Original Article

MOLECULAR METABOLISM

Foxa2 and Pdx1 cooperatively regulate postnatal maturation of pancreatic β-cells

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ABSTRACT

Objective: The transcription factors (TF) Foxa2 and Pdx1 are key regulators of beta-cell (β-cell) development and function. Mutations of these TFs or their respective cis-regulatory consensus binding sites have been linked to maturity diabetes of the young (MODY), pancreas agenesis, or diabetes susceptibility in human. Although Foxa2 has been shown to directly regulate Pdx1 expression during mouse embryonic development, the impact of this gene regulatory interaction on postnatal β-cell maturation remains obscure.
Methods: In order to easily monitor the expression domains of Foxa2 and Pdx1 and analyze their functional interconnection, we generated a novel double knock-in homozygous ($FVFPBF^{DHom}$) fluorescent reporter mouse model by crossing the previously described Foxa2-Venus fusion (FVF) with the newly generated Pdx1-BFP (blue fluorescent protein) fusion (PBF) mice.
Results: Although adult PBF homozygous animals exhibited a reduction in expression levels of Pdx1, they are normoglycemic. On the contrary, despite normal pancreas and endocrine development, the $FVFPBF^{DHom}$ reporter male animals developed hyperglycemia at weaning age and displayed a reduction in Pdx1 levels in islets, which coincided with alterations in β-cell number and islet architecture. The failure to establish mature β-cells resulted in loss of β-cell identity and trans-differentiation towards other endocrine cell fates. Further analysis suggested that Foxa2 and Pdx1 genetically and functionally cooperate to regulate maturation of adult β-cells.
Conclusions: Our data show that the maturation of pancreatic β-cells requires the cooperative function of Foxa2 and Pdx1. Understanding the postnatal gene regulatory network of β-cell maturation will help to decipher pathomechanisms of diabetes and identify triggers to regenerate dedifferentiated β-cell mass.
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Keywords Foxa2; Pdx1; β-Cell maturation; β-Cell identity; Trans-differentiation

1. INTRODUCTION

The pancreatic and duodenal homeobox 1 (Pdx1) transcription factor plays an essential role in the proper expansion and specification of pancreatic lineages during development [1—3]. In rodents, Pdx1 is expressed in the entire pancreatic epithelium during early development, and null mice do not develop a pancreas and die within a few days after birth [4,5]. During the formation of endocrine cells, an increase in Pdx1 levels is crucial for the commitment and differentiation of pancreatic β-cells [1,6,7]. In adulthood, Pdx1 ultimately becomes restricted to β- and δ-cells in the islets, where it maintains β-cell identity and function through the regulation of genes involved in glucose homeostasis [8,9], such as insulin, glucose transporter 2 (GLUT-2), and glucokinase (GK) [10]. Therefore, mice heterozygous for *Pdx1* develop diabetes in adulthood with glucose unresponsiveness and increased β-cell apoptosis [11—13]. Moreover, a mutation in the human *PDX1* locus generates MODY4 (Maturity onset diabetes of the young 4), which results in early diabetes occurrence without any signs of insulin resistance [14].

Another recently emerging player predisposing for diabetes is *FOXA2*, which belongs to the forkhead family of TFs. This protein is a pioneer transcription factor, which opens compact chromatin and allows DNA binding of other TFs in a higher-order gene regulatory network [15]. Although at earlier development stages Foxa2 is detectable in all pancreatic progenitor cells, the protein becomes upregulated and progressively restricted to endocrine progenitors during secondary transition and has lower expression levels in the exocrine and ductal lineages [16]. Foxa2 is not only essential for pancreas development and endocrine differentiation [7] but also for the maintenance of adult β-cell function [17,18]. Therefore, deletion of Foxa2 specifically in β-cells causes a hyperinsulinemic hypoglycemic phenotype through the misregulation of the insulin secretion machinery [19]. In line with this, a recent genetic fine mapping and genomic annotation study has revealed an association of type 2 diabetes risk alleles with FOXA2-bound enhancers in human [20].
Foxa2 and Pdx1 share several important functions, such as regulation of insulin production and modulation of the function of other transcription factors [10,21,22]. In diabetic models, the levels of both TFs

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appeared to be reduced in dedifferentiated β-cells, suggesting that these TFs act alone or in combination to induce or maintain β-cell identity [23,24]. Furthermore, mice heterozygous for both *Pdx1* and *Foxa2* display altered islet architecture and loss of β-cell identity resulting in severe distortion in insulin secretion and blood glucose regulation [2]. Foxa2 binds directly to *Pdx1* enhancer elements during development and deletion of Foxa1 and Foxa2 in the Pdx1 lineage leads to pancreas agenesis [7]. Although Foxa2-driven Pdx1 expression has also been reported in differentiating β-cells [25], the biological significance of this interconnection regarding postnatal β-cell maturation has not been thoroughly addressed.

To follow the expression domains of Foxa2 and Pdx1 easily and study the functional link between these two TFs in detail, we have generated knock-in reporter fluorescent protein (FP) fusion mouse lines, a previously reported Foxa2-Venus fusion (FVF) strain [15], and, for this study, a Pdx1-BFP fusion (PBF) model. Both single homozygous knock-in reporter mice were viable and fertile with normal glycemic condition. Surprisingly, the $FVFPBF^{DHom}$ reporter mice developed hyperglycemia at the weaning age although they were vital and healthy immediately after birth, with no obvious alteration in pancreas organogenesis and islet formation. Remarkably, the elevated blood glucose levels were only detected in male animals and coincided with significantly reduced levels of Pdx1. In addition, β-cells from $FVFPBF^{DHom}$ male mice did not induce a proper maturation program, lost their identity, and possibly transdifferentiated towards the α- and δ-cell fates. Analysis of ChIP-seq datasets showed that Foxa2 and Pdx1 co-occupy a substantial number of *cis*-regulatory regions of genes involved in β-cell development, maturation, and function. Strikingly, the expression of key players in establishing and maintaining β-cell maturation were impaired in the $FVFPBF^{DHom}$ male mice, suggesting the interference of the fused FPs with the cooperative binding and/or transactivation of these crucial factors. These results suggest that Foxa2 and Pdx1 genetically interact and cooperatively regulate a gene regulatory network of postnatal β-cell maturation.

2. MATERIAL AND METHODS

2.1. Generation of Pdx1 BFP fusion (PBF) reporter mouse line

2.1.1. Production of the Pdx1 BFP targeting construct

The Pdx1 BFP targeting construct was designed as shown in Supplementary Figure 1A. The 5′ and 3′ homology regions (HR) for the Pdx1 gene were amplified by PCR using EP-1015/1016 and EP-1017/1018 primer pairs, respectively. We used C57BL/6 BAC (RPCI22—254-G2) as the template DNA. HRs were cloned by restriction enzymes AscI, HindIII, and BamHI into the PL-254 vector [26—28]. From the same BAC, a 9.5 kb fragment was recovered using gap repair via homologous recombination in EL350 bacteria as described previously [26], resulting in the production of pL254-Pdx1. BamHI and XbaI were used as single cutters for linearization prior to the gap repair. For cloning of the knock-in cassette, the 5′ HR (EP-1006, 1007) and 3′HR (EP-1008, 1009) from exon 2 of Pdx1 were amplified by PCR using the previously described BAC as template and subcloned via NotI, XbaI, HindIII, or XhoI into the pBluescript KS- (pBKS-), respectively, to generate the pBKS-Ex2 HR. The BFP sequence (Evrogen) with translational stop codon was amplified (EP-25, 1010) and subcloned between the homology regions of the pBKS-Ex2 HR via XbaI and SpeI, resulting in the generation of pBKS-Ex2 HR BFP. Next, the neomycin resistance gene, which is driven by PGK promoter and is flanked by LoxP sites, was cloned from the PL452 [27] vector via EcoRI and BamHI into the downstream of the BFP sequence resulting in the formation of pBKS-Ex2 HR-BFP-Neo. Afterwards, the mini-targeting cassette was released by digestion with NotI and KpnI and introduced into the pL254-Pdx1 via bacterial homologous recombination in EL250 bacteria, resulting in the generation of the final targeting construct (pL-254 Pdx1-BFP Fusion targeting vector, see Supplementary Figure 1A), which was confirmed by sequencing.

2.1.2. Cell culture and homologous recombination in ES-Cells

Mouse ES cells were cultured as previously described [29]. The AscI-linearized pL-254 Pdx1-BFP fusion (PBF) targeting vector was electroporated into the IDG3.2 ES cells [30]. 300 mg/mL of G418 (Invitrogen, 50 mg/mL) were used to select the neo-resistant clones. Homologous recombination at the Pdx1 locus was evaluated by southern blot analysis of the BamHI-digested genomic DNA using the Pdx1 3′ probe located outside the targeting vector (3′ (EP1013, 1014) was subcloned into and later released by PstI and HindIII digestion from pBKS). Through the southern blot analysis, three positive clones were identified out of 110 total selected clones (Supplementary Figure 1B). The homologous recombined-clones were aggregated with CD1 morulae and the resulting chimeric mice passed the Pdx1BFPneo allele through their germline cells. Finally, by intercrossing with the ROSA-Cre mouse line, the Neo cassette flanked by LoxP sites was deleted. The excision of the Neo cassette was confirmed through the genotyping PCR using the primers EP-536, 1189, and 1193, generating a 339 bp product for the $Pdx1^{BFP\ Neo}$ allele and a 421 bp product for the Pdx1BFPdelta Neo allele (Supplementary Figure 1C). To genotype the homozygous and heterozygous PBF mice, the primers EP 1188, 1189, and 1193 were used. The WT mice generated a 211 bp PCR product, distinguished from the 421 bp band of the homozygous animals. Two products of 211 and 421 bp were identified for the heterozygous mice (Supplementary Figure 1D). The mouse line described here will be available to the research community upon acceptance of this manuscript.

2.2. Western blot analysis

Western blot analysis was performed according to the standard protocols. Briefly, lysates from embryonic pancreata or isolated islets from adult mice were prepared as described [31] and subjected to the SDS-PAGE electrophoresis and transferred to the nitrocellulose membranes. After blocking, the membranes were incubated with the appropriate primary antibodies (Supplementary Table 2) overnight followed by incubation with HRP-conjugated secondary antibodies. The signals were detected by enhanced chemiluminescence (Thermo Scientific).

2.3. Immunostaining and imaging

2.3.1. Whole-mount staining

Mouse embryos were fixed using 2% PFA in PBS for 20 min. After permeabilization (0.1% Triton, 0.1 M Glycin) for 15 min, they were subjected to the blocking solution (10% FCS, 3% Donkey serum, 0.1% BSA and 0.1% Tween-20 in PBS) for 1 h at room temperature (RT). Next, the primary antibodies (Supplementary Table 2) diluted in the blocking solution were added to the samples overnight at 4 °C. After extensive washing with PBST (0.1% Tween-20 in PBS), the embryos were stained by secondary antibodies diluted in the blocking solution for 3—5 h at RT. The samples were then incubated with 4′,6-diamidin-2-phenylindol (DAPI), followed by washing with PBST and embedding in the commercial medium (Life Tech., ProLong Gold).

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2.3.2. Pancreas section staining

Embryonic or adult pancreata were dissected and fixed in 4% PFA in PBS for 2 h at 4 °C. The tissues were merged in 7.5, 15 and 30% sucrose-PBS solutions at RT (2 h incubation for each solution). Afterwards, they were embedded in cryoblocks using tissue-freezing medium (Leica 14020108926). Next, sections of 20 μm thickness were cut from each sample and subjected to immunostaining as described for Whole-mount staining. All images were obtained with a Leica microscope of the type DMI 6000 using the LAS AF software. Images were analyzed using ImageJ and Imaris imaging software programs.

2.4. Islet isolation

The isolation of islets was performed by collagenase P (Roche) digestion of the adult pancreas. Briefly, 3 mL of collagenase P (1 mg/mL) was injected into the bile duct and the perfused pancreas was consequently dissected and placed into another 3 mL collagenase P for 15 min at 37 °C. 10 mL of G-solution (HBSS + 1% BSA) was added to the samples followed by centrifugation at 1600 rpm at 4 °C. After another washing step with G-solution, the pellets were resuspended in 5.5 mL of gradient preparation (5 mL 10% RPMI + 3 mL 40% Optiprep/per sample), and placed on top of 2.5 mL of the same solution. To form a 3-layers gradient, 6 mL of G-solution was added on the top. Samples were then incubated for 10 min at RT before subjecting to centrifugation at 1700 rpm. Finally, the interphase between the upper and the middle layers of the gradient was harvested and filtered through a 70 μm Nylon filter and washed with G-solution and the islets were handpicked under the microscope.

2.5. RNA isolation and amplification

RNA isolation was performed using the miRNA micro kit (Qiagen) according to the manual. On a column, DNase I treatment was applied to degrade DNA. 14 μl of nuclease-free water were added to elute the RNA fractions, which were used immediately or stored at −80 °C. Due to the low amounts of RNA, RNA amplification was carried out using the Ovation® PicoSL WTA SystemV2 (Nugen). The amplification process was done according to the manual using 50 ng total RNA.

2.6. Quantitative PCR (qPCR)

The qPCR was carried out using TaqMan™ probes (Life Technologies) (Supplemental Table 3) and the Viia7 Real Time PCR System (Thermo Fisher Scientific). Each reaction contained 25 ng of cDNA. For analysis, the C_t-values were transformed to the linear expression values and normalized to the reference genes and to the control samples.

2.7. Glucose-stimulated insulin secretion (GSIS)

For GSIS analysis, the isolated islets were cultured overnight before transferring to a 96-well plate containing modified Krebs Ringer phosphate Hepes (KRPH) buffer with 2 mM glucose for 1 h. Different glucose concentrations (2 and 16.5 mM) were added to the islets (2 h for each). The supernatant were used for insulin measurement. At the end, the islets were lysed in RIPA buffer and samples were kept at −20 °C. Insulin concentrations were measured and quantified using an ultrasensitive insulin ELISA kit (Cristal Chem). The analysis was performed using a standard curve, and the data were normalized to the total insulin content. The islet insulin content was obtained by normalizing the insulin content to the total protein content in the islets.

2.8. Blood glucose level measurement

Mice were maintained in standard conditions and starved 6 h before the measurement. Blood glucose values were determined from venous blood using an automatic glucose monitor (Glucometer Elite, Bayer).

2.9. ChIP-seq dataset analysis

Raw reads from Foxa2 ChIP-seq (GSM1306337) and Pdx1 ChIP-seq (GSM1824088) were obtained from public databases and subsequently processed with Trimmomatic (0.35) to remove low quality bases and adapter contamination. Next, reads were aligned to mm10 genome using bowtie2 (2.2.6) with very-sensitive option and duplicate reads were removed using samtools (1.3). Binding sites were then called using GEM [32] and filtered after visual inspection using a q value cut-off of 10^{-4} and 10^{-12} for Pdx1 and Foxa2, respectively. For pathway analysis, binding sites within 20 kb of a TSS or within a gene body were mapped to the respective gene using bedtools (2.18). Pathway enrichment analysis was performed using HOMER [33].

2.10. Statistical analysis

All data are presented as Means ± Standard Deviation. The data were analyzed using a two-tailed unpaired *t*-test.

3. RESULTS

To circumvent development of MODY4 due to *Pdx1* haploinsufficiency [34], we generated a PBF reporter mouse line by removing the translational stop codon and fusing *BFP* in frame with *Pdx1* (Supplementary Figure 1A). Southern blot analysis with a 3′ probe revealed a targeting efficiency of 2.72% at the *Pdx1* locus (Supplementary Figure 1B). We generated chimeric mice and removed the *LoxP*-flanked neomycin selection cassette by Cre recombinase-mediated excision in the germ line (Supplementary Figure 1C). Genotyping of the offspring from heterozygous intercrosses revealed normal Mendelian distribution, suggesting that the Pdx1-BFP fusion protein does not interfere with pancreas development (Supplementary Figure 1D and data not shown). Western blot analysis of embryonic day (E) 14.5 pancreata and adult islets confirmed the endogenous expression of both Pdx1 and Pdx1-BFP fusion protein in heterozygous animals. Furthermore, a reduction in Pdx1 and PBF levels was observed in both heterozygous and homozygous PBF mice compared to the WT animals during development and adulthood (Supplementary Figure 1E and F).

Next, we assured that the PBF mirrors the endogenous Pdx1 expression patterns during primary and secondary transitions of pancreas development. Using whole-mount staining of embryos, we confirmed that PBF is expressed in a spatio-temporal manner as endogenous Pdx1 during primary transition (E9.0—11.5) (Figure 1A—D). During the secondary transition, the expression of Pdx1 is restricted to the endocrine lineage [35]. Analysis of the E13.5—15.5 pancreata indicated that the PBF expression becomes gradually limited to a subpopulation of pancreatic cells (Supplementary Figure 1G—I). In addition, immunostaining of E15.5 pancreatic sections using endocrine and exocrine markers revealed the expression of PBF in the endocrine compartment (Figure 1E—H). Furthermore, we showed that β- but not α-cells exhibit PBF reporter activity (Figure 1I). Finally, we found that PBF expression is restricted to β- and δ-cells in adult islets (Figure 1J). Collectively, these data indicate that the PBF protein resembles the spatio-temporal expression pattern of endogenous Pdx1 during development and in adulthood. Moreover, PBF homozygous mice are viable and fertile and do not show any signs of MODY4.

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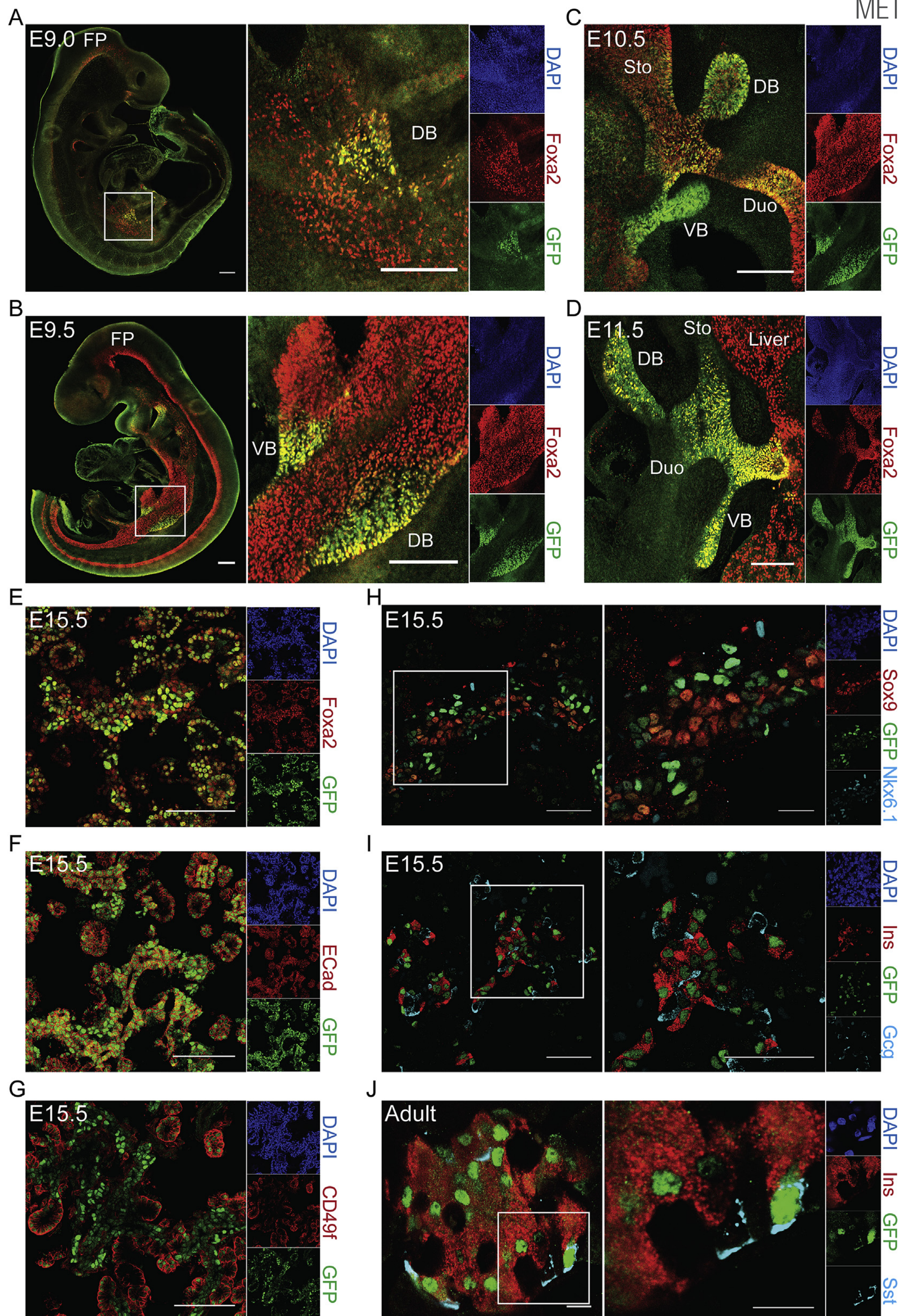

Figure 1: PBF is expressed in a spatio-temporal pattern as endogenous Pdx1 during pancreas development and in adulthood. (A, B) Whole-mount immunostaining of embryos indicating the expression of PBF during early stages of pancreas organogenesis at E9.0 and E9.5. Whereas the BFP signal is detected at the dorsal buds at E9.0, it is visible at both ventral and dorsal buds at E9.5. (C, D) The expansion of the BFP-positive buds through the primary transition of pancreas development, indicating the similar expression pattern of PBF and the endogenous Pdx1 protein. (E, F) Immunostaining of pancreata for Foxa2 and E-Cadherin suggesting the endocrine identity of the PBF^{+} cells at E15.5. (G) Co-staining of the PBF and CD49f (α*6 integrin* subunit), as a marker for exocrine cells, excludes the exocrine characteristics of PBF^{+} cells. (H) PBF-positive cells are negative for Sox9 and positive for Nkx6.1, which mark ductal epithelial and a subpopulation of endocrine cells, respectively. (I) Co-staining of PBF with insulin and glucagon indicating the β- but not α-cells identity of the PBF^{+} cells. (J) Immunostaining of PBF mice-derived isolated islets showing the expression of Pdx1-BFP in $insulin^{+}$ β- and $somatostatin^{+}$ δ-cells in adulthood. All analyses have been performed using heterozygous animals. Scale bars, A-D, 200 μm; E-G, 100 μm; H and I, 50 μm; J, 10 μm. FP, floor plate; DB, dorsal bud; VD, ventral bud; Sto, stomach; Duo, duodenum; ECad, E-Cadherin; Ins, Insulin; Gcg, Glucagon and Sst, Somatostatin.

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To analyze the functional link between Foxa2 and Pdx1 in detail, we created FVF and PBF double homozygous knock-in (FVFPBF^{DHom}) animals. Early postnatal FVFPBF^{DHom} mice were viable and healthy with normal pancreas development and growth when compared to WT, FVF homozygous, and PBF homozygous mice. However, further analysis of 3-month-old animals revealed remarkably high blood glucose levels (>450 mg/dL) in males FVFPBF^{DHom} (Figure 2A), but only slightly increased levels of 130 mg/dL in females, suggesting that females are protected from developing diabetes (Supplementary Figure 2A). In comparison, PBF single homozygous animals were normoglycemic. For this study, we focused on understanding the pathomechanisms of developing diabetes in the FVFPBF^{DHom} male animals. First, we analyzed gene expression by qPCR in isolated isles from 3-month-old FVFPBF^{DHom} and controls, which indicated a reduction in the expression of *Pdx1* but not in *Foxa2* mRNA (Figure 2B). This was further confirmed by western blot analysis in isolated islets from FVFPBF^{DHom} mice, in which we found no change in Foxa2 but a notable decrease in Pdx1 protein levels (Figure 2C and D). Moreover, whereas PBF homozygous mice exhibited a reduction in Pdx1 levels, the FVF single homozygous animals expressed normal levels of this protein (Figure 2C and D and Supplementary Figure 2B). Finally, immunostaining of isolated islets from FVFPBF^{DHom} mice also revealed a reduction in the Pdx1, but not in Foxa2 levels (Figure 2E and F). These results confirm the upstream function of Foxa2 on Pdx1 in a gene regulatory network [7] and the presence of an autoregulatory, positive feedback loop of Pdx1 [36]. Therefore, the fusion of the FPs to these TFs impacts the expression levels of Pdx1 but not of Foxa2.

To assess whether the reduction in the Pdx1 levels in FVFPBF^{DHom} mice leads to impaired β-cell function [9], we measured the mRNA levels of the downstream *Ins1* gene. We found a striking reduction in the expression of this gene at mRNA level in islets from 3-month-old FVFPBF^{DHom} mice (Figure 3A). Furthermore, enzyme-linked immunosorbent assay (ELISA) of total islet-precipitated protein indicated a significant reduction in insulin content in the FVFPBF^{DHom} animals compared to the controls (Figure 3B). In line with this, glucose-stimulated insulin secretion (GSIS) of pancreatic islets from FVFPBF^{DHom} animals showed a significant decreased insulin release upon high glucose when compared to control islets (Figure 3C). These changes in islet insulin content and GSIS coincided with remarkable alterations in islet morphology. We observed that islets from FVFPBF^{DHom} mice lose their typical spherical shape and exhibit the presence of α-cells intermingled with β-cells in the islet core (Figure 3D). Finally, we analyzed the maturation status of β-cells by analyzing the maturation markers Urocortin 3 (Ucn3) [37] and MafA [38] and found an almost complete lack of expression in 3-month-old FVFPBF^{DHom} male animals when compared to controls (Figure 3E and F). Taken together, these data suggest that the combined defect in FVF and PBF activity results in impaired β-cell maturation and function.

To better understand if β-cells lose their identity or fail to mature, we monitored the newly born β-cells in control and FVFPBF^{DHom} mice from postnatal day 9 (P9) until 1.5 months of age. We selected these time points to cover the postnatal maturation period of β-cells. In mice, β-cells undergo two major steps of maturation, postnatally. The first step occurs at P14 and is marked by the expression of Ucn3 [37], whereas

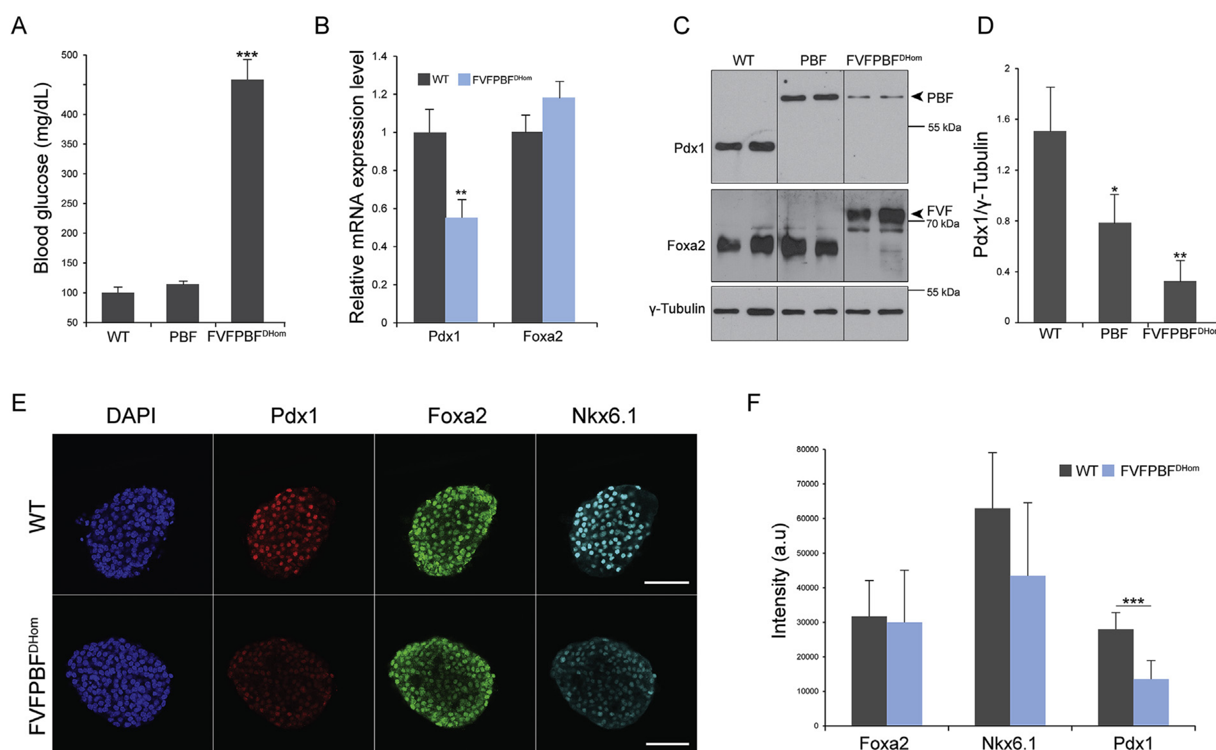

Figure 2: FVFPBF^{DHom} male mice exhibit reduction in Pdx1 levels postnatally and develop hyperglycemia at the weaning age. (A) Fasted blood glucose levels (mg/dL) for 3-month-old male FVFPBF^{DHom}, PBF, and WT mice (n = 3, ***P < 0.001). (B) qPCR analysis for *Foxa2* and *Pdx1* of isolated islets from 3-month-old male WT and FVFPBF^{DHom} mice. Data were normalized according to 18S. (n = 4 for WT and 3 for FVFPBF^{DHom}, **P < 0.01). (C, D) Expression of Foxa2 and Pdx1 proteins using western blot analysis from isolated adult islets. (n = 3 for, *P < 0.05; **P < 0.01) (E) Immunostaining of isolated islets exhibits a significant reduction in the expression levels of Pdx1 but not Foxa2 in the male FVFPBF^{DHom} mice compared to the controls. (F) Quantification of signal density of Foxa2, Nkx6.1, and Pdx1 in the FVFPBF^{DHom} and WT islets (n = 3, ***P < 0.001). Scale bars, 50 μm.

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the second step takes place when the animal changes from a high-fat milk diet from the mother to a high-carbohydrate chow after weaning (around P25) [39]. Blood glucose levels were comparable between controls and FVFPBF^{DHom} mice at P9 and P14, but significantly increased from P25 onwards in FVFPBF^{DHom} animals. Remarkably, this elevation in blood glucose levels was not affected by the diet change and increase in metabolic demands during weaning, suggesting that the genetic predisposition rather than environmental factors causes β-cell dysfunction (Figure 4A). When we quantified the endocrine composition of islets, we found a significant reduction in β-cells along with an increase in α-cell number in 1.5-month-old FVFPBF^{DHom} mice. However, at P14 the number of both cell types remained unaffected (Figure 4B and Supplementary Figures 3A and B), suggesting that normal number of endocrine cells are formed during development. We could also detect subtle but not significant changes in the δ-cell number in the FVFPBF^{DHom} mice (Supplementary Figure 3B). Next, we investigated the expression of β-cell maturation marker and found that FVFPBF^{DHom} animals exhibited lower levels of Ucn3 at P14, and almost no expression at P25 and 1.5 months. Importantly, the switch from high-fat milk diet to carbohydrate-enriched chow did not prevent the loss of Ucn3, indicating that the failure in β-cell maturation occurs before the weaning age (Figure 4C). Furthermore, we analyzed the expression levels of Ucn3 in isolated islets from FVF and PBF single homozygous mice and found normal levels of this proteins compared to the WT islets (Figure 4D). The changes in Ucn3 levels in FVFPBF^{DHom} mice were accompanied by sporadic appearance of insulin-glucagon and insulin-somatostatin double-positive cells within the islets (Figure 4E and F), suggesting that β-cells lose their identity and transdifferentiate towards other endocrine cell types as previously reported [8]. This phenotype was not detected in the FVF and PBF single homozygous islets (Supplementary Figure 3C).

Since Foxa2 and Pdx1 induce several similar target genes in β-cells, it is possible that they function, cooperatively. To identify Pdx1 and Foxa2 binding sites in β-cell-specific enhancers and promoters, we analyzed the ChIP-seq dataset from mouse pancreatic β-cell lines [40,41]. In agreement to previous results [42], we found 5976 sites to be co-

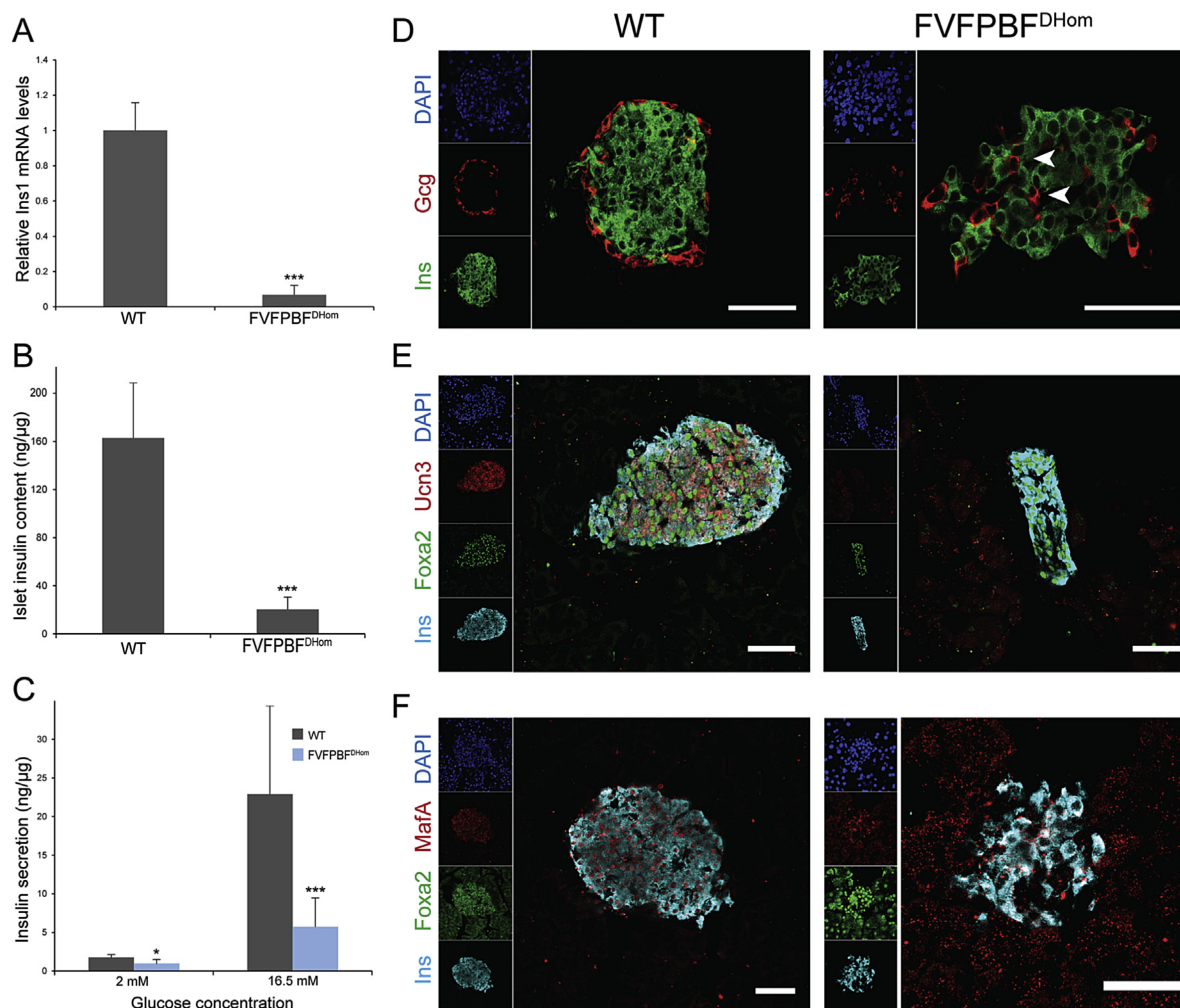

Figure 3: Impairment in insulin biosynthesis and release coincides with loss of islet architecture integrity in adult FVFPBF^{DHom} mice. (A) qPCR analysis for *Ins1* levels in isolated islets from male FVFPBF^{DHom} and WT mice (n = 4 for WT and 3 for FVFPBF^{DHom}, ***$P < 0.001$). (B) Measurement of total insulin content normalized to protein content from isolated islets from 3-month-old male FVFPBF^{DHom} and control animals (n $\geq$ 3, ***$P < 0.001$). (C) Quantification of insulin secretion in response to low (2 mM) and high (16 mM) glucose concentrations determined by GSIS assay in islets from adult male FVFPBF^{DHom} and WT mice (n $\geq$ 3, ***$P < 0.001$). (D) Immunostaining of pancreatic sections showing the disturbed islet structure in the FVFPBF^{DHom} male compared to the WT mice. (E) Ucn3 staining in islets from 3-month-old male FVFPBF^{DHom} and WT mice. (F) Immunostaining of MafA in male FVFPBF^{DHom} and WT adult islets. Scale bars, 50 μm.

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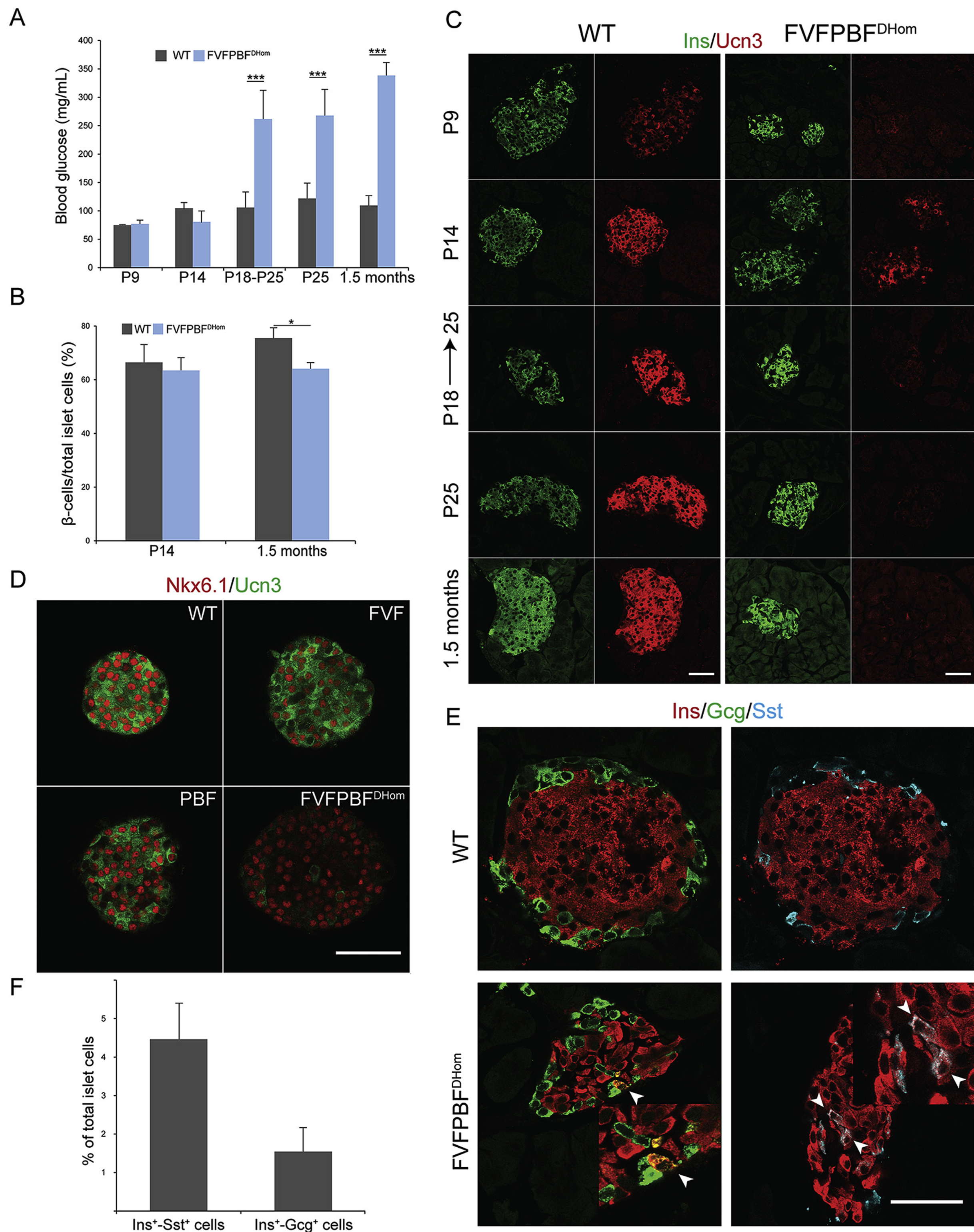

Figure 4: FVFPBF^{DHom} β-cells fail to induce maturation step and consequently lose their identity. (A) Fasted blood glucose levels (mg/dL) from animals of age P9 through 1.5 months old ($n \geq 3$, ***$P < 0.001$). (B) Quantification of β-cell number in islets from the male FVFPBF^{DHom} and WT mice ($n = 3$, *$P < 0.05$). (C) FVFPBF^{DHom} male mice show a gradual reduction in the Ucn3 expression levels starting from P25 till 1.5 months. (D) Ucn3 staining of isolated islets from 1.5 month single homozygous FVF and PBF mice shows normal levels of this protein compared to the WT control. (E) Islets derived from the FVFPBF^{DHom} male mice exhibit the presence of poly-hormonal cells within the islet core. (F) Quantification of poly-hormonal cells within the islet from the male FVFPBF^{DHom} animals ($n = 3$). Scale bars, 50 μm.

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occupied by Foxa2 and Pdx1, suggesting that a substantial number of β-cell-specific enhancers and promoters are co-occupied by both TFs (Figure 5A). More general, genes with co-occupied sites are involved in insulin secretion, β-cell development and function, and can be related to diabetes susceptibility (Figure 5B). This suggests that the cooperative action of Foxa2 and Pdx1 is critical to maintain β-cell maturation and identity (Figure 5C). Furthermore, genes nearby these co-occupied sites also included those important for mature β-cell function, such as MafA, Slc2a2, and Ins1. To test whether these co-occupied genes are indeed directly regulated by Foxa2 and Pdx1, we next performed qPCR analysis and found a significant reduction in the levels of MafA, Slc2a2, and Ucn3 in the islets from the $FVFPBF^{DHom}$ mice when compared to WT. Interestingly, we found a striking increase in the levels of MafB in the $FVFPBF^{DHom}$ islets, suggesting that failure to switch from MafB to MafA impairs β-cell maturation (Figure 5D) [38]. Another β-cell signature gene is glucose transporter, Glut2 (also known as Slc2a2), which is essential for glucose sensing of mature β-cells [43]. Immunostaining for Glut2 revealed failure of synthesis of this protein in the $FVFPBF^{DHom}$ islets supporting the loss of β-cell maturation and function in these mice (Figure 5E).

4. DISCUSSION

The Pdx1 and Foxa2 TFs are two crucial players during multiple stages of pancreas development and are also important for adult β-cell function; however, their role in early postnatal maturation of β-cells is

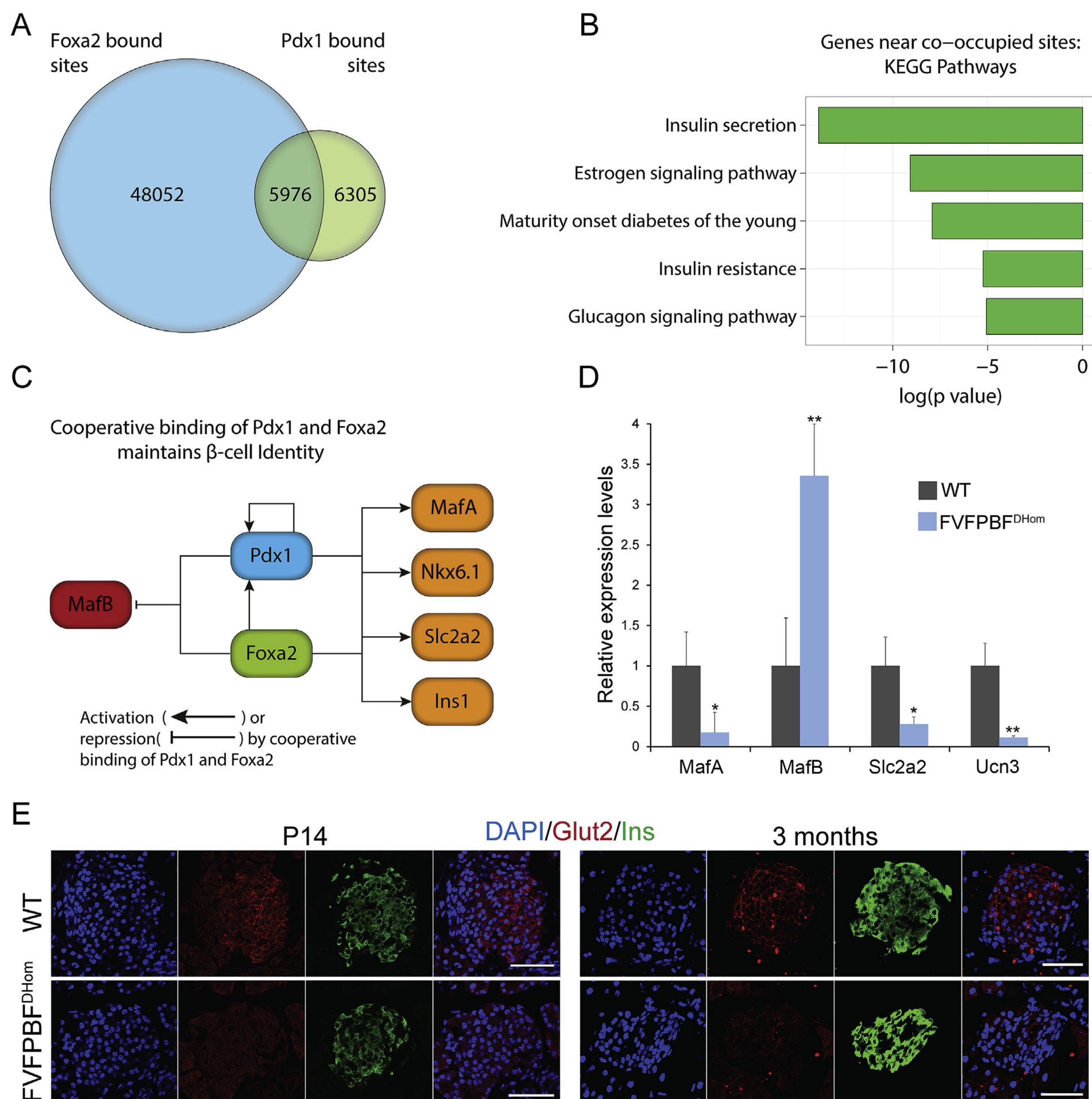

Figure 5: Cooperative function of Foxa2 and Pdx1 regulates β-cell maturation. Foxa2 and Pdx1 target genes were identified using raw reads from Foxa2 ChIP-seq (GSM1306337) and Pdx1 ChIP-seq (GSM1824088) obtained from pancreatic β-cell lines [40,41]. (A) Venn diagram representing the co-occupation of 5976 enhancer and promoter sites by Foxa2 and Pdx1. (B) KEGG ontology pathway of gene category near to the co-binding sites of Foxa2 and Pdx1. (C) Cooperative binding of Foxa2 and Pdx1 regulates expression of key genes involved in β-cell maturation and function. (D) qPCR analysis of crucial genes regulating β-cell maturation in the $FVFPBF^{DHom}$ and WT islets. ($n = 4$ for WT and 3 for $FVFPBF^{DHom}$. *$P < 0.05$; **$P < 0.01$). (E) Immunostaining of Glut2 in P14 and 3-month-old islets from $FVFPBF^{DHom}$ and WT mice. Scale bars, 50 μm.

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less well studied. The synergistic effect of these TFs in pancreatic islets has been demonstrated in mice heterozygous for both Pdx1 and Foxa2 [2]. Although the results so far point to a combinatorial effect of both TFs on β-cell function, the precise functional relationship between these two TFs during postnatal β-cell maturation is not well understood. Therefore, we generated a double reporter knock-in mouse line to easily track the expression domains and investigate the functional interconnection between Foxa2 and Pdx1 in adult islets. In these mice, FVF and PBF proteins mirror the expression of endogenous Foxa2 and Pdx1, respectively. Whereas, the single homozygous reporter mice did not show blood glucose alterations, the male FVFPBF^{DHom} homozygous mice developed hyperglycemia, postnatally. Furthermore, they displayed a reduction in the Pdx1 levels which coincided with alteration in β-cell number and islet architecture.

It is well known that the expression of several endocrine-specific TFs, including Nkx6.1, Pax6, Nkx2.2, and Pdx1, is driven by Foxa1 and Foxa2 (Foxa1/2) during development [7]. Therefore, the absence of obvious defects in early pancreas development in the FVFPBF^{DHom} animals is likely due to the compensatory function of Foxa1 [44]. To regulate the Pdx1 expression, Foxa1/2 bind to multiple *cis*-regulatory elements, which precisely regulate and maintain the expression levels of Pdx1 gene [7,45]. This enhancer domain consists of a proximal region close to the transcriptional start site called Area I—II—III and a distal district termed Area IV, containing binding sequences for different *trans*-acting factors, including Foxa1/2 [45—52]. Although, the interaction of Foxa1/2 with Pdx1 enhancer domain increases during development, the increase is more robust for Foxa2 than for Foxa1 [7]. Thus, it is tempting to speculate that the fusion of Venus to Foxa2 perhaps reduces DNA binding or cooperative binding with other TF and cofactors to the Pdx1 enhancer elements that consequently decreases the expression levels of Pdx1. This might explain why the FVFPBF^{DHom} mice exhibit a severe postnatal phenotype when Foxa2 plays a major role in regulating Pdx1 levels [7]. In addition, it has been shown that by binding to *cis*-regulatory elements (Area I and IV), Pdx1 regulates its own expression through an autoregulatory positive feedback loop [36,47,53]. Therefore, it is possible that the fusion of the bulky BFP to Pdx1 reduces the autoregulatory function of this TF in a similar manner as described above for FVF and contributes to the reduction of Pdx1 levels. This is also supported by the finding of reduced Pdx1 levels in PBF homozygous mice.

We found almost normal blood glucose levels in the female FVFPBF^{DHom} compared to the male mice, which can be due to the gender difference in diabetes susceptibility and has been reported previously [54]. This sexual dimorphism has been attributed to the protective role of hormones, specifically estrogen, in the development of diabetes [55—57]. Recently, Foxa2 has been shown to dictate sexual dimorphism in liver cancer by opposing interactions with the androgen and estrogen receptors [58]. Of note, genes acting in the estrogen signaling pathway showed significant enhanced Foxa2 and Pdx1 binding in their enhancer and promoter regions (Figure 5B). However, the interaction of Foxa2 (and Pdx1) with sex hormones in pancreatic islets and its potential role in diabetes need further investigation.

Even though the FVFPBF^{DHom} male mice showed normal pancreas morphogenesis, they developed hyperglycemia at weaning age, suggesting either the involvement of diet-induced metabolic changes or failure in generating or maintaining functional mature β-cells. When we pre-weaned the mice at P18 and compared them to those that remained on high-fat milk diet, we could not distinguish any differences regarding blood glucose homeostasis, indicating that the observed phenotype is due to genetic predisposition to develop diabetes rather than environmental factors. Indeed, we found a massive reduction in the levels of the β-cell maturation marker Ucn3 in the FVFPBF^{DHom} male mice before and at weaning age independent from the diet switch. These data indicate that β-cells not only undergo a poor maturation process but also fail to maintain and preserve their maturation program in FVFPBF^{DHom} mice.

The defect in β-cell maturation concurred with the appearance of poly-hormonal endocrine cells within the islets from the FVFPBF^{DHom} mice. In particular, we could detect a significant number of insulin-glucagon and insulin-somatostatin double-positive cells in the FVFPBF^{DHom} islets. In addition, immunostaining analysis revealed an increase in the α-cell number in the FVFPBF^{DHom} mice. It has been shown that *Insulin*-Cre-induced Pdx1 removal in β-cells during development results in increase in the ratio of glucagon-positive to insulin-expressing cells [9,59]. Moreover, β-cell-specific deletion of Pdx1 leads to hyperglycemia through the reprograming of these cells towards α-cells [8]. This occurs because Pdx1 is not only required for preserving β-cell identity but also for repressing a α-cell program [8]. This is supported by the finding that Pdx1 converts perinatal α-cells into β-cells through a glucagon-insulin double positive state, upon forced expression in the embryonic endocrine progenitors [60]. Furthermore, Pdx1 represses the *MafB* and *glucagon* genes in β-cells. Therefore, in Pdx1-depleted β-cells, this inhibitory function is removed, leading to the formation of cells exhibiting ultrastructural and physiological features of endogenous α-cells [8]. The fact that the levels of *MafB* are increased in the FVFPBF^{DHom} mice suggests that β-cells undergo the trans-differentiation towards the α-cells partially through the derepression of this gene. Thus, trans-differentiation of β-cells might be the main mechanism reducing the number of these cells in the FVFPBF^{DHom} islets. We detected no cleaved caspase3 signal in the FVFPBF^{DHom} mice (Supplementary Figure 3D), proposing a minor contribution of cell death to the decreased number of β-cells. This is also supported by the absence of apoptotic cells in the Pdx1-depleted β-cells [8]. Therefore, the impairment in β-cell maturation along with hyperglycemia in FVFPBF^{DHom} mice likely leads to reduction in β-cell number through loss of identity and trans-differentiation towards other endocrine cells.

Because Foxa2 and Pdx1 activate many similar genes in β-cells, it is likely that they trigger their targets, cooperatively. Indeed, using ChIP-seq analysis, 5976 loci were identified to be co-occupied by both Foxa2 and Pdx1 in the islets. Among these, were those involved in insulin secretion, estrogen signaling pathway, MODY, insulin resistance, and the glucagon signaling pathway. This cooperative function is highly tissue-specific, and in the islets, it regulates several target genes involved in β-cell maturation and function, such as *MafA*, *Ins1*, and *Slc2a2*. The expression of these genes that are important for mature β-cell function is impaired in the FVFPBF^{DHom} islets. Furthermore, the function of Foxa2 and Pdx1 in preserving β-cell maturation and identity is partially through the repression of genes necessary for the α-cell fate, such as *MafB*, which is up-regulated in the FVFPBF^{DHom} mice. Therefore, it is possible that the fused bulky fluorescence tags prevent the co-binding of Foxa2 and Pdx1 to the regulatory elements of their target genes through the steric hindrance. Notably, the binding of FOXA2 and PDX1 to *cis* regulatory elements has also been identified in human. For instance, both TFs occupy a sequence acting as a developmental enhancer of *PTF1A* in hESC-derived pancreatic progenitors. The binding of FOXA2 and PDX1 is eliminated upon mutations in this *cis*-regulatory enhancer region and leads to isolated pancreatic agenesis [61].

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In summary, we generated a FVFPBF^{DHom} reporter mouse line that develops hyperglycemia postnatally. This phenotype occurs due to the failure to generate and preserve mature β-cells, which ultimately undergo trans-differentiation towards other endocrine cells, mainly α- and δ-cells. The reduction in Pdx1 levels along with the possible destruction of the cooperative function of this TF with Foxa2 are the responsible mechanisms underlying loss of β-cell maturation, identity, and function.

AUTHOR CONTRIBUTIONS

A.B. and S.R. researched data, contributed to discussion and reviewed/edited manuscript. I.B., E.B. and M.S. researched data and reviewed/edited manuscript. M.B. researched data and wrote the manuscript. H.L. wrote the manuscript and conceived the work. H.L. is the guarantor of this work and takes full responsibility for the work as a whole. The authors declare no conflicts of interest.

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CONFLICT OF INTEREST

None declared.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molmet.2017.03.007.

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