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ATF5 regulates β-cell survival during stress

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The stress response and cell survival are necessary for normal pancreatic β-cell function, glucose homeostasis, and prevention of diabetes. The homeodomain transcription factor and human diabetes gene pancreas/duodenum homeobox protein 1 (*Pdx1*) regulates β-cell survival and endoplasmic reticulum stress susceptibility, in part through direct regulation of activating transcription factor 4 (*Atf4*). Here we show that *Atf5*, a close but less-studied relative of *Atf4*, is also a target of Pdx1 and is critical for β-cell survival under stress conditions. *Pdx1* deficiency led to decreased *Atf5* transcript, and primary islet ChIP-sequencing localized PDX1 to the *Atf5* promoter, implicating *Atf5* as a PDX1 target. *Atf5* expression was stress inducible and enriched in β cells. Importantly, *Atf5* deficiency decreased survival under stress conditions. Loss-of-function and chromatin occupancy experiments positioned Atf5 downstream of and parallel to Atf4 in the regulation of eIF4E-binding protein 1 (*4ebp1*), a mammalian target of rapamycin (mTOR) pathway component that inhibits protein translation. Accordingly, *Atf5* deficiency attenuated stress suppression of global translation, likely enhancing the susceptibility of β cells to stress-induced apoptosis. Thus, we identify ATF5 as a member of the transcriptional network governing pancreatic β-cell survival during stress.

ATF5 | 4EBP1 | pancreatic β cell | stress | apoptosis

Reduced pancreatic β-cell number and function characterize all forms of diabetes. Insulin-secreting β cells are notoriously susceptible to stress, including endoplasmic reticulum (ER), cytokine, and oxidative stress (1–4). Thus, understanding apoptotic cell-fate decisions during stress could provide new targets that could be exploited for the prevention or amelioration of diabetes.

In secretory cells such as the β cell, the unfolded protein response (UPR) and regulation of translation, particularly in response to stress, are key factors in maintaining cellular homeostasis, as clearly demonstrated in mouse models with deficiencies of critical regulators such as protein kinase R-like ER kinase (PERK) and EIF2α (5–7). In humans, PERK mutation causes Wolcott–Rallison syndrome, a rare autosomal recessive disorder characterized by permanent neonatal diabetes (8, 9). Downstream of PERK, the basic leucine zipper (bZIP) transcription factor activating transcription factor 4 (ATF4) regulates the expression of *4ebp1*, a member of the eukaryotic translation initiation factor 4E (eIF4E)-binding protein family (4EBPs). 4EBP1 is the most abundant mammalian isoform in the pancreas (10) and functions as an inhibitor of translation initiation by binding the cap-binding protein eIF4E, thereby preventing formation of the eIF4F translational initiation complex (11, 12). Expression of 4EBP1 is induced by stress, and *4ebp1* deficiency results in deregulated translational control and increased susceptibility to ER stress-mediated apoptosis in β cells (13).

We previously demonstrated that the homeodomain transcription factor and human diabetes gene pancreas/duodenum homeobox protein 1 (*Pdx1*) regulates pancreatic β-cell susceptibility to ER stress. Although PDX1 expression itself is not induced by stress, PDX1 does function to orchestrate islet compensation for insulin resistance, at least in part through direct transcriptional regulation of the UPR mediators ATF4 and Wolfram syndrome 1 (*Wfs1*) (14–16). A cDNA microarray of $Pdx1^{+/-}$ islets indicated *Atf5* as a possible downstream target of *Pdx1* (15). *Atf5* is a close but less well-studied homolog of *Atf4* that also contains a bZIP domain. The interconnected nature of their functions, homology, and expression make it necessary to elucidate the overlapping and independent functions of ATF4 and ATF5 in cell survival. ATF5 binds to CCAAT/enhancer-binding protein (C/EBP)-ATF response element (CARE) sites (17) and is itself a direct transcriptional target of ATF4 and C/EBP homologous protein (CHOP) in response to ER stress (18). Similar to ATF4, ATF5 expression is regulated by both transcriptional and translational mechanisms that allow selective translation in response to ER stress controlled by upstream ORFs (19, 20). ATF4 has established roles in cell susceptibility to ER stress through the regulation of CHOP, whereas ATF5 has been found to have antiapoptotic roles in several tissues, including cartilage, hematopoietic cells, cancers, and olfactory sensory neurons (18–25). Interestingly, ATF5 regulates expression and promotes cell death downstream of CHOP in mouse embryonic fibroblasts (18, 26). Two direct transcriptional targets of ATF5 have been identified in transformed cells, the antiapoptotic factor B-cell chronic lymphocytic leukemia/lymphoma 2 (BCL-2) and mammalian target of rapamycin (*mTOR*), a conserved serine/threonine kinase that controls cell growth (22, 23). The tissue-specific duality of both adaptive and maladaptive roles in cell survival and stress response position ATF5 as an interesting target for study in the β cell, which requires precise regulation of protein homeostasis and stress response for survival.

Here, we examine the β-cell–specific role of ATF5, independent of ATF4, in cell survival. Our results position ATF5 in a cross-regulatory transcriptional network governing protein translation and survival of pancreatic β cells during stress.

Significance

The survival and function of insulin-secreting β cells is critical for the prevention of diabetes. We identify a previously unknown function for activating transcription factor 5 (ATF5) in the apoptotic susceptibility of β cells. ATF5 expression in β cells is stress inducible, and ATF5 deficiency results in a significant increase in β-cell apoptosis in response to stress. Further, eukaryotic translation initiation factor 4E-binding protein 1 (EIF4EBP1), a component of the mammalian target of rapamycin (mTOR) pathway that inhibits protein translation through interaction with EIF4E, is regulated by ATF5 and likely is involved in apoptotic susceptibility through regulation of global translation. Understanding the role of ATF5 deepens our understanding of the complex mechanisms governing β-cell fate decisions, providing a potential pathway for the prevention of diabetes.

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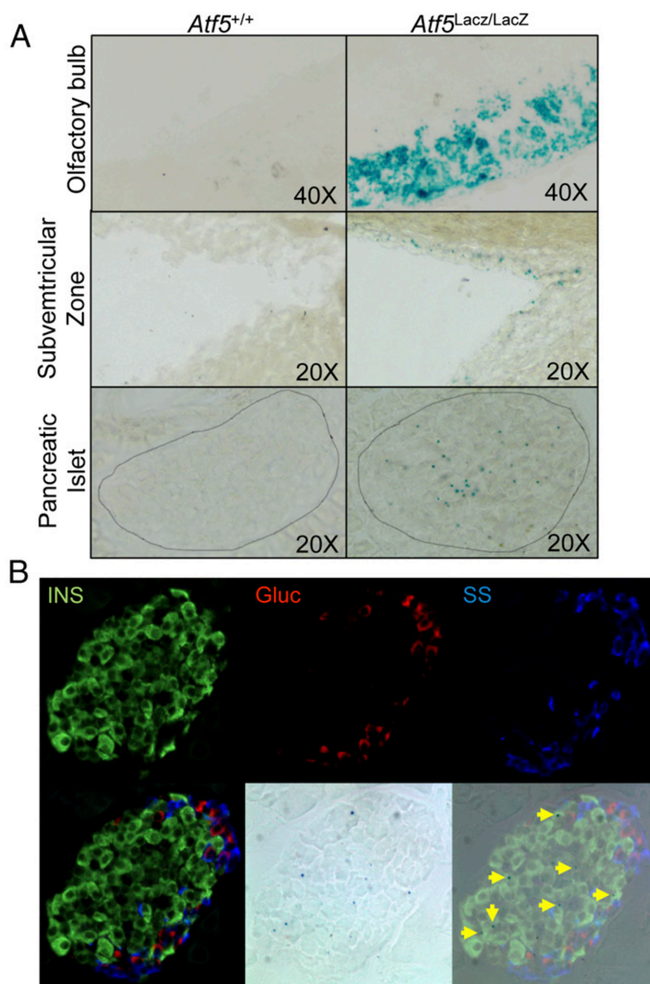

Fig. 1. ATF5 is expressed in pancreatic islets. (*A*) β-Gal activity assessed by X-Gal staining of the olfactory bulb (*Top*), subventricular zone (*Center*), and islets (*Bottom*) from $Atf5^{+/+}$ (*Left*) and $Atf5^{LacZ/LacZ}$ (*Right*) mice. (*B*) β-Gal and immunofluorescent staining of parallel sections from $Atf5^{LacZ/LacZ}$ pancreata for insulin (*Upper Left*), glucagon (*Upper Center*), and somatostatin (*Upper Right*). The lower row shows merged (*Lower Left*), β-gal bright-field (*Lower Center*), and merged IF and X-Gal (*Lower Right*) images. Arrows indicate insulin-positive, β-gal$^+$ cells.

Results

Stress- and PDX1-Regulated Expression of ATF5 in Murine β Cells. We assessed β-gal enzymatic activity in tissues of $Atf5^{LacZ/LacZ}$ mice in which the *LacZ* reporter was homologously recombined into the endogenous *Atf5* locus (25). As previously reported, β-gal activity was observed in the olfactory bulb and the subventricular zone in the brain of $Atf5^{LacZ/LacZ}$ mice but not in $Atf5^{+/+}$ littermate controls (Fig. 1*A*) (21, 25). We observed a similar level of β-gal activity in a subset of insulin-positive cells in the pancreatic islets of $Atf5^{LacZ/LacZ}$ mice, which was not seen in $Atf5^{+/+}$ littermate control islets (Fig. 1). This pattern of β-gal activity was unchanged after treatment with thapsagargin (Tg) (Fig. S1). The expression of ATF5 in β cells of the murine pancreatic islet is consistent with several single-cell transcriptomic studies that identified *Atf5* transcript in murine and human β cells (27–29).

ER stress has an established role in β-cell apoptosis and diabetes development (2, 7, 30, 31). Signs of ER stress have been observed in the β cells of patients with type 2 diabetes, and ER stress is thought to contribute to the β-cell failure and insulin resistance leading to disease progression (32–34). A previous study demonstrated stress induction of ATF5 by arsenite (35). To determine whether ATF5 expression is stress responsive in the β cell, we induced ER stress by Tg or the fatty acid palmitate. Both *Atf5* and *LacZ* mRNA showed a significant increase in $Atf5^{+/+}$ and $Atf5^{LacZ/+}$ islets during the time course of Tg administration, and the protein encoded by the ATF5–LacZ fusion, expressed under the control of endogenous *Atf5* regulatory elements, was induced also (Fig. 2 *A*, *B*, and *D*). $Atf5^{LacZ/+}$ islets expressed wild-type levels of *Atf5* and *LacZ* transcript, suggesting the possibility of autoregulation. Palmitate treatment significantly induced Atf5 mRNA in Min6 cells (Fig. 2*C*). Taken together, these results indicate that ATF5 is expressed in the murine pancreatic islet and that its expression is induced by stress.

A cDNA microarray of $Pdx1^{+/-}$ islets revealed *Atf5* as downstream of *Pdx1* (15). ChIP-sequencing (ChIP-seq) analysis of isolated murine islets demonstrated enrichment of PDX1 near the ATF5 transcriptional start site at an upstream CARE site previously shown to be enriched for ATF4 and CHOP (Fig. 3*A*) (18), suggesting that Pdx1 might regulate *Atf5* expression directly. Indeed, siRNA-mediated reduction of *Pdx1* in Min6 cells reduced the levels of *Atf5* transcript (Fig. 3*B*) and protein (Fig. 3 *C* and *D*). These results are consistent with ATF5 being a direct transcriptional target of PDX1. It is important to note that it has been shown previously that PDX1 expression is not induced by ER stress (16). However, ATF4 and CHOP are selectively translated in response to stress, so it is possible, particularly because they are enriched at similar loci, that PDX1 cooperates with these or other transcription factors to regulate ATF5 expression in the context of stress stimuli.

ATF5 Regulates the Susceptibility of β Cells to Stress-Induced Apoptosis. We observed a significant reduction in cell number and total protein in Min6 cells 72 h after siRNA-mediated reduction of *Atf5* (Fig. 4*A*), and this reduction was corroborated by an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reduction assay (Fig. 4*B*). As compared with nonimmortalized cell lines, immortalized cell lines can be susceptible to stresses induced by high cell density (36). Therefore we hypothesized that ATF5 regulates β-cell viability in the immortalized Min6 insulinoma cell line in response to stress caused by high cell

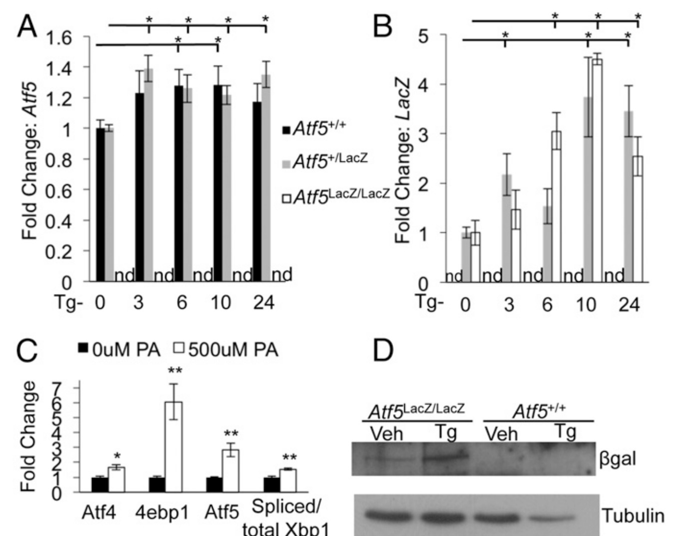

Fig. 2. ATF5 expression is induced by stress. (*A* and *B*) Murine pancreatic islets were isolated from $Atf5^{+/+}$, $Atf5^{+/LacZ}$, and $Atf5^{LacZ/LacZ}$ mice, cultured and treated with Tg (1 uM) over time (expressed in hours), and then were collected and harvested for RNA. qPCR results are shown for *Atf5* (*A*) and *LacZ* (*B*). $n = 3$; nd, not detected. (*C*) Min6 cells were treated with 500 uM palmitate (PA) for 48 h and then were harvested for RNA. qPCR results are shown for *Atf4*, *4ebp1*, *Atf5*, and spliced/total *Xbp1*. $n = 3$. (*D*) Western blot analysis of whole-cell lysate from isolated islets cultured and treated with Tg (1 μM) for 6 h and then harvested for protein. $n = 3$. Bars in *A*–*C* show the mean, and error bars indicate the SEM. *P* values were calculated with Student's *t* test; $^*P \leq 0.02$, $^{**}P \leq 0.01$.

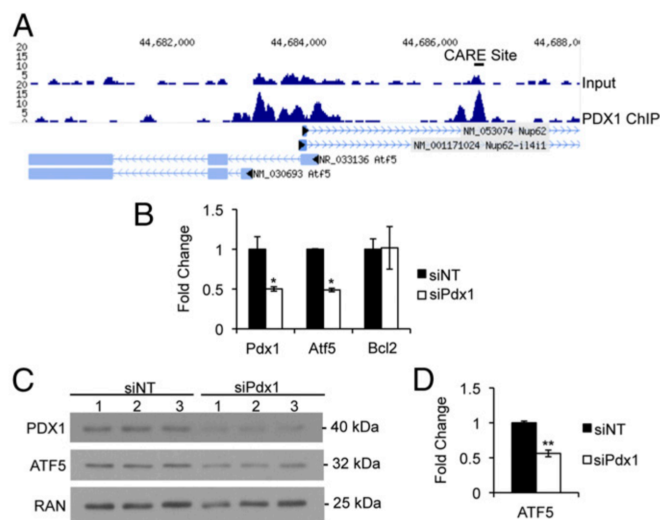

Fig. 3. *Atf5* is a transcriptional target of PDX1. (*A*) Mouse islet PDX1 ChIP-seq profile for the *Atf5* locus. (*B* and *C*) Min6 cells nucleofected with siRNA duplexes targeting PDX1 were harvested 96 h after nucleofection for RNA (*B*) and protein with loading control ras-related nuclear protein (RAN) (*C*). qPCR results are shown for *Pdx1*, *Atf5*, and *Bcl2*. $^{*}P < 0.05$. (*D*) Quantification of Western blot analysis. $n = 6$. Bars in *B* and *D* show the mean, and error bars indicate the SEM. *P* values were calculated with Student's *t* test; $^{**}P < 0.01$.

density. Indeed, we observed a significant increase in the cleavage of caspase-3, which normally is increased during apoptosis, in *Atf5*-deficient Min6 cells over that seen in control cells in the setting of ER stress induced by Tg or amino acid deprivation (Fig. 4*C*). The degree of caspase-3 cleavage in these cells was notably greater than that seen with *Atf4* deficiency (Fig. 4*C*). These observations suggest a role for ATF5 independent of ATF4 in susceptibility to apoptosis.

To assess the potential role of ATF5 in primary β-cell survival, we examined $Atf5^{LacZ/LacZ}$ mice. No overt glucose homeostasis phenotype was observed; the results of glucose and insulin tolerance tests of $Atf5^{LacZ/LacZ}$ mice were indistinguishable from those of control littermates (Fig. S2). Although we previously demonstrated the prosurvival role of Pdx1 in the context of a C57Bl6 genetic background and diet-induced insulin resistance (15), $Atf5^{LacZ/LacZ}$ mice on a C57Bl6 background exhibit high neonatal lethality (25), and the strain of $Atf5^{LacZ/LacZ}$ mice on the Balb/C genetic background used in our study is resistant to a range of metabolic stresses (37–40), including diet-induced metabolic phenotypes (37). These factors precluded the establishment of an in vivo stress model. Therefore, we challenged isolated $Atf5^{LacZ/LacZ}$ islets with Tg to induce ER stress. $Atf5^{LacZ/LacZ}$ islets exhibited a significant enhancement of caspase-3 cleavage compared with $Atf5^{+/+}$ islets after Tg exposure, indicating an increased susceptibility to ER stress-induced apoptosis (Fig. 4*D*). To assess β-cell apoptosis in vivo further, droplet digital PCR was used to measure circulating levels of unmethylated cell-free insulin 2 (*Ins2*) DNA in serum (41–43). Importantly, $Atf5^{LacZ/LacZ}$ mice had significantly more circulating unmethylated *Ins2* DNA than $Atf5^{+/+}$ controls (Fig. 4*E*), strongly supporting a role for ATF5 in reducing β-cell susceptibility to apoptosis.

Identification of *4ebp1* as an ATF5 Transcriptional Target. BCL-2 and mTOR have been identified as direct targets of ATF5 regulation in glioblastoma cells and in BCR-ABL–transformed myeloid progenitor cells, respectively (22, 23). In contrast, *Atf5* deficiency in Min6 cells was not associated with a change in the transcript levels of *Bcl2*, *mTor*, or the mTOR pathway components *S6K* and *Eif4e* (Fig. 5*A*). We did, however, observe a significant reduction in the level of *4ebp1* transcript and protein (Fig. 5 *A* and *B*). Similarly, quantitative PCR (qPCR) and Western blot analysis of $Atf5^{LacZ/LacZ}$ isolated islets demonstrated a significant decrease in *4ebp1* transcript and 4EBP1 protein, respectively (Fig. 5 *C* and *D*). Tg treatment of $Atf5^{LacZ/LacZ}$ islets still induced *4ebp1* transcript; however, the degree of induction was significantly less than in $Atf5^{+/+}$ control islets (Fig. 5*E*). Notably, the reduction of 4ebp1 occurred in the absence of any change in *Atf4* expression in *Atf5*-deficient Min6 cells and primary islets (Fig. 5 *A* and *C*). siRNA-mediated silencing of *Atf4* resulted in a significant decrease in *Atf5* transcript, indicating that ATF4 regulates *Atf5* in Min6 cells, in agreement with previous findings in mouse embryonic fibroblasts (Fig. 5*F*) (18). Deficiency of *Atf4* led to a decrease in *Atf5* and *4ebp1*, whereas *Atf5* deficiency affected *4ebp1* but not *Atf4*, thus positioning *Atf5* both downstream of and parallel to *Atf4* in the regulation of *4ebp1*.

To determine whether ATF5 could regulate *4ebp1* directly, we performed ChIP. Because ATF5 antisera suitable for ChIP are not available, we generated a Min6 cell line stably expressing a C-terminally epitope-tagged form of ATF5 (HA-ATF5) at approximately twofold above endogenous levels (Fig. S3 *A* and *B*). Protein levels expressed by the CMV-driven HA-ATF5 construct were induced by Tg. The induction of HA-ATF5 protein expression by Tg correlated with induction of the *HA-Atf5* transcript, suggesting transcriptional regulation of the expression construct by Tg. Indeed, the CMV promoter can be induced by Tg through the JNK pathway (19, 44, 45). We also noted a significant induction of endogenous Atf5, similar to our observations in primary islets (Fig. 3 and Fig. S3 *B* and *C*). HA-ATF5 was enriched at the CARE site near the transcriptional start site of *4ebp1* by ChIP PCR (Fig. 5*G*). Enrichment also was observed at the CARE site near the *Atf5* transcriptional start site, consistent with autoregulation. HA-ATF5

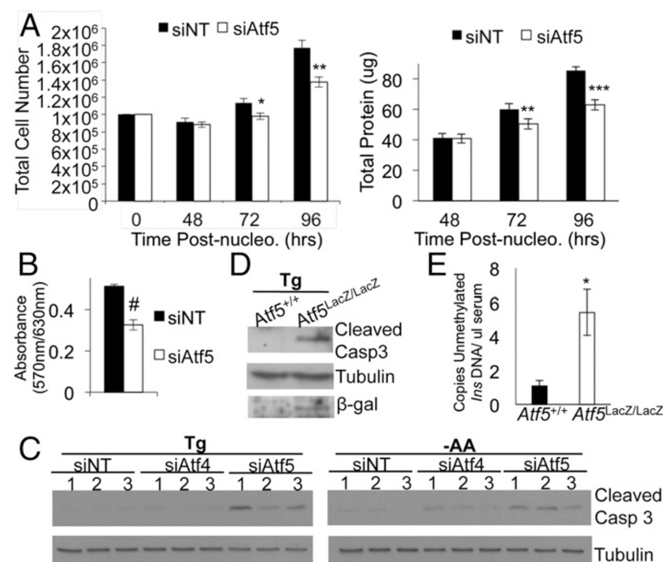

Fig. 4. ATF5 deficiency decreases cell viability and increases stress-induced susceptibility to apoptosis independent of ATF4. (*A*) Growth curve assay. Min6 cells were nucleofected with siRNA targeting *Atf5* (siAtf5) or with nontargeting control siRNA (siNT) and were harvested for cell counting (*Left*) and protein (*Right*). $n = 3$. (*B*) MTT assay performed 96 h after nucleofection. $n = 3$. (*C*) Min6 cells were nucleofected with siRNA targeting *Atf5* or *Atf4* or with control siRNA, were treated at 96 h after nucleofection with either Tg (1 μM) or amino acid deprivation (–AA) for 6 h, and then were harvested for protein and analyzed by Western blot for cleaved caspase-3 and tubulin. Lanes were run on same gel but were noncontiguous. $n = 3$. (*D*) Islets isolated from $Atf5^{+/+}$ or $Atf5^{LacZ/LacZ}$ mice were cultured, treated with 1 μM Tg for 6 h, and then were harvested for protein and analyzed by Western blot for cleaved caspase-3, tubulin, and β-gal. $n = 3$. (*E*) Blood serum was collected from $Atf5^{LacZ/LacZ}$ mice and from $Atf5^{+/+}$ controls. Levels of circulating unmethylated mouse insulin DNA levels were measured by droplet PCR and are denoted as copies per microliter. $n = 6$. Bars in *A*, *B*, and *E* show the mean, and error bars indicate the SEM. *P* values were calculated with Student's *t* test; $^{*}P < 0.02$; $^{**}P < 0.0007$; $^{***}P = 6.1 \times 10^{-8}$; $^{\#}P < 0.001$.

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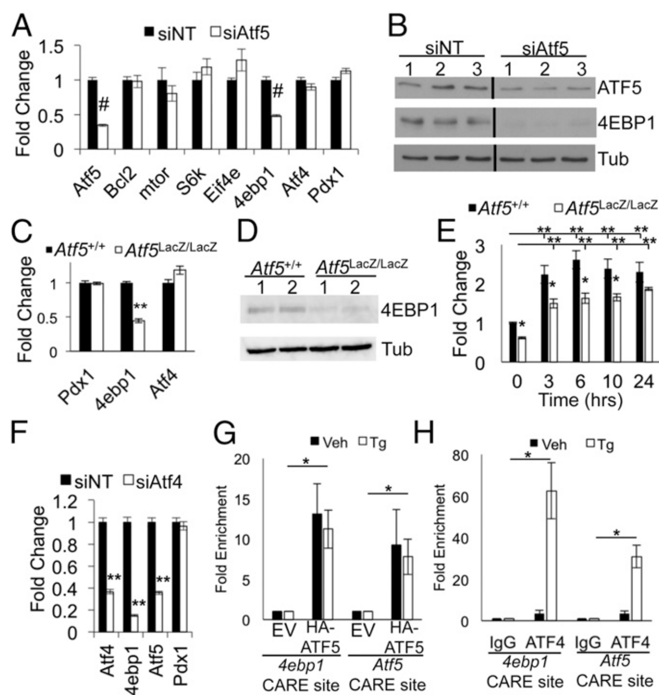

Fig. 5. ATF5 regulates 4ebp1. (*A* and *B*) Min6 cells were nucleofected with siRNA targeting *Atf5* (siAtf5) or with nontargeting control siRNA (siNT) and were harvested 96 h later for RNA (*A*) and protein (*B*). Lanes were run on same gel but were noncontiguous. qPCR results are shown for *Atf5*, *mTor*, *S6k*, *Eif4e*, *4ebp1*, *Atf4*, and *Pdx1*. $n = 6$. (*C* and *D*) Islets isolated from *Atf5*$^{+/+}$ and *Atf5*$^{LacZ/LacZ}$ mice were harvested for RNA (*C*) and protein (*D*). qPCR results are shown for *Pdx1*, *4ebp1*, and *Atf4*. ($n = 5$) (*E*) Islets isolated from *Atf5*$^{+/+}$ and *Atf5*$^{LacZ/LacZ}$ mice were cultured, treated with Tg (1 μM) for the denoted times and then were harvested for RNA. qPCR results are shown for *4ebp1*. $n = 5$. (*F*) Min6 cells were nucleofected with siRNA targeting Atf4 or with nontargeting control siRNA and were harvested for RNA. qPCR results are shown for ATF4, 4ebp1, Atf5, and Pdx1. $n = 3$. (*G* and *H*) Min6 cells were infected with lentivirus expressing C-terminal HA-tagged ATF5 or empty vector control. ChIP PCR was performed on chromatin isolated from the cells expressing HA-ATF5 (*G*) and from wild-type Min6 cells (*H*), with or without treatment with Tg (1 μM) for 6 h using anti-HA, anti-ATF4, or anti-IgG. qPCR results are shown for CARE sites near the transcriptional start sites of *4ebp1* and *Atf5*. *P* values compare TG-treated empty vector (EV) and TG-treated HA-ATF5 cells. $n = 3$. Bars in *A*, *C*, and *E*–*H* show the mean, and error bars indicate the SEM. *P* values were calculated with Student's *t* test; $^{*}P \leq 0.03$; $^{**}P < 2 \times 10^{-4}$; $^{\#}P < 1 \times 10^{-7}$.

enrichment at these CARE sites was not altered by Tg exposure, despite the induction of protein expression, implying that the role of ATF5 at these loci is in basal, and not induced, expression. In contrast, ATF4 was significantly enriched in a Tg-induced manner at the CARE sites of both *4ebp1* and *Atf5*, consistent with previous reports (Fig. 5*H*) (13, 18). Taken together, these findings suggest that ATF5 directly regulates the transcription of *4ebp1* and that ATF5 recruitment is not ATF4 dependent.

***Atf5* Deficiency Increases Global Protein Translation.** Several studies have elucidated the importance of 4E-BP1 expression and translational regulation for β-cell survival, particularly in response to stress (5, 6, 13). A central role for the PERK arm of the UPR response is the down-regulation of global translation to allow recovery by reducing the protein load on the ER while simultaneously and selectively translating transcripts required for recovery, e.g., *Atf4* (5, 19). Perturbations of these translational regulation mechanisms are detrimental to cell survival, particularly in highly secretory cells such as β cells that normally function at augmented levels of protein synthesis (5–8). To assess global translation rates under basal and stressed conditions, we treated *Atf5*-deficient and control Min6 cells with Tg over a time course and measured global translation by puromycin incorporation followed by Western blot analysis. Under basal conditions, ATF5 deficiency induced an increase in basal global translation (Fig. 6*A*). After Tg exposure, rates of translation are initially suppressed at 1 h and then recover over 8–10 h in control cells; in *Atf5*-deficient Min6 cells a notable enhancement of translational recovery was observed beginning at 4 h, and the maximal level of translation at 10 h also was visibly increased (Fig. 6*B*). Similarly, an increased rate of translational recovery after Tg was seen in primary isolated *Atf5*$^{LacZ/LacZ}$ islets compared with *Atf5*$^{+/+}$ islets, with global translation at 10 h surpassing that observed under basal conditions (Fig. 6*C*). The results indicate that *Atf5* deficiency abrogates the reduced protein load in the ER that normally allows the restoration of cellular homeostasis under stress conditions.

Discussion

ER stress is particularly detrimental to highly secretory cells such as pancreatic β cells, because they are dependent on optimal protein synthesis for survival and function. In normal cells, stress and perturbations in protein synthesis lead to the activation of the UPR and eventual restoration of homeostasis. We find that ATF5 is induced by stress and is critical for the survival of β cells during stress. Loss-of-function studies and ChIP assays further positioned *Atf5* in the transcriptional network governing β-cell survival during stress and suggest that 4EBP1 is a direct transcriptional target of ATF5 in this context. Deficiency of ATF5 resulted in an enhancement of global

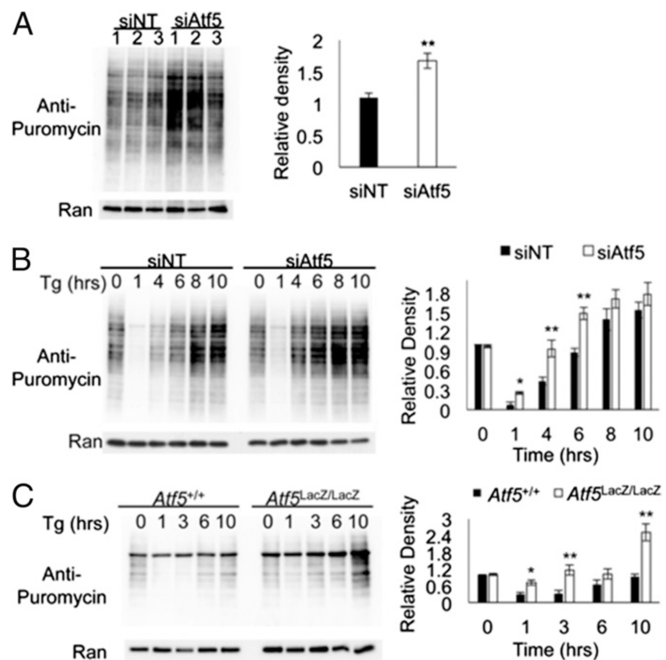

Fig. 6. *Atf5* deficiency enhances the recovery of global translation under stress conditions. (*A*) Min6 cells were nucleofected with siRNA targeting Atf5 (siAtf5) or with nontargeting control siRNA (siNT). At 96 h after nucleofection, cells were treated with puromycin (1 μg/mL) for 30 min and then were harvested for protein. (*Left*) Western blot analysis with anti-puromycin for the basal level of global translation. (*Right*) Measurement of the relative density of blots. $n = 3$. (*B*) Min6 cells were nucleofected with siAtf5 or with siNT. At 96 h after nucleofection, cells were treated with Tg (1 μM) for the denoted time (in hours) and with puromycin (1 μg/mL) for the last 30 min and were harvested for protein. (*Left*) Western blot analysis with anti-puromycin for global translation by puromycin incorporation. (*Right*) Quantitation of relative density. $n = 3$. (*C*, *Left*) Islets harvested from *Atf5*$^{+/+}$ and *Atf5*$^{LacZ/LacZ}$ mice were cultured, treated with Tg (1 μM) for the denoted time, and harvested for protein and Western blot analysis. (*Right*) Quantitation of relative density. $n = 3$. Bars show the mean, and error bars indicate the SEM. *P* values were calculated with Student's *t* test; $^{*}P \leq 0.05$; $^{**}P \leq 0.01$.

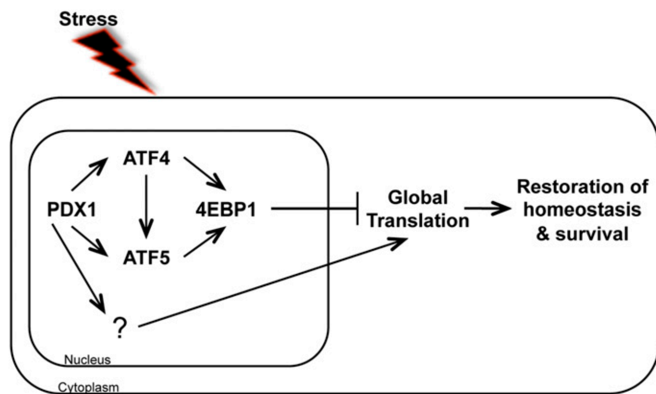

Fig. 7. Role of ATF5 in the survival of β cells during stress. Our findings place ATF5 within the PDX1–ATF4–4EBP1 regulatory pathway, downstream of and parallel to ATF4. ATF5 regulates *4ebp1*, a negative regulator of translation. In response to stress, 4EBP1 binds to EIF4E, preventing it from joining the cap-dependent translation initiation complex, thereby inhibiting global translation. Global translation and protein load on the ER decrease, allowing the cell to restore homeostasis and survive.

translation, explaining, at least in part, the increase in apoptotic susceptibility of β cells during stress.

Loss of ATF5 appears to "prime" the β cell for apoptotic susceptibility by impairing its ability to down-regulate translation in response to stress (Fig. 7). ER stress has been observed in the islets of patients with both type 1 and type 2 diabetes, and multiple studies have demonstrated the importance of translational regulation for β-cell survival (2, 7, 13, 30–34, 46). 4EBP1 is an important negative regulator of translation that reduces the ER protein load during ER stress, allowing the cell an opportunity for recovery and restoration of homeostasis. Droplet digital PCR results measuring circulating cell-free unmethylated Ins2 DNA demonstrated a significant increase in β-cell apoptosis in *Atf5*$^{LacZ/LacZ}$ mice as compared with *Atf5*$^{+/+}$ controls even before stress exposure. The preservation of *Atf5*$^{LacZ/LacZ}$ β-cell mass under basal conditions is likely related to the Balb/C genetic background, possibly through a small adaptive increase in proliferation. We speculate that on a susceptible genetic background such as C57BL6, β-cell mass and function would deteriorate under stress, analogous to the normal glucose homeostasis of *4ebp1*$^{-/-}$ mice that deteriorates under stress imposed by Wfs1 deficiency or expression of the Akita misfolding mutation in *Ins2* (13). This experiment is not yet possible because of the neonatal lethality of *Atf5*$^{LacZ/LacZ}$ mice on a C57Bl6 genetic background and the unavailability of a conditional allele to date. Overall, our findings indicate that *Atf5* deficiency leads to reduced *4ebp1* expression and impaired adaptive translational suppression, which likely contribute to enhanced β-cell susceptibility to stress-induced apoptosis.

The close ATF5 homolog ATF4 has been studied extensively in cellular stress responses, and its downstream targets include both negative and positive regulators of translation and protein synthesis (Fig. 5*G*) (13, 26, 47). There appear to be distinct temporal dynamics and thresholds of ATF4 expression, and thereby protein synthesis, that are beneficial to cell survival. ATF4 is one member of a set of mRNAs specifically translated in the context of stress when there is global translation reduction of most other mRNAs. Translational regulation of ATF4 is controlled by the presence of an inhibitory ORF in the 5′ UTR, which is skipped through delayed ribosomal reinitiation during stress (19). Examination of the ATF5 mRNA sequence demonstrates that its mRNA transcript has a similar dual upstream ORF that could regulate translation in a similar stress-dependent manner. The complexity and overlapping levels of translational regulation highlight the importance of tightly controlled protein synthesis and the specific expression of stress-response components. Our results, consistent with findings in other systems, suggest that *Atf5* regulation of *4ebp1* downstream of *Atf4* may serve as a threshold checkpoint for protein synthesis during cellular stress recovery.

Our chromatin occupancy and loss-of-function experiments position ATF5 both downstream of and parallel to ATF4 in the regulation of *4e-bp1*. Overlapping chromatin occupancy of the CARE sites of *Atf5* and *4ebp1* strongly suggests a level of stress-induced cooperation between ATF4 and ATF5, but the molecular mechanism remains to be determined. It is not yet clear whether they interact in a complex or have a sequential role in DNA binding and transcriptional regulation. One defining characteristic of the bZIP domains is the ability to heterodimerize with other bZIP domain-containing proteins. Although the exact mechanisms of bZIP-domain specificity are not defined, this ability for protein–protein interaction and the plethora of possible bZIP partners creates the potential for diverse interactions. In particular, the bZIP domain containing the C/EBP transcription factor C/EBPβ is highly expressed in β cells (48) and can be induced by ER stress in other cell types (49). Further investigation will be required to determine whether and how C/EBPβ and/or other bZIP and related factors participate with ATF4 and ATF5 in the transcriptional network governing protein translation and cell survival in β cells.

Although the stresses may be distinct, the survival of pancreatic β cells is impaired in both type 1 and type 2 diabetes, and islet inflammation has been observed during the progression of both forms of diabetes (50, 51). In addition to the prosurvival roles we identify here, ATF5 has been identified as a transcriptional regulator of the proapoptotic CHOP transcription factor as well as TXNIP, a proinflammatory component that increases the expression of IL-1β in response to oxidative stress and ER stress (52, 53). The effect of stress on the β cell has been reported to depend on the length and severity of the stresses (2, 54, 55). Reduction of the global translational load allows recovery and return to homeostasis, but long-term suppression of translation would not be beneficial to the β cell. Our findings suggest that ATF5 is necessary for survival in the setting of acute stress through suppression of translation; however, the overall function of ATF5 in the regulation of the proinflammatory and proapoptotic components may be to serve as a threshold checkpoint for chronic stresses that cannot be overcome. The involvement of ATF5 in both inflammatory signaling and translational regulation in response to environmental stresses suggests a nuanced role at the apex of β-cell survival that requires further investigation. The identification of ATF5 as a potential gatekeeper of decisions regarding β-cell fate during stress introduces a potential node for therapeutic intervention to prevent or ameliorate diabetes by improving β-cell durability.

Materials and Methods

Mice. The *Atf5*-knockout allele (herein referred to as *Atf5*$^{LacZ/LacZ}$) was generated by inserting a LacZ-Neo cassette in frame, replacing most of exon 1 and all of exons 2 and 3 (26). To assess β-cell apoptosis, droplet digital PCR (Bio-Rad QX200 Droplet Digital PCR System) of serum was used to measure unmethylated insulin DNA (44). All animal procedures were approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

Cell Culture and siRNA Nucleofection. Passage 25-35 Min6 cells were cultured in DMEM (Invitrogen) supplemented with 10% (vol/vol) FBS. For siRNA experiments, cells were nucleofected by AMAXA with predesigned siRNAs targeting *Pdx1*, *Atf5*, or *Atf4* or with a nontargeting control (Ambion) and were harvested 72–96 h after nucleofection for protein or RNA.

Islet Isolation and Culture. Islets were isolated from 8- to 10-wk-old mice by collagenase digestion (56). After hand-picking and overnight culture in RPMI 1640, size-matched islets were treated with vehicle (DMSO), 1 μM Tg (Sigma), or 500 μM palmitate for the denoted times.

Immunofluorescence and β-Gal Staining. Immunofluorescence (IF) was carried out as previously described (57). Additional information is given in *SI Materials and Methods*.

Western Blot Analysis. Protein lysates were prepared using Nonidet P-40 lysis buffer (0.4% Nonidet P-40, 150 mM NaCl, 50 mM Tris base, pH 7.6). Western blot analysis was carried out as previously described (15). Use and dilution

information for antisera is found in Table S1. Additional information is given in *SI Materials and Methods*.

ChIP. ChIP was carried out as previously described (15). Primer sequences are found in Table S2. Additional information is given in *SI Materials and Methods*.

RNA Isolation and qPCR. Min6 cells and isolated islets were harvested and stored in TRIzol (Invitrogen). Min6 RNA was extracted using ethanol precipitation. Islet RNA was extracted using the RNeasy Mini Kit (QIAGEN). Min6 samples were reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and random primers. Islet samples were reverse transcribed using SuperScript III (Invitrogen) and oligo(dT). Transcript was analyzed by qPCR (Bio-Rad CFX384) and normalized to hypoxanthine-guanine phosphoribosyltransferase (*Hprt*). Primer sequences are given in Table S2.

Measurement of Global Translation. Min6 cells were nucleofected with siRNA targeting Atf5 or with nontargeting (NT) control siRNA. Nucleofected cells or primary $Atf5^{+/+}$ and $Atf5^{LacZ/LacZ}$ islets were treated with 1 μM Tg for the denoted times or were left untreated. Puromycin (1 μg/mL) was applied during the last 30 min of incubation. Cells were washed with 1× PBS and collected for protein.

Statistics. Data are presented as mean ± SEM. Differences between compared groups were achieved by two-tailed Student's *t* tests and were considered significant when *P* values were less than 0.05.

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