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Type 2 diabetes is thought to involve a compromised  $\beta$  cell differentiation state, but the mechanisms underlying this dysfunction remain unclear. Here, we report a key role for the TF PAX6 in the maintenance of adult  $\beta$  cell identity and function. PAX6 was downregulated in  $\beta$  cells of diabetic *db/db* mice and in WT mice treated with an insulin receptor antagonist, revealing metabolic control of expression. Deletion of *Pax6* in  $\beta$  cells of adult mice led to lethal hyperglycemia and ketosis that were attributed to loss of  $\beta$  cell function and expansion of  $\alpha$  cells. Lineage-tracing, transcriptome, and chromatin analyses showed that PAX6 is a direct activator of  $\beta$  cell genes, thus maintaining mature  $\beta$  cell function and identity. In parallel, we found that PAX6 binds promoters and enhancers to repress alternative islet cell genes including ghrelin, glucagon, and somatostatin. Chromatin analysis and shRNA-mediated gene suppression experiments indicated a similar function of PAX6 in human  $\beta$  cells. We conclude that reduced expression of PAX6 in metabolically stressed  $\beta$  cells may contribute to  $\beta$  cell failure and  $\alpha$  cell dysfunction in diabetes.

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# PAX6 maintains $\beta$ cell identity by repressing genes of alternative islet cell types

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**Type 2 diabetes is thought to involve a compromised  $\beta$  cell differentiation state, but the mechanisms underlying this dysfunction remain unclear. Here, we report a key role for the TF PAX6 in the maintenance of adult  $\beta$  cell identity and function. PAX6 was downregulated in  $\beta$  cells of diabetic *db/db* mice and in WT mice treated with an insulin receptor antagonist, revealing metabolic control of expression. Deletion of *Pax6* in  $\beta$  cells of adult mice led to lethal hyperglycemia and ketosis that were attributed to loss of  $\beta$  cell function and expansion of  $\alpha$  cells. Lineage-tracing, transcriptome, and chromatin analyses showed that PAX6 is a direct activator of  $\beta$  cell genes, thus maintaining mature  $\beta$  cell function and identity. In parallel, we found that PAX6 binds promoters and enhancers to repress alternative islet cell genes including ghrelin, glucagon, and somatostatin. Chromatin analysis and shRNA-mediated gene suppression experiments indicated a similar function of PAX6 in human  $\beta$  cells. We conclude that reduced expression of PAX6 in metabolically stressed  $\beta$  cells may contribute to  $\beta$  cell failure and  $\alpha$  cell dysfunction in diabetes.**

## Introduction

Mature pancreatic  $\beta$  cells are highly specialized for sensing blood glucose levels and secreting insulin. Extensive efforts have resulted in a detailed understanding of the transcriptional cascades leading to differentiation of  $\beta$  cells from progenitor cells during embryonic development and their subsequent maturation (1–3). More recently, it has emerged that, even after differentiation, the maintenance of adult  $\beta$  cell identity and function requires the continuous activity of multiple transcription factors (TFs) (4–6). Importantly, some of these factors are sensitive to metabolic insults. For example, oxidative stress reduces the activity of the  $\beta$  cell TFs PDX1, NKX6.1, and MAFA, suggesting a pathogenic mechanism for the development of diabetes (7). Perturbation of such factors by either genetic or environmental insults may result in  $\beta$  cell death, or, alternatively, in loss of  $\beta$  cell identity.

Interestingly, the loss of  $\beta$  cell differentiation is often accompanied by acquisition of alternative cellular identities (5, 8, 9), reflecting the retention of some developmental plasticity in differentiated  $\beta$  cells. Typically, these alternative fates remain within the endocrine lineage. They may include, for example, a switch from expression of insulin to expression of glucagon or somatostatin, indicating that  $\beta$  cell plasticity is largely confined to the islet program. The potential of metabolically stressed  $\beta$  cells to dedifferentiate and then redifferentiate into non- $\beta$  cell fates was proposed as

a novel mechanism underlying reversible  $\beta$  cell failure in diabetes (8, 10–13). Plasticity between  $\alpha$  and  $\beta$  cell fates is supported by the remarkably similar epigenetic states of the 2 cell types (14). Other islet cell types also show such intraislet plasticity, and may, in some instances, reprogram into functional  $\beta$  cells. For example, it was shown that near-total ablation of  $\beta$  cells in mice results in the spontaneous conversion of some  $\delta$  cells (15) or  $\alpha$  cells (16) to functional  $\beta$  cells, suggesting novel approaches for regenerative therapy in diabetes. Thus, the molecular mechanisms that govern the maintenance of adult islet cell identity are of great interest, with implications for the prevention of  $\beta$  cell failure as well as expansion of  $\beta$  cell mass in diabetes.

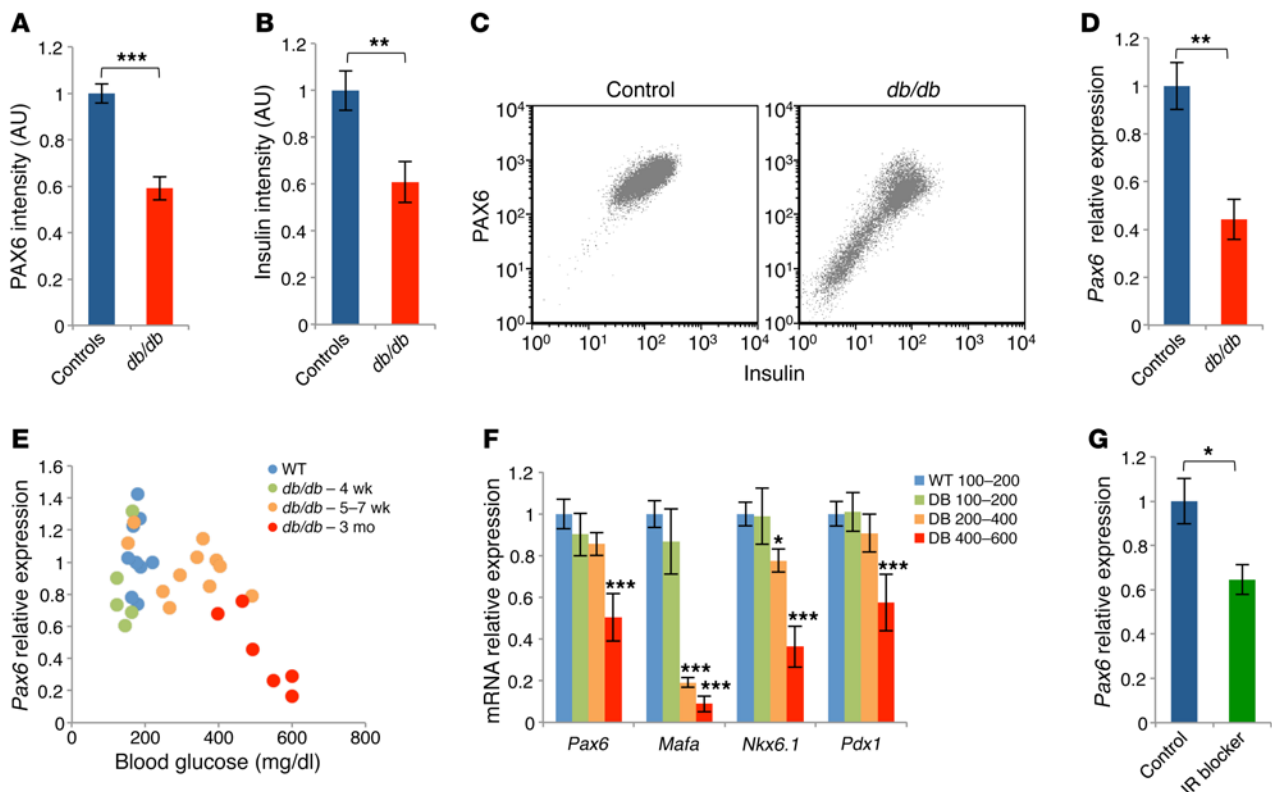
In this study, we focus on the role of the paired and homeodomain TF PAX6 in adult  $\beta$  cells. PAX6 is crucial for the generation of neuronal lineages in the CNS including the cortex and retina, as well as the differentiation of non-neuronal lineages of the eye (17–19). This TF acts in these situations as both a transcriptional activator and repressor via complex gene regulatory networks that are only partly resolved (20–23). In the pancreas, PAX6 is required for normal islet development. In the absence of PAX6, the production of  $\alpha$  cells and  $\beta$  cells is greatly reduced; instead, there is a dramatic increase in the expression of ghrelin, a gut hormone normally expressed only transiently in the fetal pancreas. Thus, during development of the pancreas, PAX6 acts to direct the differentiation of endocrine-committed progenitor cells to correct fates. The molecular targets of PAX6 in mediating these developmental decisions are only partly understood (24–26). Notably, mice and humans heterozygous for *PAX6* show defects in nervous system development as

## ► Related Commentary: p. 94

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**Figure 1. PAX6 expression in adult  $\beta$  cells from *db/db* mice and after treatment with an insulin receptor antagonist.** (A) PAX6 protein levels in  $\beta$  cells from *db/db* mice and controls. Dissociated islet cells were costained for insulin and PAX6 and analyzed by flow cytometry. Graph shows the mean of PAX6 intensity in insulin<sup>+</sup> cells isolated from 3 control and 5 *db/db* mice at 3 months of age. (B) Mean insulin intensity for the same samples as in A. (C) Representative plots of PAX6 versus insulin protein levels in the control and *db/db* mice depicted in A and B. PAX6 levels correlated with insulin levels in both control and *db/db* mice. *db/db* mice in panels A–C had, on average, glucose levels of 480 mg/dl. (D) *Pax6* mRNA levels in 3-month-old control and *db/db* islets.  $n = 6$  animals per group. (E) Negative correlation between *Pax6* mRNA and blood glucose levels in *db/db* mice at different ages (4 weeks, 5–7 weeks, and 3 months, by Pearson's correlation test,  $R = -0.9$ ,  $P < 0.05$ ). (F) mRNA levels of *Pax6*, *Mafa*, *Nkx6.1*, and *Pdx1* in the same *db/db* (DB) mice as in E, averaged per blood glucose ranges (100–200, 200–400, and 400–600 mg/dl).  $n = 9, 7, 8$ , and 7 mice, respectively. (G) *Pax6* mRNA levels of islets isolated from WT mice treated with the insulin receptor (IR) antagonist 5961 for 4 days. Average blood glucose level was 434 mg/dl in the treated group.  $n = 3$  per each group. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ , by 2-tailed Student's  $t$  test.

well as perturbed glucose homeostasis (27–29), underscoring the importance of tight control over its expression level. In addition, a common variant in the *PAX6* gene was associated with a reduction in both PAX6 expression and insulin secretion (30).

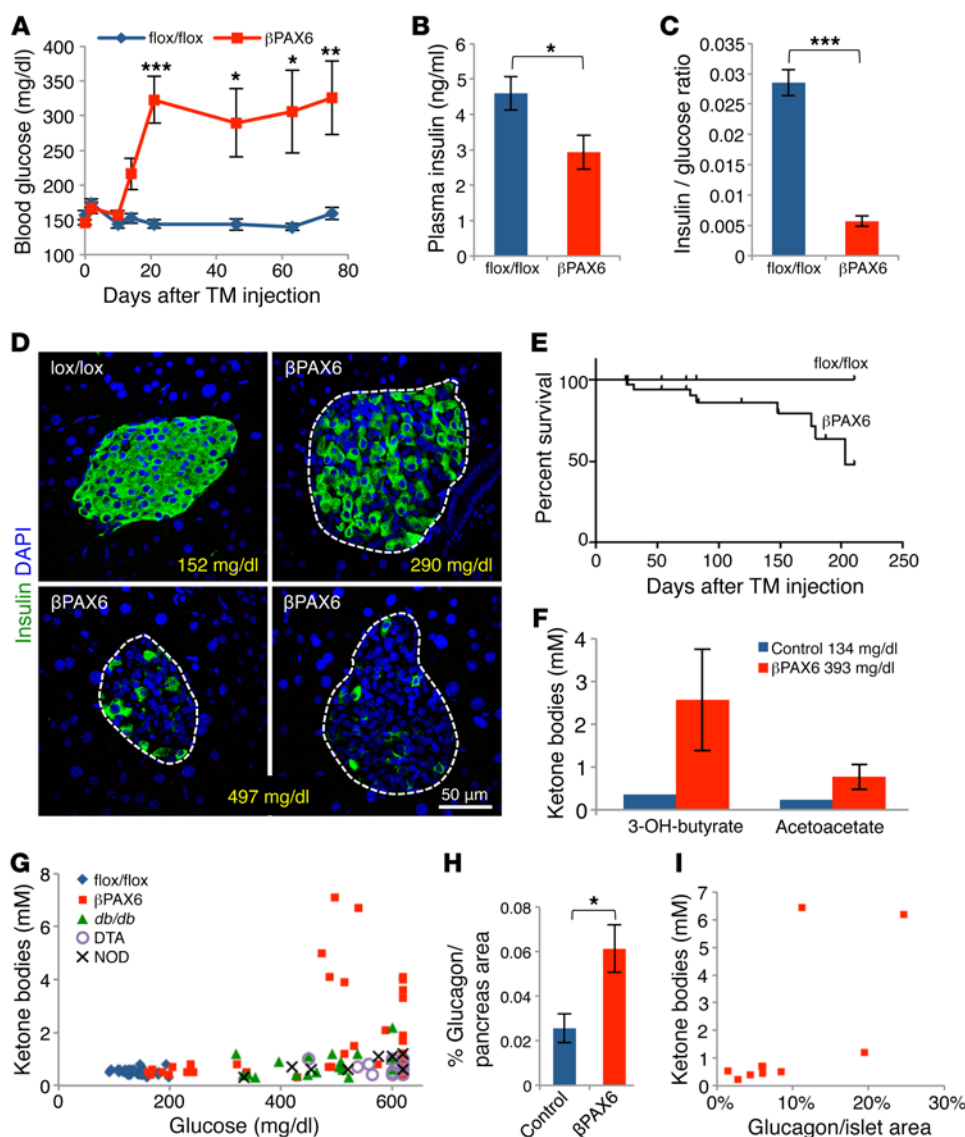
In the adult pancreas, PAX6 is expressed in all hormone-producing cells, suggesting a general role in terminally differentiated endocrine cells (24, 25). In immortalized  $\beta$  cell lines, PAX6 was shown to bind and activate the promoters of insulin and additional  $\beta$  cell genes (26, 31). Deletion of *Pax6* in adult murine islet cells causes hyperglycemia, with reduced expression of insulin, glucagon, and somatostatin and upregulated expression of ghrelin (32). A more recent study showed that deletion of *Pax6* in adult  $\alpha$  or  $\beta$  cells induces ghrelin expression in mutant cells (33). However, the molecular mechanisms underlying these phenotypes were not studied.

Here, we report that PAX6 is downregulated in  $\beta$  cells in 2 models of hyperglycemia and insulin resistance: *db/db* mice, a model for type 2 diabetes, and WT mice treated with an insulin receptor antagonist. Deletion of *Pax6* in adult murine  $\beta$  cells led to lethal hyperglycemia and ketosis due to loss of  $\beta$  cell differentiation, combined with expansion of WT  $\alpha$  cells. Genome-wide molecular analysis revealed that PAX6 is an essential direct acti-

vator of key genes responsible for  $\beta$  cell identity and function; in parallel, PAX6 binds promoters and enhancers of genes normally silenced in  $\beta$  cells to directly repress alternative islet cell programs. We also provide evidence that these functions of PAX6 are conserved in human  $\beta$  cells. The findings highlight the fragility of  $\beta$  cell identity and establish PAX6 as a central determinant of adult  $\beta$  cell differentiation state and function. We propose that downregulation of PAX6 in metabolically stressed  $\beta$  cells may contribute to both  $\beta$  cell failure and  $\alpha$  cell dysfunction in diabetes.

## Results

**Reduced expression of PAX6 in  $\beta$  cells of insulin-resistant, hyperglycemic mice.** We determined PAX6 levels in hyperglycemic *db/db* mice, a model for type 2 diabetes. FACS analysis showed that PAX6 protein levels were reduced by 40% in  $\beta$  cells of diabetic *db/db* mice compared with levels in controls (Figure 1A). The reduction in PAX6 protein levels mirrored the reduced insulin content in  $\beta$  cells of *db/db* mice (Figure 1B). Strikingly, PAX6 levels correlated with cellular insulin content in individual  $\beta$  cells from both control and diabetic mice (Figure 1C). These results suggest, but do not prove, that PAX6 is an important determinant of insulin content in



**Figure 2. Hyperglycemia, hypoinsulinemia, hyperketonemia and loss of  $\beta$  cells in  $\beta$ PAX6 mice.** (A) Blood glucose levels over time in  $Pax6^{f/f}$  (flox/flox) and  $\beta$ PAX6 mice following tamoxifen (TM) injection. Tamoxifen was injected into mice at 6 weeks of age.  $n = 13$  flox/flox and 17  $\beta$ PAX6 mice. (B) Plasma insulin levels in  $\beta$ PAX6 and flox/flox mice at the age of 4 months and 2 months after tamoxifen injection.  $n = 5$  mice in each group. (C) Ratio of plasma insulin to blood glucose levels in the same mice as represented in B. (D) Representative insulin staining (green) of pancreatic sections from 1 flox/flox mouse and 2  $\beta$ PAX6 mice. Blood glucose of each mouse is indicated. Note that despite a loss of insulin-stained cells, islets retained typical morphology and size. Original magnification,  $\times 400$ . (E) Survival of  $\beta$ PAX6 mice.  $n = 13$  flox/flox and 35  $\beta$ PAX6 mice. Graph shows the percentage of surviving mice up to 7 months following tamoxifen administration at 1 month of age.  $P < 0.05$ , by log-rank (Mantel-Cox) test. (F) Ketone bodies in plasma of control and  $\beta$ PAX6 mice ( $n = 6$ ). Mice were injected with tamoxifen at 1 month of age and analyzed 3–5 months later. (G) Blood ketone bodies as a function of blood glucose levels in  $\beta$ PAX6 mice and other models of murine diabetes. Note that the highest levels of ketone bodies were not always detected in the most hyperglycemic mice. (H) Quantification of  $\alpha$  cell proportion of the total pancreatic area in  $\beta$ PAX6 mice aged 3–6 months. Tamoxifen was injected at 1 month of age.  $n = 4$  flox/flox and 7  $\beta$ PAX6 mice. (I) Positive correlation between blood ketone bodies and the fraction of glucagon-stained area in  $\beta$ PAX6 mice 3–6 months after tamoxifen injection ( $P < 0.05$ , by Spearman's correlation). Glucagon was measured and calculated from histological sections as the ratio between the area stained for glucagon and the total islet area. Each point represents 1 mouse. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ , by 2-tailed Student's  $t$  test.

adult  $\beta$  cells and that its downregulation in diabetes may contribute to loss of insulin and  $\beta$  cell dysfunction. This idea is particularly attractive, given the established genetic haploinsufficiency of PAX6 for glucose tolerance (27, 29, 34). Consistent with the reduction

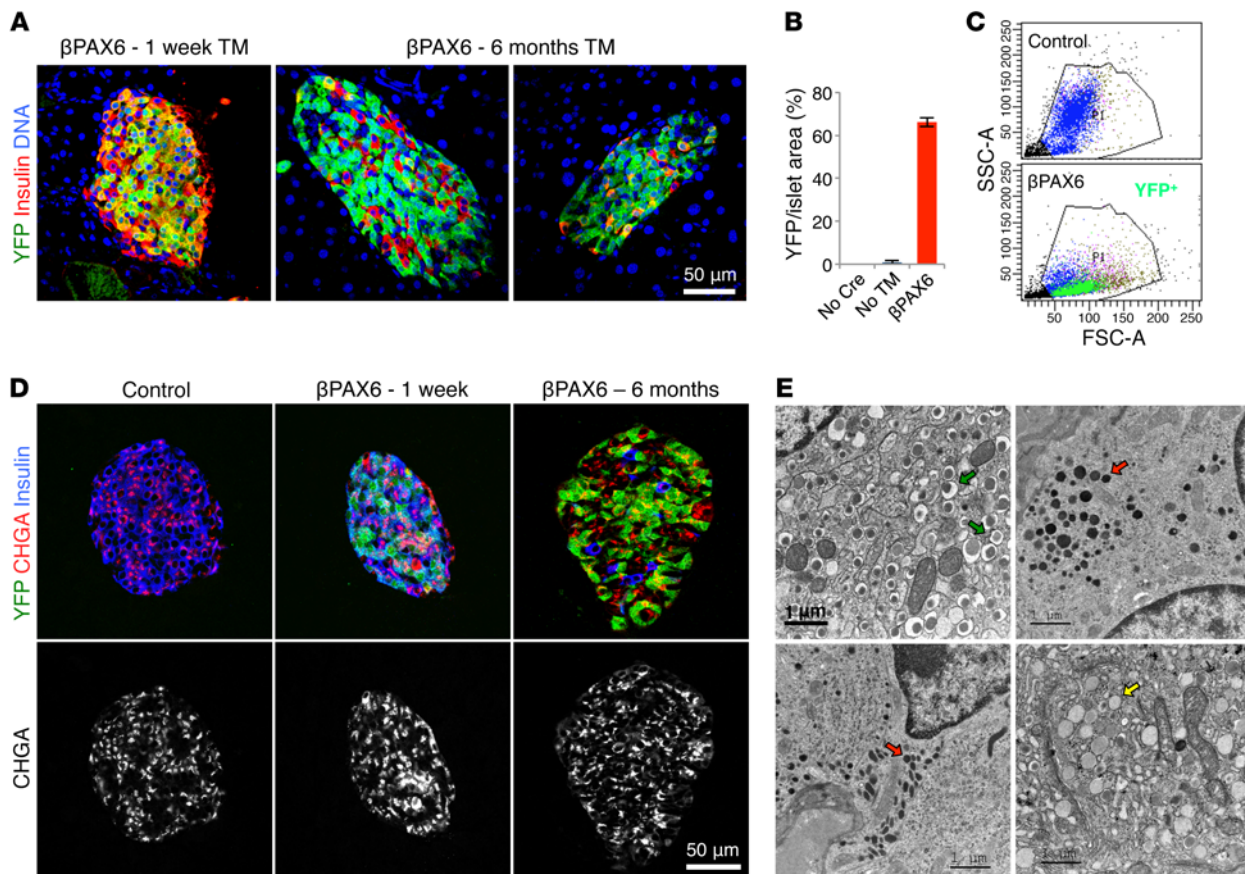
in protein levels, *Pax6* mRNA was reduced by approximately 55% in islets of diabetic *db/db* mice (Figure 1D), indicating regulation at the level of transcription or mRNA stability. Importantly, measurements of *Pax6* mRNA at various stages during the progression of *db/db* mice to diabetes revealed that it was downregulated in severely hyperglycemic (400–600 mg/dl glucose), but not in mildly hyperglycemic (200–400 mg/dl), mice (Figure 1E). A similar pattern was observed for other key  $\beta$  cell TFs — *Pdx1*, *Nkx6.1*, and *Mafa* — although *Mafa* levels were reduced in much lower levels of hyperglycemia, indicating a higher sensitivity to glucose (Figure 1F and Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI88015DS1). These findings suggest that PAX6 downregulation is probably not a primary driver of  $\beta$  cell failure and diabetes, but rather a responder and potential contributor to glucotoxicity.

To further investigate the control of *Pax6* expression in diabetes, we treated WT mice with the insulin receptor antagonist S961 (35). Four days of treatment led to severe hyperglycemia and resulted in a 40% decrease in *Pax6* mRNA levels compared with levels in controls (Figure 1G). These results indicate that downregulation of PAX6 in diabetes is not a peculiarity of *db/db* mice, but rather a response to metabolic changes associated with insulin resistance and hyperglycemia. Further work is required to pinpoint the molecular mechanisms that control PAX6 expression in diabetes.

**Efficient tamoxifen-induced deletion of *Pax6* in adult  $\beta$  cells.** To understand the function of PAX6 in mature  $\beta$  cells, we deleted the gene in  $\beta$  cells of adult mice. We generated *MIP-CreER Pax6<sup>f/f</sup> Rosa26-LSL-YFP* mice (referred to hereafter as  $\beta$ PAX6 mice) with a conditional deletion of *Pax6* and performed simultaneous genetic

lineage tracing of  $\beta$  cells. PAX6 was expressed in most  $\beta$  cells in WT mice as well as in *Pax6<sup>f/f</sup>* and  $\beta$ PAX6 mice in the absence of tamoxifen, confirming tight control of Cre recombinase activity (Supplemental Figure 2). Injection of tamoxifen into  $\beta$ PAX6 mice (2 doses of





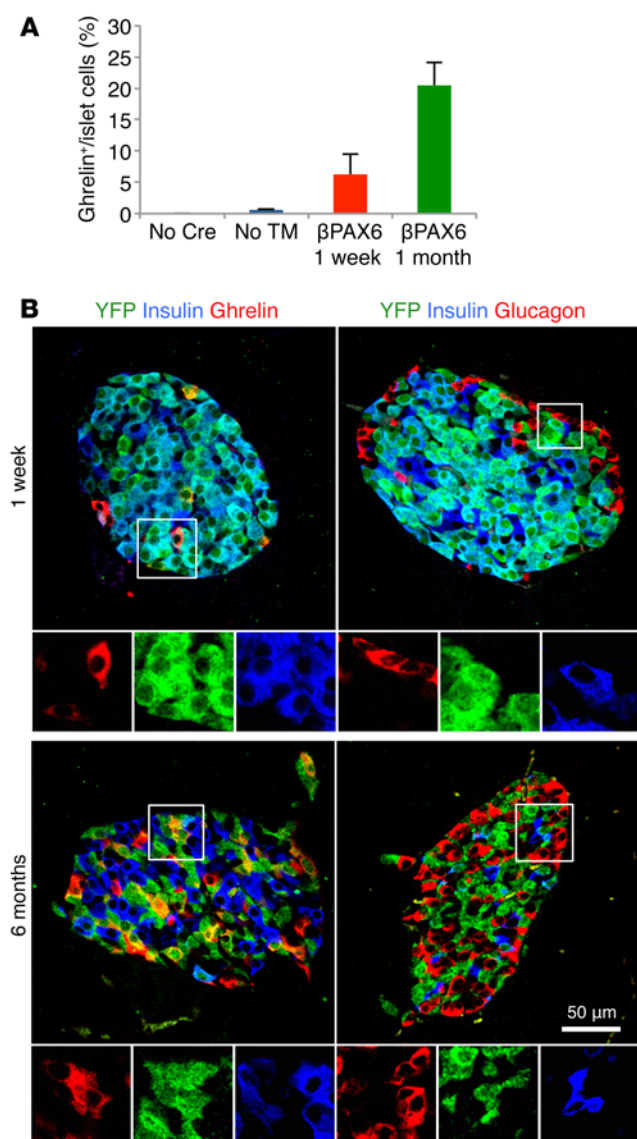
**Figure 3. Lineage tracing of *Pax6*-deleted  $\beta$  cells.** (A) Loss of insulin in *Pax6*-deleted  $\beta$  cells, labeled with YFP, after tamoxifen injection. Immunostaining for YFP (green), insulin (red), and DNA (blue). Note that most remaining insulin<sup>+</sup> cells at 6 months were YFP<sup>+</sup> (i.e., had probably escaped deletion of *Pax6*). Mice were injected with tamoxifen at 2 months of age and sacrificed 1 week or 6 months later as indicated. Original magnification,  $\times 400$ . (B) Quantification of YFP area versus total islet area in *Pax6*<sup>fl/fl</sup> mice (No Cre), *MIP-CreER Pax6*<sup>fl/fl</sup> *Rosa26-YFP* mice without tamoxifen (No TM), and *MIP-CreER Pax6*<sup>fl/fl</sup> *Rosa26-YFP* mice 1 week after tamoxifen injection ( $\beta$ PAX6).  $n = 3$  mice in each group. (C) FACS analysis of sorted YFP<sup>+</sup>  $\beta$  cells 3 weeks after tamoxifen injection.  $\beta$ PAX6 cells show a low side scatter (SSC) signal, probably indicating degranulation of  $\beta$  cells. The control sample was from a *Pax6*<sup>fl/fl</sup> (flox/flox) mouse. Analysis was performed on 4 samples of each genotype. FSC-A, forward scatter. (D) Pancreatic sections stained for chromogranin A (CHGA, red), YFP (green), and insulin (blue). Chromogranin was retained following long-term deletion of *Pax6*, suggesting retention of islet cell identity. Original magnification,  $\times 400$ . (E) Representative electron micrographs of pancreatic sections from a  $\beta$ PAX6 mouse at the age of 3 months, 2 months after tamoxifen injection. Top left: normal insulin granules in WT islet (green arrow); top right: atypical cell type with dark granules (red arrows); bottom left: another atypical cell (red arrows); bottom right:  $\beta$  cell with empty granules (yellow arrow). Samples from 3 flox/flox and 3  $\beta$ PAX6 mice were imaged by electron microscopy. Original magnification,  $\times 3,000$ .

8 mg) resulted in a rapid and efficient loss of PAX6 protein in more than 90% of  $\beta$  cells as soon as 1 week after injection. PAX6 expression persisted in insulin<sup>+</sup> cells in the periphery of the islet where  $\alpha$  and  $\delta$  cells are located, indicating  $\beta$  cell-specific deletion of *Pax6* (Supplemental Figure 2). We also sorted yellow fluorescent protein-positive (YFP<sup>+</sup>)  $\beta$  cells from  $\beta$ PAX6 and control mice and measured the mRNA levels of *Pax6* using quantitative reverse transcription PCR (qRT-PCR). One week after tamoxifen administration, *Pax6* mRNA was reduced by 80% in  $\beta$  cells isolated from  $\beta$ PAX6 islets compared with levels in controls, as expected (Supplemental Figure 2).

*Pax6* deletion in adult  $\beta$  cells leads to progressive, lethal diabetes. Blood glucose levels started to rise 12 days after tamoxifen injection, and by 21 days,  $\beta$ PAX6 mice became severely hyperglycemic (Figure 2A). As expected with a primary defect in  $\beta$  cells, hyperglycemia correlated with a reduction in plasma insulin levels in  $\beta$ PAX6 mice (Figure 2B) as well as a reduction of the insulin-to-glucose ratio (Figure 2C). Immunostaining of pancreatic

sections revealed a near-complete loss of insulin expression in many  $\beta$ PAX6 islets (Figure 2D). The number of remaining insulin<sup>+</sup> cells varied between mice, probably due to a variation in recombination efficiency, and was correlated with the glycemic state of the mice (i.e., mice with fewer insulin<sup>+</sup> cells had more severe hyperglycemia, Figure 2D).

Interestingly, 50% of the  $\beta$ PAX6 mice died 1–7 months after tamoxifen injection (Figure 2E). This finding is difficult to explain by hyperglycemia alone, since multiple mouse models of diabetes, in our laboratory and elsewhere, survive well with similar or even higher levels of blood glucose (36, 37). Since ketoacidosis is known to be an acute cause of death in diabetes, we hypothesized that  $\beta$ PAX6 mice had elevated levels of ketone bodies in their blood. Indeed, we found very high levels of both 3-hydroxybutyrate and acetoacetate in the plasma of  $\beta$ PAX6 mice compared with levels in control animals (Figure 2, F and G). By contrast, hyperglycemic mice expressing diphtheria toxin A (DTA) under an insulin-



**Figure 4. Origins of ghrelin- and glucagon-expressing cells in  $\beta$ PAX6 mice.** (A) Gradual increase in the fraction of islet cells expressing ghrelin in  $\beta$ PAX6 mice compared with expression in controls (No Cre and No TM, as in Supplemental Figure 2). All mice were 3–4 months of age, 1 week after tamoxifen injection, except for mice in the “1 month” group, which were analyzed 1 month after tamoxifen administration (green bar).  $n = 3, 6, 3,$  and  $2$  mice per group, respectively. (B) *Pax6*-deleted  $\beta$  cells strongly expressed ghrelin, but not glucagon, as shown by costaining of YFP (green), insulin (blue), and ghrelin or glucagon (red). Islets shown were from  $\beta$ PAX6 mice 1 week or 6 months after tamoxifen injection. Original magnification,  $\times 400$ .

driven, rtTA-responsive promoter (insulin-rtTa TET-DTA mice) (36), *db/db* mice, and NOD mice had minimal elevation of circulating ketones, supporting — though not proving — a link between ketones and death of diabetic  $\beta$ PAX6 mice (Figure 2G).

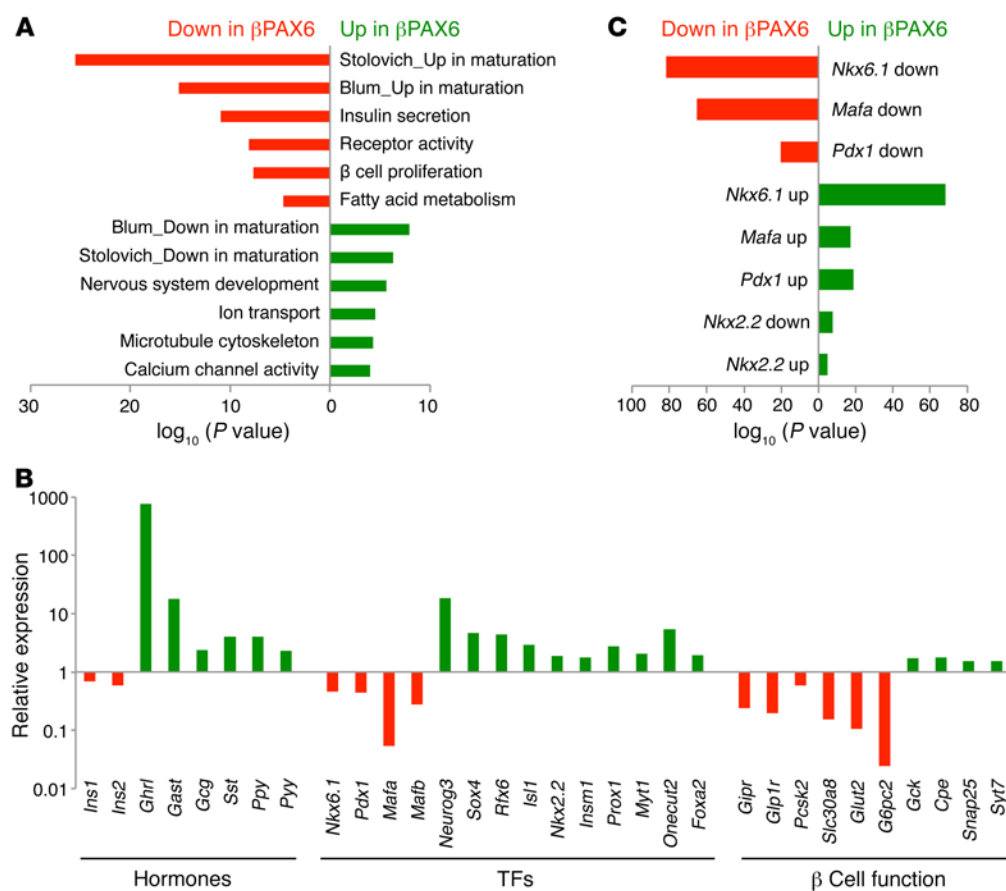
$\beta$ PAX6 mice with severe hyperglycemia or low levels of plasma insulin did not always show high blood ketone levels (Figure 2G and Supplemental Figure 3). Glucagon produced by  $\alpha$  cells is a central driver of ketone formation, acting by inducing the expression of ketone biosynthesis enzymes in liver and adipose tissue

(38–40). To test whether hyperactivity of  $\alpha$  cells could account for ketosis in  $\beta$ PAX6, we measured plasma glucagon levels and the area stained by glucagon in the pancreas. Mutant mice with high ketone levels had higher levels of circulating glucagon than did controls (Supplemental Figure 3), and  $\beta$ PAX6 mice had a higher proportion of pancreas area stained by an anti-glucagon antibody (Figure 2H). Moreover, plasma ketone body levels correlated with the abundance of  $\alpha$  cells in islets ( $P < 0.05$ , Spearman’s correlation test, Figure 2I, and see below). Thus, *Pax6* deletion in adult  $\beta$  cells causes loss of  $\beta$  cells, leading to hypoinsulinemia and hyperglycemia. In addition, this deletion results in hypersecretion of glucagon and severe ketosis, a feature rarely seen in mouse models of diabetes driven by insults to adult  $\beta$  cells.

*Pax6*-deleted  $\beta$  cells lose insulin but survive and maintain their endocrine identity. The loss of insulin staining in  $\beta$ PAX6 mice could result from extensive  $\beta$  cell death, or, alternatively, from loss of insulin expression and potentially other  $\beta$  cell markers, as was found upon *Foxo1* deletion (8). To distinguish between these possibilities, we monitored YFP expression, which serves as an indelible marker of  $\beta$  cells following tamoxifen injection. YFP was present in nearly all  $\beta$  cells 1 week after tamoxifen injection, and nearly all YFP<sup>+</sup> cells expressed insulin, indicating that YFP faithfully labeled  $\beta$  cells (Figure 3, A and B). Examination of islets at later time points after *Pax6* deletion revealed that YFP<sup>+</sup> cells remained abundant in islets but were insulin negative (Figure 3A). We found that the area occupied by YFP<sup>+</sup> cells was similar between  $\beta$ PAX6 and control *MIP-CreER Rosa26-LSL-YFP* mice (data not shown).  $\beta$ PAX6 islets did not show massive staining for the cell death marker TUNEL, although we observed more TUNEL<sup>+</sup> islet cells in mutant mice than in controls (Supplemental Figure 4), suggesting the possibility that a lack of PAX6 compromises  $\beta$  cell viability to some extent. While this remains to be further explored, we focused on the majority population of  $\beta$  cells that survived *Pax6* deletion. Analysis by flow cytometry showed greatly diminished cytoplasmic insulin content in YFP<sup>+</sup> cells from  $\beta$ PAX6 mice, manifested as decreased granularity (Figure 3C). These results indicate that *Pax6* deletion in  $\beta$  cells causes a gradual loss of insulin content, but that PAX6-deficient  $\beta$  cells largely remain alive.

YFP<sup>+</sup> cells in  $\beta$ PAX6 pancreata stained strongly for the endocrine marker chromogranin A, indicating that PAX6-deficient  $\beta$  cells retain their basic endocrine phenotype (Figure 3D and Supplemental Figure 5). This notion was supported by electron microscopic analysis of pancreatic sections from  $\beta$ PAX6 mice, which revealed an endocrine appearance of mutant islet cells, including secretory granules (Figure 3E). However, compared with control cells,  $\beta$ PAX6 islet cells were morphologically heterogeneous, with very few typical  $\beta$  cells. Some cells had granules that lost the typical dense core appearance, while other cells had bigger, dark granules, not existing normally in islets and reminiscent of ghrelin-producing cells in the stomach (Figure 3E and Supplemental Figure 6) (41). Thus, *Pax6* deletion causes a loss of insulin and ultrastructural features of  $\beta$  cells and results in the acquisition of alternative endocrine cell morphologies.

*Adult Pax6-deleted  $\beta$  cells switch from insulin to ghrelin expression.* To determine whether *Pax6*-deleted  $\beta$  cells acquired a new endocrine identity, we stained  $\beta$ PAX6 islets for all known islet hormones, including ghrelin, normally expressed only in fetal islets in mice.



**Figure 5. Transcriptomic analysis of PAX6-deficient  $\beta$  cells.** (A) Gene set enrichment analysis of sorted YFP<sup>+</sup> cells from  $\beta$ PAX6 mice ( $n = 3$ ) compared with YFP controls ( $n = 4$ ), 1 week after tamoxifen injection. Genes that were previously shown to be upregulated in mouse  $\beta$  cell maturation (“Blum” adults vs. postnatal day 10 [ref. 76]; “Stolovich” postnatal day 35 vs. day 18 [Stolovich-Rain, unpublished observations]; see Supplemental Table 3) were downregulated in *Pax6*-deleted  $\beta$  cells, as were gene sets of insulin secretion and  $\beta$  cell proliferation. On the other hand, genes that were shown to be downregulated in maturation, as well as genes of nervous system development, were upregulated in *Pax6*-deleted  $\beta$  cells. Interestingly, gene sets associated with ion transport and microtubule and calcium channel activity were also upregulated. (B) Fold change in mRNA of specific islet cell genes. Ghrelin was the top upregulated gene in *Pax6*-deleted  $\beta$  cells. The FDR was below 0.05 for all genes, except *Ins1* and *Gcg* ( $P < 0.05$ , FDR  $< 0.15$ ). (C) Enrichment analysis of differentially expressed genes obtained in KO models of other TFs essential in cells. *Nkx6.1* deletion in  $\beta$  cells showed the closest similarity to *Pax6* deletion.

One week after *Pax6* deletion, we found no change in the abundance or distribution of cells expressing glucagon, somatostatin, and pancreatic polypeptide; however, we observed a striking appearance of cells expressing ghrelin (Figure 4A and Supplemental Figure 5). Three weeks after tamoxifen injection,  $\beta$ PAX6 islets showed a further expansion of ghrelin staining as well as a dramatic increase in cells expressing glucagon or somatostatin (Supplemental Figure 5).

The abnormal abundance of cells expressing noninsulin hormones could emerge via a cell-autonomous mechanism (reprogramming of  $\beta$  cells deficient for PAX6) or, alternatively, a non-cell-autonomous mechanism (e.g., a paracrine effect of PAX6-deficient  $\beta$  cells on neighboring cells). To distinguish between these possibilities, we costained for pancreatic hormones and the  $\beta$  cell lineage marker YFP. Strikingly, ghrelin costained with YFP, indicating that ghrelin expression was initiated in cells that had expressed insulin at the time of tamoxifen injection. By contrast, glucagon, somatostatin, and pancreatic polypeptide did not costain with YFP (Figure

4B and Supplemental Figure 7). These results indicate that ghrelin expression occurs in  $\beta$  cells following *Pax6* deletion, while the expansion of  $\alpha$  and  $\delta$  cells occurs via a non-cell-autonomous mechanism.

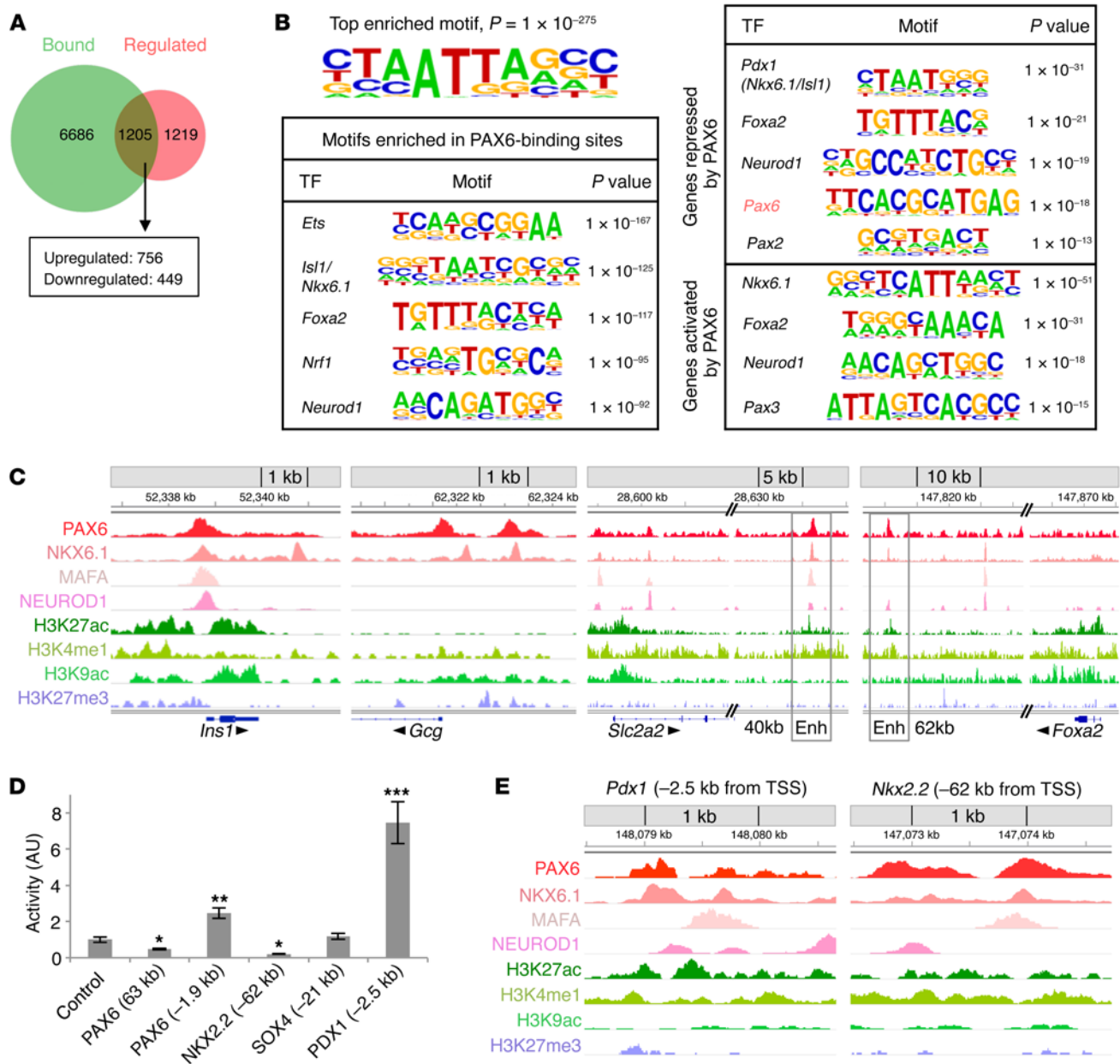
*Transcriptome-wide changes in  $\beta$  cell differentiation markers and TFs following Pax6 deletion.* To further characterize the phenotype of  $\beta$  cells following *Pax6* deletion, we determined their transcriptome. We sorted live YFP<sup>+</sup> cells from islets of control and  $\beta$ PAX6 mice 1 week after tamoxifen administration and performed RNA sequencing. Gene set enrichment analysis showed that the most significant change following *Pax6* deletion was a reduction in gene sets and genes, such as *Slc2a2* (encoding GLUT2), *G6pc2*, and *Slc30a8* (Figure 5, A and B, and Supplemental Tables 1 and 2), related to  $\beta$  cell maturation and insulin secretion. At the protein level, we observed a dramatic reduction in GLUT2 levels by immunostaining (Supplemental Figure 8). Consistent with this finding, key  $\beta$  cell TFs, including *Pdx1*, *Mafa*, and *Nkx6.1*, were downregulated (Figure 5B). The reduction of MAFA expression was evident even by immunostaining (Supplemental Figure 8).

Some gene sets associated with secretion or exocytosis,

e.g., those for nervous system development, ion transport, and calcium channels, were upregulated, in agreement with the observation that PAX6-deficient  $\beta$  cells retained a secretory phenotype (Figure 5A and Supplemental Table 1). Surprisingly, mRNAs encoding all pancreatic hormones, including ghrelin, gastrin, glucagon, somatostatin, and pancreatic polypeptide, were upregulated in PAX6-deficient cells (even though only ghrelin could be detected at the protein level; Figure 4B and Supplemental Figure 5). We also observed upregulation of multiple TFs (Supplemental Table 2), including some known regulators of non- $\beta$  cell programs, such as *Hhex* (a direct activator of somatostatin transcription) (42).

The specific downregulation of the  $\beta$  cell TFs *Mafa*, *Nkx6.1*, and *Pdx1* suggests that PAX6 maintains  $\beta$  cell identity through transcriptional activation of these genes. To examine this hypothesis, we compared transcriptomic changes that take place following deletion of each of these factors (6, 43, 44) with the changes in  $\beta$ PAX6 cells. The genes that were up- or downregulated upon





**Figure 6. PAX6 ChIP-seq analysis in  $\beta$  cells.** (A) Venn diagram showing the overlap of genes regulated by PAX6 (from the RNA-seq analysis in  $\beta$ PAX6 cells) with PAX6-bound genes (from ChIP-seq analysis in Min6 cells). (B) Top enriched motifs in all PAX6 peaks (left) and motifs differentially enriched in activated versus repressed genes in  $\beta$ PAX6 mice (right). (C) Maps of representative key  $\beta$  cell loci demonstrating the integration of PAX6 ChIP-seq data with other epigenomic data sets. Shown are PAX6-binding sites in the insulin and glucagon promoters and in *Foxa2* and *Slc2a2* (encoding GLUT2) enhancers (Enh, highlighted in rectangles; the distance from the transcription start site [TSS] is indicated). Note the active histone marks in insulin, *Foxa2*, and *Slc2a2* versus the absence of active marks in the glucagon (*Gcg*) promoter. (D) Luciferase activity upon cloning of different PAX6-bound elements to a pGL4.23 luciferase reporter vector and transfection into Min6 cells.  $n = 3$  per element. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ , by 2-tailed Student's  $t$  test. (E) Integrated map of histone marks and  $\beta$  cell TFs binding at PAX6-bound regions found by luciferase assay (see Figure 6D) to have enhancer (*Pdx1*) and silencer (*Nkx2.2*) activity. Note the higher enrichment of H3K27ac in the *Pdx1* enhancer region.

deletion of *Nkx6.1*, *Mafa*, and *Pdx1* were significantly enriched in the differential  $\beta$ PAX6 transcriptome (Figure 5C), consistent with the idea that the phenotype of  $\beta$ PAX6 cells is at least partly caused by downregulation of these TFs. Interestingly, there was no overlap with genes altered upon *Nkx2.2* deletion (45), a finding that was consistent with the lack of a major change in the expression of *NKX2.2* itself (which was, in fact, mildly induced). *NKX2.2* deficiency was previously shown to trigger massive expansion of

ghrelin cells at the expense of cells (46). The fact that *NKX2.2* and its targets were not significantly changed in  $\beta$ PAX6 cells suggests that PAX6 prevents ghrelin expression via an *NKX2.2*-independent mechanism. Overall, the transcriptomic changes observed strongly suggest that PAX6 is required to maintain the specific differentiation state of  $\beta$  cells in adults and to repress alternative islet cell expression programs. However, PAX6 is not involved in maintaining the general endocrine identity of cells or in their survival.



**Table 1. Histone marks and binding of other  $\beta$  cell TFs in genes bound and activated or bound and repressed by PAX6**

	H3K27ac	H3K4me1	H3K27me3	H3K9ac	MAFA	PDX1	NEUROD1	FOXA2	NKX6.1
Genes activated by PAX6	41%	21%	7%	3%	6%	28%	14%	17%	21%
Genes repressed by PAX6	26%	23%	15%	2%	2%	22%	11%	13%	15%

Numbers represent the percentage of PAX6 peaks that contain the indicated mark (see Supplemental Table 4 for details).

**Direct transcriptional targets of PAX6 in  $\beta$  cells.** To study the mechanism of gene regulation by PAX6 in  $\beta$  cells, we determined its direct targets using ChIP-sequencing (ChIP-seq) analysis in Min6 (mouse insulinoma) cells. PAX6 was physically associated with 9,789 genomic sites that were assigned to 7,891 genes (bound site and coding sequence located <100 kb apart). PAX6 was bound to either the promoters of genes (11% of bound sites, 1 kb from the transcription start site) or to distal sites. Among the PAX6-bound genes, 15% also showed significant changes in their steady-state mRNA levels in  $\beta$  cells lacking PAX6. The preferential binding of PAX6 to genes that it regulates was statistically significant (PAX6 was bound to 50% of the genes that were regulated by it transcriptionally, but to only 32% of all nonregulated genes;  $P < 0.005$  by hypergeometric test), suggesting a direct regulation of these genes by PAX6 (Figure 6A).

While recognized as both a transcriptional activator and repressor in the nervous system, PAX6 has thus far been shown to be only an activator of gene expression in  $\beta$  cells (26, 31). If this were the case, PAX6-bound genes in  $\beta$  cells should be downregulated upon its deletion. Surprisingly, two-thirds (756 of 1,205) of the genes bound and regulated by PAX6 were in fact activated in *Pax6*-deficient  $\beta$  cells (Figure 6A and Supplemental Table 4). This suggests that PAX6 functions as both a transcriptional activator and repressor in  $\beta$  cells. Genes directly repressed by PAX6 in  $\beta$  cells included the hormones glucagon, somatostatin (both of which were also upregulated nonautonomously in  $\beta$ PAX6 islets; Figure 4 and Supplemental Figure 7), and ghrelin and TFs including *Pax6* itself, *Isl1*, and *Foxa2* (Supplemental Table 5).

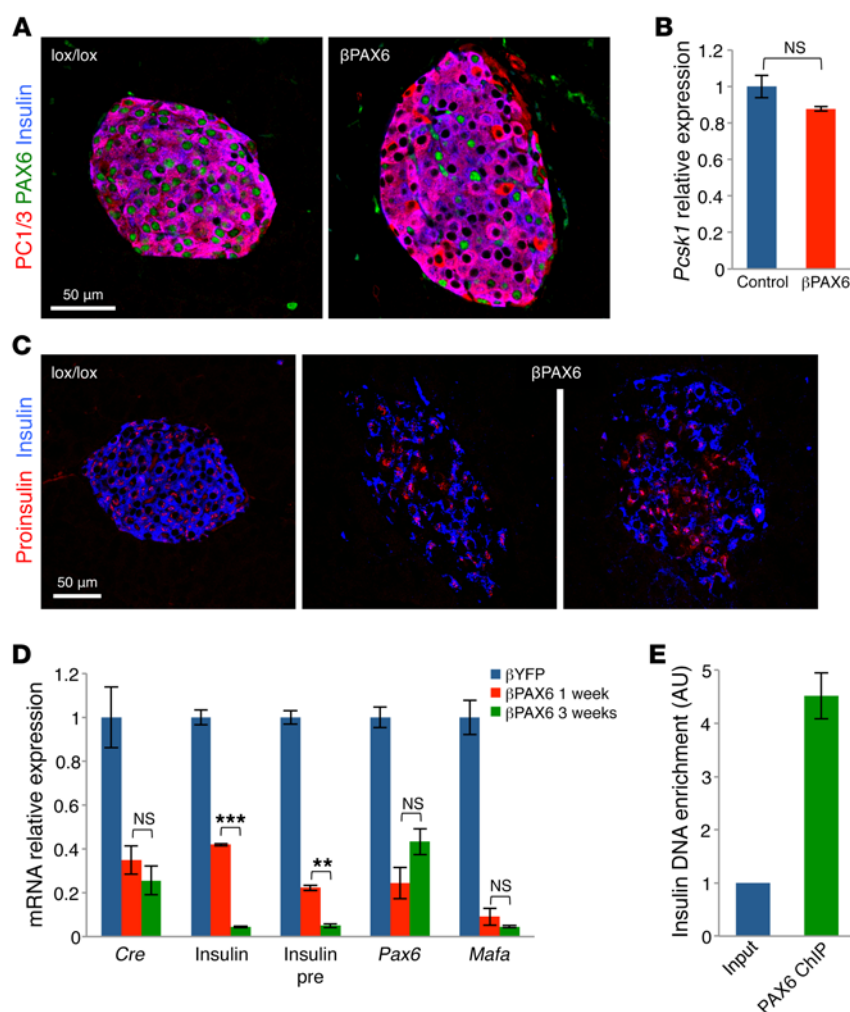
How PAX6 represses transcription is not fully understood. Studies in the developing eye and various cell lines have shown that 2 different splice variants of PAX6 bind distinct DNA motifs, leading to either transcriptional activation or repression (47–50). We found both PAX6 splice variants to be expressed at high and equivalent levels in  $\beta$  cells (data not shown), consistent with the idea that this mechanism is operative.

Our analysis of PAX6 ChIP-seq peaks for enrichment of recognition sequences identified a homeobox element highly similar to a known LIM homeobox (Lhx) and PAX4 motif, not previously recognized as a PAX6 motif, as the top enriched motif ( $P = 1 \times 10^{-275}$ ) (Figure 6B). The motif was enriched in both upregulated and downregulated genes. In contrast, the known PAX6 motif (51) was enriched only among the genes upregulated by *Pax6* deletion, suggesting that this motif is associated with PAX6-mediated transcriptional repression in  $\beta$  cells. Additional motifs overrepresented in PAX6-bound regions resembled the known targets of other  $\beta$  cell TFs such as ISL1, NKX6.1, FOXA2, NEUROD1, and PDX1, consistent with the notion that PAX6 cooperates with these factors in the regulation of  $\beta$  cell expression programs.

Next, we asked whether other  $\beta$  cell TFs and histone marks are differentially associated with PAX6-bound sites in the activated versus repressed genes. We overlapped the PAX6-bound sites with previously published whole-genome maps of monomethylated histone H3 at lysine 4 (H3K4me1, associated with enhancers); acetylated histone H3 at lysine 27 (H3K27ac, associated with active enhancers); acetylated histone H3 at lysine 9 (H3K9ac, associated with active promoters); and histone H3 lysine 27 trimethylation (H3K27me3, associated with repression) — all obtained from sorted mouse  $\beta$  cells (52). In addition, we overlapped PAX6 ChIP-seq peaks with whole-genome chromatin-binding maps of MAFA, NKX6.1, PDX1, FOXA2, and NEUROD1 (6, 52, 53). Genes activated by PAX6 (downregulated in mutant  $\beta$  cells) were enriched for the active enhancer mark H3K27ac, while genes repressed by PAX6 were enriched for the repressive mark H3K27me3, consistent with the idea that PAX6 functions as a direct repressor of these genes. The same pattern was observed in promoters and distal elements ( $P < 0.01$ ) (Figure 6C and Table 1). Interestingly, while key  $\beta$  cell TFs (PDX1, MAFA, NEUROD1, FOXA2, and NKX6.1) bind both PAX6-activated and PAX6-repressed genes (Supplemental Table 4), the genes activated by PAX6 were more likely to be bound by these factors (Table 1). These results raise the possibility that the effect of PAX6 on a particular gene that it binds — i.e., activation or repression — is related to the number and identity of additional  $\beta$  cell TFs occupying the same regulatory region.

Finally, to examine whether PAX6-bound sites can indeed function to enhance or silence gene expression, we selected several distal PAX6-bound elements associated with key islet genes and cloned them in front of a luciferase reporter gene driven by a minimal promoter. We transfected the reporters into Min6 cells and determined the potential regulatory activity of the cloned elements. Strikingly, we identified PAX6-bound sites that either activated or repressed luciferase expression, supporting the idea that PAX6 can function as a direct activator as well as a direct repressor of gene expression (Figure 6D). We then tested whether PAX6-bound regulatory elements that functioned in the luciferase assay as enhancers or silencers carried relevant histone marks in Min6 cells. Indeed, the bound site associated with *Nkx2.2* (which is repressed by PAX6 according to our RNA-seq analysis) showed a silencing activity in the reporter assay and was relatively depleted of the H3K27ac active enhancer mark; in contrast, the region associated with *Pdx1* (activated by PAX6, according to our RNA-seq analysis) showed enhancer activity and was relatively enriched for H3K27ac (Figure 6E). These findings support the idea that PAX6 functions as a direct activator as well as a direct repressor of gene expression in  $\beta$  cells.

**A mechanism for indirect negative regulation of *Neurog3* by PAX6 via direct repression of *Foxa2*.** Multiple TFs were upregulated in mutant



**Figure 7. Regulation of insulin expression and processing by PAX6.** (A) PC1/3 (encoded by *Pcsk1*) immunostaining was not reduced in *Pax6*-deleted  $\beta$  cells. Staining for PC1/3 (red), PAX6 (green), and insulin (blue) in cells from 3-month-old mice, 1 week after tamoxifen administration. Original magnification,  $\times 400$ . (B) *Pcsk1* mRNA level was not changed in sorted YFP $^{+}$   $\beta$  cells from  $\beta$ PAX6 mice, 1 week after tamoxifen administration.  $n = 4$  control, 3  $\beta$ PAX6 mice. (C) Proinsulin staining of  $\beta$ PAX6 and control pancreatic section showed no accumulation of proinsulin (red) in insulin $^{-}$  (blue) cells. Mice were littermates of those represented in A. Original magnification,  $\times 400$ . (D) Transcriptional downregulation of insulin after *Pax6* deletion. Reduced *Ins1* mRNA, *Ins1* pre-mRNA, *Cre* recombinase mRNA (driven by a transgenic insulin gene promoter), *Pax6* mRNA, and *Mafa* mRNA after deletion of *Pax6*. Analysis was performed on sorted YFP $^{+}$  cells from control ( $\beta$ YFP) and  $\beta$ PAX6 mice injected with tamoxifen at 1 month of age and sacrificed 1 or 3 weeks later.  $n = 3$  for each group of mice. (E) ChIP with PAX6 antibody in Min6 cells showing enrichment for the *Ins1* promoter ( $n = 2$ ) compared with input.  $**P < 0.01$  and  $***P < 0.001$ , by 2-tailed Student's *t* test.

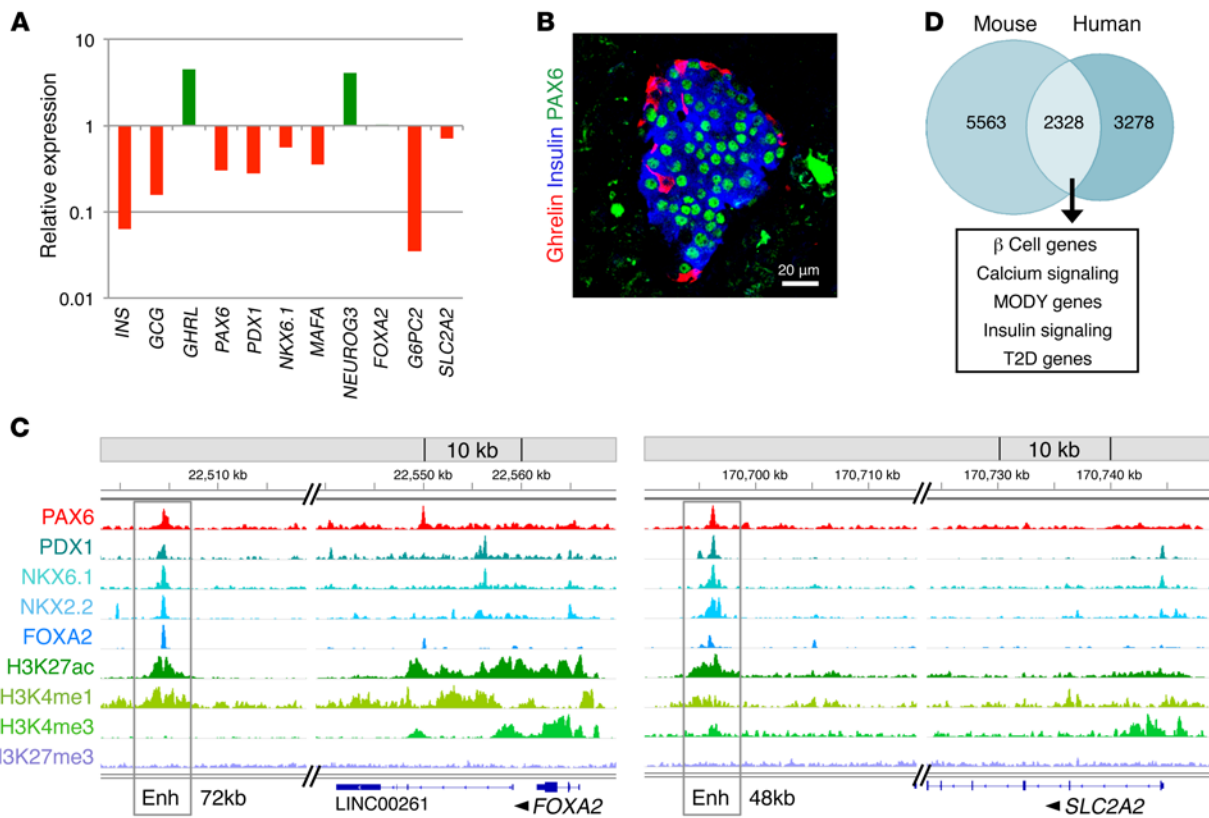
$\beta$  cells (Figure 5B). Most notably, the fetal endocrine progenitor cell marker *Neurog3* was elevated by 16-fold at the mRNA level, a finding that we confirmed by immunostaining (Supplemental Figure 8). PAX6 could directly repress the *Neurog3* promoter or, alternatively, could affect a transcriptional regulator of *Neurog3*. Since the ChIP-seq data did not reveal direct binding of PAX6 to the *Neurog3* gene, we examined the expression levels of factors, including HES1, SOX9, ONECUT1, TCF2, and FOXA2, known to regulate *Neurog3* expression during embryonic development (54, 55). Of these, *Foxa2* mRNA levels were 2-fold higher in PAX6-deficient  $\beta$  cells (Figure 5B), suggesting a scenario in which PAX6 contributes to the silencing of *Neurog3* transcription in adult  $\beta$  cells by repressing *Foxa2*. Consistent with this model, our ChIP-seq data revealed that PAX6 was bound to a *Foxa2* enhancer (52) (Figure 6C). Thus, PAX6 represses *Neurog3* expression in adult  $\beta$  cells, and this probably occurs, at least in part, via repression of the *Neurog3* regulator FOXA2. Whether derepressed *Neurog3* in PAX6 mutants contributes to the phenotype of mutant cells remains to be determined via genetic loss-of-function experiments. Finally, *Neurog3* is normally activated only transiently before shutting off its own promoter. The persistence of *Neurog3* expression in PAX6-deficient  $\beta$  cells raises the possibility that PAX6 participates in the autoregulation of *Neurog3*.

In summary, *Pax6* deletion in adult  $\beta$  cells leads to the downregulation of  $\beta$  cell-specific expression programs and their

upstream TFs. In parallel, PAX6 deficiency derepresses alternative islet hormone genes and alternative islet TFs.

*PAX6 directly regulates transcription from the insulin gene promoter.* We performed experiments to better understand the loss of insulin in PAX6-deficient  $\beta$  cells. Previous studies suggested that PAX6 regulates insulin by direct binding of the gene promoter as well as by regulating the expression of prohormone convertase 1/3 (PC1/3), which is essential for insulin processing (22, 26, 31, 56, 57). PAX6-deficient  $\beta$  cells expressed PC1/3 strongly at both the mRNA and protein levels (Figure 7, A and B), indicating that PC1/3 is not activated by PAX6 in adult  $\beta$  cells. In addition, no accumulation of proinsulin was observed in  $\beta$ PAX6 islets, again, arguing against a block in insulin processing. In fact, proinsulin levels were decreased in  $\beta$ PAX6 islets (Figure 7C), suggesting that PAX6 regulates insulin expression at a stage prior to translation.

Next, we examined the effect of PAX6 on insulin transcription. We used the *CreER* transgene, driven by the mouse insulin promoter, as a reporter for insulin promoter activity. *CreER* mRNA was significantly reduced as early as 1 week after tamoxifen administration (Figure 7D), suggesting that PAX6 is needed for activation of the insulin promoter. Insulin mRNA levels decreased by only 2-fold 1 week after tamoxifen administration but dropped further 2 weeks later. A similar pattern was observed for insulin pre-mRNA levels (Figure 7D). The different kinetics of decay of



**Figure 8. PAX6 function in human  $\beta$  cells.** (A) Change in  $\beta$  cell gene expression following PAX6 knockdown in human EndoC- $\beta$ H2 cells. (B) Ghrelin<sup>+</sup> cells in human islets did not express PAX6, as shown by costaining of human pancreatic sections for PAX6 (green), ghrelin (red), and insulin (blue). Material was from a 9-year-old healthy donor (obtained from nPOD). Original magnification,  $\times 400$ . (C) PAX6-binding sites in *FOXA2* and *SLC2A2* (encoding GLUT2) enhancers (Enh) (enhancers are highlighted in rectangles; the distance from the transcription start site is indicated). Note the active enhancer histone marks as well as the binding of all other  $\beta$  cell TFs in the same genomic position. (D) Venn diagram showing the overlap of genes bound by PAX6 in murine and human  $\beta$  cells. Note the  $\beta$  cell-specific gene sets enriched in the common group (with *P* values of  $1.58 \times 10^{-21}$ ,  $1.5 \times 10^{-16}$ ,  $6.05 \times 10^{-14}$ ,  $6.65 \times 10^{-12}$ , and  $5.07 \times 10^{-10}$ , respectively). MODY, maturity-onset diabetes of the young; T2D, type 2 diabetes.

steady-state mRNA levels of the *CreER* and insulin mRNAs are likely the result of very different rates of mRNA degradation, as the insulin mRNA has a half-life in excess of 3 days in rodent  $\beta$  cells (58). We also examined the expression dynamics of *Mafa*, a direct activator of insulin transcription and a known target of PAX6 (59). The reduction in *Mafa* mRNA was rapid and occurred maximally already 1 week after tamoxifen administration, as with the kinetics of *Pax6* loss. These results suggest that *Pax6* deletion in  $\beta$  cells shuts off insulin transcription rapidly and completely.

PAX6 can activate insulin transcription indirectly, through other TFs such as MAFA, or directly, by binding to the insulin gene promoter. The ChIP-seq data revealed that PAX6 binds directly to the insulin promoter (Figure 6C), which was also validated by qRT-PCR (Figure 7E), consistent with previous reports (26, 31). These results indicate that PAX6 is an essential positive regulator of insulin gene transcription, acting directly, by binding to the insulin gene promoter, as well as indirectly, by activating MAFA and potentially other insulin TFs.

**PAX6 expression and function in human  $\beta$  cells.** To evaluate the role of PAX6 in human cells, we suppressed PAX6 levels in human EndoC- $\beta$ H2 cells (60) using retroviral delivery of PAX6-specific siRNA. Reducing PAX6 expression in these cells yielded transcriptional changes similar to those observed in mouse  $\beta$  cells. In

particular, ghrelin and *NEUROG3* were upregulated, while insulin, *G6PC2*, and other  $\beta$  cell TFs were downregulated (Figure 8A). Unlike in adult mouse  $\beta$  cells, glucagon mRNA was downregulated in EndoC- $\beta$ H2 cells. Consistent with ghrelin upregulation upon PAX6 silencing, immunostaining of human pancreatic sections showed that PAX6 was expressed in all islet cells except for ghrelin cells, suggesting that PAX6 represses ghrelin expression in human islets, as it does in murine islets (Figure 8B).

To determine the genomic binding locations of PAX6 in human  $\beta$  cells, we performed ChIP-seq on EndoC- $\beta$ H2 cells (Figure 8C and Supplemental Table 6). By overlapping PAX6-bound sites with previously published data on ChIP-seq of histone modifications and  $\beta$  cell TFs (14, 61), we identified distal regulatory elements. Importantly, some of these elements, such as those associated with *FOXA2* and *SLC2A2*, are conserved between mice and humans (Figure 8C). Of 5,606 genes annotated to EndoC PAX6 ChIP-seq peaks, 2,328 (41.5%) are also bound by PAX6 in mouse Min6 cells. Strikingly, this group of genes commonly bound by PAX6 in mice and humans is highly enriched for  $\beta$  cell function genes such as *PDX1*, *NKX6.1*, *HNF1A*, and *FOXA2* (Figure 8D and Supplemental Table 6). These findings suggest that the functions of PAX6 as an activator and repressor of genes in adult  $\beta$  cells are conserved from mice to humans.



## Discussion

Our experiments show that PAX6 expression is reduced in  $\beta$  cells of hyperglycemic and insulin-resistant mice and demonstrate the importance of proper expression levels of PAX6 for the maintenance of  $\beta$  cell identity and glucose homeostasis. PAX6 emerges as a key regulator of the differentiated state of  $\beta$  cells, with additional non-cell-autonomous effects on other islet cells, and as a novel candidate effector of  $\beta$  cell failure under metabolic stress. The molecular functions of PAX6 appear to be conserved from mice to humans.

*Pax6 and transcriptional regulation of  $\beta$  cell identity.* We found that PAX6 is needed for maintaining the proper expression of  $\beta$  cell genes as well as for repressing alternative islet cell genes, most notably hormones and transcriptional regulators of other islet cell types. This adds to the growing recognition that adult islet cells maintain a considerable degree of plasticity within the islet endocrine lineage (4–7, 15, 16). In fact, the promiscuous expression of genes typical of multiple islet cell types appears to be the default of  $\beta$  cells, and PAX6 activity is required to repress this default; this is most clearly seen in the case of ghrelin, a fetal islet hormone typically shut off in adult  $\beta$  cells that is dramatically upregulated in  $\beta$  cells lacking PAX6. Conceptually similar findings were reported for PDX1 (needed to prevent expression of glucagon in adult  $\beta$  cells) (5) and NKX6.1 (needed to prevent somatostatin expression in adult  $\beta$  cells) (6). We speculate that islet cells have a stable “ground state,” which includes expression of the generic endocrine machinery, as well as low-level expression of markers of multiple islet cell types (including different hormone genes). TFs such as PAX6 sharpen this generic program into a mature endocrine cell type, with high expression of relevant genes and repression of irrelevant genes. Repression of *Neurog3*, a key regulator of the generic endocrine identity during development (which is derepressed in PAX6-deficient  $\beta$  cells), could be involved in the progression from a ground endocrine state to a mature, fully differentiated islet cell. More experiments are needed to test this idea, which could be relevant for efforts to drive stem cell-derived endocrine cells into functional  $\beta$  cells.

In  $\beta$  cells, PAX6 is typically described as a transcriptional activator (26, 31), although studies in other tissues and cell lines have shown that it can also act as a direct repressor (47, 49, 50). Our results from RNA-seq, ChIP-seq, and luciferase reporter experiments provide strong evidence that PAX6 acts simultaneously as a direct repressor and activator of transcription in adult  $\beta$  cells via DNA elements in promoters, enhancers, and silencers. More work is needed to understand what determines whether a particular gene will be repressed or activated in  $\beta$  cells by PAX6 binding. Moreover, PAX6 is ubiquitously expressed in hormone-producing islet cells, and in each islet cell type, it is required for maintaining a particular identity, sometimes by controlling opposing genes and pathways in different cell types (26, 32). The differential effects of PAX6 in different islet cell types likely involve cooperation with cell-type-specific factors or coregulators. In the case of  $\beta$  cells, we identified a striking resemblance between the genes controlled by PAX6 and the genes regulated by PDX1, MAFA, and NKX6.1. The similarity in the transcriptomes of mutants is at least in part driven by the fact that PAX6 controls the expression of all these TFs; we speculate that, in addition, PAX6 cooperates with these TFs

to directly control the expression of target genes. Indeed, PAX6-bound sites contain DNA motifs recognized by these TFs and are physically bound by these factors.

*PAX6 in glucose homeostasis and diabetes.* Our finding that PAX6 levels are reduced in islets of diabetic *db/db* mice as well as in WT mice rendered hyperglycemic by an insulin receptor antagonist suggests that metabolic downregulation of PAX6 expression may play a role in the pathogenesis of diabetes. Previous studies reported a modest downregulation of PAX6 in  $\beta$  cells cultured in high glucose (62) and in  $\beta$  cells exposed to oxidative stress (7). However, in our hands, a 2-day treatment of murine and human islets with 25 mM glucose or palmitate failed to reduce *Pax6* mRNA levels (data not shown), suggesting a more complex mechanism underlying the regulation of expression in hyperglycemia *in vivo*. Haploinsufficiency of PAX6 in humans does interfere with  $\beta$  cell function and leads to abnormal glucose homeostasis, suggesting that even partial effects on PAX6 expression may have glyce-mic consequences (27, 28). We note, however, that in expression profiles obtained from type 2 diabetic donors using both whole islets and single  $\beta$  cells, no significant reduction in PAX6 expression levels was observed compared with levels detected in non-diabetic donors (7, 63, 64). This may be attributed to euglycemia in those patients. More work is required to definitely determine whether PAX6 expression is altered in human diabetes.

How can reduced PAX6 expression contribute to diabetes? Our findings suggest several potential mechanisms. First, *Pax6* deletion causes a massive loss of  $\beta$  cell identity (potentially in addition to increased  $\beta$  cell death), leading to hypoinsulinemia and hyperglycemia. Second, ghrelin secretion from mutant  $\beta$  cells may further inhibit insulin secretion from remaining functional  $\beta$  cells via mechanisms proposed elsewhere (65–67). We note, however, that thus far, there is no evidence for expression of ghrelin in islets of human patients with diabetes. Third, the non-cell-autonomous expansion of somatostatin-expressing cells may further reduce insulin secretion (68). Fourth, the expansion of  $\alpha$  cells may contribute to a worsening of hyperglycemia and to the unique metabolic feature of  $\beta$  cell PAX6 deficiency, namely severe ketosis.

The latter possibility may relate to the emerging view that the pathogenesis of diabetes involves simultaneous  $\beta$  and  $\alpha$  cell dysfunction (69, 70) and that hypersecretion of glucagon is a key driver of diabetic symptoms (71). It has been difficult to determine the cause of  $\alpha$  cell hyperfunction in diabetes.  $\alpha$  Cell hyperfunction could result from a non-cell-autonomous effect of defective  $\beta$  cells (71) or from reprogramming of  $\beta$  cells into glucagon-expressing cells (8). Diabetes in the  $\beta$ PAX6 model includes key features resulting from abnormal function of  $\alpha$  cells (i.e., hyperglucagonemia and the resulting ketosis). Given the nature of this genetic model, the source of the defect has to be the  $\beta$  cell. Moreover, the use of genetic lineage tracing showed that, in this case, the  $\beta$  cell defect was affecting  $\alpha$  cells via a non-cell-autonomous mechanism. It will be interesting to determine why PAX6 deficiency and some types of diabetes (e.g., human type 1 diabetes), but not other insults to  $\beta$  cells (e.g., DTA-mediated  $\beta$  cell killing) (36), induce neighboring  $\alpha$  cells to expand and hypersecrete glucagon to produce ketones.

Regardless of the mechanism leading to  $\alpha$  cell dysfunction, the phenotype of mice deficient for PAX6 in  $\beta$  cells raises the possibility that there might be different types of  $\beta$  cell failure in diabetes,



resulting from different molecular miswirings of the  $\beta$  cell, which may trigger distinct aspects of diabetes. Clinically, a wide range of diabetic phenotypes is observed (e.g., including or not including ketoacidosis, with or without severe hyperglycemia). We propose that these phenotypes do not reflect a simple graded scale of severity (modulated by peripheral tissue biology), but rather distinct molecular types of  $\beta$  cell failure.

In conclusion, our work, together with recent studies (32, 33), highlights PAX6 as a key transcriptional regulator of adult  $\beta$  cell identity and function and suggests that reduced PAX6 expression may contribute to  $\beta$  cell failure in diabetes.

## Methods

**Mice.** The mouse strains used in this study were *Pax6*<sup>fl/fl</sup> (72), *MIP-CreER* (73), and *Rosa26-LSL-EYFP* (all from The Jackson Laboratory). Tamoxifen (20 mg/ml in corn oil; Sigma-Aldrich) was injected s.c. into 1- to 2-month-old adult mice. Two daily doses of 8 mg were used to achieve near-total deletion of *Pax6* in  $\beta$  cells. PAX6-deficient mice are referred to herein as  $\beta$ PAX6 mice. Littermate controls are referred to herein as flox/flox (for *Pax6*<sup>fl/fl</sup> *Rosa26-LSL-EYFP*) or  $\beta$ YFP (for *MIP-CreER* *Rosa26-LSL-EYFP*) mice.

*db/db* mice (purchased from Envigo) and their C57BL/6J control littermates were used at the age of 3 months. Only hyperglycemic (blood glucose >300 mg/dl) *db/db* mice were used for the experiments.

Measurements of blood glucose and plasma insulin levels were performed as described elsewhere (36). Plasma glucagon was measured with the Crystal Chem Glucagon ELISA Kit. Ketone bodies were measured with FreeStyle Optium  $\beta$  Ketone Test Strips.

**Immunostaining.** Paraffin sections (5- $\mu$ m-thick) were prepared from formalin-fixed, paraffin-embedded pancreata. Sections were rehydrated, and antigen retrieval was performed. A list of the primary antibodies used and their details are provided in Supplemental Table 7. Secondary antibodies were conjugated to CY2 (1:200), CY3 (1:500), or CY5 (1:500; Jackson ImmunoResearch). Immunofluorescence images were captured using a Nikon C2 confocal microscope.

**Isolation of islets of Langerhans.** Islets were isolated using collagenase P (Roche) injected into the pancreatic duct, followed by Histopaque gradient (1119 and 1077; Sigma-Aldrich). Dissociation into single cells was performed by standard trypsinization.

**Flow cytometry.** For FACS analysis, dissociated islet cells were fixed and stained by the Cytofix/Cytoperm method (BD Biosciences). Cells were stained with guinea pig anti-insulin (1:500; Dako, Agilent Technologies) and rabbit anti-PAX6 (1:200; EMD Millipore), followed by secondary antibodies (Cy2 or Cy5 conjugated; Jackson ImmunoResearch).

**Transmission electron microscopy.** Pancreas was fixed with 4% paraformaldehyde and 2.5% glutaraldehyde (Electron Microscopy Sciences [EMS]), post fixed with 1% osmium tetroxide (Sigma-Aldrich), and dehydrated with increasing concentrations of ethanol, followed by propylene oxide (Sigma-Aldrich). For embedment, we used Agar 100 Resin (Agar Scientific). For imaging, we used 80-nm sections stained with 5% uranyl acetate for 10 minutes, followed by 10 minutes with lead citrate. Samples were visualized with a JEM-1400 Plus transmission electron microscope (Jeol) equipped with a Gatan CCD camera.

**RNA.** RNA was isolated and purified from sorted YFP<sup>+</sup>  $\beta$  cells with TRI Reagent (Sigma-Aldrich) and an RNeasy Micro Kit (QIAGEN). For gene expression profiling, RNA-seq libraries were prepared and sequenced as previously described (14).

Gene set enrichment analysis based on the hypergeometric test was performed using Genomica Software (<http://genomica.weizmann.ac.il/>). Gene sets with a *P* value below 0.01 and an FDR below 0.05 were considered significant. Gene sets were from Gene Ontology (GO) or compiled from the literature as described in the text.

For qPCR assays, cDNA was synthesized using 1 ng RNA and a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). For qRT-PCR, we used SYBR Green Mix (Quanta Biosciences) and the primers listed in Supplemental Table 7. Reactions were performed in triplicate in 96-well plates using a CFX96 Real-Time System (Bio-Rad).

**Luciferase reporter assays.** PAX6-bound genomic regions (140–200 bp around the peak center; see the primer sequences listed in Supplemental Table 7) were cloned into a pGL4.23 luciferase reporter plasmid upstream of a minimal promoter and transiently transfected into Min6 cells. Luciferase activity was normalized against the activity of a cotransfected *Renilla* construct, and the mean values  $\pm$  SEM are shown relative to empty vector.

**ChIP.** Chromatin was obtained from mouse Min6 cells (cultured in DMEM with 15% FCS and 25 mM glucose) and from human EndoC- $\beta$ H2 cells (cultured as previously described) (60). Chromatin was prepared and immunoprecipitated with rabbit anti-PAX6 (Bio-Legend). ChIP-seq libraries were prepared and sequenced as previously described (14). Enrichment of the mouse insulin promoter fragments was also tested by qPCR with the following primers: forward, 5'-CTGGGGAATGATGTGGAAAT-3' and reverse, 5'-GACCAGATGGCCTGATGAAC-3'. DNA motif enrichment was performed using HOMER (74). Integrative Genomics Viewer (IGV) software was used for visualization of PAX6 peaks and additional epigenomic data (75). For integration of PAX6 genomic binding sites with whole-genome histone modifications and TF maps, we overlapped PAX6-bound sites revealed by ChIP-seq analysis of Min6 and EndoC-H2 cells with previously published ChIP-seq data for mouse and human islets or sorted  $\beta$  cells, including the histone modifications H3K4me1, H3K4me3, H3K27me3, H3K27ac, and H3K9ac and the  $\beta$  cell TFs PDX1, NKX6.1, FOXA2, MAFA, MAFB, NEUROD1, and NKX2.2. We used an overlap of 1 bp. For histone modifications, wings of 400 bp were added to PAX6-bound sites.

**Pax6 gene suppression.** Proliferating EndoC- $\beta$ H2 cells were infected with a lentivirus expressing Cre (pTRIPAU3-CMV-nlsCre) (60). Two weeks after Cre infection, cells were infected with a pLKO.1-Puro-shPAX6 plasmid carrying the targeting sequence CGTC-CATCTTTGCTTGGGAAA to silence PAX6. One week after puromycin selection, RNA was extracted from the cells and used to demonstrate efficient suppression of PAX6 and changes in the expression of additional genes.

**Accession numbers.** The genomic data reported here were deposited in the NCBI's Gene Expression Omnibus (GEO) database (GEO GSE87530).

**Statistics.** Statistical analyses were performed using an unpaired 2-tailed Student's *t* test. Data are presented as mean  $\pm$  SEM, unless otherwise indicated.

**Study approval.** The joint ethics committee (IACUC) of the Hebrew University and Hadassah Medical Center approved the study protocol for animal welfare. The Hebrew University is an American Association for the Accreditation of Laboratory Animal Care (AAALAC) International-accredited institute.

## Author contributions

AS, RA-P, and YD designed the study. AS, NE, DA, JZ, RA-P, KHK, BG, and YD designed the experiments. AS, DA, NE, EF, TD, MS-R, YC-T, and RA-P conducted the experiments and analyzed the data. JZ conducted the ChIP experiments. AS, DA, and YD wrote the manuscript.

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