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Postnatal β-Cell Proliferation and Mass Expansion Is Dependent on the Transcription Factor Nkx6.1

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All forms of diabetes are characterized by a loss of functional β-cell mass, and strategies for expanding β-cell mass could have significant therapeutic benefit. We have recently identified the transcription factor Nkx6.1 as an essential maintenance factor of the functional β-cell state. In addition, Nkx6.1 has been proposed to control β-cell proliferation, but a role for Nkx6.1 in regulating β-cell mass has not been demonstrated. Here, we show that Nkx6.1 is required for postnatal β-cell mass expansion. Genetic inactivation of *Nkx6.1* in newly formed β-cells caused a drastic decrease in early postnatal β-cell proliferation, leading to reduced β-cell mass and glucose intolerance. Interestingly, Nkx6.1 was dispensable for prenatal β-cell proliferation. We found that Nkx6.1 regulates the expression of several β-cell maturation markers as well as expression of the nutrient sensors Glut2 and Glp1r. Manifestation of the β-cell mass defect at the transition to postnatal feeding suggests that Nkx6.1 could regulate β-cell growth by enabling β-cells to respond to nutrient-dependent proliferation signals, such as glucose and Glp1. Identification of β-cell-intrinsic regulators that connect nutrient-sensing and proliferation suggests new therapeutic targets for expanding functional β-cell mass.

The establishment of sufficient β-cell mass depends on the rapid expansion of β-cell numbers during early postnatal life (1–5). The extent of this early postnatal β-cell growth is postulated to influence later susceptibility to type 2 diabetes (6). Postnatal β-cell mass expansion is driven by β-cell proliferation (7), which is controlled by the cell cycle regulators *Cyclin D1* or *Cyclin D2* (encoded by *Ccnd1* and *Ccnd2*, respectively) (2,3). It has been shown that β-cells are highly proliferative in the perinatal period and that this early proliferation is necessary to establish sufficient β-cell mass for maintaining glucose homeostasis (1–5). However, the cell extrinsic and intrinsic factors that drive β-cell proliferation and mass expansion during the perinatal period are still poorly defined.

Glucose has been identified as a systemic factor that stimulates β-cell proliferation (8,9), and recent studies suggest that glucose is a significant driver of early postnatal β-cell proliferation (10). Furthermore, it has been shown that glucose metabolism in β-cells produces signals that increase *Cyclin D2* expression and β-cell proliferation (11–13). Independent of glucose, β-cell proliferation is also stimulated by gut-derived hormone GLP-1 (Glp1), which is secreted by intestinal enteroendocrine cells in response to food intake (14,15). Thus there is an established link between feeding, increases in blood glucose levels, and β-cell proliferation. However, β-cells also exhibit significant proliferation during fetal life, when blood glucose concentrations are low and glucose has little effect on β-cell proliferation (16). The distinct mechanisms used in prenatal and postnatal β-cells to regulate proliferation remain unclear.

The β-cell-restricted transcription factor Nkx6.1 is essential for maintaining the functional state of β-cells during adulthood (17). Both in vitro and in vivo experiments have suggested a role for Nkx6.1 in β-cell proliferation (17–19), but whether it is required for β-cell growth in

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vivo is unknown. To reveal a possible role for Nkx6.1 in β-cell mass expansion, we inactivated *Nkx6.1* in newly formed β-cells of the embryo and examined the effects on β-cell proliferation and mass during the prenatal and postnatal period.

RESEARCH DESIGN AND METHODS

RIP-Cre (20), *Nkx6.1*^{flox} (21), *Nkx6.1* null (22), and *R26-YFP* mice (23) have been described. *RIP-Cre;Nkx6.1*$^{flox/+}$; *R26-YFP* mice served as control mice in all experiments. All experiments were approved by the Institutional Animal Care and Use Committee of the University of California.

Methods for tissue preparation, immunofluorescence staining, and terminal deoxynucleotidyl transferase dUTP nicked end labeling (TUNEL) have been previously described (21). The following primary antibodies were used: guinea pig anti-insulin (Dako), 1:2,000; mouse anti-Nkx6.1 (BCBC #2023), 1:500; rabbit anti-Glut2 (Millipore), 1:1,000; rabbit anti-Glp1r (S. Heller, Novo Nordisk), 1:2,000; rat anti-GFP (C. Kioussi, Oregon State University), 1:1,000; rabbit anti-Ki67 (Laboratory Vision), 1:500; rabbit anti-Ucn3 (M. Huising, University of California, Davis), 1:500; rabbit anti-MafA (Bethyl), 1:200; and rabbit anti-Pdx1 (Abcam), 1:500. Staining with antibodies raised in mice was performed using the M.O.M. Kit (Vector Laboratories). When necessary, nuclei were counterstained with DAPI (Sigma) at 0.1 μg/mL. Primary antibodies were detected with donkey-raised secondary antibodies conjugated to Cy3, Cy5, or Alexa 488 (Jackson ImmunoResearch). β-Cell mass and marker^{+} area were determined as described (21). Images were captured on a Zeiss Axio Observer Z1 microscope with an ApoTome module and processed with Zeiss AxioVision 4.8 software. All images were processed in accordance with *Diabetes* journal guidelines.

The quantitative RT-PCR (qRT-PCR) analysis was performed as previously described (17) on total RNA isolated from postnatal day 2 pancreata from individual mice. Primers used are as follows: Nkx6.1 (f-CTTCTGGCCCGGAGT GATG; r-GGGTCTGGTGTGTTTTCTCTTC), Ucn3 (f-GCTGT GCCCCTCGACCT; r-TGGGCATCAGCATCGCT), Adh1 (f- GCA AAGCTGCGGTGCTATG; r-TCACACAAGTCACCCCTTCTC), Angptl7 (f- TGACTGTTCTTCCCTGTACCA; r-CAAGGCCACTC TTACGTCTCT), Dlk1 (f-CCCAGGTGAGCTTCGAGT; r-GGA GAGGGGTACTCTTGTTGAG), Gstm2 (f-ACACCCGCATACAG TTGGC; r-TGCTTGCCCAGAAACTCAGAG), Zyx (f-TCCCACC GCAGGTATCATC; r-GGAGCTAGAAGGGGTCTTCCA), and Gapdh (f-CATGTTCCAGTATGACTCCACTC; r-GGCCTCACCC CATTTGATGT).

Glucose tolerance tests and blood glucose measurements were performed as described (17). For glucose tolerance tests, a 1.5 g/kg body weight intraperitoneal injection of glucose was administered after overnight fasting.

All values are shown as mean ± SEM; *P* values were calculated using a two-tailed Student *t* test in Microsoft Excel. $P < 0.05$ was considered significant.

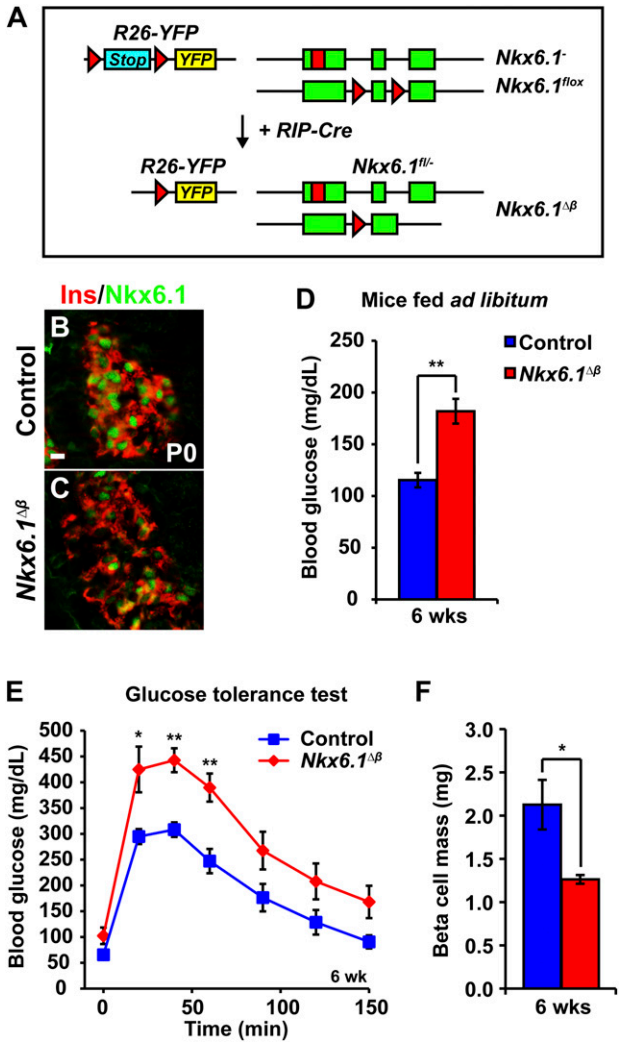

Figure 1—*Nkx6.1* deletion in newly formed β-cells leads to glucose intolerance and reduced β-cell mass. *A*: Schematic of alleles and transgenes used to inactivate *Nkx6.1* in fetal β-cells. Rectangles show coding sequences; triangles show *loxP* sites; red rectangle shows *DsRed* coding sequence. *B* and *C*: Immunofluorescence staining for Nkx6.1 and insulin reveals loss of Nkx6.1 in most β-cells of *Nkx6.1*$^{\Delta\beta}$ mice at P0. *D*: Blood glucose levels in 6-week-old *Nkx6.1*$^{\Delta\beta}$ mice fed ad libitum compared with control mice (n = 6). *E*: Intraperitoneal glucose tolerance test shows glucose intolerance in 6-week-old *Nkx6.1*$^{\Delta\beta}$ mice as compared with control mice ($n = 6$). *F*: Quantification of β-cell mass reveals decreased β-cell mass in *Nkx6.1*$^{\Delta\beta}$ mice at 6 weeks of age ($n = 3$). Data shown as mean ± SEM. Scale bars = 20 μm. Ins, insulin; YFP, yellow fluorescent protein. *$P < 0.05$; **$P < 0.01$.

RESULTS

Nkx6.1 Inactivation in Embryonic β-Cells Causes Hyperglycemia and Reduced β-Cell Mass

To investigate the role of Nkx6.1 in perinatal β-cell development, we intercrossed mice to generate progeny carrying a *Nkx6.1* null allele (*Nkx6.1*$^{-}$), a *Nkx6.1* conditional loss of function allele (*Nkx6.1*^{flox}), and the *rat insulin2-Cre* transgene (*RIP-Cre*). Additionally, the mice carried a conditional *YFP* reporter gene targeted to the *Rosa-26* locus (*R26-YFP*), resulting in heritable YFP expression upon

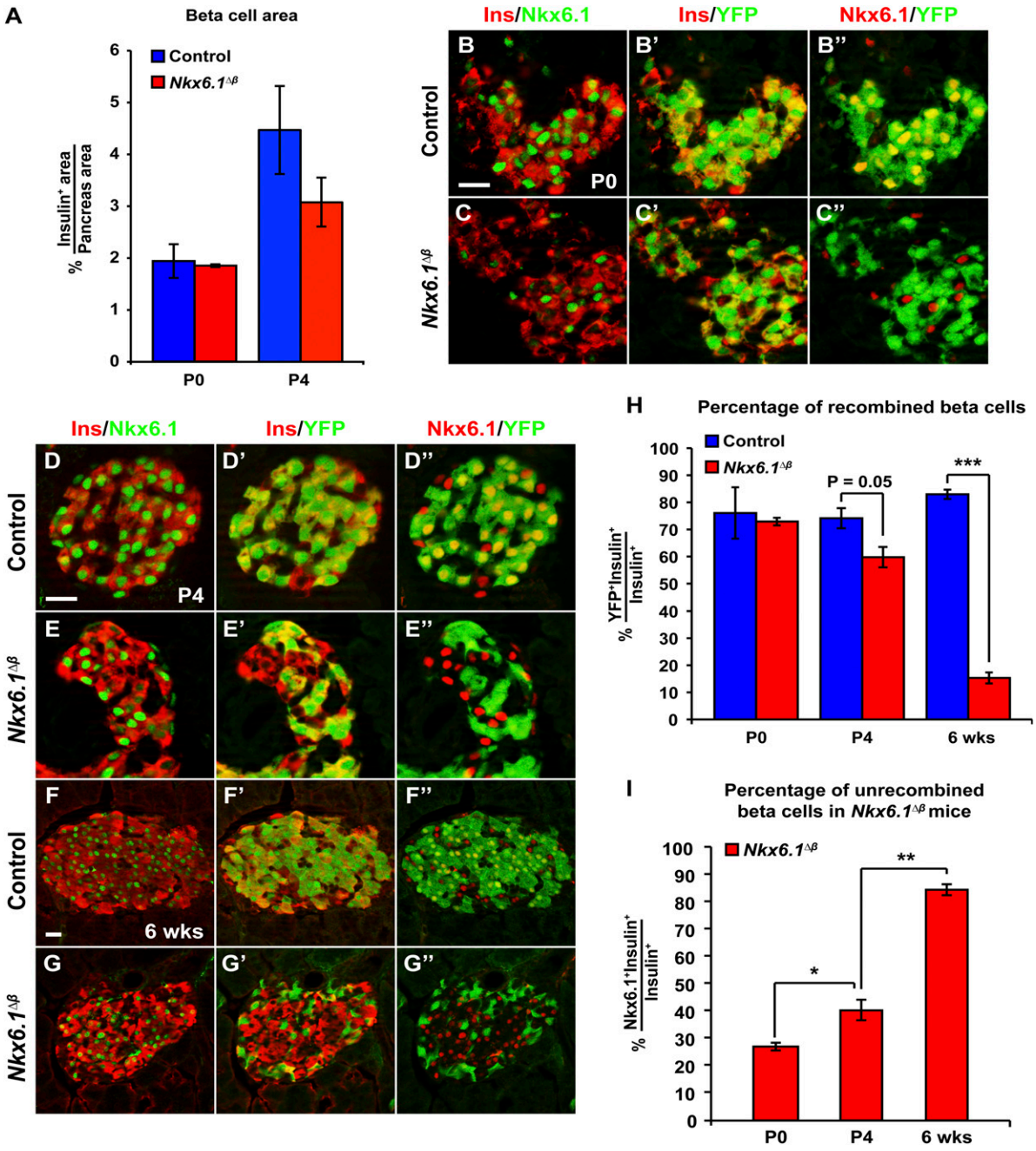

Figure 2—Nkx6.1 is required for postnatal β-cell mass expansion. *A*: Quantification of the insulin immunofluorescent area relative to total pancreatic area reveals no difference in β-cell mass between *Nkx6.1*$^{\Delta\beta}$ and control mice at P0 and a slight but not significant decrease at P4 (n = 3). *B–G''*: Immunofluorescence staining for insulin, Nkx6.1, and YFP at P0 (*B–C''*), P4 (*D–E''*), and 6 weeks of age (*F–G''*). *H*: Quantification of insulin$^+$ cells expressing YFP at P0, P4, and 6 weeks shows a progressive decrease of YFP$^+$ recombined β-cells in *Nkx6.1*$^{\Delta\beta}$ mice postnatally (n = 3). *I*: Quantification of insulin$^+$ cells expressing Nkx6.1 reveals a progressive increase of Nkx6.1-expressing unrecombined β-cells in *Nkx6.1*$^{\Delta\beta}$ mice between P0 and 6 weeks of age (n = 3). Data shown as mean ± SEM. Scale bar = 20 μm. Ins, insulin; YFP, yellow fluorescent protein. *P < 0.05; **P < 0.01; ***P < 0.001.

RIP-Cre-mediated recombination of a translational stop signal. Thus, in *RIP-Cre;Nkx6.1*$^{flox/-}$*;R26-YFP* (hereafter referred to as *Nkx6.1*$^{\Delta\beta}$) mice, YFP labels all cells in which *Nkx6.1* has been inactivated (Fig. 1*A*).

Nkx6.1$^{\Delta\beta}$ mice were born with the expected Mendelian frequency (data not shown). Consistent with previous reports showing incomplete targeting of β-cells by the *RIP-Cre* transgene (20), most but not all β-cells were devoid of Nkx6.1 at birth (Fig. 1*B* and *C*). At 6 weeks of age, *Nkx6.1*$^{\Delta\beta}$ mice exhibited significantly elevated blood glucose levels (Fig. 1*D*) and impaired glucose tolerance after intraperitoneal injection of a glucose bolus (Fig. 1*E*). To investigate whether Nkx6.1 deficiency affects postnatal β-cell growth, we examined β-cell mass in *Nkx6.1*$^{\Delta\beta}$ mice. Compared with

littermate controls, 6-week-old *Nkx6.1*$^{\Delta\beta}$ mice exhibited a 40% reduction in β-cell mass (1.26 ± 0.05 mg in *Nkx6.1*$^{\Delta\beta}$ mice vs. 2.13 ± 0.29 mg in controls) (Fig. 1*F*). Thus Nkx6.1 is necessary to establish appropriate β-cell mass.

Nkx6.1 Is Required for Postnatal, but Not Prenatal, β-Cell Mass Expansion

To determine when β-cell mass is first affected in *Nkx6.1*$^{\Delta\beta}$ mice, we measured the relative insulin$^+$ area in *Nkx6.1*$^{\Delta\beta}$ mice immediately after birth. In contrast to 6-week-old mice, β-cell mass in neonatal *Nkx6.1*$^{\Delta\beta}$ mice was indistinguishable from control mice (Fig. 2*A*), showing that Nkx6.1 is required for postnatal expansion but not for establishing prenatal β-cell mass.

Because *RIP-Cre*-mediated recombination of the *Nkx6.1*^{flox} allele is mosaic and did not delete *Nkx6.1* in all β-cells (Fig. 1*C*), both unrecombined Nkx6.1$^+$ and recombined Nkx6.1-deficient β-cells can contribute to β-cell growth in *Nkx6.1*$^{\Delta\beta}$ mice. To investigate the contribution of recombined β-cells to postnatal β-cell mass expansion, we quantified the percentage of recombined β-cells in *Nkx6.1*$^{\Delta\beta}$ and control mice. In line with our observation that Nkx6.1 is dispensable for prenatal β-cell growth (Fig. 2*A*), the percentage of recombined YFP$^+$ β-cells was similar in newborn *Nkx6.1*$^{\Delta\beta}$ and control mice (73 ± 1.4% in *Nkx6.1*$^{\Delta\beta}$ mice vs. 76 ± 9.5% in control mice) (Fig. 2*B–C”* and *H*). Consistent with a slight decrease in overall β-cell mass in *Nkx6.1*$^{\Delta\beta}$ mice at postnatal (P) day 4 (Fig. 2*A*), a reduction in the percentage of recombined β-cells was discernable in *Nkx6.1*$^{\Delta\beta}$ mice by P4 (Fig. 2*D–E”* and *H*). At 6 weeks of age, the reduction of YFP$^+$ β-cells was highly significant (15 ± 2.02% of β-cells in *Nkx6.1*$^{\Delta\beta}$ mice vs. 83 ± 1.72% in control mice) (Fig. 2*F–H*). The decrease of YFP$^+$ β-cells in *Nkx6.1*$^{\Delta\beta}$ mice was accompanied by an age-dependent increase in the percentage of β-cells expressing Nkx6.1 (Fig. 2*B–G”* and *I*). Closely mirroring the reported 82% recombination efficiency of the *RIP-Cre* transgene (20), 27 ± 1.41% of β-cells expressed Nkx6.1 in newborn *Nkx6.1*$^{\Delta\beta}$ mice (Fig. 2*I*). This percentage increased significantly to 40 ± 3.75% at P4 (Fig. 2*I*). These findings indicate that a selective disadvantage becomes apparent for Nkx6.1-deficient β-cells shortly after birth.

Postnatal, but Not Prenatal, β-Cell Proliferation Depends on Nkx6.1

We next investigated whether the postnatal β-cell growth defect in *Nkx6.1*$^{\Delta\beta}$ mice is caused by reduced β-cell proliferation and/or survival. First, we examined the possibility that Nkx6.1 deficiency causes increased β-cell apoptosis by performing TUNEL assays on pancreatic sections. Virtually no TUNEL$^+$ β-cells were detected in either *Nkx6.1*$^{\Delta\beta}$ or control mice at P4 (Fig. 3*A–C*), indicating that apoptosis does not account for the negative selection of Nkx6.1-deficient β-cells. By contrast, analysis of β-cell proliferation by immunofluorescence staining for Ki67, insulin, and YFP in *Nkx6.1*$^{\Delta\beta}$ mice at P4 revealed reduced numbers of Ki67$^+$ β-cells (Fig. 3*F–H*). Quantification of

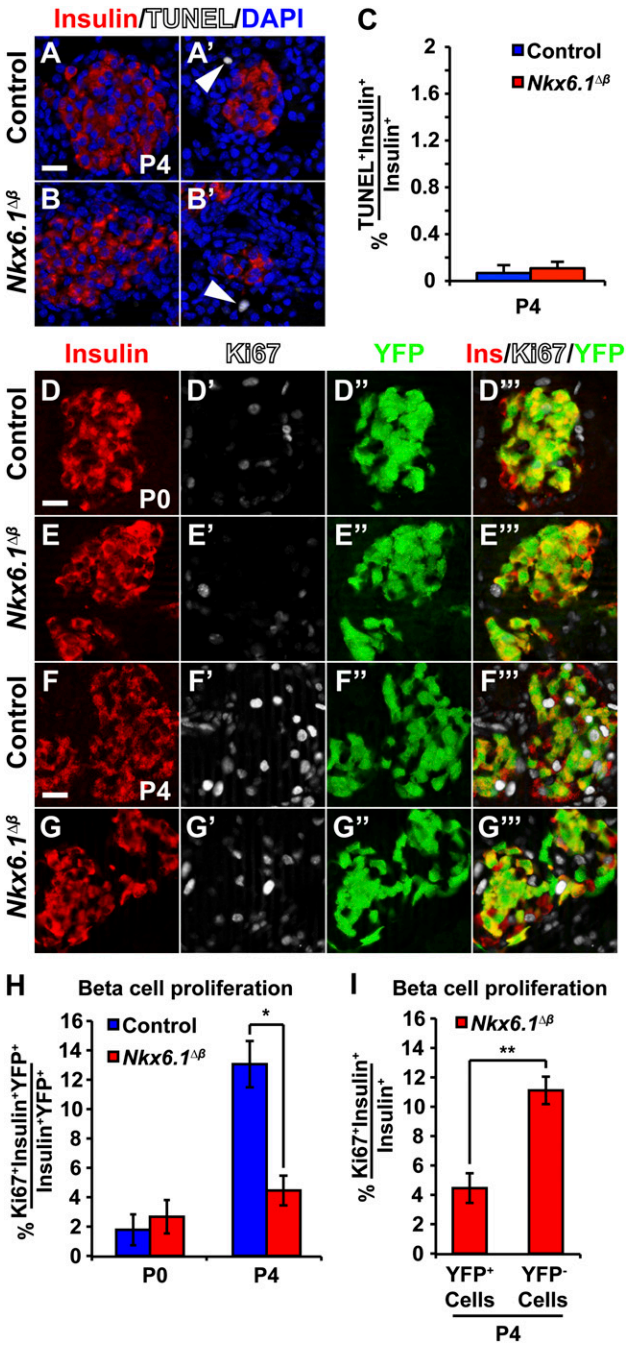

Figure 3—Nkx6.1 is required for postnatal β-cell proliferation. *A–C*: β-Cells are not apoptotic at P4 in *Nkx6.1*$^{\Delta\beta}$ or control mice based on TUNEL combined with immunofluorescence staining for insulin and DAPI. TUNEL$^+$ cells in the pancreas are shown as a positive control (arrowheads) and TUNEL$^+$insulin$^+$ cells were quantified. *D–G'''*: Immunofluorescence staining for insulin, Ki67, and YFP at P0 and P4. *H*: Quantification of the percentage of insulin$^+$YFP$^+$ cells expressing Ki67 shows decreased β-cell proliferation in *Nkx6.1*$^{\Delta\beta}$ mice at P4, but not at P0 (n = 3). *I*: Quantification of Ki67-expressing YFP$^+$insulin$^+$ cells and YFP$^-$insulin$^+$ cells in *Nkx6.1*$^{\Delta\beta}$ mice at P4 reveals a selective decrease in proliferation of recombined compared with unrecombined β-cells within the same animal (n = 3). Data shown as mean ± SEM. Scale bar = 20 μm. Ins, insulin; YFP, yellow fluorescent protein. *P < 0.05; **P < 0.01.

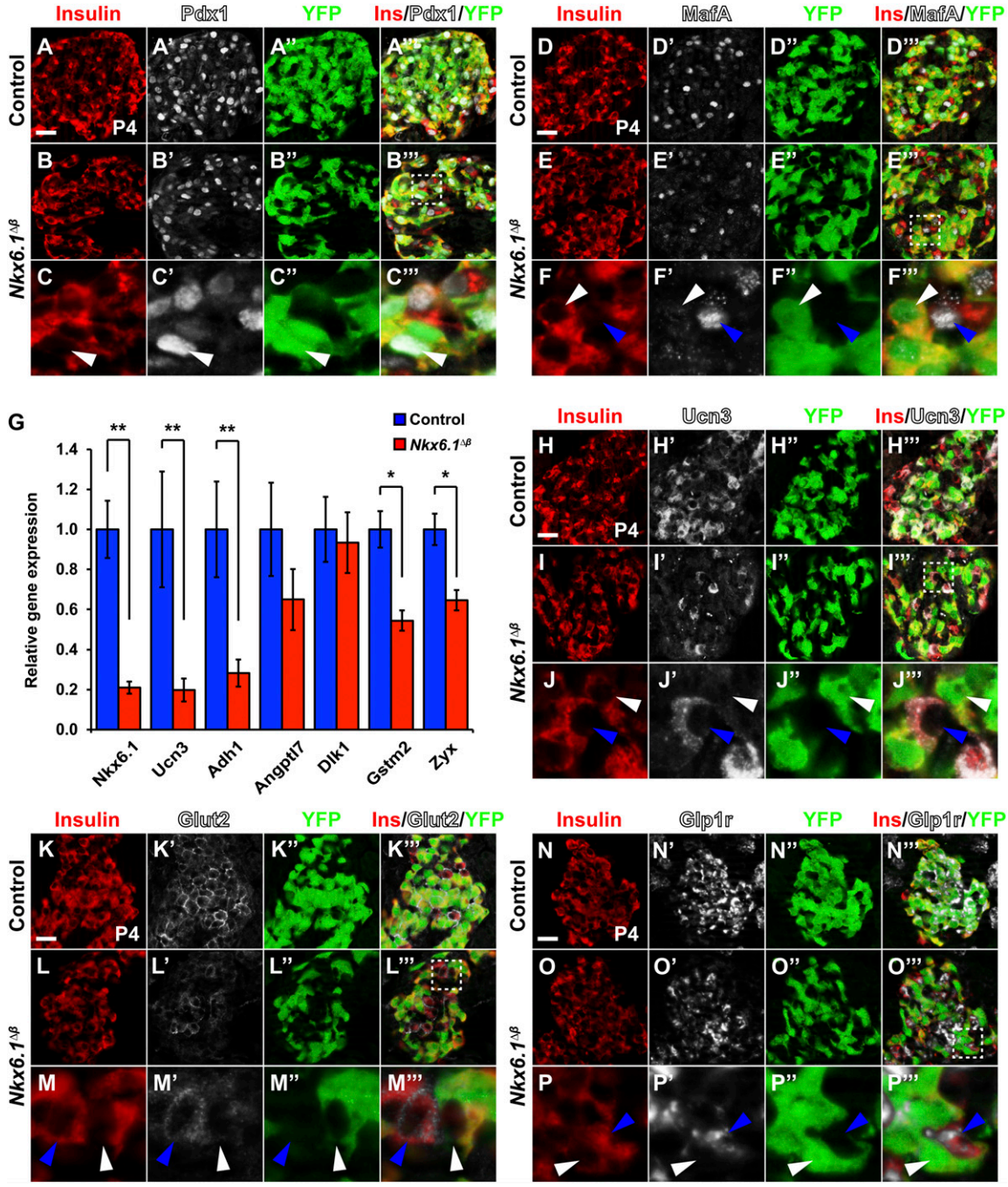

Figure 4—*Nkx6.1* inactivation leads to a cell autonomous loss of β-cell maturation and nutrient sensing markers. Immunofluorescence staining for insulin, Pdx1, and YFP (*A–C'''*) or insulin, MafA, and YFP (*D–F'''*) shows Pdx1 but not MafA expression in recombined YFP$^+$insulin$^+$ cells of *Nkx6.1*$^{\Delta\beta}$ mice at P4. Unrecombined YFP$^-$insulin$^+$ cells express Pdx1 and MafA in *Nkx6.1*$^{\Delta\beta}$ mice. *G*: qRT-PCR analysis of pancreata from *Nkx6.1*$^{\Delta\beta}$ and control mice at P2 for genes associated with β-cell maturation (n = 3). Immunofluorescence staining for insulin, Ucn3, and YFP (*H–J'''*); insulin, Glut2, and YFP (*K–M'''*); or insulin, Glp1r, and YFP (*N–P'''*) shows loss of Ucn3, Glut2, and Glp1r expression in recombined YFP$^+$insulin$^+$ cells but not in unrecombined YFP$^-$insulin$^+$ cells of *Nkx6.1*$^{\Delta\beta}$ mice at P4. For each marker, representative areas are shown in lower panels for *Nkx6.1*$^{\Delta\beta}$ mice, as indicated by a dashed box in the merged middle panel. White arrowheads point to recombined YFP$^+$insulin$^+$ cells and blue arrowheads to unrecombined YFP$^-$insulin$^+$ cells. Data shown as mean ± SEM. Scale bar = 20 μm. Ins, insulin; YFP, yellow fluorescent protein. *P < 0.05; **P < 0.01.

Ki67$^+$YFP$^+$ β-cells showed a threefold decrease in β-cell proliferation in 4-day-old *Nkx6.1*$^{\Delta\beta}$ compared with control mice (4.48 ± 1.01% in *Nkx6.1*$^{\Delta\beta}$ mice vs. 13.00 ± 1.58% in control mice) (Fig. 3*H*). Consistent with our finding that *Nkx6.1* inactivation does not affect prenatal β-cell growth (Fig. 2*A*), the frequency of Ki67$^+$ β-cells did not differ between *Nkx6.1*$^{\Delta\beta}$ and control mice at P0 (2.67 ± 1.14% in *Nkx6.1*$^{\Delta\beta}$ mice vs. 1.78 ± 1.05% in control mice)

(Fig. 3*D–E‴* and *H*). Thus Nkx6.1 is required for β-cell proliferation and expansion during early postnatal life but is dispensable prenatally. Furthermore, the effect of *Nkx6.1* deletion on β-cell proliferation is cell autonomous, as revealed by comparing proliferation rates between recombined and unrecombined β-cells in *Nkx6.1*$^{\Delta\beta}$ mice at P4 (4.48 ± 1.01% YFP$^+$insulin$^+$ cells vs. 11.0 ± 0.93% YFP$^-$insulin$^+$ cells expressed Ki67) (Fig. 3*I*).

Nkx6.1 Deletion Causes a Cell Autonomous Loss of Markers for β-Cell Maturation and Nutrient Sensing

To determine whether loss of Nkx6.1 affects other β-cell markers, we performed immunofluorescence staining for Pdx1 and MafA. While Pdx1 was unaffected, MafA was lost in recombined Nkx6.1-deficient β-cells (Fig. 4*A–F‴*). We further assessed whether Nkx6.1 regulates β-cell maturation markers. To this end, we selected genes found to be significantly changed between immature and mature postnatal β-cells (24) and performed qRT-PCR analysis on pancreata from control and *Nkx6.1*$^{\Delta\beta}$ mice at P2, when β-cell mass is similar between *Nkx6.1*$^{\Delta\beta}$ and control mice (Fig. 2*A*). Of these genes, *Ucn3, Adh1*, *Gstm2*, and *Zyx* were expressed at significantly lower levels in *Nkx6.1*$^{\Delta\beta}$ mice, while *Angptl7* and *Dlk1* were unchanged (Fig. 4*G–J‴*). These results suggest that Nkx6.1 regulates a subset of genes associated with β-cell maturation. Given the postnatal onset of the β-cell proliferation defect in *Nkx6.1*$^{\Delta\beta}$ mice, we next investigated whether Nkx6.1-deficient β-cells are able to receive feeding-induced signals that stimulate β-cell proliferation. We analyzed the expression of Glut2 and the Glp1 receptor (Glp1r), which are known to have a role in the regulation of postnatal β-cell growth (14,16). In accordance with *Glut2* being a direct Nkx6.1 target gene (17), *Nkx6.1*$^{\Delta\beta}$ mice exhibited a selective loss of Glut2 expression only in recombined β-cells (Fig. 4*K–M‴*). Similarly, recombined β-cells displayed a cell autonomous reduction in Glp1r expression (Fig. 4*N–P‴*). The cell autonomous role of Nkx6.1 in regulating β-cell proliferation, Glut2, and Glp1r expression argues against an Nkx6.1-dependent paracrine or systemic factor affecting β-cell proliferation in *Nkx6.1*$^{\Delta\beta}$ mice. These findings demonstrate that Nkx6.1-deficient β-cells lack key sensors for extrinsic stimuli of postnatal β-cell growth.

DISCUSSION

The role of Nkx6.1 in β-cell proliferation has been controversial. While in vitro studies have suggested a direct role of Nkx6.1 in stimulating β-cell proliferation through the regulation of *Cyclin* gene expression (18), in vivo overexpression of Nkx6.1 in β-cells showed no effect on β-cell proliferation or mass (19). Moreover, we have recently reported that β-cell-specific inactivation of *Nkx6.1* in adult mice has no overt effect on β-cell mass (17). However, due to the extremely low proliferation rate of β-cells in adult animals (1), the role of Nkx6.1 in β-cell mass expansion could not be rigorously tested in this model. By ablating *Nkx6.1* in newly formed β-cells of the embryo, we here show that postnatal β-cell proliferation and mass expansion depends on Nkx6.1 activity. We found that Nkx6.1-deficient β-cells begin to exhibit reduced proliferation between P0 and P4, which manifests in a measurable decrease in the contribution of Nkx6.1-deficient β-cells to β-cell mass as early as P4. We have previously reported that Nkx6.1 deficiency leads to a loss of β-cell identity and, ultimately, their conversion into delta cells (21). It is important to note that this fate conversion occurs later and is not yet observed at P4 (see Fig. 2*E–E″*; all YFP$^+$ cells express insulin). Thus the reduced contribution of Nkx6.1-deficient β-cells to β-cell mass is caused by the proliferation defect and cannot be attributed to a β-to-delta–cell fate conversion.

Using chromatin immunoprecipitation sequencing analysis, we have recently shown that Nkx6.1 does not bind to *Cyclin* gene regulatory regions (17). Therefore, Nkx6.1 is likely an indirect regulator of β-cell proliferation. Consistent with this idea, our current work shows that prenatal β-cell proliferation is unaffected in *Nkx6.1*$^{\Delta\beta}$ mice. Interestingly, we found that the onset of reduced β-cell proliferation in *Nkx6.1*$^{\Delta\beta}$ mice coincides with birth and thus the beginning of food intake, suggesting that Nkx6.1 could enable β-cells to respond to nutrient-dependent inducers of β-cell proliferation. Supporting this notion, *Nkx6.1*-deleted β-cells fail to express two important nutrient sensors, Glut2 and Glp1r. At the transition from prenatal to postnatal life, glucose becomes an important stimulus of β-cell proliferation (16) and similar to *Nkx6.1*$^{\Delta\beta}$ mice, Glut2-deficient mice exhibit reduced β-cell proliferation during the early postnatal period (25). Since Glp1 regulates β-cell proliferation independent of glucose (15), loss of Glut2 and Glp1r in *Nkx6.1*$^{\Delta\beta}$ mice likely have additive effects on β-cell proliferation. In addition to regulating nutrient sensors, we found that Nkx6.1 also regulates several markers associated with postnatal β-cell maturation (24). It is still largely unclear whether and how these genes affect β-cell maturation, but the regulation of several of these genes by Nkx6.1 suggests a role for Nkx6.1 in β-cell maturation. Collectively, our results demonstrate that Nkx6.1 controls multiple relevant pathways for postnatal β-cell development.

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Author Contributions. B.L.T. and J.B. designed and performed experiments, analyzed data, prepared figures, and wrote the manuscript. M.S. wrote the manuscript. M.S. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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