaked at 12 hr after treatment with thapsigargin or tunicamycin, 4E-BP1 protein was further

increased at 24 hr post-treatment (Figure 1D). 4E-BP1 protein induction appeared to result from transcriptional 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 (Figure S1), suppressed 4E-BP1 induction by thapsigargin. Conversely, expression of wild-type ATF4 highly induced 4E-BP1 expression (Figure 2B). Furthermore, *4ebp1* mRNA levels were not increased by thapsigargin in *Atf4*$^{-/-}$ mouse embryonic fibroblasts (MEFs) (Harding et al., 2003) (Figure 2C).

A survey of the mouse *Eif4ebp1* gene using the luciferase assay identified a segment in intron 1, which conferred thapsigargin sensitivity to a luciferase reporter (Figure S2). Indeed, we found two potential ATF4 binding sequences (C/EBP-ATF composite site) 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).

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, have been 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 was accompanied by enhanced caspase 3 cleavage (Figure 3B), indicating reduced MIN6*Eif4ebp1*$^{-/-}$ cell viability to be due, at least partly, to increased apoptosis. In addition, DNA fragmentation under ER stress was greater in *Eif4ebp1*$^{-/-}$ 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 analogue, 7-methyl-GTP, revealed that thapsigargin-induced 4E-BP1 expression resulted in marked increases in 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% ($n = 4$, $p < 0.05$). 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*$^{-/-}$ 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-deficienct cells (Figure 3D).

Translations of newly synthesized mRNA molecules are reportedly much more dependent on eIF4E availability than those of pre-existing 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 with those of HFD-fed wild-type mice. These data suggest *Eif4ebp1*$^{-/-}$ mice to 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 the defect of β cells lacking 4E-BP1.

We then 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 altered neither body weight (Figures S5A and S5B) nor 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 (Figure 4C), with the majority of islets being smaller (Figure 4D), that in *Ins2*$^{WT/C96Y}$ mice at 5 weeks of age. We also observed a 38% decrease in pancreatic insulin content in *Eif4ebp1*$^{-/-}$*Wfs1*$^{-/-}$ as compared to *Wfs1*$^{-/-}$ mice (Figure 4E). Importantly, the insulin positive area was smaller in *Eif4ebp1*$^{-/-}$*Wfs1*$^{-/-}$ than in *Wfs1*$^{-/-}$ mice at 27-30 weeks of age (Figure 4F), indicating ER stress-mediated β cell loss to be exacerbated by 4E-BP1 deficiency in vivo.

Global protein synthesis was studied in these mouse islets. A tendency for decreasing protein synthesis was observed in both *Ins2*$^{WT/C96Y}$ (Figure 4G, hatched column, P = 0.074) and *Wfs1*$^{-/-}$ islets (Figure 4H, hatched column, P = 0.079) as compared to that in wild-type islets. *Eif4ebp1* deletion ablated this regulation and caused significantly higher protein synthesis in *Eif4ebp1*$^{-/-}$*Ins2*$^{WT/C96Y}$ (P = 0.013) and *Eif4ebp1*$^{-/-}$*Wfs1*$^{-/-}$ (P = 0.045) islets than in corresponding single mutants (Figures 4G and 4H). These data suggest accelerated β cell loss under ER stress to be 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 were 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 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 pro-survival proteins

involved in the UPR are stable, while pro-apoptotic 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. Especially, expression of CHOP was augmented in 4E-BP1 deficiency. Enhanced CHOP expression in 4E-BP1-deficient cells suggests that in wild-type cells a reduction in eIF4E availability due to 4E-BP1 induction suppresses CHOP translation during ER stress, 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, 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, of great interest is a recent report demonstrating a gene encoding eIF4A2, a component of eIF4F, to possibly be linked to type 2 diabetes in French families (Cheyssac et al., 2006). Furthermore, our findings raised the possibility of 4E-BP1 being a potential target for diabetes mellitus treatment.

EXPERIMENTAL PROCEDURES

Animal experiments

The Tohoku University Institutional Animal Care and Use Committee approved all animal experiments. *Wfs1*$^{-/-}$ mice were backcrossed to a 129S6 (Taconic) background for six generations. *Ins2*$^{WT/C96Y}$ mice (Charles River) were backcrossed to a 129S6 background for five generations. *Eif4ebp1*$^{-/-}$ mice were maintained on a 129S6 background. Only male mice were used. For in vivo studies, littermates from mating between male *Ins2*$^{WT/C96Y}$*Eif4ebp1*$^{+/-}$ and female *Ins2*$^{WT/WT}$*Eif4ebp1*$^{+/-}$ mice (Figures 4A, 4C and 4D) and littermates from mating of *Eif4ebp1*$^{+/-}$ mice and littermates from mating of *Eif4ebp1*$^{+/-}$*Wfs1*$^{-/-}$ mice (Figures 4B, 4E and 4F) were used. For isolated islet experiments (Figures 4G and 4H), age-matched non-littermate 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, 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, generating 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 non-essential amino acid mixture and 10% FCS. Cells seeded in 24-well plates 2 days before were treated with thapsigargin or tunicamycin

and then 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 as described previously (Ishihara et al., 2004).

Northern, Western blotting and Cap-affinity binding assay

Total RNA extracted using ISOGEN (Nippon Gene) was probed with ^{32}P-labelled 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 2 or 3 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 (Perkin Elmer) (1.0 MBq/tube) for 15 min and then resolved in a 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 x 10^6 cells treated with thapsigargin for 12 hr were washed with Met/Cys-free DMEM containing 15% dialyzed FCS and labeled with [^{35}S]Met/Cys (20 MBq/bottle) for 2 hr. Cells were then resolved

in a lysis buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 2 mM $MgCl_2$, 0.1% Triton X-100, protease inhibitors (Roche)). Lysates were pre-cleared 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 (*Bam*HI-*Sal*I, Promega). MIN6 cells were transfected with luciferase reporters using LipofectAMINE (Invitrogen). Luciferase activities were assayed with a Dual-Luciferase system (Promega) using a luminometer (Berthold).

Chromatin immunoprecipitation assay

The proteins bound to DNA were cross-linked 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 cross-links at 65°C for 6 hr, the DNA was purified on a DNA purification column (Qiagen). PCR was performed with primers, 5'-GATGAGGAAGAGGAAGCTGAGTTG-3' and 5'-AGTTGTAAGAG GAGTAGTTGGGGG -3'.

Statistical analysis

Data are presented as means ± SEM. Differences between groups were assessed by Student's *t-test*. A P value of < 0.05 was considered significant.

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Figure legends

Figure 1. ER stress induces 4E-BP1 expression

(A) Expressions 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 which had received intraperitoneal injections of tunicamycin (Tm) 96 hr before.

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

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

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 the wild-type ATF4 at the indicated multiplicity of infection (m.o.i.).

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