Dynamics of genomic H3K27me3 domains and role of EZH2 during pancreatic endocrine specification

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Abstract

Endoderm cells undergo sequential fate choices to generate insulin-secreting beta cells. *Ezh2* of the PRC2 complex, which generates H3K27me3, modulates the transition from endoderm to pancreas progenitors, but the role of *Ezh2* and H3K27me3 in the next transition to endocrine progenitors is unknown. We isolated endoderm cells, pancreas progenitors, and endocrine progenitors from different staged mouse embryos and analyzed H3K27me3 genome-wide. Unlike the decline in H3K27me3 domains reported during embryonic stem cell differentiation *in vitro*, we find that H3K27me3 domains increase in number during endocrine progenitor development *in vivo*. Genes that lose the H3K27me3 mark typically encode transcriptional regulators, including those for pro-endocrine fates, whereas genes that acquire the mark typically are involved in cell biology and morphogenesis. Deletion of *Ezh2* at the pancreas progenitor stage enhanced the production of endocrine progenitors and beta cells. Inhibition of EZH2 in embryonic pancreas explants and in human embryonic stem cell cultures increased endocrine progenitors *in vitro*. Our studies reveal distinct dynamics in H3K27me3 targets *in vivo* and a means to modulate beta cell development from stem cells.

Keywords embryogenesis; endocrine; *Ezh2*; H3K27me3; pancreas

Subject Categories Chromatin, Epigenetics, Genomics & Functional Genomics; Development & Differentiation; Stem Cells

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Introduction

The generation of insulin-secreting beta cells in the pancreas involves sequential cell fate choices. Pancreas progenitor cells, which initially express *Pdx1*, a pancreatic determination gene, arise

from the definitive endoderm at the 7 somite pair (7S) stage of mouse embryogenesis, or embryonic day 8.5 (E8.5) (Jonsson *et al*, 1994; Offield *et al*, 1996) as an alternate fate from that of liver progenitors (Deutsch *et al*, 2001). The early differentiated PDX1⁺ cells are multipotent progenitors that can give rise to ductal cells, acinar cells, and endocrine progenitors (NGN3⁺) (Jorgensen *et al*, 2007). From about E13.5–E14.5, endocrine progenitors are specified and then differentiate into all five hormone-expressing endocrine lineages (α , β , δ , ϵ , and PP) which will comprise the pancreatic islets (Oliver-Krasinski & Stoffers, 2008; Zaret & Grompe, 2008; Pan & Wright, 2011). Due to technical limitations in working with the small cell numbers that can be harvested from embryos, progress in mapping chromatin transitions during beta cell development has been primarily from embryonic stem cell (ESC) cultures. The extent to which chromatin dynamics *in vitro* mirror those *in vivo* has yet to be explored. Since chromatin modifications are created by enzymes, and enzymes can be inhibited by small molecules, understanding chromatin dynamics can help control cell fates and thus enhance the generation of desired cell types, such as beta cells.

Progress in understanding chromatin states relevant to beta cell development includes the discovery that the H3K27me3 demethylases UTX (KDM6A) and JMJD3 (KDM6B) regulate endoderm differentiation from human ESCs by modulating the WNT signaling pathway (Jiang *et al*, 2013). Mouse ESC studies further showed that JMJD3 cooperates with the transcription factors Tbx3 and Eomes to promote endoderm induction (Kartikasari *et al*, 2013). We found a functional “prepattern” of chromatin states whereby regulatory elements of the *Pdx1* gene, but not regulatory elements of liver genes, are marked by H3K27me3 in mouse embryonic endoderm, where all of these genes are silent and the cells are not yet committed to one fate or another (Xu *et al*, 2011). The histone H3K27 methyltransferase EZH2, which binds to the *Pdx1* regulatory elements in endoderm, was found to modulate the pancreas versus liver fate choice by suppressing the pancreas lineage (Xu *et al*, 2011), consistent with a generally repressive role for PRC2 (Conerly *et al*, 2011; Schwartz & Pirrotta, 2013).

In a human ESC model of pancreatic development, the global number of H3K27me3 peaks declined during *in vitro* differentiation to

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endoderm and pancreas progenitor stages [see Fig 3D of Xie *et al* (2013)], with transcriptional regulatory genes being among those losing the mark, over time. Whether a cumulative loss of H3K27me3 occurs globally *in vivo* is unknown. Another study of *in vitro* huESC differentiation to endoderm and posterior foregut progenitors, including pancreatic progenitors, observed a wide diversity of chromatin mark patterns that did not cohesively predict classes of enhancers as being prepatterned or common gene sets at each multipotent progenitor stage (Loh *et al*, 2014). Due to challenges in maturing huESC cultures to endocrine progenitors *in vitro*, this latter step was not explored.

A cross-tissue study found that genomic locations of the active H3K4me3 mark are similar between beta cells and neural tissue, whereas locations of H3K27me3 displayed a similar profile between beta cells and acinar cells, thereby better reflecting a shared developmental lineage (van Arensbergen *et al*, 2010). Notably, this study also showed that H3K4me3/H3K27me3 bivalency in pancreas progenitors at E10.5 did not predict competency of genes to be activated later in beta cells. Therefore, a primary focus on H3K27me3 dynamics seems most informative for endocrine development. A subsequent *in vivo* study showed that Ring1b, a PRC1 complex subunit, establishes repressed domains in pancreas progenitors but is not required to maintain them in insulin cells (van Arensbergen *et al*, 2013). Taken together, the above studies show that H3K27me3 dynamics are crucial to early pancreatic development, but roles at the endocrine induction step are unclear.

Prior studies of native embryonic cells compared ES chromatin profiles with that in pancreas progenitors and fully differentiated beta cells. Here, we present the first assessment of H3K27me3 dynamics in the step-by-step transitions between foregut endoderm cells, pancreas progenitor cells, and endocrine progenitor cells isolated from mouse embryos. Notably, the overall H3K27me3 peak dynamics in embryos differ from that observed with ESCs *in vitro*. With regard to gene networks that change in H3K27me3 coverage during the induction of pancreas progenitors into endocrine cells, H3K27me3 was acquired predominantly at genes involved in cell biological and morphogenetic changes. We genetically deleted *Ezh2* during the pancreatic endocrine induction step in embryos and pharmacologically inhibited EZH2 in human ESC cultures *in vitro* and observed an increased yield of functional beta cell progenitors. These findings reveal gene networks specific to cells undergoing organogenesis *in vivo* and demonstrate how a detailed analysis of chromatin during native embryonic development provides insight that can be applied to stem cell differentiation.

Results

Net increase of H3K27me3 peaks during pancreas progenitor and endocrine progenitor specification *in vivo*

To obtain a genomic view of H3K27me3 locations during the transitions from endoderm to pancreas progenitors and from pancreas progenitors to endocrine progenitors, we used FACS to isolate cells and performed ChIP-Seq with an antibody for H3K27me3 (Supplementary Fig S1). For the pancreatic progenitor specification step, we isolated undifferentiated endoderm cells from E8.25 mouse embryos with the antibody ENDM1 (Gadue *et al*, 2009; Xu *et al*, 2011) and PDX1⁺/Liv2⁻ pancreatic progenitor cells at E10.5 from *Pdx1-GFP* transgenic

embryos (Supplementary Fig S2, Q3) (Gu *et al*, 2004). The selection of Liv2⁻ cells eliminated about a fifth of the Pdx1-GFP⁺ population that co-expresses the Liv2⁺ hepatoblast surface antigen (Supplementary Fig S2, Q2) (Xu *et al*, 2011); these cells were not characterized further. For the endocrine specification step, we isolated PDX1⁺/Liv2⁻ cells at E10.5 and Ngn3⁺ endocrine progenitor cells at E14.5 from *Ngn3-GFP* embryos (Lee *et al*, 2002; White *et al*, 2008) (Supplementary Fig S2). The experiments employed 5×10^4 to 10^5 cells per ChIP with an antibody against H3K27me3, pooled from about 100 E8.25, 50 E10.5 embryos, and 40 E14.5 pancreata (Fig 1A, Supplementary Fig S3, Supplementary Table S1). We mapped the H3K27me3 peaks in the genome at each stage (see Supplementary Methods, Supplementary

Figure 1. Increasing numbers of H3K27me3 peaks during pancreatic progenitor specification and endocrine specification.

- A** Scheme employed to use FACS to obtain ENDM1⁺ endoderm progenitors (EN), Pdx1-GFP⁺/Liv2⁻ pancreatic progenitors (PP), and Ngn3-GFP⁺ endocrine progenitors (EP) for H3K27me3 ChIP-Seq.
- B** Top, schematic of mouse chromosome 9; red boxed region is expanded below, showing a local genomic view of 2 Mb of input-depleted H3K27me3 aligned sequence tags at the EN, PP, and EP stages. Note the clustering of tags over the gene-dense regions. Chromosomal coordinates (mm8) are shown above the tag display.
- C** Expanded view of the *Hnf6/Onecut1* locus, showing a local diminution of sequence tags at the PP stage, when the *Hnf6* gene is expressed (Jacquemin *et al*, 2000).
- D, E** Venn diagrams depicting the unique and overlapping H3K27me3 aligned tag counts, illustrating a progressive increase in aligned tags at each of the pancreatic progenitor and endocrine progenitor transitions.

Table S1). Called peaks appeared visually as “patches” (Barski *et al*, 2007) of broad-spread H3K27me3 binding. Notably, among all chromatin samples, the number of patches was not a simple function of the number of aligned reads (Supplementary Table S1).

As seen in a typical 2-megabase view of the genome in Fig 1B, H3K27me3 aligned sequence tags were inhomogenous, being more concentrated over gene-dense regions at each stage. A magnified view (Fig 1C, shown by red lines above tracks) centered on the *Hnf6* (*Onecut1*) locus showed denser tags over the promoter and first exon in endoderm (EN, ENDM1⁺) and endocrine progenitors (EP, *Ngn3-GFP*⁺), where *Hnf6* is silent, and fewer tags over the region in pancreatic progenitors (PP, *Pdx1-GFP*⁺/*Liv2*[−]), where the gene is active (Jacquemin *et al*, 2000) (Fig 1C, see green dotted line). Indeed, *Hnf6/Onecut1* was called as an H3K27me3⁺ target in EN and EP cells and not in PP cells (see Supplementary Methods and Fig 2A, below).

Comparing the total number of all H3K27me3 peaks at each stage (Fig 1D and E), 4,043 peak locations were common between the endoderm (EN) and pancreas progenitor (PP) stages and 5,399 peak locations were common between the PP and endocrine progenitor (EP) stages. However, there were 847 peaks unique to endoderm cells that were lost during pancreas progenitor induction, while 1,635 peaks were gained in the pancreas progenitor cells (Fig 1D). Similarly, 279 peaks in pancreas progenitors were lost during endocrine progenitor induction, while 2,949 peaks were gained in the endocrine progenitor cells (Fig 1E). The substantial increase in H3K27me3 peaks at each step of early beta cell development in embryos differs markedly from that seen with differentiation of human ESCs, where progressive loss of the mark occurs to the pancreatic endoderm stage *in vitro* [see Fig 3D of Xie *et al* (2013)].

H3K27me3 gained and lost at distinct gene networks during pancreatic endocrine specification *in vivo*

To broadly assess the dynamics of the relevant gene networks marked by H3K27me3, we also mapped H3K27me3 targets for the alternate fates of ENDM1[−] cells, that is, non-endoderm fate, and *Liv2*⁺ cells, that is, the hepatoblast (non-pancreatic) fate (Xu *et al*, 2011). This provided a collection of genes marked by H3K27me3 at either outcome of fate choices leading to endocrine cells. Of the H3K27me3 peaks from all of the above populations, 4,347 overlapped RefSeq genes in at least one tissue or stage examined (Fig 2A). We then created a heat map of the dynamics of H3K27me3 peaks at the genes during the EN, PP, and EP stages (Fig 2A). In agreement with the Venn diagrams in Fig 1D and E, the heat map in Fig 2A shows that while about 60% of the genes marked by H3K27me3 in endoderm remain marked into the endocrine progenitor stage, a large number of genes acquire H3K27me3 during the two underlying transitions. In addition, many genes positive in all three stages gained in H3K27me3 tag density by the endocrine progenitor stage (more red in Fig 2A, “EP”). This latter feature, that genes that retain H3K27me3 in development exhibit increased tag density over time, was also seen in the *in vitro* human ESC data [see Fig 3D of Xie *et al* (2013)].

We then examined the genes that lost H3K27me3 when pancreas progenitors became *Ngn3*⁺ endocrine cells (115 genes, “++−”) or that gained H3K27me3 during the transition (598 genes, “−−+”), where the state of positive or negative for H3K27me3 had been

stable for the previous endoderm to pancreas progenitor transition (Fig 2C). This focused the analysis on genes that underwent their first H3K27me3 transition at the endocrine step within the pancreatic endoderm lineage. Boxplots showing the distribution of fold-changes of gene expression scores for *Ngn3*-YFP⁺ versus *Ngn3*-YFP[−] pancreatic cells (E15.5) (Soyer *et al*, 2010) revealed that genes in the “++−” category showed a net increase in the overall expression level (Fig 2B, red box) while genes in the “−−+” category showed a net decrease in expression level (Fig 2B, green box), consistent with a repressing effect of the H3K27me3 mark. Genes that did not change their + or − status of H3K27me3 in these three populations exhibited no effect at the median (Fig 2B, “others”, gray). This analysis revealed an inverse correlation between our calls for H3K27me3 and gene expression during endocrine pancreas development, as expected for a repressive chromatin mark.

To understand the cellular networks that could explain the increase in H3K27me3 peaks seen *in vivo*, but not *in vitro*, we performed Gene Ontology (GO) analyses. Strikingly, the category of “−−+” typically encompassed genes related to cytoskeletal structure, membrane proteins, and cell adhesion (Fig 2C, “gained in EP”, Supplementary Table S2, Supplementary Dataset S1). It would therefore appear that the acquisition of H3K27me3 at the endocrine progenitor stage helps extinguish cell functions that are associated with cell biology and morphogenesis, features that are prominent during organogenesis *in vivo* and could be missing in cell culture.

By contrast, the category of “++−” predominantly encompassed genes related to transcriptional regulation (Fig 2D, “lost in EP”, Supplementary Table S3, Supplementary Dataset S1). This agrees with the data from human ESC differentiation *in vitro*, where loss of the H3K27me3 mark was seen extensively for transcriptional regulators (Xie *et al*, 2013). Notably, the “++−” category, where the H3K27me3 mark is lost during endocrine cell induction, includes the transcription factor genes *Ngn3*, *Nkx6.1*, *Nkx2.2*, and *Neurod1* (Supplementary Table S3; GO:0031018), which are necessary for the specification of endocrine progenitors and for establishing beta cell identity (Gradwohl *et al*, 2000; Gu *et al*, 2002; Schaffer *et al*, 2010, 2013; Papizan *et al*, 2011; Mastracci *et al*, 2013).

For example, inspection of the aligned sequence tags shows that H3K27me3 spans the *Ngn3*, *Nkx2.2*, and *NeuroD1* genes at the endoderm and pancreatic progenitor stages, but is depleted during endocrine induction stage, when the genes are activated (Fig 3A). While *Nkx6.1* is first activated in pancreatic progenitors and is needed for an endocrine versus acinar fate (Schaffer *et al*, 2010), recent studies indicate that *Nkx6.1* also has a secondary role downstream of *Ngn3* (Schaffer *et al*, 2013). In this context, we observe marked levels of H3K27me3 on *Nkx6.1* in both endoderm and pancreatic progenitors, and then a loss of such in endocrine progenitors (Fig 3A). By contrast, the *Hoxb5* gene is inactive and is blanketed by H3K27me3 at all stages tested and the *Gapdh* gene is expressed at all stages and lacks patches of H3K27me3. To validate these results, we performed ChIP-qPCR on the enhancer and promoter elements of *Ngn3* and the promoters of *Nkx2.2*, *Neurod1*, and *Nkx6.1* (Fig 3; at location of red boxes in panels A, B), with the *Hoxb5* promoter as a positive control and a *Gapdh* exon as a negative control (Fig 3). We found that in the endoderm and pancreatic progenitors, H3K27me3 was present at the *Ngn3*, *Nkx2.2*, *NeuroD1*, and

Figure 2. Dynamic patterns of H3K27me3 during pancreatic progenitor specification and endocrine specification.

- A** Heat map indicating intensity of H3K27me3-bound genes (red, more tags per positive gene; black, called as negative) at the endoderm (EN), pancreas progenitor (PP), and endocrine progenitor (EP) stages. The number of genes in each sequential dynamic expression category is shown to the right of the heat map.
- B** Boxplots with y-axis indicating gene expression fold change; that is, net increase in the overall RNA expression level of genes in the “+ + -” category, that lose H3K27me3 at the endocrine induction step, while genes in the “- - +” category, that gain H3K27me3, show a net decrease. Genes not changed in H3K27me3 + or - in these three populations exhibit no effect at the median.
- C, D** Gene Ontology analysis of the category of “- - +” (gain in H3K27me3 in *Ngn3-GFP*⁺ endocrine progenitor cells, panel C) and of the category of “+ + -” (loss of H3K27me3 in *Ngn3-GFP*⁺ endocrine progenitor cells, panel D). *P*-values are shown. See Supplementary Tables S2 and S3 for details.

Nkx6.1 regulatory sequences (Fig 3C, blue, red bars) and at the positive control site, but not at the negative control site. In *NGN3*⁺ cells, H3K27me3 was depleted from the *Ngn3*, *Nkx2.2*, *NeuroD1*, and *Nkx6.1* elements, consistent with the activation of

the respective genes (Fig 3C, green bars). In summary, H3K27me3 is gained at differentiation and morphogenesis genes and is lost from key transcriptional regulatory genes in a stepwise manner during pancreatic endocrine development *in vivo*.

Figure 3. Changes of H3K27me3 modification at *Ngn3*, *Nkx6.1*, *Nkx2.2*, and *NeuroD1* elements during the endocrine specification.

A Genome browser images of H3K27me3 patches covering the indicated loci at the Endoderm (EN), Pancreatic Progenitor (PP), and Endocrine Progenitor (EP) stages. *Hoxb5* is blanketed at all stages and *Gapdh* at none of them, as positive and negative controls. The *Ngn3*, *Nkx6.1*, *Nkx2.2*, and *NeuroD1* loci are blanketed in EN and EP stages, but not in the PP stage, coincident with their transcriptional activation at PP. Red bars show locations of ChIP-qPCR analysis.

B Regulatory elements of *Ngn3*, *Nkx6.1*, *Nkx2.2*, and *NeuroD1* genes. Red bars show locations of ChIP-qPCR analysis.

C H3K27me3 ChIP-qPCR assays ($n = 3$) were performed on FACS-isolated EN, EP, and PP cells, validating the ChIP-Seq data in (A). Results are mean $\pm$ SD. * $P \leq 0.05$ determined by t-test; ** $P \leq 0.01$; *** $P \leq 0.001$.

Ezh2 suppresses the normal extent of endocrine cell induction

Given the loss of H3K27me3 at key endocrine transcription factor genes during endocrine cell induction, we tested whether precocious loss of *Ezh2*, which catalyzes the H3K27me3 mark (Cao et al, 2002), would affect the timing or extent of endocrine induction. Since the *Ezh2* null mouse is embryonic lethal during gastrulation (O'Carroll et al, 2001), we used an *Ezh2* conditional allele (*Ezh2*^{CA}) (Su et al, 2003) and a *Pdx1-Cre* transgene (Gu et al, 2002; Hingorani et al, 2003), the latter of which starts to express the Cre recombinase around E9.5, in an *Ngn3-GFP* background. This would cause precocious loss of *Ezh2* prior to E12.5, when endocrine cell production begins. In sorted GFP⁺ cells from E14.5 *Ezh2*^{fl/fl}; *Pdx1-Cre*; *Ngn3-GFP* pancreata, the expression level of *Ezh2* is greatly diminished from that seen in wild-type or heterozygous floxed embryos

(Fig 4A). In heterozygous and homozygous floxed *Ezh2* embryos, the *Ngn3* gene is modestly up-regulated in *Ngn3-GFP*⁺ cells, possibly because precocious expression causes increased accumulation of the *Ngn3* mRNA (Fig 4A). Importantly, H3K27me3 is greatly diminished in the *Ezh2* knockout pancreas and undetectable in nascent endocrine progenitors expressing endogenous NGN3 (Fig 4B). In addition, *Ngn3-GFP*⁺ cells displayed a higher fluorescence intensity in *EZH2* heterozygous and homozygous pancreas tissue (Fig 4C). Flow cytometry analysis provided a quantitative view of the three cell populations, *Ngn3-GFP*^{neg}, *Ngn3-GFP*^{low} and *Ngn3-GFP*^{high} in E14.5 pancreases. In *Ezh2*^{fl/fl} pancreases, *Ngn3-GFP*^{low} cells were increased nearly threefold and *Ezh2*^{fl/+} pancreases showed about a two-fold increase (Fig 4D and E). Together, these data show that *Ezh2* depletion prior to the normal time of endocrine cell induction causes more endocrine progenitors to develop. Furthermore, the

Figure 4. Histone methyltransferase *Ezh2* restrains endocrine pancreatic progenitor specification.

- A *Ezh2* and *Ngn3* mRNA levels in sorted E14.5 *Ngn3-GFP*⁺ cells (*Ngn3*-H) as analyzed by qRT-PCR, normalized to *Gapdh* within each sample, relative to the levels in *Ezh2*^{+/+} cells (set to 1). **P* ≤ 0.05; ****P* ≤ 0.001.
- B Immunofluorescence staining of NGN3 (green) and H3K27me3 (red). Arrows point to the NGN3⁺, H3K27me3[−] cells. In *Pdx1-Cre;Ezh2*^{fl/fl} E14.5 pancreas, H3K27me3 modification cannot be detected.
- C Fluorescence images of *Ezh2* wild-type (upper left), *Pdx1-Cre;Ezh2*^{fl/+} (middle), and *Pdx1-Cre;Ezh2*^{fl/fl} (right) pancreas, each in an *Ngn3-GFP* background. *Ngn3-GFP*⁺ cells displayed a higher fluorescence intensity in *Ezh2* heterozygous and homozygous pancreas.
- D FACS analysis shows *Ngn3-GFP*[−] (neg), *Ngn3-GFP* low (L), and *Ngn3-GFP* (H) high cells in WT versus *Ezh2* heterozygous KO and homozygous KO embryos; the y-axis shows FSC/forward scatter.
- E Statistics of the percent of *Ngn3-GFP*[−] (neg), *Ngn3-GFP* low (L), and *Ngn3-GFP* high (H) cells to total pancreatic cells in *Ezh2* WT, heterozygous, and homozygous embryos, expressed as mean ± SEM; *n* = number of embryos. WT embryos lacking *Ngn3-GFP* had virtually no fluorescent cells scoring positive (right panel).

rate of bromodeoxyuridine (BrdU) incorporation in the *Ezh2*^{fl/fl} pancreas was indistinguishable from WT (Supplementary Fig S4). In prior studies (Jacquemin et al, 2006), we showed that there is essentially no apoptosis in early pancreatic epithelial cells. Thus, the increase in *Ngn3-GFP*⁺ cells in *Ezh2*^{fl/fl} pancreases was not due to increased cell proliferation or programmed cell death and therefore apparently by enhanced specification.

During pancreas development, multipotent progenitors undergo cell fate choices to differentiate into endocrine and exocrine pancreatic cells, the latter including acinar and duct cells. The exocrine compartment is highly branched, with the branches capped by acinar cells at the tips and connecting to duct trunks (Zhou et al, 2007). To address where the excess *Ngn3-GFP*⁺ endocrine cells arise in the *Ezh2*^{fl/fl} pancreas, we performed immunohistochemistry on E14.5 pancreases for the acinar cell marker amylase or the trunk marker SOX9 (Seymour et al, 2007), combined with NGN3 antibodies. In both WT and *Ezh2*^{fl/fl} pancreas, NGN3⁺ cells were evident in the central trunk domains, as expected (Supplementary Fig S5, upper panels, blue cells). We did not observe any NGN3⁺ cells in the peripheral, amylase⁺ tip areas (Supplementary Fig S5, upper panels, brown cells); NGN3⁺ cells in *Ezh2*^{fl/fl} were only evident in the Sox9⁺ duct trunk area (Supplementary Fig S5, lower panels). Taken together, the genetic data show that *Ezh2* normally suppresses the extent of endocrine cell induction in the trunk cells of the developing pancreas. *Ezh2* diminution at an earlier stage, due to *Pdx1-Cre*, increases the number of endocrine cells that develop.

***Ezh2* loss at pancreas progenitor stage leads to increased beta cells**

To assess the developmental consequence of an increase in NGN3⁺ endocrine cells, we examined the mass of islets at postnatal day 9 (P9) by insulin immunohistochemistry. In the *Pdx1-Cre;Ezh2*^{fl/fl} pancreas, we observed a 1.5-fold increase in the beta cell mass in the P9 animals, compared to wild type (Supplementary Fig S6A and B). These findings revealed that deleting *Ezh2* at the pancreas progenitor stage enhances endocrine cell induction, allowing a greater number of beta cells to develop. Consistent with these data, in 2-month-old *Pdx1-Cre;Ezh2*^{fl/+} pancreases, the mass of islets shows a 1.34-fold increase compared to wild type (Fig 5A and B). However, surprisingly, in 2-month-old *Pdx1-Cre;Ezh2*^{fl/fl} pancreases, the mass of islets was significantly reduced from WT (Fig 5A and B). Islet structure and beta cell morphologies appeared normal in the knockouts (data not shown).

Chen et al (2009) previously employed the *Ezh2*^{fl/fl} model, but deleted *Ezh2* later, at the beta cell stage, with a *RIP-Cre*. They found that the homozygous loss of *Ezh2* after beta cells are formed induces

Ink4a/Arf expression, which impairs beta cell proliferation and leads to mild diabetes (Chen et al, 2009). To test whether the loss of *Ezh2* in the earlier stage, *Pdx1-Cre* model induces *Ink4a/Arf* in the islet, we isolated islets from about 2-month-old *Pdx1-Cre;Ezh2*^{fl/fl} and *Pdx1-Cre;Ezh2*^{fl/+} male mice and their wild-type male littermates, and performed gene expression analysis. The expression of the *Ezh2* gene could not be detected in *Pdx1-Cre;Ezh2*^{fl/fl} islets, while the *Ink4a/Arf* gene, which was severely repressed in wild-type islets, was highly expressed in *Pdx1-Cre;Ezh2*^{fl/fl} islets but not in *Ezh2* heterozygotes (Fig 5C). *Insulin1* and *Insulin2* gene expression, on a per islet basis, did not show significant differences in these three genotypes (Fig 5C). Consistent with Chen's study, we detected a mild defect in glucose normalization in 2-month-old *Pdx1-Cre;Ezh2*^{fl/fl} male mice (Fig 5D, lower, green line), where overnight fasted mice were injected intraperitoneally with glucose and blood glucose levels were measured at various time points thereafter. But in *Pdx1-Cre;Ezh2*^{fl/+} mice, where the remaining EZH2 is still sufficient to repress *Ink4a/Arf* in the islet (Fig 5C), we detected enhanced glucose normalization compared to 2-month-old littermate controls (Fig 5D, red line). It appears that a gain in endocrine progenitor cell numbers in the *Ezh2*^{fl/+} pancreas, without *Ink4a/Arf* induction, is sufficient to enhance islet function, whereas in the *Ezh2*^{fl/fl} pancreas, aberrant *Ink4a/Arf* induction results in impaired function.

Histone methyltransferase inhibitors modulate two stages of pancreas differentiation

Previously we showed that *Pdx1* regulatory sequences contain H3K27me3 in undifferentiated ENDM1⁺ endoderm cells and that *Ezh2* loss, via an endoderm Cre, causes an increase in the development of pancreatic progenitors at the expense of liver progenitors (Xu et al, 2011). To test small molecule inhibitors of EZH2 for their ability to mimic the *Ezh2* conditional knockout at this stage, we set up half-embryo cultures that allow foregut tissue development *in vitro* (Wandzioch & Zaret, 2009). Briefly, "halves" of 2-7S embryos were taken anterior to the first somite and cultured for 48 h, allowing gut tube closure, the induction of early liver and pancreatic genes, and heart development (Wandzioch & Zaret, 2009). We cultured *Pdx1-GFP* half-embryos in the presence of 3-deazaneplanoncin A (DZNep), which inhibits the methyl donor pathway for EZH2 (Fiskus et al, 2009), and the EZH2-specific inhibitor GSK-126 (McCabe et al, 2012). Notably, we observed a significant increase in the number of *Pdx1-GFP*⁺ cells in DZNep- and GSK-126-treated half-embryos (Supplementary Fig S7A and B). These data show that inhibitors of EZH2 can regulate a step of pancreatic differentiation similar to that seen in prior genetic studies with *Ezh2*^{fl/fl} and an endoderm Cre (Xu et al, 2011).

Figure 5. Beta cell mass expands in adult *Ezh2* heterozygotic knockout pancreas, but reduces in *Ezh2* homozygotic knockout pancreas.

- A** Immunohistochemistry for insulin (brown) shows beta cell area increases in 2-month-old male *Ezh2* heterozygous KO pancreas, while decreases in homozygous KO pancreas, compared to WT counterparts.
- B** Statistics of the percentage of beta cell area to total pancreas area. Intermittent sections in 100 μ m intervals were analyzed for each pancreas, and 2–3 middle sections were measured for their beta cell area over total pancreas area; *n* = number of animals. Asterisks denote *P* < 0.05.
- C** *Ezh2* and *Ink4a/Arf* mRNA levels in isolated islet cells as analyzed by qRT-PCR, normalized to *Gapdh* within each sample and relative to levels in *Ezh2* normal islets (*Ezh2* expression) or *Pdx1*-Cre;*Ezh2*^{fl/fl} (*Ink4a/Arf* expression) islets; nd means not detected.
- D** Glucose tolerance test in littermate sets of WT and *Pdx1*-Cre;*Ezh2*^{fl/+} male mice (*n* = 4 each) and WT and *Pdx1*-Cre;*Ezh2*^{fl/fl} mice (*n* = 5, 6 respectively) were analyzed at different times and gave slightly different peak glucose levels for the different cohorts of WT animals, but the glucose levels were consistent within each experiment (see error bars). Asterisks denote *P* < 0.05. *Ezh2* heterozygous KO mice suppressed glucose levels more effectively than WT whereas homozygous KO mice were less effective than WT.

Given this validation of the action of the EZH2 inhibitors in explants, we sought to determine whether treatment with DZNep can modulate the later step of endocrine progenitor induction. We dissected dorsal pancreatic tissue from E12.5 *Ngn3-GFP* embryos and treated cultured explants with DZNep for 4 days. The DZNep-treated *Ngn3-GFP* pancreas explants showed a higher GFP intensity compared to non-treated controls (Fig 6A). A 1.8-fold increase in the percentage of GFP⁺ cells in DZNep-treated explants, without a decrease in GFP[−] cells, was confirmed by flow cytometry analysis (Fig 6B). Hence, both the genetic and pharmacologic data indicate that EZH2 normally restrains the pancreatic progenitor and the endocrine progenitor induction steps of beta cell development.

Histone methyltransferase inhibitors enhance the induction of beta-like cells *in vitro*

We next tested EZH2 inhibitors with a human endodermal progenitor cell differentiation system *in vitro*. The endodermal progenitor cells are a recently described, pan-endodermal stem cell population derived from human ES or induced pluripotent stem (iPS) cells that can efficiently generate mono-hormonal pancreatic beta-like cells *in vitro* (Cheng *et al*, 2012). Differentiation of endodermal progenitor cells follows a stepwise developmental progression that mimics endocrine cell development *in vivo*, including pancreatic induction, endocrine specification, and subsequent expression of insulin (Fig 7A).

To determine whether methyltransferase inhibition could enhance endocrine differentiation of human cells *in vitro*, we treated endodermal progenitor cell cultures at distinct stages from differentiation initiation to endocrine specification. We used doses of DZNep

and GSK-126 that could decrease H3K27me3 enrichment at the *NEUROG3* promoter in undifferentiated human EP cells (Supplementary Fig S8). While treatment with DZNep early in the differentiation cultures had slight inhibitory effect on the later generation of C-peptide⁺ cells, treatment at days 8–10, during endocrine specification, led to an approximate doubling of the percentage of C-peptide⁺ cells in the end stage cultures without inducing the generation of poly-hormonal insulin⁺/glucagon⁺ cells, as determined by intracellular flow cytometry (Fig 7B and D). The increase in C-peptide⁺ cells correlated with an increase in *NGN3* mRNA expression at day 10 of the differentiation culture (Fig 7C). We found that treatment with GSK-126 had effects similar to DZNep both in *NGN3* and in C-peptide induction (Fig 7C and D). Similar results were found with differentiation of an endoderm progenitor cell line derived from iPS cells (data not shown). These findings demonstrate that the positive effects of manipulating EZH2 function on pancreatic beta cell induction in mouse models can be applied to human stem cell differentiation cultures.

Discussion

The stepwise development of pancreatic beta cells is regulated by cell signaling, transcription factors, and chromatin modifiers. As noted in the Introduction, abundant evidence demonstrates a general role for the Polycomb complexes (PRCs) in lineage commitment (Lee *et al*, 2006; Surface *et al*, 2010) in general, and in endoderm and pancreatic progenitor development in particular. In addition, *Ezh2* is necessary in the adult to maintain the physiological function of beta cells (Chen *et al*, 2009). Yet there was a gap in

Figure 6. Histone methyltransferase inhibitor improves pancreatic endocrine cell induction.

A Pancreas tissue was dissected from E12.5 *Ngn3-GFP* embryos and cultured in the presence or absence (control) of 0.1 μ M DZNep, an inhibitor of the methyl donor pathway for EZH2 activity (right, schema). The DZNep-treated tissues displayed more GFP⁺ cells (left).
B After 4 days, cells were dissociated and GFP⁺ cells over the total number of cells in the explants were quantitated by FACS, as displayed in the bar chart. *n* = number of embryos' pancreases assessed; mean and SEM shown; the difference (*) is significant (*P* < 0.05).

Figure 7. Histone methyltransferase inhibitors enhance pancreatic beta-like cell development from human endoderm progenitor cells.

- A** Experimental scheme of beta-like cell differentiation, showing the times of DZNeP treatment (red bars).
- B** The % of C-peptide⁺ endocrine cells at days 16–18 of differentiation when treated with DZNeP at the times indicated by red bars in (A), expressed as mean $\pm$ SEM (n = 10) (***)P-value < 0.001.
- C** *Ngn3* mRNA was quantified by qRT-PCR at day 10 of differentiation, comparing control DZNeP- and GSK-126-treated cultures at days 8–10, expressed as mean $\pm$ SEM (n = 4) (*P-value < 0.05).
- D** FACS analysis of cells expressing C-peptide versus glucagon on day 18 of differentiation in cultures treated at days 8–10 with DZNeP or GSK-126.

understanding PRC function in endocrine induction. Analogous to our earlier finding that *Ezh2*, encoding the enzyme for H3K27me3 modification, restrains the differentiation of endoderm cells into pancreatic lineage (Xu et al, 2011), we now find that *Ezh2* restrains the induction of the later, endocrine commitment step. The existence of inhibitors of EZH2 allowed us to employ such to enhance endocrine and beta cell induction in embryonic tissue explants and in human embryonic stem cell cultures.

To gain a dynamic view of H3K27me3 modification during the cell fate transitions from endoderm progenitors to pancreatic progenitors, and from pancreatic progenitors to endocrine progenitors, we performed H3K27me3 ChIP-Seq in cells isolated by FACS from mouse embryos. Prior studies had mapped H3K27me3 specifically at

promoters in pancreatic progenitors (van Arensbergen et al, 2010), but genome-wide assessments in such cells and in native endoderm and endocrine progenitors had not been performed. Three features of our dataset stand out, when compared to the *in vitro* ESC differentiation studies of (Xie et al, 2013). First, both *in vitro* and *in vivo*, genes that retain H3K27me3 throughout the developmental stages tested exhibit an increase in tag intensity over time. While the functionality of such changes remains to be determined, we suggest that it may reflect enhanced commitment to early-established lineage decisions. Second, both *in vitro* and *in vivo*, loss of H3K27me3 at each developmental stage frequently occurs at genes involved in transcriptional control. This would be consistent with a need to activate tissue-specific regulators that are silent in early development. The third feature reveals a disparity; we saw a clear increase in the number of H3K27me3 peaks, or patches, in the genome during the progression to the endocrine state, which contrasts markedly with a diminution of H3K27me3 peaks seen during the progression of embryonic stem cell cultures differentiated *in vitro* (Xie et al, 2013).

What could serve as the basis for the much greater number of genes gaining the H3K27me3 mark in embryonic development than in cell culture? It was striking that at the transition to endocrine progenitors, the genes gaining H3K27me3 largely fell into the categories of cell biology and morphogenesis. Since these functions are among the major determinants of embryonic organogenesis and are greatly minimized in cell culture, if they occur at all, we suggest that the deposition of H3K27me3 across the genome could be governed substantially by signaling that occurs during morphogenesis *in vivo* and that is largely absent *in vitro*.

The low cell number ChIP-Seq method from sorted mouse embryo cells currently works best, in our laboratory, for chromatin marks such as H3K27me3 that exist in patches and thus are relatively easy to map to peaks. The disparity between our *in vivo* results and those reported from *in vitro* studies for H3K27me3 indicates that it is worth the effort to advance low cell number technologies further, in order to assess other chromatin modifications *in vivo*.

Releasing H3K27me3 from the key endocrine and beta cell transcriptional factor genes of *Ngn3*, *Nkx6.1*, *Nkx2.2*, and *NeuroD1* is a prominent feature of the transition from pancreatic progenitors to endocrine progenitors. Concordantly, we found that precocious diminution of *Ezh2* in pancreatic progenitor cells led to an increase in endocrine progenitors. Eviction of the Polycomb complex from key target genes was recently found to promote the developmental timing of gene expression in *Arabidopsis* (Sun et al, 2014). The mechanism of how the EZH2 targets the development-specific genes remains elusive. The broad H3K27me3 patches we observed made it unproductive to screen for correlating DNA sequence motifs.

Upon precociously deleting the *Ezh2* gene in pancreatic progenitors, we found that the expression of *Ngn3* is elevated and the number of endocrine progenitors increased to a similar extent in E14.5 *Ezh2*^{fl/fl} and *Ezh2*^{fl/+} pancreas. This suggests that the precise expression level of *Ezh2* is critical to restrain endocrine pancreas development. Since the increased NGN3⁺ endocrine progenitors occurred in the trunk domains, it appears that *Ezh2* normally restrains endocrine induction in the natural trunk population, as opposed to a putative role in the exocrine compartment. Also, the lack of change in proliferation in the *Ezh2*^{fl/fl} pancreases is consistent with a cell fate control change instead of a putative non-specific increase in all cells of the organ.

At the adult stage, to our surprise, the mice with *Ezh2*^{fl/+} had enhanced glucose tolerance compared to their wild-type littermate, while the mice with *Ezh2*^{fl/fl} pancreas showed mild diabetes. We suggest that the gain in beta cell numbers, in the *Ezh2*^{fl/+} pancreas, without *Ink4a/Arf* induction, is sufficient to enhance islet function, whereas in the *Ezh2*^{fl/fl} pancreas, aberrant *Ink4a/Arf* induction results in impaired islet function. Thus our study is consistent with previous work (Chen *et al*, 2009) indicating that the proper amount of *Ezh2* is necessary to maintain beta cell function in the adult pancreas. Based on these findings, we propose that during pancreas development, the expression of the *Ezh2* gene itself and/or EZH2 enzymatic activity are under strict control.

Given the role *Ezh2* plays at the endocrine induction step *in vivo*, we used small molecule inhibitors of EZH2 on human embryonic stem cell cultures *in vitro* and discovered an enhanced yield of insulin-expressing cells. Since *Ezh2* is also necessary to maintain beta cell function (Chen *et al*, 2009), EZH2 inhibitors should be used in a short window prior to endocrine progenitor induction and then removed, to enhance the yield of beta cells. Our studies show that understanding mechanisms that underlie beta cell development *in vivo* can reveal processes that are not presently recapitulated *in vitro*, but yet can be used to enhance the *in vitro* approach.

Materials and Methods

Mouse strains and explant cultures

Pdx1-GFP (Gu *et al*, 2004), *Ngn3-GFP* (Lee *et al*, 2002; White *et al*, 2008), *Ezh2*^{fl/fl} (Su *et al*, 2003), and *Pdx1-Cre* (Hingorani *et al*, 2003) mice were used. Mice deleted for *Ezh2* in pancreatic progenitor cells were generated by mating *Pdx1-Cre*^{+/+}; *Ezh2*^{fl/+} mice to one another.

For half-embryo cultures, E8.25 (2–7 somite pair) embryos were dissected, the posterior half was removed from the first somite site, and the anterior half was cultured at 37°C for 48 h in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum (Hyclone) (Wandzioch & Zaret, 2009). For pancreas explant cultures, E14.5 pancreata were dissected and cultured on Nuclepore Track-Etch Membranes (Whatman, 110414) floating on DMEM containing 10% FBS for 4 days (Metzger *et al*, 2012).

FACS analysis, cell sorting, RNA analysis

Endoderm cells were purified by FACS with the ENDM1 and Liv2 antibodies as described (Xu *et al*, 2011) (Supplementary Fig S2). Embryos or pancreas tissue at later developmental stages was dissected from *Pdx1-GFP* or *Ngn3-GFP* strains, dissociated with trypsin for 5 min at 37°C then stopped with 0.4 volumes of FBS. The cells were filtered through a nylon mesh. GFP⁺ cells were then analyzed or purified on a BD FACSDiva cell sorter.

Human beta-like cells were harvested at day 18 of differentiation and trypsinized into single cells. Cells were fixed with 1.6% paraformaldehyde and stained with anti-C-peptide antibody (Cell Signaling) and anti-GCG antibody (Sigma). Secondary antibodies were goat-anti-mouse-IgG1-PE (Jackson ImmunoResearch) and goat-anti-rabbit-IgG-647 (Invitrogen). The stained cells were analyzed with the BD FACS Canto II Flow Cytometer (BD Biosciences), and the data were analyzed using Flowjo software (TreeStar).

Total RNA was isolated from cells isolated by FACS with the RNeasy Micro Kit (Qiagen) and was reverse transcribed with the iScript cDNA Synthesis kit (Bio-Rad). Real-time PCR was with the Power SYBR Green Supermix (Applied Biosystems) on the Applied Biosystems StepOnePlus. For RT-qPCR, primers were: *Gapdh* ATGGTGAAGGTCGGTGTGAAC, GCCTTGACTGTGCCGTTGAAT; *Ngn3* AGCTCTTGGCCCATAGATGATG, AAGAAGGCAGATCACCTTCGTG; *Ezh2* CTGCTGGCACCGTCTGA, GTTGCATCCACCACAAA. For human stem cell RT-qPCR total RNA was extracted with the RNeasy Micro kit (Qiagen). RNA was reversely transcribed into cDNA using random hexamers with Superscript III Reverse Transcriptase (Invitrogen). QPCR was performed on the LightCycler 480 II (Roche) using LightCycler 480 SYBR Green I Master Mix (Roche). Expression levels were normalized to the housekeeping gene PPIG (CYCLOPHILIN G). Human primers were: PPIG GAAGAGTGGCATCAAGAACCCATGAC, GTCTCTCCTCCTTCTCCTCTATC TTTACTT; NGN3 TCGAATGCACAACCTCAAC, AAGCTGTGGTCCGCTAT.

Low cell number ChIP-qPCR and ChIP-Seq

H3K27me3 ChIP-qPCR using about 10,000 embryonic cells was as described (Xu *et al*, 2011). One μ l of antibody against H3K27me3 (Millipore 07-449) was added per ChIP assay. The Western blot in Supplementary Fig S1 demonstrates the quality of the H3K27me3 antibody. All ChIP-qPCR data are from at least triplicate assays, normalized to input DNA. Primer sets: *Hoxb5* ACGACTGGTCAACAAAAGCA, GCGATGCACTCTACTTCGTT; *Gapdh* exon TCAACGGCACAGTCAAGGC, CTCCACGAATACTCAGCACC; *Ngn3* enh TCGCCTTAGGAGCAGGTGAT, TTGTAAAGCGGGATGC TTTG; *Ngn3* pro GAGAGTTGCTGGGACTGAGC, GGGAGCAGCACT CTGTTTGT; *Nkx6.1* pro GAGCCCCCTCATAAGTGATAATG, TCCGT CTCCTGCTCTTTTCTG; *Nkx2.2* pro CATCTTGCTCTAGAGGGCT GTT, ATTTGCAGATGTGAAATTGTGG; *NeuroD1* pro GTCCGCGGA GTCTCTAACTG, GAACCACGTGACCTGCCTAT.

For ChIP-Seq, we used chromatin from 5×10^4 to 1×10^5 sorted cells, pooled from many embryos harvested on multiple dates (see main text). Five μ l of H3K27me3 antibody was used with the MAGnify™ Chromatin Immunoprecipitation System (Invitrogen 49-2024). The preparation of multiplex libraries for sequencing (Supplementary Fig S3) was as per the "NEBNext ChIP-Seq Library Prep Set for SOLiD" (NEB E6260S). Sequencing was performed on the SOLiD4.0 platform with input and H3K27me3-immunoprecipitated libraries. See Supplementary Methods for a detailed low cell number ChIP-Seq protocol.

ChIP-Seq data analysis

Alignment

Colorspace sequence tags were aligned to the mouse genome (assembly mm8, NCBI 36) using Bowtie v0.12.5. Up to three seed mismatches were allowed and 10 bp were trimmed from the 3' end of each sequence tag prior to alignment. See Supplementary Table S1 for a summary of total and aligned reads for each sample.

Peak assessment

H3K27me3 peaks were called using the sliding window approach used to identify regions of H3K36me3 enrichment in (Guttman *et al*, 2009). Briefly, a 500-bp sliding window is scored for aligned tags

genome-wide; then, each window is assessed for its likelihood of enrichment given a Poisson model of the genomic background. A Bonferroni correction was applied to remove false discoveries (the effective alpha is 0.05 after correction), and overlapping peaks (at least 1 bp) were merged. Additionally, peaks were called in input using the same criteria. H3K27me3 peaks having 50% or more overlap to input peaks were discarded. The remaining peaks were then joined if the gap between them was less than 1 kb, and regions under 1 kb were discarded. A unified “super” peak set was constructed by pooling and merging overlapping peaks from each tissue (at least 1 bp overlap); then, an H3K27me3:input area under the curve (AUC) measurement was assessed for each unified peak in each tissue. In a given tissue, if a peak region had an AUC > 45, it was said to be a true enrichment. Each RefSeq gene-transcript was then assessed for overlap with true enrichments in each tissue; if any overlap could be detected the transcript was said to have H3K27me3 in that tissue. A detailed summary and analysis of the H3K27me3 target genes is presented in the Supplementary Dataset S1 in an Excel spreadsheet with multiple tabs.

Heat map

Each transcript therefore has an array of three values, one each for endoderm, pancreas progenitors, and endocrine cells, with “true” meaning “has H3K27me3 in this tissue” and “false” meaning “lacking H3K27me3 in this tissue”. To map H3K27me3 dynamics, transcripts with “true” or “false” in all tissues were filtered out. The remaining transcripts were recorded with their H3K27me3 profile and annotation information. The 90th percentile value for H3K27me3 AUC enrichment was set to the “maximum red” for the heat map.

Gene ontology

We assessed the genes that gained H3K27me3 upon the pancreas progenitor-to-endocrine transition and genes that lost the mark upon the transition, where the genes lacked or possessed H3K27me3, respectively, in the prior endoderm state. This focused the analysis on genes that underwent their first H3K27me3 transition at the endocrine step within the pancreatic endoderm lineage. GO analysis was performed on the two gene groups. Details of the GO analysis are presented in the Supplementary Dataset S1 in an Excel spreadsheet with multiple tabs.

Data sharing

Aligned tags, mapped peaks, and gene targets are being made available on GEO (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE56617>).

Immunohistochemistry

We used a citrate-based antigen unmasking solution (Vector Labs) and performed immunohistochemistry with the ABC Kit (Vector Labs) (Xu et al, 2011). We also used antibodies against NGN3 (BCBC, ab2013), Sox9 (Millipore AB5535), amylase (Santa Cruz sc-46657), and biotinylated anti-mouse IgG or anti-rabbit IgG (Vector Labs).

Glucose tolerance analysis

Sixteen hours overnight fasted mice were injected intraperitoneally with glucose (2 mg/kg body weight), and blood glucose levels were

measured at 0, 15, 30, 60, 90 and 120 min after injection. The investigator was blinded as to the identity of the samples during the glucose assays. Littermate sets of WT and *Pdx-Cre;Ezh2^{fl/+}* mice ($n = 4$ each) and WT and *Pdx-Cre;Ezh2^{fl/fl}* mice ($n = 5, 6$ respectively) were analyzed at different times and gave slightly different peak glucose levels for the different cohorts of WT animals, but the glucose levels were consistent within each experiment (see error bars in Fig 5D).

Human ES cell culture, differentiation, drug treatments, ChIP-qPCR

The human EP cell line used was from derived the H9 human ESC line (WiCell) as described previous (Cheng et al, 2012). EP cell maintenance and pancreatic differentiation were performed as described previously (Cheng et al, 2012). Differentiation cultures were treated with DZNep (0.1 μ M) or GSK-126 (2 μ M) for 2-day periods as indicated. Five million EP cells were used for each ChIP assay with 5 μ l of anti-H3K27me3 (Millipore 07-449). Primers used for the human *NEUROG3* promoter are GTGAGACGATGCACACAT-CACAAACAAG and AGCGATTGCGACCATAGTGGA.

Supplementary information for this article is available online: <http://emboj.embopress.org>

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Author contributions

C-RX, GD and KSZ designed the research; C-RX, L-CL, LY and Y-WZ performed the research; C-RX, L-CL, GD, LY, PG and KSZ analyzed the data; and C-RX, PG and KSZ wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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