

# Sustained *Neurog3* expression in hormone-expressing islet cells is required for endocrine maturation and function

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**Neurog3 (Neurogenin 3 or Ngn3) is both necessary and sufficient to induce endocrine islet cell differentiation from embryonic pancreatic progenitors. Since robust *Neurog3* expression has not been detected in hormone-expressing cells, *Neurog3* is used as an endocrine progenitor marker and regarded as dispensable for the function of differentiated islet cells. Here we used 3 independent lines of *Neurog3* knock-in reporter mice and mRNA/protein-based assays to examine *Neurog3* expression in hormone-expressing islet cells. *Neurog3* mRNA and protein are detected in hormone-producing cells at both embryonic and adult stages. Significantly, inactivating *Neurog3* in insulin-expressing  $\beta$  cells at embryonic stages or in *Pdx1*-expressing islet cells in adults impairs endocrine function, a phenotype that is accompanied by reduced expression of several *Neurog3* target genes that are essential for islet cell differentiation, maturation, and function. These findings demonstrate that *Neurog3* is required not only for initiating endocrine cell differentiation, but also for promoting islet cell maturation and maintaining islet function.**

endocrine progenitor | maintenance | pancreas | diabetes | sugar metabolism

It is well established that the basic helix–loop–helix transcription factor *Neurog3* has an essential role in pancreatic endocrine cell differentiation. All such endocrine islet cells are derived from *Neurog3*<sup>+</sup> (positive) precursors (1, 2) and *Neurog3* deficiency virtually abolishes islet cell differentiation (3, 4). Moreover, ectopic *Neurog3* expression converts early endodermal progenitor cells into endocrine islet cells (5–8), and *Neurog3* controls the expression of multiple genes that influence both endocrine differentiation and function (3, 9, 10). Because *Neurog3* has not been detected in differentiated islet cells, its expression in the adult pancreas is proposed as a marker for putative endocrine progenitors (2, 11).

Contradictory findings exist regarding *Neurog3* expression in the adult pancreas. Several reports have shown *Neurog3* expression in WT adult islet cells (2, 12–14), and this expression is enhanced by regenerative conditions (11–13). Yet these analyses have failed to establish whether *Neurog3* expression is restricted to only a few putative endocrine progenitor cells at a high level, or whether *Neurog3* is also present in differentiated islet cells at a low level. Nor is it clear how the sustained *Neurog3* expression impacts endocrine function. Here we have used a combination of knock-in reporter mice, immunoassays, and loss-of-gene-function studies to show that differentiated hormone<sup>+</sup> islet cells continue to express *Neurog3*, and that *Neurog3* is important for both islet cell production and function.

## Results

### Knock-In Reporter Mice Reveal *Neurog3* Expression in Adult Islet Cells.

Three independent *Neurog3* knock-in mice were used to examine *Neurog3* expression in the adult pancreas (Fig. 1A). In

*Neurog3*<sup>tTA</sup>, an IRES-tTA-PolyA cassette replaced the endogenous *Neurog3* coding sequences (Fig. S1A and B). Production of tTA, as a surrogate of *Neurog3* expression, was examined with the strictly tTA-dependent reporter line, *TetO*<sup>LacZ</sup> (15, 16). At embryonic days (E) 12.5 and 16.5 and postnatal day 7, and in the absence of doxycycline (Dox; the presence of which inhibits tTA activity), *Neurog3*<sup>tTA</sup>;*TetO*<sup>LacZ</sup> pancreata expressed *LacZ* in a subset of pancreatic cells reminiscent of *Neurog3*-expressing cells or their descendants (Fig. S1C and D), suggesting that expression of the *Neurog3*<sup>tTA</sup> allele faithfully recapitulates that of endogenous *Neurog3*.

The pancreata of 9-week-old *Neurog3*<sup>tTA</sup>;*TetO*<sup>LacZ</sup> animals were stained for  $\beta$ -galactosidase ( $\beta$ -Gal) expression in the absence of Dox. Large proportions of islet cells expressed robust levels of  $\beta$ -Gal (Fig. 1B). Because  $\beta$ -Gal has a half-life of  $\approx$ 30 h in mammalian cells (17), and islet cells are constantly dividing, the detected  $\beta$ -Gal molecules were likely produced within a short period before our assay instead of the residual protein being made during embryogenesis. Indeed, when *Neurog3*<sup>tTA</sup>;*TetO*<sup>LacZ</sup> animals were treated with Dox until 1 week of age (to repress *LacZ* expression during embryogenesis and first week of postnatal life), a large number of islet cells were found to activate *LacZ* expression at 8 weeks (Fig. S1E), demonstrating that  $\beta$ -Gal could be expressed in islet cells 1 week after birth in the *Neurog3*<sup>tTA</sup>;*TetO*<sup>LacZ</sup> animals. We examined a large number of tissue sections from pancreata of 9-week-old *Neurog3*<sup>tTA</sup>;*TetO*<sup>LacZ</sup> animals and did not detect any exocrine cells with  $\beta$ -Gal.

The above findings were verified by using a *Neurog3*<sup>CreERT</sup> allele in which the 5' 150 base pairs of the *Neurog3* coding region were replaced by *CreERT* cDNA (4). *CreERT* remains cytoplasmic and inactive, and unable to recombine LoxP sites in the absence of tamoxifen (TM). The conditional *R26R*<sup>EYFP</sup> (18) reporter line was used to monitor for the presence of *CreERT*. In *R26R*<sup>EYFP</sup> mice, enhanced yellow fluorescent protein (EYFP) is ubiquitously expressed under *Rosa26* promoter control, but in a strictly Cre-dependent manner. In *Neurog3*<sup>CreERT</sup>;*R26R*<sup>EYFP</sup> adult mice, no pancreatic cells expressed EYFP without TM (6). Seven days after the administration of TM to 7-week-old adult

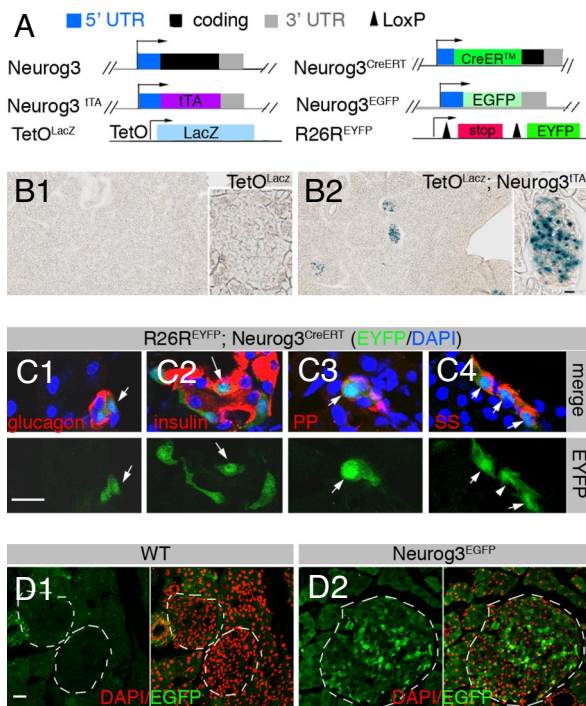
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The authors declare no conflict of interest.

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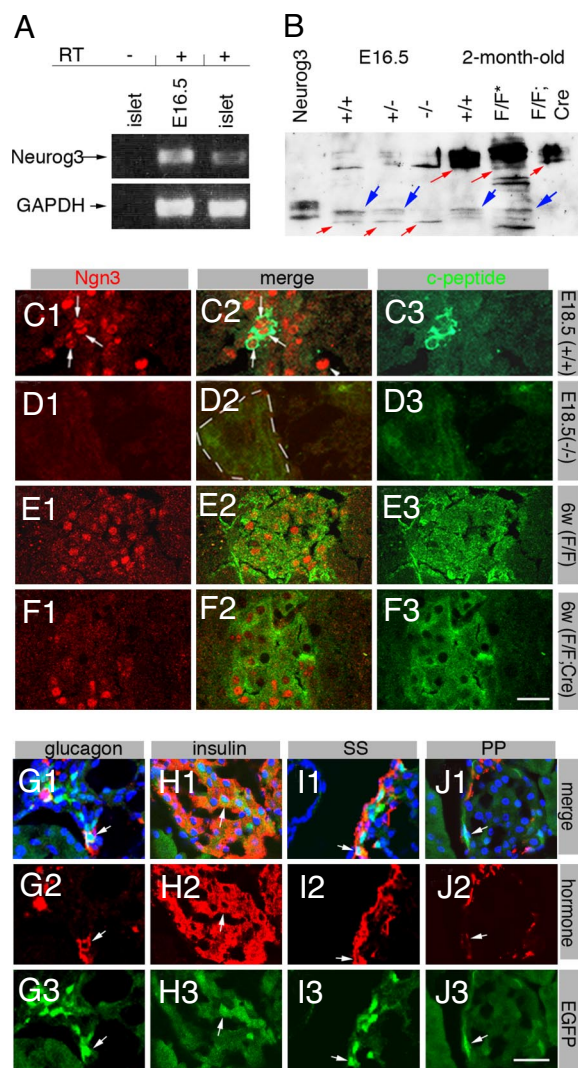


**Fig. 1.** Knock-in reporter mice reveal *Neurog3* expression in adult islet cells. (A) Knock-in and reporter alleles. *TetO<sup>LacZ</sup>* and *R26R<sup>EYFP</sup>* are reporter alleles of tTA and Cre, respectively. (B)  $\beta$ -Gal expression in 9-week-old *TetO<sup>LacZ</sup>* (i) and *TetO<sup>LacZ</sup>;**Neurog3<sup>tTA</sup>* (ii) pancreata. (Insets) Islets at a higher magnification. (C) Coexpression of endocrine hormones (red) with EYFP in 8-week-old *R26R<sup>EYFP</sup>;**Neurog3<sup>CreERT</sup>* males that received TM at 7 weeks of age. Two panels (as a column) for each staining are shown: a merged image of EYFP (green), hormone (red), and DAPI (blue) signal, and a single channel of EYFP. Arrows indicate EYFP<sup>+</sup>hormone<sup>+</sup> cells. (D) EGFP recognized by a rabbit anti-EGFP antibody in 6-month-old *Neurog3<sup>EGFP</sup>* pancreata. Only EGFP alone and EGFP-DAPI merged images are shown. (Scale bars, 20  $\mu$ m.)

*Neurog3<sup>CreERT</sup>;**R26R<sup>EYFP</sup>* mice, up to 4.5% of the 4 major islet cell types expressed EYFP (Fig. 1C).

The above findings led us to examine enhanced green fluorescent protein (EGFP) expression in the pancreata of *Neurog3<sup>EGFP</sup>* knock-in mice (19), a line in which EGFP expression was reportedly absent in the adult pancreas (13, 20). By using confocal microscopy, weak yet visible EGFP fluorescence (enriched in nuclei) was seen in a large number of islet cells from *Neurog3<sup>EGFP</sup>* animals at 2, 4, and 6 months of age (Fig. S2). A rabbit anti-EGFP antibody further verified the presence of EGFP in a large portion of adult islet cells (Fig. 1D). Notably, no exocrine cells were found to express EGFP (Fig. S2). Not all islet cells exhibit EGFP signals. It is not clear whether this lack of EGFP signal in all islet cells is due to limited EGFP detection sensitivity or variation in *Neurog3* expression between different islet cells.

***Neurog3* mRNA and Protein can be Detected in Hormone-Expressing Islet Cells.** The above analyses demonstrate that *Neurog3* expression is maintained in the adult pancreas, albeit at low levels, and with the caveat that all 3 knock-in alleles studied inactivate *Neurog3* and thus may exhibit a *Neurog3* haploinsufficiency phenotype. Additionally, because there could be a time-lag between *Neurog3* activation (as represented by CreERT or tTA expression) and the EYFP and  $\beta$ -Gal production, it is not clear whether *Neurog3* expression is restricted to differentiated islet cells or putative islet progenitors (which express *Neurog3* and quickly relocalize to the islets) or both. For this reason, we sought to directly examine the expression of *Neurog3* in WT adult islet



**Fig. 2.** Differentiated islet cells express *Neurog3*. (A) RT-PCR detection of *Neurog3* mRNA in 2-month-old WT (+/+) islets and E16.5 embryonic pancreas. GAPDH expression was used as RT control. (B) Western blot detection of Neurog3. Recombinant Neurog3 produced in HEK293 cells, wild-type (+/+), and *Neurog3<sup>-/-</sup>* E16.5 embryonic pancreata were used as positive controls. E16.5 *Neurog3<sup>-/-</sup>* (-/-) total pancreas and 2-month-old *Neurog3<sup>F/F</sup>;**Ins2<sup>Cre</sup>* (F/F; Cre) islets were used to verify the specificity of the Neurog3 antibodies. All pancreatic samples except the *Neurog3<sup>F/F</sup>* lane (F/F, labeled with \*) were nuclear extract. Note the presence of the nonspecific bands (red arrows), which serve as internal loading controls of total proteins. The Neurog3 proteins were marked with blue arrows. (C–F) IF detection of Neurog3 in embryonic (E18.5) and 6-week-old pancreata. C-peptide costaining localizes the endocrine compartment. Also note that the *Neurog3<sup>H1</sup>* cells in Cii (arrowheads) do not express insulin c-peptide, but some *Neurog3<sup>lo</sup>* cells do (white arrows). Broken lines in Dii show a duct. (G–J) Coexpression of *Neurog3<sup>EGFP</sup>* with endocrine hormones in three-month-old pancreas. Note that all 4 major islet cell types express detectable EGFP. White arrows point to examples of EGFP<sup>+</sup>hormone<sup>+</sup> cells. In the “merge” channel, DAPI signal (blue) is included to show the enriched EGFP presence in nuclei. (Scale bars, 20  $\mu$ m.)

cells by using RT-PCR, protein blot, and immunofluorescence (IF) methods in 2-month-old mice.

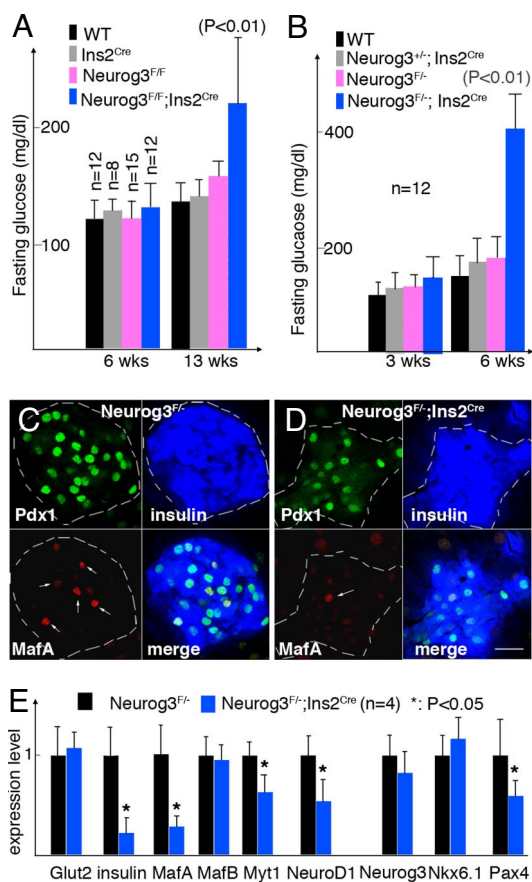
Adult islets were isolated and analyzed for *Neurog3* transcription. Consistent with published findings (12, 13), *Neurog3* transcripts were readily detected by RT-PCR in the WT adult islet cells (Fig. 2A).

When blotted with a guinea pig anti-Neurog3 or a rabbit

anti-Neurog3 antibody (2, 21), multiple protein bands were detected in islet nuclear extract (Fig. 2B and Fig. S3A). The mobility of the protein closely matches that of recombinant Neurog3 produced in HEK293 cells and Neurog3 in E15.5 or E16.5 WT pancreatic buds. Importantly, the putative Neurog3 bands were absent in the *Neurog3*<sup>-/-</sup> pancreas and substantially reduced in the islets of *Neurog3*<sup>F/F</sup>;*Ins2*<sup>Cre</sup> adults, where *Neurog3* should be inactivated in most, if not all,  $\beta$  cells (see below). Consequently, preabsorbing the Neurog3 antibodies by using Neurog3 produced in HEK293 cells caused a specific block in the detection of the Neurog3 signal (Fig. S3B). Interestingly, Neurog3 produced in transfected cells resolved into 4 major bands, whereas the putative Neurog3 in isolated islets and in embryonic pancreases appeared as 3 species (Fig. 2B). Although the presence of multiple Ngn3 species suggests the possibility of posttranslational modification of Neurog3, neither the basis nor implications of this finding are currently known.

IF-based assays on mildly fixed pancreatic cryosections (see *Materials and Methods*) using a guinea pig anti-Neurog3 antibody showed that a substantial number of hormone-expressing cells express *Neurog3* at E18.5 and in the adult pancreas (Fig. 2C and E). Notably, 2 types of *Neurog3*<sup>+</sup> cells were found in the E18.5 pancreas. A small population of cells ( $\approx 2$ –5% of all endocrine clusters) produces a relatively higher level of *Neurog3* based on their brighter IF signal (Fig. 2C). These cells do not express detectable *insulin* c-peptide or other islet hormones. A majority of cells express a low level of *Neurog3* and they coexpress hormones. As expected, the *Neurog3*<sup>-/-</sup> null pancreas showed no *Neurog3* signal in any cells, verifying the specificity of the guinea pig anti-Neurog3 antibody (Fig. 2D). Similar to E18.5, large portions of islet cells in WT, *Neurog3*<sup>F/F</sup>, and *Ins2*<sup>Cre</sup> adult pancreata express *Neurog3* (Fig. 2E). In some cells the *Neurog3* nuclear signal appears higher than in others, yet we could not find any cells that express *Neurog3* at a level as high as that in embryonic endocrine progenitors. We verified the *Neurog3* signal by inactivating *Neurog3* in *Neurog3*<sup>F/F</sup>;*Ins2*<sup>Cre</sup> animals. This arrangement allows  $\beta$  cells to be generated, yet not produce *Neurog3*. Indeed, the number of *Neurog3*<sup>+</sup> islet cells was largely reduced, although some *Neurog3*<sup>+</sup> cells remained at the periphery of islets (presumably non- $\beta$  cells, or  $\beta$  cells escaping recombination) (Fig. 2F). Because of the light fixation, endocrine hormone signals appeared diffuse, and we could not identify the exact islet cell type(s) that express *Neurog3* by using the guinea pig antibody. We also examined *Neurog3* production in the adult islet cells by using a mouse monoclonal *Neurog3* antibody (22) and a rabbit anti-*Neurog3* (2). Both antibodies produced high background in lightly fixed postnatal islets, making it impossible to discern whether a true *Neurog3* signal exists.

**Neurog3 Expression Is Maintained in All Islet Cell Types.** To understand the potential functional significance of *Neurog3* expression in hormone<sup>+</sup> cells, we determined which islet cell type(s) maintain *Neurog3* expression, and we chose EGFP production as a marker for *Neurog3* expression. Because EGFP has a half-life of  $\approx 26$  h in mouse cells (23), detectable EGFP is unlikely to survive beyond 2 months after birth if *Neurog3*<sup>EGFP</sup> expression is turned off after birth. EGFP can survive harsh fixation, which is required for clear cellular resolution hormone detection. In the 2- and 6-month-old pancreata, double immuno-IF assays showed that all islet cell types maintain *Neurog3* expression (Fig. 2G–J), suggesting that *Neurog3* may have a general role for endocrine cell maintenance, either for survival or function. We also examined whether any hormone<sup>-</sup> (negative) cells express *Neurog3*, which may potentially represent adult islet progenitor cells. Only rarely do we find EGFP<sup>+</sup> cells remain hormone<sup>-</sup> (<1% of total EGFP<sup>+</sup> cells; see Fig. S4). At present, we do not know whether these EGFP<sup>+</sup>hormone<sup>-</sup> cells are putative endocrine progenitors.



**Fig. 3.**  $\beta$ -cell-specific *Neurog3* inactivation impairs endocrine function and endocrine gene expression. (A and B) Fasting blood glucose levels in males. The genotypes and ages of animals are labeled. "P" is calculated between the *Neurog3*<sup>F/F</sup> and *Neurog3*<sup>F/F</sup>;*Ins2*<sup>Cre</sup> mice in A and *Neurog3*<sup>F/F</sup> and *Neurog3*<sup>F/F</sup>;*Ins2*<sup>Cre</sup> animals in B. (C and D) Insulin, MafA, and Pdx1 expression in E18.5 *Neurog3*<sup>F/F</sup> and *Neurog3*<sup>F/F</sup>;*Ins2*<sup>Cre</sup> pancreata. Three single channels and a merged image are presented. Note the percentage of islet cells (Pdx1<sup>+</sup>) that express *MafA* (arrows). (Scale bar, 20  $\mu$ m.) (E) QRT-PCR analysis of the expression of several genes in *Neurog3*<sup>F/F</sup> and *Neurog3*<sup>F/F</sup>;*Ins2*<sup>Cre</sup> pancreata (E18.5).

**Neurog3 Plays a Functional Role in Newly Born  $\beta$  Cells.** We next sought to determine whether *Neurog3* expression in differentiated  $\beta$  cells plays a role in islet cells. *Neurog3* was efficiently inactivated in newly born insulin<sup>+</sup> cells in *Neurog3*<sup>F/F</sup>;*Ins2*<sup>Cre</sup> animals (Fig. 2F) via Cre recombination, which we verified to be restricted to insulin-producing  $\beta$  cells (24) (Fig. S5). WT, *Ins2*<sup>Cre</sup>, and *Neurog3*<sup>F/F</sup> animals were used as controls. The fasting blood glucose levels of these 4 groups of animals showed no significant variation at 6 weeks of age (Fig. 3A). By 13 weeks after birth, male *Neurog3*<sup>F/F</sup>;*Ins2*<sup>Cre</sup> animals displayed significantly higher fasting blood glucose levels than the control groups (Fig. 3A), suggesting that loss of *Neurog3* in  $\beta$  cells compromises islet function. These findings demonstrate that *Neurog3* expression in insulin-producing  $\beta$  cells affects overall/global glucose homeostasis, which was most clearly evident in male animals. Male mice were chosen because they tend to be more sensitive to attenuation of endocrine function (25).

During our analysis of *Neurog3* dosage (WT versus *Neurog3*<sup>+/-</sup>) on islet cell differentiation, we found that although glucose homeostasis in *Neurog3*<sup>F/F</sup> animals was normal, *Neurog3*<sup>+/-</sup> and *Neurog3*<sup>F/F</sup> adult animals displayed significantly impaired glucose tolerance. Thus, *Neurog3*<sup>F/F</sup> animals provide a sensitized background to examine the consequences of *Neurog3*

deficiency in  $\beta$  cells. Indeed, six-week-old *Neurog3<sup>F1/-</sup>;Ins2<sup>Cre</sup>* animals, in this case both males and females, displayed significantly higher fasting blood glucose levels compared with *Neurog3<sup>+/-</sup>;Ins2<sup>Cre</sup>* and *Neurog3<sup>F1/-</sup>* control animals (Fig. 3B). These findings further demonstrate that *Neurog3* expression in  $\beta$  cells contributes to endocrine function.

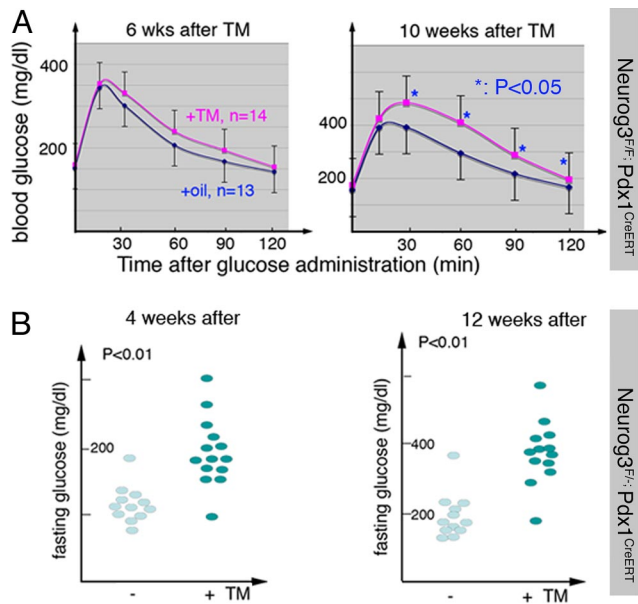
We also examined whether *Neurog3* deficiency in insulin<sup>+</sup> cells affects the expression of *insulin*, *Glut2*, *Myt1*, *MafA*, *MafB*, *NeuroD1*, *Nkx6.1*, *Pax4*, and *Pdx1* in E18.5 embryonic pancreata. These genes were chosen because their expression depends on *Neurog3* and they are known to play roles in endocrine cell differentiation and function. E18.5 embryos were selected to examine the primary effect of *Neurog3* inactivation before the pancreatic function was required for postnatal life, which may feed back, resulting in secondary effects on expression of these genes.

We first scrutinized possible *Ins2<sup>Cre</sup>* toxicity to  $\beta$  cells. E18.5 pancreata of *Neurog3<sup>+/-</sup>* and *Neurog3<sup>+/-</sup>;Ins2<sup>Cre</sup>* animals were collected for section-based immunoassays or quantitative RT-PCR (QRT-PCR). IF staining and QRT-PCR results showed that the presence of Cre does not significantly affect the transcription of the above genes (Fig. S6A, B, and J), demonstrating the lack of overt Cre toxicity on gene expression in  $\beta$  cells at this stage.

Protein production of the above genes in E18.5 *Neurog3<sup>F1/-</sup>* and *Neurog3<sup>F1/-</sup>;Ins2<sup>Cre</sup>* pancreata was analyzed. Qualitative evaluation from side-by-side IF staining suggested that the levels of insulin, glucagon, *Glut2*, *MafB*, *Myt1*, *Nkx6.1* and *Pdx1*, on a per-cell basis, were not appreciably affected by loss of *Neurog3* in insulin-expressing  $\beta$  cells (Fig. S6C–H), whereas *MafA* expression was considerably reduced in the *Neurog3<sup>F1/-</sup>;Ins2<sup>Cre</sup>* pancreas compared with controls (Fig. 3C and D). *NeuroD1* and *Pax4* were not examined because of the lack of suitable, well-characterized antibodies.

QRT-PCR assays showed that *Glut2* and *MafB* mRNA levels were not significantly affected by *Neurog3* inactivation in  $\beta$  cells. Yet the mRNA levels of *insulin*, *MafA*, *Myt1*, *NeuroD1*, and *Pax4*, all of which are required for  $\beta$  cell differentiation/maturation, in *Neurog3<sup>F1/-</sup>;Ins2<sup>Cre</sup>* pancreas, were significantly reduced compared with the *Neurog3<sup>F1/-</sup>* pancreas (Fig. 3E). We could not detect *Neurog3* mRNA reduction at this stage (Fig. 3E), possibly due to the presence of a significant number of *Neurog3<sup>+</sup>* progenitor cells whose high *Neurog3* expression could not be inactivated by *Ins2<sup>Cre</sup>*. Because we did not detect significant  $\beta$  cell mass variation between *Neurog3<sup>F1/-</sup>;Ins2<sup>Cre</sup>* and *Neurog3<sup>F1/-</sup>* animals (Fig. S6I), this reduction in gene expression indicates that a loss of *Neurog3* in insulin-producing cells delayed the maturation of the  $\beta$  cells (26). One notable finding here is that, although we detected significant reduction in *insulin* and *Myt1* mRNA levels in the *Neurog3<sup>F1/-</sup>;Ins2<sup>Cre</sup>* animals over that of the controls, we did not observe any apparent decrease in either insulin or *Myt1* protein by using confocal microscopy. The underlying reason(s) for this discrepancy are not known, although one possibility is the low sensitivity of IF-based assays for protein level quantification.

**Sustained *Neurog3* Expression in Young Adult Islets Contributes to Endocrine Maintenance.** We next examined whether sustained *Neurog3* expression in mature islet cells contributes to endocrine function/maintenance. We used the TM-inducible Cre deleter, *Pdx1<sup>CreERT</sup>* (2) for this study. One-month-old *Neurog3<sup>F1/F</sup>;Pdx1<sup>CreERT</sup>* animals were administered with TM. At this stage, *Pdx1<sup>CreERT</sup>*-based recombination was mostly restricted to insulin-expressing  $\beta$  cells (2). As controls, *Neurog3<sup>F1/F</sup>;Pdx1<sup>CreERT</sup>* mice were administered with vehicle, and *Neurog3<sup>F1/F</sup>* animals were administered with TM. Six weeks after TM administration, TM-administered male *Neurog3<sup>F1/F</sup>;Pdx1<sup>CreERT</sup>* animals showed a trend of compromised glucose tolerance capability, although this glucose tolerance reduc-



**Fig. 4.** *Neurog3* inactivation in mature islet cells compromises endocrine function. (A) IPGTT assays after *Neurog3* inactivation in *Neurog3<sup>F1/F</sup>;Pdx1<sup>CreERT</sup>* female animals. (B) Fasting blood glucose levels in male *Neurog3<sup>F1/F</sup>;Pdx1<sup>CreERT</sup>* animals 4 and 12 weeks after *Neurog3* inactivation. Each dot represents 1 animal. Control animals received corn oil vehicle instead of TM.

tion did not attain statistical significance (Fig. 4A). Ten weeks after TM administration, TM-administered males, but not females, showed a significant decrease in glucose tolerance (Fig. 4A).

We also tested the effect of *Neurog3* inactivation in *Neurog3<sup>F1/-</sup>* animals, in which *Neurog3* haploinsufficiency renders them more sensitive to a loss of endocrine function. One-month-old *Neurog3<sup>F1/-</sup>;Pdx1<sup>CreERT</sup>* animals were administered with TM. Four weeks later, TM-treated male animals displayed a significant ( $P < 0.01$ ) increase in their fasting blood glucose levels compared with control littermates (Fig. 4B). The differences between the experimental and control groups increased with time, so that by 12 weeks after TM injection, hyperglycemia (a blood glucose level higher than 250 mg/dL) occurred in most of the TM-treated males but rarely in controls (Fig. 4B). Fasting blood glucose levels of female *Neurog3<sup>F1/-</sup>;Pdx1<sup>CreERT</sup>* animals did not significantly vary with or without TM administration (Fig. S7A, data at “0” min). However, intraperitoneal glucose tolerance testing (IPGTT) showed that 2 and 3 months after TM administration, TM-treated *Neurog3<sup>F1/-</sup>;Pdx1<sup>CreERT</sup>* females displayed significantly decreased glucose tolerance compared with control animals (Fig. S7A). Consistent with this phenotype, postglucose-challenge-serum-insulin levels in TM-treated *Neurog3<sup>F1/-</sup>;Pdx1<sup>CreERT</sup>* males were significantly reduced 12 weeks after treatment compared with control animals (Fig. S7B).

Next we examined whether sustained *Neurog3* expression in the adult  $\beta$  cells regulates cell division and cell survival. Ten days after TM administration, both mitotic and cell-death indices remained similar in islets of *Neurog3<sup>F1/F</sup>* and *Neurog3<sup>F1/F</sup>;Pdx1<sup>CreERT</sup>* animals (Fig. S8). These data suggest that *Neurog3* in mature islet cells regulates  $\beta$  cell function, but not cell division or cell death.

Finally, we investigated whether *Neurog3* regulates the same set of genes in adult  $\beta$  cells as in newly born insulin<sup>+</sup> cells (see above). Ten days after TM administration in 1-month-old *Neurog3<sup>F1/F</sup>;Pdx1<sup>CreERT</sup>* animals, a time at which the animals still showed normal glucose homeostasis, gene expression in the islets was examined by QRT-PCR and IF. As expected, QRT-PCR showed that *Neurog3* mRNA in TM-treated *Neurog3<sup>F1/F</sup>*;



Ascl + XhoI fragment from pGpx1-IRES<sup>TA</sup> (4.6kb) was ligated into Ascl + XhoI-digested pSL1180-Neurog3-Spe-EGFP vector to obtain the targeting vector pSL1180Neurog3-IRES<sup>TA</sup>. The vector was linearized and electroporated into TL1 mouse ES cells. Targeted clones were screened by Southern blot (Fig. S1). Blastocyst injection and germ line transmission testing followed standard techniques. Additional information on materials and methods used can be found in *SI Materials and Methods*.

**Immunohistochemistry/Immunofluorescence and Western Blot.**  $\beta$ -Gal detection followed standard protocol (16). Immunoassays followed established protocols with minor modifications. For MafA (1:500 dilution) and Neurog3 antibody (preincubated with Neurog3<sup>Fl-/-</sup>;Ins2<sup>Cre</sup> adult pancreas, 1:1000 dilution) staining, pancreata were frozen in OCT (optimum cutting temperature compound, Sakura Finetek) after dissection. Air-dried sections of 10–20  $\mu$ m thickness were fixed in 4% paraformaldehyde in PBS (with 1 mM EGTA and 1.5 mM MgCl<sub>2</sub>) for 15–20 min. Sections were permeabilized in 0.1% Triton-X100 (in PBS) for 20 min and blocked with antibody solution (0.5% BSA + 5% donkey serum + 0.1% Tween-20 in PBS; pH 7.6–7.8) for 30 min. Slides were incubated overnight at 4 °C with primary antibodies. After 4 washes with PBS containing 0.1% Tween-20, fluorophore-conjugated secondary antibodies were used to visualize signals. Glut2 staining used paraffin sections. Guinea pig anti-insulin, goat anti-C-peptide, guinea pig anti-glucagon, guinea pig anti-PP, and rabbit anti-SS were obtained from Dako. Rabbit anti-MafA was a gift from R. Stein (Vanderbilt University Medical Center, Nashville, TN) (31) and goat anti-Pdx1 was a gift from C. Wright (Vanderbilt University Medical Center, Nashville, TN). Guinea pig anti-Neurog3 was described previously (21), rabbit anti-Glut2 was from Alpha Diagnostics, and Rabbit anti-EGFP was from Clontech. FITC-conjugated donkey anti-rabbit IgG, Cy3-conjugated donkey anti-rabbit IgG, Cy3-conjugated donkey anti-mouse IgG, Cy3-conjugated donkey anti-guinea pig IgG, Cy3-conjugated donkey anti-goat, Cy5-conjugated donkey anti-rabbit, and Cy5-conjugated donkey anti-guinea pig were all from Jackson ImmunoResearch. All transcription factor antibodies (except Neurog3) were used at a 1:500 dilution. Hormone antibodies were diluted at 1:1,000. Secondary antibodies were used at 1:2,000 dilutions. For Western assays, islets from 6–8 adult animals of desired genotypes were hand-picked (25) and used

for nuclear extract preparation by using NuPer following manufacturer's protocol (Pierce). For blot, nuclear extract from  $\approx$ 50–100 islets was loaded into each well ( $\approx$ 5–10  $\mu$ g protein). E16.5 pancreatic nuclear extract was prepared as individual samples from each bud. After genotyping, extract from 3 buds of identical genotype were pooled and loaded for Western ( $\approx$ 5  $\mu$ g protein per lane). Recombinant Neurog3 protein from whole 293HEK cell lysate was used as positive control. Acrylamide gels (18%) were used to resolve the protein for blotting.

**Glucose Tolerance Test, Microscopy, QRT-PCR, and Statistical Analysis.** IPGTT followed published procedures (25). All fluorescent images were obtained by using confocal microscopy. QRT-PCR used the Bio-Rad Icyler. For PCR analysis of embryonic tissues, 2 or 3 embryos of the same genotype were prepared as a single RNA sample. Four individual RNA samples of control and 4 samples of experimental animals were prepared. For adult islets, 12 controls or 12 experimental pancreata were perfused. Four or five islet pools were handpicked from both groups and were used to extract total RNA. For each RNA preparation, duplicated reverse transcriptase reactions were used to prepare cDNA. Each cDNA was used to assay the abundance of each transcript as duplicated PCRs. DNA oligos used are listed in Table S1. Statistical analyses used the standard student's 2-tailed *t* test. A *P* value of <0.05 was considered significant. Data are presented as the mean  $\pm$  SEM.

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