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Research Article

Metabolism

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Inactivation of specific β cell transcription factors in type 2 diabetes

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Type 2 diabetes (T2DM) commonly arises from islet β cell failure and insulin resistance. Here, we examined the sensitivity of key islet-enriched transcription factors to oxidative stress, a condition associated with β cell dysfunction in both type 1 diabetes (T1DM) and T2DM. Hydrogen peroxide treatment of β cell lines induced cytoplasmic translocation of MAFA and NKX6.1. In parallel, the ability of nuclear PDX1 to bind endogenous target gene promoters was also dramatically reduced, whereas the activity of other key β cell transcriptional regulators was unaffected. MAFA levels were reduced, followed by a reduction in NKX6.1 upon development of hyperglycemia in *db/db* mice, a T2DM model. Transgenic expression of the glutathione peroxidase-1 antioxidant enzyme (GPX1) in *db/db* islet β cells restored nuclear MAFA, nuclear NKX6.1, and β cell function in vivo. Notably, the selective decrease in MAFA, NKX6.1, and PDX1 expression was found in human T2DM islets. MAFB, a MAFA-related transcription factor expressed in human β cells, was also severely compromised. We propose that MAFA, MAFB, NKX6.1, and PDX1 activity provides a gauge of islet β cell function, with loss of MAFA (and/or MAFB) representing an early indicator of β cell inactivity and the subsequent deficit of more impactful NKX6.1 (and/or PDX1) resulting in overt dysfunction associated with T2DM.

Introduction

Oxidative stress appears to contribute to pancreatic islet β cell dysfunction in both type 1 (T1DM) and type 2 (T2DM) diabetes (1–6). As a consequence, understanding how oxidative stress impacts β cells is clearly of therapeutic relevance. Compelling evidence indicates that the accumulation of ROS, such as hydroxyl radical and hydrogen peroxide (H_2O_2) generated by increased glucose and/or lipid metabolism, causes cell inactivation and death (7). For example, the levels of oxidative stress markers are significantly higher in human T2DM islets (e.g., 3-nitrotyrosine and 8-hydroxy-2'-deoxyguanosine) (8, 9). Notably, islet β cells have unusually low antioxidant enzyme levels (e.g., glutathione peroxidase-1 [GPX1] and catalase), thus exposing their proteins, lipids, and/or DNA to oxidative modifications (10, 11). Importantly, antioxidant treatment can prevent the onset of diabetes in animal models of T1DM (6, 12) as well as improve β cell function in T2DM animal models (3–6, 12, 13) and human T2DM islets (8, 9, 14).

Maturity-onset diabetes of the young (MODY) typically results from mutations in islet-enriched transcription factors, with 6 of 9 MODY genes encoding transcription factors that are required in β cell development and/or function (15, 16). Furthermore, mutations in other distinct islet transcription factors decrease β cell function in vivo (e.g., MAFA, refs. 17, 18; NGN3, ref. 19; and PAX6, refs. 20, 21). Collectively, these observations indicate that islet β cell transcription factors could be primary targets of oxidative stress, with reduced (or induced) expression of their target genes resulting in cell dysfunction.

Experiments with β cell lines have demonstrated that 2 transcription factors, MAFA and PDX1, are inactivated under the oxidative stress conditions imposed by supraphysiological glucose levels (22). For example, the reduction in insulin-driven reporter activity and insulin mRNA levels coincided with a specific reduction in PDX1 and MAFA gel-shift binding activity, although the change in MAFA occurred earlier than in PDX1 and correlated more closely with the loss in insulin expression (23). Notably, mice only lacking MAFA in the pancreas (i.e., termed *Mafa^{ΔPanc}*) (18) are glucose intolerant, but have normal fasting glucose levels, whereas loss of PDX1 from islet β cells causes overt hyperglycemia (24–27). These data suggest that β cell inactivity results from the stepwise loss of MAFA and then PDX1 under glucotoxic conditions (22, 23, 28). Interestingly, the N-acetyl-L-cysteine antioxidant improved both MAFA (referred to as RIPE3b1 activator) and PDX1 gel-shift-binding ability as well as β cell function in HIT-T15 β cells and in the ZDF T2DM rat model (3, 4). Moreover, transgenic β cell-specific *Gpx1* expression profoundly increased β cell function in the T2DM *db/db* mouse model, coinciding with the recovery of nuclear MAFA (5). The insulin secretion defects in human T2DM islets were also improved in vitro upon treatment with ROS scavengers (8, 9, 14).

In this study, islet-enriched transcription factor levels and activity in β cell lines were first screened for sensitivity to H_2O_2 , an effector of oxidative stress. Our results demonstrated that MAFA, MAFB, PDX1, and NKX6.1 were selectively inactivated. We further observed that MAFA and NKX6.1, the latter being essential for islet β cell development and function (29–31), were sequentially and selectively lost upon induction of hyperglycemia in *db/db* mice. Nuclear NKX6.1 and MAFA were restored by transgenic *Gpx1* antioxidant enzyme production in *db/db* islet β cells. Importantly, MAFA, MAFB, PDX1, and NKX6.1 levels were also severely

Conflict of interest: The authors have declared that no conflict of interest exists.

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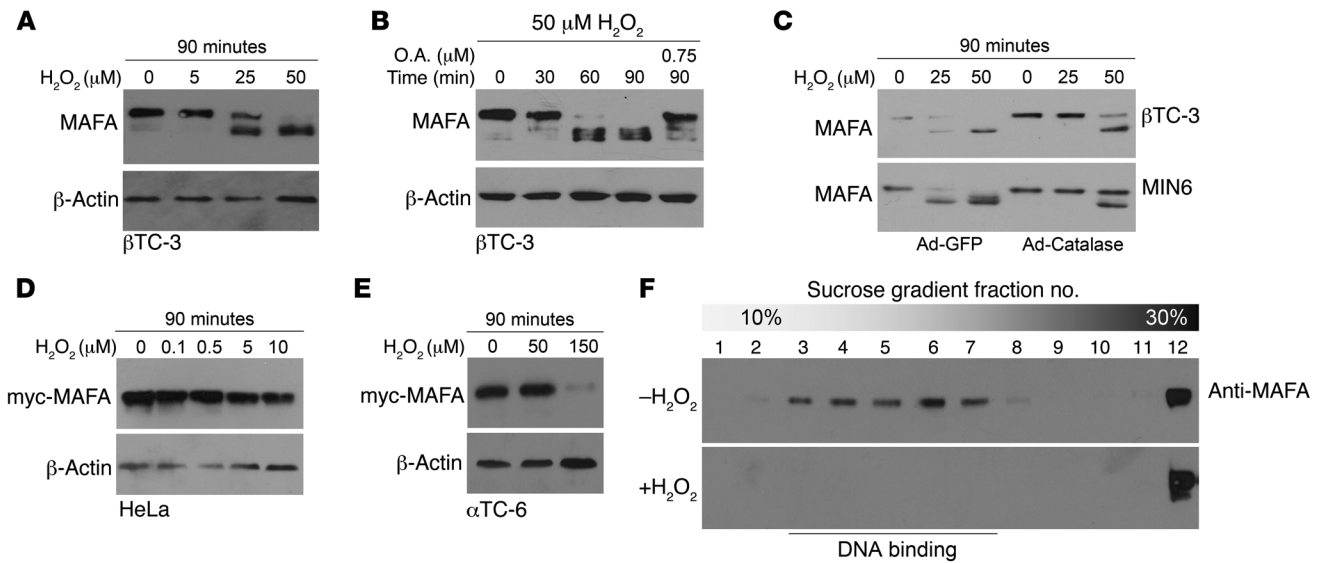


Figure 1

H₂O₂ treatment induces MAFA dephosphorylation in βTC-3 cells but not in HeLa or αTC-6 cells. (A) βTC-3 cells treated with H₂O₂. (B) βTC-3 cells incubated with 0.75 μM okadaic acid (O.A.) for 30 minutes before H₂O₂ addition. (C) Adenovirus-driven catalase decreases the effect of H₂O₂. (D and E) Transfected myc-MAFA is not dephosphorylated after H₂O₂ treatment in HeLa cells or αTC-6 cells. (F) βTC-3 cells were treated with (+) or without (–) 50 μM H₂O₂ for 90 minutes, and the whole-cell extract was separated by sucrose gradient ultracentrifugation (35). The MAFA DNA-binding species (fractions 3–7) was not detected after H₂O₂-induced dephosphorylation (data not shown and ref. 35). (A–F) MAFA protein was analyzed by immunoblotting.

compromised in human T2DM islets. Overall, our results strongly suggest that inactivation of a specific subset of islet-enriched transcriptional regulators contributes to β cell dysfunction in T2DM. We propose the possibility that disease progression involves the early loss of MAFA (and/or MAFB), with debilitating β cell function resulting from increased production of stress factors and subsequent loss of PDX1 and/or NKX6.1.

Results

MAFA is translocated to the cytoplasm and dephosphorylated under oxidative stress conditions. MAFA is heavily phosphorylated in vivo, a post-translational modification regulating dimerization and DNA binding, transactivation capacity, and stability (32–35). To determine whether oxidative stress affects MAFA phosphorylation, βTC-3 cells were briefly treated with H₂O₂ and whole-cell extracts were probed for MAFA by immunoblotting. The SDS-PAGE mobility of MAFA was substantially faster after exposure to H₂O₂ (Figure 1A), migrating in a manner observed after serine/threonine protein phosphatase treatment (35). Pretreatment of βTC-3 cells with okadaic acid, an inhibitor of endogenous protein phosphatase (PP) 1 and 2A (36), prevented this change in mobility (Figure 1B). Infecting βTC-3 or MIN6 β cells with adenovirus-driven catalase, an H₂O₂ scavenger, attenuated the impact of H₂O₂ on MAFA dephosphorylation (Figure 1C). In addition, the sucrose gradient sedimentation properties of MAFA were impacted by H₂O₂ treatment, with localization now exclusively in the fraction containing high molecular weight proteins and not in the approximately 92 kD MAFA species capable of DNA binding (Figure 1F) (35).

MAFA is found in the *db/db* β cell cytoplasm under hyperglycemic conditions (5). In addition, PDX1 shuttling between the nuclear periphery and nucleoplasm is phosphorylation dependent and regulated by external stimuli (i.e., glucose and insulin) (37).

Cytoplasmic and nuclear fractions from H₂O₂-treated βTC-3 cells were collected and probed for MAFA by immunoblotting to determine whether H₂O₂ treatment affected subcellular distribution. MAFA was found in the cytoplasm within 30 minutes after H₂O₂ treatment, while little remained in the nucleus after 90 minutes (Figure 2A). Cytoplasmic accumulation was dependent on exposure time and dosage (Figure 2, A and B). In addition, cytoplasmic MAFA was clearly detectable by immunofluorescence staining in treated βTC-3 cells (Figure 2C).

The susceptibility of MAFA to oxidative inactivation was compared with MAFB, a closely related islet-enriched protein (18, 38, 39). MAFB was equally responsive to H₂O₂ in βTC-3 cells (Figure 2E), with C-terminal sequences spanning the conserved MAFA and MAFB DNA-binding/dimerization domain sufficiently to mediate nuclear-to-cytoplasmic translocation (see myc-MAFA [210–323] and myc-MAFB [210–323] in Supplemental Figure 1A). Notably, translocation was unaffected by pretreatment with okadaic acid, a phosphatase inhibitor that prevents MAFA dephosphorylation in vivo (Supplemental Figure 1B), or by C-terminal phosphorylation site mutants (Supplemental Figure 1, A and B). These results demonstrate that MAFA dephosphorylation is not necessary for cytoplasmic translocation, with dephosphorylation likely catalyzed by PP1 and/or PP2A in the cytoplasm (Figure 2A).

Oxidation-mediated inactivation of MAFA results from covalent dimer formation by C-terminal cysteines. MAFA was only dephosphorylated (Figure 1, D and E) and translocated to the cytoplasm (Figure 2, F and G) after H₂O₂ treatment of β cell lines, and not non-β cell lines (i.e., HeLa or αTC-6). However, the non-β cell lines were responsive to H₂O₂ treatment (40), as noted, for example, by the translocation of the FOXO1 transcription factor from the cytoplasm to the nucleus in αTC-6 cells (Figure 2D), which occurs under a variety of different stress conditions (41, 42). The ability of exogenously

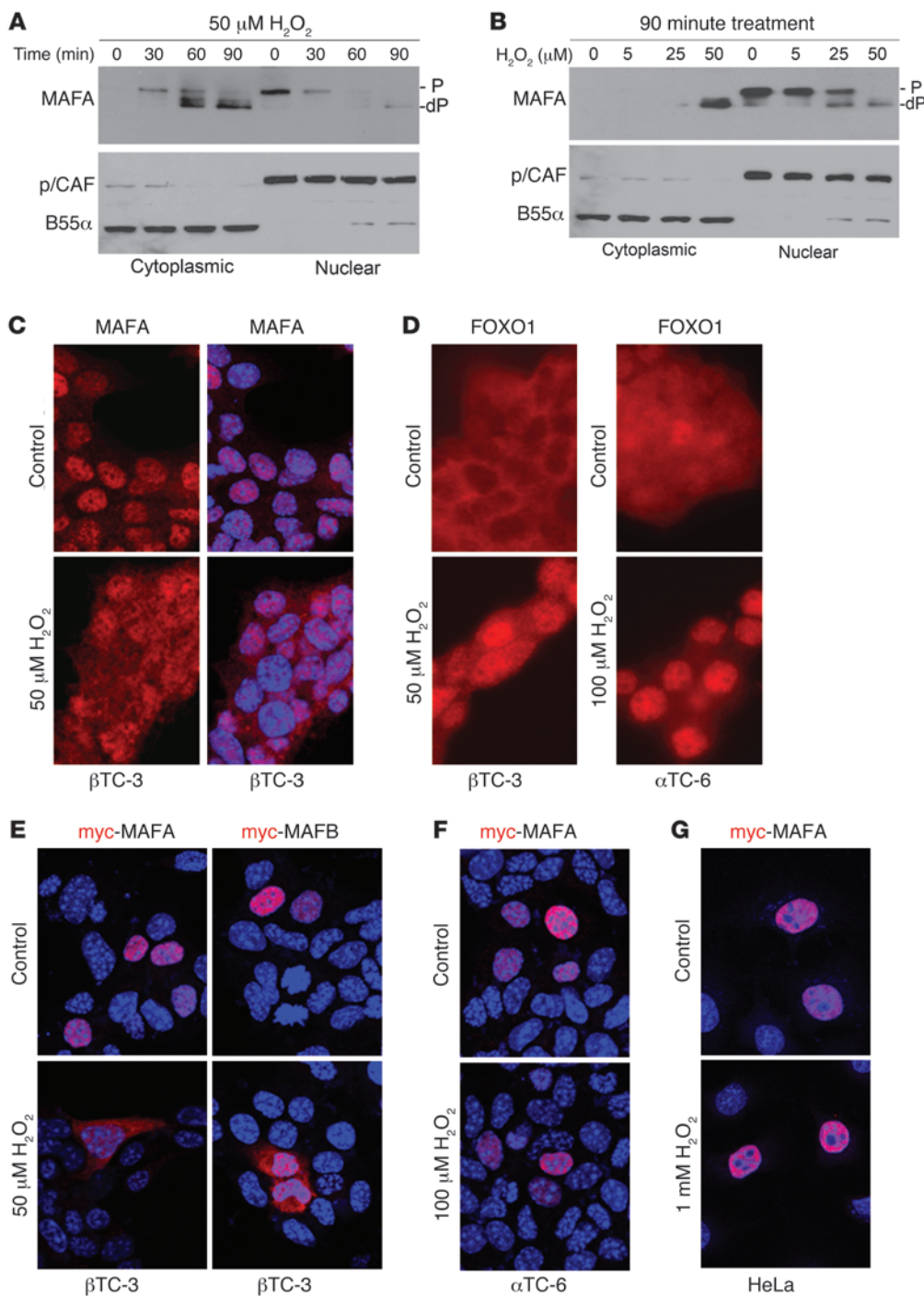


Figure 2 MAFA is translocated to the cytoplasm after H₂O₂ treatment. (**A** and **B**) Immunoblotting to determine MAFA levels in the cytoplasmic and nuclear fractions of β TC-3 cells. Subcellular marker controls: nuclear, p/CAF coactivator; cytoplasm, B55 α regulatory subunit of PP2A. The immunosignal shows (**C**) MAFA, (**D**) FOXO1, (**E–G**) myc-MAFA, or (**E**) myc-MAFB levels in cells treated with H₂O₂ for 90 minutes. Yopro (blue) staining labels the nuclei. Notably, myc-MAFA is not cytoplasmic in non- β cell lines (**F** and **G**) after H₂O₂ treatment, although endogenous FOXO1 (**D**) is relocalized to the nucleus. P, phosphorylated; dP, dephosphorylated.

expressed catalase to attenuate H₂O₂ effects on MAFA in β TC-3 and MIN6 cells suggests a relatively low antioxidant enzyme level of β cells (Figure 1C). As a further test, we analyzed whether MAFA and/or PDX1 DNA-binding activity in β TC-3 nuclear extracts was dependent on the DTT-reducing agent. Notably, only MAFA activity required the presence of DTT (Figure 3A). However, MAFA activity was also sensitive to these conditions in HeLa-transfected extracts, with inactivation coinciding with covalent dimer formation (MAFA)₂ (Figure 3B). Analysis of cysteine mutations throughout MAFA, an amino acid particularly responsive to oxidative

conditions, demonstrated a distinct sensitivity of C277 and C293 to H₂O₂ treatment (Figure 3C). Thus, (MAFA)₂ was not produced, and the DNA-binding activity of MAFA was unaffected by the C277/293A mutation in HeLa-transfected cell extracts, but was affected by the C42/59/69S mutation. Moreover, these same conserved basic leucine-zipper dimerization region cysteines in MAFB were solely responsive to oxidative stress (data not shown). Notably, only the migration and native MAFA levels were compromised in β TC-3 nuclear extracts prepared in the absence of DTT [Figure 3D, note the (MAFA)₂ production] compared with other islet-enriched

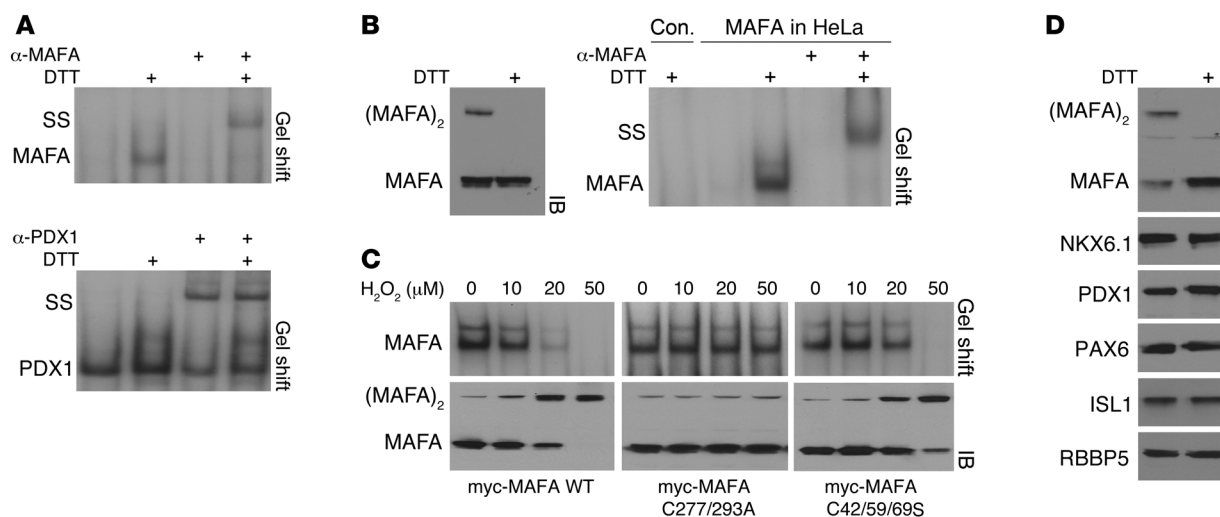


Figure 3 MAFA forms a covalent dimer species unable to bind DNA under oxidative stress conditions. **(A)** MAFA and PDX1 DNA-binding activity in β TC-3 nuclear extract reactions conducted in the presence (+) and absence of 20 mM DTT. **(B)** Transfected myc-MAFA containing HeLa nuclear extracts prepared in the absence of DTT was analyzed in gel-shift (right panel: [+] 20 mM DTT) and immunoblotting assays (left panel: [+] 300 mM DTT). **(C)** The HeLa-produced myc-MAFA C277/C293A mutant does not lose gel-shift activity or form (MAFA)₂ after H₂O₂ treatment, in contrast to WT or mutant C42/59/69S myc-MAFA. **(D)** Immunoblotting shows that only native MAFA (i.e., ~46 kD) levels and few, if any, other islet-enriched transcription factors were reduced in β TC-3 nuclear extract prepared in the absence of DTT. IB, immunoblotting; SS, antibody super-shifted complex.

factors in β TC-3 nuclear extracts prepared in the absence of DTT (Figure 3D). These results clearly illustrate the increased sensitivity of MAFA to oxidative stress changes. In addition, the ability to produce the dysfunctional (MAFA)₂ species in both HeLa and β TC-3 extracts suggests that intact β cell sensitivity reflects differences in antioxidant defense mechanisms compared with non- β cells.

NKX6.1 and PDX1 are also inactivated by H₂O₂ treatment in β cell lines. Previous studies have shown that only nuclear MAFA levels, and not PDX1 levels, are reduced in diabetic *db/db* mouse islets (5, 42). Furthermore, PDX1 gel-shift binding activity is not decreased in these extracts when compared with normoglycemic controls (43). However, since *Mafa*^{ΔPanc} mice are euglycemic (18), the loss of this transcription factor alone would not cause hyperglycemia in *db/db* mice (5, 44). Thus, we next screened to determine whether the subcellular distribution and/or activity of other islet-enriched transcription factors was sensitive to H₂O₂ exposure in β cell lines. Strikingly, in addition to the positive indicators of oxidative stress, MAFA and FOXO1, only the nuclear content of the islet β cell-enriched NKX6.1 transcription factor was affected (Figure 4A). MAFA was more responsive to H₂O₂ than was NKX6.1, as nearly 90% was in the cytoplasm compared with approximately 60% for NKX6.1. In addition, immunostaining also showed NKX6.1 translocation to the cytoplasm and few other islet-enriched transcription factors or cofactors (Figure 4B and Supplemental Figure 2). The oxidative stress-induced increase in NKX6.1 SDS-PAGE mobility also resulted from dephosphorylation (Figure 4A and Supplemental Figure 1C), and like MAFA, the phosphosite mutants did not prevent cytoplasmic accumulation in H₂O₂-treated β TC-3 cells (Supplemental Figure 1, D and E).

Altered PDX1 activity is commonly associated with β cell dysfunction under chronically high glucose conditions, as revealed by loss of in vitro gel-shift binding ability in diabetic ZDF rat islets and HIT-T15 β cell extracts (4). However, nuclear PDX1

levels were unchanged in H₂O₂-treated β TC-3 cells (Figure 4A), with protein removed from the nuclear periphery during extraction being the probable source of the control and experimental cytoplasmic signal (37). ChIP assays were next performed to determine whether nuclear PDX1-binding activity to endogenous target gene promoter sequences was impacted by H₂O₂ in β TC-3 cells. The time course of inactivation was indistinguishable between PDX1, MAFA, and NKX6.1 (Figure 4C), implying that these stress conditions had a similar negative effect on endogenous promoter-binding ability. In contrast, the endogenous binding ability of other islet-enriched transcription factors was unchanged (Figure 4C). Collectively, these cell line-based experiments provide insight into both the complexity and selectivity of islet-enriched transcription factor inactivation under conditions simulating those of T2DM in vivo.

Nuclear NKX6.1 levels are profoundly reduced in diabetic db/db β cells and rescued by GPX1 expression. The *db/db* mouse is a model of obesity and T2DM due to a deficiency in leptin receptor activity (45, 46). Translocation of FOXO1 to the nucleus is a barometer of oxidative stress in these mice (Figure 5B) (41, 42, 47). The nuclear content of MAFA and NKX6.1 was drastically and specifically reduced in islet β cells of hyperglycemic 10-week-old *db/db* mice, seen both by immunostaining and immunoblotting (WT mice blood glucose levels were 163 ± 22 mg/dl; *db/db* mice blood glucose levels were 500 ± 73) (Figure 5). As observed in H₂O₂-treated β cell lines, the nuclear levels of many other transcription factors and coregulators were unchanged in *db/db* islets (Figure 5A and Supplemental Figure 2). Islet β cell function and nuclear MAFA content were rescued upon transgenic expression of *Gpx1* in *db/db* mice (5), which functions to detoxify the H₂O₂ generated under hyperglycemic conditions. *Gpx1* expression also returned NKX6.1 and GLUT2 subcellular levels to normal (Figure 6); GLUT2 is the β cell's principal glucose transporter and is MAFA regulated (17). Of significant note, NKX6.1 affects adult β cell function more pro-

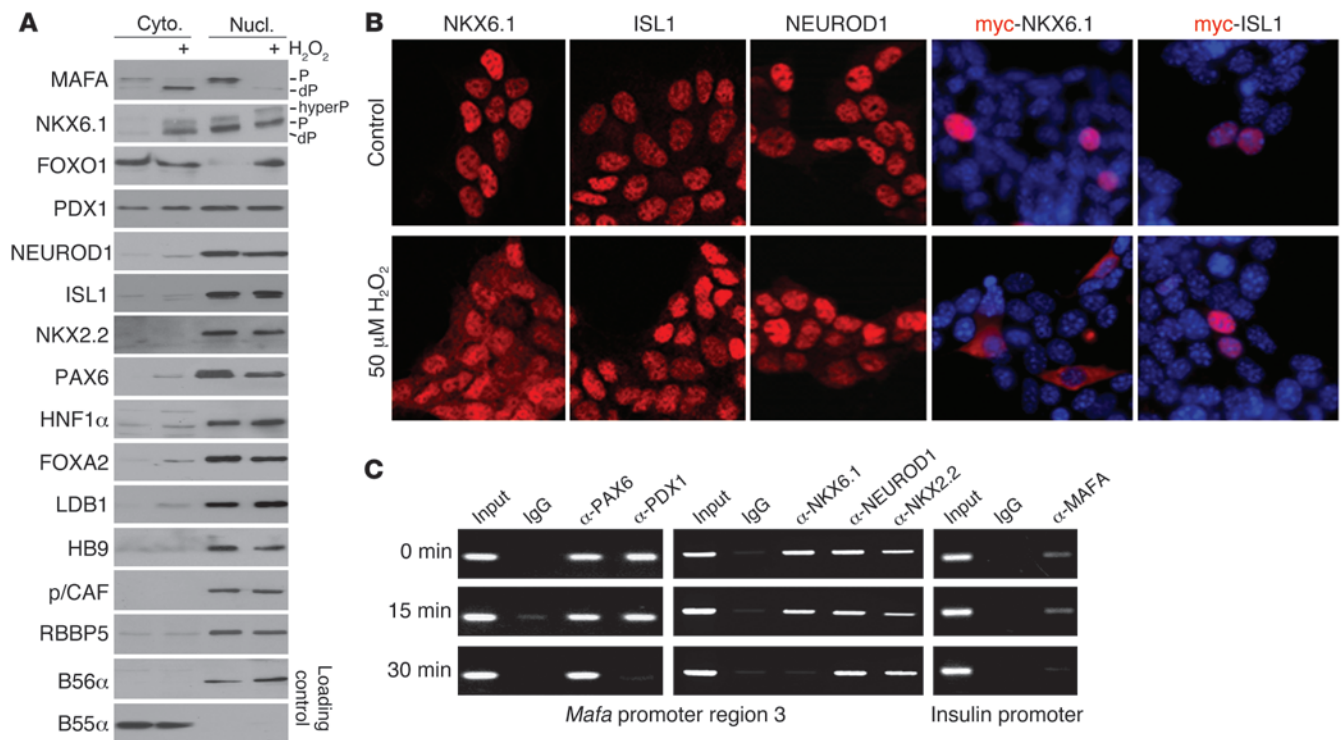


Figure 4
 NKX6.1 and PDX1 are also inactivated in H₂O₂-treated βTC-3 cells. **(A)** Cytoplasmic and nuclear distribution of a variety of regulatory factors in βTC-3 cells treated with or without H₂O₂ for 90 minutes. The change in NKX6.1 and MAFA mobility and subcellular localization is shown by immunoblotting, with levels quantitated using NIH ImageJ software. Dephosphorylation causes MAFA (35) and NKX6.1 to run at a faster mobility on SDS-PAGE (see also Supplemental Figure 1C). **(B)** Immunostaining shows endogenous or myc-tagged transfected NKX6.1, ISL1, and/or NEUROD1 subcellular localization in control and treated cells. **(C)** Oxidative stress results in the specific loss of endogenous MAFA, NKX6.1, and PDX1 binding to target gene regulatory sequences in ChIP assays. hyperP, hyperphosphorylated.

foundly than MAFA, with overt hyperglycemia observed within days of deletion in adult *Pdx1-Cre^{ER};Nkx6.1^{f/f}* mice (B. Taylor and M. Sander, unpublished observations).

Islet β cell proliferation precedes the sequential loss of nuclear MAFA and NKX6.1 in db/db mice. Elevated blood glucose levels are first observed at 4 weeks, and hyperglycemia plateaus at around 12 weeks in *db/db* mice (5). The early rise in glucose is due to insulin resistance primarily in muscle, liver, and adipose tissues. A normal response to conditions of reduced peripheral insulin sensitivity, also seen during pregnancy, is increased β cell proliferation to improve insulin availability (48–51). Here we have compared the temporal change in MAFA, NKX6.1, and FOXO1 levels with β cell proliferation in 4-, 6-, 8-, and 10-week-old *db/db* mice. The early nuclear activation of FOXO1 protects β cells against oxidative damage, in part, through stimulation of *Mafa* expression (41).

Immunostaining results showed that FOXO1 nuclear translocation occurs at around 6 weeks (Supplemental Figure 3), preceding the major changes in MAFA and NKX6.1 nuclear content at 8 and 10 weeks, respectively (Figure 7, A and B). The loss of GLUT2 levels closely correlated with the decrease in MAFA (Supplemental Figure 3), an expected result considering the importance of MAFA to *Glut2* expression (17). Notably, increases in β cell proliferation preceded FOXO1 nuclear translocation, as more than 20% of 4-week-old insulin-positive cells were positive for Ki67, a cell replication marker (Figure 7C). However, only 1% of these cells were still Ki67⁺

by 6 weeks. These results indicated that an initial stress signal(s) in *db/db* mice mediates adaptive β cell expansion and FOXO1 translocation (41), while hyperglycemia and oxidative stress lead to β cell dysfunction by targeting MAFA and then NKX6.1.

MAFA, MAFB, PDX1, and NKX6.1 levels are decreased in human T2DM islet β cells. The data collected in animal models of T2DM suggest that β cell dysfunction could reflect the loss of MAFA (5) and NKX6.1 (Figure 5) in *db/db* mice or MAFA and PDX1 in ZDF rats (4). Islets from 8 normal control and 8 T2DM donors were obtained to examine whether a similar association existed in humans. As expected, the T2DM islets had poorer glucose-stimulated insulin secretion properties than the normal controls (Supplemental Figure 4A). The mRNA and protein levels of human MAFA, MAFB, PDX1, and NKX6.1 were compared with a variety of transcription factors unaffected by oxidative stress in β cell lines and *db/db* islets. MAFB was also assessed, since this large MAF factor is coexpressed with MAFA in human islet β cells (52, 53), a situation not found in rodents (38, 54). In addition, we also analyzed NEUROGENIN3 (i.e., NEUROG3), OCT4 (POU5F1), NANOG, and L-MYC (MYCL1) levels, transcription factors associated with progenitor cell development that were induced in islet β cells of T2DM animal models, including *db/db* mice (47).

Steady-state MAFA, MAFB, NKX6.1, and PDX1 mRNA and protein levels were significantly lower in human T2DM islets, whereas no difference was observed in HNF1α, ISL1, and NEUROD1 (Figure 8,

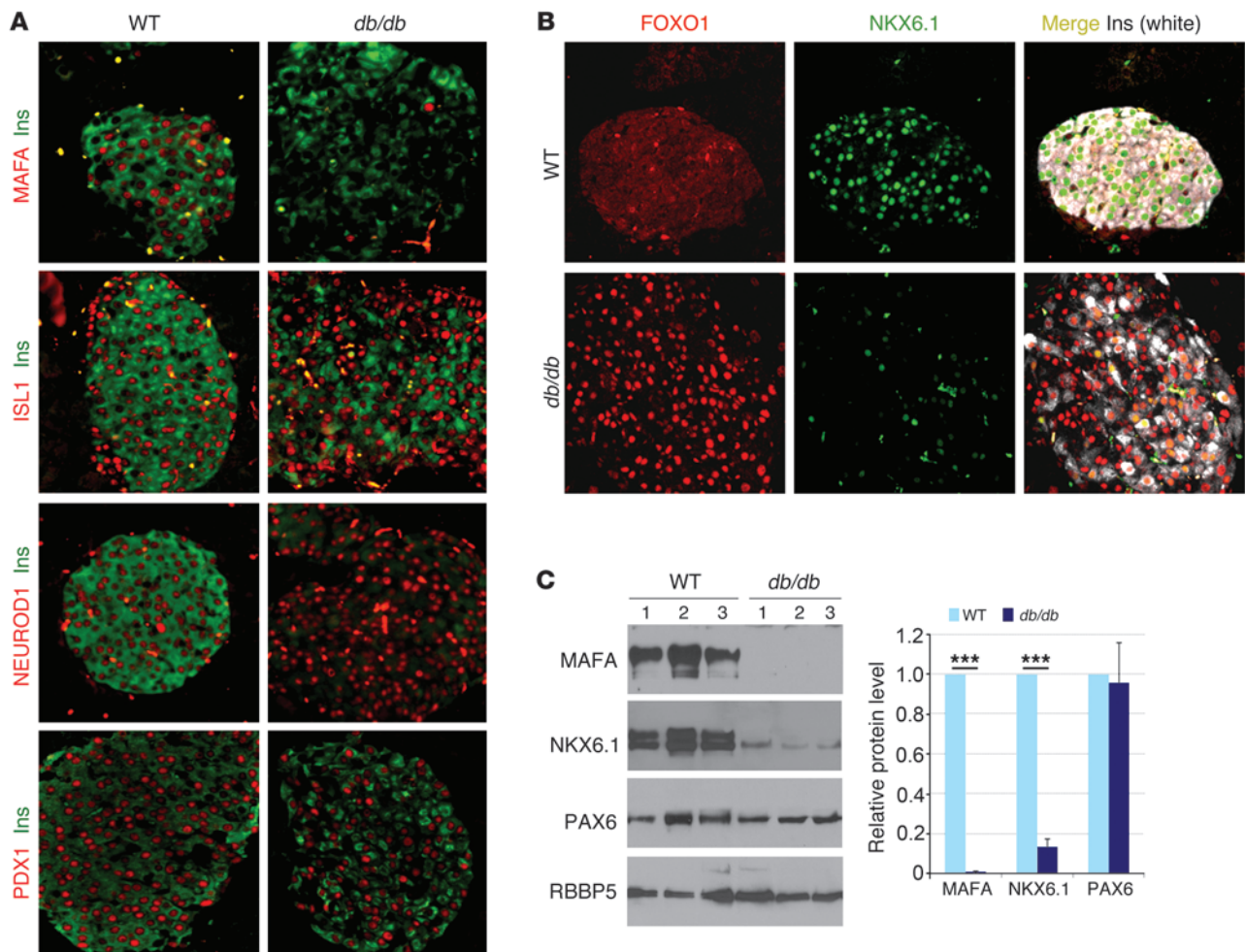


Figure 5 Nuclear NKX6.1 levels are profoundly reduced in diabetic *db/db* islet β cells. **(A)** Immunostaining for MAFA, ISL1, NEUROD1, PDX1, and insulin in 10-week-old WT and *db/db* mouse pancreas. **(B)** FOXO1, NKX6.1, and insulin immunosignals in WT and diabetic *db/db* pancreas. **(C)** Immunoblotting of MAFA and NKX6.1 in islet extracts prepared from 10-week-old WT and *db/db* mice ($n = 3$). Quantified MAFA, NKX6.1, and PAX6 levels were normalized to RBBP5. The relative change in each *db/db* mouse was compared with 3 controls. *** $P < 0.001$.

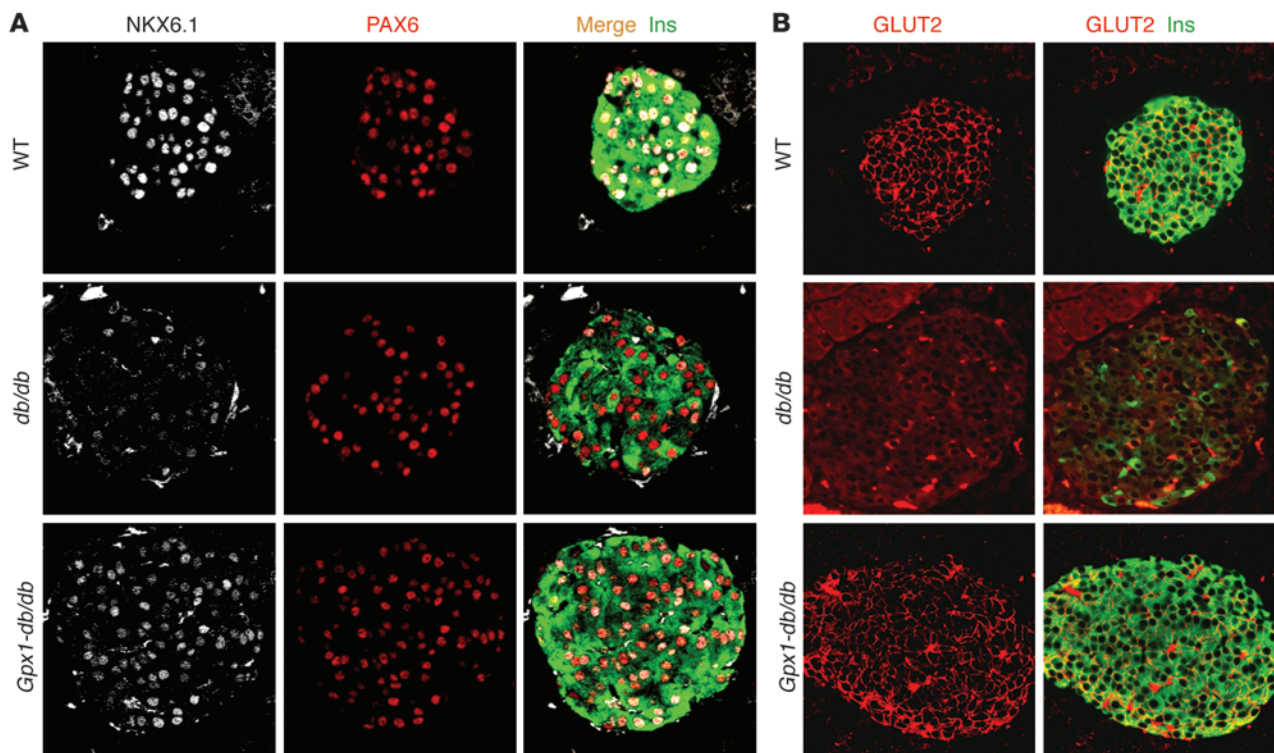
A and B). As predicted, the expression level of many physiologically important genes activated by these factors was compromised in T2DM islets, including insulin, glucose transporter type 1 (*SLC2A1*), and *GLPI* receptor (Figure 8C). However, the protein levels and/or nuclear distribution of factors unaffected in H_2O_2 -treated β TC-3 cells and/or hyperglycemic *db/db* mice were also unaltered in human T2DM islets (Figure 8B and Supplemental Figure 4B). Moreover, there was no change in mRNA expression levels in human T2DM islets of the progenitor markers induced in rodents (see *NEUROG3*, *POU5F1*, *NANOG*, and *MYCL1* in Figure 8A).

A profound loss of MAFA, MAFB, PDX1, and NKX6.1 protein levels was also revealed upon quantitation of nuclear β cell staining (Figure 8D). Furthermore, the MAFB nuclear content was reduced to a similar low level in human islet α cells (i.e., normal, $53 \pm 7.2\%$ of glucagon-positive cells and only $1.51 \pm 2.3\%$ in T2DM; Supplemental Figure 5A), whereas no change was observed in diabetic *db/db* islet α cells (Supplemental Figure 5B). Collectively, our analysis of β cell lines, the *db/db* mice, and human T2DM islets illustrates that disease stress conditions cause the selective loss of

MAFA, MAFB, NKX6.1, and/or PDX1, which we propose plays a principal role in compromising β cell function in T2DM.

Discussion

Oxidative stress induces modifications within proteins, and these modifications impact activity (55, 56), stability (57, 58), and/or subcellular distribution (40, 41). Such conditions are also induced within T1DM and T2DM islets, contributing to β cell dysfunction. In this study, β cell lines, *db/db* mice, and human T2DM islets were used to determine whether a fundamental cause of β cell dysfunction was a result of loss in islet-enriched transcription factor activity. Our results suggest that the β cell's inability to produce sufficient insulin to overcome the resistance of peripheral tissues is caused by the gradual loss of a specific subset of transcription factors essential to glucose sensing and insulin secretion. Specifically, we propose that MAFA (and/or MAFB) represents early and very sensitive targets of oxidative stress, potentially manifested as postprandial hyperglycemia in human prediabetics (59, 60). Subsequent damaging events in NKX6.1 and/or PDX1 generated over

**Figure 6**

NKX6.1 and GLUT2 subcellular levels are rescued by transgenic *Gpx1* expression in *db/db* islet β cells. Immunostained images of (A) NKX6.1, PAX6, and (B) GLUT2 in 20-week-old WT, *db/db*, and *Gpx1-db/db* pancreas.

the course of many years lead to disease progression, drastically reducing insulin production and causing overt diabetes (Figure 9).

Several islet-enriched transcription factors have been linked to MODY (e.g., HNF4 α [MODY1], HNF1 α [MODY3], PDX1 [MODY4], NEUROD1 [MODY6]) (15, 61), a rare autosomal dominant form of diabetes. With the exception of PDX1, we found that these transcription factors and many others were unaffected by diabetic stress conditions in human or rodent β cells. This included gene products associated with progenitor cell formation recently found produced by physiological pressures such as multiparity and aging in mice lacking FOXO1 in β cells (47). Thus, lineage-tracing analysis showed that hyperglycemia imposed by such conditions caused β cells to become insulin⁻ NEUROG3⁺ L-MYC (MYCL1)⁺ NANOG⁺ OCT4 (POU5F1)⁺, described as dedifferentiation to an endocrine progenitor-like stage. In contrast, we did not detect any changes in NEUROG3, POU5F1, NANOG, or MYCL1 levels in human T2DM islets (Figure 8A), presumably because such changes do not occur during the progression of the disease. However, our results are consistent with the view that FOXO1 plays an early and influential role in the gradual decline of β cell function, preceding the loss of MAFA and MAFB.

MAFB is expressed in both α and β cells in human islets (52, 53), but is silenced in rodent β cells soon after birth (18, 38). It is not only essential for glucagon and insulin transcription in the development of α and β cells, but also for adult gene products required for glucose sensing and hormone secretion (18, 38). Postnatal repression of MAFB and the predominance of MAFA appear to be required for rodent β cell maturation (38), while it is unknown what impact the coexpression of MAFA and MAFB has on human islet cells.

Both MAFA and MAFB were compromised to a similar low level in human T2DM β cells (Figure 8D and Supplemental Figure 5) and transfected H₂O₂-treated β TC-3 cells (Figure 2E). However, MAFB mRNA and protein expression was only affected in human T2DM islet α cells (Figure 8 and Supplemental Figure 5). It will be interesting to explore whether this reduction in MAFB contributes to dysregulated glucagon secretion in human T2DM patients (62).

In contrast to the MODY proteins, whose inactivation results from coding region mutations, our studies suggest that transcription factor dysfunction can also be caused by environmental factors. Oxidative stress likely represents a principal means of disabling β cells in T2DM, as deduced by the posttranslational modifications commonly found (63) and the effectiveness of antioxidants at improving cell function (5, 14). Moreover, using a transgenic model, we showed that *Gpx1* restored NKX6.1 nuclear levels under circumstances that also improved blood glucose homeostasis and MAFA content (Figure 6). Significantly, *Gpx1-db/db* mice are still morbidly obese despite improved β cell function (5), so the damaging signals leading to cell dysfunction continue being produced from insulin-resistant peripheral tissues.

Although precisely how MAFA, MAFB, NKX6.1, or PDX1 become inoperative *in vivo* is unclear, both the H₂O₂ treatment data from β cell lines and the time course analysis in *db/db* mice strongly suggest that MAFA is more sensitive to this stressor than NKX6.1. Moreover, earlier work also indicates that MAFA activity precedes the loss of PDX1 during glucotoxicity (23). The initial step of MAFA and MAFB inactivation appears to be the formation of a DNA-binding defective covalent dimer species, as shown upon incubation of MAFA containing HeLa and β TC-3 cell extracts with

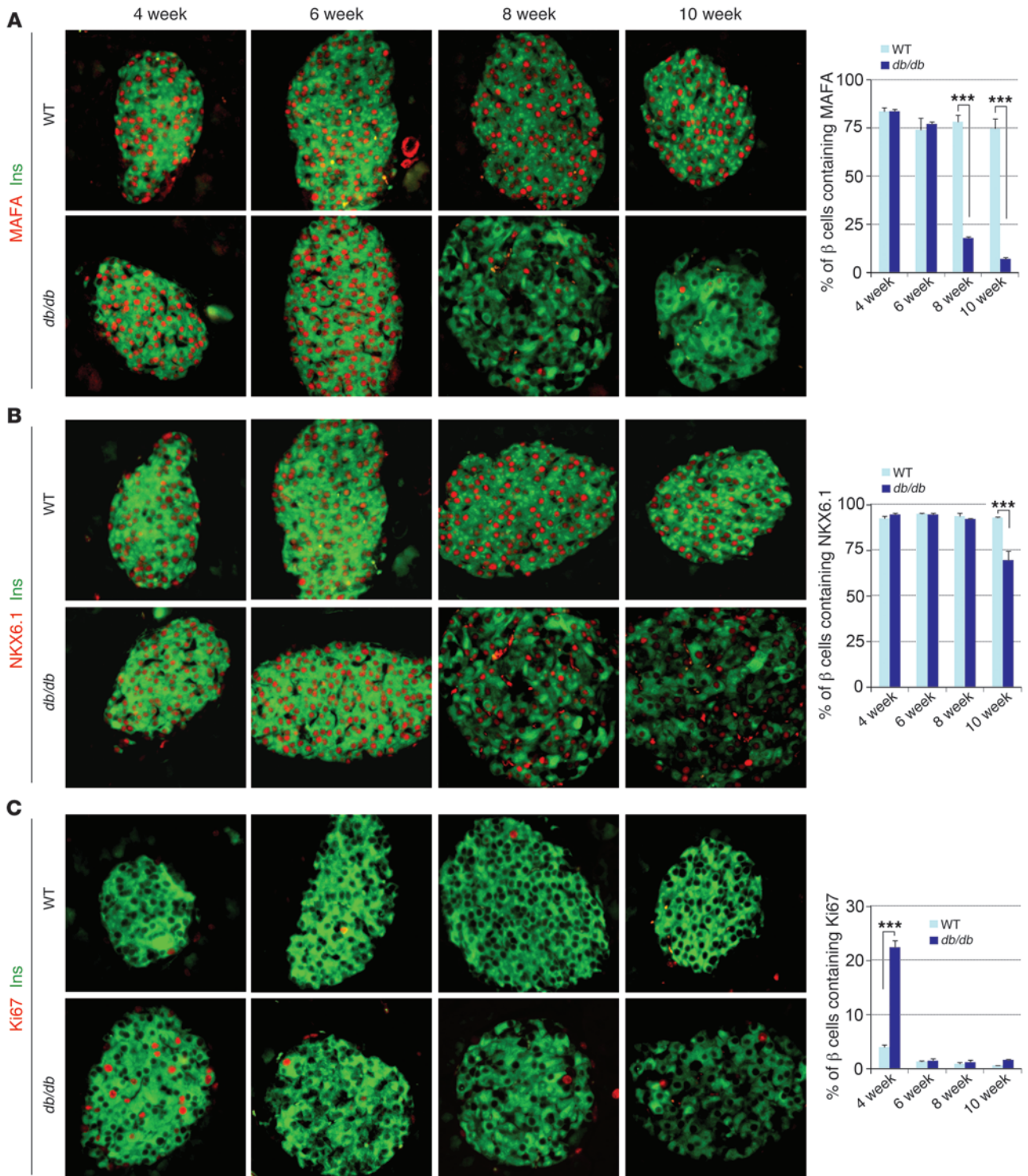


Figure 7 Islet β cell proliferation precedes the loss of MAFA and NKX6.1 in *db/db* islets of (A) MAFA, (B) NKX6.1, and (C) Ki67 in 4-, 6-, 8-, and 10-week-old WT and *db/db* pancreas. Quantification of the percentage of insulin-positive (β) cells costaining for MAFA, NKX6.1, and Ki67 is shown ($n = 4$). *** $P < 0.001$.

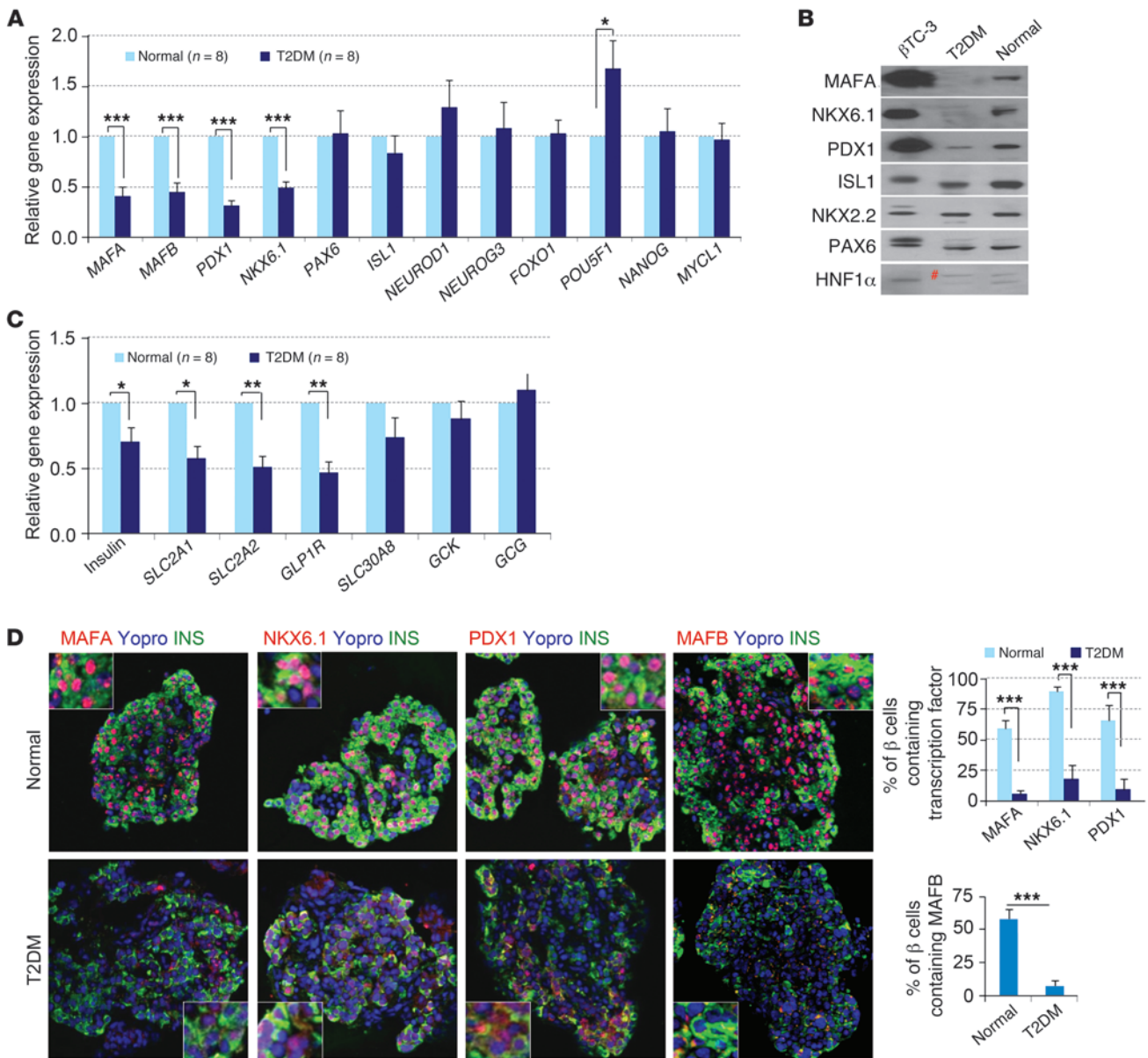


Figure 8

MAFA, MAFB, NKX6.1, and PDX1 expression levels are compromised in human T2DM islet β cells. (A and C) Real-time PCR analysis of normal human and T2DM human islets. (B) MAFA, PDX1, and NKX6.1 protein levels were reduced in human T2DM islets when compared with other islet-enriched transcription factors. #Predicted molecular weight of human HNF1 α . (D) Reduction in MAFA, NKX6.1, PDX1, and MAFB in human T2DM islet insulin-positive cells by immunostaining. Quantification of the percentage of β cells containing nuclear MAFA, NKX6.1, PDX1, and MAFB is shown. * $P < 0.10$; ** $P < 0.01$; *** $P < 0.001$.

H₂O₂ alone or in the absence of the DTT antioxidant (Figure 3). Notably, these conditions did not affect PDX1 SDS-PAGE mobility or DNA-binding properties. A future objective will be to determine whether (MAFA)₂ can be detected during β cell inactivation in vivo, which was probably undetectable here due to the relatively long time window between *db/db* sample collections (i.e., 1 week) and the limited availability of human T2DM samples. In addition, we are working to determine how oxidative stress leads to PDX1 and NKX6.1 incapacitation, presumably through oxidation at lysines, cysteines, and/or histidines found to be targeted in islet β cell proteins in diabetic GK rats (64).

An important feature of our findings was how selectively MAFA, MAFB, PDX1, and NKX6.1 were deactivated as compared with other key islet regulators in T2DM β cells (Figure 8, A and D, and Supplemental Figure 4B). Such findings strongly suggest that the decrease in glucose-stimulated insulin secretion properties in these islets (Supplemental Figure 4A) primarily results from reduced levels of these key transcriptional regulators. Moreover, we propose that their progressive decline leads to both “ β cell exhaustion” and loss of insulin-positive cell mass associated with T2DM (65). We postulate that restoring the activity of these transcription factors is critical to the improved β cell function and blood

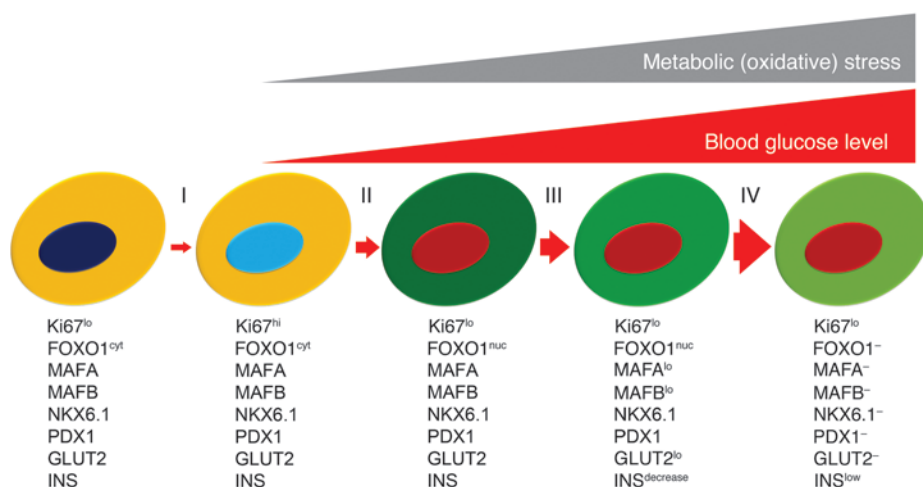


Figure 9

Events leading up to islet β cell dysfunction in T2DM. Adaptive responses: (I) β cell replication and (II) FOXO1 nuclear localization (41). Stress responses: (II) FOXO1 nuclear localization/inactivation (47) and sequential loss of (III) MAFA (and/or MAFB), and (IV) NKX6.1 (and/or PDX1) in response to hyperglycemia and oxidative stress. These circumstances lead to a decline in β cell function, also termed “the stunned β cell” (65).

glucose control observed within weeks after bariatric surgery in T2DM patients (66, 67), a proposal supported by the increase in PDX1 protein levels seen upon recovery (66). It is also striking that human embryonic stem cell-derived insulin-positive cells produced in vitro are dysfunctional and fail to express NKX6.1, PDX1, and MAFA (68–70). Consequentially, the identification of small molecules that increase expression and/or restore the nuclear content of these transcription factors could be very useful in T2DM treatment as well as in generating β cells for T1DM therapy.

Methods

DNA constructs. MAFA C277/293A, MAFA C42/59/69S, as well as all of the other MAFA and MAFB mutants described in Supplemental Figure 1, were prepared in a cytomegalovirus-driven (CMV-driven) pCMV4-myc vector using the primers listed in Supplemental Table 1 and the QuikChange site-directed mutagenesis kit (Stratagene, Agilent Technologies) (35).

H₂O₂ treatment, cellular fractionation, and immunoblotting analysis. HeLa (71), α TC-6 (72), MIN6 (73), and β TC-3 (74) cells were grown as described. H₂O₂-treated cells were resuspended in lysis buffer (100 mM Tris-HCl, pH 8.0, 0.5% NP-40), with the centrifuged supernatant serving as the cytoplasmic fraction. The pellet was suspended in extraction buffer (20 mM HEPES, pH 7.4, 400 mM NaCl, 1 mM EGTA, 1 mM EDTA, 2 mM DTT, and 1 \times Protease inhibitor [Roche]), with the centrifuged supernatant serving as the nuclear fraction. Isolated islets were suspended in passive lysis buffer (0.15 μ l/islet; Promega), and the supernatant protein was obtained after sonication and centrifugation. Adenovirus-expressed catalase was provided by Chris Rhodes (University of Chicago, Chicago, Illinois, USA), and infection of β TC-3 and MIN6 cells occurred prior to H₂O₂ treatment. Proteins (10 μ g loaded) were separated by 10% SDS-PAGE and tested by immunoblotting with the following antibodies: rabbit α -MAFA (Bethyl Laboratories, 1225, 1:2,000); mouse α -NKX6.1 (Developmental Studies Hybridoma Bank, 1:2,000); mouse α -ISL1 (Developmental Studies Hybridoma Bank, 1:1,000); goat α -HNF1 α (Santa Cruz Biotechnology, 1:1,000); goat α -FOXA2 (Santa Cruz Biotechnology, 1:1,000); goat α -B56 α (Santa Cruz Biotechnology, 1:1,000); goat α -LDB1 (CLIM-2) (Santa Cruz Biotechnology, 1:1,000); rabbit α -NEUROD1 (Epitomics, 1:1,000); mouse α -p/CAF (Santa Cruz Biotechnology, 1:1,000); mouse α -RBBP5 (RBQ3) (Santa Cruz Biotechnology, 1:1,000); mouse α -HB9 (Developmental Studies Hybridoma Bank, 1:1,000); mouse α -NKX2.2 (Developmental Studies Hybridoma Bank, 1:1,000); rabbit α -PDX1 (provided by Chris Wright, Vanderbilt University, Nashville, Tennessee, USA; 1:10,000); rabbit α -PAX6

(Covance, 1:1,000); mouse α -B55 α (Santa Cruz Biotechnology, 1:1,000); rabbit α -FOXO1 (Cell Signaling Technology, 1:1,000); HRP-conjugated α -rabbit IgG (Promega, 1:2,000); and HRP-conjugated α -mouse IgG (Promega, 1:2,000). The experiments were performed at least 3 times, with representative results quantitated using NIH ImageJ software.

DNA binding assays. Nuclear extracts were prepared from β TC-3 or HeLa cells transfected using the Lipofectamine reagent (Invitrogen) with myc-tagged MAF plasmids as described previously (75), except DTT was not added to the extraction buffer. MAFA was tested for DNA-binding activity with ³²P-labeled rat insulin II C1 element (-¹²⁶TGGAACTGCAGCTTCAGCCCCCTCTG⁻¹⁰¹) (76), and PDX1 with *Mafa* region 3 site (-⁸⁰⁸ATTACCCATTTCTGTAAATTTGTTCTGGG⁻⁸⁰⁶) (75). HeLa nuclear extract was also prepared from cells transfected with myc-MAFA, myc-MAFA C277/293A, or myc-MAFA C42/59/69S in the presence of 20 mM of dithiothreitol (DTT) and then treated with increasing concentrations of H₂O₂.

Immunostaining analysis. β TC-3, α TC-6, or HeLa cells were cultured on cover slides and either analyzed directly or transfected overnight with 0.5 μ g of DNA using the Lipofectamine reagent (Invitrogen). The cells were treated for 90 minutes in MEM containing either 50 μ M H₂O₂ (β TC-3 cells), 100 μ M H₂O₂ (α TC-6 cells), or 1 mM H₂O₂ (HeLa cells). Tissue fixation, embedding, and immunofluorescence labeling of WT, *db/db*, and *Gpx1-db/db* pancreas were performed as described previously (5). WT C57BLKS/J (000662) and *db/db* homozygous (000642) BKS.Cg-*m*^{+/-} *Lep^{db}*/J mice were purchased from The Jackson Laboratory. Isolated human islets were embedded and immunostained as described (53). An islet cell was scored positive for nuclear factor only when at least 75% of the nucleus was surrounded by the cytoplasm labeled for a given hormone. Immunostaining was performed with the following antibodies: mouse α -myc (9E10; Santa Cruz Biotechnology, 1:500); rabbit α -MAFA (1:1,000), mouse α -ISL1 (Developmental Studies Hybridoma Bank, 1:500); rabbit α -MAFB (Bethyl Laboratories, 658, 1:1,000); mouse α -NKX6.1 (1:2,000); mouse α -NKX2.2 (1:1,000); rabbit α -PAX6 (1:500); rabbit α -FOXO1 (1:300); mouse α -HB9 (1:500); goat α -FOXA2 (1:1,000); rabbit α -Ki67 (BD Pharmingen, 1:400); and rabbit α -NEUROD1 (1:500). The secondary Cy2-, Cy3-, or Cy5-conjugated donkey α -goat, α -guinea pig, α -rabbit, or α -mouse IgGs were obtained from Jackson ImmunoResearch Laboratories. Immunofluorescence images were acquired using confocal microscopy (LSM510, Carl Zeiss) or immunofluorescence microscopy (Imager.M2, Carl Zeiss) and presented at a magnification of \times 200.

Human islet functional analysis. Islets were obtained from multiple isolation centers supported by the NIH, the Juvenile Diabetes Research Foundation (JDRF), the Islet Cell Resource Centers, and the Integrated Islet



Distribution Program (<http://iidp.coh.org>). Islets were handpicked on the day of arrival and then cultured at 37°C for 24 hours in RPMI-1640 containing 10% FBS and 5 mM glucose (53). Islet health was determined as described (53). Glucose-stimulated insulin secretion (GSIS) was evaluated in an islet perfusion apparatus using 2 secretagogues and 60 medium-size islets (77). Islet preparations were obtained from 8 normal and 8 T2DM cadaver donors: normal, 4 male and 4 female (44.0 ± 3.4 years [range: 23 to 52], weight 74.8 ± 5.1 [range: 56 to 94], BMI 26.3 ± 0.7 [range: 24.3 to 29.4], cold ischemia time, *n* = 6, 8.33 ± 1.65 hours [range: 1.5–13.5 hours], and 2 unknown); T2DM, 4 male, 3 female, and 1 unknown (49.1 ± 2.5 years [range: 38 to 59], weight 114 ± 11.7 [range: 71 to 168], BMI 37.3 ± 2.9 [range: 27 to 52.4], cold ischemia time, *n* = 5: 8.89 ± 0.98 hours [range: 6–12 hours], and 3 unknown).

Quantitative RT-PCR analysis. Cellular RNA from human islets was isolated using the RNeasy Lysis Buffer (Qiagen). RNA assessment was determined in the Vanderbilt Functional Genomics Shared Resource Laboratory (28S/18S > 1.4, RNA integrity number > 8.2). DNA was removed with the TURBO DNA-free kit (Ambion). cDNA was prepared from RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), and quantitative PCR was performed using the One-Step system and TaqMan primers (Applied Biosystems). The experimental data were normalized to β2 microglobulin, and relative mRNA changes were calculated by the comparative ΔCt method. Each T2DM sample was compared with the 8 controls. Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines were followed (78).

ChIP assay. βTC-3 cells were formaldehyde cross-linked after H₂O₂ treatment, and the sonicated protein-DNA complexes were isolated under conditions described previously (75, 79). Sonicated chromatin was incubated for 12 to 14 hours at 4°C with α-MAFA, α-PDX1, α-PAX6, α-NKX6.1, α-NEUROD1, or α-NKX2.2 antibody, and the complexes were isolated with protein A/G-agarose (Santa Cruz Biotechnology). PCR was performed on one-tenth of the purified immunoprecipitated DNA using 5 PRIME MasterMix (5 PRIME) and 15 pmol of the insulin (⁻³⁷⁸GGAAGTGTGAAACAGTCCAAGG and ⁻⁴⁶CCCCTGGACTTTG CTGTTTG) and *Mafa* (⁻⁸¹²⁰CACCCAGCGAGGGCTGATTTAATT and ⁻⁷⁷⁵⁰AGCAAG-

CACTTCAGTGTGCTCAGTG) transcriptional control region primers. The amplified products were resolved on a 1.4% agarose gel in Tris-acetate EDTA buffer containing ethidium bromide.

Statistics. Data are expressed as the means ± SEM. *P* values were calculated with a Student's 2-tailed test. Results were considered significant at **P* < 0.10, ***P* < 0.01, or ****P* < 0.001.

Study approval. All studies involving the use of animals or human islets were approved by the IACUC or the IRB of Vanderbilt University Medical Center (Nashville, Tennessee, USA).

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