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The MafA Transcription Factor Becomes Essential to Islet β -Cells Soon After Birth

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The large Maf transcription factors, MafA and MafB, are expressed with distinct spatial-temporal patterns in rodent islet cells. Analysis of *Mafa*^{-/-} and pancreas-specific *Mafa* ^{Δ panc} deletion mutant mice demonstrated a primary role for MafA in adult β -cell activity, different from the embryonic importance of MafB. Our interests here were to precisely define when MafA became functionally significant to β -cells, to determine how this was affected by the brief period of postnatal MafB production, and to identify genes regulated by MafA during this period. We found that islet cell organization, β -cell mass, and β -cell function were influenced by 3 weeks of age in *Mafa* ^{Δ panc} mice and compromised earlier in *Mafa* ^{Δ panc};*Mafb*^{+/-} mice. A combination of genome-wide microarray profiling, electron microscopy, and metabolic assays were used to reveal mechanisms of MafA control. For example, β -cell replication was produced by actions on cyclin D2 regulation, while effects on granule docking affected first-phase insulin secretion. Moreover, notable differences in the genes regulated by embryonic MafB and postnatal MafA gene expression were found. These results not only clearly define why MafA is an essential transcriptional regulator of islet β -cells, but also why cell maturation involves coordinated actions with MafB.

The pancreatic β -cell is one of several hormone-producing cell types critical to glucose homeostasis present within

the islet of Langerhans. However, hyperglycemia is specifically caused by the loss or inability of β -cells to synthesize and secrete the insulin hormone, causing diabetes mellitus, a life-threatening condition associated with millions of people worldwide. Current therapies for diabetes involve external insulin treatment either through injection or a pump, but this still often results in long-term medical complications, including cardiovascular disease, chronic renal failure, and retinopathy. These complications are likely due to the inability of exogenously supplied insulin to regulate blood glucose levels in the same facile manner as endogenous β -cells. As a consequence, efforts have been focused on understanding the molecular mechanisms underlying normal β -cell development and function, with the hope that such knowledge will provide insight into therapeutic ways of either sustaining endogenous β -cell function or engineering replacement cells.

Genetic and biochemical studies have demonstrated that islet-enriched transcription factors play a central role in orchestrating the intricate series of events required in the biosynthesis of islet hormone-producing α (i.e., glucagon), β , δ (somatostatin), ϵ (ghrelin), and pancreatic polypeptide cells (reviewed elsewhere [1–3]). The functional significance of these proteins was reinforced upon observing that six of nine genes associated with a monoallelic form of diabetes, maturity-onset diabetes of the young, encode for transcription factors essential to islet cells (reviewed elsewhere [4,5]). Moreover, altering the

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expression of individual islet-enriched transcription factors often influences the presence and/or activity of a particular islet cell type (e.g., Arx [6], Pax4 [7], Pdx1 [maturity-onset diabetes of the young, type 4] [8]). In addition, the combinatorial actions of these proteins are capable of reprogramming nonislet cells to β -like cells, exemplified by misexpression of MafA, Pdx1, and Ngn3 in pancreatic acinar cells (9).

Interestingly, members of the same transcription factor family often contribute to β -cell formation, including winged-helix/forkhead-containing FoxA1 and FoxA2 (10), zinc finger Gata4 and Gata6 (11,12), NK6 homeodomain Nkx6.1 and Nkx6.2 (13), paired box homeodomain Pax4 and Pax6 (7,14), as well as basic leucine-zipper MafA and MafB (15). FoxA1/2, Nkx6.1/6.2, Gata4/6, and Pax4/6 are expressed broadly in early pancreatic epithelial cells and then become principally confined to islet cells (e.g., Nkx6.1 [β], FoxA1/A2 [all], Gata6 [all], Pax6 [all]) or cease to be expressed after birth (Gata4, Pax4, Nkx6.2) (2,3). However, MafA and MafB are distinct in being produced relatively late during development and primarily (i.e., MafB [16]: α , β , Ngn3⁺ islet cell progenitors), if not exclusively in hormone⁺ cells (β only, MafA [17]). Consequently, analysis of *Mafa* and *Mafb* mutant mice has revealed more specialized roles in islet cell maturation than other islet-enriched transcription factors. Thus islet cell identity and/or numbers are often profoundly affected in islet-enriched transcription factor knockout mice (1–3), whereas only terminal β - and α -cell maturation is defective in *Mafb*^{-/-} embryos (16,18). In contrast, islet cell development was unchanged in *Mafa*^{-/-} (19) or pancreas-specific knockout *Mafa* ^{Δ panc} (20) mice, although glucose-regulated insulin secretion and islet cell architecture was compromised in adults. Remarkably, human embryonic-stem-derived insulin⁺ cells do not become glucose responsive until the appearance of MafA (21,22), with induction currently requiring transplantation of in vitro programmed cells into mice (22).

Rodent β -cells switch from producing primarily MafB during development to exclusively MafA postnatally (18,20). Hence, MafB is expressed in all embryonic glucagon⁺ cells and insulin⁺ cells produced during the first and secondary wave of hormone⁺ cell formation and then disappears from β -cells roughly 2 weeks after birth (16,18). In contrast, MafA is produced exclusively in the second or principal wave of insulin⁺ cells arising from embryonic (E) day 13.5 (17), which become the β -cells of the islet. Notably, MafA controls many embryonically activated MafB genes in adults (20). Here we have more precisely defined the functional interrelationship between these closely related large Maf proteins by answering the following questions: 1) When does MafA become important to postnatal β -cells? 2) Is transient expression of MafB postnatally significant to islet β -cell activity? 3) How does MafA regulate islet β -cells? 4) Are there genes regulated by MafA in adults that are unaffected by MafB during development?

A time course study performed with mice lacking MafA in the pancreas (termed *Mafa* ^{Δ panc}) revealed that loss of MafA first significantly affected β -cell mass, β -cell function, and islet architecture by 3 weeks of age. Large-Maf-regulated gene expression in islet β -cells was compromised even earlier in *Mafa* ^{Δ panc}; *Mafb*^{+/-} than *Mafa* ^{Δ panc} mice. Gene profiling analysis revealed that MafA regulates many key aspects of the glucose-stimulated insulin secretion pathway, including glucose metabolism, insulin production, and insulin granule docking. These findings substantially expand our knowledge of how MafA affects islet β -cell activity and provide a greater awareness into why this factor is so critical to the production of functional β -cells.

RESEARCH DESIGN AND METHODS

Animals

Pancreas-wide MafA deletion mutant mice (*Mafa* ^{Δ panc}) were generated by breeding *Mafa*^{fl/fl} with *Pdx1*^{5.5-Cre} mice (20). *Mafa*^{fl/fl} mice served as experimental wild-type controls. *Mafb*^{+/-} mice have been described previously (20). Compound *Mafa*^{fl/fl}; *Mafb*^{+/-}; *Pdx1*^{5.5-Cre} mutant mice were referred to as *Mafa* ^{Δ panc}; *Mafb*^{+/-}. All mice used are on a mixed genetic background (C57BL/6J, I129, Balb/c). The Vanderbilt University Institutional Animal Care and Use Committee approved all of these studies.

Islet Isolation, RNA Extraction, and Quantitative Real-Time PCR Analysis

Mice of 2, 3, or 4 weeks of age were anesthetized and then killed via cervical dislocation. The abdominal cavity was exposed and collagenase P (Roche) in PBS was injected into the common bile duct with the end connecting to the duodenum blocked. The pancreas was then isolated and digested further with collagenase P and islets isolated by hand picking under 10 \times magnification. Islet RNA was isolated using TRIzol (Invitrogen) and further treated with the DNA-Free RNA Kit (Zymo Research). RNA quality was determined using the Agilent 2100 Bioanalyzer in the Vanderbilt Functional Genomics Shared Resource Core Laboratory; RNA was only used if the integrity value was 7 or greater. Quantitative real-time PCR (qPCR) was conducted as previously described on a Light Cycler 480 II (Roche) (20); primer sequences are available upon request. The RNA data presented in Figs. 4 and 6 were performed with islet preparations free of acinar cell contamination, as determined by amylase analysis.

Tissue Preparation and Immunohistochemistry

The conditions used for paraffin embedding pancreatic sections and immunofluorescence labeling were described previously (20). The following primary antibodies were used for immunostaining: guinea pig α -insulin (1:2,000; Linco Research), guinea pig α -glucagon (1:2,000; Linco Research), rabbit α -glucagon (1:2,000; Linco Research), rabbit α -MafB (1:10,000; Bethyl Laboratories), rabbit α -MafA (1:1,000, Bethyl Laboratories), rabbit α -Slc30a8 (1:1,000, Mellitech), and rabbit α -Glut2 (1:1,000; Chemicon). Species-matched secondary antibodies were used for

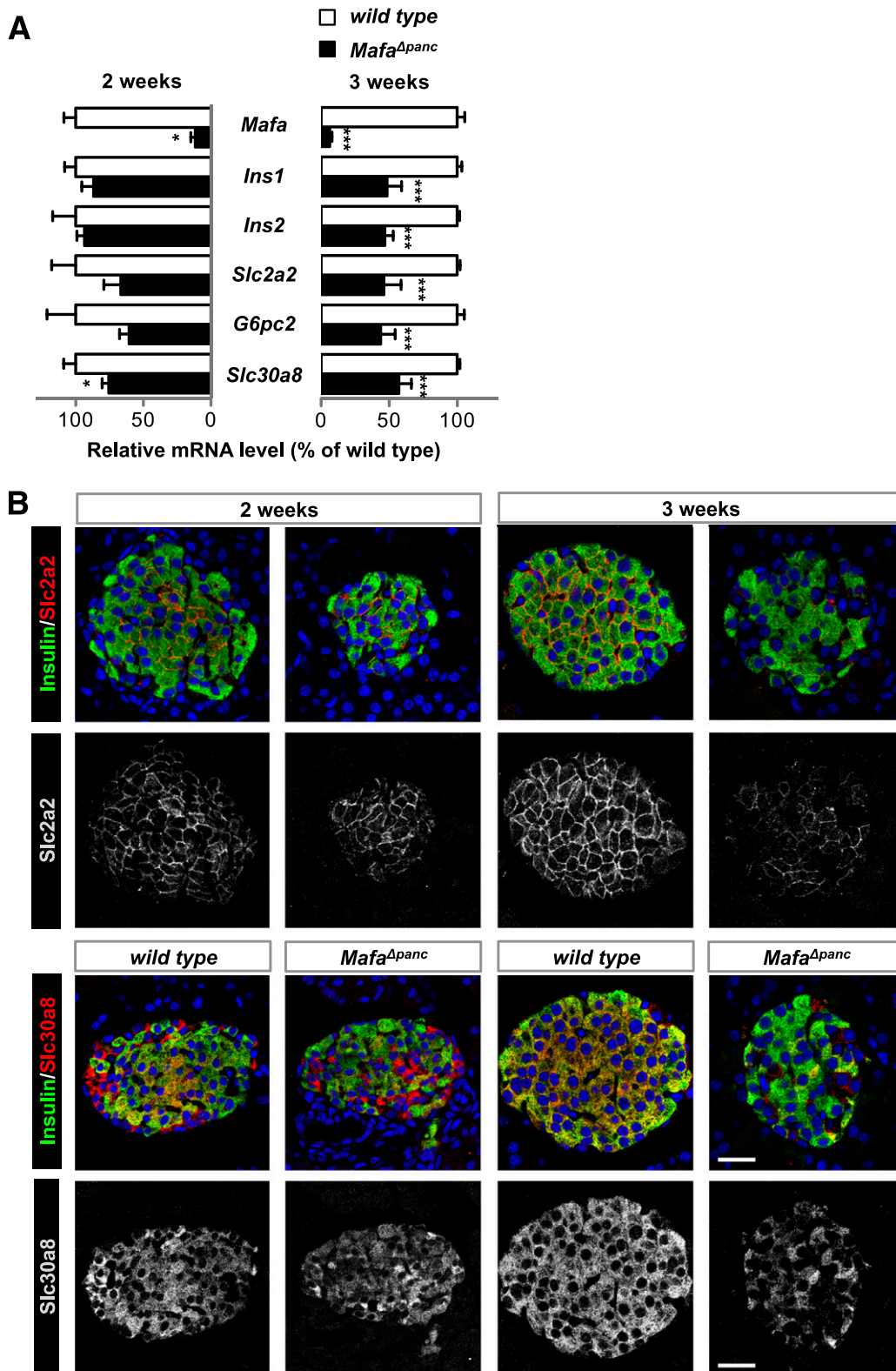


Figure 1—MafA begins to affect β -cell gene expression by 3 weeks after birth. (A) The mRNA levels of MafA-regulated genes in 2- and 3-week-old *Mafa* Δ *panc* and control islets. All were significantly diminished by 3 weeks, with only *Slc30a8* statistically changed at 2 weeks. Results were presented as mean \pm SEM. * $P < 0.05$ ($n \geq 3$); *** $P < 0.001$. (B) The 2- and 3-week-old *Slc2a2* and *Slc30a8* protein staining pattern in wild-type and *Mafa* Δ *panc* islets. The change in protein levels was most evident in 3-week-old *Mafa* Δ *panc* β -cells. Representative images are shown; nuclei were stained with Yopro1 (blue). The red channel (*Slc2a2* or *Slc30a8*) was presented in gray scale next to each figure. Scale bar = 20 μ m.

immune detection (Cy5-conjugated donkey anti-guinea pig, Cy5-conjugated donkey anti-sheep, Cy2-conjugated donkey anti-guinea pig, Cy2-conjugated donkey anti-rabbit, Cy3-conjugated donkey anti-rabbit [all 1:500; Jackson ImmunoResearch Laboratories]). Cy3-conjugated tyramide signal amplification (1:400, PerkinElmer) was used for detecting MafB labeling with the biotin-conjugated donkey anti-rabbit secondary antibody (1:500; Jackson ImmunoResearch Laboratories). Nuclear counterstaining was performed using YoPro1 or DAPI (Invitrogen). Immunofluorescence images were acquired with a Carl Zeiss LSM510 confocal microscope.

Islet Morphology, β - to α -Cell Ratio, and β -Cell Area Analyses

Evenly spaced pancreatic sections at 150 and 240 μm apart were used from 1- or 2- and 3-week-old mice, respectively. Insulin⁺ cells and glucagon⁺ cell images were counted at 400 \times magnification. The β - to α -cell ratio was calculated by dividing the total number of insulin⁺ cells by the total number of glucagon⁺ cells. The relative β -cell mass was determined in pancreatic sections where

insulin⁺ cells were visualized by DAB labeling, and pancreatic tissue was counterstained with eosin. Images of pancreatic sections were captured at 20 \times magnification using a ScanScope CS (Aperio), and the relative β -cell area was calculated by dividing the area of insulin⁺ cells by that of the whole pancreas.

Intraperitoneal Glucose Tolerance Test

Four-week-old mice ($n \geq 7$) were fasted from 1800 to 0800 h, and blood glucose levels from the sampled saphenous vein were determined using a FreeStyle glucometer (Abbott Diabetes Care). The mice were then weighed, and 1 mg dextrose/g body weight (FisherBiotech) in sterile PBS was injected intraperitoneally. Blood glucose levels were measured at 15, 30, 60, 90, and 120 min post-injection. Fed blood glucose levels were measured prior to fasting.

Islet Zn²⁺ Content

Freshly isolated islets were washed in Ca²⁺-free Hanks' balanced salt solution and frozen at -80°C in size-matched 20-islet aliquots. Islets were lysed in 1 mL lysis buffer (1% Triton X-100 in 10 mmol/L Tris-HCl, pH = 7.4,

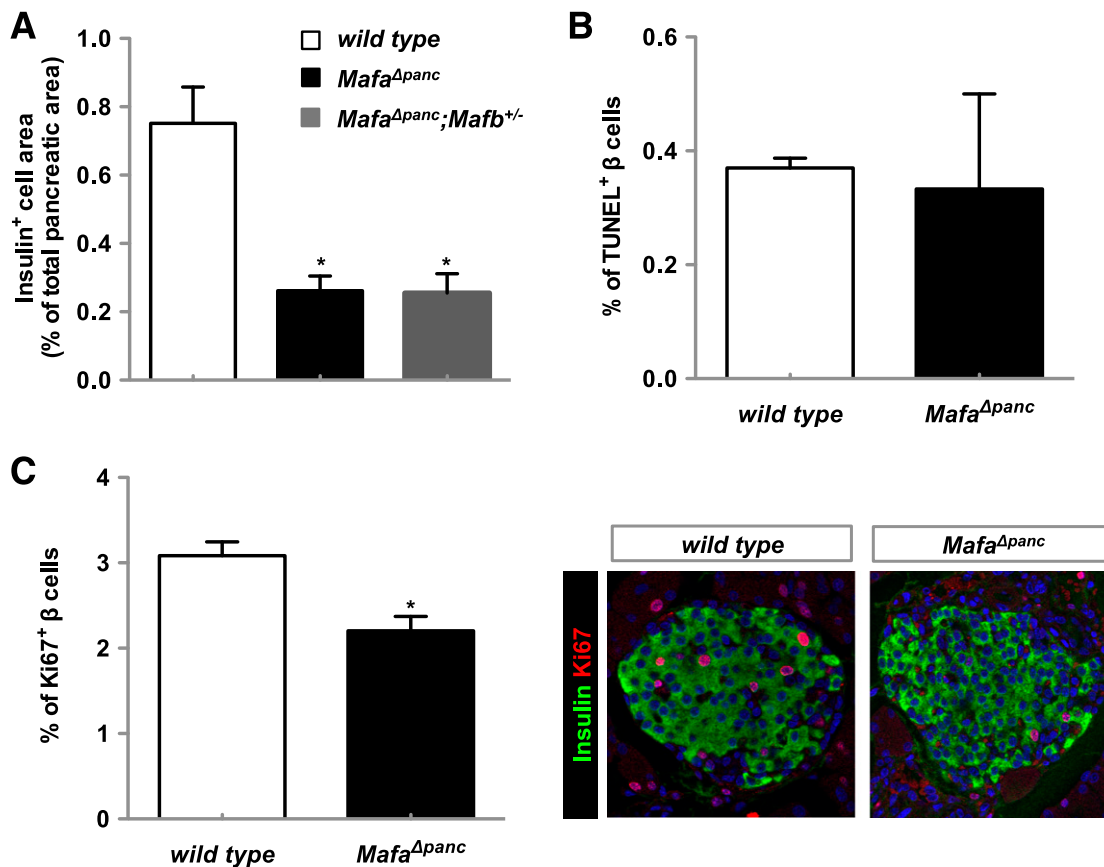


Figure 2—MafA influences β -cell proliferation. (A) Islet β -cell area is reduced in 3-week-old *Mafa*^{Δpanc} mice when compared with littermate wild-type mice ($n = 3$). $*P < 0.05$. No change in (B) β -cell apoptosis was detected in the TUNEL assay ($n = 4$; $P = 0.8406$), whereas the percentage of (C) Ki67⁺ proliferating insulin⁺ cells decreased in 3-week-old *Mafa*^{Δpanc} islets ($n = 4$). $*P < 0.05$. Representative images of Ki67 and insulin labeling were included in C. Approximately 3,000 insulin⁺ cells were counted per mouse in the Ki67 studies. Results were presented as mean \pm SEM.

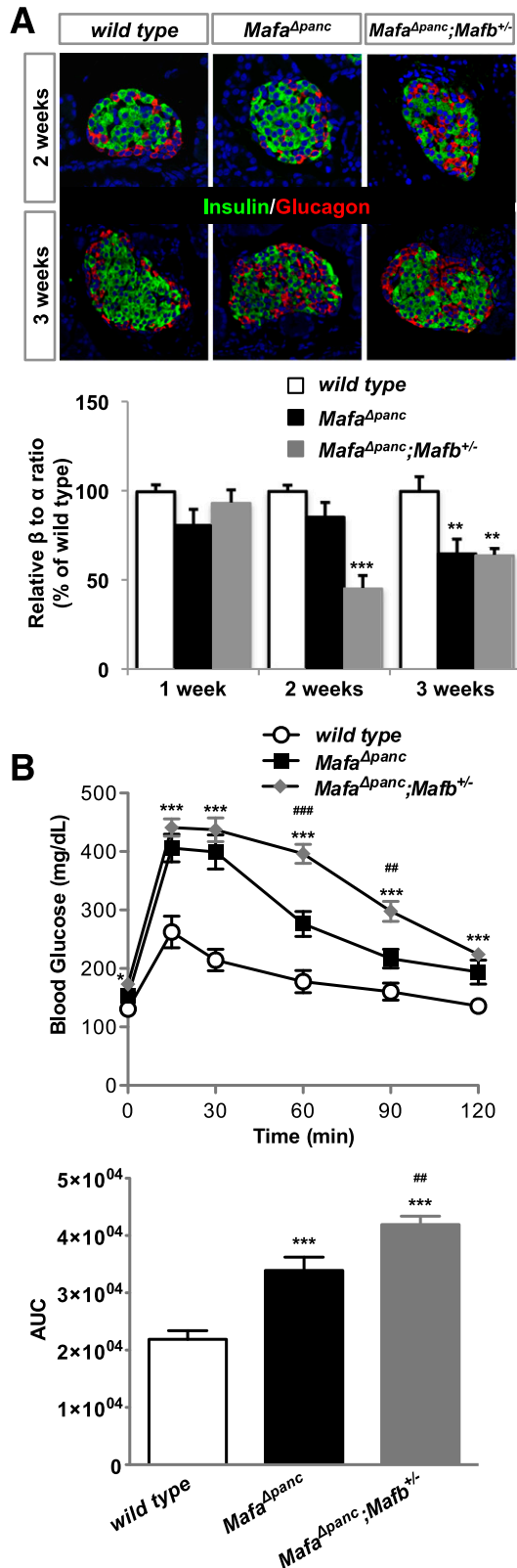


Figure 3—The changes in islet architecture and glucose tolerance in *Mafa* ^{Δ panc} mice are first observed at 3 weeks, and these defects appear more profound and earlier in *Mafa* ^{Δ panc};*Mafb*^{+/-} mice. (A, top) The glucagon⁺ and insulin⁺ staining pattern in 2- and 3-week-old wild-type, *Mafa* ^{Δ panc}, and *Mafa* ^{Δ panc};*Mafb*^{+/-} pancreas. Unlike wild-type, α -cells were commonly found in the islet core in 3-week-old *Mafa* ^{Δ panc} and *Mafa* ^{Δ panc};*Mafb*^{+/-} mice. (A, bottom) In

made in double-distilled H₂O [18.2 M Ω], avoiding any glassware), and the Zn²⁺ concentration in the lysate was measured using a FluoZin-3 (Invitrogen) fluorescent dye assay (23). The Zn²⁺ concentration per islet (micromoles per islet) was determined by comparing the fluorescent signal at 516 nm to a standard curve generated from serial dilutions of ZnCl₂ in lysis buffer.

NAD(P)H Imaging

Freshly isolated islets were incubated in imaging medium (125 mmol/L NaCl, 5.7 mmol/L KCl, 2.5 mmol/L CaCl₂, 1.2 mmol/L MgCl₂, 10 mmol/L HEPES, 2 mmol/L glucose, 0.1% BSA, pH = 7.4) for 90 min and placed in a polydimethylsiloxane microfluidic flow device (24) on an LSM710 microscope (Zeiss). NAD(P)H autofluorescence was imaged under two-photon excitation using a 710 nm mode-locked Ti:sapphire laser oscillator (Coherent) and a custom 380–500 nm band-pass filter (Chroma) and nondescanned detector. Z-stacks of six images were acquired at 2 μ m spacing 10 min after a change in glucose stimulation. All microscope and laser settings were kept constant between measurements.

mRNA Microarray

Total isolated islet RNA was prepared from four independently derived 3-month-old littermate *Mafa* ^{Δ panc} and wild-type mice. Total RNA (50 ng) was amplified using the Ovation RNA Amplification System V2 (Nugen Inc.), labeled with the Bio-Prime Array CGH Genomic Labeling System (Invitrogen Life Technologies), purified, and then analyzed on the whole mouse genome G4122A oligo microarray (Agilent Technology). The microarray analysis was performed at the Functional Genomics Core at the University of Pennsylvania.

Islet Perfusion

Islet function was studied in a dynamic cell perfusion system at a perfusate flow rate of 1 mL/min (25,26). The effluent was collected at 3-min intervals using an automatic fraction collector. Insulin concentration in each fraction was measured by radioimmunoassay (RI-13K, Millipore). Insulin content in islet extracts was determined by radioimmunoassay (insulin, RI-13K, Millipore).

Electron Microscopy

Islet β -cell ultrastructure was assessed by transmission electron microscopy as described previously (25). The density of β -cell secretory granules was determined using

addition, the β - to α -cell ratio is reduced in 3-week-old *Mafa* ^{Δ panc} mice and by 2 weeks in *Mafa* ^{Δ panc};*Mafb*^{+/-} mice. (B, top) Impaired glucose clearance was observed in 4-week-old *Mafa* ^{Δ panc} mice as expected from the changes in islet architecture and β -cell gene expression (Fig. 1A). Glucose intolerance is more severe in the *Mafa* ^{Δ panc};*Mafb*^{+/-} mutant of same age. (B, bottom) The area under the curve of the glucose tolerance test curve is shown in the bar graph. All data are presented as mean \pm SEM. **P* < 0.05; ***P* < 0.01; ****P* < 0.001 compared with wild-type control. ###*P* < 0.01; ####*P* < 0.001 compared with *Mafa* ^{Δ panc}. AUC, area under the curve.

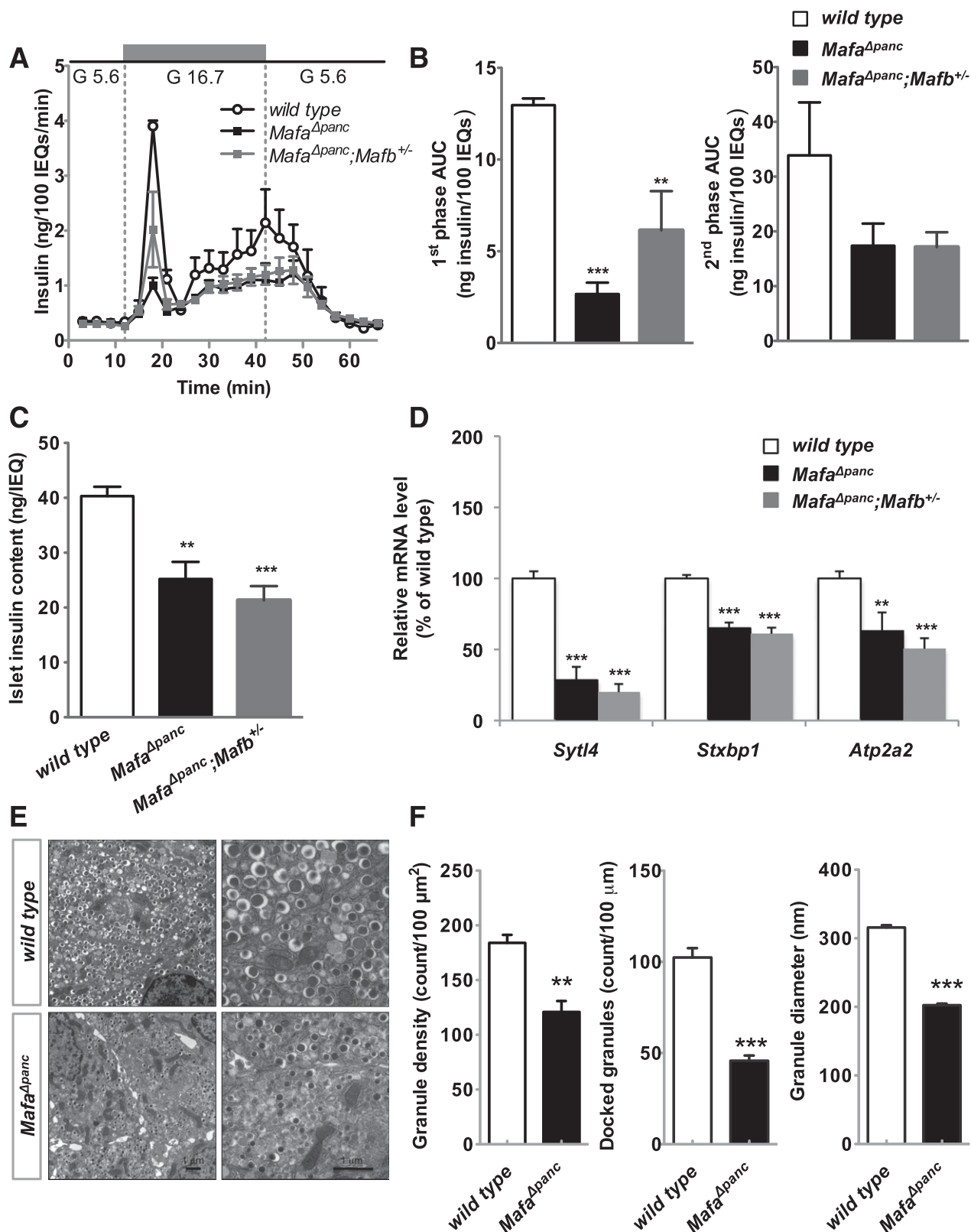


Figure 4—MafA regulates both insulin content and glucose-stimulated insulin secretion levels. (A) Dynamic glucose-regulated insulin secretory characteristics of perfused 12-week-old *Mafa*^{Δpanc}, *Mafa*^{Δpanc};*Mafb*^{+/-}, and control islets (*Mafa*^{Δpanc}, *n* = 5; *Mafa*^{Δpanc};*Mafb*^{+/-}, *n* = 5; wild-type, *n* = 4). (B) Integrated area under the curve showed that the first phase of glucose-stimulated insulin secretion was decreased by 79% in *Mafa*^{Δpanc} versus the control ($***P < 0.0001$), with only second-phase secretion trending lower ($P = 0.1319$). Similarly, only first-phase insulin secretion was impaired in *Mafa*^{Δpanc};*Mafb*^{+/-} mutant islets (45% of wild-type). $**P < 0.001$; $***P < 0.001$. (C) Islet insulin content in 12-week-old *Mafa*^{Δpanc} mice was down by 37% and *Mafa*^{Δpanc};*Mafb*^{+/-} by 47% when compared with controls. $**P < 0.01$. (D) Critical regulators of insulin secretion pathway were compromised in adult *Mafa*^{Δpanc} and *Mafa*^{Δpanc};*Mafb*^{+/-} mouse islets, including

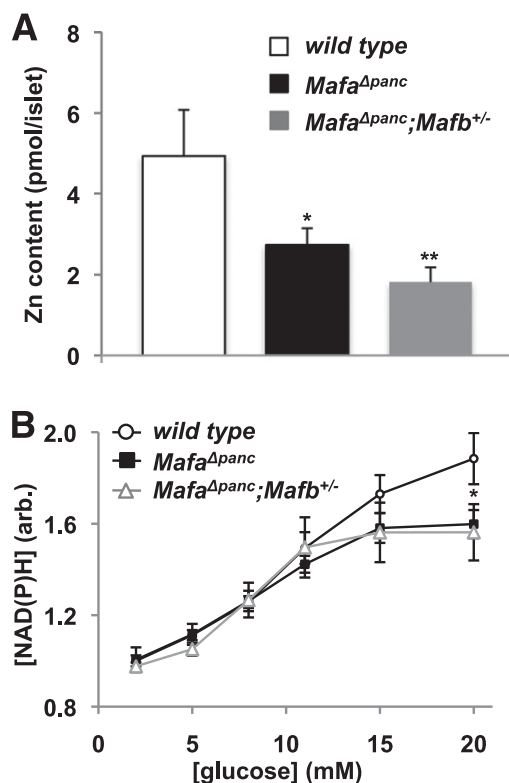


Figure 5—Zn²⁺ and NAD(P)H content in *Mafa*^{Δpanc} and *Mafa*^{Δpanc};*Mafb*^{+/-} β -cells. (A) Total islet Zn²⁺ content was reduced to 56% in 3-month-old *Mafa*^{Δpanc} islets and 37% in *Mafa*^{Δpanc};*Mafb*^{+/-} islets (wild-type, $n = 10$; *Mafa*^{Δpanc}, $n = 9$; *Mafa*^{Δpanc};*Mafb*^{+/-}, $n = 5$). * $P < 0.05$; ** $P < 0.01$. (B) Normalized glucose dose-response curve of NAD(P)H levels in 3-month-old wild-type, *Mafa*^{Δpanc}, and *Mafa*^{Δpanc};*Mafb*^{+/-} islets ($n = 4$). The NAD(P)H response was not significantly different from base line until 20 mmol/L glucose. * $P < 0.05$, independent samples. arb., arbitrary.

MetaMorph v7.7 software (Universal Imaging, Downingtown, PA) of calibrated images captured at 5,600 \times magnification. The number of docked β -cell granules was expressed per membrane length and β -cell granule size measured using ImageScope software (Aperio, Vista, CA) of calibrated images captured at 5,600 \times . The granule size was measured using ImageScope software of calibrated images captured at 25,000 \times magnification. Each analysis was based on 12–15 images per genotype.

Quantification and Statistical Analysis

All data were presented as mean \pm SEM, and mean differences were tested for statistical significance using the Student two-tail t test unless otherwise noted.

RESULTS

Impact of MafA Loss on β -Cell Function, β -Cell Mass, and Islet Architecture Is not Observed Until 3 Weeks of Age

Genes important to β -cell function are regulated by MafB at E18.5 and by MafA in 3-month-old islets, including those involved in glucose sensing (i.e., *Slc2a2*), vesicle maturation (*Slc30a8*), Ca²⁺ signaling (*Camk2b*), and glucose metabolism (*G6pc2*) (20). To more precisely define the postnatal period when MafA becomes essential for regulating β -cells, a time course study was performed. Quantitative PCR analysis revealed that *Insulin 1* (*Ins1*), *Insulin 2* (*Ins2*), *Slc2a2*, *G6pc2*, and *Slc30a8* mRNA expression levels were reduced in 3-week-old *Mafa*^{Δpanc} islets (Fig. 1A), with only *Slc30a8* significantly reduced within 2 weeks. Notably, MafB is barely detectable in 2-week-old β -cells (20). In addition, there were clearly fewer insulin⁺ cells producing the *Slc2a2* and *Slc30a8* proteins in 3-week-old *Mafa*^{Δpanc} islets (Fig. 1B), which, respectively, represent the glucose and zinc transporters required for normal β -cell function (27–29). The number of islet β -cells was also diminished in 3-week-old *Mafa*^{Δpanc} islets (Fig. 2), whereas the glucagon⁺ α -cell population was unchanged (Supplementary Fig. 1). Notably, this resulted from diminished β -cell replication and not increased apoptosis (Fig. 2B and C).

The presence of α -cells within the islet β -cell-enriched core is commonly observed in pancreatic transcription factor-deficient mice (e.g., Pdx1 [30,31] and Pax6 [14]). Conspicuously, MafA (but not MafB) expression is compromised in many, if not all, of these mutants (e.g., Pdx1 [32], Pax6 [33], data not shown). The previously observed intermingling of glucagon⁺ α -cells within the β -cell enriched islet core (19) was also regulated within the 3-week temporal period in *Mafa*^{Δpanc} islets, as was the decrease in the islet insulin⁺ to glucagon⁺ cell ratio (Fig. 3A). Furthermore, glucose clearance was impaired in 4-week-old *Mafa*^{Δpanc} mice (Fig. 3B). Collectively, these data highlighted how indispensable MafA is in postnatal islet β -cells after the loss of MafB expression.

Islet Morphology and Glucose Clearance Are Impaired Earlier in Compound *Mafa*^{Δpanc};*Mafb*^{+/-} Mice

Unfortunately, the functional significance of MafB in neonatal β -cells remains unknown since *Mafb*^{-/-} mice die at birth from central apnea (34). To obtain a greater awareness of MafB actions during this period, changes in islet morphology and function were compared between *Mafa*^{Δpanc};*Mafb*^{+/-} and *Mafa*^{Δpanc} littermates. The reduction in the β - to α -cell ratio started 2 weeks after birth

granule docking (*Syt14*), vesicle exocytosis (*Stxbp1*), and calcium transport (*Atp2a2*). The mRNA levels were measured by qPCR in *Mafa*^{Δpanc}, *Mafa*^{Δpanc};*Mafb*^{+/-}, and wild-type islets ($n = 3$). ** $P < 0.01$; *** $P < 0.0001$. (E) Representative electron micrographs of a 12-week-old *Mafa*^{Δpanc} and wild-type β -cell. (F) Insulin granule size ($n \geq 320$ granules/genotype), granule density ($n \geq 64$ β -cells/genotype), and number of docked granules ($n \geq 41$ β -cells/genotype) are reduced in *Mafa*^{Δpanc} β -cells. ** $P < 0.01$; *** $P < 0.0001$. AUC, area under the curve; G, glucose; IEQ, islet equivalent.

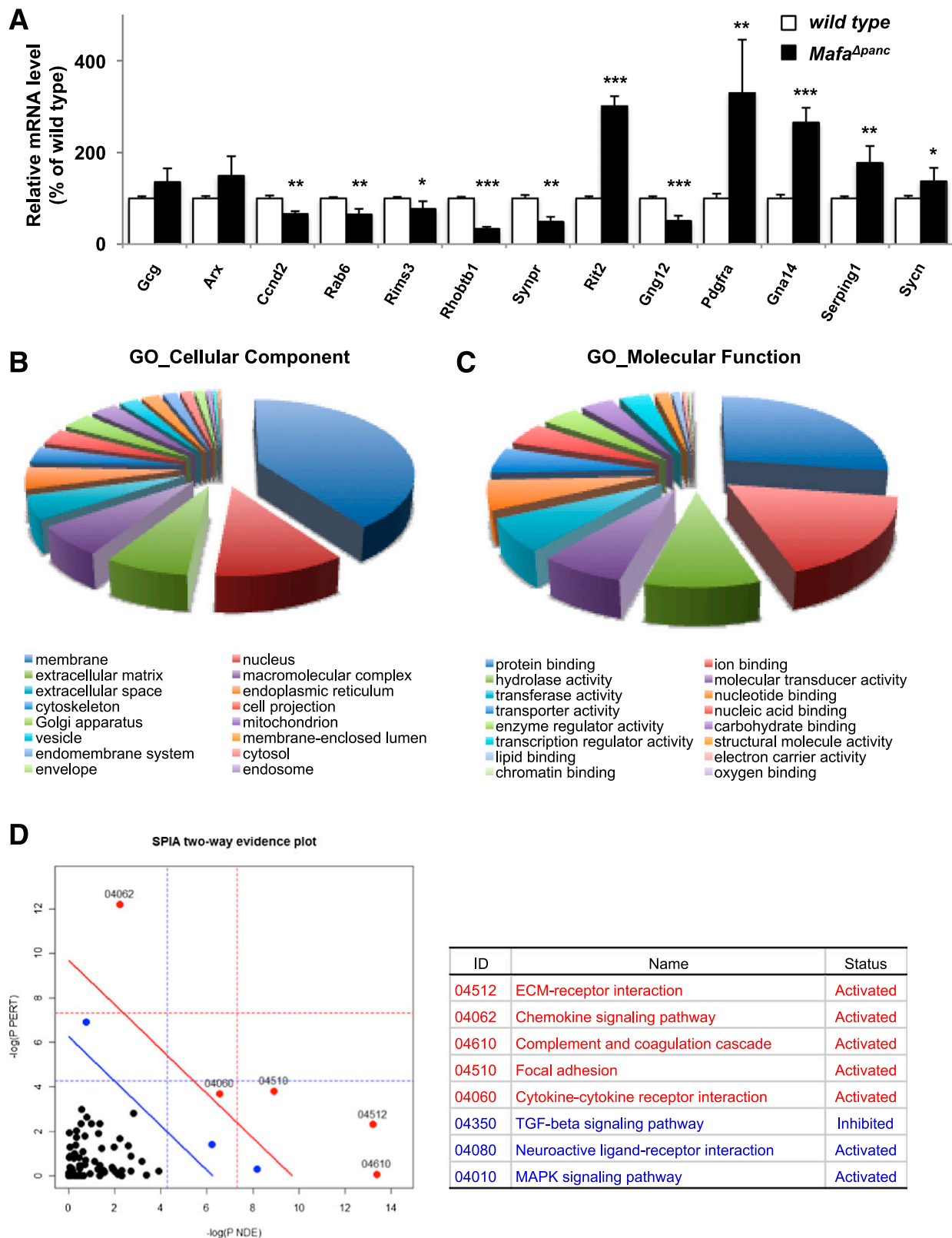


Figure 6—Gene set enrichment analysis reveals MafA regulation of many key β -cell activities. (A) The mRNA expression levels of 13 MafA microarray identified candidate genes in 3-week-old *Mafa* ^{Δ panc} and control islets ($n \geq 3$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Gene ontology analysis showing the (B) cellular component and (C) molecular function enrichment obtained from the microarray mRNA studies performed on 3-month-old wild-type and *Mafa* ^{Δ panc} islets. Each pie chart section is proportional to the number of genes in the category. The acinar cell signals in the *Mafa* ^{Δ panc} microarray data are contamination, since these were not detected in the independently isolated *Mafa* ^{Δ panc} islet preparations used for Figs. 4 and 6. (D) Signaling pathway impact analysis illustrates those significantly altered in *Mafa* ^{Δ panc} islets. Each dot represents a unique signaling pathway, with those labeled with an identification number most significantly perturbed. A red dot denotes

in *Mafa* ^{Δ panc};*Mafb*^{+/-} islets, 1 week earlier than the *Mafa* ^{Δ panc} mutant (Fig. 3A). Such changes were not observed at any postnatal time point in *Mafb*^{+/-} islets (data not shown). Additionally, *Mafa* ^{Δ panc};*Mafb*^{+/-} mice displayed more profound defects in acute glucose clearance (Fig. 3B). Taken together, these data indicate a role for MafB in postnatal β -cell maturation.

First-Phase Insulin Secretion, Insulin Granule Density, and the Number of Docked Insulin Granules Are Regulated by MafA and MafB in Islets

Because blood glucose clearance was compromised after an intraperitoneal glucose challenge in *Mafa* ^{Δ panc} mice (20), islet perfusion assays were performed to determine whether this resulted from an effect on first- and/or second-phase insulin granule release. In relation to wild-type littermates, first-phase insulin secretion was significantly reduced in *Mafa* ^{Δ panc} islets, with second-phase trending in this direction (Fig. 4A and B). Islet insulin content was also decreased by ~40% in *Mafa* ^{Δ panc} β -cells (Fig. 4C). Analogous findings were also observed in *Mafa* ^{Δ panc};*Mafb*^{+/-} islets.

Given that biphasic insulin secretion reflects the recruitment and release of geographically distinct pools of insulin-containing granules, the ultrastructure appearance of control and *Mafa* ^{Δ panc} β -cells were compared by transmission electron microscopy. *Mafa* ^{Δ panc} β -cells contained 40% less mature insulin granules with a characteristic dark core appearance (Fig. 4E). Granule density and size was also reduced to a similar extent (Fig. 4F). Moreover, the number of docked insulin granules that are exocytosed during the first phase of glucose-stimulated insulin secretion was decreased by 55% in *Mafa* ^{Δ panc} β -cells. A similar deficiency in mature insulin granule formation was found in *Slc30a8*^{-/-} islets (35), circumstances that preclude Zn²⁺ transport into granules for insulin crystallization and the dense-core appearance. Both *Slc30a8* expression (Fig. 1B) and Zn²⁺ levels (Fig. 5A) were also significantly reduced in *Mafa* ^{Δ panc} and *Mafa* ^{Δ panc};*Mafb*^{+/-} β -cells, with the compound mutant being effected more dramatically. In contrast, NAD(P)H-sensitive metabolic activity in *Mafa* ^{Δ panc} and *Mafa* ^{Δ panc};*Mafb*^{+/-} islets was not impaired until high glucose concentrations (Fig. 5B). These data support a role for MafA in regulating processes directly involved in insulin synthesis, storage, and secretion.

MafA Impacts Genes Involved in Many Aspects of Islet β -Cell Function

To obtain an unbiased and comprehensive perspective on MafA control in mouse islets, Affymetrix whole mouse genome profiling was performed on isolated 3-month-old

Mafa ^{Δ panc} and control islets. There was a significant difference in 1,093 array transcripts in the *Mafa* ^{Δ panc} sample (with a false discovery rate of 10% and a fold change >2), with 741 of these mapped to distinct EntrezGene identifiers (Supplementary Table 1). However, there were several exocrine-enriched genes within the differentially expressed group (e.g., elastase, carboxypeptidase, chymotrypsinogen), indicating possible contamination of the islet RNA preparation. This was verified by analyzing expression for exocrine marker gene levels in other independently isolated *Mafa* ^{Δ panc} RNA samples (data not shown). Consequentially, 13 candidate genes were selected from the microarray to determine the accuracy of the data. Importantly, qPCR studies performed on 3-week-old islet RNAs confirmed the same changes between wild-type and *Mafa* ^{Δ panc} (Fig. 6A), with 11 genes altered significantly in the expected manner and 2 showing the same trend. These results indicate that much of the microarray data reflects MafA control of islet β -cell gene expression. This included cyclin D2 (i.e., *Ccnd2*), a primary cell cycle regulator in islets (36), contributing to the decrease in *Mafa* ^{Δ panc} β -cell mass (Fig. 2). Notably, these results also revealed early control by MafA in postnatal β -cells, suggesting that MafA is influencing transcription directly.

Gene ontology enrichment analysis performed on the *Mafa* ^{Δ panc} and control islet microarray data disclosed that protein-binding and ion-binding activity were affected most profoundly, although MafA appears to influence a broad range of cellular functions found in many distinct subcellular compartments (Fig. 6B and C). Importantly, established effectors of granule docking (*Syt14*, granuphilin [37]), granule release (*Stxbp1*, *Munc18-1* [38]), and calcium influx (*Atp2a2* [39,40]) were identified (Supplementary Table 1), with regulation confirmed by qPCR (Fig. 4). When these data are also considered in the context of the decrease in *Ins1*, *Ins2*, *Slc30a8*, *Slc2a2* (the major β -cell GLUT [27]), and *G6pc2* (a key gluconeogenic enzyme [41]) levels in *Mafa* ^{Δ panc} islet β -cells (Fig. 1), these combined deficiencies provide an explanation as to why the first phase of glucose-stimulated insulin secretion was particularly impaired.

Interestingly, while many genes appear to be commonly regulated by MafA and MafB (20), expression-profiling analyses identified genes only affected in 3-month-old *Mafa* ^{Δ panc} islets and not E18.5 *Mafb*^{-/-} pancreata (e.g., *Ccnd2*, *Nqo1*, and *Atp2a2*). Moreover, pathway analysis further supported MafA control in many aspects of β -cell signaling, including those involved with the extracellular matrix receptor (see 4512), chemokines (4062),

focal adhesion (4510), cytokine–cytokine receptor (4060), and mitogen-activated protein kinase signaling (4010). (The numbers in the parentheses refer to pathways described in Fig. 6, Supplementary Fig. 2, and Supplementary Table 2.) Collectively, these gene expression studies provide a broader perspective into the many modes of action used by MafA to regulate β -cell function.

DISCUSSION

Our principal objective here was to precisely define when MafA first became functionally significant to mouse β -cells postnatally and to explore the nature of the dependency. Experiments performed in *Mafa* ^{Δ panc} mice revealed that MafA was important to β -cells from 3 weeks of age, just after their loss of MafB. This included changes in β -cell mass, islet cell architecture, and glucose-stimulated insulin secretion. The proteins likely mediating these changes were identified by candidate and microarray analysis. These studies provide an in-depth perspective into the many ways MafA influences islet β -cells and mechanistic insight into why it is so necessary in cell maturation and adult activity.

MafA only begins to influence β -cells around weaning time in laboratory rodents. The switch from high-fat-based mother's milk to a chow diet results in higher MafA expression and significant changes in the metabolic profile of rodent β -cells (42). Notably, MafB⁺ neonatal β -cells were functional immature, characterized by higher basal insulin secretion and little response to glucose. The MafB-to-MafA transition appears to be key to β -cell maturation. This is illustrated not only by the islet β -cell deficiencies of *Mafa*^{-/-} and *Mafa* ^{Δ panc} mice, but also from studies examining the glucose-sensitive regulatory properties of in vitro generated insulin⁺ cells derived during human embryonic stem differentiation (21,22,43). Thus these Ins⁺ cells are only MafB⁺ and do not secrete insulin in a glucose-stimulated manner until after transplantation into mice, when the cells become MafA⁺ (22). The present studies predict many likely inadequacies in the human embryonic stem–derived MafB⁺ Ins⁺ cells due to their lack of MafA, including diminished expression of key effectors of insulin granule secretion (*Stxbp1*, *Syt14*), calcium signaling (*Atp2a2*), and glucose signaling (*G6pc2*, *Slc2a2*).

MafA has a clear effect on insulin granule release. Hence glucose-stimulated insulin secretion, insulin granule density, and the number of predocked insulin granules on the plasma membrane were decreased in *Mafa* ^{Δ panc} β -cells (Fig. 4). The reduction in the total number and size of mature dense-core insulin granules per cell is presumably attributable to diminished production of both insulin and the Slc30a8 zinc transporter (Figs. 1 and 4). Despite an ~30% paucity of insulin granules in *Mafa* ^{Δ panc} β -cells, there was a >50% loss predocked granules. Consistent with the concept that predocked granule release supports first-phase insulin secretion, *Mafa* ^{Δ panc} β -cells were only significantly

decreased during this phase. Presumably, this was due to limited expression of key MafA-regulated docking effectors (e.g., granuphilin [*Syt14*] and Munc18–1 [*Stxbp1*]). Importantly, Munc18–1 was recently found to specifically regulate first-phase insulin secretion (38).

The architecture of the *Mafa* ^{Δ panc} islet is similar to human (44), with fewer β -cells and α -cells present within the core (Fig. 3). The reduced number of β -cells in *Mafa* ^{Δ panc} islets reflected a change in replication and not cell survival, likely involving cyclin D2. Potentially, the coexpression of MAFA and MAFB in human islets (26) contributes to the difference in islet architecture and β -cell composition between species. This is supported by the lack of any observable change in endocrine cell numbers, replication rate, or cyclin D2 levels in the embryonic *Mafb*^{-/-} pancreas (16) (data not shown). On the other hand, adult MafA controls many genes activated by MafB during mouse embryonic development (20). The more severe defects of the compound *Mafa* ^{Δ panc};*Mafb*^{+/-} mutant relative to *Mafa* ^{Δ panc} further indicates that MafB is also beneficial to β -cells prior to its loss postnatally.

The overall mechanistic similarities and differences between MafA and MafB are still unclear. Notably, only 35 genes were identified in the PancChip 6 microarray performed on E18.5 *Mafb*^{-/-} pancreata (i.e., ≥ 1.5 -fold; 47 in *Mafa* ^{Δ panc};*Mafb*^{-/-}; none in *Mafa* ^{Δ panc} [20]), while 741 mapped to EntrezGene identifiers using the Affymetrix platform on 3-month-old *Mafa* ^{Δ panc} islets here. Much of the difference is likely due to the relative insensitivity of the PancChip 6 microarray. It is expected that reinspection of *Mafb*^{-/-} pancreata will reveal both common and distinct regulatory targets in relation to the *Mafa* mutant, especially considering that *Mafa* ^{Δ panc} β -cells are dysfunctional despite the presence of many MafB⁺ Ins⁺ cells (20). Both biochemical and cell-line-based studies also provide evidence supporting functional differences between islet cell expressed MafA and MafB (15,45,46). Consequentially, human MAFA:MAFB heterodimer activity in islet β -cells may contribute to observed differences in glucose-stimulated insulin secretion properties with rodents (26).

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