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STEM CELLS AND REGENERATION

RESEARCH ARTICLE

The mammal-specific *Pdx1* Area II enhancer has multiple essential functions in early endocrine cell specification and postnatal β-cell maturation

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ABSTRACT

The transcription factor Pdx1 is required for multiple aspects of pancreatic organogenesis. It remains unclear to what extent *Pdx1* expression and function depend upon *trans*-activation through 5′ conserved *cis*-regulatory regions and, in particular, whether the mammal-specific Area II (−2139 to −1958 bp) affects minor or major aspects of organogenesis. We show that Area II is a primary effector of endocrine-selective transcription in epithelial multipotent cells, nascent endocrine progenitors, and differentiating and mature β cells *in vivo*. $Pdx1^{\Delta AREAII/-}$ mice exhibit a massive reduction in endocrine progenitor cells and progeny hormone-producing cells, indicating that Area II activity is fundamental to mounting an effective endocrine lineage-specification program within the multipotent cell population. Creating an Area II-deleted state within already specified *Neurog3*-expressing endocrine progenitor cells increased the proportion of glucagon$^+$ α relative to insulin$^+$ β cells, associated with the transcriptional and epigenetic derepression of the α-cell-determining *Arx* gene in endocrine progenitors. There were also glucagon and insulin co-expressing cells, and β cells that were incapable of maturation. Creating the $Pdx1^{\Delta AREAII}$ state after cells entered an insulin-expressing stage led to immature and dysfunctional islet β cells carrying abnormal chromatin marking in vital β-cell-associated genes. Therefore, *trans*-regulatory integration through Area II mediates a surprisingly extensive range of progenitor and β-cell-specific *Pdx1* functions.

KEY WORDS: *Cis*-regulatory function, Lineage diversification, Pancreatic endocrine progenitors, *Pdx1* enhancer Area II, Mouse

INTRODUCTION

Various pancreas-enriched transcription factors have been linked to the programs that direct the differentiation of early and later pancreas progenitors into functional islet β cells (e.g. Sox9, Nkx6.1, Neurog3, Pdx1) (reviewed by Pan and Wright, 2011). Although the integrated, likely highly cross-regulatory gene-regulatory networks are not well defined, the dynamic expression pattern of *Pdx1*, and profound defects incurred with global or cell type-specific inactivation (e.g. Fujitani et al., 2006; Gannon et al., 2001; Hale et al., 2005; Kodama et al., 2016; Offield et al., 1996; Swift et al., 1998), clearly point to its pervasive and orchestrating role during organogenesis. Thus, a *Pdx1* null mutation in human (Stoffers et al., 1997a,b) or mouse (Jonsson et al., 1994; Offield et al., 1996) results in pancreatic agenesis, and a heterozygous mutation leads to human early-onset diabetes (Stoffers et al., 1997a,b). Moreover, conditional deletion of *Pdx1* has revealed the requirement for this transcription factor in several of the later stages of pancreatic endocrine cell development and in adult islet β-cell function (reviewed by Pan and Wright, 2011).

Much of *Pdx1* transcriptional regulation appears to be exerted by *trans*-acting factors acting within four conserved upstream *cis*-regulatory regions (termed Areas I-IV), located within 6.5 kb of the transcriptional start site (Gannon et al., 2001; Gerrish et al., 2000; Van Velkinburgh et al., 2005). Whereas Areas I, III and IV are present in widely differing vertebrate species, Area II is restricted, somewhat surprisingly, to mammals (Gerrish et al., 2000). In mouse, combined deletion of Areas I-II-III ($Pdx1^{\Delta I\text{-}II\text{-}III}$) *in vivo* produces severely deficient *Pdx1* expression and impairs formation of the early pancreatic buds (Fujitani et al., 2006), an effect similar to the pancreatic agenesis in *Pdx1* germline nulls (Offield et al., 1996). Complementary experiments showed that *Pdx1* expression driven by Areas I-II-III, with only a small portion of Area IV, restored full pancreatic development to *Pdx1* null mice (Boyer et al., 2006; Gannon et al., 2001). These results imply that the embryonic *Pdx1* expression required for complete production of a differentiated pancreatic organ is principally, if not exclusively, regulated by Areas I-II-III.

Enhancer-like activities for Areas I, II and III have been documented in reporter assays in β-cell lines and a limited number of transgenic mouse assays. Such studies assigned β-cell-specific enhancer-like activities to Area II. For example, while Area I or Area II imparted β-cell-specific activation in cell lines (Gerrish et al., 2000), only Area II independently directed expression to islet β cells *in vivo*, although expression was variegated. When placed together, Areas I and II seemed to show functional interactions that were now able to induce high *Pdx1* expression throughout the entire β-cell population from around embryonic day (E) 13.5, which represents the start of the major phase of insulin$^+$ cell production (Van Velkinburgh et al., 2005). Whereas the region representing Areas I-II-III is bivalently marked in early endodermal progenitors, it is subsequently derepressed in nascent pancreatic progenitors leading to a relative deficit of repressive chromatin markings (van Arensbergen et al., 2010; Xie et al., 2013; Xu et al., 2011). Together with Area I-II-III transgene analysis (Wiebe et al., 2007), these findings supported the idea that Areas I-II-III are involved in driving *Pdx1* expression in pancreatic endocrine as well as exocrine progenitors.

Although these combined findings support a central role for Area II in driving *Pdx1* transcription, the effect of removing just Area II

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from the endogenous gene remained untested. It was therefore uncertain whether this mammal-specific *cis*-regulatory region operates broadly over multiple steps of the entire organogenesis process, or perhaps plays a more nuanced or 'tuning role' directed at producing the correct number of islet β cells or keeping them functioning normally. We addressed this issue *in vivo* with a newly derived targeted allele carrying a precise Area II deletion, termed $Pdx1^{\Delta AREAII}$ ($Pdx1^{\Delta II}$). By combining $Pdx1^{\Delta II}$ with null and conditionally inactivated floxed *Pdx1* alleles, we established that the mammal-restricted Area II is essential to *Pdx1* transcription during several distinct phases of pancreatic organogenesis and islet endocrine cell ontogeny. Although previous findings pointed to a β-cell-selective role for Area II, a germline global deletion massively affected all pancreatic endocrine progenitors and progeny. Endocrine-selective reduction of gene activity by removing Area II affected endocrine cell-type allocation, and severely debilitated maturation of β cells. We report effects on chromatin marking status of *Pdx1* and key genes directly or indirectly targeted by Pdx1 caused by reducing the level of Pdx1. These studies establish that Area II is a potent contributor to all endocrine-specific functions of *Pdx1*, including endocrine progenitor specification, β-cell versus α-cell lineage allocation, and β-cell maturation. Our findings are discussed with respect to the possible unique regulatory significance of this compact *cis*-regulatory region in pancreas formation in mammals as compared with other vertebrates.

RESULTS

Loss of Area II does not affect *Pdx1* regulation of overall pancreas size

An Area II-specific deletion was generated within the endogenous locus ($Pdx1^{\Delta II}$) to determine the requirement for this transcriptional control region in *Pdx1* expression and function (Fig. S1). Mice of several genotypes were derived (Fig. 1A-C).

Homozygous $Pdx1^{\Delta II/\Delta II}$ mice showed no gross changes during development nor postnatal physiological abnormalities in younger adults, with pancreas size and islet cluster size/number being normal, although the various hormone-secreting islet cell types were arranged differently from control islets, exhibiting the well-known abnormal 'mixed-islet' phenotype (Fig. S2A,B). $Pdx1^{\Delta II/\Delta II}$ β cells were immature at 4 weeks of age, suggested by the lack of the key maturation markers MafA and Glut2 (Slc2a2), along with abnormal maintenance of the adult α-cell marker MafB (Fig. S2C-E). However, older, 5- to 6-month-old male $Pdx1^{\Delta II/\Delta II}$ mice became distinctly hyperglycemic (Fig. 1E,E′), indicating an age-dependent requirement for this control domain in islet β-cell function.

Crossing existing $Pdx1^{+/-}$ mice with $Pdx1^{\Delta II/+}$ mice led to the production of $Pdx1^{\Delta II/-}$ animals, in which Pdx1 protein is only derived from one $Pdx1^{\Delta II}$ allele, thereby creating a more sensitized condition compared with $Pdx1^{+/-}$. $Pdx1^{\Delta II/-}$ neonates were also indistinguishable from control $Pdx1^{+/-}$ littermates by outward appearance, and their gastrointestinal tract anatomy was indistinguishable from control littermates, including a pancreas of normal size (Fig. 2A,B). They did not, however, survive beyond postnatal day (P) 3 and were hyperglycemic at all time points measured before death (Fig. 1D,D′). Pdx1 protein production in $Pdx1^{\Delta II/-}$ pancreatic tissue was markedly diminished compared with the $Pdx1^{+/-}$ state (Fig. 2C-D′), and there was a large decrease in the total number of all endocrine cells, and a greater representation of glucagon (Gcg)-expressing cells (Fig. 2E,F,O).

Because Pdx1 autoregulates its own expression through the neighboring Area I control region (Gerrish et al., 2000), we used a *Pdx1* exon 2 *lacZ* knock-in null allele ($Pdx1^{2E3\text{-}lacZ}$; Offield et al., 1996) and whole-mount embryo analysis to examine whether this reduced Pdx1 condition affected the spatiotemporal pattern of *Pdx1* expression in $Pdx1^{\Delta II/2E3\text{-}lacZ}$ embryos. E13.5 control embryos ($Pdx1^{2E3\text{-}lacZ/+}$) showed β-galactosidase expression throughout the normal *Pdx1* expression domain, spanning from caudal stomach to the rostral duodenum and including the pancreas and bile duct (Fig. 2G). The spatial pattern in $Pdx1^{\Delta II/2E3\text{-}lacZ}$ embryos was

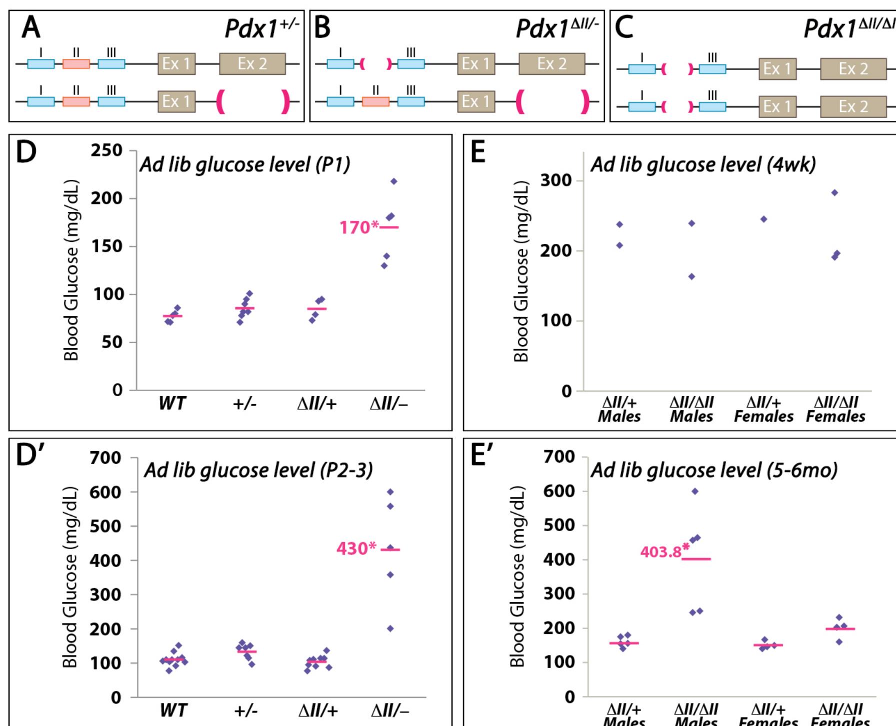

Fig. 1. Glucose levels of different *Pdx1* mutant classes. (A-C) Schematic of $Pdx1^{+/-}$, $Pdx1^{\Delta II/-}$ and $Pdx1^{\Delta II/\Delta II}$ genotypes. (D-E′) Glucose level (mg/100 ml) of different *ad libitum*-fed *Pdx1* mutant classes at early postnatal stages (D,D′), 4 weeks (E) and 5-6 months (E′) of age. *P<0.05.

equivalent, but of lower intensity (Fig. 2H), suggesting decreased β-galactosidase production related to the known interactions between Areas I and II and between Areas I, II and III (Gerrish et al., 2001).

The total size of the *Pdx1*$^{\Delta II/-}$ pancreatic epithelium was not significantly changed compared with *Pdx1*$^{+/-}$ (Fig. S3C,E,H) and was essentially equivalent to the wild type (Fujitani et al., 2006; Offield et al., 1996). In addition, the pancreatic epithelium in E12.5 *Pdx1*$^{\Delta II/-}$ and *Pdx1*$^{+/-}$ embryos was indistinguishable in the formation and developmental advancement of the epithelial plexus (Fig. 2J, Fig. S3B, Fig. S4A-D). The production of several progenitor stage transcription factors required for key aspects of pancreas growth and differentiation was also normal [e.g. Sox9, HNF6 (Onecut1), Gata4; Fig. S4A-D]. Moreover, similar numbers of lineage-labeled pancreatic epithelial cells were found at E12.5 in *Pdx1*$^{\Delta II/-}$ and *Pdx1*$^{+/-}$ pancreatic tissues (using embryos carrying the *Pdx1*^{TgCre}-*R26R*^{EYFP} system for *Pdx1* lineage tracing; Fig. S3C) (Gu et al., 2002). Pdx1 lineage-labeled cells from both *Pdx1*$^{\Delta II/-}$ and *Pdx1*$^{+/-}$ genotypes at E13.5 were flow-sorted and *Pdx1* expression was determined by qRT-PCR using allele-specific primers that do not detect transcript from the *Pdx1* null allele (Table S2). Whereas mRNA from the *Pdx1*$^{\Delta II}$ allele was ∼32% of the control level (Fig. S3D), mRNAs of several other lineage-selective factors found in multipotent progenitor cells were unchanged (e.g. Sox9, HNF1β, HNF6; Fig. S3D) (Haumaitre et al., 2005; Lynn et al., 2007; Zhang et al., 2009), which was corroborated by immunoanalysis (Fig. S4). Interestingly, only the α-cell-determining factor *Arx* (Collombat et al., 2007, 2003) was significantly upregulated in qRT-PCR analysis (Fig. S3D). Further, normal expression of ductal and acinar markers was found in P1 *Pdx1*$^{\Delta II/-}$ pancreas (Fig. S5). These results indicate that the greatly reduced *Pdx1* mRNA of the *Pdx1*$^{\Delta II/-}$ mutant was still sufficient for

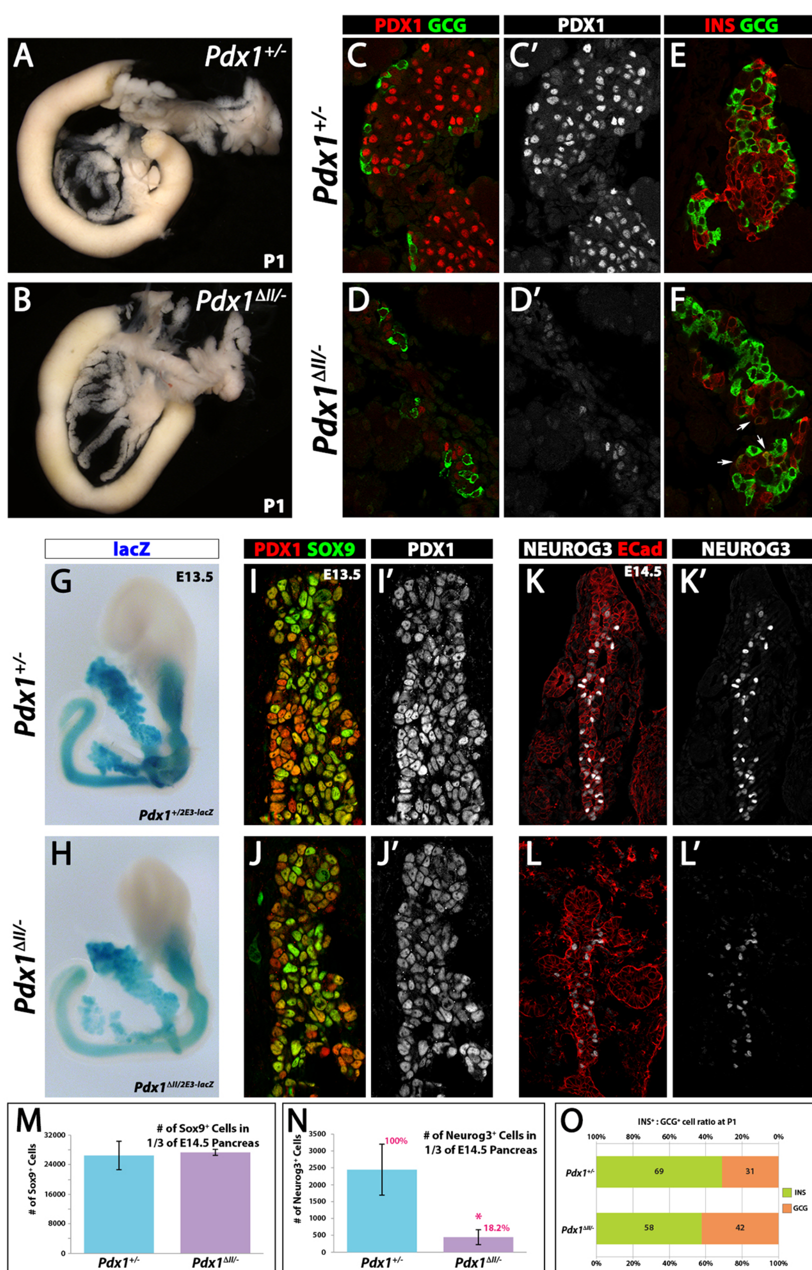

Fig. 2. Endocrine-specific defects in the *Pdx1*$^{\Delta II/-}$ pancreas. (A,B) Overall morphology of the *Pdx1*$^{+/-}$ and *Pdx1*$^{\Delta II/-}$ pancreatic/duodenal region at P1. (C-D′) Gcg and Pdx1 expression (single Pdx1 channel and merge) in P1 endocrine clusters. (E,F) Ins and Gcg expression at P1. Arrows indicate Ins$^+$ Gcg$^+$ co-expressing cells. Note that the large cluster of hormonal cells shown in F was not commonly found in *Pdx1*$^{\Delta II/-}$ and is shown for comparison. (G,H) *Pdx1*$^{2E3\text{-}lacZ}$ reporter for *Pdx1* transcriptional activities in E13.5 *Pdx1*$^{+/-}$ and *Pdx1*$^{\Delta II/-}$ pancreata. (I-J′) Sox9 and Pdx1 expression at E13.5. (K-L′) Decreased Neurog3$^+$ cell numbers are observed in E14.5 *Pdx1*$^{\Delta II/-}$ pancreas (L,L′). (M,N) Morphometric analysis of Sox9$^+$ cells and Neurog3$^+$ cells at E14.5. **P*<0.05. (O) Ins$^+$:Gcg$^+$ cell ratios in *Pdx1*$^{+/-}$ and *Pdx1*$^{\Delta II/-}$ at P1.

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the formation of the exocrine acinar and ductal cell lineages, which constitute ~98% of the organ mass.

Endocrine islet cell formation is severely compromised in the *Pdx1*$^{\Delta II/-}$ state

The severe hyperglycemia in *Pdx1*$^{\Delta II/-}$ newborns (Fig. 1), suggestive of β-cell dysfunction and/or reduced β-cell mass, prompted an examination of endocrine cell formation and differentiation. The E14.5-E15.5 *Pdx1*$^{\Delta II/-}$ pancreata showed a massively decreased number of Neurog3$^+$ endocrine progenitor cells (20-40% of control; Fig. 2N, Fig. S3F). Neurog3$^+$ cells give rise to postmitotic endocrine precursors that express *Pax6* (Sander et al., 1997; St-Onge et al., 1997), and both *Neurog3* and *Pax6* mRNA levels were specifically reduced in *Pdx1*$^{\Delta II/-}$ epithelium (Fig. S3D). This large decrease in endocrine progenitors is likely to have resulted in the 70% decline in total endocrine cell number at P1 (Fig. S3G).

Any one of several scenarios could result in a reduction in endocrine progenitor numbers. First, because Neurog3$^+$ cells arise from a Sox9$^+$ bipotent progenitor pool (Kopp et al., 2011), reduced Sox9$^+$ cell production would lead to fewer endocrine progenitors. However, the number of Sox9$^+$ cells in E13.5 pancreata was similar between genotypes (Fig. 2M) and the development of the epithelial plexus was relatively normal, as described above. In addition, the 16% decrease in the mitotic index of Sox9$^+$ cells (as assessed by phospho-histone H3 staining; Fig. S4F) would not account for the profound loss of endocrine progenitors observed in the *Pdx1*$^{\Delta II/-}$ mutant. Second, general epithelial or endocrine progenitor stage apoptosis is unlikely to contribute to the failure of cells to enter the endocrine lineage in *Pdx1*$^{\Delta II/-}$ pancreata, as only rare TUNEL$^+$ cells were detected (Fig. S4C,D). Third, and most likely, the threshold of Pdx1 protein production in the *Pdx1*$^{\Delta II/-}$ pancreatic epithelium was insufficient to activate *Neurog3* (Oliver-Krasinski et al., 2009). Pertinent here is the previous finding that Pdx1 augments the expression of other essential early epithelial regulatory factors, such as Sox9 and HNF1β, which together are required for normal *Neurog3* transcription and endocrine specification (Oliver-Krasinski et al., 2009), yet the Pdx1^{LOW} condition in the *Pdx1*$^{\Delta II/-}$ pancreata did not affect Sox9 or HNF1β production (Fig. 2M, Fig. S3D), only Neurog3$^+$ cell formation (Fig. 2N,L′). Since the birth of endocrine cells affects islet size significantly (Jo et al., 2012), the formation of smaller islets in the *Pdx1*$^{\Delta II/-}$ pancreas is likely to have resulted from the massive reduction of endocrine progenitors.

Area II deletion reduces Pdx1 levels and disturbs α versus β lineage allocation

Although the greatly reduced numbers of endocrine cells produced in *Pdx1*$^{\Delta II/-}$ mutants were nonetheless assembled into islet-like clusters by P1, there was impairment in the normal core versus mantle distribution of the various hormone cell types in the rarely found larger clusters (Fig. 2F), again yielding the mixed-islet phenotype. In addition, the majority of hormone-producing cells were found in small clusters of ~5-7 cells, broadly dispersed throughout the pancreas (Fig. 2D). Many insulin (Ins) and Gcg co-expressing cells were also detected in P1 *Pdx1*$^{\Delta II/-}$ mutants (arrows in Fig. 2F), representing 5.3% of total Ins$^+$ or Gcg$^+$ cells, as compared with only 0.5% in *Pdx1*$^{+/-}$. This phenotype suggests that the lower level of Pdx1 produced in mutant β cells fails to fully repress the α-cell program (Gao et al., 2014).

The Pdx1^{LOW} condition associated with the *Pdx1*$^{\Delta II/-}$ state caused a significant alteration in the proportion of α (Gcg$^+$) and β (Ins$^+$) cell types. At P1, whereas islets in *Pdx1*$^{+/-}$ control pancreas contained β and α cells in a 69:31% ratio, in *Pdx1*$^{\Delta II/-}$ it was 58:42% (Fig. 2O). Unfortunately, *Pdx1*$^{\Delta II/-}$ animals died too soon after birth to assess the quality of these β cells by expression of various β-cell maturation markers. However, the overall hyperglycemia and increased number of Ins and Gcg co-expressing cells strongly suggested far-reaching defects in β-cell lineage progression and, consequently, in the postnatal physiological activity of the islet β-cell population.

We next analyzed pancreas with the genotype *Neurog3*^{Cre}*;Pdx1*$^{FloxE2/\Delta II}$ (referred to as *Pdx1*$^{endo\text{-}\Delta II}$) in which inactivation of the conditional null *Pdx1*^{FloxE2} allele (Gannon et al., 2008) leads to Pdx1 protein being derived only from the *Pdx1*$^{\Delta II}$ allele in the endocrine progenitors and their descendants (Fig. 3B). These mice lived much longer than *Pdx1*$^{\Delta II/-}$ mice, indicating a less profound deficit when restricting the loss of Area II to the endocrine lineage. The total endocrine cell number at P1 in *Pdx1*$^{endo\text{-}\Delta II}$ pups was 72% of that in controls (Fig. 3F). Nonetheless, there was a noticeable restoration of islet size distribution in favor of a size increase and now with an essential absence of the many small clusters found in *Pdx1*$^{\Delta II/-}$ pancreata (compare Fig. 3D with Fig. 2D). There was an alteration in the relative proportion of β versus α cells that was strikingly similar to that seen in P1 *Pdx1*$^{\Delta II/-}$ animals: a 60:40% β to α ratio in *Pdx1*$^{endo\text{-}\Delta II}$ mutant tissue as compared with 73:27% for controls (Fig. 3E). In addition, ~5.5% of these endocrine cells showed Ins and Gcg co-expression, versus 0.5% in controls. The

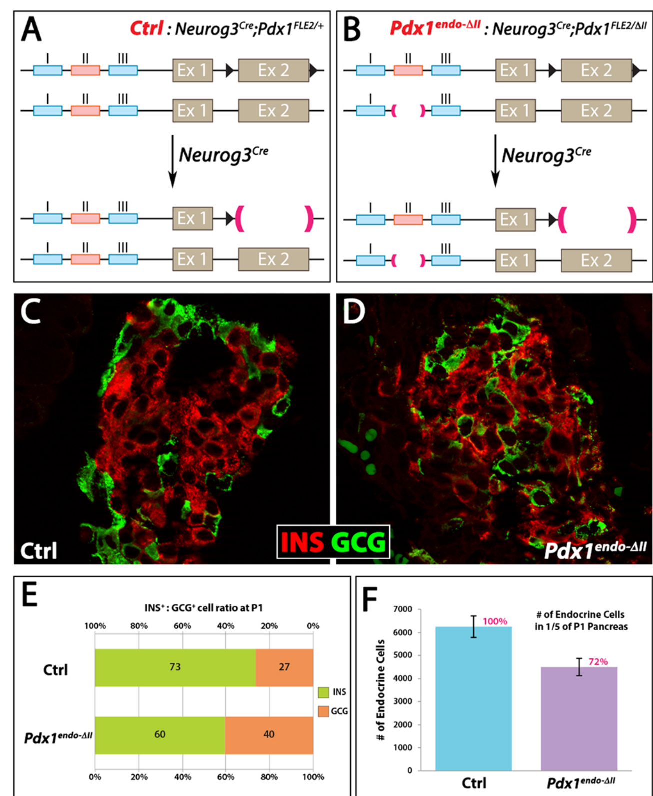

Fig. 3. *Pdx1*$^{endo\text{-}\Delta II}$ mice show defects in islet organization and in the β:α cell ratio. (A,B) Schematic presentation of *Neurog3*^{Cre}*;Pdx1*$^{FloxE2/\Delta II}$ (*Pdx1*$^{endo\text{-}\Delta II}$) and *Neurog3*^{Cre}*;Pdx1*$^{FloxE2/+}$ control. In *Pdx1*$^{endo\text{-}\Delta II}$, the endocrine progenitors and their descendant endocrine cells were *Pdx1*$^{\Delta II/-}$ upon *Neurog3*-mediated recombination. (C,D) Ins and Gcg expression in P1 islets. The mantle and core structure (C) was not obvious in *Pdx1*$^{endo\text{-}\Delta II}$ islets (D). (E) Quantitative estimation of β:α cell ratio at P1 (by Ins and Gcg expression, respectively). (F) Total numbers of endocrine cells in P1 pancreata.

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effect on lineage allocations seemed to be restricted to α and β cells, as we found no quantifiably significant difference in the numbers of somatostatin or pancreatic polypeptide cell types (Fig. S6). *Pdx1*$^{endo\text{-}ΔII}$ mice were hyperglycemic from P1 until at least 4 weeks of age (Fig. 4A,B), with islet dysfunction probably reflecting the inability of the Pdx1^{LOW} condition to facilitate β-cell maturation. For example, although expression of the β-cell factor Nkx6.1 was relatively normal (Fig. 4D′), mutant β cells at 4 weeks of age lacked an important transcriptional regulator of this process, MafA, while a factor that is normally enriched in mouse α but not β cells, MafB, was still apparent (Fig. 4E,F). Moreover, the size spectrum of *Pdx1*$^{endo\text{-}ΔII}$ islets, although relatively similar to controls at P1 (Fig. 3), appeared at 4 weeks to have shifted noticeably in favor of being much smaller (e.g. Fig. 4C′), suggesting postnatal defects in islet expansion.

Derepression of *Arx* in *Pdx1*$^{endo\text{-}ΔII}$ endocrine progenitors

We further probed the function of *Pdx1* in controlling β-cell versus α-cell fate choice by introducing into *Pdx1*$^{endo\text{-}ΔII}$ mice a *Neurog3*^{GFP} knock-in allele (Lee et al., 2002) and combining this with immunodetection of CD133 (Prom1), a lumenal apical surface marker (Benitez et al., 2014; Sugiyama et al., 2007), to flow-sort *Neurog3*-expressing endocrine progenitors. This process enriched for endocrine progenitor cells by guarding against the inclusion of cells that had passed well beyond the endocrine progenitor state but still contained a long-lived GFP signal from the *Neurog3*^{GFP} knock-in reporter (Fig. S7).

Certain islet-enriched transcription factors have been linked to hormone-selective endocrine lineage commitment, acting within or shortly after the *Neurog3*-expressing state (Desgraz and Herrera, 2009; Nelson et al., 2007). *Arx* has been linked to α-cell fate (Collombat et al., 2007, 2003), whereas *Pdx1*, *Pax4* and *Nkx6.1* are potent instructors of the β-cell fate (Collombat et al., 2009; Nelson et al., 2007; Schaffer et al., 2013; Yang et al., 2011). Consequently, we flow-sorted *Neurog3*^{GFP} and CD133 co-positive progenitors from E14.5 *Pdx1*$^{endo\text{-}ΔII}$ pancreata to assess abnormalities in the early-stage lineage bias toward the α-cell pathway. *Pdx1* mRNA was substantially decreased (∼50%) in this progenitor population, as were *Neurog3*, *Nkx6.1* and *Hnf6* (Fig. 5A). Although in a slightly different cellular context, Pdx1 has been shown to be a direct effector of *Nkx6.1* and *Hnf6* expression (Schaffer et al., 2013; Teo et al., 2015), and as early-stage endocrine progenitors these cells are expected to express both *Nkx6.1* and *Hnf6*. It is likely that the reduction in expression of these other factors was at least partly caused by a direct transcriptional effect because, for example, both Pdx1 and HNF6 are reported to positively control *Neurog3* expression (Jacquemin et al., 2000; Oliver-Krasinski et al., 2009). *Arx* mRNA was notably elevated over controls. We cannot explain why *Pax4* was unaltered (Fig. 5A), but the degree of alteration in Arx might not be translated into the known negative transcriptional effect on *Pax4* (Collombat et al., 2003). At the level of chromatin marking, we always found a significant decrease in three marks examined within Area II in *Pdx1*$^{endo\text{-}ΔII}$ endocrine progenitors, an expected outcome because of the deletion of Area II (that is, there is

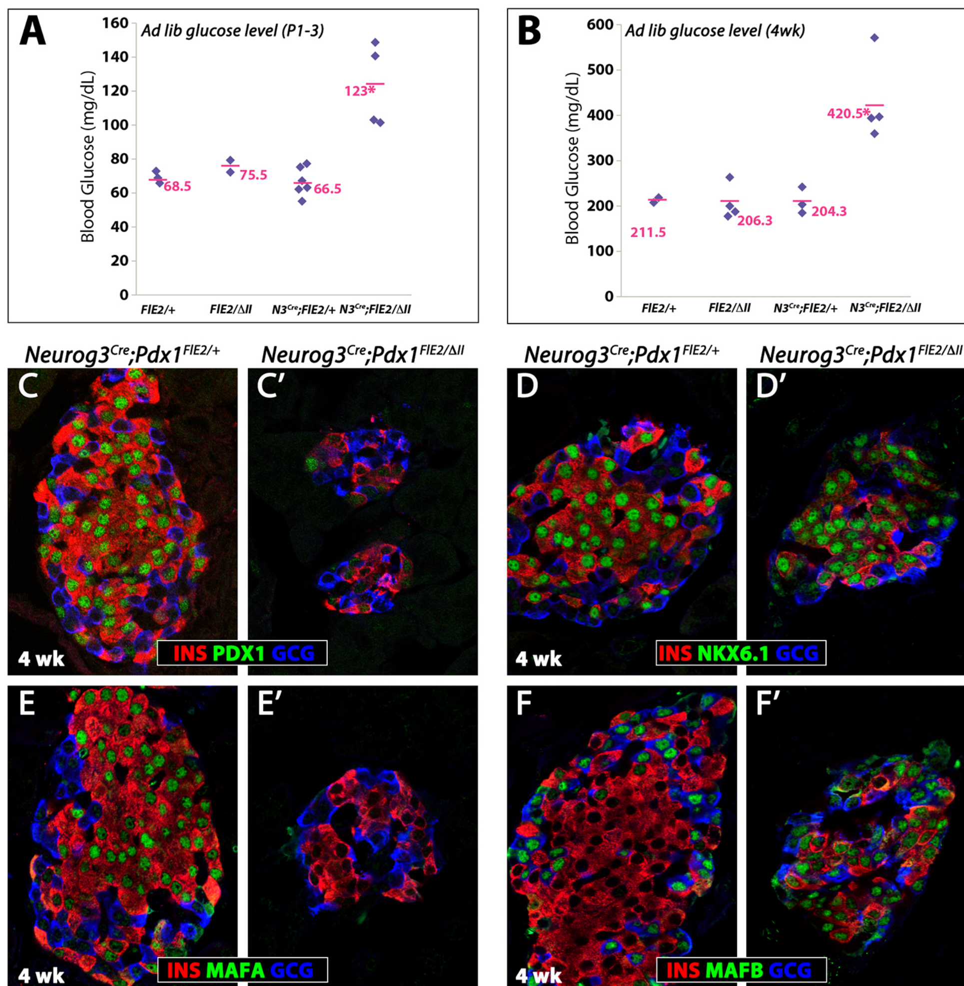

Fig. 4. Immature β cells in *Pdx1*$^{endo\text{-}ΔII}$ mice. (A,B) Glucose level at P1-3 (A) and 4 weeks of age (B) for the indicated genotypes. *P<0.05. (C-F′) Four-week-old *Pdx1*$^{endo\text{-}ΔII}$ β cells showed low to no Pdx1 (C′) and MafA (E′), abnormally sustained MafB (F′), and no changes in Nkx6.1 (D′).

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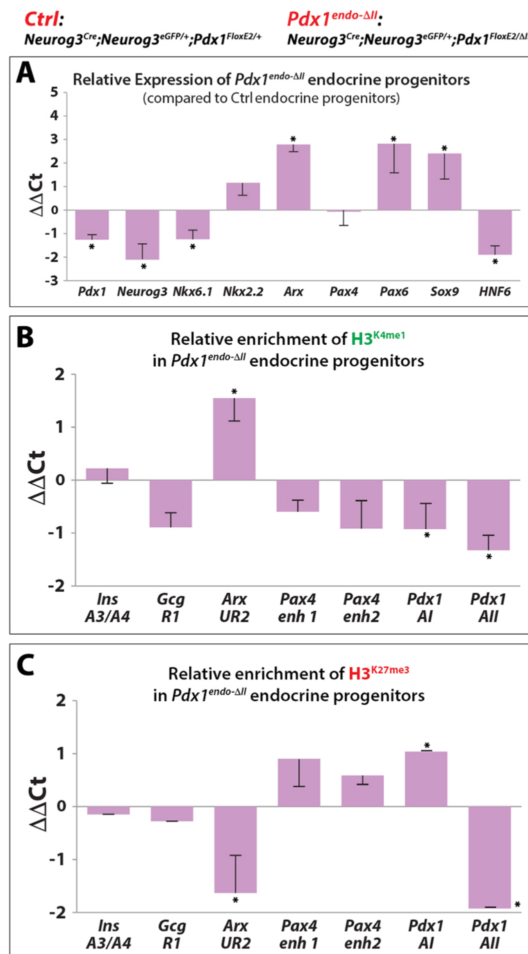

Fig. 5. Loss of *Pdx1* Area II leads to derepression of *Arx* in *Pdx1*$^{\text{endo-}\Delta\text{II}}$ endocrine progenitors. (A) qRT-PCR assessment of relative expression of key epithelial factors comparing sorted *Pdx1*$^{\text{endo-}\Delta\text{II}}$ endocrine progenitor cells with control. (B,C) ChIP assays showing relative enrichment of H3K4me1 and H3K27me3 (comparing mutant endocrine progenitor cells with the control) at enhancer regions of key transcription factors. *P<0.05.

one copy of Area II in *Pdx1*$^{\text{endo-}\Delta\text{II}}$ versus two copies in control). There was a significant increase in the histone activating marks H3K4me1 and H3K27ac, and a decrease in the H3K27me3 repressive mark, within the *Arx* UR2 enhancer (Fig. 4B,C, Fig. S8), which is a control region important in maintaining the mature β-cell state (Dhawan et al., 2011). Consistent with the unaltered *Pax4* mRNA levels (Fig. 5A), no significant alterations in the levels of activating or repressive histone marks were detected within its respective enhancer regions in *Neurog3*$^{\text{GFP}}$ and CD133 co-positive progenitors at E14.5 (Fig. 5B,C, Fig. S8).

Area II mediates the higher *Pdx1* expression essential for β-cell maturation

As a more selective test of the requirement for Area II within β cells that have progressed relatively further into their lineage, we generated a β-cell-specific *Pdx1*$^{\Delta\text{II}/-}$ condition (*Pdx1*$^{\text{beta-}\Delta\text{II}}$) by placing the *RIP2*$^{\text{Cre}}$ transgene (in which Cre is driven by the rat *Ins2* promoter) in combination with *Pdx1*$^{\text{FloxE2}/\Delta\text{II}}$, and examining the effect on islet composition and function (Fig. 6A,A′). These mice showed normal pancreatic organogenesis prior to β-cell specification, as expected. Postnatal *Pdx1*$^{\text{beta-}\Delta\text{II}}$ animals were hyperglycemic, with very rare Ins^{+} Gcg^{+} cells observed (Fig. 6B). Not all β cells showed the marked decrease in Pdx1 protein because of variegated Cre production (Fig. 6D′, Fig. S9C) (Pan et al., 2015). However, in β cells that were YFP^{+} flow-sorted from *Pdx1*$^{\text{beta-}\Delta\text{II}}$; *R26R*$^{\text{EYFP}}$ pancreas at P1, we recorded an 88% reduction in *Pdx1* mRNA compared with controls (Fig. 6F).

Although there were no obvious defects in endocrine cell formation or β/α proportional representation (data not shown), *Pdx1*$^{\text{beta-}\Delta\text{II}}$ β cells displayed classical characteristics of immaturity, including low levels of MafA (Fig. 6B′,F) and of the β-cell-specific glucose transporter *Glut2* (Fig. 6E′,F), and sustained MafB expression (Fig. 6C′,F). The effect on MafA and MafB is likely to be direct because Pdx1 binds to control regions to either positively or negatively influence transcription (Gao et al., 2014; Raum et al., 2006). In addition, *Gcg*, *Brn4* (*Pou3f4*) and *Arx* expression was increased in YFP-sorted *Pdx1*$^{\text{beta-}\Delta\text{II}}$ β cells (Fig. 6F). The *Pdx1* Area I enhancer showed an enrichment of the activating marks H3K4me1 and H3K27ac, whereas *Pdx1* transcription was significantly decreased (Fig. 6G-I). This suggests that Area I alone, in the absence of Area II, is insufficient to drive adequate *Pdx1* transcription. There was a reduction in the activating marks H3K4me1 and H3K27ac and an increase in repressive H3K27me3 levels within the *Mafa* and *Ins* enhancers (Fig. 6G-I). Reciprocally, increased H3K4me1/H3K27ac and reduced H3K27me3 were observed at enhancers of the pro-α-cell loci *Arx*, *Mafb* and *Gcg* (Fig. 6G-I). Therefore, the action of the mammal-specific Area II is apparently essential to produce enough Pdx1 to repress a cohort of α-cell-associated genes, with a central influence being the Pdx1-MafA positive-feedback loop.

DISCUSSION

Overall, our data demonstrate that the Area II enhancer-like module within the *Pdx1* gene functions to augment the role of Pdx1 at three key stages of mammalian pancreatic endocrine and β-cell differentiation. These include: (1) the creation of an epithelial state to allow the emergence of the correct number of *Neurog3*-expressing endocrine lineage cells during the secondary transition period of organogenesis; (2) Pdx1-mediated repression of *Arx* for directing lineage selection as cells move into the β-cell lineage; and (3) Pdx1$^{\text{HIGH}}$ expression within β cells that drives switching of *Mafb* to *Mafa* expression, and affects other genes acting as principal components of the developmental maturation and physiological maintenance of the β-cell state. These conclusions were supported by the observation that reducing Pdx1 production disturbs the epigenetic configuration and expression of fate-determining genes that have been strongly connected to endocrine cell differentiation and/or islet cell function.

Because Area II is only found in mammals, the degree of conservation of the mechanisms that ensure appropriate spatiotemporal *Pdx1* expression in lower vertebrates, such as chickens, for producing the correct number and type of islets of Langerhans, or directing entry into and long-term pursuance of β-cell versus α-cell programs, emerges as an issue of evolutionary interest. We speculate that Area II imparts unique properties to *Pdx1* expression, not only affecting the gene itself but also by influencing the expression of other key regulators through distinct transcriptional interactions (Papantonis et al., 2012; Schoenfelder et al., 2010). The Area II control region could have been evolutionarily acquired in mammals to increase the absolute number of β cells or islets, thereby affecting the spectrum of islet sizes, relative intra-islet organization of the various hormone-secreting cell types, or functional β-cell heterogeneity. It is possible that such effects could then indirectly lead to altered cellular communication with the neural and vascular systems. Presumably,

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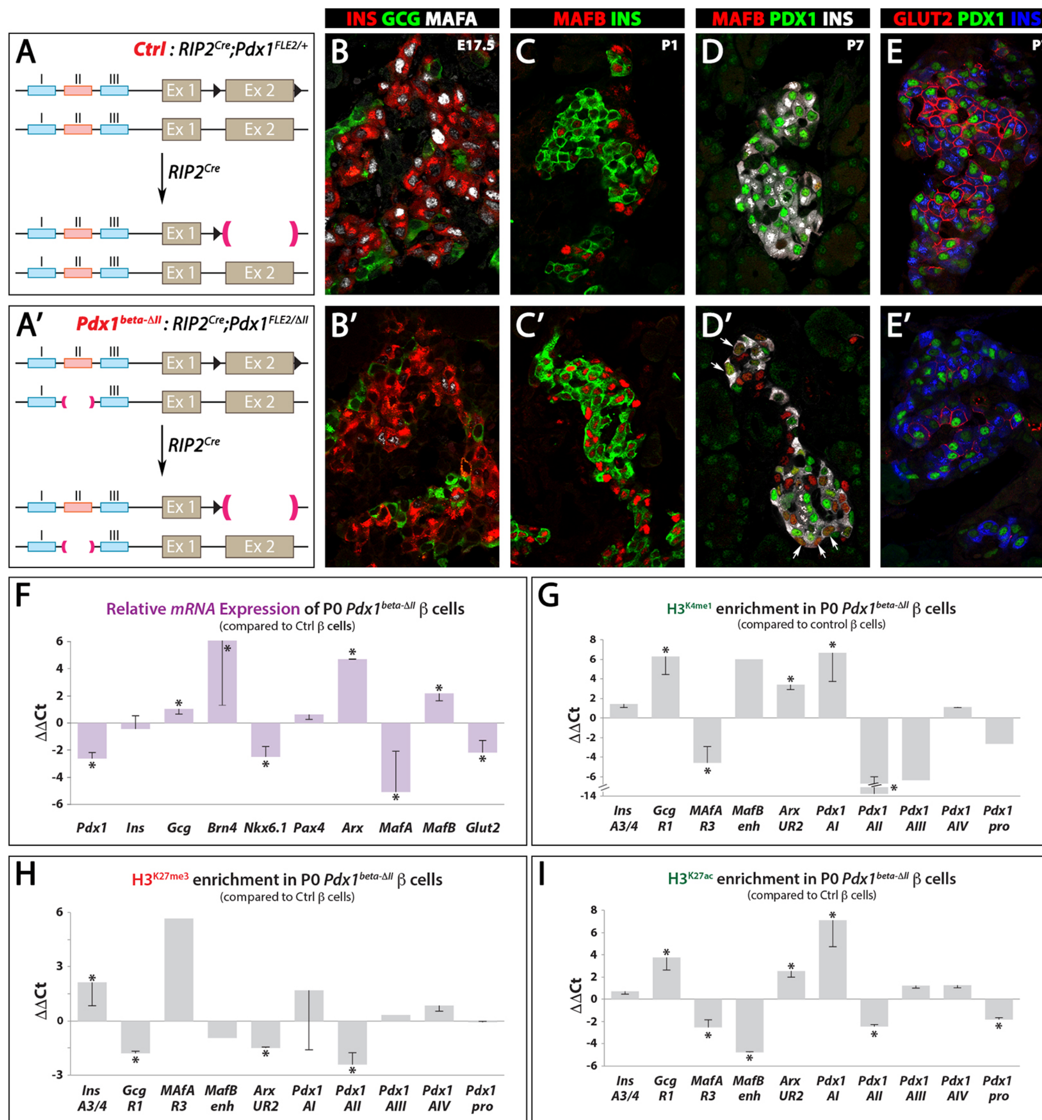

Fig. 6. *Pdx1*$^{\text{beta-}\Delta\text{II}}$ β cells are immature and show transcriptional and epigenetic alterations. (A,A′) Schematic presentation of *RIP2*$^{\text{Cre}}$*;Pdx1*$^{\text{FloxE2/}\Delta\text{II}}$ (*Pdx1*$^{\text{beta-}\Delta\text{II}}$) and *RIP2*$^{\text{Cre}}$*;Pdx1*$^{\text{FloxE2/+}}$ (Ctrl). In *Pdx1*$^{\text{beta-}\Delta\text{II}}$, the β cells were *Pdx1*$^{\Delta\text{II/−}}$ upon recombination. (B,B′) MafA expression was lost in E17.5 *Pdx1*$^{\text{beta-}\Delta\text{II}}$ β cells (B′). (C-E′) MafB, Ins and Pdx1 expression at P1 (C,C′) and P7 (D-E′). Arrows (D′) indicate abnormal Ins^{+} MafB^{+} Pdx1$^{\text{LOW}}$ cells. Glut2 was largely diminished in *Pdx1*$^{\text{beta-DII}}$ β cells (E′) compared with the control (E). (F) qRT-PCR analysis of key factors involved in α/β identity and β-cell maturation of *Pdx1*$^{\text{beta-}\Delta\text{II}}$ β cells isolated by FACS. (G-I) ChIP-qPCR analysis of the enrichment of histone marks H3K27me3, H3K4me1 and H3K27ac at the enhancer region of key factors involved in α/β identity and β-cell maturation from isolated *Pdx1*$^{\Delta\text{II/−}}$ β cells. **P*<0.05.

non-mammals use non-Area II-dependent processes to produce sufficient appropriately structured islets to function efficiently under the normal physiological parameters and rigors of their own ecological niche. An anomaly within mammals is the desert sand rat *Psammomys obesus*, which is proposed not to have a *Pdx1* gene (Leibowitz et al., 2001). We speculate that genome sequencing might detect a highly divergent version, in which case it will be interesting to determine the degree of conservation of its putative Areas I-II-III-IV *cis*-regulatory domains.

Pdx1 expression in pancreas organogenesis

Whereas complete removal of *Pdx1* Areas I-II-III causes pancreatic agenesis (Fujitani et al., 2006; Offield et al., 1996), the remaining Areas I, III and IV working synthetically in the *Pdx1*$^{\Delta\text{II}}$ state were sufficient to drive early pancreatic multipotent progenitor specification and apparently full-scale exocrine acinar and ductal differentiation. Thus, the organization of the *Pdx1* transcriptional regulatory landscape in the *Pdx1*$^{\Delta\text{II}}$ mutant does not prevent transcription factor and co-factor interactions essential for the early specification and differentiation of non-endocrine cell types, presumably including genomic regulatory interactions that are mediated by chromatin looping (e.g. Pasquali et al., 2014). Previous studies indicate that the broad *Pdx1* expression within embryonic pancreatic buds and in the developing acinar compartment involves *trans*-acting contributions from the Ptf1a transcription factor within Area III and likely also Area IV (Gannon et al., 2001; Wiebe et al., 2007).

Pdx1 and Area II function in endocrine fate selection and progression into maturity

The massive failure of endocrine progenitor formation in the ‘pancreas global’ *Pdx1*$^{\Delta\text{II/−}}$ germline heteroallelic animals is likely to be due to failures in Pdx1-mediated *trans*-activation of *Neurog3* in

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the developing pancreatic epithelium. These results indicate that a relatively high Pdx1 level within the epithelium is needed to engage the Neurog3^{HIGH} state, leading to subsequent commitment toward all endocrine cell lineages (Yang et al., 2011). Moreover, the data from the endocrine-specific deletion of Area II function suggest that increased *Pdx1* works within endocrine progenitors to direct progeny cells away from the α-cell fate, prominently through the repression of *Arx* expression and a negative influence via chromatin marking. It is possible that the degree of reduction in the relative number of β cells (versus α cells) in the *Pdx1*$^{endo\text{-}\Delta II}$ condition was constrained by our method of creating this state, which used Cre-based inactivation of a conditional protein null allele. Pdx1 protein could thus have persisted in lineage-committing cells at a high enough level, over sufficient time, for large numbers of cells to enter the β-cell lineage. Subsequently, at later stages of ontogeny, the lower *Pdx1*$^{\Delta II}$-derived protein level could be sufficient to prevent large-scale drift to another endocrine fate (note that substantial numbers of Ins and Gcg co-expressing cells were found) but be inadequate for driving proper maturation to the final β-cell state. These widespread effects under lowered Pdx1 production occurred in the absence of any apparent effect on the level of mRNA for the β-cell determinant Pax4 (Fig. 4A); this Pdx1-Pax4 discrepancy has been discussed previously (e.g. by Yang et al., 2011), but caveats here include possible heterogeneous effects across the cell population, and the current unavailability of reliable antibodies to assess Pax4 protein levels at cellular resolution. The different chromatin marking on the enhancer regions of *Arx* and *Pax4* in *Pdx1*$^{endo\text{-}\Delta II}$ endocrine progenitor cells is likely to reflect the potential recruitment of different co-regulator complexes at these loci. It has been reported that Pdx1-recruited Brg-containing versus Brm-containing SWI/SNF complexes play opposing regulatory roles in β cells *in vitro* (McKenna et al., 2015). Therefore, we speculate that Pdx1 recruits its co-regulator complexes in a cell type- and gene-specific manner, leading to a diversified outcome in the chromatin architecture and transcriptional status of different target genes.

Finding that a reduced proportion of β versus α cells formed in the absence of Area II suggests that other transcription factors (such as Nkx6.1, Nkx2.2 and MafB) working together with the low level of Pdx1 are involved in keeping embryonic endocrine cells on their correct differentiation tracks. In fact, Pdx1 has been shown to directly repress MafB expression in β cells, preventing entry, although possibly incomplete, to the α-like program (Gao et al., 2014; Yang et al., 2011). In terms of deriving the various islet cell types in their correct proportions, the principal effect of removing Area II function seemed to be on the β and α populations, without significantly affecting the other hormone-expressing cell types (although we did score an overall ∼25% reduction in endocrine cell numbers in the *Pdx1*$^{endo\text{-}\Delta II}$ condition). Even though the amount of Pdx1 from a *Pdx1*$^{\Delta II}$ allele could, with other β-cell lineage-driving factors, function to preserve largely appropriate lineage allocation, β-cell maturation was nonetheless prevented. Consistent with this idea, we found widespread β-cell dysfunction, without the increased proportional representation of α versus β cells or Ins^{+} Gcg^{+} bihormonal cells, with the *Pdx1*$^{\Delta II/-}$ condition generated in insulin-expressing β cells via *RIP2*^{Cre}. This demonstrates that, once the β-cell lineage is ‘appropriately entered’, reductions in Pdx1 protein level as observed in this mutant only affect β-cell maturation and functional activity and not cell identity per se. The β cells under this condition did not drift *en masse* toward an α-like condition, unlike those under the complete inactivation of *Pdx1* described by Gao et al. (2014).

The mixed-islet phenotype, with normally peripheral non-β cells improperly located more centrally within the islet, was detected in all Area II mutant classes (*Pdx1*$^{\Delta II/-}$, *Pdx1*$^{\Delta II/\Delta II}$, *Pdx1*$^{endo\text{-}\Delta II}$ and *Pdx1*$^{beta\text{-}\Delta II}$). We found no evidence that the intermingled α cells came from *trans*-differentiation of β cells (Fig. S10), but it is unclear what caused this islet architecture disturbance. This islet phenotype is relatively common and often ill-defined regarding the degree of intermixing, and it arises under multiple genetic or physiological perturbations, including β-cell-specific deletion of *Pdx1*, *Foxa2* or *Nkx6.1* (Gannon et al., 2008; Schaffer et al., 2013; Sund et al., 2001), persistent *Hnf6* expression (Gannon et al., 2000), or under chronic hyperglycemia caused by altering K_{ATP} channel function or diet (Brereton et al., 2014; Roat et al., 2014; Shiota et al., 2005). Although the large effects at the mRNA level for the fate-instructive gene *Arx* (Fig. 4) played out into a more modest effect on β- versus α-cell numbers, this increase in α cells could contribute to islet cell intermixing. In addition, a large number of β cells that are profoundly deficient in moving along the final steps of β-cell differentiation might display altered preferences for other islet cell types as neighbors, allowing extensive intermingling of normally peripheral endocrine cells.

Interestingly, although all *Pdx1*$^{\Delta II/\Delta II}$ animals showed abnormal expression of key β-cell genes (Fig. S2C-E), only males developed hyperglycemia at a later stage (Fig. 1E′). In the present study, the cause of this sexual dimorphism phenotype is unclear. This dimorphism is observed in multiple rodent models (Amrani et al., 1998; Bell et al., 1994; Östenson et al., 1989; Yoshioka et al., 1997), possibly because of the protective effect of estrogen (Efrat, 1991) and the tendency for females in general to show increased glucose tolerance (Bonnevienielsen, 1982; Geisler et al., 2002).

MafA/B and Area II interactions in driving acquisition of β-cell maturity

Although MafA and MafB share high protein domain similarity, expression in Ins^{+} cells during early organogenesis, and the same binding specificity within *Pdx1* Area II (Vanhoose et al., 2008), they have different functional requirements in β-cell development. In mouse, MafB predominantly functions embryonically and during early postnatal stages, whereas MafA is only required in postnatal β cells (Artner et al., 2010; Hang et al., 2014). It is likely that MafB normally occupies Area II motifs before *Mafa* expression is initiated in pro-β cells. The lower level of Pdx1 protein produced under Area II deficiency had a strong negative effect on *Mafa* but not *Mafb* expression, suggesting a relatively selective and reciprocal positive feedback between *Pdx1* and *Mafa* in the β-cell lineage. This situation represents an interaction that normally functions to stabilize progression along the maturation phase of the β-cell differentiation program, via the activation of additional target genes.

MATERIALS AND METHODS

Mice

Information on mouse strains, including generation of the *Pdx1*$^{\Delta II}$ allele, is provided in the supplementary Materials and Methods. All animals and embryos were PCR genotyped. Animal handling was under protocols approved by the Vanderbilt University Medical Center Institutional Animal Care and Use Committee.

Immunohistochemistry, β-galactosidase staining and morphometric analysis

Tissues were prepared and β-galactosidase staining staining was performed as described (Fujitani et al., 2006). Information on antibodies and morphometric methods are provided in the supplementary Materials and Methods and Table S1. For data shown in Figs 2–6, no gender-specific phenotypes were detected. Statistical analysis was performed using single-factor ANOVA tests and significance determined by $P<0.05$.

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FACS analysis, cell sorting, and low cell number ChIP

To generate single cells for FACS, embryos were dissected and dissociated with Accumax (Sigma) for 20-40 min at 37°C, then stopped with an equal volume of L15 (Gibco). The cells were filtered through a nylon mesh, mixed with CD133-PE (0.05 µg per 100 µl; eBioscience 12-1331) for 30 min at 4°C, then washed and purified on a BD FACSDiva cell sorter. ChIP was performed using the Low Cell Number ChIP Kit (Diagenode) as described (Xu et al., 2011), with antibodies against H3K4me1 (Millipore 07-436), H3K27ac (Millipore 07-360) or H3K27me3 (Millipore 07-449; 1 µg per ChIP). Primers are listed in Table S3. All ChIP-qPCR data were normalized to IgG controls.

qRT-PCR

RNA was isolated from flow-sorted pancreatic cells using Trizol (Invitrogen), followed by DNase treatment (Ambion), cDNA synthesis (iScript, Bio-Rad) and qPCR (SsoFast, Bio-Rad) using the primers listed in Table S2. Three samples per genotype per stage were collected and qPCR was performed at least twice on each sample to determine ΔC_T. Results are shown as ΔC_T±s.e.m. and were subjected to Student's *t*-test to determine significance ($P<0.05$).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Y.-P.Y. and C.V.E.W. developed the concept, designed and/or performed experiments, interpreted data and wrote the manuscript. M.A.M. designed and generated mice. R.S. developed the concept, designed experiments and critically read the manuscript.

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Supplementary information

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