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Preserving Mafa Expression in Diabetic Islet β-Cells Improves Glycemic Control *in Vivo*[*] [S]

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Background: MAFA expression is markedly decreased in islet β-cells of type 2 diabetes mellitus.
Results: Mis-expression of Mafa in mouse diabetic *db*/*db* β-cells ameliorated glucose-stimulated insulin secretion and β-cell mass.
Conclusion: Mafa alone is sufficient to improve β-cell function and mass under diabetic conditions.
Significance: These results establish how consequential this transcription factor is to islet β-cells under pathological conditions.

The murine Mafa transcription factor is a key regulator of postnatal islet β-cell activity, affecting *insulin* transcription, insulin secretion, and β-cell mass. Human MAFA expression is also markedly decreased in islet β-cells of type 2 diabetes mellitus (T2DM) patients. Moreover, levels are profoundly reduced in *db*/*db* islet β-cells, a mouse model of T2DM. To examine the significance of this key islet β-cell-enriched protein to glycemic control under diabetic conditions, we generated transgenic mice that conditionally and specifically produced Mafa in *db*/*db* islet β-cells. Sustained expression of Mafa resulted in significantly lower plasma glucose levels, higher plasma insulin, and augmented islet β-cell mass. In addition, there was increased expression of *insulin*, *Slc2a2*, and newly identified Mafa-regulated genes involved in reducing β-cell stress, like Gsta1 and Gckr. Importantly, the levels of human GSTA1 were also compromised in T2DM islets. Collectively, these results illustrate how consequential the reduction in Mafa activity is to islet β-cell function under pathophysiological conditions.

Type 2 diabetes mellitus (T2DM)[2] is caused by insufficient insulin production from pancreatic islet β-cells in the setting of insulin resistance, with the latter principally reflecting the inability of cells in muscle, liver, and fat to respond adequately to normal insulin levels. Precisely why β-cells fail to produce sufficient quantities of insulin under these conditions is unclear. Notably, a subset of β-cell-enriched transcription factors essential to β-cell development and/or function were recently shown to be inactivated under T2DM stress conditions in rodent models and human islet β-cells (1–3), specifically MAFA, PDX1, and NKX6.1. Compelling evidence indicates that reactive oxygen species generated by increased glucose metabolism causes β-cell inactivation and even death in T2DM. Significantly, islet β-cells have unusually low antioxidant enzyme levels (*e.g.* glutathione peroxidase-1 and catalase) (4–6), with antioxidant treatment improving β-cell function in human T2DM islets (7–9) and T2DM animal models (10–12). For example, transgenic β-cell-specific expression of glutathione peroxidase-1 improved Mafa, Nkx6.1, and blood glucose levels in *db*/*db* mice, a model of T2DM (2, 12).

The change in Mafa was found to occur earlier than Nkx6.1 in mouse *db*/*db* β-cells, correlating closely with decreased expression of essential regulators involved in cell proliferation, glucose sensing, and insulin secretion (2, 13). Reduced levels of such effectors were also found in pancreas-specific $Mafa^{\Delta panc}$ deletion mutant mice (*e.g. Insulin*, *CyclinD2*, and *Munc18-1* (14)). In addition, Mafa is only produced in embryonic insulin$^+$ cells destined to populate the adult, which represents an unusually late and highly specific expression pattern in relationship to other islet-enriched transcription factors (15). Islet β-cell dysfunction under T2DM stress conditions likely results from the gradual loss of MAFA followed by either PDX1 or NKX6.1, because $Mafa^{\Delta panc}$ mice are only glucose intolerant (14), whereas islet β-cell-specific loss of Pdx1 or Nkx6.1 almost immediately causes overt hyperglycemia (16–19).

In the present study, we directly evaluated the impact of Mafa insufficiency in T2DM by generating transgenic *db*/*db* mice that conditionally expressed this transcription factor in only islet β-cells. The Mafa producing *db*/*db* mice demonstrated improved glycemic control and β-cell function, with restoration coinciding with expression of proteins that reduce oxidative stress. These studies not only provide keen insight into the prominence of Mafa activity *in vivo*, but also shed light on the significance of developing T2DM therapeutics to ameliorate β-cell function by preventing transcription factor inactivation.

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[S] This article contains supplemental Figs. S1–S10, Table S1, and Appendix S1.
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[2] The abbreviations used are: T2DM, type 2 diabetes mellitus; GO, gene ontology; TM, tamoxifen; Gckr, glucokinase regulatory protein; GSTA1, glutathione *S*-transferase α1.

EXPERIMENTAL PROCEDURES

Human Pancreas Samples—Pancreatic tissue was obtained from patients at Osaka University Hospital who were undergoing a partial pancreatectomy to remove pancreatic or distal bile duct tumor cells. Glucagon tolerance tests were performed before the surgery. The pancreas sample was fixed in 4% paraformaldehyde and 4-μm sections were prepared using routine procedures. The study protocol was approved by the Osaka University Hospital Ethics Committee, and informed consent was obtained from each patient. The clinical donor data are provided in supplemental Table 1.

Generation of Pdx1^{PB}-CreER^{TM};CAG-CAT-Mafa^{myc};db/db Mice (termed βMafa^{myc}:db/db)—p*CAG-CAT-Mafa^{myc}* was constructed from *pCAG-CAT-lacZ* (20) by replacing the *lacZ* sequences with a fragment containing mouse *Mafa* coding sequences linked to a *myc* tag and the *bovine growth hormone* polyadenylation signal. A 5.0-kb SalI-SacI *CAG-CAT-Mafa^{myc}* spanning fragment of this plasmid was purified and microinjected into fertilized eggs of BDF1 mice. A total of 13 lines of *CAG-CAT-Mafa^{myc}* mice were generated, and the high TM-inducible signal to sham-treated Mafa^{myc} expression properties of the b, d, and f lines were selected for further analysis. *Pdx1^{PB}-CreER^{TM}* transgenic mice (21), which express TM-activated Cre recombinase under the control of the islet β-cell specific *Pdx1$^{Area\ I/II}$* enhancer, were crossed with the b, d, and f *CAG-CAT-Mafa^{myc}* lines to generate *Pdx1^{PB}-CreER^{TM};CAG-CAT-Mafa^{myc}* (*i.e. βMafa^{myc}*) mice. These mice were then backcrossed with *C57BL/KsJ-db/m* (*db/m*) mice for more than 10 generations to eventually obtain *βMafa^{myc};db/db* mice. Subcutaneous injections of 0.1 mg/1.0 g of BW TM were performed three times within 5 days for the induction of Mafa^{myc} expression. The efficacy of islet β-cell expression was determined by anti-myc epitope staining. Because all of three lines demonstrated a similar improvement of plasma glucose levels after crossing with *db/db* mice, we mainly used the b line of *CAG-CAT-Mafa^{myc}*. All animal procedures were approved by the Ethics Review Committee for Animal Experimentation of Osaka University Graduate School of Medicine.

Glucose Tolerance Tests—Glucose tolerance tests (0.5 g/kg of BW) were performed 4 and 8 weeks after TM injection on mice fasted overnight. Glucose and insulin levels were measured from tail vein sampled blood with a portable glucose meter and the insulin ELISA Kit (Morinaga Biochemicals, Yokohama, Japan).

Immunohistochemical and Islet β-Cell Mass Analysis—Mouse pancreata were dissected and fixed overnight in 4% paraformaldehyde and 4-μm thick sections were prepared under standard procedures. After blocking with 3% donkey serum, immunolabeling was performed with the following antibodies: rabbit Mafa, 1:500 (Bethyl Laboratories, Inc., Montgomery, TX); rabbit myc tag, 1:200 (Cell Signaling Technology, Inc., Danvers, MA); guinea pig insulin, 1:1000 (DAKO, Glostrup, Denmark); mouse Ki-67, 1:500 (BD Biosciences); rabbit cleaved caspase-3, 1:500 (Cell Signaling Technology, Inc., Danvers, MA); mouse 4–4-hydroxy-2-nonenal, 1:200 (Japan Institute for the Control of Aging, Shizuoka, Japan); and rabbit GSTA1 (Thermo Fisher Scientific, Rockford, IL). The secondary antibodies used for fluorescent imaging on an Olympus FV1000-D confocal microscope were 488- or 555-conjugated donkey anti-guinea pig and anti-rabbit (Jackson ImmunoResearch Laboratories, 1:200). Biotinylated goat anti-guinea pig, donkey anti-rabbit, or anti-mouse antibodies were diluted 1:200 for 3,3′-diaminobenzidine staining. Sections were counterstained with Mayer's hemalum solution. Insulin^{+} islet β-cell mass was determined by insulin and 3,3′-diaminobenzidine staining as reported previously (22) using WinRoof® (Mitani Corporation, Japan) and ImageJ software.

Islet Perifusion Analysis—Isolated islets were first cultured overnight in 10% FCS RPMI medium containing 5 mM glucose, and then 20 islets were placed in a chamber and perifused for 1 h with 40 mg/dl of glucose, followed by 30 min with 400 mg/dl of glucose. The effluent was collected every 30 s for 5 min, followed by 1 min for 5 min, and 2 min for 8 min. The sample insulin concentration was normalized to that of the whole cell protein.

Real-time PCR Analysis—Real-time RT-PCR analysis was performed as described previously (23) with the following primer sets: mouse *insulin 1* (mRNA numbering relative to ATG, forward, −47 GACCAGCTATAATCAGAGACC; reverse +331 AGTTGCAGTAGTTCTCCAGCTG, 378 bp product), mouse *insulin 2* (forward, −57 AGCCCTAAGTGATCCGCTACAA; reverse, +331 AGTTGCAGTAGTTCTCCAGCTG, 388 bp), mouse total *Mafa* (forward, +757 TTCAGCAAGGAGGAGGTCAT; reverse, +973 CCGCCAACTTCTCGTATTTC; 217 bp), mouse *Pdx1* (forward, +192 CATCTCCCCATACGAAGTGC; reverse, +526 GGGGCCGGGAGATGTATTTG; 335 bp), mouse *Gck* (forward, +1893 CTTTCCAGGCCACAAAACATT; reverse, +2079 TGAGTGTTGAAGCTGCCATC; 187 bp), mouse *Gckr* (forward, +1429 CAGCGTGAGTTAAGCACCAA; reverse, +1649 TCAGTGATGGAGCACCTGAG; 221 bp), mouse *Gsta1* (forward, +225 CGCCACCAAATATGACCTCT; reverse, +456 CCTGTTGCCCACAAGGTAGT; 232 bp), and mouse *β-actin* (forward, +778 GCTCTTTTCCAGCCTTCCTT; reverse, +945 CTTCTGCATCCTGTCAGCAA; 168 bp). To quantify only endogenous *Mafa* mRNA levels, the TaqMan MGB Gene Expression Kit (Applied Biosystems, Foster City, CA) was used with primers spanning unique 3′-flanking region sequences (forward, +1360 TCCGAGCCAGGTCTGACTTC; reverse, +1414 TGCGCTCCACGTCTGTACA; 55 bp, probe +1381 TCGGCAGCGTCCAC).

Preparation of shMafa-expressing Adenoviruses—Recombinant adenoviruses expressing short hairpin RNA against Mafa (Ad-shMafa) was constructed using the pAdEasy system and the following oligonucleotides: 5′-GTTTAG*T*GGGAC*T*TGTACA*G*GGAACGTGTGCTGTCCGTTCCTTGTACAGGTCCCGCTTTTTT-3′ and 5′-ATGCAAAAAAGCGGGACCTGTACAAGGAACGGACAGCACACGTTCC*C*TGTACA*A*GTCC*C*A*C*T-3′ (wild type *Mafa* sequences are underlined and mutant in italics). These oligonucleotides or control oligonucleotides (5′-GTTTTTTTTTT-3′ and 5′-ATGCAAAAAAA-3′; T7stop) were inserted downstream of the mouse U6 promoter of piGENE^{TM}mU6 (iGENE Therapeutics, Inc., Tokyo, Japan).

Microarray Analysis—The quality of islet RNAs was determined using an Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA), and samples with RNA integrity number more than 7.0 were used for microarray analysis. Total RNA was

TABLE 1
GO terms up-regulated in double-TG *db/db* islets

GO Term	Counts in selection[a]	Counts in total[b]	Corrected *p* value
	%		
Endopeptidase inhibitor activity	7.94	0.53	3.67.E-16
Peptidase inhibitor activity	7.94	0.57	1.45.E-15
Serine-type endopeptidase inhibitor activity	6.50	0.39	4.06.E-14
Enzyme inhibitor activity	8.66	0.86	4.36.E-14
Oxidoreductase activity	13.72	2.76	4.90.E-13
Monooxygenase activity	6.50	0.48	1.64.E-12
Catalytic activity	40.07	20.03	9.26.E-12
Iron ion binding	6.50	0.70	7.06.E-10
Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, reduced flavin or flavoprotein as one donor, and incorporation of one atom of oxygen	3.25	0.11	7.18.E-09
Enzyme regulator activity	11.19	2.72	1.46.E-08
Heme binding	4.69	0.43	8.54.E-08
Aromatase activity	2.89	0.11	1.66.E-07
Tetrapyrrole binding	4.69	0.46	1.90.E-07
Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	5.05	0.55	1.92.E-07
Lipid binding	6.86	1.34	2.80.E-06
Lipid transporter activity	2.89	0.16	4.31.E-06
Electron carrier activity	3.61	0.42	6.53.E-05
Glutathione transferase activity	2.17	0.11	9.97.E-05
Cofactor binding	5.05	0.95	1.11.E-04
Fatty acid transporter activity	1.44	0.03	1.31.E-04

[a] Refers to the percentage of genes in the input entity list that have that GO term.
[b] Refers to the percentage of genes in the all entities list that have that GO term.

amplified with the WT-Ovation™ RNA Amplification system (NuGEN Technologies, San Carlos, CA) and labeled with cyanine 3. Each hybridization contained 1.65 μg of fragmented cyanine 3-labeled cDNA, and was hybridized at 65 °C for 17 h to the Agilent Mouse GE 4 × 44K v2 microarray (Design ID 026655). Signal intensity was determined with an Agilent DNA microarray scanner. Normalization was performed using Agilent GeneSpring GX version 11.0.2 (per chip, normalization to 75 percentile shift; per gene, normalization to median of all samples). Data filtration was performed, resulting in a total of 30,161 probes as a valid probe set where at least one of the four total samples had a present flag. A 2-fold or greater change in signal intensity was considered a significant difference.

Gene Ontology (GO) Analysis—Gene ontogeny analyses on microarray data were performed with GeneSpring GX software using the annotations provided in the database of the Gene Ontology Consortium. The data were processed with Fisher's exact test and multiple test correction to identify significant over-representation of Gene Ontology annotations belonging to Molecular Functions, and the top 20 of significantly up-regulated (≥ 2.0-fold) GO terms were listed in Table 1.

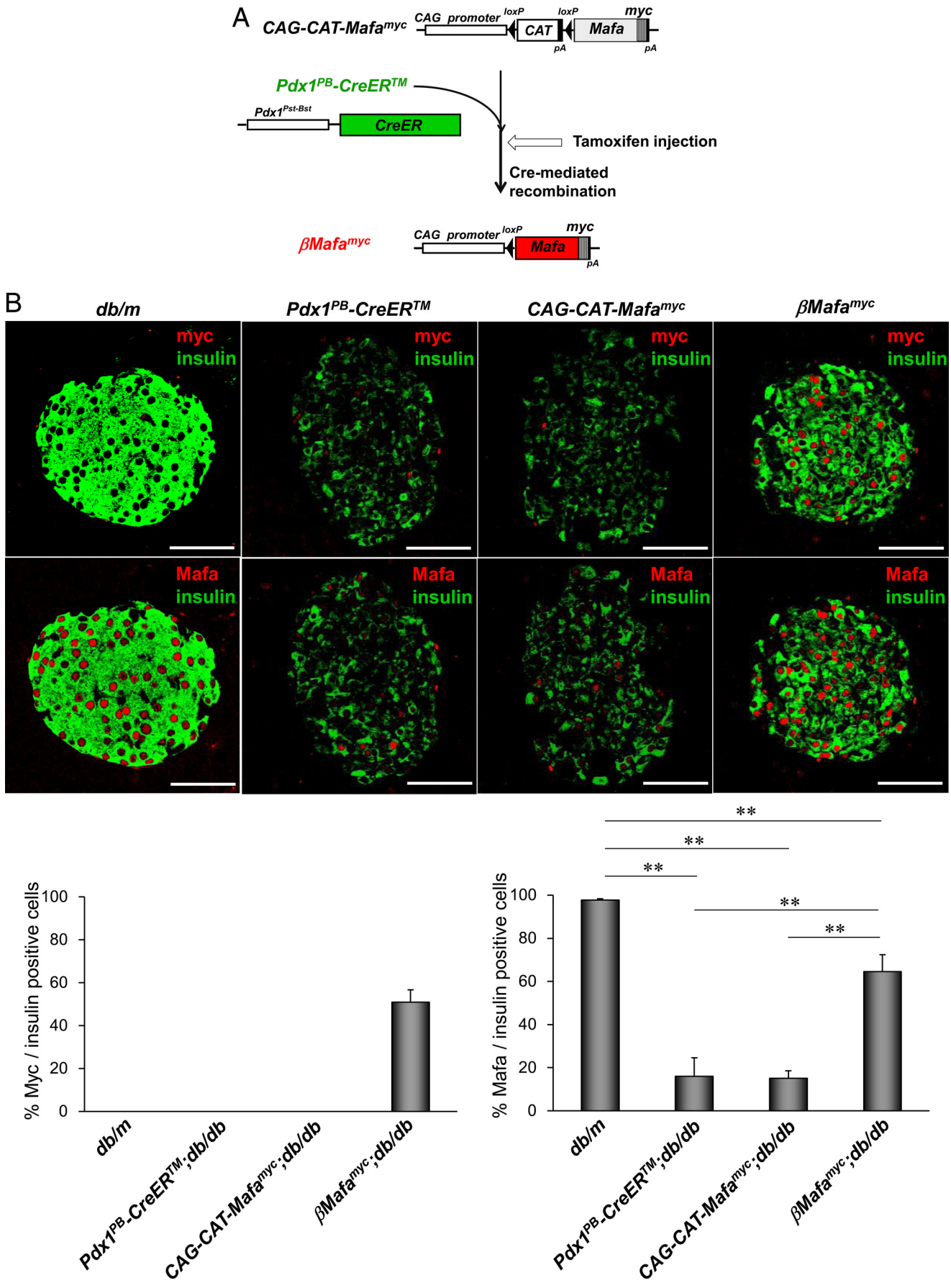
Statistical Analysis—Data are expressed as mean ± S.D. Statistical analysis was performed using one-way analysis of variance followed by the Scheffe's test. A value of $p < 0.05$ was considered to be statistically significant.

RESULTS

Transgenic Islet β-cell Mafa^{myc} Improved Glycemic Control in Insulin-resistant and Diabetic βMafa^{myc}:db/db Mice—To obtain insight into significance of the reduction of human MAFA levels in T2DM islets (supplemental Fig. S1) (2, 24), we generated *CAG-CAT*-Mafa-myc epitope-tagged (Mafa^{myc}) expressing mice to conditionally and specifically produce sustained factor activity in *db*/*db* β-cells (Fig. 1*A*). Significantly, the gel-shift binding and *insulin*-reporter driven properties of Mafa^{myc} was indistinguishable from the wild type protein (supplemental Fig. 2). *CAG-CAT-Mafa^{myc}* mice were crossed with *Pdx1^{PB}-CreER^{TM}* mice (21) to produce Mafa^{myc} in pancreatic β-cells upon tamoxifen (TM) treatment (*βMafa^{myc}* in Fig. 1*A*). Mafa^{myc} was detected in 59% of islet β-cells in TM-induced *βMafa^{myc}* mice (supplemental Fig. S3, *A* and *B*). Moreover, immunoblot analysis of nuclear extract from double transgenic islets demonstrated that Mafa^{myc} was produced at roughly non-diabetic, control levels (52%, supplemental Fig. S3*C*).

βMafa^{myc} mice were crossed more than 10 generations into the C57BL/6KsJ *db*/*db* background, a T2DM model characterized by insulin resistance and β-cell failure due to severe obesity (25). TM injections were performed in 9-week-old *Pdx1^{PB}-CreER^{TM};db/db*, *CAG-CAT-Mafa^{myc};db/db,* and *βMafa^{myc}*; *db*/*db* mice, roughly when Mafa levels decline in *db*/*db* β-cells (1, 2). In contrast, Pdx1 expression was unaffected under these circumstances, another key islet-enriched transcription factor sensitive to glucotoxic conditions associated with T2DM (supplemental Fig. S4). Transgenic Mafa^{myc} in *βMafa^{myc};db/db* mice was clearly detectable in insulin^{+} cells within 1 week of TM induction (Fig. 1*B*), and expression was sustained during the remaining 8 weeks of analysis. In contrast, Mafa^{myc} was not produced in non-diabetic (*db/m*) and diabetic (*Pdx1^{PB}-CreER^{TM};db/db*, *CAG-CAT-Mafa^{myc};db/db*) control islets. The total number of Mafa^{+} cells was not only much greater than in diabetic control *db*/*db* β-cells, but exceeded the number of Mafa^{myc} producing cells (Fig. 1*B*). These results imply that Mafa^{myc} restored expression of endogenous Mafa.

Blood glucose levels in *βMafa^{myc};db/db* mice were significantly lower than either control diabetic *Pdx1^{PB}-CreER^{TM}*; *db*/*db* or *CAG-CAT-Mafa^{myc};db/db* mice (Fig. 2*A*). Notably, this improvement was observed after only 1 week of TM treatment, which was associated with Mafa^{myc} production in β-cells and the reduction in fed blood glucose levels in *βMafa^{myc}*; *db*/*db* mice to 327.0 ± 59.4 mg/dl, whereas control diabetic and


A
CAG-CAT-Mafa^myc
CAG promoter
loxP
CAT
loxP
Mafa
myc
pA
pA
Pdx1^PB-CreER^TM
Pdx1^Pst-Bst
CreER
Tamoxifen injection
Cre-mediated recombination
βMafa^myc
CAG promoter
loxP
Mafa
myc
pA
B
db/m
Pdx1^PB-CreER^TM
CAG-CAT-Mafa^myc
βMafa^myc
myc insulin
Mafa insulin
% Myc / insulin positive cells
% Mafa / insulin positive cells
0
20
40
60
80
100
**
db/m
Pdx1^PB-CreER^TM;db/db
CAG-CAT-Mafa^myc;db/db
βMafa^myc;db/db

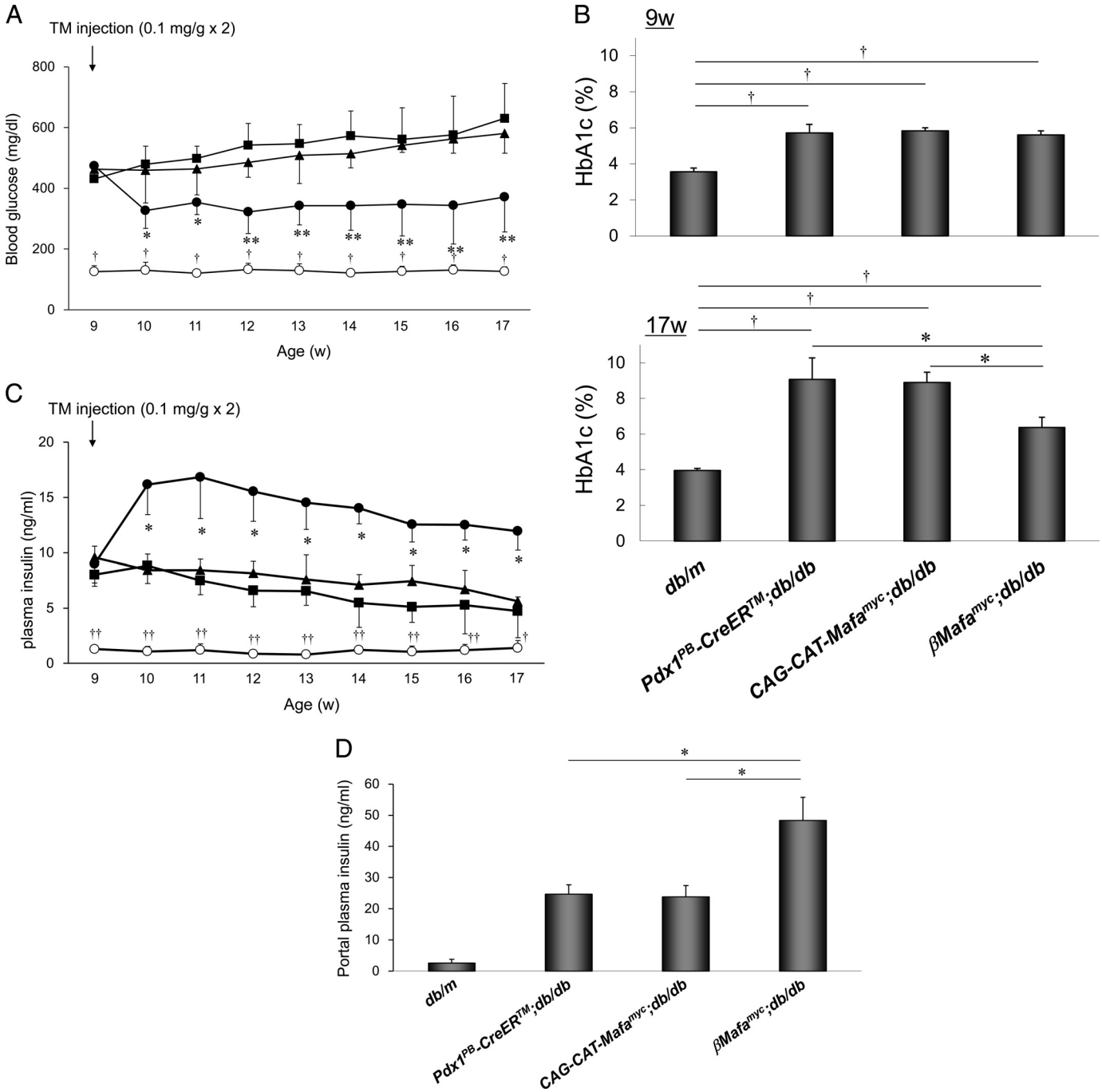


FIGURE 2. **Islet β-cell Mafa^{myc} production improves glycemic control in *βMafa^{myc};db/db* mice.** *A,* fed blood glucose levels were measured every week at 4 p.m. before and after TM injections. Control, *db/m* (○); *Pdx1^{PB}-CreER^{TM};db/db* (■); *CAG-CAT-Mafa^{myc};db/db* (▲); *βMafa^{myc};db/db* (●). *, $p < 0.05$; **, $p < 0.01$ *versus* control *db/db* mice. †, $p < 0.01$ *versus db/m* mice ($n = 6–8$). *B*, HbA1c levels were measured at 9 and 17 weeks. *, $p < 0.01$ *versus* control *db/db* mice. †, $p < 0.01$ *versus db/m* mice ($n = 6–8$). *C,* fed plasma insulin levels. *, $p < 0.01$ *versus* control *db/db* mice. †, $p < 0.05$; ††, $p < 0.01$ *versus db/m* mice ($n = 6–8$). *D,* plasma blood insulin levels sampled from the hepatic portal vein of 13 week-old mice. *, $p < 0.001$ *versus* control *db/db* mice ($n = 4$). All of *error bars* represent S.D.

non-diabetic (*db/m*) were 471.3 ± 89.1 and 130.2 ± 26.8 mg/dl, respectively. This resulted from higher peripheral and portal plasma insulin levels in the double transgenic *db*/*db* mice (Fig. 2, *C* and *D*), which also manifested reduced HbA1c values (Fig. 2*B*).

Improved plasma glucose clearance and higher insulin secretion levels were observed in *βMafa^{myc};db/db* mice in intra-peritoneal glucose tolerance tests (Fig. 3, *A* and *B*), whereas insulin sensitivity (Fig. 3*C*) and body weight (supplemental Fig. 5) did

FIGURE 1. **Generating islet β-cell-specific Mafa^{myc} expressing *db*/*db* mice.** *A,* schematic representation of transgenic strategy used to specifically express Mafa^{myc} in islet *db/db* β-cells. Exogenous Mafa^{myc} is expressed via Cre-mediated excision of the *loxP* flanked stuffer *CAT* cassette of *CAG-CAT-Mafa^{myc}*, with TM injection at 9 weeks causing activation of the ER-fused Cre recombinase produced from the islet β-cell-specific *Pdx1$^{Pst-Bst}$* promoter of *Pdx1^{PB}-CreER^{TM}*. *B,* pancreatic *db*/*db* islets from 18-week-old *βMafa^{myc}*, *CAG-CAT-Mafa^{myc}*, *Pdx1^{PB}-CreER^{TM}* mice were immunostained with myc (*red*), insulin (*green*), and/or Mafa (*red*) antibodies. The percent of insulin^{+} cells synthesizing *Mafa^{myc}* or *Mafa* is shown as average ± S.D. *Scale bars*: 50 μm.

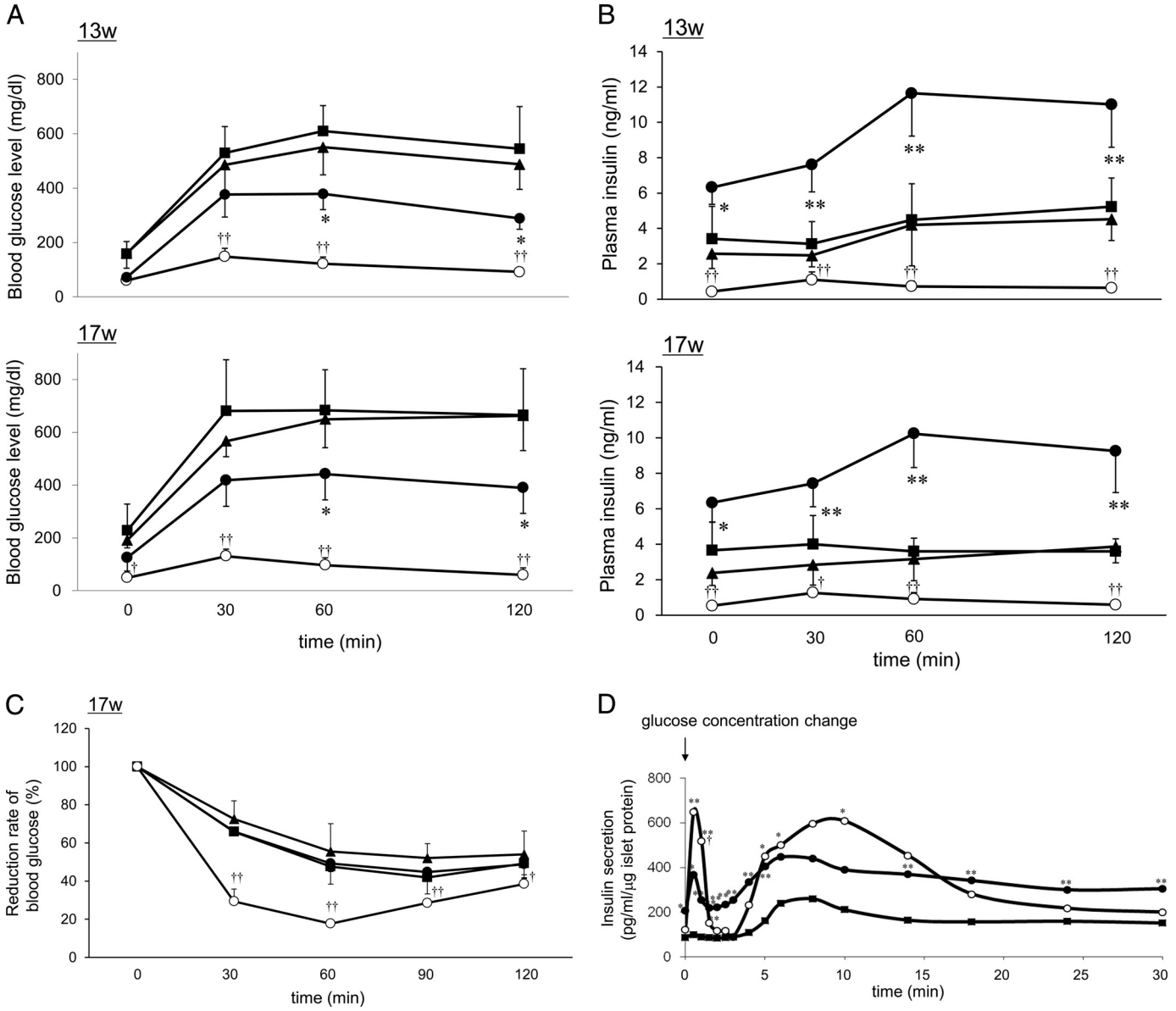
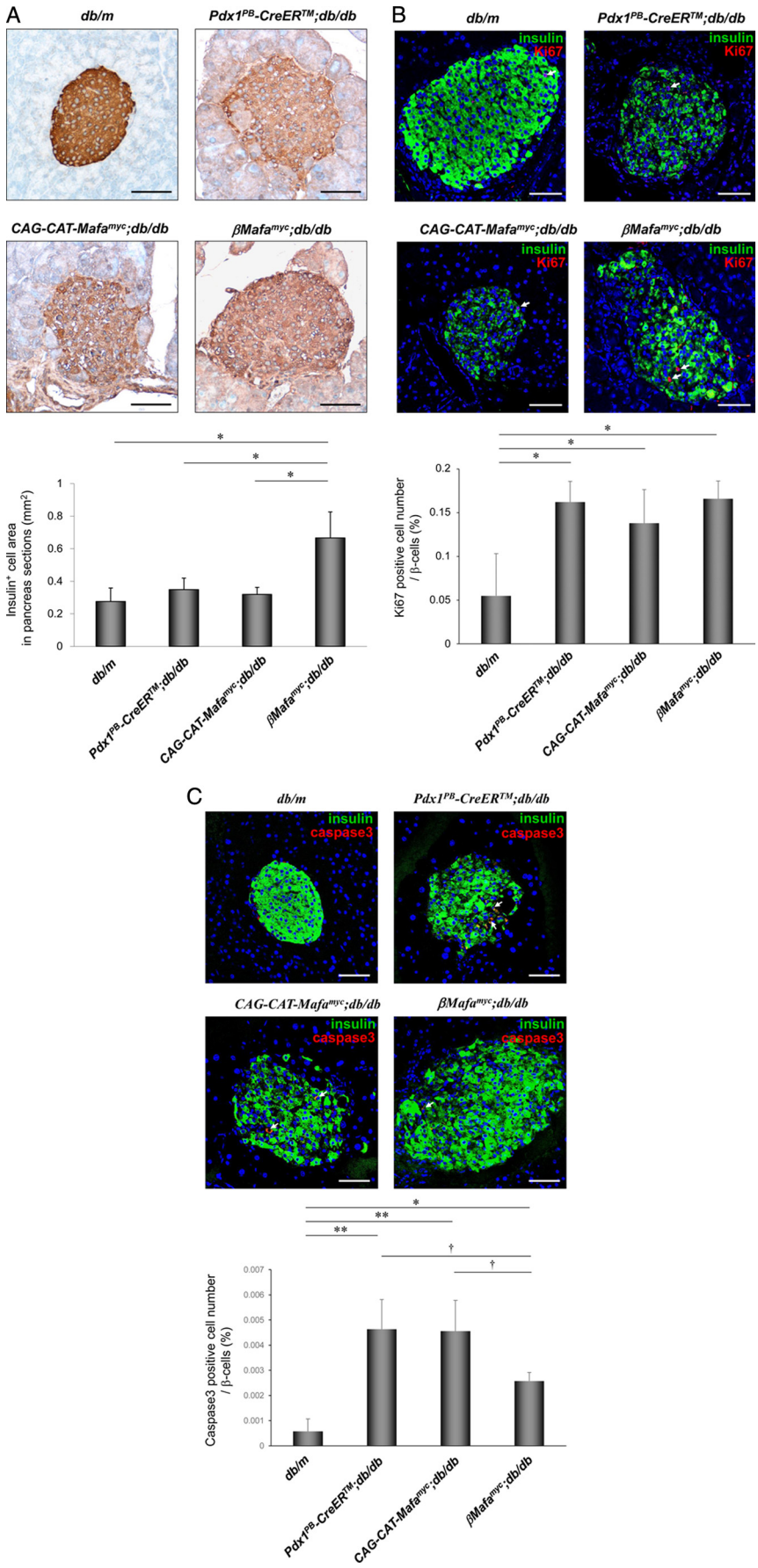


FIGURE 3. **Improved glucose-stimulated insulin secretion in *βMafa^{myc};db/db* mice.** Intraperitoneal glucose tolerance tests were performed on 13- and 17-week-old mice after a 14-h fast. Blood glucose (A) and plasma insulin (B) levels in *βMafa^{myc};db/db* (●), *Pdx1^{PB}-CreER^{TM};db/db* (■), *CAG-CAT-Mafa^{myc};db/db* (▲), and *db/m* (○) mice. *, $p < 0.05$; **, $p < 0.01$ *versus* control *db/db* mice. †, $p < 0.05$; ††, $p < 0.01$ *versus db/m* mice ($n = 4–5$). *C,* blood glucose levels in 17-week-old mice injected intraperitoneally with insulin (2.0 units/kg) after a 2-h fast. †, $p < 0.05$; ††, $p < 0.01$ *versus db/m* mice. *D,* islets from 13-week-old *βMafa^{myc};db/db* (●), *Pdx1^{PB}-CreER^{TM};db/db* (■), and *db/m* mice (○) perifused with normal and high glucose. *, $p < 0.05$; **, $p < 0.01$ *versus Pdx1^{PB}-CreER^{TM};db/db*, †, $p < 0.05$ *versus βMafa^{myc};db/db* ($n = 4$). All of *error bars* represent S.D.

not differ between the transgenic Mafa^{myc} expressing and non-expressing *db*/*db* mice. In addition, both 1st and 2nd phase insulin secretion increased in isolated *βMafa^{myc};db*/*db* islets (Fig. 3*D*). Collectively, these results show that Mafa^{myc} synthesis in 9-week-old *db*/*db* mice enabled quick recovery of β-cell function even in the context of persistent insulin insensitivity and obesity, resulting in better glucose utilization in peripheral tissues.

Islet β-Cell Mass Increases in Mafa^{myc} Producing db/db Mice—Insulin resistance and elevated blood glucose levels are first observed at around 4 weeks in *db*/*db* mice, whereas hyperglycemia plateaus at around 12 weeks. High-level β-cell proliferation occurs at 4 weeks in response (2), with sustained hyperglycemia decreasing islet insulin^{+} cell mass (25). Significantly, β-cell mass improved in *βMafa^{myc};db*/*db* islets relative to diabetic controls (Fig. 4*A* and supplemental Fig. S6). This resulted from reduced cellular caspase-3-mediated apoptosis, and not a change in proliferation rate of islet β-cells (Fig. 4, *B* and *C*). These findings illustrated the importance of Mafa in preserving β-cell levels in T2DM islets.

Identification of Gene Products Impacted by Maintaining Mafa Expression in βMafa^{myc};db/db Islet β-Cells—We first investigated how Mafa^{myc} influenced the expression of genes known to be regulated by this factor in *βMafa^{myc};db*/*db* islets, specifically *insulin I*, *insulin II*, *Pdx1*, and *glucokinase* (*Gck*), and *Mafa* itself (13). As expected from immunostaining (Fig. 1*B*), total *Mafa* mRNA was greatly induced over the non-transgenic diabetic controls, essentially now equivalent to euglycemic


A
db/m
Pdx1PB-CreERTM;db/db
CAG-CAT-Mafamyc;db/db
βMafamyc;db/db
B
db/m
Pdx1PB-CreERTM;db/db
CAG-CAT-Mafamyc;db/db
βMafamyc;db/db
insulin
Ki67
Insulin+ cell area in pancreas sections (mm2)
Ki67 positive cell number / β-cells (%)
db/m
Pdx1PB-CreERTM;db/db
CAG-CAT-Mafamyc;db/db
βMafamyc;db/db
C
db/m
Pdx1PB-CreERTM;db/db
CAG-CAT-Mafamyc;db/db
βMafamyc;db/db
insulin
caspase3
Caspase3 positive cell number / β-cells (%)
db/m
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βMafamyc;db/db

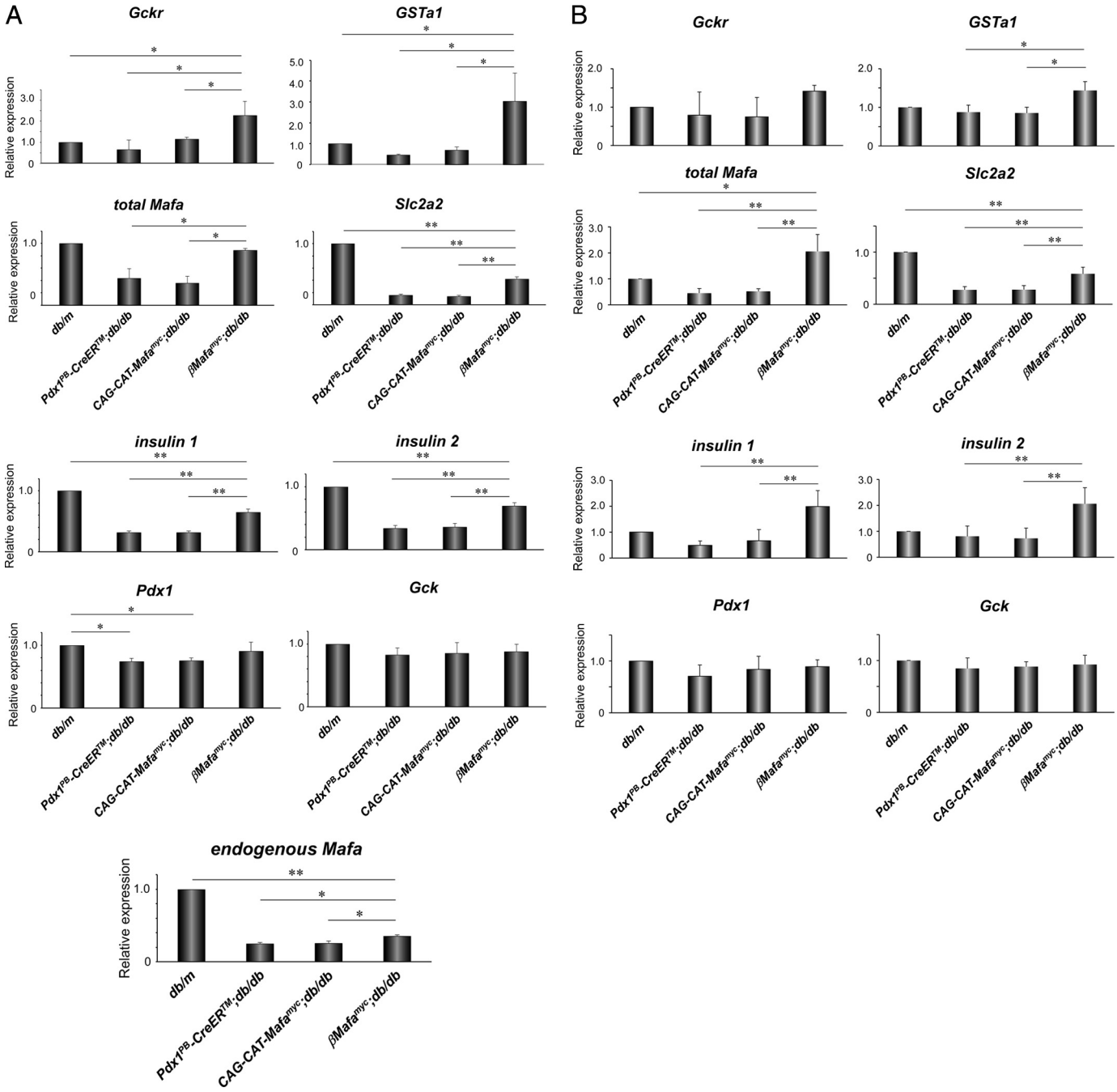

FIGURE 5. **Sustained expression of *bona fide* Mafa target genes in *βMafa*^{myc}*;db/db* islet β-cells.** Real-time RT-PCR was used to measure *Gckr, GSTa1,* total *Mafa* (*Mafa*^{myc} + endogenous *Mafa*), *Slc2a2*, *insulin 1*, *insulin 2*, *Pdx1*, *Gck*, and endogenous *Mafa* expression with islets at (*A*) 14 weeks, and (*B*) 9 weeks or 3 days after TM injections. *, $p < 0.05$; **, $p < 0.01$ ($n = 3–4$). All of *error bars* represent S.D.

db/m islets (Fig. 5). Moreover, *insulin 1, insulin 2*, and *Slc2a2* were also increased in *Mafa*^{myc} expressing *db/db* samples, with *Pdx1* trending toward recovery. In contrast, *Gck* expression was unaffected by diabetic conditions. These results demonstrated that functionally important Mafa-regulated gene products were limiting in diabetic *db*/*db* islet β-cells.

To identify other significant targets of Mafa^{myc} control, Agilent Mouse GE 4 × 44K v2 microarray analysis was performed with RNA extracted from 14-week-old *βMafa*^{myc}*;db*/*db* and diabetic control *Pdx1*^{PB}*-CreER*^{TM}*;db*/*db* islets. Approximately 340 genes had a more than 2-fold level increase in Mafa^{myc} producing islets (supplemental Appendix S1), including, as

FIGURE 4. **Mafa^{myc} preserves islet β-cell mass in *db*/*db* mice.** *A,* diaminobenzidine peroxidase immunodetection was used to measure the islet insulin$^+$ cell area in the 18-week-old *βMafa*^{myc}*;db/db*, *Pdx1*^{PB}*-CreER*^{TM}*;db/db*, *CAG-CAT-Mafa*^{myc}*;db/db*, and *db/m* pancreas. *, $p < 0.01$ *versus db/db* or *db/m* ($n = 3–4$). Pancreatic sections from 18-week-old mice immunostained for (*B*) insulin and Ki-67 or (*C*) cleaved caspase-3. The percent of Ki-67$^+$ or caspase-3$^+$ cells per total insulin$^+$ β-cells. *Arrows* mark Ki-67$^+$ or caspase-3$^+$ cells. *Scale bars*: 50 μm. *, $p < 0.05$; **, $p < 0.01$ *versus db/m* mice. †, $p < 0.01$ *versus* control *db/db* mice ($n = 3–4$). All of *error bars* represent S.D.

expected, *Mafa* and *Slc2a2*. Notably, gene ontology analysis indicated that proteins involved in reducing oxidative stress in β-cells were up-regulated, like the *glucokinase regulatory protein (Gckr*) that inactivates Gck by translocating the enzyme into the nucleus (26, 27), and *glutathione S-transferase α1* (*Gsta1*), which catalyzes the conjugation of glutathione to various molecules for the purpose of detoxification (28, 29).

The selective increase in *Gckr* and *Gsta1* expression levels was confirmed upon comparing RNA expression in 14-week-old Mafa^{myc} *db*/*db* islets to control *db*/*db* and *db*/*m* islets (Fig. 5*A*). This is likely caused by Mafa directly activating *Gckr* and *Gsta1* transcription, because expression of both was significantly reduced upon shRNA-mediated knock-down of Mafa in mouse MIN6 β-cells (supplemental Fig. S7). These results imply that factors reducing *db*/*db* β-cell stress, such as Gckr and Gsta1, were important effectors of *βMafa^{myc};db*/*db* recovery.

Induction of Gckr and Gsta1 Levels Coincides with Mafa^{myc} Production and Reduced Oxidative Stress in βMafa^{myc};db/db β-Cells—Further support of a direct role for Mafa^{myc} in recovery of *db*/*db* β-cells, *Gckr,* and *Gsta1* expression increased within 3 days of TM treatment in *βMafa^{myc};db*/*db* islets (Fig. 5*B*). *Insulin 1*, *insulin 2*, and *Slc2a2* levels were also elevated within this early period, although there was not yet a change in blood glucose levels (supplemental Fig. S8). Because elevated Gckr and Gsta1 could reduce oxidative stress in β-cells, for example, by the Gckr protein sequestering Gck in the nucleus and reducing glucose flux (27), production of the oxidative stress marker 4-hydroxy-2-nonenal was compared in non-diabetic, diabetic, and *Mafa^{myc}* containing *db*/*db* islets (supplemental Fig. S9). As predicted from elevated *Gckr* and *Gsta1*, 4-hydroxy-2-nonenal staining levels were decreased in *Mafa^{myc}* islets in comparison to diabetic *CAG-CAT-Mafa^{myc}*; *db*/*db* and *Pdx1^{PB}-CreER^{TM}* islets. However, *Mafa^{myc}* islet staining was still elevated in relationship to euglycemic *db*/*m* islets, implying that restoration of *Mafa* alone is unable to completely prevent glucotoxicity in *db*/*db* β-cells. Such was expected from the difference in blood glucose and HbA1c levels between *βMafa^{myc};db*/*db* and *db*/*m* mice (Fig. 2*B*). Collectively, these results strongly suggest that *Mafa^{myc}* improves *db*/*db* β-cell by reducing toxic stress conditions, presumably by elevating expression of factors like *Gckr* and *Gsta1*.

There Is a Marked Decrease of GSTA1 in Human T2DM Islets—The presence of MAFA in insulin^{+} cells is markedly decreased in T2DM islets (see supplemental Fig. S1 and Refs. 2 and 24). Immunostaining for GSTA1 was performed in normal and T2DM pancreatic samples to determine whether compromised expression was also associated with the disease state in humans. Gsta1 was clearly detected in normal human β-cells, whereas almost undetectable in T2DM islet (Fig. 6). Collectively, our analysis in *db*/*db* mice and human T2DM islets illustrates that reduction of MAFA in T2DM not only influences normal mediators of β-cell function and mass, but also stress effector levels in islet cells.

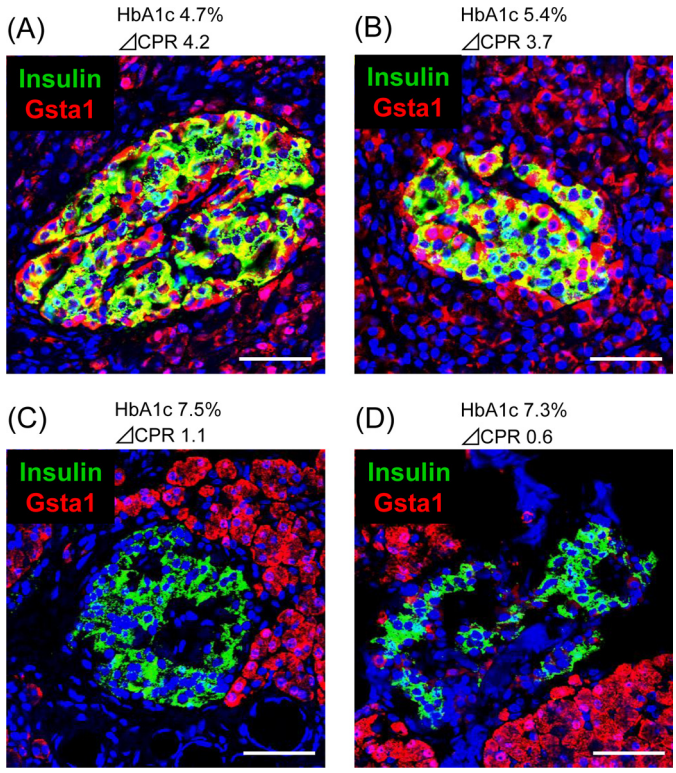

FIGURE 6. **GSTA1 levels are compromised in human T2DM islets.** Pancreatic section from (*A* and *B*) normal non-diabetic and (*C* and *D*) T2DM donors were costained with GSTA1 (*red*) and insulin (*green*). Nuclei (*blue*) were stained with DAPI. *Scale bars*: 50 μm.

DISCUSSION

Transcription factors have been demonstrated to play essential roles in both the embryonic formation and adult function of islet cells in mice. The association of many of these islet-enriched proteins to MODY implies that their function is also conserved within humans (30). Notably, Mafa is distinct among this group of transcription factors in being uniquely produced at the onset of islet β-cell formation during development and exclusively within this embryonic and adult islet population. Moreover, gene knock-out studies have demonstrated that Mafa only affects islet β-cell maturation, but not euglycemia. Human MAFA has also been found to be markedly decreased in T2DM β-cells, with overt cell dysfunction likely caused by decreased NKX6.1 and/or PDX1, whose loss from mouse islets quickly results in hyperglycemia (2, 16, 19). However, because the expression and/or activity of a variety of factors were affected in islet β-cells under these glucotoxic conditions, it is difficult to elucidate what factor(s) is pathophysiological. In this report, we directly examined the significance of Mafa activity in insulin-resistant, diabetic *db*/*db* mice by conditionally and specifically producing Mafa^{myc} in islet β-cells. Expression levels comparable with endogenous normoglycemic Mafa improved β-cell function, β-cell mass, and blood glucose levels. These results support MAFA in the molecular underpinnings of islet β-cell inactivation during T2DM.

The induction of Mafa^{myc} in *βMafa^{myc};db*/*db* mice was initiated at 9 weeks of age to maintain production during the period when endogenous stressors would normally cause Mafa inactivation. The expression of many target genes of Mafa increased in Mafa^{myc} β-cells relative to diabetic β-cells. Improvement of glycemic control by Mafa^{myc} β-cells was produced in the context of continued production of obesity- and

dyslipidemia-induced stress signals instigated by the leptin receptor mutation in *db*/*db* mice. Microarray studies were performed to identify the proteins enhancing β-cell activity. As expected, a variety of gene products normally required in glucose sensing and insulin secretion were elevated in Mafa^{myc} β-cells, like the Slc2a1 and Slc2a2 (supplemental Fig. S10), glucose transporters essential to rodent and human glucose-stimulated insulin secretion (31). Interestingly, genes encoding proteins protective to the β-cell were also found, with Gsta1 and Gckr representative examples (supplemental Appendix S1). Notably, Gsta1 and Gckr expression increased immediately after Mafa^{myc} production, prior to the reduction in blood glucose levels. Moreover, their expression was sustained throughout the time course of experimentation, as predicted of important neutralizing effectors of β-cell stress.

Islet β-cells have very low hydrogen peroxide scavenging enzyme levels relative to other cell types, like glutathione peroxidase-1 and catalase (4–6). Significantly, transgenic β-cell-specific glutathione peroxidase-1 expression profoundly increased β-cell function in *db*/*db* mice, coinciding with recovery of nuclear Mafa and Nkx6.1 (2). The insulin secretion defects in human T2DM islets are also improved upon *in vitro* treatment with reactive oxygen species scavengers (7). Induction of Gsta1 and Gckr by Mafa^{myc} would reduce oxidative stress in β-cells, for example, by preventing oxidation of cysteine 277 and 293 in Mafa that cause loss in *cis*-acting DNA binding activity (2). This could explain why endogenous Mafa levels were also increased in *βMafa^{myc}*;*db*/*db* islets (Fig. 1*B*).

Interestingly, the mechanisms of action of these antioxidants are distinct. Thus, the Gsta1 enzyme detoxifies reactive oxygen species by direct conjugation with glutathione, whereas Gckr inhibits glucokinase by binding non-covalently to form an inactive complex (26). As glucokinase is the principal regulator of glucose flux and insulin secretion in β-cells (32), Gckr binding would reduce cellular metabolism, and consequentially the generation of active oxygen species. *Gckr* was identified as a T2DM susceptible gene in genome-wide association studies (33). The increased oxoreductase activity in *βMafa^{myc}*;*db*/*db* islet β-cells would also reduce thioredoxin-interacting protein levels, a cellular redox regulator that both induces cell death and inhibits *Mafa* mRNA expression (34).

GSTA1 levels were decreased in T2DM islets (Fig. 6). Consequently, it will be important to investigate precisely how expression of antioxidant factors like Gckr and Gsta1 influence β-cell activity. These findings suggest that screening of pharmacological agents to either enhance antioxoreductase levels is warranted for treatment of pre- and established T2DM patients. For example, could induction of antioxidant factors be protective in islet β-cells of obese individuals, as the majority will not become T2DM (35)? Perhaps expression is also limiting within the insulin^{+} cells produced during *in vitro* differentiation of human embryonic stem cells, which express many transcriptional regulators associated with mature islet cells, yet remain dysfunctional with regards to glucose responsiveness and high insulin production until able to express MAFA (36). Notably, oxidative destruction is still evident in *βMafa^{myc}*;*db*/*db* islets (supplemental Fig. S9), presumably limiting β-cell activity and the complete restoration of blood glucose levels. Presumably, the failure to produce normal Nkx6.1 levels in *βMafa^{myc}*;*db*/*db* β-cells results in relative inactivity, and prevents the return to euglycemia.

Interestingly, improved blood glucose levels and islet β-cell function are observed upon treatment of *db*/*db* mice with either phloridzin (37) or insulin (38), which act by effecting glucose utilization (*i.e.* insulin) or clearance (phloridzin) in peripheral tissues. The consequential reduction in blood glucose levels likely enhances β-cell mass and activity by decreasing cellular glucose flux and stress effector levels. The present work strongly suggests that Mafa is a direct mediator of improved β-cell activity under these conditions. Thus, sustaining Mafa expression in diabetic *βMafa^{myc}*;*db*/*db* β-cells led to lowered blood glucose levels, increased β-cell mass, and improved β-cell function. Future efforts will be directed at determining how to prevent MAFA inactivation in T2DM β-cells.

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