MafA and MafB Regulate Genes Critical to β-Cells in a Unique Temporal Manner

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OBJECTIVE—Several transcription factors are essential to pancreatic islet β-cell development, proliferation, and activity, including MafA and MafB. However, MafA and MafB are distinct from others in regard to temporal and islet cell expression pattern, with β-cells affected by MafB only during development and exclusively by MafA in the adult. Our aim was to define the functional relationship between these closely related activators to the β-cell.

RESEARCH DESIGN AND METHODS—The distribution of MafA and MafB in the β-cell population was determined immunohistochemically at various developmental and perinatal stages in mice. To identify genes regulated by MafB, microarray profiling was performed on wild-type and *MafB*$^{-/-}$ pancreata at embryonic day 18.5, with candidates evaluated by quantitative RT-PCR and in situ hybridization. The potential role of MafA in the expression of verified targets was next analyzed in adult islets of a pancreas-wide *MafA* mutant (termed *MafA*$^{\Delta Panc}$).

RESULTS—MafB was produced in a larger fraction of β-cells than MafA during development and found to regulate potential effectors of glucose sensing, hormone processing, vesicle formation, and insulin secretion. Notably, expression from many of these genes was compromised in *MafA*$^{\Delta Panc}$ islets, suggesting that MafA is required to sustain expression in adults.

CONCLUSIONS—Our results provide insight into the sequential manner by which MafA and MafB regulate islet β-cell formation and maturation. ***Diabetes*** **59:2530–2539, 2010**

Insulin-secreting islet β-cells play a pivotal role in the regulation of fuel metabolism. Loss or dysfunction of β-cells causes an imbalance in glucose homeostasis that leads to the development of diabetes. Efforts aimed at understanding the molecular processes underlying β-cell development and function have provided perspectives into new diabetes treatment strategies. For example, a hierarchy of transcription factors has been found to regulate β-cell differentiation during development and adult islet cell function, a few of which are mutated in type 2 diabetic patients as discussed by others (1–3). The significance of these proteins was recently reinforced upon observing their expression during the stepwise differentiation of human embryonic stem cells to β-like cells (4,5) and the reprogramming of adult acinar cells to β-like cells upon misexpression of a unique subset of transcription factors, specifically MafA, Pdx1, and Ngn3 (6).

Among the transcription factors vital to the pancreas, there are instances when members of the same gene family contribute to β-cell formation, including winged-helix/forkhead (e.g., FoxA1/2) (7–9), NK6 homeodomain (Nkx6.1 and Nkx6.2) (10–12), paired box homeodomain (Pax4/6) (13–17), and basic leucine-zipper (MafA and MafB) (18,19) proteins. FoxA1/2, Nkx6.1/6.2, and Pax4/6 are expressed broadly in pancreatic epithelial cells in both islet hormone$^+$ and hormone$^-$ cells before or near the onset of pancreatic morphogenesis (3) and then become confined to more specific cellular domains (e.g., Nkx6.1 [β] and Pax6 [all islet cells]) or disappear entirely late in development (Pax4, Nkx6.2). The large MafA/B factors are distinct in being produced relatively later in development and essentially only in hormone$^+$ cells. Thus, MafB is present in developing α-(glucagon$^+$) cells, β-cells, and a very small number of Ngn3$^+$ islet hormone$^-$ progenitors and then becomes restricted to α-cells soon after birth (20,21). MafA is found exclusively in developing and adult insulin$^+$ cells, with expression first detected at embryonic day (E) 13.5 during the secondary and principal wave of insulin$^+$ cell production (22).

A comparison of the properties of islet-enriched transcription factor mutant mice reveals a novel role for MafA and MafB in β-cell maturation and function. Thus, islet α, β, δ, ε, or pancreatic polypeptide producing cells are either lost or respecified in most transcription factor knockout mice (1–3), whereas the principal defect in *MafB*$^{-/-}$ embryos is reduced insulin and glucagon expression (18). Furthermore, this change does not affect total endocrine cell numbers, supporting a distinct importance in one or more late steps in α- and β-cell differentiation (18). In contrast, MafA is solely required for glucose-regulated insulin secretion in adult islet β-cells and is not involved in islet cell development (19). Notably, MafB$^+$ insulin$^+$ cells generated during human embryonic stem cell differentiation were dysfunctional until becoming MafA$^+$ insulin$^+$ (5), suggesting that the transition from MafB to MafA is critical to β-cell function.

To obtain a mechanistic understanding of the association between MafB and MafA in β-cell formation and function, gene-profiling studies were performed with E18.5 pancreata from wild-type, *MafB*$^{-/-}$, *MafA*$^{\Delta Panc}$, and *MafA*$^{\Delta Panc}$;*MafB*$^{-/-}$ embryos. (*MafA*$^{\Delta Panc}$ mice were generated by crossing *MafA*$^{fl/fl}$ mice with transgenic mice producing Cre recombinase from the *Pdx1*$^{5.5}$ promoter fragment early in development and in a pancreas-wide

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TABLE 1
The mRNA expression profile of E18.5 wild-type and mutant pancreata

	% of wild-type		
	MafA$^{\Delta panc}$	*MafB*$^{-/-}$	*MafA*$^{\Delta panc}$;*MafB*$^{-/-}$
MafA	1 ± 1*	16 ± 1*	2 ± 1*
MafB	168 ± 1*	10 ± 6*	9 ± 4*
Insulin	99 ± 16	34 ± 18*	11 ± 3*
Glucagon	132 ± 23	55 ± 28*	31 ± 10*
Isl1	112 ± 12	118 ± 50	117 ± 42
Slc30a8	90 ± 23	4 ± 3*	0 ± 0*
G6pc2	59 ± 20*	18 ± 9*	4 ± 0*
Rbp4	84 ± 21	196 ± 54*	188 ± 21*
Nnat	50 ± 11*	31 ± 7*	26 ± 4*

Data are means ± SEM. *Change from wild-type littermates is significant, $P < 0.05$.

pattern.) Multiple genes were differentially expressed in the *MafB*$^{-/-}$ mutant, but not in *MafA*$^{\Delta Panc}$ mutant, consistent with the much more critical role of MafB in α- and β-cell development (18,21). Significantly, MafB-dependent genes were associated with adult β-cell function, including glucose sensing and insulin secretion. Interestingly, many of these target genes were affected in a similar manner in adult *MafA*$^{\Delta Panc}$ islets, even though MafB was retained in a fraction of the mutant insulin^{+} cell population. These findings provide insight into why the β-cell is dysfunctional in *MafA* mutant mice, and they illustrate the unusual interrelationship between closely related transcription factors in the generation of a particular islet cell type.

RESEARCH DESIGN AND METHODS

Animals. Pancreas-wide *MafA* deletion mutant mice were generated using the Cre-*loxP* mediated recombination system. A conditional *MafA* allele was generated using a targeting vector consisting of two *loxP* sites inserted into the 5′ (*Pst*I site [−699 bp] and 3′ (*Kpn*I site [1,658 bp]) end of the *MafA* exon (supplementary Fig. 1*A* in the online appendix available at http://diabetes.diabetesjournals.org/cgi/content/full/db10-0190/DC1). The *herpes simplex virus-thymidine kinase (TK)* gene was placed outside of the *MafA* gene homology region for *neomycin*^{R} selection. After electroporation of 129S6-derived mouse embryonic stem cells, 327 clones survived chemical selection. Fifteen clones were correctly targeted, as determined by Southern blotting hybridization. Two clones were independently injected in mouse blastocysts, and then chimeric mice were bred with C57BL/6J mice for germline transmission screening. The FRT-flanked *neomycin*^{R} gene was then removed in transmitted *MafA*$^{fl/+}$ mice by crossing with FLPe mice (JAX human β-actin FLPe deleter strain; more information online at http://www.mmrrc.org/strains/29994/029994.html). To delete *MafA* in the developing pancreas, *MafA*$^{fl/fl}$ animals were bred with *Pdx1*$^{5.5}$*-Cre* mice (a gift from Dr. Guoqiang Gu, Vanderbilt University), which produce Cre recombinase by E10.5 in a pattern analogous to endogenous *Pdx-1* early in development (23, supplementary reference 1). *MafA*$^{fl/fl}$;*Pdx1*$^{5.5}$*-Cre* mice were referred to as *MafA*$^{\Delta Panc}$, with MafA effectively removed from the pancreas by E18.5 (Table 1, supplementary Fig. 1*B*). *MafB*$^{-/-}$ mice were generated by homologous recombination (24). For embryonic analysis, noon of the day of the vaginal plug discovery was designated E0.5. The Vanderbilt University Institutional Animal Care and Use Committee approved all of our studies in mice.

Microarray and quantitative RT-PCR analysis. E18.5 pancreas anlagen from five independently derived wild-type *MafA*$^{\Delta Panc}$, *MafB*$^{-/-}$, and *MafA*$^{\Delta Panc}$;*MafB*$^{-/-}$ mice were dissected in PBS and stored in RNA*later* (Ambion). Tissue RNA was isolated using the ToTALLY RNA isolation kit (Ambion). RNA was further purified over RNeasy columns (Qiagen), and RNA quality was analyzed using an Agilent 2100 Bioanalyzer. Each RNA sample (100 ng) was amplified using the Ovation Aminoallyl RNA Amplification and Labeling System (NuGEN Technologies), and PancChip 6.1 (http://www.betacell.org/ma, supplementary reference 2) microarray analysis was performed at the Functional Genomics Core at the University of Pennsylvania.

Quantitative RT-PCR was conducted on total RNA from E18.5 pancreata or 12-week-old islets ($n \geq 3$ for each genotype). The RNA (1 μg) was transcribed using iScript reverse transcriptase and iScript reaction mix (Bio-Rad). The PCRs were performed using the SYBR Green (with dissociation curve) program on an Applied Biosystems 7900 machine. All reactions were performed in duplicate with reference dye normalization, and median cycling threshold values were used for analysis. Primer sequences are available on request.

Immunohistochemistry and in situ hybridization. Tissue fixation, embedding, and immunofluorescence labeling were performed as previously described (25). The primary antibodies used were guinea pig α-insulin (1:2,000; Linco Research), mouse α-insulin (1:2,000; Invitrogen), guinea pig α-glucagon (1:2,000; Linco Research), sheep α-somatostatin (1:2,000; American Research), guinea pig α-pancreatic polypeptide (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), mouse α-Rbp4 (1:1,000, Abnova), rabbit α-G6PC2 (1:500, a gift from Dr. John Hutton at the University of Colorado), rabbit α-Pdx1 (1:5,000, a gift from Dr. Chris Wright at Vanderbilt University), mouse α-Isl1 (1:300, Developmental Studies Hybridoma Bank), rabbit α-Nkx6.1 (1:1,000, Beta Cell Biology Consortium), and rabbit α-Pax6 (1:200, Covance Research Products). The secondary Cy2-, Cy3-, or Cy5-conjugated donkey α-rabbit, α-guinea pig, and α-sheep IgGs were obtained from Jackson ImmunoResearch Laboratories. Nuclear counterstaining was performed using YoPro1 or DAPI (Invitrogen). Immunofluorescence images were acquired using confocal microscopy (LSM510, Carl Zeiss).

In situ hybridization assays were performed using *Neuronatin* (base pair 119 to 1,163 relative to coding ATG) and *Slc30a8* (base pair 166 to 1,269) gene probes according to Henrique et al. (26). The *Rbp4* cDNA plasmid (clone ID IMAGp998P0711141Q1) was obtained from the resource center of the human genome project (www.rzpd.de) and used to generate the probe (base pair 295–927).

Quantification and statistical analysis. Immunolabeling for insulin and MafA (or MafB) was performed on serial 6-μm pancreatic sections that were collected at 60-μm (i.e., at E14.5), 84-μm (E18.5), or 150-μm (postnatal) intervals. All insulin^{+} cells were imaged by confocal microscopy and presented as the percentage of MafA^{+}insulin^{+} or MafB^{+}insulin^{+} to total insulin^{+} cells. At least 10 random fields were counted from wild-type and *MafA*$^{\Delta Panc}$ pancreata ($n = 3$) to calculate the percentage of MafB^{+}insulin^{+} cells in adult islets. Immunolabeling for insulin, glucagon, somatostatin, and pancreatic polypeptide-producing cells was performed on 12-week islet sections at 300-μm intervals. At least 10 random fields were counted from wild-type and *MafA*$^{\Delta Panc}$ pancreata ($n = 4$) to calculate the proportion of somatostatin^{+} δ-cells within islets. Mean differences were tested for statistical significance using a two-tailed Student *t* test.

RESULTS

MafA and MafB are dynamically expressed in developing and adult β-cells. There are two distinct waves of insulin^{+} and glucagon^{+} cell production within the pancreatic epithelium during embryogenesis, with the massive expansion of secondary transition cells starting at E13.5 populating the adult islet (27). Previous studies established that MafB is expressed in both waves (18,21), whereas MafA is only in the insulin^{+} cells of the second wave (22). Quantitative immunohistochemical analysis was performed at E15.5, E18.5, P14, and P28 to precisely define the MafA and MafB expression pattern during embryonic and postnatal β-cell differentiation.

MafB was found in almost all insulin^{+} cells produced at E14.5 and E18.5, whereas the fraction of MafA^{+} insulin^{+} cells increased during this period (Fig. 1*A*). Our results are in accordance with previous data showing that MafA was expressed in only 54% of the insulin^{+} cells at E15.5 and MafB in nearly 90% (21). However, MafB was present only in very few insulin^{+} cells soon after birth (P14, 2.70 ± 0.11%) and was essentially absent in insulin^{+} cells after 4 weeks (P28, 0.69 ± 0.06%). In contrast, MafA was detected in most islet β-cells by 4 weeks (Fig. 1*A*). The postnatal switch in β-cells from principally MafB to exclusively MafA occurs during a period of cell maturation in regards to islet architecture, islet cell mass, and glucose-sensitive insulin secretion.

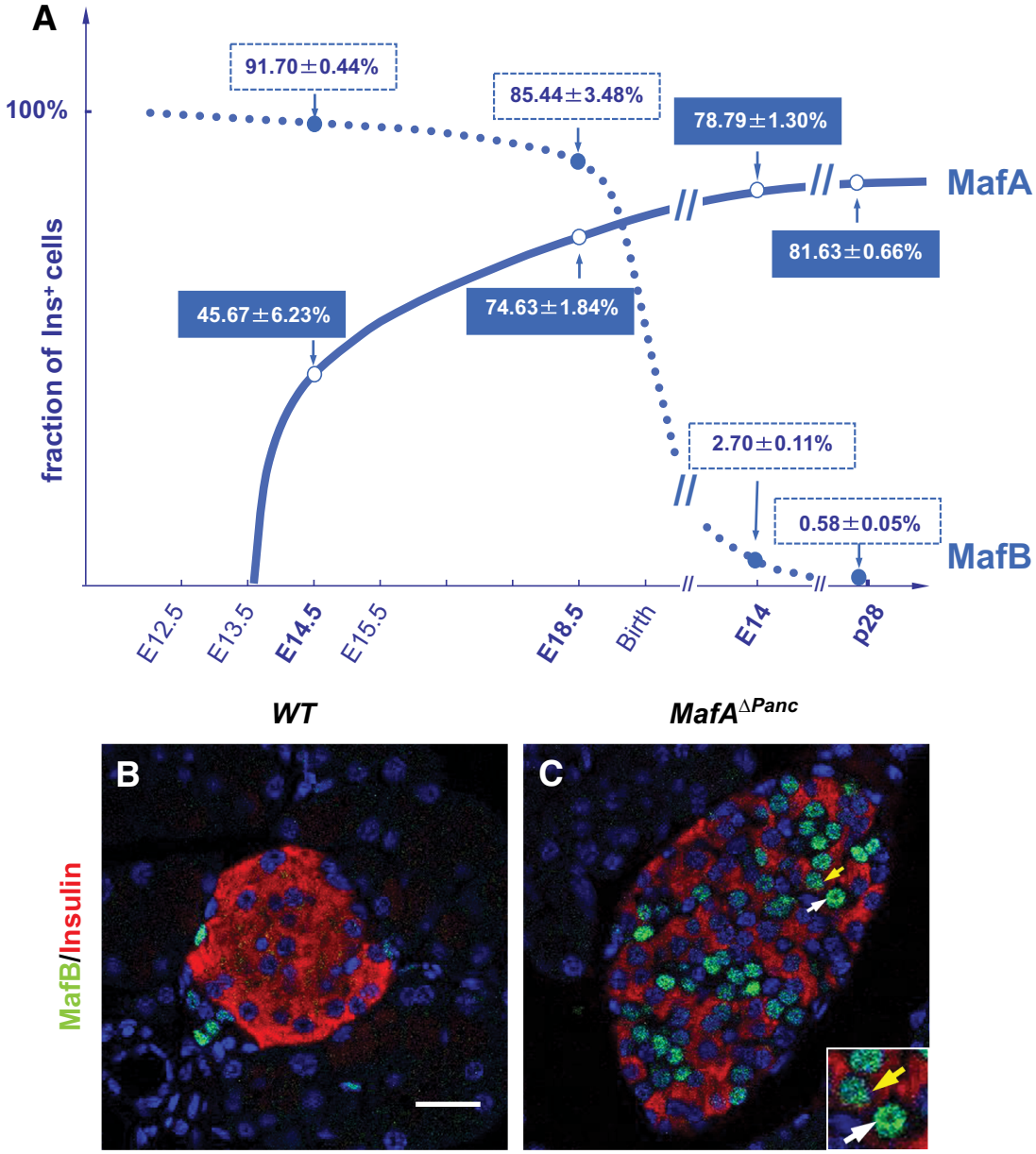


FIG. 1. MafA and MafB expression is dynamically regulated in developing and adult β-cells. *A*: The percentage of β-cells coexpressing MafA or MafB was assessed at E14.5, E18.5, P14, and P28 by quantification of the number of insulin$^+$ cells costaining with either MafA or MafB. Sections spanning the entire pancreas of wild-type mice were used; the results are presented as the mean ± SEM. *B* and *C*: MafB is sustained in a fraction of the insulin$^+$ cells in *MafA*$^{\Delta Panc}$ islets. Wild-type (*B*) and mutant (*C*) pancreatic sections were stained for MafB (green) and insulin (red). Notably, MafB$^+$ is not found in wild-type insulin$^+$ cells, but is found in *MafA*$^{\Delta Panc}$. *Yellow arrowhead* denotes representative MafB$^+$ insulin$^+$ cells, and *white arrowhead* denotes MafB$^+$ insulin$^-$ cells. Nuclei were stained with YO-PRO-1 in blue. MafB was found in 33.4 ± 5.6% of *MafA*$^{\Delta Panc}$ insulin$^+$ cells (n = 3). (A high-quality digital representation of this figure is available in the online issue.)

MafB is retained in some adult islet MafA$^{\Delta Panc}$ β-cells. *MafB* mRNA expression at E18.5 was elevated 1.7 ± 0.1-fold in *MafA*$^{\Delta Panc}$ pancreata, yet no overt alterations in islet hormone$^+$ cell development or Isl1, Nkx6.1, Pax6, or Pdx1 expression were found (supplementary Fig. 2). Also similar to *MafA*$^{-/-}$ mice (19), changes in *MafA*$^{\Delta Panc}$ β-cell function and islet morphology were first observed postnatally (data not shown). Pan-endocrine Isl1 expression was unaffected in the *MafA*$^{\Delta Panc}$, *MafA*$^{\Delta Panc}$;*MafB*$^{-/-}$, and *MafB*$^{-/-}$ mutants at E18.5 (Table 1), supporting evidence linking MafB to α- and β-cell maturation, but not islet cell formation or viability (18,21). As expected, insulin mRNA levels were compromised in *MafB*$^{-/-}$ (18) and *MafA*$^{\Delta Panc}$;*MafB*$^{-/-}$ samples from E18.5, but not the *MafA*$^{\Delta Panc}$ (Table 1) or *MafA*$^{-/-}$ mutants (19). The larger effect on *Insulin* mRNA expression in *MafA*$^{\Delta Panc}$;*MafB*$^{-/-}$ pancreata implies that *MafA* contribution to embryonic *Insulin* transcription becomes significant only in the absence of MafB. This was also indicated by the delay in residual insulin$^+$ cell production until the beginning of the secondary transition at E13.5 in *MafB*$^{-/-}$ mice (18).

Strikingly, immunohistochemical analyses revealed that MafB protein expression was retained in a fraction of insulin$^+$ cells in adult *MafA*$^{\Delta Panc}$ mice (compare Fig. 1*B* with *C*; 33.4 ± 5.6% of insulin$^+$ cells express MafB). These results showed that the MafB protein produced in adult *MafA*$^{\Delta Panc}$ β-cells does not compensate for the loss of *MafA*.

MafB regulates the expression of genes critical to β-cell function during embryogenesis. Gene expression profiling studies of E18.5 wild-type, $MafA^{\Delta Panc}$, $MafB^{-/-}$, and $MafA^{\Delta Panc}$;$MafB^{-/-}$ pancreata were performed to more broadly define the regulatory roles of MafB and MafA in β-cell differentiation. Thirty-five genes with mRNA levels altered by ≥1.5-fold ($P \leq 0.01$, false discovery rate ≤25%) were identified using the PancChip 6 microarray platform in the $MafB^{-/-}$ mutant, and 47 were identified in $MafA^{\Delta Panc}$;$MafB^{-/-}$ (supplementary Table 1). The profiles were similar between $MafB^{-/-}$ and $MafA^{\Delta Panc}$;$MafB^{-/-}$ (29 genes were differentially expressed in both genotypes), with most changes more pronounced in $MafA^{\Delta Panc}$;$MafB^{-/-}$ (supplementary Table 1). In contrast, no differences were found between $MafA^{\Delta Panc}$ and the wild-type gene expression profile.

Gene ontology analysis revealed that the differentially expressed genes were mainly involved in aspects of mature β-cell function, such as ion binding and transport, signal transduction, and hormone secretion (supplementary Table 2). The mRNA changes of many candidate genes (like *Slc30a8*, *G6pc2*, *Rbp4*, *Nnat*, *Insulin*, and *Slc2a2*) were independently verified in E18.5 $MafB^{-/-}$ and $MafA^{\Delta Panc}$;$MafB^{-/-}$ pancreata by quantitative RT-PCR (Table 1; data not shown). The studies described below examined the significance of MafA in adult expression of genes initially regulated by MafB. Expression within specific islet cell types was examined by in situ hybridization during development and by immunohistochemistry in adults; unfortunately, limitations in the technique (e.g., in situ) or protein abundance precluded examination of these temporal periods using both methods. Importantly, our results provide mechanistic insight into the mutually beneficial relationship between MafB and MafA and provide an explanation for why β-cell activity decreases in $MafA^{-/-}$ and $MafA^{\Delta Panc}$ mice.

MafB activates *Slc30a8*-encoded zinc transporter expression in developing α- and β-cells, whereas MafA is essential in adult β-cells. *Slc30a8* or *ZnT8* encodes an islet-specific zinc transporter that facilitates the accumulation of zinc from the cytoplasm into intracellular vesicles for insulin maturation and storage (28,29). Overexpression of Slc30a8 enhances glucose-stimulated insulin secretion in INS1 β-cells (29), whereas variants in this locus are linked to type 2 diabetes in humans (30,31). The transporter is also a type 1 diabetes autoantigen in humans (32).

The normal *Slc30a8* expression pattern during pancreatic development was first determined by in situ hybridization. *Slc30a8* mRNA transcripts were found in both developing β- and α-cells (Fig. 2*A*–*D*, supplementary Fig. 3). Notably, *Slc30a8* expression was lost in α-cells at E18.5 in the $MafB^{-/-}$ mutant, but retained in the remaining insulin$^+$ cells (Fig. 2*E* and *F*, supplementary Fig. 3). Significantly, *Slc30a8* expression was undetectable in the $MafA^{\Delta Panc}$;$MafB^{-/-}$ mutant (Fig. 2*G* and *H*, supplementary Fig. 3), demonstrating that expression was mediated by MafA in the residual insulin$^+$ cells in the *MafB* mutants. These results suggest that MafB is the primary activator of *Slc30a8* expression in α- and β-cells during development.

Next, immunohistochemical staining was performed to assess Slc30a8 expression in adult wild-type and $MafA^{\Delta Panc}$ islets. As reported while this work was in progress (33), Slc30a8 is present in both α- and β-cells (Fig. 2*J* and *K*). (The specificity of the Slc30a8 antibody was confirmed upon comparing pancreatic samples from wild-type and $Slc30a8^{-/-}$ mice [supplementary Fig. 4]). Expression was essentially absent in 3-month-old islet insulin$^+$ cells from the $MafA^{\Delta Panc}$ mutant (Fig. 2*L*). The remaining Slc30a8 in $MafA^{\Delta Panc}$ islets is primarily (if not exclusively) in α-cells (Fig. 2*M*). These data imply that MafA and MafB function at distinct temporal stages to promote *Slc30a8* expression in β-cells.

Both MafA and MafB activate islet-specific glucose-6-phosphatase catalytic subunit-2 protein expression. Glucose-6-phosphatase catalytic subunit-2 protein (G6pc2, also known as IGRP) is a major autoantigen in type 1 diabetes (34) and may regulate fasting plasma glucose levels in humans (35). Expression of *G6pc2* mRNA was significantly compromised in E18.5 pancreata from $MafB^{-/-}$, $MafA^{\Delta Panc}$;$MafB^{-/-}$, and $MafA^{\Delta Panc}$ embryos. However, the impact of MafA appears greater in adult $MafA^{\Delta Panc}$ islets than during development (see Fig. 3*A* and Table 1), as a much lower level of *G6pc2* was present in 3-month-old $MafA^{\Delta Panc}$ islet β-cells than wild-type (Fig. 3, compare *D* with *G*). These data indicate that both MafA and MafB play a role in *G6pc2* expression and complement recent cell-line–based studies showing that MafA stimulates *G6pc2* transcription by binding to the −177/−164 bp element in its promoter region (36).

Neuronatin expression is principally regulated by MafB. Neuronatin (*Nnat*) is involved in modulating ion channel activity in β-cells, with overexpression increasing insulin secretion by elevating intracellular Ca^{2+} levels (37,38). In situ hybridization analysis showed that *Nnat* mRNA was expressed at E15.5 and E18.5 in insulin$^+$ cells and in a population that was neither insulin$^+$ nor glucagon$^+$ (Fig. 4, see WT panel, supplementary Figs. 5 and 6). *Nnat* synthesis was predominately in insulin$^+$ cells by E18.5. A profound reduction in steady-state *Nnat* levels was observed in E18.5 $MafB^{-/-}$ and $MafA^{\Delta Panc}$;$MafB^{-/-}$ pancreata (Table 1), with the enduring *Nnat* detected only in the remaining mutant insulin$^+$ cells (Fig. 4, supplementary Figs. 5 and 6).

These results suggest that *Nnat* expression in developing β-cells is only partially dependent on MafB. This was also true of *Pdx1* transcription wherein the ~50% reduction in E18.5 mRNA levels reflected the loss in the $MafB^{-/-}$ insulin$^-$ cell population (18). Notably, steady-state *Nnat* (Fig. 4*M*) and *Pdx1* (19) levels were unchanged in $MafA^{\Delta Panc}$ adult islets, a distinction from *Slc30a8* (Fig. 2*I*) or *G6pc2* (Fig. 3*A*). These data demonstrate that only *MafB* regulates *Nnat* expression.

Rbp4 expression is upregulated in E18.5 *MafB* mutant and $MafA^{\Delta Panc}$ adult islets. Rbp4 contributes to the pathogenesis of type 2 diabetes, as its release from adipocytes causes systemic insulin resistance in both mice and humans (39). This circulating protein is also produced in the liver, although little was known about its effect on islets until recently. Rbp4 has now been found to repress glucose-stimulated insulin secretion in both isolated rat islets and in vivo (P. Fueger, C. Newgard, unpublished data).

In distinction to the genes described previously, *Rbp4* mRNA expression was increased in $MafB^{-/-}$ and $MafA^{\Delta Panc}$;$MafB^{-/-}$ pancreata at E18.5 (Table 1). In situ RNA analysis was performed to determine the distribution of *Rbp4* in hormone$^+$ cells during development. *Rbp4* transcripts were found in the majority of wild-type insu-

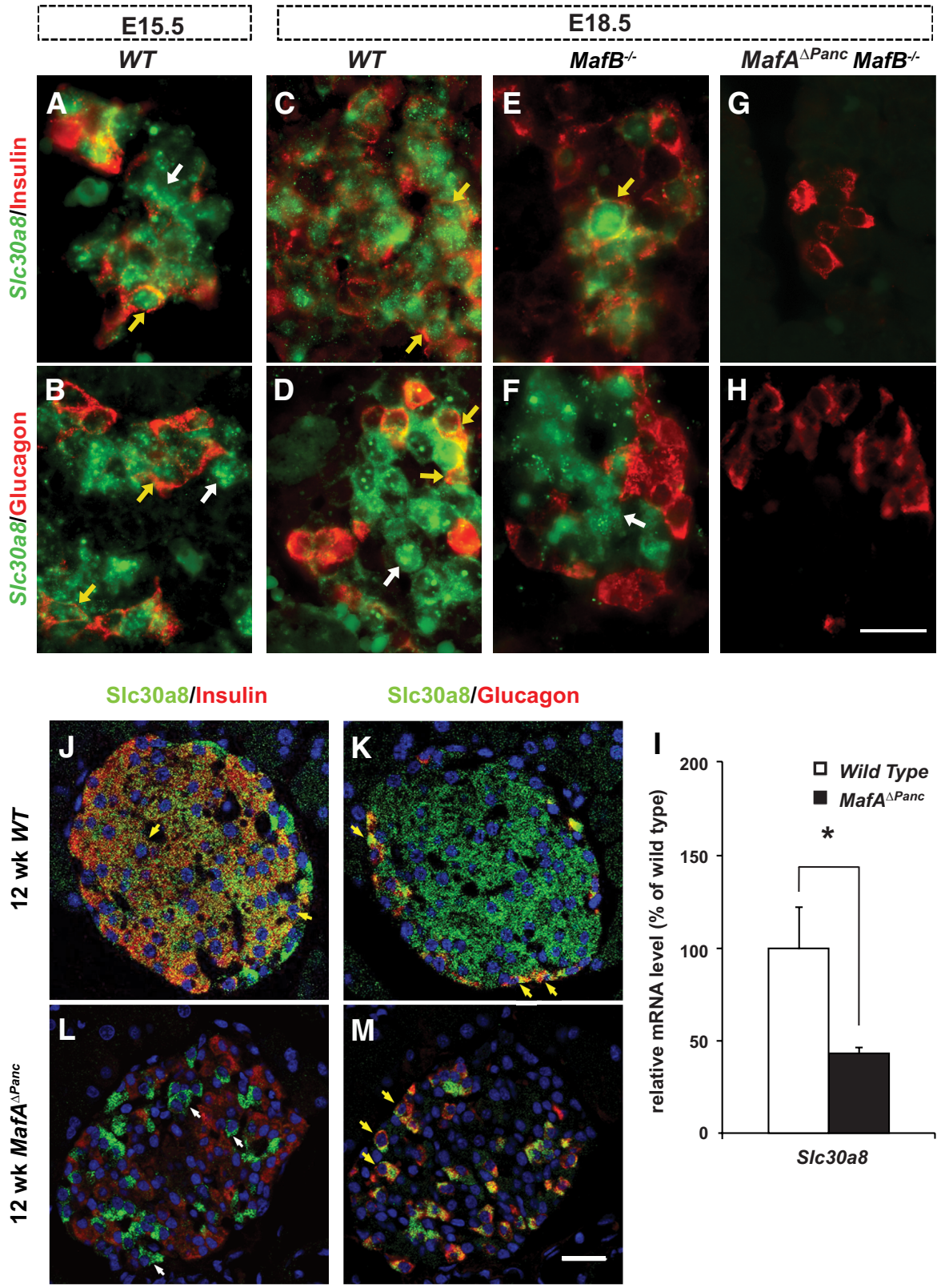


FIG. 2. Islet zinc transporter Slc30a8 expression is activated by MafB during embryogenesis and by MafA in islet β-cells. *Slc30a8* transcripts (*green*) were detected by in situ analysis in wild-type insulin$^+$ (*red*, *A* and *C*) and glucagon$^+$ (*red*, *B* and *D*) cells at E15.5 and E18.5. Expression is still detected in the remaining insulin$^+$ cells (*E*), but lost in glucagon$^+$ (*F*) cells in *MafB*$^{-/-}$ pancreata. No *Slc30a8* expression was detected in the MafA and MafB compound mutant (*MafA*$^{\Delta Panc}$; *MafB*$^{-/-}$; *G* and *H*). *I*: RT-PCR data illustrate the change in *Slc30a8* mRNA expression between 3-month-old *MafA*$^{\Delta panc}$ and wild-type islets. *$P < 0.05$ ($n = 3$). Slc30a8 was detected in islet β-cells (*J*) and α-cells (*K*) of 12-week-old wild-type islets by immunofluorescence. Expression was profoundly reduced in *MafA*$^{\Delta Panc}$ β-cells (*L*) and unaffected in α-cells (*M*). The (*L*) Slc30a8$^+$ insulin$^-$ cells labeled with *white arrows* correspond to (*M*) Slc30a8$^+$ glucagon$^+$ cells denoted by *yellow arrows*. Scale bar = 20 μm. Nuclei were stained in blue in panels *J*–*M*. (A high-quality digital representation of this figure is available in the online issue.)

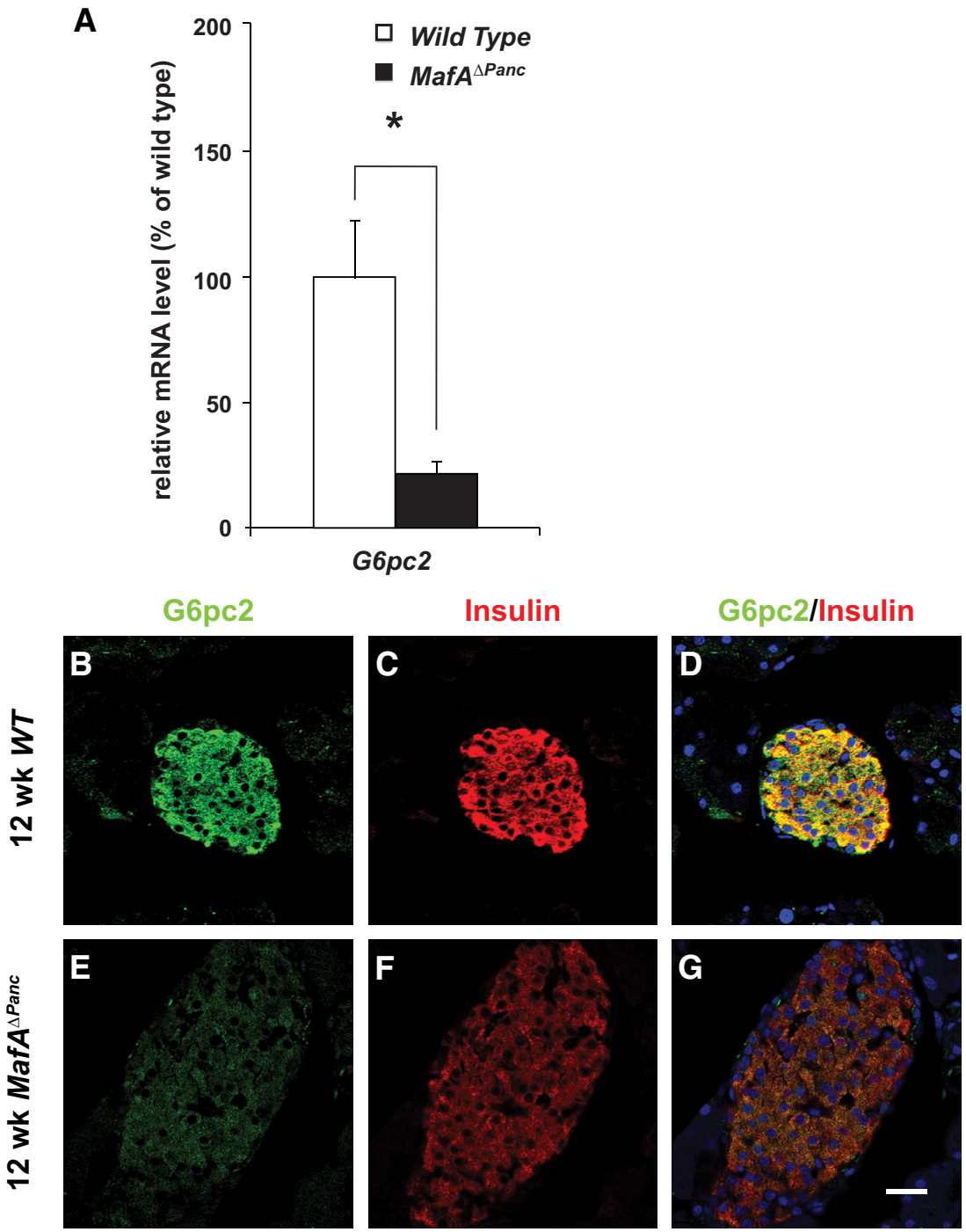


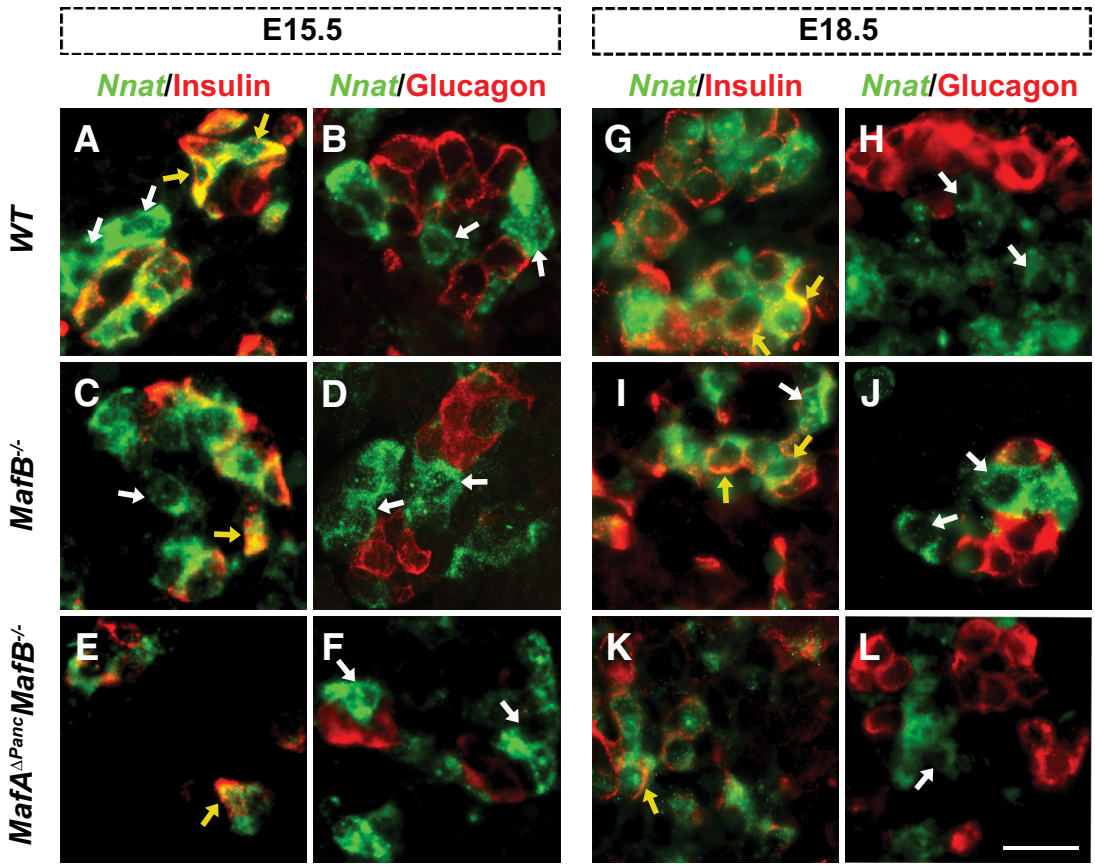
FIG. 3. G6pc2 expression is compromised in adult islets lacking MafA. *A*: Quantitative RT-PCR analysis revealed that *G6pc2* mRNA levels were significantly downregulated in adult *MafA*$^{\Delta Panc}$ islets. **P* < 0.05 (*n* = 3). G6pc2 expression (*green*) in islet insulin^{+} cells (*B–D*) (*red*) was shown by immunostaining to be profoundly reduced in *MafA*$^{\Delta panc}$ islets (*E–G*). Scale bar = 20 μm. Nuclei were stained with YO-PRO-1 (*blue*). (A high-quality digital representation of this figure is available in the online issue.)

lin^{+} and glucagon^{+} cells at E15.5, but only in a small portion of insulin^{+}, glucagon^{+}, and somatostatin^{+} (δ) cells by E18.5 (Fig. 5, WT panel, supplementary Figs. 7 and 8). In contrast, most of the remaining hormone^{+} cell types, as well as hormone^{-} islet cells, synthesize *Rbp4* in the *MafB*$^{-/-}$ and *MafA*$^{\Delta Panc}$;*MafB*$^{-/-}$ mutants (Fig. 5*C–F*, *J–O*, supplementary Figs. 7 and 8).

Quantitative RNA analysis demonstrated that adult *MafA*$^{\Delta Panc}$ islets contained elevated *Rbp4* mRNA levels (Fig. 5*P*). Notably, Rbp4 protein expression was detected only in somatostatin-producing δ-cells in both wild-type and *MafA*$^{\Delta Panc}$ islets (Fig. 5*Q–X*). However, there was no change from the wild-type in the somatostatin^{+} δ-cell proportion produced in mutant islets (wild-type: 8.94 ± 0.73% vs. *MafA*$^{\Delta Panc}$: 8.59 ± 0.89%). Collectively, the data indicate that MafA and MafB negatively regulate production of this effector of insulin secretion and insulin resistance in developing and mature islet cell types.

DISCUSSION

The expression pattern and functional characteristics of MafA and MafB is unusual when compared with all other



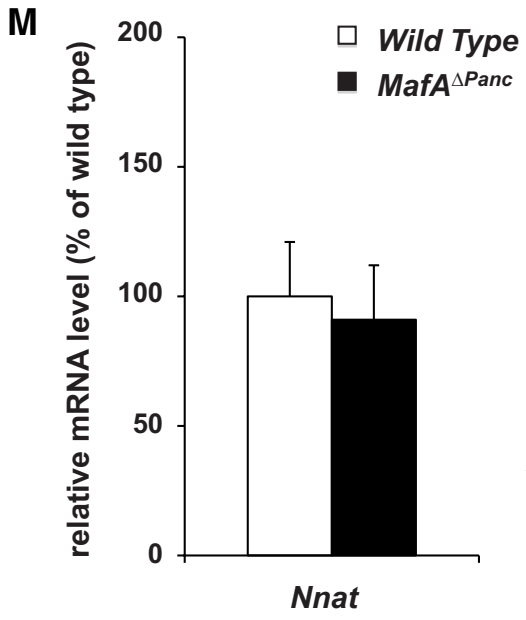


FIG. 4. *Nnat* expression in β-cells is regulated only by MafB. Appreciable levels of *Nnat* (*green*) were detected in insulin$^+$ (*red*, *A*, *yellow arrows*), insulin$^-$ (*A*, *white arrows*), but not glucagon$^+$ (*red*, *B*) cells by in situ analysis. *Nnat* was essentially restricted to β-cells by E18.5 in the wild-type (*G* and *H*) and the remaining insulin$^+$ cells in the *MafB*$^{-/-}$ and *MafA*$^{\Delta Panc}$;*MafB*$^{-/-}$ mutants (*I* and *L*). Scale bar = 20 μm. *Nnat* expression was unaffected in *MafA*$^{\Delta panc}$ islets (*M*). *Yellow arrows* denote *Nnat*$^+$hormone$^+$ cells and *white arrows* denote *Nnat*$^+$hormone$^-$ cells. (A high-quality digital representation of this figure is available in the online issue.)

islet transcriptional regulators. Hence, the *MafA*$^{-/-}$ mutant affects only adult islet architecture and β-cell activity, whereas MafB and essentially all other pancreatic transcriptional regulators (at least) affect islet cell development. Here we have examined the relationship between these closely related factors in islet β-cell formation and function, an especially important issue considering that MafB is not produced within this islet cell population in adults.

Histologic and gene expression analyses of regulated genes identified from a microarray screen performed on E18.5 *MafB*$^{-/-}$, *MafA*$^{\Delta Panc}$;*MafB*$^{-/-}$, and *MafA*$^{\Delta Panc}$ pancreata clearly illustrated that MafB is a more potent regulator of β-cell development than MafA, with the majority of differentially expressed genes identical in *MafB*$^{-/-}$ and *MafA*$^{\Delta Panc}$;*MafB*$^{-/-}$ samples. Our data also indicate that MafB may serve in a compensatory capacity in the embryonic *MafA*$^{\Delta Panc}$ mutant, since MafB levels were amplified at E18.5. Notably, persistent MafB expression in a fraction of adult *MafA*$^{\Delta Panc}$ β-cells did not prevent the change in islet architecture and β-cell function first described in *MafA*$^{-/-}$ mice (19). MafB may not compensate in this setting because of limiting protein

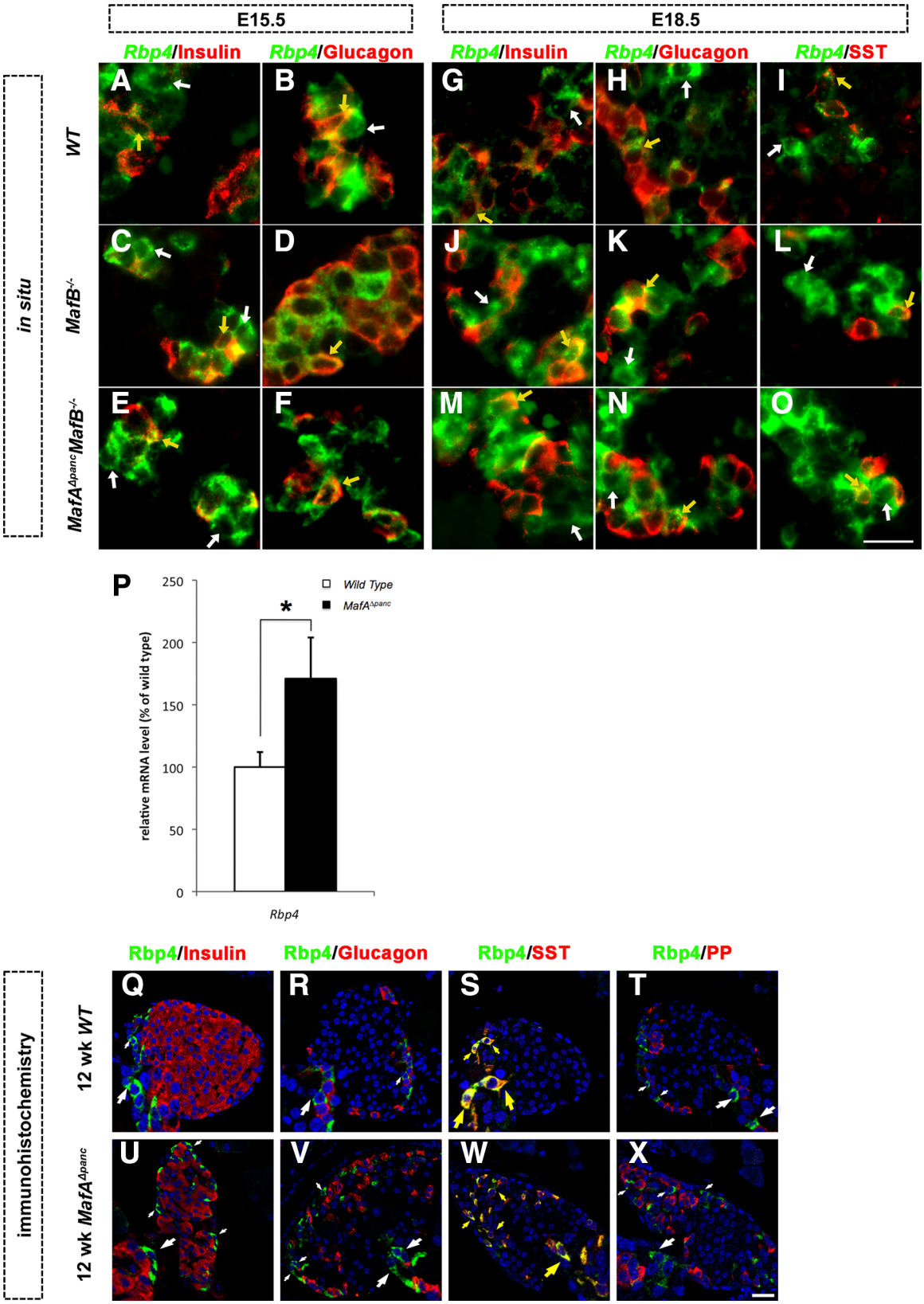


FIG. 5. Rbp4 is induced in the embryonic *MafB*$^{-/-}$ and islet *MafA*$^{\Delta Panc}$ mutants. *Rbp4* transcripts (*green*) are present in some (*A* and *G*) insulin^{+} (*red*, *yellow arrows*) and (*B* and *H*) glucagon^{+} (*red*) cells (*A* and *B*, *yellow arrows*) at both E15.5 and E18.5, with expression also found in (*I*) somatostatin^{+} δ-cells (*red*, *yellow arrows*) by E18.5. The number of hormone^{+} Rbp4^{+} cells increased in *MafB*$^{-/-}$ and *MafA*$^{\Delta Panc}$;*MafB*$^{-/-}$ mutant pancreata (*C–F*; *J–O*). *P*: Rbp4 mRNA expression was elevated in 12-week *MafA*$^{\Delta Panc}$ islets. *S–X*: The Rbp4 protein is found in somatostatin (SST)$^{+}$ δ-cells in (*S*) wild-type and (*W*) *MafA*$^{\Delta Panc}$ islets, and not (*Q* and *U*) insulin^{+}, (*R* and *V*) glucagon^{+}, or (*T* and *X*) pancreatic peptide^{+} cells. Scale bar = 20 μm. Nuclei were stained in blue in panels *Q–W*. *White arrows* denote Rbp4^{+} hormone^{-} cells. (A high-quality digital representation of this figure is available in the online issue.)

levels, or more interestingly, because of differences in activation properties with MafA. In this regard, it is noteworthy that MafA was found to activate *Insulin* expression, whereas MafB specifically stimulated *glucagon* in experiments performed in endocrine cells (20,22,40).

Expression profiling analysis showed that MafB activates genes involved in mature endocrine function, including those significant to glucose sensing (e.g., *Slc2a2*), vesicle maturation (*Slc30a8*), Ca^{2+} signaling (*Camk2b*), and insulin secretion (*Nnat*) (Table 1, supplementary Table 2; data not shown). In contrast, the levels of transcription factors associated with β-cell differentiation (e.g., *Pax6*, *NeuroD1*, *Pax4*, and *Isl1*) were unaffected in the $MafB^{-/-}$ (or $MafA^{\Delta Panc}$;$MafB^{-/-}$) mutant, supporting a role for MafB in late events essential to cell maturation and not early islet cell specification (e.g., *Ngn3*) or cell lineage commitment steps (e.g., *Arx*, *Isl1*, *Nkx2.2*, *Pax4*, and *Pax6*) (18). This proposal is also supported by the fact that $MafB^{-/-}$ mutant retained the expression of some α- and β-cell–enriched developmental markers while undergoing a loss in insulin and glucagon production (18). Interestingly, profiling E18.5 $MafB^{-/-}$ or $MafA^{\Delta Panc}$;$MafB^{-/-}$ pancreata did not pick up the *Gck* (i.e., glucokinase), *Pcsk1*, or *Glp1R* products that were previously associated with MafA control in a candidate gene study performed with INS1 β-cells (41). These genes may be preferentially under later stage MafA control, since, for example, *Gck* is produced in high levels postnatally only in β-cells (42).

Strikingly, our results demonstrate that islet MafA controls many genes first regulated by MafB in developing β-cells. This conclusion is based on immunohistological and mRNA analysis of selected $MafB^{-/-}$ microarray candidate genes within the embryonic $MafB^{-/-}$ pancreas and adult $MafA^{\Delta Panc}$ islet. For example, *Slc30a8* expression was compromised in MafB mutant glucagon$^+$ and insulin$^+$ cells during development and specifically in adult MafA mutant insulin$^+$ cells (Fig. 2). Instances were also found when MafB was the primary (if not exclusive) activator, such as *Nnat* (Fig. 3) and *Pdx1* (data not shown).

Given the significance of MafA and MafB mutual target genes (e.g., *Slc30a8*, *Insulin*, and *Slc2a2*) to β-cell function, insulin secretion defects associated with the $MafA^{-/-}$ (19) and $MafA^{\Delta Panc}$ mutants would be expected. The recent studies showing that Rbp4 produced from rat islet cells inhibits glucose-stimulated insulin secretion (P. Fueger, C. Newgard, unpublished data) suggests that activation in δ-cells of $MafA^{\Delta Panc}$ mouse would also act to diminish β-cell function. (Rbp4 is also principally expressed in human islet δ-cells [supplementary Fig. 9]). Rbp4 attenuates insulin-induced phosphorylation of insulin receptor substrate-1 in human adipocytes (supplementary reference 3). Presumably, it also inhibits insulin-responsive signalling that is essential to β-cell function (supplementary reference 4). We believe that the increased Rbp4 expression in δ-cells is a byproduct of $MafA^{\Delta Panc}$ β-cell inactivity. Further investigation may reveal that islet Rbp4 expression is also induced in mouse models of type 2 diabetes wherein islet MafA expression was selectively lost (43,44).

Although other closely related transcription factors are synthesized within the pancreas (e.g., Pax4/Pax6, Nkx6.1/Nkx6.2, FoxA1/FoxA2), none act as specifically and in such a coordinated manner to affect islet cell function as MafA and MafB. For instance, Nkx6.1 and Nkx6.2 are expressed more broadly and earlier, with only Nkx6.1 significantly influencing β-cell development and function (10,12,45). Likewise, FoxA1 and FoxA2 have a similar early expression pattern to Nkx6.1/6.2 in the forming pancreas, with the dominant and general role in islet cell formation and function served by FoxA2 (7–9). Perhaps parallels can be made between islet MafA and MafB and the basic helix-loop-helix Myf-5, MyoD, and Myogenin factors through their actions in skeletal muscle formation. Hence, gene knockout experiments in mice have shown that myogenin plays an essential role in the late differentiation of myoblasts to myotubes (46) (analogous to MafA in the maturing β-cell), whereas both Myf-5 and MyoD act upstream and are critical to myoblast formation (47) (acting similarly to MafB). However, expression of any of these myogenic factors can convert a variety of tissue culture cells into muscle cells (48,49), whereas only MafA appears capable of activating *Insulin* transcription in vitro (22,40). Interestingly, differences in myogenic potential was revealed when *Myogenin* was expressed from the *Myf5* locus in vivo (48), suggesting that a *knock-in* analysis might also reveal elementary regulatory differences between the MafA and MafB proteins.

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