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*J Clin Invest.* 2017;127(1):230-243. [https://doi.org/10.1172/JCI88015](https://doi.org/10.1172/JCI88015).

Type 2 diabetes is thought to involve a compromised β 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 β cell identity and function. PAX6 was downregulated in β 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 β cells of adult mice led to lethal hyperglycemia and ketosis that were attributed to loss of β cell function and expansion of α cells. Lineage-tracing, transcriptome, and chromatin analyses showed that PAX6 is a direct activator of β cell genes, thus maintaining mature β 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 β cells. We conclude that reduced expression of PAX6 in metabolically stressed β cells may contribute to β cell failure and α cell dysfunction in diabetes.

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PAX6 maintains β cell identity by repressing genes of alternative islet cell types

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Type 2 diabetes is thought to involve a compromised β 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 β cell identity and function. PAX6 was downregulated in β 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 β cells of adult mice led to lethal hyperglycemia and ketosis that were attributed to loss of β cell function and expansion of α cells. Lineage-tracing, transcriptome, and chromatin analyses showed that PAX6 is a direct activator of β cell genes, thus maintaining mature β cell function and identity. Importantly, 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 β cells. We conclude that reduced expression of PAX6 in metabolically stressed β cells may contribute to β cell failure and α cell dysfunction in diabetes.

Introduction

Mature pancreatic β 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 β 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 β 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 β 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 β cell death, or, alternatively, in loss of β cell identity.

Interestingly, the loss of β cell differentiation is often accompanied by acquisition of alternative cellular identities (5, 8, 9), reflecting the retention of some developmental plasticity in differentiated β 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 β cell plasticity is largely confined to the islet program. The potential of metabolically stressed β cells to dedifferentiate and then redifferentiate into non-β cell fates was proposed as a novel mechanism underlying reversible β cell failure in diabetes (8, 10–13). Plasticity between α and β 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 β cells. For example, it was shown that near-total ablation of β cells in mice results in the spontaneous conversion of some δ cells (15) or α cells (16) to functional β 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 β cell failure as well as expansion of β cell mass in diabetes.

In this study, we focus on the role of the paired and homeodomain TF PAX6 in adult β 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 α cells and β 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

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Conflict of interest: The authors have declared that no conflict of interest exists.
Submitted: April 11, 2016; Accepted: October 13, 2016.
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 β cell lines, PAX6 was shown to bind and activate the promoters of insulin and additional β 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 α or β 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 β 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 β cells led to lethal hyperglycemia and ketosis due to loss of β cell differentiation, combined with expansion of WT α cells. Genome-wide molecular analysis revealed that PAX6 is an essential direct activator of key genes responsible for β cell identity and function; in parallel, PAX6 binds promoters and enhancers of genes normally silenced in β cells to directly repress alternative islet cell programs. We also provide evidence that these functions of PAX6 are conserved in human β cells. The findings highlight the fragility of β cell identity and establish PAX6 as a central determinant of adult β cell differentiation state and function. We propose that downregulation of PAX6 in metabolically stressed β cells may contribute to both β cell failure and α cell dysfunction in diabetes.

Results

Reduced expression of PAX6 in β 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 β 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 β cells of db/db mice (Figure 1B). Strikingly, PAX6 levels correlated with cellular insulin content in individual β 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 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 = –0.3, 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 S961 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.

Figure 1. PAX6 expression in adult β cells from db/db mice and after treatment with an insulin receptor antagonist. (A) PAX6 protein levels in β 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+ 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 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 = –0.3, 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 S961 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.
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 β 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 β cell failure and diabetes, but rather a responder and potential contributor to glucoxicity.

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.

**Figure 2. Hyperglycemia, hypoinsulinemia, hyperketonemia and loss of β cells in βPAX6 mice.** (A) Blood glucose levels over time in Pax6fl/fl (flox/flox) and βPAX6 mice following tamoxifen (TM) injection. Tamoxifen was injected into mice at 6 weeks of age. n = 13 flox/flox and 17 βPAX6 mice. (B) Plasma insulin levels in βPAX6 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 βPAX6 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, ×400. (E) Survival of βPAX6 mice. n = 13 flox/flox and 35 βPAX6 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 βPAX6 mice. (G) Quantification of ketone bodies as a function of blood glucose levels in βPAX6 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 a cell proportion of the total pancreatic area in βPAX6 mice aged 3–6 months. Tamoxifen was injected at 1 month of age. n = 4 flox/flox and 7 βPAX6 mice. (I) Positive correlation between blood ketone bodies and the fraction of glucagon-stained area in βPAX6 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.
sections revealed a near-complete loss of insulin expression in many βPAX6 islets (Figure 2D). The number of remaining insulin+ 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+ cells had more severe hyperglycemia, Figure 2D).

Interestingly, 50% of the βPAX6 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 βPAX6 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 βPAX6 mice compared with levels in control animals (Figure 2, F and G).

8 mg) resulted in a rapid and efficient loss of PAX6 protein in more than 90% of β cells as soon as 1 week after injection. PAX6 expression persisted in insulin+ cells in the periphery of the islet where α and δ cells are located, indicating β cell–specific deletion of Pax6 (Supplemental Figure 2). We also sorted yellow fluorescent protein–positive (YFP+) β cells from βPAX6 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 β cells isolated from βPAX6 islets compared with levels in controls, as expected (Supplemental Figure 2).

Pax6 deletion in adult β cells leads to progressive, lethal diabetes. Blood glucose levels started to rise 12 days after tamoxifen injection, and by 21 days, βPAX6 mice became severely hyperglycemic (Figure 2A). As expected with a primary defect in β cells, hyperglycemia correlated with a reduction in plasma insulin levels in βPAX6 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 βPAX6 islets (Figure 2D). The number of remaining insulin+ 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+ cells had more severe hyperglycemia, Figure 2D).

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driven, rTA-responsive promoter (insulin-rTa 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 βPAX6 mice (Figure 2G).

βPAX6 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 α 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 α cells could account for ketosis in βPAX6, 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 βPAX6 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 α cells in islets (P < 0.05, Spearman’s correlation test, Figure 2I, and see below). Thus, Pax6 deletion in adult β cells causes loss of β 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 insulin in adult β cells.

Pax6-deleted β cells lose insulin but survive and maintain their endocrine identity. The loss of insulin staining in βPAX6 mice could result from extensive β cell death, or, alternatively, from loss of insulin expression and potentially other β 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 β cells following tamoxifen injection. YFP was present in nearly all β cells 1 week after tamoxifen injection, and nearly all YFP+ cells expressed insulin, indicating that YFP faithfully labeled β cells (Figure 3, A and B). Examination of islets at later time points after Pax6 deletion revealed that YFP+ cells remained abundant in islets but were insulin negative (Figure 3A).

We found that the area occupied by YFP+ cells was similar between βPAX6 and control MIP-CreER Rosa26-LSL-YFP mice (data not shown). βPAX6 islets did not show massive staining for the cell death marker TUNEL, although we observed more TUNEL+ islet cells in mutant mice than in controls (Supplemental Figure 4), suggesting the possibility that a lack of PAX6 compromises β cell viability to some extent. While this remains to be further explored, we focused on the majority population of β cells that survived Pax6 deletion. Analysis by flow cytometry showed greatly diminished cytoplasmic insulin content in YFP+ cells from βPAX6 mice, manifested as decreased granularity (Figure 3C). These results indicate that Pax6 deletion in β cells causes a gradual loss of insulin content, but that PAX6-deficient β cells largely remain alive.

YFP+ cells in βPAX6 pancreata stained strongly for the endocrine marker chromogranin A, indicating that PAX6-deficient β cells retain their basic endocrine phenotype (Figure 3D and Supplemental Figure 5). This notion was supported by electron microscopic analysis of pancreatic sections from βPAX6 mice, which revealed an endocrine appearance of mutant islet cells, including secretory granules (Figure 3E). However, compared with control cells, βPAX6 islet cells were morphologically heterogeneous, with very few typical β 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 β cells and results in the acquisition of alternative endocrine cell morphologies.

Adult Pax6-deleted β cells switch from insulin to ghrelin expression. To determine whether Pax6-deleted β cells acquired a new endocrine identity, we stained βPAX6 islets for all known islet hormones, including ghrelin, normally expressed only in fetal islets in mice.
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, βPAX6 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 β cells deficient for Pax6) or, alternatively, a non–cell-autonomous mechanism (e.g., a paracrine effect of PAX6-deficient β cells on neighboring cells). To distinguish between these possibilities, we cotained for pancreatic hormones and the β cell lineage marker YFP. Strikingly, ghrelin co-stained 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 co-stain with YFP (Figure 5B and Supplemental Figure 7). These results indicate that ghrelin expression occurs in β cells following Pax6 deletion, while the expansion of α and δ cells occurs via a non–cell-autonomous mechanism.

Transcriptome-wide changes in β cell differentiation markers and TFs following Pax6 deletion. To further characterize the phenotype of β cells following Pax6 deletion, we determined their transcriptome. We sorted live YFP+ cells from islets of control and βPAX6 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 β 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 β cell TFs, including Pdx1, Mafa, and Nkx6.1 (Figure 5A and Supplemental 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 β 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-β cell programs, such as Hhex (a direct activator of somatostatin transcription) (42).

The specific downregulation of the β cell TFs Mafa, Nkx6.1, and Pdx1 suggests that Pax6 maintains β 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 βPAX6 cells. The genes that were up- or downregulated upon
deletion of \textit{Nkx6.1}, \textit{Mafa}, and \textit{Pdx1} were significantly enriched in the differential \textit{βPAX6} transcriptome (Figure 5C), consistent with the idea that the phenotype of \textit{βPAX6} cells is at least partly caused by downregulation of these TFs. Interestingly, there was no overlap with genes altered upon \textit{Nkx2.2} deletion (45), a finding that was consistent with the lack of a major change in the expression of \textit{Nkx2.2} itself (which was, in fact, mildly induced). \textit{Nkx2.2} deficiency was previously shown to trigger massive expansion of ghrelin cells at the expense of cells (46). The fact that \textit{Nkx2.2} and its targets were not significantly changed in \textit{βPAX6} cells suggests that \textit{PAX6} prevents ghrelin expression via an \textit{Nkx2.2}-independent mechanism. Overall, the transcriptomic changes observed strongly suggest that \textit{PAX6} is required to maintain the specific differentiation state of \textit{β} cells in adults and to repress alternative islet cell expression programs. However, \textit{PAX6} is not involved in maintaining the general endocrine identity of cells or in their survival.
Table 1. Histone marks and binding of other β cell TFs in genes bound and activated or bound and repressed by PAX6

<table>
<thead>
<tr>
<th></th>
<th>H3K27ac</th>
<th>H3K4me1</th>
<th>H3K27me3</th>
<th>H3K9ac</th>
<th>MAFA</th>
<th>PDX1</th>
<th>NEUROD1</th>
<th>FOXA2</th>
<th>NKX6.1</th>
</tr>
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<tbody>
<tr>
<td>Genes activated by PAX6</td>
<td>41%</td>
<td>21%</td>
<td>7%</td>
<td>3%</td>
<td>6%</td>
<td>28%</td>
<td>14%</td>
<td>17%</td>
<td>21%</td>
</tr>
<tr>
<td>Genes repressed by PAX6</td>
<td>26%</td>
<td>23%</td>
<td>15%</td>
<td>2%</td>
<td>2%</td>
<td>22%</td>
<td>11%</td>
<td>13%</td>
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Numbers represent the percentage of PAX6 peaks that contain the indicated mark (see Supplemental Table 4 for details).

Direct transcriptional targets of PAX6 in β cells. To study the mechanism of gene regulation by PAX6 in β 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 β cells lacking PAX6. The preferential binding of PAX6 to genes that it regulates was statistically significant (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 β cells (26, 31). If this were the case, PAX6-bound genes in β 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 β cells (Figure 6A and Supplemental Table 4). This suggests that PAX6 functions as both a transcriptional activator and repressor in β cells. Genes directly repressed by PAX6 in β cells included the hormones glucagon, somatostatin (both of which were also upregulated nonautonomously in βPAX6 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, suggesting that this motif is associated with PAX6-mediated transcriptional repression in β cells. Additional motifs overrepresented in PAX6-bound regions resembled the known targets of other β 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 β cell expression programs.

Next, we asked whether other β 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 β 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 β 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 β 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 β 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 β cells.

A mechanism for indirect negative regulation of Neurog3 by PAX6 via direct repression of Foxa2. Multiple TFs were upregulated in mutant
levels were 2-fold higher in PAX6-deficient β cells during embryonic development (54, 55). Of these, Neurog3, ONECUT1, TCF2, and FOXA2, known to regulate expression, we examined the expression levels of factors, including HES1, SOX9, seq data did not reveal direct binding of PAX6 to the Neurog3 could affect a transcriptional regulator of promoter or, alternatively, PAX6 could directly repress the Neurog3 expression in PAX6-deficient β 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). Since the CHIP-seq data did not reveal direct binding of PAX6 to the Foxa2 enhancer (52) (Figure 6C). Thus, PAX6 represses Neurog3 expression in adult β 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 β cells raises the possibility that PAX6 participates in the autoregulation of Neurog3.

In summary, Pax6 deletion in adult β cells leads to the down-regulation of β 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 β 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 β 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 β cells. In addition, no accumulation of proinsulin was observed in βPAX6 islets, again, arguing against a block in insulin processing. In fact, proinsulin levels were decreased in βPAX6 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
particular, ghrelin and NEUROG3 were upregulated, while insulin, G6PC2, and other β cell TFs were downregulated (Figure 8A). Unlike in adult mouse β cells, glucagon mRNA was downregulated in EndoC-βH2 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 β cells, we performed ChIP-seq on EndoC-βH2 cells (Figure 8C and Supplemental Table 6). By overlapping PAX6-bound sites with previously published data on ChIP-seq of histone modifications and β cell TFs (14, 61), we identified distal regulatory elements. Importantly, some of these elements, such as those associated with 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 β cell TFs in the same genomic position. (D) Venn diagram showing the overlap of genes bound by PAX6 in murine and human β cells. Note the β cell–specific gene sets enriched in the common group (with P values of 1.58 × 10−21, 1.5 × 10−16, 6.05 × 10−14, 6.65 × 10−12, and 5.07 × 10−10, respectively). MODY, maturity-onset diabetes of the young; T2D, type 2 diabetes.

Figure 8. PAX6 function in human β cells. (A) Change in β cell gene expression following PAX6 knockdown in human EndoC-βH2 cells. (B) Ghrelin+ 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, ×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 β cell TFs in the same genomic position. (D) Venn diagram showing the overlap of genes bound by PAX6 in murine and human β cells. Note the β cell–specific gene sets enriched in the common group (with P values of 1.58 × 10−21, 1.5 × 10−16, 6.05 × 10−14, 6.65 × 10−12, and 5.07 × 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 β 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 β 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 β cells. To evaluate the role of PAX6 in human cells, we suppressed PAX6 levels in human EndoC-βH2 cells (60) using retroviral delivery of PAX6-specific siRNA. Reducing PAX6 expression in these cells yielded transcriptional changes similar to those observed in mouse β cells. In particular, ghrelin and NEUROG3 were upregulated, while insulin, G6PC2, and other β cell TFs were downregulated (Figure 8A). Unlike in adult mouse β cells, glucagon mRNA was downregulated in EndoC-βH2 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).

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Discussion

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

Pax6 and transcriptional regulation of β cell identity. We found that PAX6 is needed for maintaining the proper expression of β 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 β 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 β cells that is dramatically upregulated in β cells lacking PAX6. Conceptually similar findings were reported for PDX1 (needed to prevent expression of glucagon in adult β cells) (5) and NKX6.1 (needed to prevent somatostatin expression in adult β 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 β 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 β cells.

In β 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 β 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 β 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 β 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 β cells cultured in high glucose (62) and in β 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 β cell function and leads to abnormal glucose homeostasis, suggesting that even partial effects on PAX6 expression may have glycemic consequences (27, 28). We note, however, that in expression profiles obtained from type 2 diabetic donors using both whole islets and single β 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 β cell identity (potentially in addition to increased β cell death), leading to hypoinsulinemia and hyperglycemia. Second, ghrelin secretion from mutant β cells may further inhibit insulin secretion from remaining functional β 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 α cells may contribute to a worsening of hyperglycemia and to the unique metabolic feature of β cell PAX6 deficiency, namely severe ketosis.

The latter possibility may relate to the emerging view that the pathogenesis of diabetes involves simultaneous β and α 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 α cell hyperfunction in diabetes. α Cell hyperfunction could result from a non–cell-autonomous effect of defective β cells (71) or from reprogramming of β cells into glucagon-expressing cells (8). Diabetes in the βPAX6 model includes key features resulting from abnormal function of α cells (i.e., hyperglucagonemia and the resulting ketosis). Given the nature of this genetic model, the source of the defect has to be the β cell. Moreover, the use of genetic lineage tracing showed that, in this case, the β cell defect was affecting α 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 β cells (e.g., DTA-mediated β cell killing) (36), induce neighboring α cells to expand and hypersecrete glucagon to produce ketones.

Regardless of the mechanism leading to α cell dysfunction, the phenotype of mice deficient for PAX6 in β cells raises the possibility that there might be different types of β cell failure in diabetes,
resulting from different molecular miswirings of the β 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 β cell failure.

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

**Methods**

**Mice.** The mouse strains used in this study were Pax6<sup>−/−</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 β cells. Pax6-deficient mice are referred to herein as βPAX6 mice. Littermate controls are referred to herein as flox/flox (for Pax6<sup>−/−</sup> Rosa26-LSL-EYFP) or βYFP (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 β Ketone Test Strips.

**Immunostaining.** Paraffin sections (5-µm-thick) were prepared from formalin-fixed, paraffin-embedded pancreata. Sections were dehydrated, 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 (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> β 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).
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.

Acknowledgments
We thank the Next Generation Sequencing Core at the University of Pennsylvania (Philadelphia, Pennsylvania, USA) for RNA-seq and analysis; Agnes Klochendler (Hebrew University Medical School, Jerusalem, Israel) for help with transcriptome analysis and cell sorting; Hava Glickstein (interdepartmental equipment unit, Hebrew University Medical School, Jerusalem, Israel) for excellent help with electron micro-scopic analysis; Dana Barequet (Tel Aviv University, Tel Aviv, Israel) for initial experiments on PAX6 in endocrine cells; Ran Avrahami for whole-genome data analyses; Shalev Itzkovitz and Matan Golan (Weizmann Institute of Science, Rehovot, Israel) for help with single-molecule RNA-FISH experiments; and Raphael Scharfmann (INSERM, Institut Cochin, Paris, France) for providing EndoC-βH2 cells. This work was supported by grants from the Juvenile Diabetes Research Foundation (JDRF); the Beta Cell Biology Consortium and the Human Islet Research Network of the NIH (DK104216); the Helmsley Charitable Trust; the European Research Commission (ERC consolidator grant); the Britain Israel Research and Academic Exchange Partnership (BIRAX); the Diabetes Onderzoek Nederland (DON) Foundation; and the Israel Science Foundation and I-CORE Program of The Israel Science Foundation (ISF) (41.11, to YD). Research was performed with the support of the Network for Pancreatic Organ Donors with Diabetes (nPOD), a collaborative type 1 diabetes research project sponsored by the JDRF. The Organ Procurement Organizations (OPOs) partnering with nPOD to provide research resources are listed on the nPOD/JDRF partners website (http://www.jdrfnpod.org/for-partners/npod-partners/). This work was supported in part by a grant from USAID’s American Schools and Hospitals Abroad Program for the upgrading of the Hebrew University Medical School’s Flow Cytometry Laboratory. RA-P was supported by the United States–Israel Binational Science Foundation (BSF; grant 2013016) and the ISF (grant 228/14). AS received fellowships from the Adams Foundation and the Ariane de Rothschild Women Doctoral Program.

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