Neurogenin3 inhibits proliferation in endocrine progenitors by inducing Cdkn1a

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During organogenesis, the final size of mature cell populations depends on their rates of differentiation and expansion. Because transient expression of Neurogenin3 (Neurog3) in progenitor cells in the developing pancreas initiates their differentiation to mature islet cells, we examined the role of Neurog3 in cell cycle control during this process. We found that mitotically active pancreatic progenitor cells in mouse embryos exited the cell cycle after the initiation of Neurog3 expression. Transcriptome analysis demonstrated that the Neurog3-expressing cells dramatically up-regulated the mRNA encoding cyclin-dependent kinase inhibitor 1a (*Cdkn1a*). In *Neurog3* null mice, the islet progenitor cells failed to activate *Cdkn1a* expression and continued to proliferate, showing that their exit from the cell cycle requires Neurog3. Furthermore, induced transgenic expression of Neurog3 in mouse β-cells in vivo markedly decreased their proliferation, increased *Cdkn1a* levels, and eventually caused profound hyperglycemia. In contrast, in *Cdkn1a* null mice, proliferation was incompletely suppressed in the Neurog3-expressing cells. These studies reveal a crucial role for Neurog3 in regulating the cell cycle during the differentiation of islet cells and demonstrate that the subsequent down-regulation of Neurog3 allows the mature islet cell population to expand.

The mature structure of an organ depends on its constituent cell populations and how those populations expand and organize as the organ grows. Within the pancreas, the size of the islets of Langerhans and especially how many insulin-producing β-cells they contain is a critical determinant of pancreatic function and the risk of developing diabetes. The number of islet cells depends on the rate at which new endocrine cells [α-, β-, δ-, ε-, and pancreatic polypeptide (PP) cells] differentiate from progenitors, the size of the progenitor population, and the rates of proliferation of the progenitors and mature endocrine cells. Understanding the mechanisms that control these rates will help explain how distinct cell populations assemble into functional organs.

The coordinated activity of numerous transcription factors regulates the differentiation of the islet cells (1, 2). Among these factors, Neurogenin3 (Neurog3/Neurog3), a member of the basic helix–loop–helix (bHLH) transcription factor family, transiently marks the progenitor cells that will become islet cells and initiates endocrine differentiation during embryonic development, regeneration, and transdifferentiation (3–10).

Although we know that most descendants of Neurog3-expressing cells exit the cell cycle (6, 11), we do not know whether or how Neurog3 might drive cell cycle exit. To address these questions, we used several mouse models with loss- and gain-of-function mutations of Neurog3 and demonstrated that Neurog3 is both necessary and sufficient to promote cellular quiescence in pancreatic progenitors. Furthermore, transcriptome analysis with high time resolution using the Neurog3-Timer mouse model identified the cell cycle inhibitor Cdkn1a (p21/CIP1) as a downstream target of Neurog3 in the endocrine progenitors.

Results

Cell Cycle Exit Follows Neurog3 Induction. To quantify cell division during endocrine differentiation, we performed triple-labeled immunohistochemistry with antibodies against Neurog3, eGFP, and BrdU at embryonic day (E)15.5 in pancreases from heterozygous mice in which one copy of the coding sequence for Neurog3 was replaced with eGFP (*Neurog3*$^{eGFP/+}$ mice; ref. 12) (Fig. 1 *A*–*D*). Cells labeled by the anti-Neurog3 antibody but not anti-GFP antibody (Neurog3$^+$/eGFP$^-$ cells) represent the newest endocrine progenitors that will shortly become Neurog3$^+$/eGFP$^+$ double-positive cells (13). Subsequently, the Neurog3$^+$/eGFP$^+$ cells lose Neurog3 immunoreactivity but temporarily retain eGFP immunoreactivity (Neurog3$^-$/eGFP$^+$ cells) because of the long half-life of green fluorescent protein (24 h or more; ref. 14) (Fig. 1*E*).

As outlined at the bottom of Fig. 1*E*, when Neurog3 expression starts, the first cells are Neurog3$^+$, but eGFP$^-$, because maturation of the eGFP fluorophore takes time. These Neurog3$^+$/eGFP$^-$ cells progress to Neurog3$^+$/eGFP$^+$ double-positive cells after eGFP maturation. Then a few hours later when these Neurog3$^+$/eGFP$^+$ cells lose Neurog3 expression, the long half-life eGFP leads to Neurog3$^-$/eGFP$^+$ cells. Three hours after BrdU labeling, 22.0% of Neurog3$^+$/eGFP$^-$ cells, 7.2% of Neurog3$^+$/eGFP$^+$ cells, and 1.2% of Neurog3$^-$/eGFP$^+$ cells were labeled with BrdU (Fig. 1*E*). The differences in BrdU labeling rates in these three populations indicate that cells stop entering S-phase shortly after the initiation of Neurog3 expression.

To confirm the changes in cell cycle during endocrine maturation, embryonic pancreases were dissected from *Neurog3-Timer* embryos at E15.5 and E17.5, stained with the DNA dye Hoechst 33342, and analyzed by flow cytometry. The Timer (DsRed-E5) fluorescent protein shifts its fluorescence emission peak from green to red over time (15) and, thereby, overcomes the problems caused by the long half-life of GFP (16, 17) and allows the determination of the time because activation of Neurog3 in individual cells based on the ratio of green/red fluorescence (13). As shown in Fig. 1 *F* and *G*, at both E15.5 and E17.5, >98% of endocrine progenitors (gates A and B) were in G_0/G_1 phase and, thus, quiescent. In the mature endocrine cells in gate C, however, a significant increase in cells in $S/G_2/M$ demonstrates the reentry of cells into the cell cycle at E17.5, but not at E15.5 (Fig. 1*G*).

Neurog3 Is Required for Cell Cycle Exit. The studies in Fig. 1 correlate cell cycle exit with Neurog3 expression, but do not indicate whether Neurog3 drives the cells to exit the cell cycle, or whether, alternatively, cells already programmed to exit the cell cycle may preferentially activate Neurog3 expression. To address this question, we analyzed Neurog3-deficient mice by histology and flow cytometry. In Neurog3 null embryos (*Neurog3*$^{eGFP/eGFP}$), eGFP expression identifies those pancreatic progenitor cells in which Neurog3 expression would have been activated. Immunostaining for eGFP and BrdU demonstrated higher rates of BrdU labeling in the eGFP-expressing cells of *Neurog3*$^{eGFP/eGFP}$

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Conflict of interest statement: M.S.G. is an inventor on patents held by the University of California covering Neurog3 and its use.

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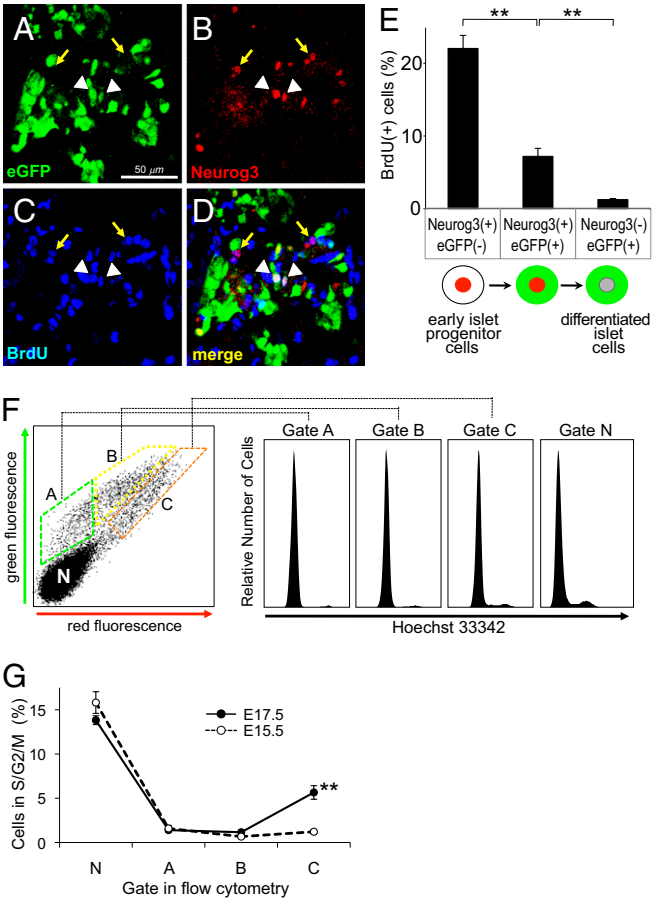

Fig. 1. Proliferation in endocrine progenitor cells. After 3 h of BrdU labeling at E15.5, pancreases were dissected from *Neurog3*$^{eGFP/+}$ embryos, and immunofluorescence costaining was performed for eGFP (green; *A* and *D*), Neurog3 (red; *B* and *D*), and BrdU (blue; *C* and *D*). Neurog3$^+$/BrdU$^+$/eGFP$^-$ and Neurog3$^+$/BrdU$^+$/eGFP$^+$ cells are denoted by yellow arrows and white arrowheads, respectively, and quantified in a bar graph (*E*). Three embryos were analyzed, and data are presented as the mean ± SEM (**$P < 0.01$) between two cell populations, by two-tailed Student's *t* test. (*F* and *G*) Dissociated cells from E15.5 and E17.5 *Neurog3-Timer* embryos were stained with the DNA-specific dye Hoechst 33342 and analyzed by flow cytometry (*F*). The percentage of proliferating cells at S/G_2/M phases at each population is shown in *G*. At least eight embryos were analyzed from three different litters for each stage, and data are presented as the mean ± SEM. **$P < 0.01$, E17.5 vs. E15.5, by two-tailed Student's *t* test.

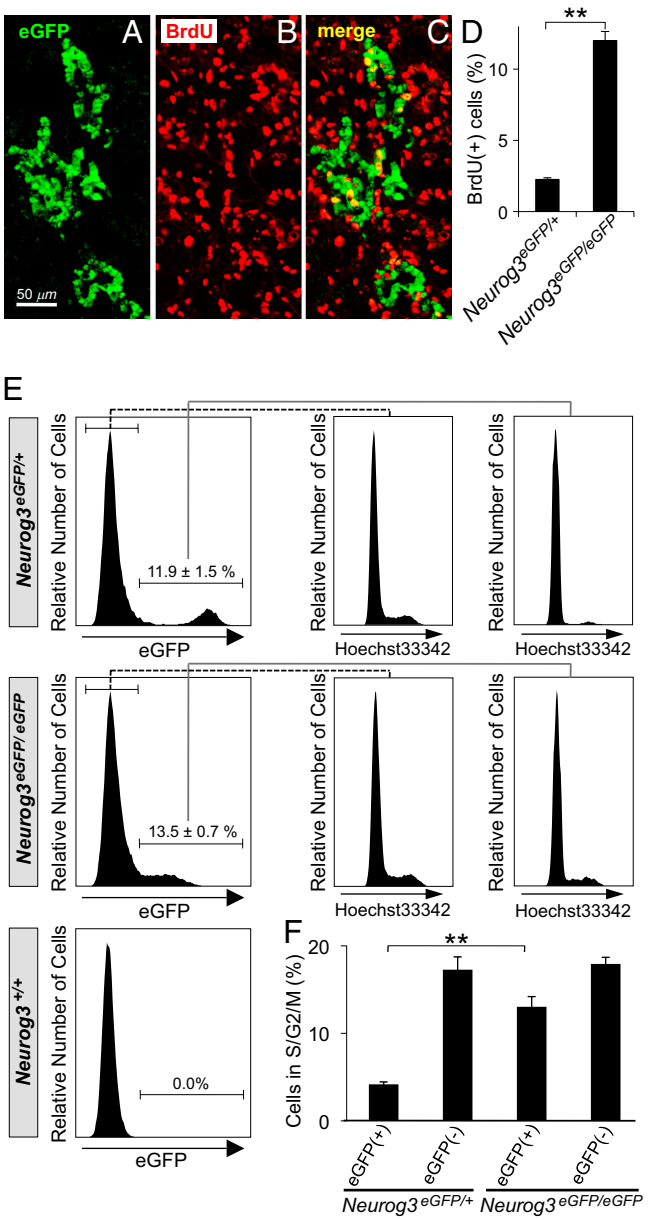

Fig. 2. Proliferation of endocrine lineage cells in Neurog3-deficient embryos. (*A*–*C*) After 3 h of BrdU labeling at E15.5, the pancreas was dissected from *Neurog3*$^{eGFP/eGFP}$ embryos, and immunofluorescence costaining was performed for eGFP (green; *A* and *C*) and BrdU (red; *B* and *C*). Costaining cells, which appear yellow in the merged panel (*C*), were quantified, expressed as percentage of total eGFP$^+$ cells, and compared with *Neurog3*$^{eGFP/+}$ pancreases in the bar graph in *D*. In *E*, pancreatic cells were dissociated from *Neurog3*$^{eGFP/+}$, *Neurog3*$^{eGFP/eGFP}$, and *Neurog3*$^{+/+}$ littermates at E14.5, stained with the DNA dye Hoechst 33342, and analyzed by flow cytometry. The percentage of eGFP$^+$ cells proliferating in S/G_2/M phases for each genotype is shown in *F*. Each data point represents the mean ± SEM of at least three independent experiments. **$P < 0.01$, *Neurog3*$^{eGFP/eGFP}$ *vs.* *Neurog3*$^{eGFP/+}$, by two-tailed Student's *t* test.

embryos at E15.5 than in those of *Neurog3*$^{eGFP/+}$ heterozygous littermates (Fig. 2 *A*–*D*). In addition, the eGFP-expressing cells of *Neurog3*$^{eGFP/eGFP}$ embryos expand within the epithelium and do not delaminate from the epithelial layer as in the *Neurog3*$^{eGFP/+}$ embryos at E15.5 (Fig. S1), although later in development some of these cells can adopt an alternate exocrine fate (18).

In flow cytometric analysis, a significantly higher fraction of eGFP-expressing cells were proliferating (in S/G_2/M phases) in *Neurog3*$^{eGFP/eGFP}$ embryos than in *Neurog3*$^{eGFP/+}$ heterozygous littermates (Fig. 2 *E* and *F*; 13.0 vs. 4.2%; $P < 0.01$). Taken together, these data demonstrate that Neurog3 is necessary for the shift to cellular quiescence that occurs when pancreatic progenitors switch to endocrine progenitors and start the process of differentiation into islet cells.

Cell Cycle Regulators Controlled by Neurog3. To gauge the expression of known cell cycle regulators in the differentiating endocrine cells, we performed TaqMan RT-PCR arrays with RNA from cells isolated by FACS from E17.5 *Neurog3-Timer* pancreases. Of the 78 cell cycle-related genes assayed, the mRNA most robustly induced in cells that recently activated Neurog3 (gate A) was *Cdkn1a*, which encodes the cyclin-dependent kinase inhibitor p21/CIP1 (Fig. 3 and Table S1). *Cdkn1a* increased 13.4-fold in gate A cells relative to the nonfluorescent cells (gate N) and then decreased again in the more mature endocrine cells in gate B and C ($P < 0.001$ by ANOVA). Moreover, the absolute expression level of *Cdkn1a* exceeded all other cell cycle-related

mRNAs in gate A cells (Fig. S2). In parallel, other negative regulators of cell cycle progression, such as *Cdkn1b* (p27/Kip1), *Trp53*, *Rb1*, and *Rbl1*, also peaked in the endocrine progenitors in gate A (Fig. 3*B*, Fig. S2, and Table S1). In contrast, many positive regulators of cell cycle progression, such as *Ccnb2*, *Ccnd2*, *Cdk6*, and *Cdc25c*, and a cellular marker of proliferation, *Mki67* (Ki67), decreased in the endocrine progenitors (Fig. S2 *C–H* and Table S1).

To determine whether these changes in gene expression require Neurog3, we used the same TaqMan arrays to assess gene expression in eGFP-positive and -negative cells sorted from *Neurog3*$^{eGFP/+}$ and *Neurog3*$^{eGFP/eGFP}$ embryos at E14.5. *Cdkn1a* levels were significantly elevated in eGFP-expressing cells from the *Neurog3*$^{eGFP/+}$ embryonic pancreases ($P < 0.001$; eGFP-expressing cells vs. nonfluorescent cells by Student's *t* test), but not from the *Neurog3*$^{eGFP/eGFP}$ pancreases (Fig. 3*A* and Table S2), indicating that *Cdkn1a* up-regulation in these cells requires Neurog3. In addition, *Cdkn1b* and *Rb1* were robustly expressed only in endocrine populations of *Neurog3*$^{eGFP/+}$, but not *Neurog3*$^{eGFP/eGFP}$ embryos (Fig. 3*B*, Fig. S2, and Table S2). The positive cell cycle regulators showed the opposite pattern (Fig. S2 and Table S2). Taken together, these data demonstrate that the changes in expression of the cell cycle regulators seen in the endocrine lineage in the embryonic pancreas require Neurog3.

Ectopic Expression of Neurog3. Normally, few Neurog3-expressing cells persist in the pancreas after birth (5, 19). Despite this waning of islet cell neogenesis, during the perinatal period, the β-cell population expands rapidly because of the rapid proliferation of the existing β-cells in both mice and humans (20, 21). During this period, starting from E16 and lasting through the first few weeks of life in mice, proliferation rates can exceed 25% per day (20). We hypothesized that the proliferation of mature endocrine cells requires down-regulation of Neurog3 and that continued expression of Neurog3 in terminally differentiated endocrine cells could inhibit cell cycle progression. To test this hypothesis, we generated a mouse line, *Rip-rtTA/TetO-Neurog3* mice, in which the drug doxycycline can be used to induce the expression of Neurog3 in mature β-cells (Fig. 4*A*). After administration of doxycycline to *Rip-rtTA/TetO-Neurog3* mice from birth (P0) to postnatal day 7 (P7), nuclear Neurog3 protein was detected in 71% of insulin-expressing cells, but was not detected in control littermates (Fig. S3). As assessed by Ki67 staining, the Neurog3-expressing β-cells proliferated at a significantly lower rate than the Neurog3-negative β-cells at P7 (Fig. 4*B*), indicating that exogenous expression of Neurog3 inhibited cell proliferation in β-cells. When doxycycline treatment of *Rip-rtTA/TetO-Neurog3* neonatal mice was extended to 5 wk of age, blood glucose levels dramatically increased over time (Fig. 4*C*). At the age of 6 wk, the islets of the doxycycline-treated *Rip-rtTA/TetO-Neurog3* mice contained markedly reduced numbers of insulin-expressing cells, and the serum insulin in the double mutant mice was below the level of detection by ELISA (Fig. S4).

To quantify the expression of cell-cycle regulators in the cells expressing transgenic Neurog3, we purified pancreatic β-cells by FACS from *Rip-rtTA/TetO-Neurog3/MIP-eGFP* triple transgenic

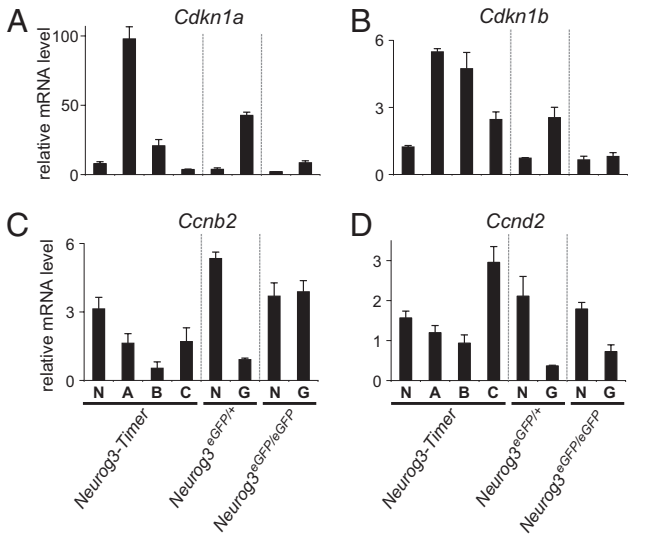

Fig. 3. Expression of cell-cycle regulators in endocrine lineage cells. Pancreases were dissected from *Neurog3-Timer* embryos at E17.5, and fluorescent cells were sorted by FACS into the four gates shown in Fig. 1*C*. In parallel, *Neurog*$^{eGFP/+}$ and *Neurog3*$^{eGFP/eGFP}$ embryos were dissected at E14.5, and GFP-positive cells (G) were separated from GFP-negative cells (N) by FACS. The sorted cell populations were analyzed by real-time RT-PCR for mRNA-encoding cell cycle-related genes, Cdkn1a (*A*), Cdkn1b (*B*), Ccnb2 (*C*), and Ccnd2 (*D*). All expression levels were normalized to glucuronidase, β (*Gusb*). Each data point represents the mean ± SEM of three independent experiments.

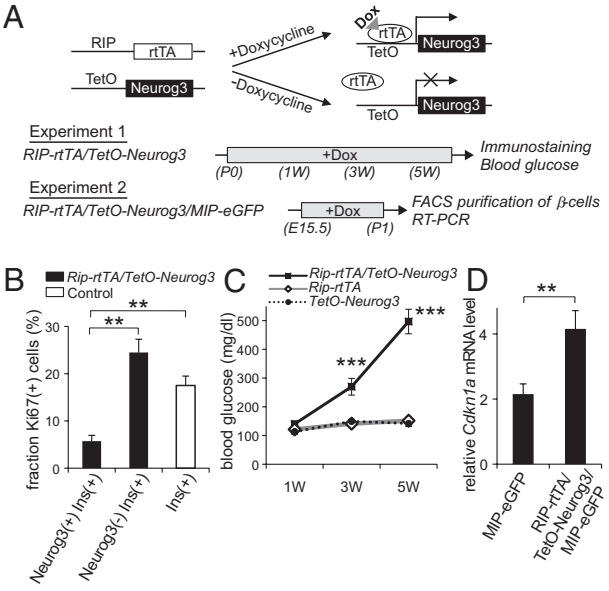

Fig. 4. Ectopic expression of Neurog3 in mature β-cells. As outlined in *A*, transgenic mice were generated with the tetracycline-regulated promoter driving *Neurog3* expression (*TetO-Neurog3* mice) and crossed with mice expressing the reverse-tetracycline receptor under the control of the rat *insulin 2* promoter (*RIP-rTtA* mice; ref. 35) to generate *Rip-rtTA/TetO-Neurog3* double transgenic mice, and these mice were crossed with mice expressing eGFP under the control of the mouse *insulin 1* promoter (*MIP-eGFP* mice; ref. 35) to generate *Rip-rtTA/TetO-Neurog3/MIP-GFP* triple transgenic mice. In experiment 1, *Rip-rtTA/TetO-Neurog3* double transgenic mice and controls (*Rip-rtTA* and *TetO-Neurog3*) received doxycycline from postnatal day 0 to 6 wk. The pancreases of some animals were harvested after 1 wk and stained for insulin, Neurog3, and the proliferation marker Ki67 (*B*). For the remaining animals, random blood glucose levels were checked every two weeks (*C*), and the pancreases were harvested at 6 wk for immunohistochemistry (Fig. S4). In experiment 2, *Rip-rtTA/TetO-Neurog3/MIP-eGFP* triple transgenic mice and *MIP-eGFP* controls (*Rip-rtTA/MIP-eGFP* and *TetO-Neurog3/MIP-eGFP*) received doxycycline from E15 for 5 d, and then on postnatal day 0, pancreases were harvested and sorted by FACS, and the levels of Cdkn1 AMRNA were analyzed by real-time RT-PCR (*D*). Each data point represents the mean ± SEM of at least three independent experiments. $^{**}P < 0.01$, Neurog3^{+}/Insulin^{+} vs. Neurog3^{-}/Insulin^{+} cells (*B*), and *MIP-eGFP* controls vs. *Rip-rtTA/TetO-Neurog3/MIP-eGFP* mice (*D*), by two-tailed Student's *t* test. $^{***}P < 0.001$ *Rip-rtTA/TetO-Neurog3* vs. control littermates by an analysis of variance (ANOVA).

mice after doxycycline treatment for 5 d between E15.5 and P1 (22) (Fig. 4*A*, Experiment 2). Measurement of mRNA levels by TaqMan RT-PCR demonstrated that the induction of transgenic Neurog3 increased the expression of *Cdkn1a* mRNA in the transgenic β-cells relative to control littermates (Fig. 4*D*). Taken together, this gain-of-function model revealed that persistent expression of Neurog3 up-regulates Cdkn1a expression, blocks β-cell proliferation, and results in severe hyperglycemia, thus demonstrating the critical importance of Neurog3 removal for the proper expansion of β-cell mass, especially during the critical perinatal period.

Role of Cdkn1a in Cell Cycle Exit. Having shown that Neurog3 induces the expression of the cell-cycle inhibitor Cdkn1, we next tested the importance of Cdkn1 in the cell-cycle exit driven by Neurog3. We generated Cdkn1a-deficient *Neurog3-Timer* reporter embryos (*Neurog3-Timer*/*Cdkn1a*$^{-/-}$) (23) and quantified proliferating cells by flow cytometry with Hoechst 33342 staining. As shown in Fig. 5*A*, the number of proliferating cells in S/G_2/M phases is significantly higher in the endocrine progenitors (gate A) of E15.5 *Cdkn1a*$^{-/-}$ embryos (5.00 ± 0.78% in gate A), compared with *Cdkn1a*$^{+/-}$ heterozygous and *Cdkn1a*$^{+/+}$ wild-type embryos (1.65 ± 0.46 and 1.35 ± 0.26% in gate A, respectively). In the *Cdkn1a*$^{-/-}$ embryos, a higher fraction of the cells in gate B also were detected in S/G_2/M phase, but the more differentiated cells in gate C showed no significant difference in proliferation rates between *Cdkn1a*$^{-/-}$ and wild-type embryos. These data demonstrate that Cdkn1a plays an essential role in promoting cell cycle exit in endocrine progenitors downstream of Neurog3.

Neurog3 Directly Binds to the Regulatory Region of the Cdkn1a Gene. To investigate whether Neurog3 directly regulates transcription of the *Cdkn1a* gene in pancreatic cells, chromatin immunoprecipitation (ChIP) assay was performed in mouse pancreatic mPAC cells transfected with FLAG-tagged Neurog3 (Fig. S5). Within regulatory sequences upstream of the first exon of *Cdkn1a*, a 0.5-kb fragment between −2227 bp and −1691 bp containing an E-box was immunoprecipitated by the anti–FLAG-tag antibody. A highly conserved E-box was also identified at −39 Kb, and ChIP confirmed binding of Neurog3 to this site in mPAC cells. Taken together, these data show that Neurog3 directly controls the expression of the *Cdkn1a* gene by binding to upstream regulatory regions of the gene.

MicroRNAs in the Cell Cycle Control. MicroRNAs miRNA-291a-3p, miRNA-291b-3p, miRNA-294, miRNA-295, and miRNA-302d promote the G_1-S transition by directly targeting the 3′ UTR of the *Cdkn1a* mRNA and inhibiting translation (24). We asked whether these miRNAs might provide further regulation of *Cdkn1a* levels in differentiating endocrine cells in addition to the fall in *Cdkn1a* mRNA levels that occurs as new endocrine progenitor cells (gate A) differentiate (gates B and C) and reenter the cell cycle at E17.5 (Fig. 3*A*). Therefore, we measured the expression levels of these miRNAs in cells purified by FACS from pancreases of E17.5 *Neurog3-Timer* embryos and found that three of the miRNAs that target *Cdkn1a*, miRNA-294, miRNA-295, and miRNA-302d, were significantly enriched in differentiated endocrine cells (Fig. 5 *B–E*). Because the increase in proliferating rate (Fig. 1*G*) parallels the increase of these miRNAs in mature endocrine cells in area C (Fig. 5 *B–D*), these miRNAs may further inhibit the expression of Cdkn1a at the level of translation and, thereby, contribute to the reactivation of the cell cycle in mature endocrine cells (Fig. 5*F*).

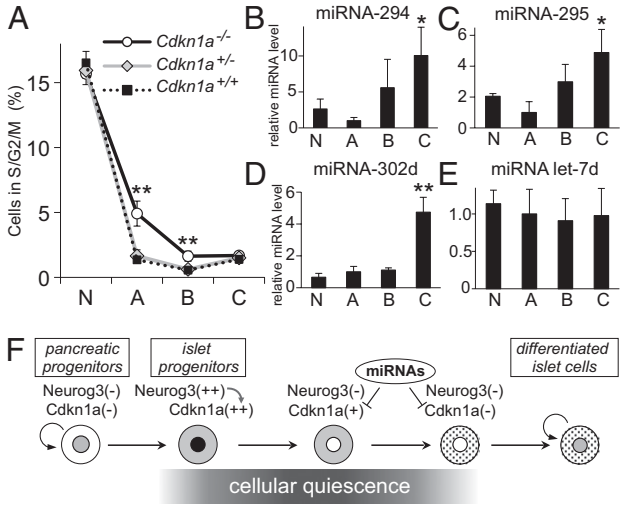

Fig. 5. Function and regulation of Cdkn1a in the pancreas. Pancreatic cells from *Neurog3-Timer* embryos with the genotypes shown were dissociated at E15.5, stained with the DNA dye Hoechst 33342, and analyzed by flow cytometry. The percentage of cells proliferating in S/G_2/M phases is shown in *A* for each of the four gates from Fig. 1C. Data are presented as the mean ± SEM of at least five independent experiments. $^{**}P < 0.01$, *Cdkn1a*$^{-/-}$ knockout *vs.* *Cdkn1a*$^{+/-}$ heterozygous or *Cdkn1a*$^{+/+}$ wild-type embryos, by an analysis of variance (ANOVA). In *B–E*, dissociated cells from pancreases from *Neurog3-Timer* embryos at E17.5 were sorted by FACS into the four gates shown in Fig. 1C. Expression levels of *miRNA-294* (*B*), *miRNA-295* (*C*), and *miRNA-302d* (*D*), and control miRNA *Let7d* were determined by TaqMan miRNA assay, normalized to snoRNA-202, and shown relative to the level in gate A. Each data point represents the mean ± SEM of at least three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. gate A, by two-tailed Student's *t* test. *F* shows a proposed model for cell cycle regulation in the pancreatic endocrine lineage.

Discussion

In these studies, several lines of evidence demonstrate that multipotent pancreatic progenitor cells exit the cell cycle when they become endocrine progenitor cells, and Neurog3 drives this exit. Although prior studies demonstrated that endocrine cells exit the cell cycle in the developing pancreas before E16 (6, 19), we used a combination of time-restricted BrdU labeling, Neurog3 staining, and transgene markers with different temporal expression patterns to demonstrate that Neurog3 expression initiates in rapidly cycling pancreatic progenitor cells, but these cells quickly exit the cell cycle while still expressing Neurog3. These cells initially remain quiescent after Neurog3 expression wanes, but late in fetal development, they renter the cell cycle.

The timing of cell cycle exit shortly after the initiation and during the peak of Neurog3 expression suggests that Neurog3 itself may force the endocrine progenitor cells out of the cell cycle, and the results with Neurog3-deficient embryos support this model. The eGFP-expressing cells in *Neurog3*$^{eGFP/eGFP}$ knockout embryos are programmed to transcribe the *Neurog3* gene but never produce Neurog3 protein. In the *Neurog3*$^{eGFP/+}$ heterozygous embryos, the GFP^{+} cells exit the cell cycle, but in the *Neurog3*$^{eGFP/eGFP}$ embryos that lack Neurog3 protein, these progenitor cells continue to proliferate within epithelium, demonstrating that cell cycle exit requires Neurog3 (Fig. 2 and Fig. S1).

In addition, data from the *Rip-rtTA*/*TetO-Neurog3* mouse model demonstrate that Neurog3 expression alone is sufficient to drive endocrine cells to exit the cell cycle. Normally, endocrine progenitor cells only express Neurog3 briefly and rapidly reduce its expression before mature endocrine gene expression commences (4–7). Our data demonstrate the critical importance of this down-regulation because it permits the mature endocrine cells to reenter the cell cycle.

Transcriptome analysis for cell cycle-related genes in the Neurog3-expressing endocrine progenitor cells revealed sharp increases of several negative regulators of cell cycle progression (Fig. 3). Among these genes, *Cdkn1a* exhibited both the largest increase relative to the non-Neurog3–expressing cells, and the

highest absolute expression level of any of the cell cycle regulators assessed in the endocrine progenitors. This up-regulation of *Cdkn1a* depended completely on Neurog3, because no increase was detected in the eGFP-expressing cells sorted from *Neurog3*$^{eGFP/eGFP}$ embryos. In addition, expression of Neurog3 in mature β-cell in the *Rip-rtTA/TetO-Neurog3* transgenic mice induced the expression of *Cdkn1a*, but not the other negative regulators of cell cycle progression (Fig. S6).

These results strongly suggest that Cdkn1a functions downstream of Neurog3. Cdkn1a can function either as a downstream target of p53 (encoded by *Trp53* in mice) or independently of p53 to inhibit cell cycle progression (23, 25). Because the up-regulation of Cdkn1a in the *Rip-rtTA/TetO-Neurog3* transgenic mice did not correlate with the expression level of p53, we conclude that Neurog3 may induce Cdkn1a expression independently of p53.

Neurog3 activates the expression of both Cdkn1a and key factors required for endocrine differentiation, such as Pax4, Nkx2.2, NeuroD1, and Rfx6 (26–28), which suggests that cell cycle exit may play a role in differentiation. We saw small increases in some of the mRNA encoding islet hormones in the *Cdkn1a*$^{-/-}$ pancreases (Fig. S7), but these changes could simply result from the increase in proliferation of the progenitors. Expression levels of transcriptions factors Neurod1, Pax4, and Rfx6 did not change in the *Cdkn1a*$^{-/-}$ pancreases (Fig. S7), suggesting that the lack of Cdkn1a does not have a large impact on differentiation. However, the release of the block in proliferation was not complete in the *Cdkn1a*$^{-/-}$ progenitor cells, probably because other cell cycle inhibitors such as Cdkn1b remain, and this redundancy may prevent any deficiency in differentiation.

During normal development in both rodents and humans, β-cell proliferation increases dramatically during the perinatal period (21, 29), establishing the size of the β-cell population and the risk of diabetes. Our data demonstrate that without normal down-regulation of Neurog3, the β-cell population does not adequately expand and diabetes ensues. Interestingly, however, a very low level of Neurog3 expression persists in adult islets (30–33). Recent studies demonstrated that this sustained low-level expression of Neurog3 contributes to endocrine function (33). Considering these data together, we conclude that sustained, but tightly regulated, expression of Neurog3 at a very low level permits the proper expansion of endocrine cells as well as their normal function. Ultimately, this balance plays a critical role in determining the size of the β-cell population, the functional structure of the mature pancreas, and the risk of diabetes.

Experimental Procedures

Animal Experiments. Mice were housed on a 12-h light–dark cycle in a controlled climate in the University of San Francisco vivarium. Timed matings were carried out with E0.5 being set as midday of the day of discovery of a vaginal plug. The UCSF Institutional Animal Care and Use Committee approved all studies involving mice.

The generation of the *Neurog3-Timer* mice was described (13). *Neurog3*$^{eGFP+}$ knock-in mice (12) were obtained through the Mutant Mouse Regional Resource Centers (University of California, Davis, CA). The *TetO-Neurog3* transgenic mouse line was generated by standard methods using DNA generated by inserting the mouse Neurog3 cDNA into pTRE2 (Clontech). *Rip-rtTA* (29) and *Cdkn1a*$^{-/-}$ (23) mice were purchased from the Jackson Laboratory. Rip-*rtTA/TetO-Neurog3* double transgenic mice and their littermates were administered chow with added doxycycline or standard chow. *MIP-eGFP* mice were obtained from M. Hara (University of Chicago) (22).

Immunostaining. Tissues were fixed in 4% paraformaldehyde in PBS at 4 °C, washed in PBS, immersed in sucrose solution, and embedded and frozen in Tissue-Tek (OCT Compound; Sakura). Sections were stained with antibodies against GFP (rabbit, 1:500; MBL International), BrdU (rat, 1:200, Serotec), Ki67 (mouse, 1:50; BD Pharmingen), Neurog3 (guinea pig, 1:1,000; made in our laboratory), insulin (guinea pig, 1:2,000; Millipore), glucagon (rabbit, 1:2,000; Millipore), E-cadherin (rat, 1:200; Abcam), laminin (rabbit, 1:1,000; Sigma), and cleaved caspase-3 (rabbit, 1:200; Cell Signaling). For BrdU experiments, BrdU (Sigma; 100 mg/kg of body weight) was injected into pregnant mice 3 h before killing, and tissues were pretreated with 1 M HCl for 30 min at room temperature before immunofluorescence. Slides were imaged on Leica SP2 AOBS confocal laser scanning microscope.

Pancreatic Cell Dispersion and Flow Cytometry. After manual dissection, pancreases were treated with 0.05% trypsin/0.53 mM EDTA (Invitrogen) at 37 °C for 5 min, and the digestion was inactivated by addition of FBS. For cell cycle analysis, the dissociated cells were incubated with medium containing 5 μg/mL Hoechst 33342 dye at 37 °C for 60 min, washed with cold PBS, then analyzed by using an LSRII flow cytometer (PerkinElmer). For sorting the fluorescent cells, a MoFlo cell sorter was used (Dako Cytomation). Dead cells were excluded with DNA dye TO-PRO-3 (Molecular Probes).

Real-Time PCR. Total RNA was extracted from FACS-sorted cells, linearly amplified, and converted into cDNA with the NuGEN WT-Ovation RNA Amplification system (NuGen) as described (13). Individual cDNAs were quantified by real-time PCR using a TaqMan Low Density array system (Applied Biosystems), designed for cell cycle-related genes (Table S1 and Table S2). Gene expression levels of the assayed genes were normalized to the expression levels of *glucuronidase,* β (*Gusb*). The mean values and SE are shown in Table S1 and Table S2. For quantitative miRNA assays, extracted RNA was converted into cDNA, amplified, and quantified with TaqMan miRNA assays according to the manufacturer's protocol (Applied Biosystems). The expression levels of miRNAs were normalized to *snoRNA202*, and the relative level of miRNAs from each gate was determined with respect to the value in nonfluorescent cells (gate A).

Chromatin Immunoprecipitation (ChIP). ChIP assays were performed as described (34) Mouse pancreatic mPAC cells were transiently transfected with plasmid vectors expressing eGFP or FLAG-tagged human NEUROG3 by using Lipofectamine PLUS reagent (Invitrogen). Cells were harvested 48 h after transfection. Proteins were cross-linked to DNA with 1% formaldehyde for 10 min at 37 °C. The cells were washed and lysed with SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris at pH 8.1). Chromatin complexes were immunoprecipitated overnight with 5 μg of preimmune serum or antibody specific for FLAG at 4 °C, and pulled down by using protein A magnetic beads (Invitrogen). Putative binding regions were amplified from precipitated DNA fragments by PCR. The Neurog3 binding site in Pax4 gene was used as a positive control (27), and the Tph1 gene promoter was used as a negative control. PCR primer sequences are available upon request.

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