

PDX1 Binds and Represses Hepatic Genes to Ensure Robust Pancreatic Commitment in Differentiating Human Embryonic Stem Cells

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SUMMARY

Inactivation of the *Pancreatic and Duodenal Homeobox 1* (*PDX1*) gene causes pancreatic agenesis, which places *PDX1* high atop the regulatory network controlling development of this indispensable organ. However, little is known about the identity of *PDX1* transcriptional targets. We simulated pancreatic development by differentiating human embryonic stem cells (hESCs) into early pancreatic progenitors and subjected this cell population to *PDX1* chromatin immunoprecipitation sequencing (ChIP-seq). We identified more than 350 genes bound by *PDX1*, whose expression was upregulated on day 17 of differentiation. This group included known *PDX1* targets and many genes not previously linked to pancreatic development. ChIP-seq also revealed *PDX1* occupancy at hepatic genes. We hypothesized that simultaneous *PDX1*-driven activation of pancreatic and repression of hepatic programs underlie early divergence between pancreas and liver. In HepG2 cells and differentiating hESCs, we found that *PDX1* binds and suppresses expression of endogenous liver genes. These findings rebrand *PDX1* as a context-dependent transcriptional repressor and activator within the same cell type.

INTRODUCTION

The adult pancreas is comprised of two major functional compartments. The endocrine pancreas contains hormone-secreting cells, with the glucose-sensing, insulin-secreting β cells garnering tremendous clinical attention for their role in the pathology of diabetes. The exocrine pancreas contains digestive enzyme-secreting acinar cells and ductal epithelial cells that produce mucin and plumb the pancreas, providing transport channels for the secretion of acinar enzymes into the duodenum. All of these tissues originate embryonically from the definitive endoderm (DE), one of the three primary germ layers formed during gastrulation (reviewed extensively in Pan and Wright, 2011; Shih et al., 2013).

By embryonic day (E) 8.5 in the mouse embryo, the DE forms a sheet of simple squamous epithelium lining the ventral surface of the lordotic embryo. Anteriorly, a specialized pit termed the anterior intestinal portal (AIP) forms and its continued invagination results in a pocket—the foregut—whose descendants give rise to the esophagus, lungs, thyroid, liver, pancreas, and biliary system. Morphologically, the incipient pancreas is recognizable as dorsal and ventral evaginations on opposing sides of the posterior foregut around E9.5. At this stage, the pancreatic primordium is conspicuously labeled by the expression of the *ParaHox* gene *Pancreatic and Duodenal Homeobox 1* (*Pdx1*) (Ahlgren

et al., 1996; Guz et al., 1995; Jørgensen et al., 2007; Offield et al., 1996). Lineage tracing experiments show that these early *Pdx1*⁺ cells are multipotent, giving rise to all three components of the mature pancreas (Gannon et al., 2000; Gu et al., 2002). Loss-of-function studies further demonstrate that *Pdx1* is not only an indelible marker of the early pancreatic lineage but also is required cell autonomously for early pancreas formation (Ahlgren et al., 1996; Jonsson et al., 1994; Offield et al., 1996). In *Pdx1* null mutant embryos, patent dorsal and ventral buds form but quickly regress, resulting in pancreatic agenesis, severe postnatal hyperglycemia, and eventual death. *PDX1* is similarly expressed in the developing human pancreas (Jennings et al., 2013), and in 1997, a case of rare human pancreatic agenesis was reported in an individual homozygous for a cytosine deletion in codon 63 of *PDX1* (Pro63fsdelC) (Stoffers et al., 1997b). This result underscores why *PDX1* activation is considered an obligatory mile marker for the successful in vitro production of therapeutically relevant β cells from pluripotent human stem cells.

Despite the central role of *Pdx1*/*PDX1* in orchestrating pancreatic morphogenesis in mice and humans, vanishingly little is known about its direct transcriptional targets. Here, we chose to address this deficit by developing an in vitro human embryonic stem cell (hESC) differentiation protocol that specifically captures robust numbers of early multipotent, proliferative *PDX1*⁺



pancreatic progenitor (ePP) cells. Based on extensive molecular marker analysis, ePP cells on day 17 of differentiation strongly resemble the early mammalian dorsal and ventral pancreatic buds. We therefore performed chromatin immunoprecipitation followed by massively parallel DNA sequencing (ChIP-seq) in an effort to elaborate the pancreatic gene regulatory network over which PDX1 presides. Our analyses identified more than 350 genes who are simultaneously bound by PDX1 (within \pm 20 kb of the transcriptional start site [TSS]) and whose expression is upregulated on day 17 of differentiation. We also unexpectedly found that PDX1 binds classic liver marker genes such as *ALB*, *AFP*, and *TTR*, which label hepatoblasts, the precursors to hepatocytes, in the developing mammalian embryo. This result suggested that acquisition of pancreatic identity requires direct and simultaneous suppression of the liver transcriptional program by PDX1. We tested this hypothesis by transiently overexpressing PDX1 in HepG2 cells and by constitutively overexpressing PDX1 in hESCs and confirmed that PDX1 can indeed bind and repress a battery of hepatic genes. These results provide fundamental insight into how organ-specific transcriptional programs are established during embryonic development and recast PDX1 as both a transcriptional activator and repressor.

RESULTS

Early Pancreatic Progenitor Cells from Differentiated hESCs

We previously reported the production of ePP cells from the differentiation of hESCs in adherent culture (Teo et al., 2012). These ePP cells were characterized by high levels of *PDX1* expression on day 12, but quantitation by fluorescence-activated cell sorting (FACS) revealed that they numbered no more than 35% of the entire culture. We therefore explored other culture methodologies and platforms aimed at improving differentiation efficiency to ePP and discovered that *PDX1*⁺ cell numbers were increased substantially by initially plating hESC on fibronectin-coated transwell dishes and by extending retinoic acid (RA) treatment by 2 days and supplementing with FGF2, nicotinamide, and DAPT (FND) (see Figure 1A). On day 14, FND was replenished, and cultures were typically harvested on day 17 (Figure 1A). In this revised protocol, hESCs expectedly form a cobblestone-like lawn of DE cells by day 5 (Figure 1A). By day 10, distinct cell clusters emerge and shortly thereafter appear to undergo microlumen formation and fusion reminiscent of the tubulogenesis that occurs in vivo in the developing mouse pancreas (Figure 1A) (Kesavan et al., 2009; Villasenor et al., 2010). With continued differentiation, thickened ridges extend and

intersect across the transwell in a honeycomb-like meshwork (Figure 1A).

To determine how faithfully ePP production in transwell culture followed the normal program of organogenesis, we used qRT-PCR to examine the expression of a panel of signature marker genes from days 0 to 17 of differentiation. These analyses document the transition from pluripotency to mesendoderm, which was characterized by the downregulation of pluripotency genes *NANOG*, *OCT4*, and *SOX2* and the upregulation of *BRACHYURY (T)*, *GOOSECOID*, *MIXL1*, and *EOMES* (Figure S1A). This event was followed shortly thereafter by upregulation of pan-DE (*GATA4*, *CXCR4*, *SOX17*, *FOXA2*, *GATA6*), ventral DE (*HHEX*), and gut tube (*HNF1B*) marker genes (Figures 1B and S1A). Between days 5 and 17, dorsal endoderm (*ISL1*, *MNX1*) and posterior foregut/pancreatic progenitor (*SOX9*, *PDX1*, *HNF6*, *NKX6-2*, *HNF1A*, *SOX4*) markers were increasingly expressed (Figures 1B and S1A). Western blot analyses on differentiated hESC from days 0 to 17 confirmed the transition from DE (*SOX17*, *FOXA2*) to pancreatic epithelial progenitors (*SOX9*, *PDX1*, E-CAD) (Figure S1B). Immunodetection analyses further showed abundant *FOXA2*-, *HNF1B*-, *SOX9*-, or E-CAD-positive cells on day 10 (Figure 1C). Two days later, significant numbers of *SOX9*;*PDX1* double-positive cells were observed, consistent with the emergence of mitogenic multipotent ePP cells in vivo (Jennings et al., 2013; Seymour et al., 2007) (Figure S1C). Significant numbers of *HNF1B*-, *SOX9*-, *PDX1*-, or E-CAD-positive cells were maintained on day 17, particularly in the thickened ridges that crisscross the transwell (Figure 1C). Based on the expression profile of hESC-derived ePP cells (Figures 1B and S1A), we reasoned that these cells closely approximate the *Pdx1*⁺ dorsal and ventral pancreatic buds in E9.5 mouse embryos, at a stage prior to the robust activation of endocrine-commitment genes such as *Ngn3* and *Nkx6-1* (Ahlgren et al., 1996; Jennings et al., 2013; Jørgensen et al., 2007; Offield et al., 1996).

PDX1 Binds a Battery of Foregut/Midgut and Early Pancreatic Genes in hESC-Derived ePP Cells

PDX1 plays a preeminent, evolutionarily conserved role in orchestrating pancreatic morphogenesis, but surprisingly little is known about the identity of its transcriptional targets during embryonic development. We therefore combined high-affinity polyclonal *PDX1* antibodies with chromatin immunoprecipitation and deep sequencing (ChIP-seq) in an effort to uncover those immediate downstream genes that govern the early growth and development of the human pancreatic anlagen. For these studies, we selected day 17 of differentiation—a time point that consistently yielded large numbers (\geq 65%) of *PDX1*⁺ ePP cells (Figure 1D). These analyses revealed 15,436 *PDX1*-bound regions that map to 6,212 genes (false discovery

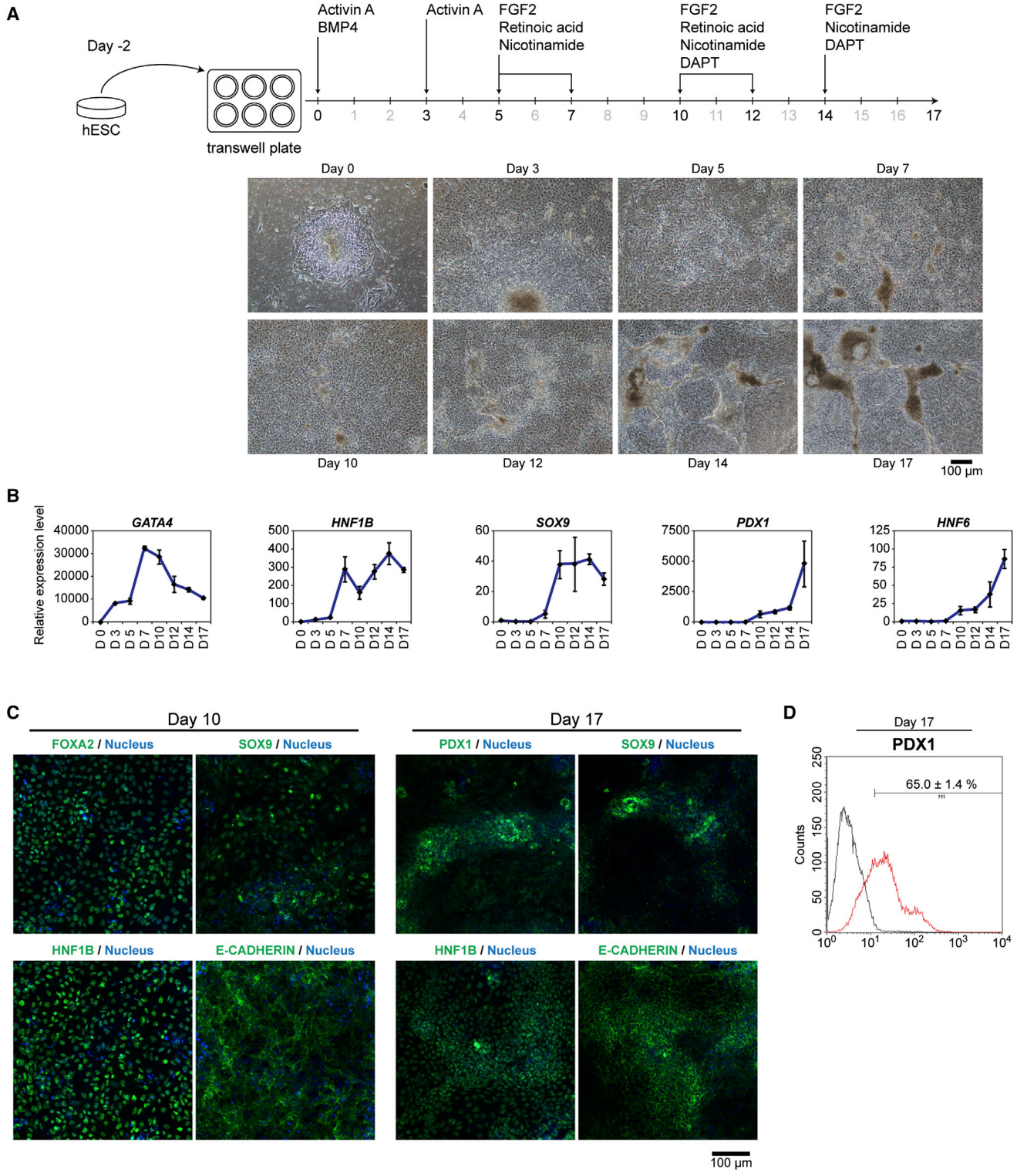


Figure 1. Directed Differentiation of hESCs into Early Pancreatic Progenitors
 (A) Schematic of 17-day pancreatic differentiation protocol. On day –2, HES3 cells are plated into fibronectin-coated transwell plates. Differentiation is initiated on day 0. Growth factors (activin A, BMP4, and FGF2) and small molecules (RA, Nic, and DAPT) were added at the indicated days (see the [Experimental Procedures](#) for additional details). The typical morphological changes that occur during differentiation are shown below the schematic. Scale bar represents 100 µm.

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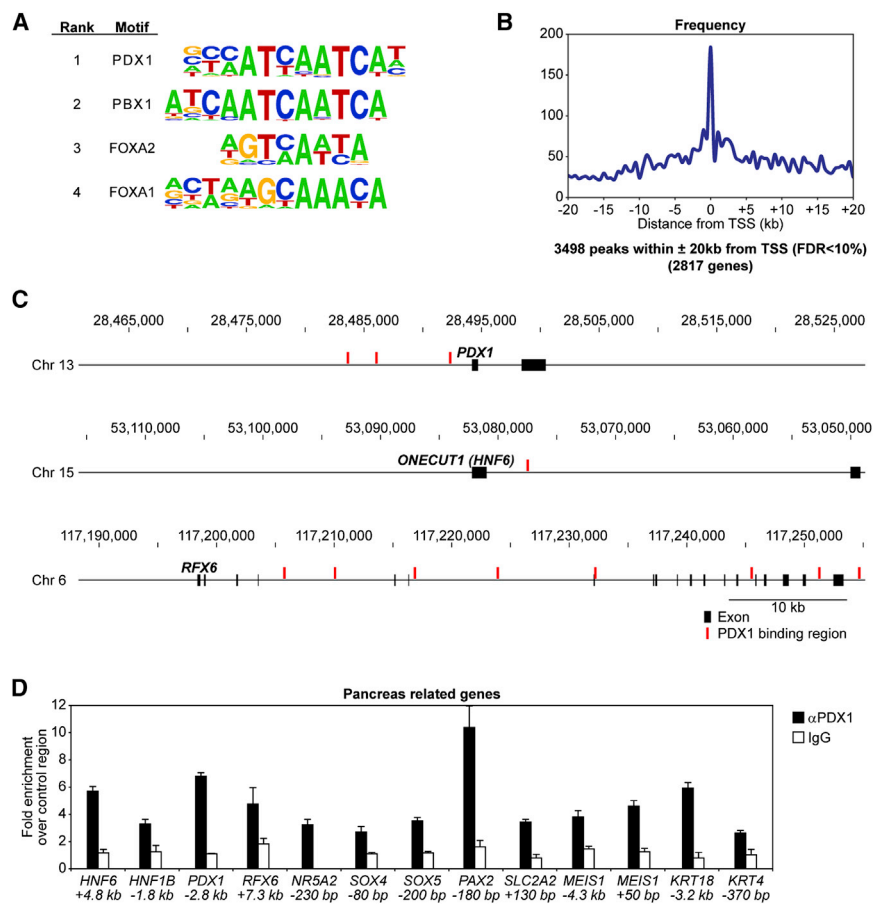


Figure 2. PDX1 Binds the Regulatory Regions of Genes Expressed in Foregut/Midgut Derivatives in hESC-Derived Human Pancreatic Progenitors

(A) De novo motif analyses identify PDX1, PBX1, FOXA2, and FOXA1 binding motifs as the top ranking motifs in PDX1-bound regions identified via ChIP-seq.

(B) Relative frequency of PDX1 binding events within ±20 kb of the TSS of candidate target genes (FDR <10%).

(C) Representative view of PDX1-binding sites within the *PDX1*, *ONECUT1* (*HNF6*) and *RFX6* genomic loci.

(D) ChIP-qPCR independently confirms the presence of PDX1 on regulatory regions of selected pancreas-related genes. Error bars indicate the SD of three technical replicates from a single ChIP-qPCR experiment. These data were independently confirmed in repeat pull-down qPCR experiments (data not shown). Normal goat IgG is the negative control.

See also [Table S1](#).

rate [FDR] < 0.1 with no distance cutoff; [Table S1](#), part A). The PDX1/PBX1-complex homeodomain-binding motif was the most highly enriched among the sequence reads, followed by the FOXA1/FOXA2 forkhead/winged helix DNA-binding motif ([Figure 2A](#)). PBX1 binds 5' to its half-site ATGAT¹, whereas PDX1/HOX binds 3' to the half-site TTAATGG, with an overlap at the middle TT (underlined), and these proteins heterodimerize to modulate gene transcription ([Dutta et al., 2001](#); [Knoepfler et al., 1996](#); [Liu et al., 2001](#); [Swift et al., 1998](#)). These findings provide strong evidence that our ChIP-seq data are highly enriched for specific PDX1 binding events and further suggest that some PDX1-bound targets in ePP cells are co-regulated by PBX1 and FOXA proteins.

We next analyzed the binding profile of PDX1 and observed an increased binding frequency near the TSS of genes, suggesting that PDX1 plays a major role in tran-

scriptional regulation ([Figure 2B](#)); 3,498 PDX1 binding sites are within ±20 kb of the TSS (FDR < 0.1), corresponding to 2,817 genes ([Figure 2B](#); [Table S1](#), part B). Among these were genes typically associated with gut endoderm, specifically the posterior foregut region where *Pdx1* shows localized expression during early mouse pancreatic development. These genes include endoderm (*CXCR4*, *LHX1*, *GATA4*, *GATA6*), pancreas (*MNX1*, *HNF1B*, *HNF1A*, *ONECUT1* (*HNF6*), *PROX1*, *SOX9*, *PDX1*, *MEIS1*, *RFX6*, *PAX2*, *GLIS3*, *NR5A2*, *NKX2-2*, *SOX4*, *SOX5*, *KRT4*, *KRT18*, *KRT19*, *CPE*, *IAPP*, *SLC2A2*, *PLA2G1B*), and numerous *HOX* (*HOXA1*, *HOXA2*, *HOXA3*, *HOXA4*, *HOXA6*, *HOXB2*, *HOXC13*, *HOXD12*) genes ([Figures 2C](#) and [S2A](#); [Table S1](#), part B). Genes typically associated with intestinal development and homeostasis (*CDX2*, *LGR5*, *DPP4*) were also identified ([Figure S2A](#)), which is consistent with the early expression of *Pdx1* in the duodenum and its genetic

(B) Kinetics of endodermal (*GATA4*), gut tube (*HNF1B*), and pancreatic progenitor (*SOX9*, *PDX1*, *HNF6*) marker gene expression by qPCR over the course of 17 days of differentiation. Error bars represent the SD of three biological replicates.

(C) Immunostaining for FOXA2, HNF1B, SOX9, E-CADHERIN, or PDX1 on days 10 or 17 of differentiation as indicated. Scale bar represents 100 μm.

(D) Quantitation of PDX1⁺ pancreatic progenitors by FACS on day 17.



requirement for normal rostral duodenum patterning (Ahlgren et al., 1996; Guz et al., 1995; Jennings et al., 2013; Offield et al., 1996). ChIP-qPCR analyses independently confirmed several of these binding events (Figures 2D and S2D). Expression analysis also revealed that *HOXA4* and *CDX2* levels continuously increased from day 10 of differentiation onward, paralleling *PDX1*, while *DPP4* peaked on day 10/12 and then declined (Figures 1B and S2E).

To gain further insight into the identity of PDX1-bound target genes, we performed Gene Ontology (GO) analyses. Statistically significant GO terms included “developmental process,” “organ development,” “embryo development,” “organ morphogenesis,” and “tube development” (Figure S2B). Genomic Regions Enrichment of Annotations Tool (GREAT) (<http://great.stanford.edu/public/html/index.php>) analysis refined the functional significance of PDX1-bound regions to GO terms such as “endocrine pancreas development” and “columnar/cuboidal epithelial cell differentiation” with the corresponding pathway designated as “regulation of gene expression in early pancreatic precursor cells” (Figure S2C). PDX1-bound targets were also generally associated with genes expressed in the foregut/midgut region when compared against the Mouse Genome Informatics (MGI) database (Figure S2C). Together, these bioinformatics approaches provided further confidence that PDX1-bound targets in day 17 ePP cells are developmentally relevant to the formation of the human pancreas.

Candidate PDX1 Transcriptional Targets through Comparative Microarray Analysis

Microarray analyses performed in quadruplicate on days 0, 10, and 17 of differentiation revealed that these selected time points corresponded to unique gene expression signatures (Figure 3A; Table S2, parts A–C). Consistent with our qPCR studies, numerous pancreas-related genes were upregulated by day 17, including *TM4SF4*, *MAFB*, *CDH1* (*E-CAD*), *SPINK1*, *PDX1*, *PROX1*, *GHRL*, *RBP4*, *RFX6*, *PCSK2*, *HOXA2*, and *HNF1A*, whereas several others such as *PITX2*, *MEIS2*, *DACH1*, *ONECUT2*, *JAG1*, *FOXA2*, *MEIS1*, *ISL1*, *SOX9*, *HNF1B*, *KRT19*, *GATA4*, *GATA6*, and *MNX1* were expressed from day 10 onward (Figure 3B). Pluripotency genes *NANOG*, *OCT4*, and *SOX2* were predictably downregulated by day 10, while mesendoderm (*EOMES*) and DE (*SOX17*) genes were downregulated on day 17 (Figure 3B). The slight increase in *SOX2* on day 17 was consistent with the expression of *Sox2* in the mouse anterior foregut (Que et al., 2007) (Figure 3B).

Next, we compared the list of PDX-bound genes to the day 17 microarray results to identify genes that are potentially directly regulated by PDX1. Among the 2,817 genes bound by PDX1 (± 20 kb from the TSS), 357 were upregu-

lated, whereas 102 were downregulated on day 17 as compared with day 0 (Figure 3C; Table S2, parts D and E). Autoregulation of *PDX1* is well documented, and *PDX1* was thus as expected among these 357 genes (Table S2, part D) (Chakrabarti et al., 2002; Marshak et al., 2000). To home in on key PDX1 targets, we took advantage of two previously published data sets that attempted to identify Pdx1-regulated genes in cells or tissue isolated directly from the developing mouse embryo. In the first, Pdx-eGFP⁺ cells were isolated by cell sorting from transgenic *Pdx1-eGFP* E10.5 mouse embryos, and their gene expression profile determined with Affymetrix microarrays (Gu et al., 2004). From 56 enriched mouse genes thus identified, only 15 human homologs are bound by PDX1 (Figure 3D; Table S3, part A). These include the DE marker *IGFBP5* and pancreatic regulator *MEIS1*, as well as a series of genes poorly characterized for their role in pancreas growth and development (*NR2F1* (*COUP-TF1*), *TNFAIP8*, *TANC1*, and *WSB1* (Figure 3D), all of which show increased expression on day 17 (Tamplin et al., 2008). Consistent with its expression in ePP cells (Pan and Wright, 2011; Shih et al., 2013), *Onecut1* (*Hnf6*) was among the 56 upregulated genes in Pdx1^{eGFP}-sorted cells reported by Gu et al. (2004). Our results showed that *ONECUT1* expression increased during ePP differentiation (Figure 1B) and that the *ONECUT1* locus was bound by PDX1 (Figures 2C and 2D).

In a second study, comparative microarrays were performed on microdissected WT and *Pdx1*^{-/-} E10.5 dorsal pancreatic buds (Svensson et al., 2007). (Beyond this stage, the unbranched *Pdx1*^{-/-} pancreatic buds growth arrest and regress [Ahlgren et al., 1996; Offield et al., 1996].) From 73 genes downregulated in *Pdx1* null mutant buds, 20 human homologs were bound by PDX1 (Figure 3E; Table S3, part B). Among these 20, 12 showed increased expression on day 17 and include known endoderm and pancreas markers (*WFDC2*, *CDH1* (*E-CAD*), *ANXA4*, *ITM2B*, *PARM1*), as well as a diverse group of genes whose functions in pancreas morphogenesis are unknown (*POSTN* (*PERIOSTIN*), *SORT1*, *SPINT2*, *SERPINB1*, and *DYNLT3*) (Figure 3E) (Hoffman et al., 2008; Tamplin et al., 2008). Taken together, these two comparative studies underscore the developmental significance of PDX1-bound target genes revealed by our in vitro studies of differentiating hESCs.

PDX1 is also expressed in adult β cells, and heterozygous mutations in *PDX1* are linked to autosomal dominant early onset type 2 diabetes (*MODY4*) (Ohlsson et al., 1993; Stoffers et al., 1997a). Moreover, in mice, *Pdx1* is genetically required for the maintenance of mature β cell function (Ahlgren et al., 1998; Gannon et al., 2008). We therefore asked whether there are common PDX1 gene targets in day 17 ePP cells and in a recently published study of human

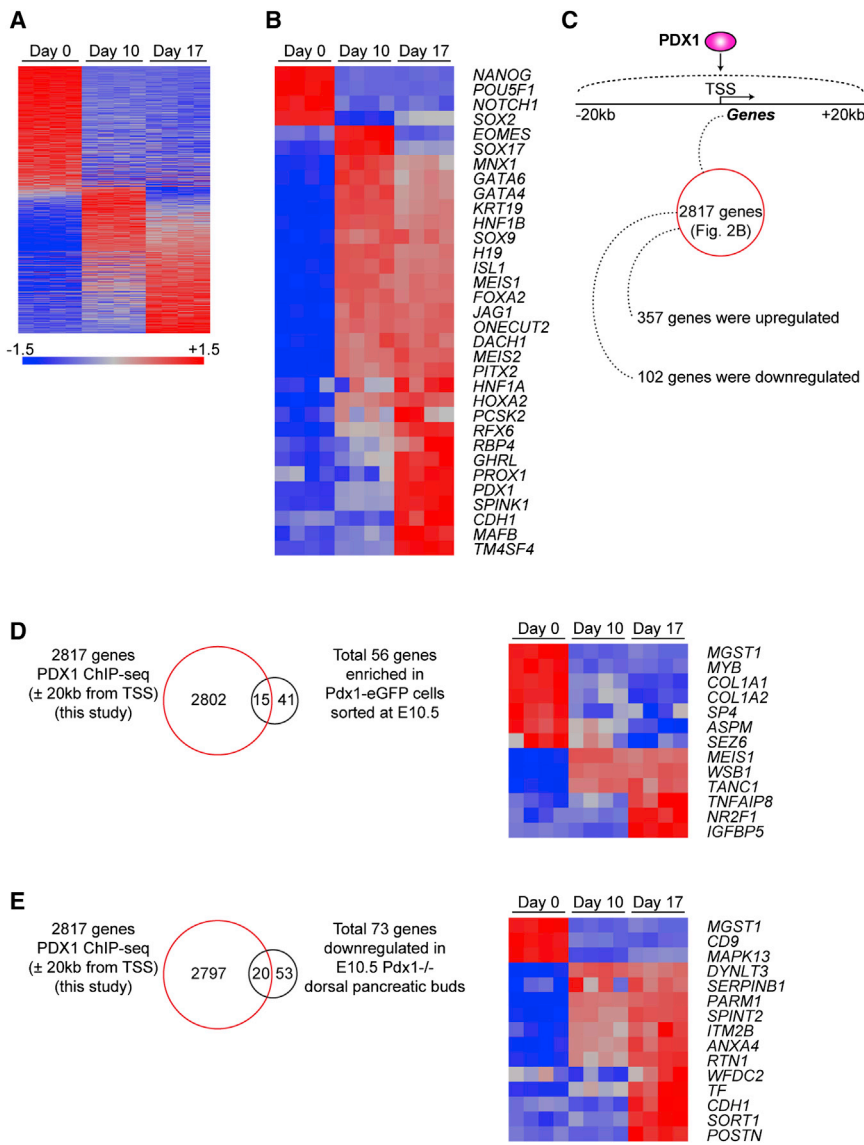


Figure 3. PDX1 Binds and Potentially Regulates Numerous Developmentally Important Pancreatic Genes

(A) Microarray gene expression heatmap of hESCs on days 0, 10, and 17 of pancreatic differentiation. Each time point is represented by four biological replicates.

(B) Microarray gene expression heatmap of selected pluripotency, gastrulation/primitive streak-related, DE, and pancreatic lineage-related genes from days 0 to 17.

(C) Schematic showing the number of PDX1-bound genes (± 20 kb from the TSS) that are upregulated and downregulated on day 17 as compared with day 0 (>2 -fold; FDR <0.05).

(D) Of the 56 genes enriched in pancreatic progenitor cells isolated from the E10.5 mouse embryo (Gu et al., 2004), 15 homologs are bound by PDX1 on day 17 of differentiation (2,817 within ± 20 kb from the TSS). Microarray gene expression heatmap showing the increase in gene expression of 6 of 15 common targets from days 0 to 17. The expression of *IGFBP5*, which encodes an endodermal marker, peaks at day 5, plummets on day 7, and continually increases beginning on day 12 by qPCR (data not shown).

(E) In E10.5 microdissected *Pdx1*^{-/-} E10.5 dorsal pancreatic buds, 73 genes were reported to be significantly downregulated (Svensson et al., 2007). Of these, 20 homologs are bound by PDX1 on day 17 of differentiation (2,817 within ± 20 kb from the TSS). Microarray gene expression heatmap depicting the increase in gene expression of 12 of 20 common targets from days 0 to 17 of pancreatic differentiation. See also Tables S1 and S2.

islets harvested from nondiabetic donors (Khoo et al., 2012). We found 1,096 PDX1-bound genes shared between the two tissue types (Figure S3A; Table S3, part C) that GREAT classifies into biological processes such as “pancreas development,” “type B pancreatic cell differentiation,” and “endoderm development” (Figure S3C). We next identified the mouse homologs of the 2,817 human genes bound by PDX1 (± 20 kb from TSS) (Figure 3C) and overlapped these with a more limited ChIP-on-Chip study using the NIT-1 mouse insulinoma cell line (Figure S3B; Table S3, part D) (Keller et al., 2007). Consistent with a β cell origin of this cell line, among the 99 shared genes were known β cell regulators, including *MEIS2*, *NKX2-2*, *PDX1*, *TLE1*, and *TLE3* (Table S3, part D). Taken together, these findings suggest

that there is substantial overlap between PDX1 transcriptional targets during development and adult β cell homeostasis.

PDX1 Binds and Represses Hepatic Genes

In the developing mouse embryo, both explant culture experiments and comprehensive lineage tracing show that the liver and ventral pancreas arise from bipotent, and possibly multipotent, precursors in the foregut endoderm (Angelo et al., 2012; Deutsch et al., 2001; Miki et al., 2012; Tremblay and Zaret, 2005). Consistent with this tight lineage relationship, closer inspection of our microarray data revealed companion upregulation of early liver lineage genes, including *APOA2*, *TBX3*, *FOXA1*, *TTR*, *AFP*, *APOB*,



HHEX, *HNF4A*, *APOC3*, *ALB*, *SERPINA1* (*A1AT*), and *TDO2* on day 17 (Figure 4A). We reasoned that this finding reflected the heterogeneous nature of our pancreatic differentiation protocol, unmasking the identity of the roughly ~35% PDX1⁻ (Figure 1D), yet endodermally derived cells present in a given differentiation. We thus confirmed by qPCR that a series of canonical hepatic markers (*FOXA1*, *FOXA3*, *HNF4A*, *AFP*, *ALB*, *TTR*, *APOA2*) were indeed upregulated in an independent differentiation experiment (Figure S4C). We were surprised to find that PDX1 bound a number of these genes (± 20 kb from the TSS), including *HHEX*, *FOXA1*, *FOXA3*, *TBX3*, *TTR*, *AFP*, *ALB*, *FABP1*, *APOA2*, *PHKA2*, *GYS2*, *ARG1*, *LEAP2*, and *CDH17* (Figures 4B and S4A; Table S1, part B). Early *Pdx1* expression in the mouse embryo exclusively labels the dorsal and ventral pancreatic buds, the caudal stomach, bile duct, and rostral duodenum (Jonsson et al., 1994; Jørgensen et al., 2007; Offield et al., 1996). Recent high-resolution immunohistochemistry and whole-mount in situ hybridization further demonstrate that *Pdx1* and *Afp* do not co-localize in the AIP in the early-somite-stage mouse embryo (Miki et al., 2012). Consistent with this, PDX1 and AFP labeled distinct cell populations on day 17 of differentiation, with small nests of AFP⁺ cells often abutting or near PDX1 labeled ridges (Figure 4C). This result raises the possibility that a critical aspect of PDX1 function is to bind and repress hepatic genes in pancreatic progenitor (PP) cells, ensuring stable commitment to the pancreatic lineage.

To test this hypothesis, we first asked whether *PDX1* overexpression could repress liver genes in the hepatocellular carcinoma cell line HepG2. We transiently overexpressed WT PDX1, or a mutant form of PDX1 (PDX1^{N196S}) that cannot bind DNA (Horb et al., 2003) and observed suppression of endogenous *AFP*, *ALB*, *TTR*, *APOA2*, and *FOXA1* gene expression by WT, but not PDX1^{N196S} (Figure 4D). In support of direct repression by PDX1, we confirmed by ChIP-qPCR that, when overexpressed, WT PDX1 binds endogenous *AFP*, *ALB*, *TTR*, and *FOXA1* regulatory sequences (Figure S4B; Table S1, part B). These results show that the DNA-binding activity of PDX1 is required for its ability to act as a strong transcriptional repressor when overexpressed in a terminally differentiated heterologous cell line.

We next asked whether PDX1 behaved similarly during hESC differentiation. On day 17, we observed that assorted liver marker genes were upregulated both by microarray and qPCR (Figures 4A and S4C). We therefore reasoned that constitutive overexpression of PDX1 in hESC may suppress or altogether eliminate the emergence of liver progenitors alongside ePP cells. Thus, we generated hESC lines that stably overexpressed either GFP or WT human PDX1. Importantly, overexpression of GFP or PDX1 neither impaired pluripotency nor the ability to sequentially acti-

vate endodermal or pancreatic marker genes (data not shown; see also Bernardo et al., 2009 and Lavon et al., 2006). Western analysis revealed that PDX1 almost entirely suppressed AFP protein expression (Figure 4E), corresponding to a nearly 90% reduction at the mRNA level by qPCR (Figure 4F). In addition, PDX1 overexpression strongly suppressed the expression of *AFP*, *ALB*, *TTR*, *APOA2*, and *FOXA1* (Figure 4F). Taken together, these data suggest that *PDX1* engages a robust commitment to the pancreatic lineage at least in part by binding the regulatory regions of liver genes and directly repressing their transcription.

DISCUSSION

Given its conspicuous expression in the early pancreatic anlagen, its ability to directly regulate the *Insulin* gene promoter and its dramatic loss-of-function phenotype, PDX1 is often designated as “master regulator” of pancreatic fate (Pan and Wright 2011; Shih et al., 2013). Consequently, numerous studies have attempted to use ectopic PDX1 to reprogram “permissive” tissues and assorted cell lines into the pancreatic lineage and, ultimately, functional beta cells for cell replacement therapy (Meivar-Levy and Ferber, 2010). For example, adenoviral approaches have overexpressed either WT PDX1 or an activated PDX1-VP16 fusion (C-terminal addition of the 80 amino acid transcriptional activation domain of the *Herpes simplex virus* VP16 protein) in the adult mouse liver, an organ whose embryonic origin is in close proximity to the ventral pancreas (Angelo et al., 2012; Deutsch et al., 2001; Miki et al., 2012; Tremblay and Zaret, 2005). Upregulation of signature pancreatic genes and islet hormones was observed to varying degrees, and depending on the study, different criteria were taken as a sign of “function” in the resulting proto-beta cells, the most stringent being the ability to positively affect blood glucose levels (Ber et al., 2003; Ferber et al., 2000; Imai et al., 2005; Kaneto et al., 2005; Kojima et al., 2003). Curiously, these in vivo studies principally focused on the acquisition of a handful of markers of pancreatic identity, but not the degree of preservation or loss of hepatic fate.

PDX1 overexpression experiments provide some evidence that this factor can partially engage the pancreatic program while conversely suppressing endogenous liver genes. In studies with primary rodent hepatocytes or liver epithelial cells, *Alb* levels were variably downregulated by overexpressing PDX1 or PDX1-VP16 (Fodor et al., 2007; Jin et al., 2008; Meivar-Levy et al., 2007; Yamada et al., 2006). Li et al. (2005) further showed that HepG2 cells expressing *TTR-XIHbox8-VP16*, encoding the *Xenopus* homolog of *PDX1* (*XIHbox8*) driven by the liver-specific *TTR* promoter, activated endocrine genes such as *Insulin*

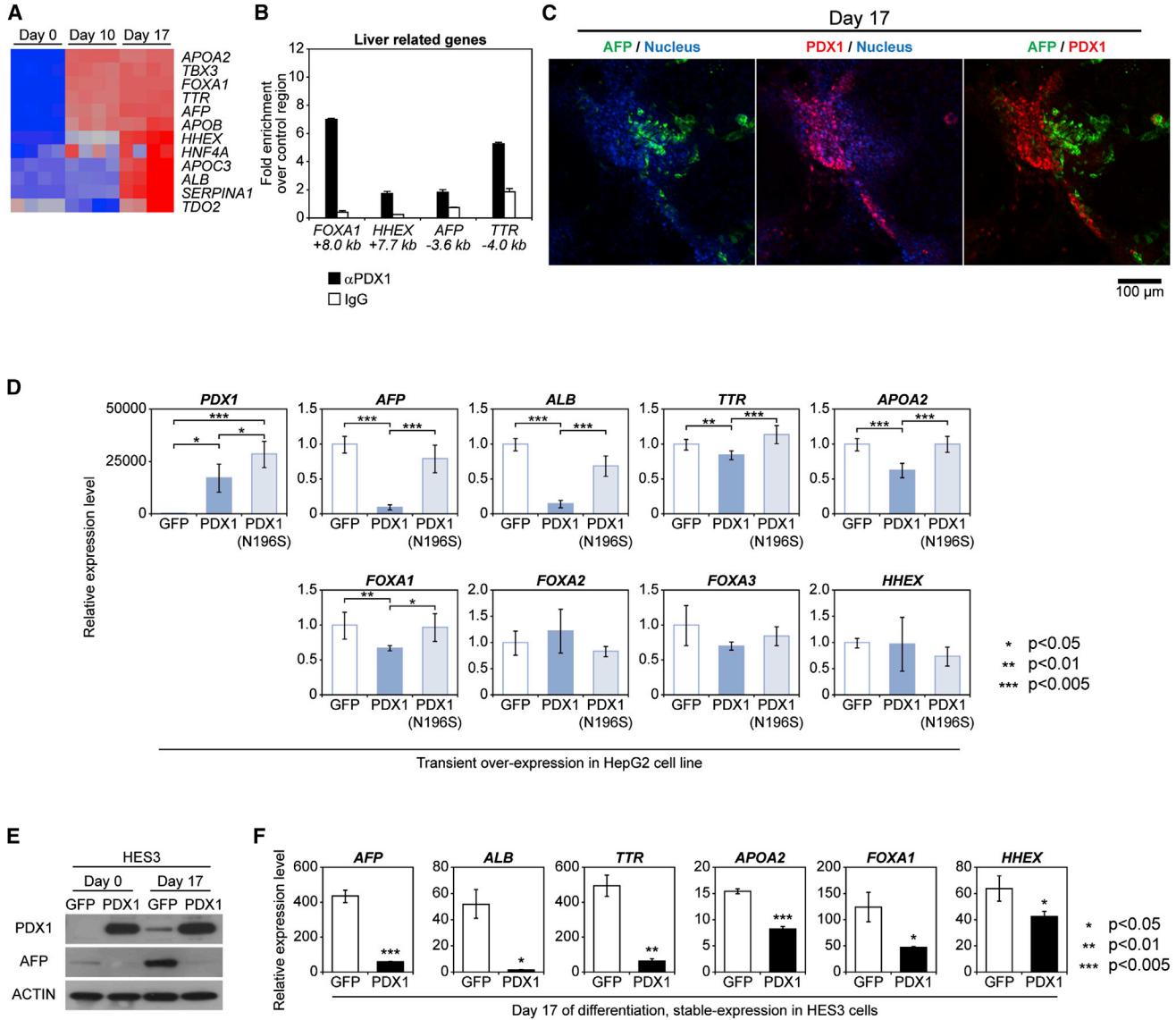


Figure 4. PDX1 Represses Liver Marker Genes

(A) Microarray gene expression heatmap showing increasing levels of liver lineage-related genes between days 0 and 17 of pancreatic differentiation.

(B) ChIP-qPCR provides independent confirmation that PDX1 binds in proximity to liver genes. Error bars indicate the SD of three technical replicates from a single ChIP-qPCR experiment. These data were independently confirmed in repeat pull-down qPCR experiments (data not shown). Normal goat IgG is the negative control.

(C) AFP and PDX1 immunostaining on day 17 of pancreatic differentiation. AFP and PDX1 label distinct cell populations that are often observed in close proximity. Scale bar represents 100 μ m.

(D) Expression of hepatic genes (*AFP*, *ALB*, *TTR*, *APOA2*, *FOXA1*, *FOXA2*, *FOXA3*, and *HHEX*) by qPCR in HepG2 cells after transient over-expression of GFP, PDX1, or the PDX1(N196S) mutant that cannot bind DNA. Both WT PDX1 and PDX1(N196S) are robustly overexpressed after transfection. All error bars indicate SD of three biological replicates. For all genes, data are shown relative to the GFP-transfected controls. p values are indicated.

(E) Western blot analyses for PDX1 and AFP on days 0 and 17 of pancreatic differentiation in GFP- or human PDX1-overexpressing hESC clones.

(F) Expression of liver markers (*AFP*, *ALB*, *TTR*, *APOA2*, *FOXA1*, *HHEX*) in hESCs stably overexpressing either GFP or human PDX1 and differentiated for 17 days. Error bars indicate the SD of three biological replicates. p values were calculated when compared with GFP control.



and suppressed *Alb*, *Transferrin (Trf)*, and α -1 *anti-trypsin (Serpin1a)* (Li et al., 2005). More akin to our PDX1 overexpression experiments in differentiating hESCs, Horb et al. (2003) reported that transgenic *TTR-XIHbox8-VP16 Xenopus* tadpoles showed some conversion of liver to pancreas, scored by reduction of *Ttr* and activation of *Ins*, *Gcg* and *Amylase (Amy)* (Horb et al., 2003). This in vivo transdifferentiation by XIHbox8-VP16 required the XIHbox8 DNA-binding homeodomain, but curiously WT XIHbox8 lacking the VP16 transactivation domain was entirely ineffective in ectopically activating pancreatic genes in the developing liver (Horb et al., 2003). In hESC-derived ePP cells, we demonstrated by ChIP-seq that WT PDX1 binds hepatic genes and formally established in HepG2 cells that such binding is necessary for the direct suppression of several cardinal liver markers (*AFP*, *ALB*, *TTR*, *APOA2*, and *FOXA1*). Given our ChIP-seq results, one prediction from work in the frog is that the XIHbox8-VP16 fusion protein should be recruited to and potently activate, rather than repress, endogenous liver genes. Such a result was not reported. We posit that the addition of the VP16 module so drastically alters WT XIHbox8 (Pdx1) that it is difficult to compare the results of Horb et al. (2003) with our own. In addition, the bulk of the studies employing XIHbox8/Pdx1-VP16 fusions did not carefully study temporally the transdifferentiation of liver to pancreas by assessing the expression of Pdx1-bound hepatic markers (e.g., *Afp*, *Ttr*, *Alb*, or *Foxa1*) at multiple time points, and consequently, one cannot exclude the possibility, ever how unlikely in our opinion, that an upregulation of hepatic genes via XIHbox8/Pdx1-VP16 did occur, even transiently. Taking together these published studies and our own findings, we favor a model whereby Pdx1-directed “transdifferentiation” of liver to pancreas requires PDX1 to act as both a transcriptional activator and repressor.

Consistent with our in vitro data, two recent genetic studies in the mouse provide strong in vivo evidence that Pdx1 is a bona fide transcriptional repressor. By E11.5, the pancreatic buds in *Pdx1* null mutant embryos arrest and begin to regress (Ahlgren et al., 1996; Offield et al., 1996). Recently, Seymour et al. (2012) performed high-resolution quantitative immunohistochemistry on *Pdx1*^{-/-} embryos and observed significant numbers of ectopic *Afp*⁺ cells within the dorsal pancreatic bud. This suggestion of partial conversion to the hepatic cell fate is consistent with prior work demonstrating that hepatic competence is not restricted to the region of the ventral foregut where the liver normally forms (Bossard and Zaret, 1998, 2000; Gualdi et al., 1996) and with our data that PDX1 represses *AFP* in vitro (Figures 4D–4F). Seymour et al. (2012) also showed that Pdx1 deficiency caused varying degrees of *Sox9* downregulation and that focal loss of *Sox9* in the developing pancreas led to elevated expression of hepatic

markers. Our results show that in hESC-derived ePP cells PDX1 binds SOX9 between exons 2 and 3 (Table S1, part A), which suggests that SOX9 is positively regulated by PDX1, as expected for these principal regulators of the pancreatic program. Phylogenetic sequence conservation in this region of mouse and human SOX9 genes (data not shown) fits the idea that this Pdx1-Sox9 regulatory relationship is central to the pro-pancreatic gene regulatory network. Taken together, these findings indicate that early-stage Pdx1⁺ progenitor cells are not stably determined (“metastable”), with Pdx1 positively regulating *Sox9* and actively repressing liver potential during a substantial period of early pancreas organogenesis.

In a second and very recent study, Gao et al. (2014) inactivated *Pdx1* in the adult β cell using Cre-Lox methods with concurrent indelible YFP labeling of the derived *Pdx1*^{-/-} cells (Gao et al., 2014). Expectedly, these mice became rapidly hyperglycemic—a result consistent with prior work (Ahlgren et al., 1998; Gannon et al., 2008)—but unexpectedly lineage-labeled *Pdx1*^{-/-} cells contained glucagon and expressed *MafB*, a transcription factor that in the adult mouse is restricted to the islet α -cells. These authors used ChIP from mouse insulinoma cell lines to detect PDX1 binding within 1.5 kb of the *MafB* TSS. Taken together, these findings strongly suggest that Pdx1 directly represses *MafB* transcription in adult β cells. Interestingly, *MafB* is required for the production of both α and β cells during pancreas development, but its expression is extinguished in β cells soon after birth (Hang and Stein, 2011). Consistent with this in vivo expression kinetic, we observed *MAFB* levels increasing from days 0 to 17 of hESC differentiation (Figure 3B). However, *MAFB* transcriptional regulation is apparently independent of PDX1 at these stages, as PDX1 binding was observed a great distance from the *MAFB* TSS (≥ 150 kb) (Table S1, part A). In addition, it is important to highlight recent data showing that, in contrast to mice, *MAFB* persists in a subset of human adult β cells (~9%) (Dai et al., 2012). This finding suggests that in mice Pdx1 adopts its role as a transcription repressor late in β cell ontogeny and that in humans PDX1⁺MAFB⁺ and PDX1⁺MAFB⁻ represent distinct β cell subtypes.

Our findings raise an important outstanding question: what is the mechanism underlying PDX1 transcriptional repression? We speculate that one answer lies in the top-ranking motifs enriched in our ChIP-seq data—PBX1 and FOXA1/A2. Nearly 20 years ago, Pdx1 was shown to bind the HOX-cofactor Pbx1 (pre-B cell leukemia factor 1), a member of the TALE (three-amino-acid loop extension) family of atypical homeodomain-containing proteins (Peers et al., 1995). *Pbx1* alternative splicing yields two isoforms differing at their C termini, the longer Pbx1a and shorter Pbx1b. Pdx1:Pbx1b complexes transcriptionally activate



target genes, while Pdx1:Pbx1a forms a repressor complex through the recruitment of co-repressor proteins such as NCoR-SMRT (or HDAC) (Asahara et al., 1999; Saleh et al., 2000). PBX1A and PBX1B are both expressed during human ePP differentiation (Figure S1A; A.K.K.T. and N.R.D., unpublished data), raising the possibility of differential recruitment within the same cell to activate or repress appropriate gene targets to direct lineage choice and stabilization. Similarly, FOXA transcription factors can also recruit HDAC via the co-repressor Groucho-related protein 3 (Grg3; formally Tle3), which is highly expressed in the pancreas during embryonic development, to silence genes central to hepatic differentiation (Lam et al., 2013; Santisteban et al., 2010). Our data also show that FOXA1/A2 binding sites were significantly enriched in sequence reads from day 17 PDX1 ChIP-seq (Figure 2A), and FOXA2:PDX1 co-binding was observed frequently in nearly 2,000 loci in mouse islets (Hoffman et al., 2010). Canonical FOXA1/A2 motifs exist close to PDX1 binding in both *AFP* and *TTR*, also suggesting context-dependent functions of FOXA proteins. Finally, it is important to note that approximately 100 genes are bound by PDX1 on day 17, but their expression is significantly downregulated by microarray (comparing day 17 to day 0) (Figure 3C; Table S2, part E). Obvious candidates for direct repression among these ~100 include *FGF8*, *TWIST2*, *ETV4*, and *ZIC3*, whose mouse orthologs are typically expressed in mesoderm or mesodermally derived tissues during embryonic development (<http://www.informatics.jax.org/genes.shtml>) (Table S2, part E). Methods to address the issue of direct repression or activation of PDX1 target loci include the development of PDX1-deficient hESC lines or lines carrying inducible knockdown tools for context and time-dependent inactivation.

EXPERIMENTAL PROCEDURES

Cell Culture

HES3 (ESO3; <http://www.nationalstemcellbank.org>) were cultured on γ -irradiated human Ortec feeders in hESC medium supplemented with 50-ng/ml FGF2 or in mTeSR 1 medium (STEMCELL Technologies) (Teo et al., 2012). Colonies were passaged every 7 days by treating with 1-mg/ml collagenase IV followed by fine scoring with a pipet tip. HepG2 cells (ATCC) were cultured in DMEM high glucose (Invitrogen), 10% fetal bovine serum (FBS), and 2-mM L-glutamine.

Transfection

Human *PDX1* cDNA was cloned into pTP6. The N196S point mutation was introduced using the Directed Mutagenesis kit (Agilent). Transfection of HepG2 and HES3 cells was performed with Lipofectamine 2000 (Invitrogen); 4 μ g of pTP6-*hPDX1* and pTP6-*hPDX1*^{N196S} were transfected into HepG2 cells for 48 hr followed by selection in puromycin (2 μ g/ml) for 6 days. Similarly, 4 μ g of pTP6-*hPDX1* was transfected into HES3 cells and 1 μ g/ml of puromycin

was included in the hESC medium for 1 week to select for *hPDX1*-overexpressing HES3 lines.

Pancreatic Differentiation

Feeder cells were depleted using a 70- μ m cell strainer (BD Falcon) prior to directed differentiation (Teo et al., 2012). hESCs were plated onto fibronectin-coated transwell plates (Corning) and cultured in hESC medium for 2 days. They were induced to differentiate in RPMI-1640, 2% B-27, 2-mM L-glutamine, 55- μ M β -mercaptoethanol, and 1 \times MEM NEAA medium supplemented with 50-ng/ml activin A and 50-ng/ml BMP4 for 3 days followed by 2 additional days in 50 ng/ml activin A (schematized in Figure 1A). Cultures were then exposed to 50-ng/ml FGF2, 3- μ M all-trans-RA, and 10-mM nicotinamide (Nic) for 5 days; 50-ng/ml FGF2, 3- μ M RA, 10-mM Nic, and 20- μ M DAPT for 4 days; and 50-ng/ml FGF2, 10-mM Nic, and 20- μ M DAPT for 3 days.

qPCR, Western Blot, Immunostaining, FACS, ChIP-Seq, and Microarray Hybridization and Data Analyses

Methods for qPCR, western blot, immunostaining, FACS, ChIP-seq, and microarray analyses were previously described (Teo et al., 2012; Tsuneyoshi et al., 2012). More detailed methods for ChIP-seq, microarray hybridization, and data analysis are provided in the Supplemental Experimental Procedures. Primers and antibodies are listed in Table S4.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and four tables and can be found with this article online at <http://dx.doi.org/10.1016/j.stemcr.2015.02.015>.

AUTHOR CONTRIBUTIONS

A.K.K.T. and N.R.D. designed the study and analyzed the data. A.K.K.T. performed hESC differentiation, qPCR, ChIP-seq, ChIP-qPCR, western blots, FACS, and comparative microarray studies. S.H. and L.W.S. provided bioinformatics support and access to deep sequencing. N.T. performed immunofluorescence, provided additional bioinformatics support, and designed all figures. E.K.T. performed subcloning, construct mutagenesis, qPCR, and transfections. C.V.E.W. provided key reagents, including the acid-purified goat anti-Pdx1 antibody used for all ChIP experiments and contributed to data analysis and interpretation. N.R.D. wrote the paper.

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REFERENCES

- Ahlgren, U., Jonsson, J., and Edlund, H. (1996). The morphogenesis of the pancreatic mesenchyme is uncoupled from that of the pancreatic epithelium in IPF1/PDX1-deficient mice. *Development* *122*, 1409–1416.
- Ahlgren, U., Jonsson, J., Jonsson, L., Simu, K., and Edlund, H. (1998). beta-cell-specific inactivation of the mouse *Ip1/Pdx1* gene results in loss of the beta-cell phenotype and maturity onset diabetes. *Genes Dev.* *12*, 1763–1768.
- Angelo, J.R., Guerrero-Zayas, M.I., and Tremblay, K.D. (2012). A fate map of the murine pancreas buds reveals a multipotent ventral foregut organ progenitor. *PLoS ONE* *7*, e40707.
- Asahara, H., Dutta, S., Kao, H.Y., Evans, R.M., and Montminy, M. (1999). Pbx-Hox heterodimers recruit coactivator-corepressor complexes in an isoform-specific manner. *Mol. Cell. Biol.* *19*, 8219–8225.
- Ber, I., Shternhall, K., Perl, S., Ohanuna, Z., Goldberg, I., Barshack, I., Benvenisti-Zarum, L., Meivar-Levy, I., and Ferber, S. (2003). Functional, persistent, and extended liver to pancreas transdifferentiation. *J. Biol. Chem.* *278*, 31950–31957.
- Bernardo, A.S., Cho, C.H., Mason, S., Docherty, H.M., Pedersen, R.A., Vallier, L., and Docherty, K. (2009). Biphasic induction of Pdx1 in mouse and human embryonic stem cells can mimic development of pancreatic beta-cells. *Stem Cells* *27*, 341–351.
- Bossard, P., and Zaret, K.S. (1998). GATA transcription factors as potentiators of gut endoderm differentiation. *Development* *125*, 4909–4917.
- Bossard, P., and Zaret, K.S. (2000). Repressive and restrictive mesodermal interactions with gut endoderm: possible relation to Meckel's Diverticulum. *Development* *127*, 4915–4923.
- Chakrabarti, S.K., James, J.C., and Mirmira, R.G. (2002). Quantitative assessment of gene targeting in vitro and in vivo by the pancreatic transcription factor, Pdx1. Importance of chromatin structure in directing promoter binding. *J. Biol. Chem.* *277*, 13286–13293.
- Dai, C., Brissova, M., Hang, Y., Thompson, C., Poffenberger, G., Shostak, A., Chen, Z., Stein, R., and Powers, A.C. (2012). Islet-enriched gene expression and glucose-induced insulin secretion in human and mouse islets. *Diabetologia* *55*, 707–718.
- Deutsch, G., Jung, J., Zheng, M., Lórá, J., and Zaret, K.S. (2001). A bipotential precursor population for pancreas and liver within the embryonic endoderm. *Development* *128*, 871–881.
- Dutta, S., Gannon, M., Peers, B., Wright, C., Bonner-Weir, S., and Montminy, M. (2001). PDX:PBX complexes are required for normal proliferation of pancreatic cells during development. *Proc. Natl. Acad. Sci. USA* *98*, 1065–1070.
- Ferber, S., Halkin, A., Cohen, H., Ber, I., Einav, Y., Goldberg, I., Barshack, I., Seiffers, R., Kopolovic, J., Kaiser, N., and Karasik, A. (2000). Pancreatic and duodenal homeobox gene 1 induces expression of insulin genes in liver and ameliorates streptozotocin-induced hyperglycemia. *Nat. Med.* *6*, 568–572.
- Fodor, A., Harel, C., Fodor, L., Armoni, M., Salmon, P., Trono, D., and Karnieli, E. (2007). Adult rat liver cells transdifferentiated with lentiviral IPF1 vectors reverse diabetes in mice: an ex vivo gene therapy approach. *Diabetologia* *50*, 121–130.
- Gannon, M., Herrera, P.L., and Wright, C.V. (2000). Mosaic Cre-mediated recombination in pancreas using the *pdx-1* enhancer/promoter. *Genesis* *26*, 143–144.
- Gannon, M., Ables, E.T., Crawford, L., Lowe, D., Offield, M.F., Magnuson, M.A., and Wright, C.V. (2008). *pdx-1* function is specifically required in embryonic beta cells to generate appropriate numbers of endocrine cell types and maintain glucose homeostasis. *Dev. Biol.* *314*, 406–417.
- Gao, T., McKenna, B., Li, C., Reichert, M., Nguyen, J., Singh, T., Yang, C., Pannikar, A., Doliba, N., Zhang, T., et al. (2014). Pdx1 maintains β cell identity and function by repressing an α cell program. *Cell Metab.* *19*, 259–271.
- Gu, G., Dubauskaite, J., and Melton, D.A. (2002). Direct evidence for the pancreatic lineage: NGN3+ cells are islet progenitors and are distinct from duct progenitors. *Development* *129*, 2447–2457.
- Gu, G., Wells, J.M., Dombkowski, D., Preffer, F., Aronow, B., and Melton, D.A. (2004). Global expression analysis of gene regulatory pathways during endocrine pancreatic development. *Development* *131*, 165–179.
- Gualdi, R., Bossard, P., Zheng, M., Hamada, Y., Coleman, J.R., and Zaret, K.S. (1996). Hepatic specification of the gut endoderm in vitro: cell signaling and transcriptional control. *Genes Dev.* *10*, 1670–1682.
- Guz, Y., Montminy, M.R., Stein, R., Leonard, J., Gamer, L.W., Wright, C.V., and Teitelman, G. (1995). Expression of murine STF-1, a putative insulin gene transcription factor, in beta cells of pancreas, duodenal epithelium and pancreatic exocrine and endocrine progenitors during ontogeny. *Development* *121*, 11–18.
- Hang, Y., and Stein, R. (2011). MafA and MafB activity in pancreatic β cells. *Trends Endocrinol. Metab.* *22*, 364–373.
- Hoffman, B.G., Zavaglia, B., Witzsche, J., Ruiz de Algora, T., Beach, M., Hoodless, P.A., Jones, S.J., Marra, M.A., and Helgason, C.D. (2008). Identification of transcripts with enriched expression in the developing and adult pancreas. *Genome Biol.* *9*, R99.
- Hoffman, B.G., Robertson, G., Zavaglia, B., Beach, M., Cullum, R., Lee, S., Soukhatcheva, G., Li, L., Wederell, E.D., Thiessen, N., et al. (2010). Locus co-occupancy, nucleosome positioning, and H3K4me1 regulate the functionality of FOXA2-, HNF4A-, and PDX1-bound loci in islets and liver. *Genome Res.* *20*, 1037–1051.
- Horb, M.E., Shen, C.N., Tosh, D., and Slack, J.M. (2003). Experimental conversion of liver to pancreas. *Curr. Biol.* *13*, 105–115.
- Imai, J., Katagiri, H., Yamada, T., Ishigaki, Y., Ogihara, T., Uno, K., Hasegawa, Y., Gao, J., Ishihara, H., Sasano, H., et al. (2005). Constitutively active PDX1 induced efficient insulin production in adult murine liver. *Biochem. Biophys. Res. Commun.* *326*, 402–409.
- Jennings, R.E., Berry, A.A., Kirkwood-Wilson, R., Roberts, N.A., Hearn, T., Salisbury, R.J., Blaylock, J., Piper Hanley, K., and Hanley, N.A. (2013). Development of the human pancreas from foregut to endocrine commitment. *Diabetes* *62*, 3514–3522.
- Jin, C.X., Li, W.L., Xu, F., Geng, Z.H., He, Z.Y., Su, J., Tao, X.R., Ding, X.Y., Wang, X., and Hu, Y.P. (2008). Conversion of immortal liver progenitor cells into pancreatic endocrine progenitor cells by persistent expression of Pdx-1. *J. Cell. Biochem.* *104*, 224–236.



- Jonsson, J., Carlsson, L., Edlund, T., and Edlund, H. (1994). Insulin-promoter-factor 1 is required for pancreas development in mice. *Nature* 371, 606–609.
- Jørgensen, M.C., Ahnfelt-Rønne, J., Hald, J., Madsen, O.D., Serup, P., and Hecksher-Sørensen, J. (2007). An illustrated review of early pancreas development in the mouse. *Endocr. Rev.* 28, 685–705.
- Kaneto, H., Nakatani, Y., Miyatsuka, T., Matsuoka, T.A., Matsuhisa, M., Hori, M., and Yamasaki, Y. (2005). PDX-1/VP16 fusion protein, together with NeuroD or Ngn3, markedly induces insulin gene transcription and ameliorates glucose tolerance. *Diabetes* 54, 1009–1022.
- Keller, D.M., McWeeney, S., Arsenlis, A., Drouin, J., Wright, C.V., Wang, H., Wollheim, C.B., White, P., Kaestner, K.H., and Goodman, R.H. (2007). Characterization of pancreatic transcription factor Pdx-1 binding sites using promoter microarray and serial analysis of chromatin occupancy. *J. Biol. Chem.* 282, 32084–32092.
- Kesavan, G., Sand, F.W., Greiner, T.U., Johansson, J.K., Kobberup, S., Wu, X., Brakebusch, C., and Semb, H. (2009). Cdc42-mediated tubulogenesis controls cell specification. *Cell* 139, 791–801.
- Khoo, C., Yang, J., Weinrott, S.A., Kaestner, K.H., Naji, A., Schug, J., and Stoffers, D.A. (2012). Research resource: the pdx1 cistrome of pancreatic islets. *Mol. Endocrinol.* 26, 521–533.
- Knoepfler, P.S., Lu, Q., and Kamps, M.P. (1996). Pbx-1 Hox heterodimers bind DNA on inseparable half-sites that permit intrinsic DNA binding specificity of the Hox partner at nucleotides 3' to a TAAT motif. *Nucleic Acids Res.* 24, 2288–2294.
- Kojima, H., Fujimiya, M., Matsumura, K., Younan, P., Imaeda, H., Maeda, M., and Chan, L. (2003). NeuroD-beta-cellulose gene therapy induces islet neogenesis in the liver and reverses diabetes in mice. *Nat. Med.* 9, 596–603.
- Lam, E.W., Brosens, J.J., Gomes, A.R., and Koo, C.Y. (2013). Forkhead box proteins: tuning forks for transcriptional harmony. *Nat. Rev. Cancer* 13, 482–495.
- Lavon, N., Yanuka, O., and Benvenisty, N. (2006). The effect of overexpression of Pdx1 and Foxa2 on the differentiation of human embryonic stem cells into pancreatic cells. *Stem Cells* 24, 1923–1930.
- Li, W.C., Horb, M.E., Tosh, D., and Slack, J.M. (2005). In vitro transdifferentiation of hepatoma cells into functional pancreatic cells. *Mech. Dev.* 122, 835–847.
- Liu, Y., MacDonald, R.J., and Swift, G.H. (2001). DNA binding and transcriptional activation by a PDX1.PBX1b.MEIS2b trimer and cooperation with a pancreas-specific basic helix-loop-helix complex. *J. Biol. Chem.* 276, 17985–17993.
- Marshak, S., Benshushan, E., Shoshkes, M., Havin, L., Cerasi, E., and Melloul, D. (2000). Functional conservation of regulatory elements in the pdx-1 gene: PDX-1 and hepatocyte nuclear factor 3beta transcription factors mediate beta-cell-specific expression. *Mol. Cell. Biol.* 20, 7583–7590.
- Meivar-Levy, I., and Ferber, S. (2010). Adult cell fate reprogramming: converting liver to pancreas. *Methods Mol. Biol.* 636, 251–283.
- Meivar-Levy, I., Sapir, T., Gefen-Halevi, S., Aviv, V., Barshack, I., Onaca, N., Mor, E., and Ferber, S. (2007). Pancreatic and duodenal homeobox gene 1 induces hepatic dedifferentiation by suppressing the expression of CCAAT/enhancer-binding protein beta. *Hepatology* 46, 898–905.
- Miki, R., Yoshida, T., Murata, K., Oki, S., Kume, K., and Kume, S. (2012). Fate maps of ventral and dorsal pancreatic progenitor cells in early somite stage mouse embryos. *Mech. Dev.* 128, 597–609.
- Offield, M.F., Jetton, T.L., Labosky, P.A., Ray, M., Stein, R.W., Magnuson, M.A., Hogan, B.L., and Wright, C.V. (1996). PDX-1 is required for pancreatic outgrowth and differentiation of the rostral duodenum. *Development* 122, 983–995.
- Ohlsson, H., Karlsson, K., and Edlund, T. (1993). IPF1, a homeodomain-containing transactivator of the insulin gene. *EMBO J.* 12, 4251–4259.
- Pan, F.C., and Wright, C. (2011). Pancreas organogenesis: from bud to plexus to gland. *Dev. Dyn.* 240, 530–565.
- Peers, B., Sharma, S., Johnson, T., Kamps, M., and Montminy, M. (1995). The pancreatic islet factor STF-1 binds cooperatively with Pbx to a regulatory element in the somatostatin promoter: importance of the FPWMK motif and of the homeodomain. *Mol. Cell. Biol.* 15, 7091–7097.
- Que, J., Okubo, T., Goldenring, J.R., Nam, K.T., Kurotani, R., Morrisey, E.E., Taranova, O., Pevny, L.H., and Hogan, B.L. (2007). Multiple dose-dependent roles for Sox2 in the patterning and differentiation of anterior foregut endoderm. *Development* 134, 2521–2531.
- Saleh, M., Rambaldi, I., Yang, X.J., and Featherstone, M.S. (2000). Cell signaling switches HOX-PBX complexes from repressors to activators of transcription mediated by histone deacetylases and histone acetyltransferases. *Mol. Cell. Biol.* 20, 8623–8633.
- Santisteban, P., Recacha, P., Metzger, D.E., and Zaret, K.S. (2010). Dynamic expression of Groucho-related genes Grg1 and Grg3 in foregut endoderm and antagonism of differentiation. *Dev. Dyn.* 239, 980–986.
- Seymour, P.A., Freude, K.K., Tran, M.N., Mayes, E.E., Jensen, J., Kist, R., Scherer, G., and Sander, M. (2007). SOX9 is required for maintenance of the pancreatic progenitor cell pool. *Proc. Natl. Acad. Sci. USA* 104, 1865–1870.
- Seymour, P.A., Shih, H.P., Patel, N.A., Freude, K.K., Xie, R., Lim, C.J., and Sander, M. (2012). A Sox9/Fgf feed-forward loop maintains pancreatic organ identity. *Development* 139, 3363–3372.
- Shih, H.P., Wang, A., and Sander, M. (2013). Pancreas organogenesis: from lineage determination to morphogenesis. *Annu. Rev. Cell Dev. Biol.* 29, 81–105.
- Stoffers, D.A., Ferrer, J., Clarke, W.L., and Habener, J.F. (1997a). Early-onset type-II diabetes mellitus (MODY4) linked to IPF1. *Nat. Genet.* 17, 138–139.
- Stoffers, D.A., Zinkin, N.T., Stanojevic, V., Clarke, W.L., and Habener, J.F. (1997b). Pancreatic agenesis attributable to a single nucleotide deletion in the human IPF1 gene coding sequence. *Nat. Genet.* 15, 106–110.
- Svensson, P., Williams, C., Lundeberg, J., Rydén, P., Bergqvist, I., and Edlund, H. (2007). Gene array identification of Ipfl1/Pdx1-/- regulated genes in pancreatic progenitor cells. *BMC Dev. Biol.* 7, 129.



- Swift, G.H., Liu, Y., Rose, S.D., Bischof, L.J., Steelman, S., Buchberg, A.M., Wright, C.V., and MacDonald, R.J. (1998). An endocrine-exocrine switch in the activity of the pancreatic homeodomain protein PDX1 through formation of a trimeric complex with PBX1b and MRG1 (MEIS2). *Mol. Cell. Biol.* *18*, 5109–5120.
- Tamplin, O.J., Kinzel, D., Cox, B.J., Bell, C.E., Rossant, J., and Lickert, H. (2008). Microarray analysis of *Foxa2* mutant mouse embryos reveals novel gene expression and inductive roles for the gastrula organizer and its derivatives. *BMC Genomics* *9*, 511.
- Teo, A.K.K., Ali, Y., Wong, K.Y., Chipperfield, H., Sadasivam, A., Poobalan, Y., Tan, E.K., Wang, S.T., Abraham, S., Tsuneyoshi, N., et al. (2012). Activin and BMP4 synergistically promote formation of definitive endoderm in human embryonic stem cells. *Stem Cells* *30*, 631–642.
- Tremblay, K.D., and Zaret, K.S. (2005). Distinct populations of endoderm cells converge to generate the embryonic liver bud and ventral foregut tissues. *Dev. Biol.* *280*, 87–99.
- Tsuneyoshi, N., Tan, E.K., Sadasivam, A., Poobalan, Y., Sumi, T., Nakatsuji, N., Suemori, H., and Dunn, N.R. (2012). The SMAD2/3 corepressor SNON maintains pluripotency through selective repression of mesendodermal genes in human ES cells. *Genes Dev.* *26*, 2471–2476.
- Villasenor, A., Chong, D.C., Henkemeyer, M., and Cleaver, O. (2010). Epithelial dynamics of pancreatic branching morphogenesis. *Development* *137*, 4295–4305.
- Yamada, S., Yamamoto, Y., Nagasawa, M., Hara, A., Kodera, T., and Kojima, I. (2006). In vitro transdifferentiation of mature hepatocytes into insulin-producing cells. *Endocr. J.* *53*, 789–795.