The Transcription Factor Rfx3 Regulates β-Cell Differentiation, Function, and Glucokinase Expression

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OBJECTIVE—Pancreatic islets of perinatal mice lacking the transcription factor Rfx3 exhibit a marked reduction in insulin-producing β-cells. The objective of this work was to unravel the cellular and molecular mechanisms underlying this deficiency.

RESEARCH DESIGN AND METHODS—Immunofluorescence studies and quantitative RT-PCR experiments were used to study the emergence of insulin-positive cells, the expression of transcription factors implicated in the differentiation of β-cells from endocrine progenitors, and the expression of mature β-cell markers during development in $Rfx3^{-/-}$ and pancreas-specific Rfx3-knockout mice. RNA interference experiments were performed to document the consequences of downregulating Rfx3 expression in Min6 β-cells. Quantitative chromatin immunoprecipitation (ChIP), ChIP sequencing, and bandshift experiments were used to identify Rfx3 target genes.

RESULTS—Reduced development of insulin-positive cells in $Rfx3^{-/-}$ mice was not due to deficiencies in endocrine progenitors or β-lineage specification, but reflected the accumulation of insulin-positive β-cell precursors and defective β-cells exhibiting reduced insulin, Glut-2, and Gck expression. Similar incompletely differentiated β-cells developed in pancreas-specific Rfx3-deficient embryos. Defective β-cells lacking Glut-2 and Gck expression dominate in Rfx3-deficent adults, leading to glucose intolerance. Attenuated Glut-2 and glucokinase expression, and impaired glucose-stimulated insulin secretion, were also induced by RNA interference–mediated inhibition of $Rfx3$ expression in Min6 cells. Finally, Rfx3 was found to bind in Min6 cells and human islets to two well-known regulatory sequences, Pal-1 and Pal-2, in the neuroendocrine promoter of the glucokinase gene.

CONCLUSIONS—Our results show that Rfx3 is required for the differentiation and function of mature β-cells and regulates the β-cell promoter of the glucokinase gene. *Diabetes* **59:1674–1685, 2010**

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Pancreatic endocrine cells are organized into clusters called islets of Langerhans. Mature mouse islets contain a central core of insulin-producing β-cells surrounded by glucagon-producing α-cells, somatostatin-producing δ-cells, and pancreatic polypeptide (PP)-producing cells. During development, these endocrine cells arise in the primitive pancreatic epithelium from progenitor cells expressing the transcription factor Ngn3 (1). Ngn3 regulates specification of the four endocrine cell lineages as a function of specific developmental time windows (2). A complex network of transcription factors directs the differentiation of Ngn3+ progenitors into mature endocrine cells (3). Key factors implicated in β-cell development include NeuroD1, Nkx2.2, Pax4, Nkx6.1, MafA, and Pdx1 (3). NeuroD1, encoded by an Ngn3-regulated gene, is required for the formation of β-cells (4). Nkx2.2 functions downstream of NeuroD1 and promotes commitment of cells to the α, β, and PP lineages at the expense of the ε-cell lineage (5,6). A balance between Arx and Pax4 expression controls specification of α/ε versus β/δ precursors (7). Nkx6.1 is expressed in cells committed to the β-lineage and participates in the developmental program leading to the generation of mature β-cells (8).

Mature β-cells acquire the capacity to synthesize and secrete insulin in response to variations in blood glucose levels. Key components of the glucose-sensing and insulin secretion machinery include the Glut-2 glucose transporter and the glucose sensor glucokinase. Several transcription factors have been implicated in the acquisition of mature β-cell functions, including Pdx1, MafA, and NeuroD1 (4,9,10).

There is growing evidence that Rfx transcription factors are implicated in islet development. There are seven Rfx factors (Rfx1–Rfx7) in mammals (11–13). With the exception of Rfx5, which is a well-known regulator in the immune system (14), the functions of mammalian Rfx factors have only started to emerge recently (15–19). Rfx6 was recently demonstrated to be crucial for islet development in zebra fish, mice, and humans (18,19). We had reported earlier that pancreatic Rfx3 expression is restricted to islets and detected in Ngn3+ progenitors and α, β, δ, and PP cells (20). Islets of perinatal $Rfx3^{-/-}$ mice contain reduced numbers of cells expressing insulin, glucagon, and ghrelin, whereas PP-producing cells are increased (20). Adult $Rfx3^{-/-}$ mice exhibit impaired glucose tolerance (20). These findings suggested that Rfx3 is, like Rfx6, implicated in the development of pancreatic endocrine cells, including β-cells. However, the mechanisms involved remained unknown.

We have performed a detailed analysis of β-cell development in $Rfx3^{-/-}$ mice and conditional pancreas-specific

Rfx3-deficient mice. β-Cell development was impaired from E15.5 onward and characterized by the accumulation of β-cell precursors and defective β-cells having reduced insulin, Glut-2, and glucokinase expression. Impaired glucose-stimulated insulin secretion and reductions in Glut-2 and glucokinase expression were induced in Min6 β-cells by RNA interference–mediated inhibition of *Rfx3* expression. Finally, we identified the glucokinase gene as a direct target of Rfx3. These results show that Rfx3 is required for the differentiation and function of mature β-cells, and that it is a key regulator of glucokinase expression.

RESEARCH DESIGN AND METHODS

Mice. Data for $Rfx3^{-/-}$ mice, and mice carrying an *Rfx3* allele in which exon 3 is flanked by *loxP* sequences ($Rfx3^{loxP}$), have been reported (16). Pancreas-specific *Rfx3* deletion ($Rfx3^{\Delta P}$) was obtained by crossing mice carrying $Rfx3^{loxP}$ with *Pdx1-Cre* mice (21). $Rfx3^{\Delta P/-}$ mice and $Rfx3^{loxP/-}$ littermates were obtained by mating $Rfx3^{loxP/-}$ and $Rfx3^{+/-}$ /*Pdx1-Cre* mice. E0.5 was defined as the morning when a vaginal plug was detected. Genotyping was done as described (16). Mice were on a C57BL/6 background. Experiments were approved by the Federal and Cantonal veterinary authorities.

Staining of sections and morphometry. For E13.5 and E15.5, pancreases were cut, respectively, into three or five consecutive series of ~10 sections. For E17.5 and E19.5, pancreases were cut into seven consecutive series of ~10 sections. Measurements were performed using one section from each series. Immunostaining of frozen sections was performed by standard procedures. Antibodies and secondary reagents are indicated in supplementary Table 1, available in the online appendix at http://diabetes.diabetesjournals.org/cgi/content/full/db09-0986/DC1. Apoptotic cells were revealed by Tdt-mediated dUTP nick end labeling (TUNEL) staining (Roche). Stained sections were visualized by confocal microscopy. Cell counting and morphometry were performed using Mertamorph v6.2 (Universal Imaging Corporation). Labeled cells were quantified within Pdx1$^+$ cells (E13.5 and E15.5) or 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI)-stained cells (E17.5 and E19.5).

Islet purification. Mouse islets were isolated as described (22). Human islets (purity >90%, viability >95%) were provided by the Islet Cell Resource Center of Geneva (Juvenile Diabetes Research Foundation, European islet distribution program).

Quantitative RT-PCR. Total RNA was extracted from pancreas with RNeasy kits (QIAGEN, Switzerland) and from Min6 cells and purified islets with TRIzol (Invitrogen). Quantitative RT-PCR (qRT-PCR) was performed as described (20). Results were normalized using TATA-binding protein (*Tbp*) mRNA. Primers are provided in supplementary Table 2.

RNA interference. Double-stranded oligonucleotides encoding *Rfx3*-specific and control (23) small interfering RNAs (siRNAs) were cloned into the pLVTHM lentiviral vector (24). Five siRNA-encoding oligonucleotides were tested for *Rfx3*. The best results were obtained with the following: 5′-CGCGTCCCCAACACTGGAGGAAATTACTTTCAAGAGAAGTAATTTCCTCC AGTGTTTTTTTTGGAAAT-3′ (sense) and 5′-CGATTTCCAAAAAAACACTGGA GGAAATTACTTCTCTTGAAAGTAATTTCCTCCAGTGTTGGGGA-3′ (antisense). Min6 cells were transduced as described (24). GFP expression was used for assessing transduction efficiencies and purifying transduced cells (FACS Vantage Sorter, Becton Dickinson).

Glucose-stimulated insulin secretion. Glucose-stimulated insulin secretion assays with Min6 cells were performed as described (25).

Rfx3 binding studies. ChIP was performed as described (26) using antibodies specific for RFX3 (13). Primer sequences are provided in supplementary Table 3. The ChIP-sequencing library was prepared from 10 ng of ChIP sample (quantified by a Qubit fluorometer; Invitrogen) using the ChIP-seq Sample Preparation Kit (Illumina). Sequencing was performed with the Genome Analyzer II (Illumina) using the 36 Cycle Sequencing Kit v2. Sequences were derived from a single channel of the flow cell. Data were processed using the Illumina Pipeline Software package v1.0. Bandshift experiments were performed as described (13) using in vitro translated Rfx3 and Min6 extracts. Oligonucleotides used as probes and competitors are provided in supplementary Table 4.

Statistical analysis. Significance was evaluated using a *t* test for independent samples, one-way ANOVA followed by post-test comparisons, and Kolmogorov-Smirnov or median nonparametric tests.
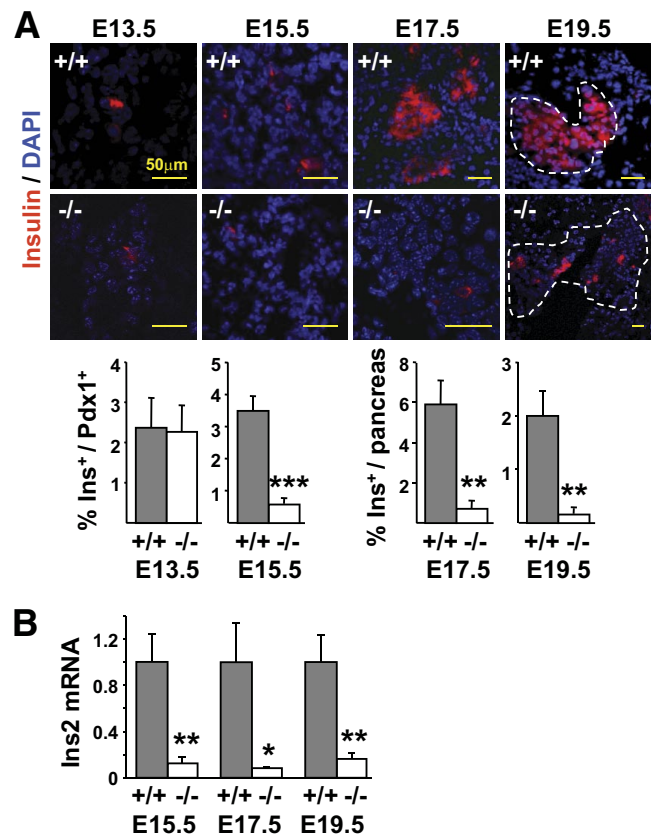

FIG. 1. Development of insulin-positive cells is severely impaired in $Rfx3^{-/-}$ embryos. *A*: Pancreas sections from WT (+/+) and $Rfx3^{-/-}$ (−/−) embryos at stages E13.5, E15.5, E17.5, and E19.5 were stained with antibodies against insulin. Nuclei were labeled with DAPI. Representative sections are shown (*top*). The percentages of insulin-positive cells were quantified in total Pdx1$^+$ cells (E13.5 and E15.5) or in the entire pancreas (E17.5 and E19.5) of WT (+/+) and $Rfx3^{-/-}$ (−/−) embryos (*bottom*). *B*: *Ins2* mRNA abundance was measured by qRT-PCR in total pancreas RNA from WT (+/+) and $Rfx3^{-/-}$ (−/−) embryos at stages E15.5, E17.5, and E19.5. The results were normalized using *Tbp* mRNA and are expressed relative to the levels found in WT embryos. The mean ± SEM derived from five embryos is shown for each stage and genotype; ***$P < 0.0001$; **$P < 0.001$; *$P < 0.05$. (A high-quality color representation of this figure is available in the online issue.)

RESULTS

Impaired pancreatic endocrine-cell development in $Rfx3^{-/-}$ embryos. Pancreas sections from E13.5, E15.5, E17.5, and E19.5 embryos were stained with antibodies against insulin (Fig. 1*A*). No differences in insulin-positive cell numbers were observed between $Rfx3^{-/-}$ and wild-type (WT) embryos at E13.5. A fourfold reduction in insulin-positive cells was observed in $Rfx3^{-/-}$ embryos by E15.5, and this reduction became accentuated at E17.5 (6-fold) and E19.5 (10-fold). A 10-fold decrease in insulin (*Ins*) mRNA expression was revealed by qRT-PCR in the pancreas of E15.5, E17.5, and E19.5 embryos (Fig. 1*B*).

Other islet defects described previously in perinatal $Rfx3^{-/-}$ mice (20) also appeared early during development (supplementary Figs. 1 and 2). Reductions in glucagon-positive cells, and glucagon and ghrelin mRNA expression, became evident by E15.5. Increases in PP$^+$ cells and PP mRNA levels were observed at E13.5. Somatostatin expression remained unchanged throughout development. In agreement with the reduction in glucagon- and insulin-positive cells, the number of cells expressing MafB—a transcription factor implicated in α- and β-cell develop-

ment—and MafB mRNA expression were decreased throughout development (supplementary Fig. 3).

Early pancreatic development is normal in $Rfx3^{-/-}$ embryos. To determine whether the reduction in insulin-positive cells in $Rfx3^{-/-}$ embryos might be secondary to a global defect in pancreas formation, we examined the pancreas at critical developmental stages. Pancreatic tissue in $Rfx3^{-/-}$ embryos was normal in size and gross morphology at E12.5, E15.5, and E19.5 (supplementary Fig. 4).

We next quantified the expression of transcription factors playing pivotal roles in multipotent pancreatic-progenitor cells (Pdx1 and Ptf1a), developing exocrine cells (Ptf1a), and endocrine-progenitor cells (Ngn3) (supplementary Figs. 5 and 6) (3). At E13.5, the developing pancreas of $Rfx3^{-/-}$ embryos appeared well branched and contained WT numbers of Pdx1$^+$ cells. The pancreas of E13.5 and E15.5 $Rfx3^{-/-}$ embryos also contained WT numbers of Ptf1a$^+$ cells concentrated in growing acini. qRT-PCR experiments confirmed that $Ptf1a$ mRNA levels were unaffected throughout development. Finally, the number of cells expressing Ngn3 was not altered in $Rfx3^{-/-}$ embryos at E13.5, E15.5, or E19.5. qRT-PCR experiments confirmed that $Ngn3$ mRNA was expressed at WT levels at all stages in $Rfx3^{-/-}$ embryos. The deficiency in Rfx3 does thus not affect early morphogenesis of the pancreatic epithelium, impair development of the exocrine pancreas, or lead to altered numbers of Ngn3$^+$ progenitors.

Normal β-lineage specification in $Rfx3^{-/-}$ embryos. As the deficiency in insulin-positive cells and reduced $Ins2$ mRNA expression first became evident in $Rfx3^{-/-}$ embryos at E15.5, this stage was examined for the expression of transcription factors implicated in β-cell development (Fig. 2A–D). Cells expressing Pdx1, Pax4, and Nkx6.1 were not reduced in E15.5 $Rfx3^{-/-}$ embryos. No significant decreases in $Pdx1$, $Pax4$, $Nkx6.1$, $Nkx2.2$, or $Neurod1$ mRNAs were observed at E15.5. These findings suggest that the emergence of cells committed to the β-lineage is not affected in $Rfx3^{-/-}$ embryos.

The frequencies of apoptotic or proliferating cells were not affected in the islets of E15.5 $Rfx3^{-/-}$ embryos (Fig. 2E). Altered islet development is therefore not due to increased apoptosis or decreased proliferation.

Defective β-cell differentiation in $Rfx3^{-/-}$ mice. To clarify the phenotype of the β-lineage cells that develop in $Rfx3^{-/-}$ embryos, we examined later developmental stages for the expression of transcription factors required for β-cell differentiation and function. $Nkx2.2$, $Nkx6.1$, $Neurod1$, and $Mafa$ mRNA levels were not reduced in the pancreas of $Rfx3^{-/-}$ embryos at E17.5 and E19.5 (Fig. 3A). $Pdx1$ mRNA expression was reduced modestly at E19.5 but not affected at E17.5 (Fig. 3A). Nkx6.1$^+$ and Pdx1$^+$ cell numbers were indistinguishable between WT and $Rfx3^{-/-}$ embryos (Fig. 3B–C).

We next analyzed the differentiation status of Nkx6.1$^+$ and Pdx1$^+$ β-lineage cells in WT and $Rfx3^{-/-}$ embryos at E17.5 and E19.5 (Fig. 3B and C). These markers become restricted to β-cells as pancreas development progresses (8,27). The fractions of Nkx6.1$^+$ cells that express or lack insulin were quantified independently at E17.5 and E19.5. In WT embryos, Nkx6.1$^+$insulin$^+$ cells increased from 44% at E17.5 to 68% at E19.5. Conversely, Nkx6.1$^+$ insulin$^-$ cells decreased from 56% at E17.5 to 42% at E19.5. In $Rfx3^{-/-}$ embryos, Nkx6.1$^+$insulin$^+$ cells attained only 22% at E19.5, whereas Nkx6.1$^+$insulin$^-$

cells constituted the major β-cell fraction (78–88%). Similar results were obtained by quantifying Pdx1$^+$insulin$^+$ and Pdx1$^+$insulin$^-$ cells at E19.5. Whereas Pdx1$^+$insulin$^+$ cells constituted the major fraction (80%) in WT embryos, Pdx1$^+$insulin$^-$ cells predominated (78%) in $Rfx3^{-/-}$ embryos.

A significant fraction of Nkx6.1$^+$ cells in $Rfx3^{-/-}$ embryos exhibited ectopic PP expression (supplementary Fig. 7A). These Nkx6.1$^+$PP$^+$ cells do not co-express insulin (supplementary Fig. 7B). Lineage-tracing experiments have suggested that β-cells develop from precursors expressing PP (28). The increase in PP$^+$ cells in Rfx3-deficient mice might thus reflect the persistence of PP-expressing β-cell precursors.

Defective β-cell differentiation in pancreas-specific $Rfx3$-knockout mice. Rfx3 is required for the formation of cilia on the embryonic node, which direct the establishment of left-right asymmetry (29). Most $Rfx3^{-/-}$ mice therefore die from developmental defects involving abnormal specification of the left-right body axis (16). We could consequently not exclude that the pancreatic phenotype observed in $Rfx3^{-/-}$ embryos might be an indirect consequence of body-patterning abnormalities. We therefore generated pancreas-specific $Rfx3$ knockout mice. Mice carrying an $Rfx3$ allele containing an essential exon flanked by $loxP$ sequences ($Rfx3^{loxP}$) were crossed with transgenic mice expressing Cre recombinase under control of the $Pdx1$ promoter ($Pdx1$-Cre) (Fig. 4A). $Rfx3^{loxP/-}$/$Pdx1$-Cre mice carrying a pancreas-specific $Rfx3$ deletion ($Rfx3^{\Delta P/-}$ mice) were born according to expected Mendelian frequencies and did not exhibit any overt developmental anomalies. Immunofluorescence staining of pancreas sections from E19.5 embryos demonstrated that the number of Rfx3$^+$ nuclei was reduced over fivefold in the islets of $Rfx3^{\Delta P/-}$ mice (Fig. 4B).

Insulin-positive islet cells were reduced threefold in E19.5 $Rfx3^{\Delta P/-}$ embryos compared with $Rfx3^{loxP/-}$ controls (Fig. 4C). As in $Rfx3^{-/-}$ mice (24), the β-cell defect in $Rfx3^{\Delta P/-}$ mice leads to impaired glucose tolerance in adults (Fig. 4D). Furthermore, the islets of E19.5 $Rfx3^{\Delta P/-}$ embryos contained fewer glucagon-positive cells and increased numbers of PP$^+$ cells (supplementary Fig. 8). Finally, cilia in the islets were shorter and reduced in number (supplementary Fig. 9). These results confirmed that all of the islet cell defects documented in $Rfx3^{-/-}$ mice result from a pancreas-intrinsic deficiency in Rfx3.

We next quantified Nkx6.1$^+$, Nkx6.1$^+$insulin$^+$, and Nkx6.1$^+$insulin$^-$ cells in pancreatic sections from $Rfx3^{\Delta P/-}$ and $Rfx3^{loxP/-}$ embryos at E19.5 (Fig. 4E). As in $Rfx3^{-/-}$ mice, the pancreas of $Rfx3^{\Delta P/-}$ embryos contained WT numbers of total Nkx6.1 cells, a marked reduction in Nkx6.1$^+$insulin$^+$ cells, and a corresponding increase in Nkx6.1$^+$insulin$^-$ cells (Fig. 4E).

$Rfx3^{-/-}$ mice exhibit a block in β-cell maturation. The accumulation of Nkx6.1$^+$insulin$^-$ and Pdx1$^+$insulin$^-$ cells in $Rfx3$-deficient islets suggested that there is a developmental block in the β-cell lineage. We therefore examined the expression of key mature β-cell markers in $Rfx3^{-/-}$ (Fig. 5) and $Rfx3^{\Delta P/-}$ (supplementary Fig. 10A) embryos. Glut-2 ($Slc2a2$) and glucokinase (Gck) mRNA levels were significantly reduced in E17.5 and E19.5 $Rfx3^{-/-}$ embryos, whereas there was no reduction in PC1/3 ($Pcsk1$) mRNA. There was a marked reduction in cells exhibiting strong Glut-2 and glucokinase expression in the islets of E19.5 $Rfx3^{-/-}$ and $Rfx3^{\Delta P/-}$ embryos. The residual insulin-posi-
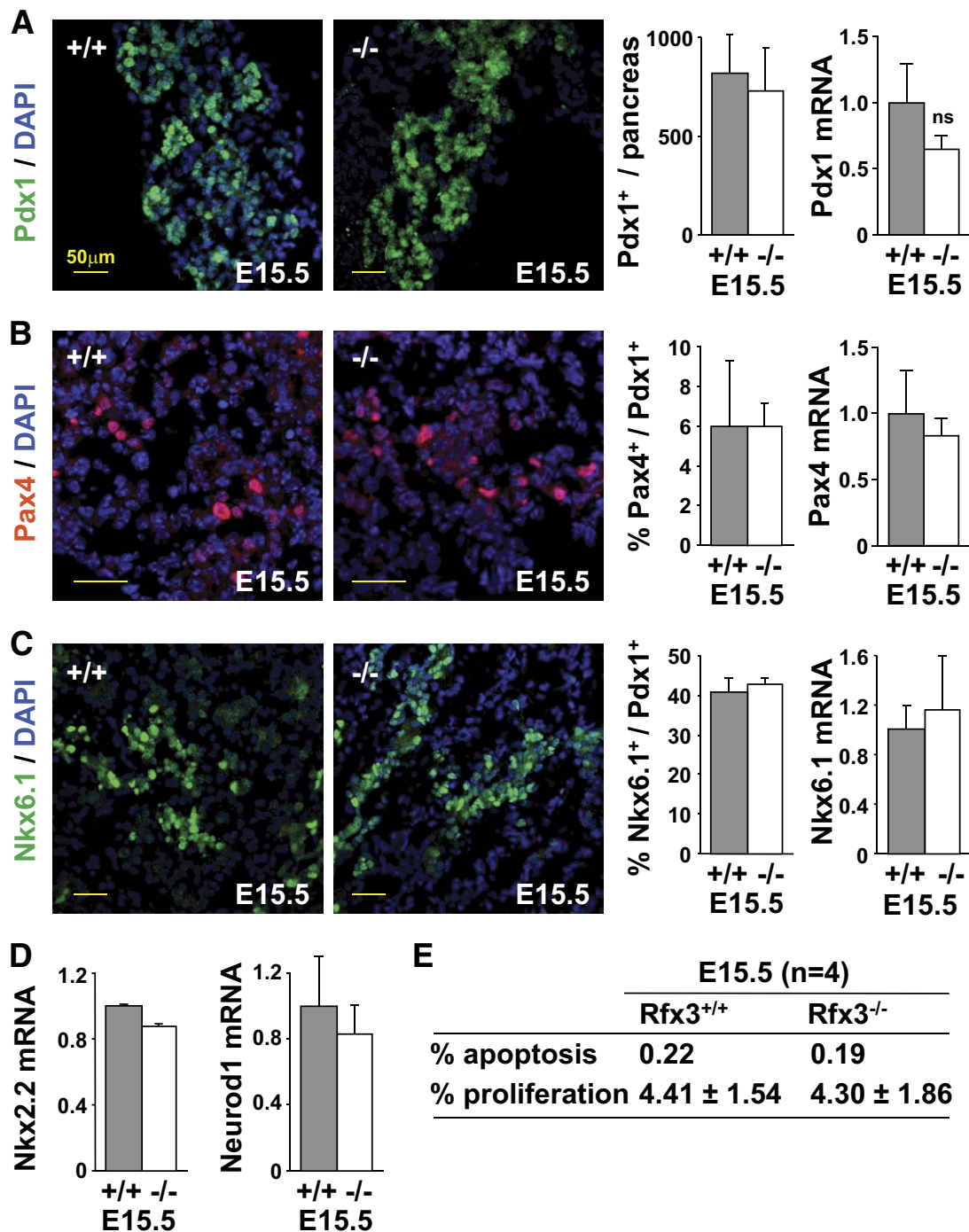
FIG. 2. The expression of key transcription factors implicated in β-cell development is normal in *Rfx3*^{−/−} embryos. *A*: Pancreas sections from WT (+/+) and *Rfx3*^{−/−} (−/−) embryos at stage E15 were stained with DAPI and antibodies against Pdx1. Representative sections are shown (*left*). Pdx1^{+} cells and *Pdx1* mRNA levels were quantified in the pancreases of WT (+/+) and *Rfx3*^{−/−} (−/−) embryos (*right*). *B*: Pancreas sections from WT (+/+) and *Rfx3*^{−/−} (−/−) embryos at stage E15.5 were stained with DAPI and antibodies against Pax4. Representative sections are shown (*left*). Pax4^{+} cells and *Pax4* mRNA levels were quantified in the pancreases of WT (+/+) and *Rfx3*^{−/−} (−/−) embryos (*right*). Pax4^{+} cells were expressed as percentage of Pdx1^{+} cells (counted on serial sections). *C*: Pancreas sections from WT (+/+) and *Rfx3*^{−/−} (−/−) embryos at stage E15.5 were stained with DAPI and antibodies against Nkx6.1. Representative sections are shown (*left*). Nkx6.1^{+} cells and *Nkx6.1* mRNA levels were quantified in the pancreases of WT (+/+) and *Rfx3*^{−/−} (−/−) embryos (*right*). Nkx6.1^{+} cells were expressed as percentage of Pdx1^{+} cells (counted on serial sections). *D*: *Nkx2.2* and *Neurod1* mRNA levels were quantified in total pancreas RNA from WT (+/+) and *Rfx3*^{−/−} (−/−) embryos at stage E15.5. The results were normalized using *Tbp* mRNA and are expressed relative to the levels found in WT embryos. The mean ± SEM derived from five embryos is shown for each stage and genotype. ns, not significant. *E*: TUNEL staining and labeling with anti–phospho-histone H3 antibodies were used to quantify the percentages of apoptotic and proliferating islet cells in pancreatic sections from E15.5 *Rfx3*^{+/+} and *Rfx3*^{−/−} embryos. Means are shown for four embryos of each genotype. (A high-quality color representation of this figure is available in the online issue.)

tive cells exhibited weak or no Glut-2 and glucokinase staining. No change in the number of PC1/3^{+} cells was evident. These findings confirmed that the deficiency in Rfx3 leads to the accumulation of cells characterized by normal expression of certain β-cell markers (Nkx6.1, Pdx1, PC1/3) but impaired expression of key proteins required for β-cell function (Glut-2, glucokinase, insulin).
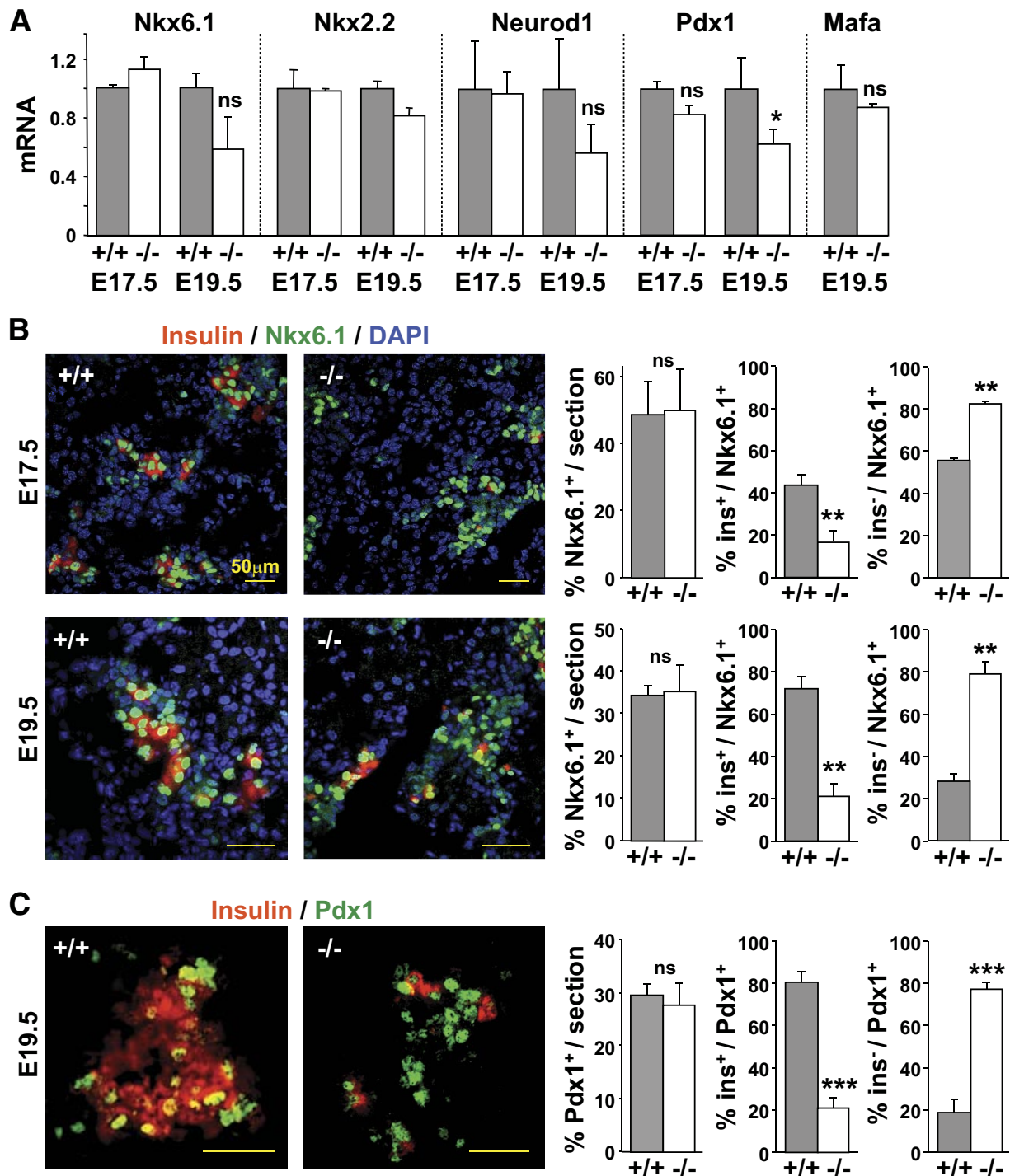
Insulin-positive cell numbers were not reduced mark-

FIG. 3. Block in β-cell differentiation in *Rfx3*^{−/−} embryos. *A*: *Nkx6.1*, *Nkx2.2*, *Neurod1*, *Pdx1*, and *Mafa* mRNA levels were quantified in the pancreases of *Rfx3*^{+/+} (+/+) and *Rfx3*^{−/−} (−/−) embryos at stages E17.5 and E19.5. The results were normalized using *Tbp* mRNA and are expressed relative to the levels found in WT embryos. *B*: Pancreas sections from WT (+/+) and *Rfx3*^{−/−} (−/−) embryos at stages E17.5 and E19.5 were co-stained with antibodies against Nkx6.1 and insulin. Nuclei were labeled with DAPI. Representative sections are shown (*left*). The percentages of total Nkx6.1⁺ cells, Nkx6.1⁺insulin⁺ cells, and Nkx6.1⁺insulin^{−} cells were quantified (*right*). *C*: Pancreas sections from WT (+/+) and *Rfx3*^{−/−} (−/−) embryos at stage E19.5 were co-stained with DAPI and antibodies against Pdx1 and insulin. Representative sections are shown (*left*). The percentages of total Pdx1⁺ cells, Pdx1⁺insulin⁺ cells, and Pdx1⁺insulin^{−} cells were quantified (*right*). The mean ± SE derived from five embryos is shown for each stage and genotype; ***$P < 0.0001$; **$P < 0.001$; ns, not significant. (A high-quality color representation of this figure is available in the online issue.)

edly in *Rfx3*^{ΔP/−} or *Rfx3*^{−/−} adults, indicating that the β-cell mass is restored in postnatal mice (supplementary Fig. 10*B*). These adult β-cells remain defective with respect to Glut-2 and glucokinase expression (supplementary Fig. 10*B*). This defect is associated with impaired

glucose tolerance in *Rfx3*^{ΔP/−} (Fig. 4*D*) and *Rfx3*^{−/−} (20) mice.

Rfx3 is required for mature β-cell function. To document a potential role of Rfx3 in mature β-cells, we inhibited *Rfx3* expression by RNA interference in the Min6

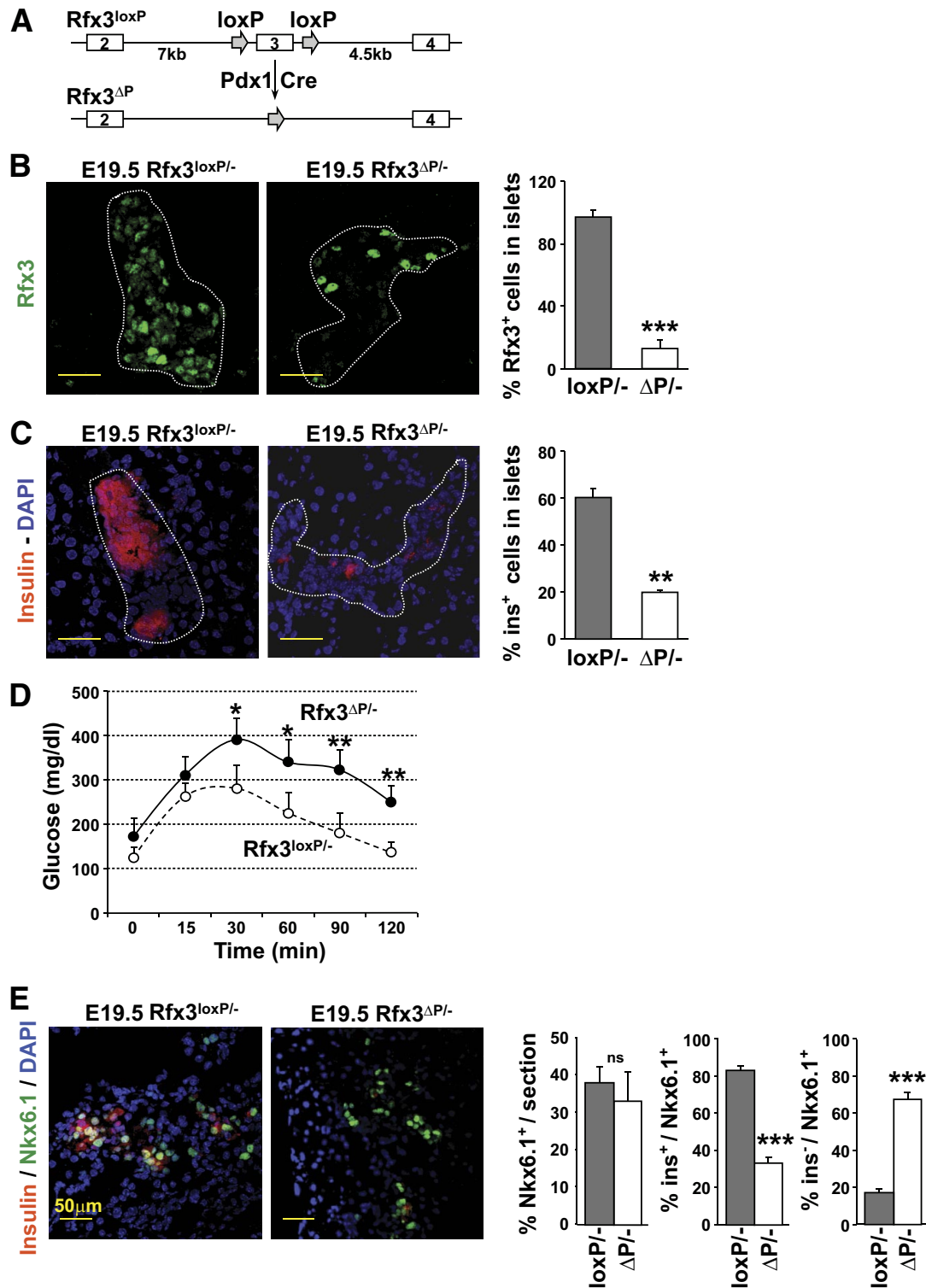FIG. 4. β-Cell differentiation is impaired in conditional pancreas-specific *Rfx3* knockout mice. *A*: Mice carrying a pancreas-specific deletion of exon 3 of the *Rfx3* gene (*Rfx3*$^{\Delta P}$) were generated by crossing mice carrying an *Rfx3* allele in which exon 3 is flanked by loxP sequences (*Rfx3*^{loxP}) with transgenic mice expressing Cre recombinase under control of the *Pdx1* promoter (*Pdx1-cre*). *B*: The efficiency of *Rfx3* deletion was monitored by staining pancreas sections with antibodies specific for Rfx3. Representative islets (dashed contours) from *Rfx3*$^{loxP/-}$ and *Rfx3*$^{\Delta P/-}$ E19.5 embryos are shown (*left*). The bar graph (*right*) shows quantifications of the percentages of Rfx3$^+$ cells in the islets of *Rfx3*$^{loxP/-}$ and *Rfx3*$^{\Delta P/-}$ E19.5 embryos. The mean ± SEM derived from five embryos is shown for each genotype; ***$P < 0.0001$. *C*: Pancreas sections from *Rfx3*$^{loxP/-}$ and *Rfx3*$^{\Delta P/-}$ E19.5 embryos were stained with antibodies against insulin. Representative islets (dashed contours) are shown (*left*). The bar graph (*right*) shows quantifications of the percentages of insulin-positive cells in the islets of *Rfx3*$^{loxP/-}$ and *Rfx3*$^{\Delta P/-}$ E19 embryos. The mean ± SEM derived from five embryos is shown for each genotype; **$P < 0.001$. *D*: *Rfx3*$^{\Delta P/-}$ mice exhibit impaired glucose tolerance. Blood glucose concentrations were measured in *Rfx3*$^{loxP/-}$ and *Rfx3*$^{\Delta P/-}$ mice at the indicated times after intraperitoneal injection of glucose. The mean ± SEM is derived from the analysis of five mice of each genotype; *$P < 0.05$; **$P < 0.001$. *E*: Pancreas sections from *Rfx3*$^{loxP/-}$ and *Rfx3*$^{\Delta P/-}$ embryos at stage E19.5 were co-stained with DAPI and antibodies against Nkx6.1 and insulin. Representative sections are shown (*left*). The percentages of total Nkx6.1$^+$, Nkx6.1$^+$insulin$^+$ cells, and Nkx6.1$^+$insulin$^-$ cells were quantified (*right*). The mean ± SEM derived from five embryos is shown for each stage and genotype; ***$P < 0.0001$; **$P < 0.001$; ns, not significant. (A high-quality color representation of this figure is available in the online issue.)
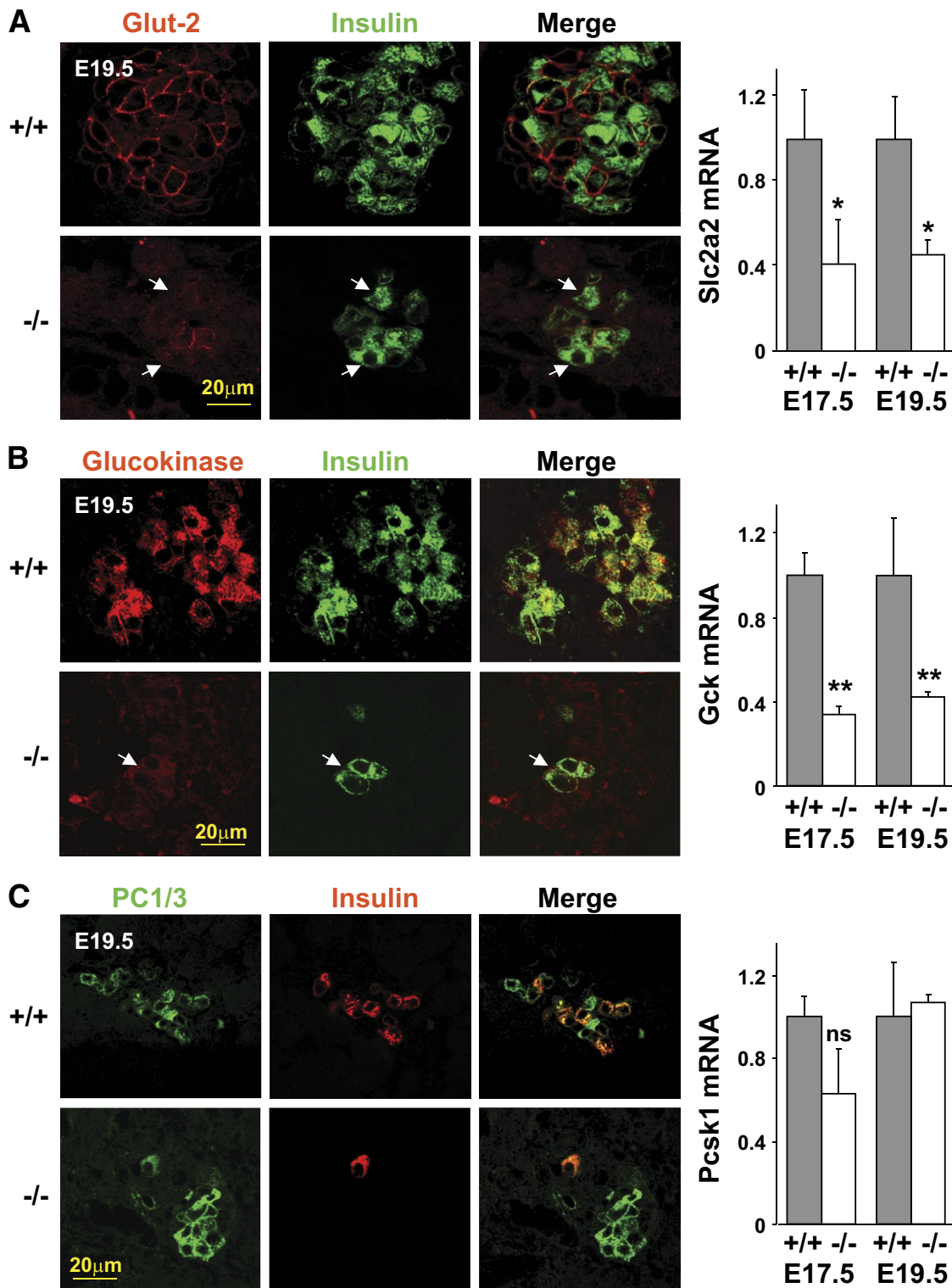
FIG. 5. β-Cells in *Rfx3*$^{-/-}$ embryos exhibit strongly decreased Glut-2 and glucokinase expression. Pancreas sections from WT (+/+) and *Rfx3*$^{-/-}$ (−/−) embryos at stage E19.5 were stained with antibodies against insulin and Glut-2 (*A*), glucokinase (*B*), or PC1/3 (*C*). Representative sections are shown (*left*). Residual insulin-positive cells in *Rfx3*$^{-/-}$ embryos show only weak Glut-2 and glucokinase expression (*arrows*). *Slc2a2* (*A*), *Gck* (*B*), and *Pcsk1* (*C*) mRNA levels were quantified in the pancreases of WT (+/+) and *Rfx3*$^{-/-}$ (−/−) embryos at stages E17.5 and E19.5 (*right*). The results were normalized using *Tbp* mRNA and are expressed relative to the levels found in WT embryos. The mean ± SEM derived from five embryos is shown for each stage and genotype; **$P < 0.001$; *$P < 0.05$; ns, not significant. (A high-quality digital representation of this figure is available in the online issue.)

cell line. Min6 cells express *Rfx3* mRNA at levels similar to those observed in mouse islets (supplementary Fig. 11*A*). Min6 cells were transduced with lentiviral vectors encoding *Rfx3*-specific or control siRNAs. Significant (albeit partial) reductions in Rfx3 protein and mRNA were induced in cells expressing the *Rfx3* siRNA (Fig. 6*A*, supplementary Fig. 11*B*).
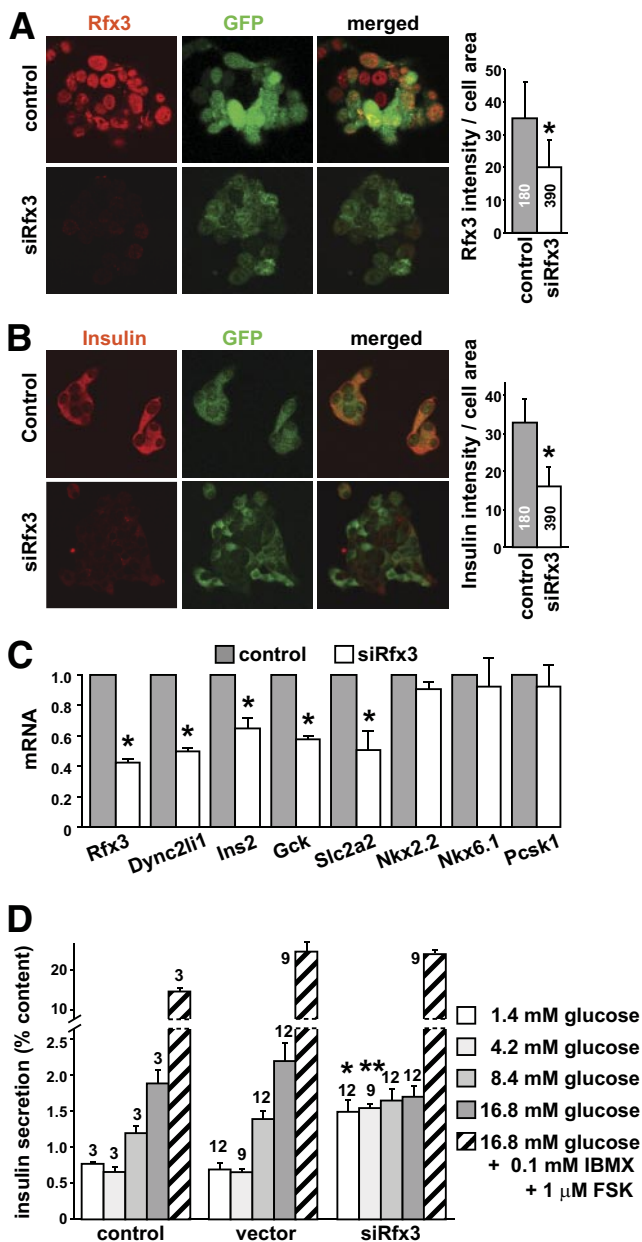
The impact of inhibiting Rfx3 expression on insulin

FIG. 6. Rfx3 is required for maintaining β-cell maturity. Min6 cells were transduced with lentiviral vectors expressing control or *Rfx3*-specific siRNAs (siRfx3). The vectors also express GFP. *A*: Transduced cells were stained with antibodies against Rfx3 and examined by fluorescence microscopy to visualize Rfx3 (*red*) and GFP (*green*) expression. Representative fields are shown (*left*). The bar graph (*right*) shows the quantification of Rfx3 expression (fluorescence intensity). The mean ± SEM was derived from analysis of the indicated numbers of cells; *$P < 0.05$. *B*: Transduced cells were stained with antibodies against insulin and examined by fluorescence microscopy to visualize insulin (*red*) and GFP (*green*) expression. Representative fields are shown (*left*). The bar graph (*right*) shows the quantification of insulin expression (fluorescence intensity). The mean ± SEM was derived from analysis of the indicated numbers of cells; *$P < 0.05$. *C*: *Rfx3*, *Dync2li1*, *Ins2*, *Gck*, *Slc2a2*, *Nkx2.2*, *Nkx6.1*, and *Pcsk1* mRNA levels were quantified in cells transduced with the control and *Rfx3*-specific siRNA vectors. The results were normalized using *Tbp* mRNA and are expressed relative to cells transduced with the control vector. The mean ± SEM derived from three independent experiments is shown; *$P < 0.05$. *D*: Insulin secretion in response to the indicated conditions was measured for Min6 cells transduced with the empty vector (vector), the control siRNA vector (control), or the *Rfx3*-specific siRNA vector (siRfx3). Results are expressed as percent of total insulin content and show the mean ± SEM derived from the number of measurements indicated at the top of the bars: *$P < 0.03$, **$P < 0.001$ vs. corresponding control value. FSK, forskolin; IBMX, isomethyl butyl xanthine. (A high-quality digital representation of this figure is available in the online issue.)

production was assessed by three complementary approaches: quantifying insulin-staining intensity of individual cells by confocal microscopy, measurement of total insulin content by acid-ethanol extraction, and quantification of *Ins2* mRNA. Although there were modest reductions in insulin staining and *Ins2* mRNA in cells expressing the *Rfx3* siRNA (Fig. 6*B* and *C*, supplementary Fig. 11*B*), this was not paralleled by a significant decrease in total insulin content (data not shown). *Slc2a2* and *Gck* mRNAs were significantly decreased in cells expressing the *Rfx3* siRNA (Fig. 6*C*). The reductions in *Slc2a2* and *Gck* mRNAs were similar to that observed for a gene (*Dync2li1*) known to be regulated by Rfx3 (Fig. 6*C*) (16,20). In contrast, as observed in Rfx3$^{-/-}$ embryos, there was no decrease in *Nkx6.1*, *Nkx2.2*, and *Pcsk1* mRNAs (Fig. 6*C*).

To test the effect of inhibiting Rfx3 expression on the secretory function of the transduced Min6 cells, we examined their insulin secretion in response to glucose or glucose plus agents raising cAMP (IBMX and forskolin) (Fig. 6*D*). The inhibition of Rfx3 expression did not affect insulin content (data not shown), but led to significantly elevated basal insulin release. As a result, stimulatory levels of glucose were unable to increase insulin output, as was observed in cells transduced with an empty vector or a vector encoding an irrelevant siRNA. This change was not due to the transduction protocol, since normal basal and glucose-stimulated insulin secretion was observed with the two control cell types. It could also not be ascribed to nonspecific inhibition of the secretory machinery, since stimulation with glucose in the presence of isomethyl butyl xanthine (IBMX) plus forskolin increased insulin release similarly in the three types of Min6 cells (Fig. 6*D*).

The β-cell promoter of the *Gck* gene is regulated by Rfx3. To determine if the *Ins2*, *Gck*, or *Slc2a2* genes might be direct targets of Rfx3, we analyzed the results of a chromatin immunoprecipitation sequencing (ChIP-seq) experiment performed with Rfx3-specific antibodies and chromatin from Min6 cells. Two peaks were observed in the promoter that drives *Gck* expression in β-cells (Fig. 7*A*). This double peak coincided with two highly conserved sequences showing homology to the consensus Rfx binding site (30). Binding of Rfx3 to the *Gck* promoter was as evident as at the control *Dync2li1* promoter. No signals were observed at the liver-specific *Gck* promoter, the *Ins2* and *Slc2a2* genes, or genes that are not expected to be targets of Rfx3 on the basis of our siRNA experiments and the analysis of *Rfx3*$^{-/-}$ mice (Fig. 7*A*, supplementary Fig. 12, data not shown).

Quantitative ChIP (qChIP) experiments confirmed that Rfx3 binds to the *Gck* promoter in Min6 cells, albeit not as strongly as at the *Dync2li1* promoter (Fig. 7*B*). Binding of Rfx3 was not observed at the *Slc2a2* or *Ins2* genes. qChIP experiments performed with purified human islets demonstrated that RFX3 also binds to the human *GCK* promoter (Fig. 7*B*).

The Rfx3 binding motifs in the *Gck* promoter lie within a region that is essential and sufficient for driving β-cell expression in vitro and in vivo (Fig. 7*C*) (31–34). They correspond to two palindromic sequences (Pal-1 and Pal-2) known to be critical for activation of the *Gck* promoter in β-cells (34,35). That Rfx3 can bind specifically to the Pal-1 and Pal-2 motifs was confirmed by bandshift experiments performed with recombinant Rfx3 and Min6 extracts (Fig. 7*D*, supplementary Fig. 13). These findings
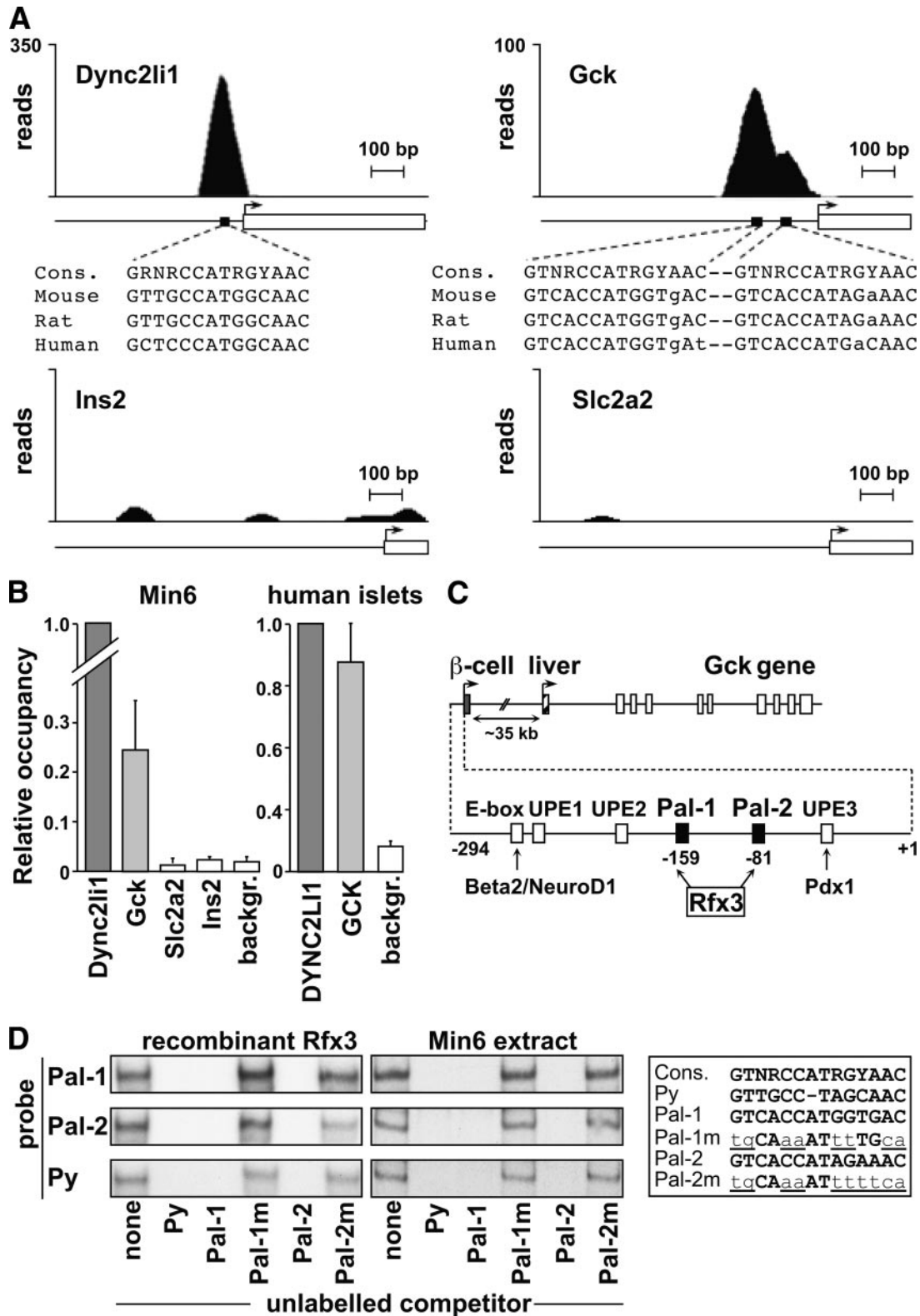
FIG. 7. Rfx3 binds to two key regulatory elements in the β-cell–specific promoter of the *Gck* gene. *A*: The profiles show the number of ChIP-seq reads mapping to the upstream regions of the *Dync2li1*, *Gck*, *Slc2a2*, and *Ins2* genes. Transcription start sites (*arrows*), transcribed regions (*open boxes*), and scales in base pairs (bp) are indicated. Peaks corresponding to binding of Rfx3 were observed in the promoters of the *Dync2li1* and *Gck* genes. The positions of these peaks coincide with conserved Rfx binding motifs (*black boxes*). The motifs found in the promoters of the mouse, rat, and human *Dync2li1* and *Gck* genes are shown aligned with the consensus Rfx-binding site. No binding of Rfx3 was observed at the promoters of the *Ins2* or *Slc2a2* genes. *B*: Binding of Rfx3 to the promoters of the *Dync2li1*, *Gck*, *Slc2a2*, and *Ins2* genes was assessed by quantitative ChIP in Min6 cells (*left*) or purified human islets (*right*). Control regions were included as background (backgr.) references. Results are expressed relative to binding of Rfx3 at the *Dync2li1* promoter and show the mean ± SEM derived from three independent experiments. *C*: Schematic map of the *Gck* gene. Transcription of the *Gck* gene is regulated by two alternative cell type–specific promoters. The first promoter drives *Gck* expression in β-cells. The second promoter, situated ~35 kb further downstream, is used in the liver. The two Rfx3 binding sites

indicate that Rfx3 is a bona fide transcriptional regulator of the human and mouse glucokinase gene.

DISCUSSION

Strongly decreased numbers of insulin-positive cells became evident in $Rfx3^{-/-}$ embryos by E15.5 and persisted until birth. This could not be attributed to a deficiency in $Ngn3^+$ progenitors, altered islet cell apoptosis or proliferation, or defective β-lineage specification. Instead, β-lineage cells develop in $Rfx3^{-/-}$ embryos, but remain incompletely differentiated. Few, if any, of these cells have a mature β-cell phenotype. A minority (~20%) correspond to insulin-positive β-cells exhibiting defective glucokinase and Glut-2 expression. The majority (~80%) have a phenotype that could correspond to late β-cell precursors ($Ngn3^-$, $NKx6.1^+$, $Pdx1^+$, insulin$^-$, glucokinase$^-$, Glut-2^-). The latter are not simply endocrine progenitors since they lack Ngn3 expression (3,21). A similar skewed distribution of β-lineage cells is observed in pancreas-specific $Rfx3$-knockout embryos, indicating that it results from an endocrine cell–intrinsic deficiency in Rfx3. Defective β-cells lacking glucokinase and Glut-2 expression persist in adult $Rfx3^{-/-}$ and conditional $Rfx3$-deficient mice, leading to intolerance to glucose. Downregulating $Rfx3$ expression in Min6 cells provoked reduced Glut-2 and glucokinase expression and an elevation of basal insulin release, which prevented the detection of a glucose-stimulated increase in insulin output. Collectively, these findings indicate that Rfx3 is critical for both β-cell differentiation and the function of mature β-cells.

The Gck gene is regulated by two cell type–specific promoters (36). The most upstream promoter drives expression in β-cells and various other cell types, including glucose-sensitive neurons in the central nervous system and endocrine cells in the gut and pituitary gland (31,37,38). This neuroendocrine promoter is essential and sufficient for driving glucokinase expression in β-cells in vitro and in vivo (31–34). Rfx3 binds to two sequences in the neuroendocrine promoter in Min6 cells. RFX3 also binds to this promoter in human islets. The Rfx3 binding sites correspond to two palindromic sequences (Pal-1 and Pal-2) that are essential for regulating β-cell activity of the neuroendocrine promoter in rodents and humans (34,35,39). Gel-retardation, footprint, methylation-interference, and cross-linking experiments have demonstrated that Pal-1 and Pal-2 are recognized by nuclear factors in β-cells (33–35,39). Until now, the identity of these factors remained unknown. Our results show that the function of Pal-1 and Pal-2 is mediated by binding of Rfx3. Rfx3 thus collaborates with other β-cell–specific transcription factors, such as Pdx1 and NeuroD1 (36,40,41), in regulating Gck expression in β-cells.

Mice heterozygous for a deletion of the neuroendocrine promoter (which present only a 50% reduction in glucokinase expression in islets) exhibit hyperglycemia, intolerance to glucose, reduced glucose-stimulated hyperinsulinemia, and impaired glucose-stimulated insulin secretion (42). Similarly, mice heterozygous for a pancreas-specific Gck deletion exhibit an impaired ability to raise plasma insulin concentrations in response to glucose (43). A modest decrease in Gck expression is thus sufficient for perturbing glucose-stimulated insulin secretion and the maintenance of glucose homeostasis. Attenuated glucokinase expression in β-cells can thus explain, at least in part, why $Rfx3^{-/-}$ and $Rfx3^{\Delta P/-}$ mice are intolerant to glucose.

Loss-of-function mutations in the human GCK gene cause one form of maturity-onset diabetes of the young (MODY-2) and permanent neonatal diabetes (44). A mutation in the neuroendocrine GCK promoter was recently found to be responsible for reduced glucokinase expression in certain MODY patients (45). Interestingly, this mutation lies immediately adjacent to the Pal-1 motif. Other forms of MODY result from mutations in genes encoding transcription factors that regulate the expression of GCK and/or play other key roles in β-cell function (45). Because we have identified Rfx3 as a critical regulator of Gck expression and mature β-cell function, it is tempting to speculate that mutations within the $RFX3$ gene, or in its regulatory regions, could give rise to MODY or other diseases characterized by pancreas dysfunction, defective insulin secretion, and/or perturbed glucose homeostasis.

We have as yet not elucidated the mechanisms responsible for reduced insulin and Glut-2 expression in β-lineage cells of $Rfx3^{-/-}$ mice. Neither gene is a direct target of Rfx3. Binding of Rfx3 was also not observed at the genes encoding NeuroD1, Pdx1, or MafA, which are known to activate insulin gene transcription (46), and the expression of these transcription factors was not affected in $Rfx3^{-/-}$ mice. Furthermore, analysis of our ChIP-seq data has not revealed other target genes known to be implicated in insulin or Glut-2 expression. Finally, the deficiencies in insulin-positive cells and insulin expression in $Rfx3^{-/-}$ mice are unlikely to be indirect consequences of reduced glucokinase expression, because no reductions in β-cell mass or pancreatic insulin content were documented in Gck-deficient mice (42).

Our ChIP-seq data have indicated that Rfx3 binds to the promoters of hundreds of genes implicated in the formation and function of cilia (data not shown). This is consistent with the fact that Rfx3 is required for the biogenesis of cilia on various cell types, including pancreatic endocrine cells (20,47). However, the functions of cilia in islets are unknown. No defects were observed in mice in which cilia were ablated in the pancreas by deletion of the $Kif3a$ gene (47). It therefore remains to be determined whether the ciliary defects in $Rfx3^{-/-}$ mice contribute directly or indirectly to perturbed islet cell development and/or function.

The islet defects in $Rfx3$-deficient mice are strikingly similar, albeit not identical, to those observed in Rfx6-deficient mice (18). Shared features include impaired development of mature endocrine cells expressing insulin, glucagon, and ghrelin; increased numbers of cells expressing PP; normal expression of transcription factors implicated in β-cell specification (Nkx6-1, Nkx2-2); the accumulation of Nkx6.1$^+$ cells lacking insulin; the

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correspond to the previously identified Pal-1 and Pal-2 sequences centered on nucleotides −159 and −81 of the β-cell promoter. The approximate positions of other known regulatory elements (E-box, UPE-1, UPE-2, UPE-3) and binding sites for transcription factors (Beta2/NeuroD1, Pdx1) implicated in Gck expression in β-cells are indicated. The map of the Gck promoter is based on the study by Im et al. (36). *D*: Bandshift experiments were performed with recombinant Rfx3 (*left gels*) and Min6 extracts (*right gels*) using double-stranded oligonucleotide probes containing Pal-1, Pal-2, and a known Rfx binding site (Py) from the polyoma-virus enhancer. The regions of the gels containing the major band corresponding to binding of Rfx3 are shown (see supplementary Fig. 11). Binding reactions were supplemented as indicated below with unlabeled competitor oligonucleotides containing the Py, Pal-1, mutated Pal-1 (Pal-1m), Pal-2, or mutated Pal-2 (Pal-2m) sites. The corresponding sequences are shown aligned with the consensus Rfx binding site. Mutated nucleotides are underlined and in lower case.

appearance of Nkx6.1$^+$ cells expressing PP; and reduced Glut-2 and Gck expression. Notable differences include the development of cells expressing somatostatin, which is defective in $Rfx6^{-/-}$ mice but normal in $Rfx3$-deficient mice, and the development of cilia on islet cells, which is impaired in $Rfx3$-deficent mice but unaffected in $Rfx6^{-/-}$ mice. A likely explanation for the partially overlapping phenotype is that Rfx3 and Rfx6 collaborate to regulate a common set of genes by binding as Rfx3-Rfx6 heterodimers and distinct sets of genes by binding as either Rfx3 and Rfx6 homodimers, or heterodimers with other Rfx factors. One common target gene is likely to be Gck, since preliminary ChIP experiments have suggested that its β-cell promoter is bound by both Rfx3 and Rfx6.

Detailed understanding of the programs governing β-cell differentiation is a prerequisite for the development of β-cell replacement therapies for diabetes relying on β-cells generated by guided differentiation of stem or progenitor cells, or trans-differentiation of other mature cells (3,48,49). Although major progress has been made, successful generation of mature β-cells has so far been mitigated because the program controlling β-cell differentiation is only partially understood. Our results contribute significantly to elucidation of this program and may therefore be valuable for the generation of functional β-cells in vitro.

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