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***Dnmt1* activity is dispensable in δ -cells but is essential for α -cell homeostasis**

Nicolas Damond^{a,b,c}, Fabrizio Thorel^{a,b,c}, Seung K. Kim^{d,e}, and Pedro L. Herrera^{a,b,c}

^a Department of Genetic Medicine & Development, Faculty of Medicine, University of Geneva, 1 rue Michel-Servet, 1211 Geneva 4, Switzerland ^b Institute of Genetics and Genomics in Geneva (iGE3), University of Geneva, Geneva, Switzerland ^c Centre facultaire du diabète, University of Geneva, Geneva, Switzerland ^d Department of Developmental Biology, Stanford University School of Medicine, California 94305, United States ^e Department of Medicine, Stanford, California 94305, United States

Abstract

In addition to β -cells, pancreatic islets contain α - and δ -cells, which respectively produce glucagon and somatostatin. The reprogramming of these two endocrine cell types into insulin producers, as observed after a massive β -cell ablation in mice, may help restoring a functional β -cell mass in type 1 diabetes. Yet, the spontaneous α -to- β and δ -to- β conversion processes are relatively inefficient in adult animals and the underlying epigenetic mechanisms remain unclear. Several studies indicate that the conserved chromatin modifiers DNA methyltransferase 1 (*Dnmt1*) and Enhancer of zeste homolog 2 (*Ezh2*) are important for pancreas development and restrict islet cell plasticity. Here, to investigate the role of these two enzymes in α - and δ -cell development and fate maintenance, we genetically inactivated them in each of these two cell types. We found that loss of *Dnmt1* does not enhance the conversion of α - or δ -cells toward a β -like fate. In addition, while *Dnmt1* was dispensable for the development of these two cell types, we noticed a gradual loss of α -, but not δ -cells in adult mice. Finally, we found that *Ezh2* inactivation does not enhance α -cell plasticity, and, contrary to what is observed in β -cells, does not impair α -cell proliferation. Our results indicate that both *Dnmt1* and *Ezh2* play distinct roles in the different islet cell types.

Keywords

Dnmt1; *Ezh2*; beta-cell regeneration; pancreatic islet; α -cells; δ -cell

Corresponding author: Pedro L. Herrera, Phone: +41 (0)22 379 52 25; pedro.herrera@unige.ch.

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Conflicts of interests. None

1. Introduction

Restoration of functional β -cell mass in type 1 diabetes is a major challenge of regenerative medicine. We have previously shown that after a near-total β -cell ablation in adult mice, a fraction of pancreatic α - and δ -cells reprogram to a β -like phenotype, thus contributing to partial restoration of insulin production (Chera et al., 2014; Thorel et al., 2010). Yet, the genetic and epigenetic mechanisms underlying this direct transdifferentiation remain unclear.

Chromatin modifications contribute to cell fate decisions during development and to fate maintenance in differentiated cells, including in the pancreas (Arnes and Sussel, 2015; Chen and Dent, 2014). Consequently, alteration of chromatin marks can facilitate cell reprogramming (Hochedlinger and Plath, 2009). DNA methyltransferases and Polycomb repressive complexes (PRCs) are two of the best studied chromatin modifiers (Di Croce and Helin, 2013; Smith and Meissner, 2013). In particular, DNA methyltransferase 1 (Dnmt1) propagates DNA methylation patterns during replication (Law and Jacobsen, 2010), whereas the catalytic subunit of PRC2, named Ezh2, tri-methylates histone H3 at lysine 27 (H3K27me3), a modification associated with gene silencing (Margueron and Reinberg, 2011).

Both DNA methylation and Polycomb-mediated gene silencing play critical roles in pancreas development and β -cell function. For instance, *Dnmt1* inactivation in pancreatic progenitors impairs their survival, resulting in pancreatic hypoplasia (Georgia et al., 2013), and *de novo* DNA methylation by Dnmt3a is important for functional β -cell maturation (Dhawan et al., 2015). Polycomb group proteins play multiple roles throughout pancreas development. In foregut endoderm, Ezh2 promotes hepatic over pancreatic fate through selective silencing of pancreas-specific genes (Xu et al., 2011). Pro-endocrine genes exhibit repressive H3K27me3 marks in pancreatic progenitors. Consequently, Ezh2 inactivation at this stage results in increased number of Ngn3⁺ endocrine progenitors, and subsequent expansion of the endocrine cell mass (Xu et al., 2014). In adult β -cells, age-dependent decline in Ezh2 expression leads to derepression of the cell cycle inhibitors p16^{Ink4a} and p19^{Arf}, thereby limiting the proliferation of aged β -cells (Chen et al., 2011; Chen et al., 2009; Dhawan et al., 2009; Krishnamurthy et al., 2006; Zhou et al., 2013). However, the role of Dnmt1 and Ezh2 in the development and maturation of glucagon-producing α -cells and somatostatin-producing δ -cells has not been studied *in vivo*.

In addition to their role in pancreas development, both Dnmt1 and Ezh2 have been associated with cell type conversion in the endocrine pancreas. In particular, *Dnmt1* inactivation in fetal mouse β -cells causes derepression of *Arx*, a master regulator of the α -cell program. This results in β -to- α cell conversion, with around 35% of β -cells expressing glucagon in 8-month-old animals (Dhawan et al., 2011). Whether the reverse conversion can occur upon inactivation of *Dnmt1* in α -cells is yet unknown. On the other hand, several genes essential for β -cell development and function, such as the transcription factors Pdx1 and MafA, exhibit bivalent activating (H3K4me3) and repressing (H3K27me3) histone marks in human α -cells. Remarkably, treating human islets with a histone methyltransferase inhibitor decreased H3K27me3 enrichment at the Pdx1 locus, leading to induction of Pdx1

and the appearance of bihormonal cells (Bramswig et al., 2013). As *Ezh2* is responsible for H3K27me3 deposition, inactivation of this protein in α -cells may lead to derepression of β -cell-specific genes, and thus facilitate α -cell conversion toward a β -cell fate. We thus hypothesized that combining *Ezh2* or *Dnmt1* inactivation with β -cell ablation, which induces the expression of β -cell-specific transcription factors in a subset of α -cells (Thorel et al., 2010), may enhance β -cell regeneration via reprogramming of other islet cell types.

To examine the role of *Dnmt1* in α - and δ -cell development and plasticity, we generated transgenic mice in which we can lineage-trace α - or δ -cells and inactivate *Dnmt1*, as well as induce massive β -cell ablation. We then took a similar approach to determine if loss of *Ezh2* could foster α -to- β cell conversion.

2. Material and Methods

2.1. Mice

RIP-DTR (Thorel et al., 2010), *Glucagon-rtTA* (Thorel et al., 2010), *TetO-Cre* (Perl et al., 2002), *Somatostatin-Cre* (Chera et al., 2014), *R26-YFP* (Srinivas et al., 2001), *Dnmt1^{fl/fl}* (Jackson-Grusby et al., 2001), and *Ezh2^{fl/fl}* (Su et al., 2003) transgenic animals were previously described. Both males and females were used for experiments. Mice were housed in 12h light/dark cycles with ad libitum access to standard chow and water. They were cared for and treated in accordance with the guidelines of the Direction Générale de la Santé, state of Geneva (license number GE/103/14).

2.2. Diphtheria toxin (DT) and Doxycycline (Dox) treatments

For β -cell ablation, DT (D0564; Sigma, St. Louis, MO) was injected i.p. in 10-week-old *RIP-DTR* mice (on days 0, 3, and 4). Each of the three injections consisted of 125 ng DT diluted in 200 μ l NaCl 0.9%. For rtTA-mediated induction of Cre recombinase in α -cells, Dox (D9891; Sigma) was added to the drinking water of breeding cages at a concentration of 1 mg/ml.

2.3. Glycemia monitoring and insulin administration

After β -cell ablation, glycemia was measured from tail-tip blood using a handheld glucometer. Diabetic animals were implanted on average every 4 weeks with a subcutaneous insulin pellet (Linbit; LinShin Canada Inc., Canada).

2.4. Immunofluorescence

Following euthanasia, collected pancreata were fixed 1h30 in cold 4% paraformaldehyde, washed in PBS, and incubated overnight in a 30% sucrose solution. After embedding in OCT compound (Sakura Finetek, Netherlands), pancreata were cut into 10 μ m sections. Immunostaining was performed as described (Desgraz and Herrera, 2009). Primary antibodies were: guinea pig anti-insulin (1:400; Dako, Denmark); chicken anti-insulin (1:750; Sigma); mouse anti-glucagon (1:1000; Sigma); rabbit anti-somatostatin (1:200; Dako); mouse anti-somatostatin (1:200, Novo Nordisk, Denmark); rabbit anti-GFP (1:300; Molecular Probes Inc., Eugene, OR); and chicken anti-GFP (1:200; Abcam, UK). For fluorescent detection, secondary antibodies were coupled to Alexa Fluor dyes 488, 568, or

647 (1:500; Molecular Probes Inc.); or to FITC, Cy3, or Cy5 (1:500; Jackson ImmunoResearch, West Grove, PA). Images were acquired on a confocal microscope (TCS SPE; Leica Microsystems, Germany). Immunomorphometry: the number of islet sections and cells counted in each experiment is indicated in **Suppl. Table 1**.

2.5. Islet isolation and cell sorting

Islet isolation was performed as described (Strom et al., 2007). In brief, pancreata were perfused with a collagenase solution, dissected, and incubated for 15' in 37°C collagenase. Islets were purified on a Histopaque density gradient and dissociated in a trypsin solution to generate a single-cell suspension. Lineage-traced α - or δ -cells were sorted based on YFP fluorescence on a Moflo Astrios (Beckman Coulter, Brea, CA) or a S3e (Bio-Rad, Hercules, CA) cell sorter.

2.6. Nucleic acid extraction

RNA from purified α - or δ -cells was extracted with the AllPrep DNA/RNA Micro Kit (Qiagen, Germany).

2.7. Reverse transcription and quantitative PCR

For RNA isolated from α *Dnmt1* and δ *Dnmt1* cells, reverse transcription and pre-amplification were performed using the RT² first strand cDNA synthesis kit and a custom PreAmp primer mix (Qiagen), according to the manufacturer's instructions. qPCR reactions were completed on a RotorGene 6000 cycler (Corbett) using a custom RT² PCR array and the RT² SYBR Green ROX FAST mastermix (Qiagen). Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*), β -actin (*Actb*), Beta-glucuronidase (*Gusb*), and Cyclophilin A (*Ppia*) were used for normalization. Data analysis was done as described (Thorel et al., 2010). The complete list of genes in the custom array is available in **Suppl. Table 2**. The expression of mouse endogenous retroviruses was measured using the same primer pairs as in (Sharif et al., 2016). RNA isolated from α *Ezh2* cells was reverse-transcribed using the QuantiTect RT kit (Qiagen). qPCR reactions and analyses were performed as described (Thorel et al., 2010). *Ppia* and *Gapdh* were used for normalization. Primers sequences are listed in **Suppl. Table 3**.

2.8. Statistical analyses

Data are presented as mean $\pm$ standard error. N in figure legends indicates the number of mice per experimental group. The data points in scatter/bar plots each represent an individual mouse. *P* values were calculated with GraphPad Prism 6 (GraphPadSoftware, La Jolla, CA). For two-sample comparisons, unpaired, two-tailed Student's *t*-test with Welch's correction was used. For multiple sample comparisons, multiple *t*-tests with Holm-Sidak post-hoc correction were used.

3. Results

3.1. Dnmt1 inactivation in fetal α -cells leads to a gradual α -cell loss in adult mice

To determine if the lack of *Dnmt1* alters α -cell development or plasticity, we generated *Glucagon-rtTA;TetO-Cre;Rosa26-YFP,Dnmt1^{fl/fl}* (*α Dnmt1^{fl/fl}*) mice (**Figure 1A**). This set of transgenes allows a doxycycline (Dox)-inducible lineage tracing and *Dnmt1* inactivation in mouse α -cells. Briefly, upon Dox exposure, the reverse tet Transactivator (rtTA) expressed in glucagon-producing α -cells binds to the TetO promoter and activates Cre recombinase; Cre in turn recombines the floxed *Dnmt1* alleles and simultaneously activates transgenic YFP expression from the *Rosa26* locus, leading to irreversible α -cell labeling and *Dnmt1* inactivation in α -cells (*α Dnmt1^{-/-}*).

To inactivate *Dnmt1* in embryonic α -cells, we added Dox to the drinking water of breeding cages for the whole developmental period (from conception to weaning) (**Figure 1B**). We observed efficient α -cell labeling in pups from Dox-treated cages: over 90% of glucagon-expressing cells were also YFP-positive in both *α Dnmt1^{-/-}* animals and *α Dnmt1^{+/+}* controls (**Suppl. Fig. 1A-B**). We noticed that 4 to 7 % of YFP⁺ cells co-expressed insulin (**Suppl. Fig. 1C**). These YFP⁺Ins⁺ cells, which were always glucagon-negative, may derive from the few cells that co-express insulin and glucagon during development (Alpert et al., 1988; Teitelman et al., 1993). To confirm loss of *Dnmt1* in α -cells, we measured *Dnmt1* expression in YFP⁺ cells sorted from *α Dnmt1^{-/-}* animals: *Dnmt1* expression was reduced by 75% as compared to YFP⁺ cells from *α Dnmt1^{+/+}* controls (**Suppl. Fig. 1D**). To confirm that *Dnmt1* inactivation induces a loss of DNA methylation, we measured the expression of CpG-rich Intracisternal A particle (IAP) endogenous retroviruses, which are known to be silenced by DNA methylation (Walsh et al., 1998). Consistent with a recent report (Sharif et al., 2016), the loss of *Dnmt1* led to derepression of IAPs, but not of other LTR and Non-LTR retrotransposons, in YFP⁺ *α Dnmt1^{-/-}* cells (**Suppl. Fig. 1E**). These data indicate that Dox administration leads to efficient YFP-labeling, *Dnmt1* deletion, and loss of DNA methylation in the α -cells of *α Dnmt1^{-/-}* mice.

We observed a similar number of glucagon-expressing cells in 1-m-o (month-old) *α Dnmt1^{-/-}* animals and *α Dnmt1^{+/+}* controls, indicating that *Dnmt1* is not required for generating a proper number of α -cells during development. Comparison of pancreatic sections from 1-, 4-, and 12-m-o *α Dnmt1^{-/-}* mice, however, revealed that *Dnmt1* deletion in fetal α -cells leads to a gradual loss of α -cells in adult mice: the number of α -cells was 5-fold lower in 12-m-o *α Dnmt1^{-/-}* mice than in age-matched *α Dnmt1^{+/+}* controls (**Figure 1C-D**). We did not observe a compensatory expansion of α -cells that had escaped recombination (Gcg⁺YFP⁻), so that the total number of glucagon-positive cells was also 5-fold reduced in 12-m-o *α Dnmt1^{-/-}* animals (**Figure 1E**). This is consistent with the finding that a massive α -cell loss is not sufficient to trigger hyperplasia of the surviving α -cells (Thorel et al., 2011).

To determine if the lack of *Dnmt1* alters α -cell maturation or fate maintenance, we compared the expression of α -cell markers in YFP⁺ cells sorted from 3-m-o *α Dnmt1^{+/+}* and *α Dnmt1^{-/-}* animals, but did not detect any significant changes (**Figure 1F**). In 12-m-o *α Dnmt1^{-/-}* mice, however, we observed that some YFP⁺ cells had lost glucagon expression,

suggesting that a subset of surviving α -cells may undergo dedifferentiation (**Figure 1C, G**). These YFP⁺Gcg⁻ cells did not express insulin or somatostatin (not shown). Together, these findings indicate that *Dnmt1* is dispensable for α -cell development and maturation. However, and surprisingly, *Dnmt1* activity in embryonic α -cells is required for α -cell maintenance in adults, and has an impact on α -cell survival in the long term, later in life.

3.2. Loss of *Dnmt1* in embryonic δ -cells neither affects δ -cell differentiation nor survival

We sought to determine whether *Dnmt1* loss causes a similar phenotype in δ -cells. To this aim, we generated *Somatostatin-Cre; Rosa26-YFP; Dnmt1^{fl/fl}* mice, in which YFP-labeling and *Dnmt1* deletion constitutively occur in somatostatin-expressing cells (**Figure 2A-B**). In $\delta Dnmt1^{-/-}$ animals, we detected YFP expression in 95% of pancreatic somatostatin-producing cells (**Suppl. Fig. 2A-C**). In comparison with $\delta Dnmt1^{+/+}$ control cells, YFP⁺ cells from $\delta Dnmt1^{-/-}$ animals exhibited a 60% downregulation of *Dnmt1* gene expression and a 3-fold upregulation of IAP retroelements, reflecting a loss of DNA methylation (**Suppl. Fig. 2D-E**).

At 4 and 12 months of age, the number of δ -cells per islet section was similar in $\delta Dnmt1^{-/-}$ mice and age-matched controls (**Figure 2C-E**). Moreover, YFP⁺ cells maintained somatostatin expression, at least until 12 months of age (**Figure 2F**). At the transcriptional level, the expression of *Sst* and *Hhex*, a transcription factor essential for maintenance of δ -cell differentiation (Zhang et al., 2014), was similar in YFP⁺ cells sorted from $\delta Dnmt1^{+/+}$ and $\delta Dnmt1^{-/-}$ mice (**Figure 2G**). These results suggest that *Dnmt1* inactivation does not impair development, maturation, or survival of δ -cells. Thus, *Dnmt1* activity in the developing pancreas is required for long-term survival of α - but not δ -cells.

3.3. Lack of *Dnmt1* causes p21 derepression in both α - and δ -cells, but *Bcl-2* downregulation only in α -cells

Global genome demethylation consecutive to loss of *Dnmt1* causes DNA damage, cell cycle arrest and apoptosis, often in a p53-dependent manner (Chen et al., 2007; Elliott et al., 2015; Georgia et al., 2013; Liao et al., 2015). To gain insight into the mechanisms that lead to loss of α - but not δ -cells after *Dnmt1* inactivation, we performed RT-qPCRs to measure the expression of genes involved in these processes in YFP⁺ cells isolated from $\alpha Dnmt1$ and $\delta Dnmt1$ mice.

Globally, we observed very few significantly regulated genes when comparing *Dnmt1* knockout cells to control cells. Because islet cells proliferate slowly and asynchronously, we can expect a large heterogeneity in *Dnmt1*-dependent loss of DNA methylation, which complicates the detection of changes in gene expression. Nevertheless, we observed upregulation of the cell cycle inhibitor p21 (*Cdkn1a*) in both $\alpha Dnmt1^{-/-}$ and $\delta Dnmt1^{-/-}$ cells (**Figure 3A-B**). The expression of other p53 target genes and cell cycle genes was not significantly altered (**Figure 3A-B** and data not shown). By contrast, the anti-apoptotic gene *Bcl-2* was downregulated in knockout α -, but not δ -cells. Thus, decreased *Bcl-2* expression may contribute to α -cell death after loss of *Dnmt1*.

3.4. Loss of *Dnmt1* does not enhance α - or δ -cell reprogramming after massive β -cell ablation

We previously reported that after a massive β -cell loss, a fraction of adult α - and δ -cells adopt a β -like fate: these cells start to produce insulin, thereby contributing to β -cell regeneration and partial diabetes recovery (Chera et al., 2014; Thorel et al., 2010). We speculated that some β -cell-specific genes may be silenced by DNA methylation in α - and/or δ -cells. Consequently, *Dnmt1* deletion combined with β -cell ablation may lead to depression of these genes and facilitate α -to- β and δ -to- β cell reprogramming. To test this hypothesis, we generated *α DNMT1^{fl/fl}* and *δ Dnmt1^{fl/fl}* mice bearing in addition the *RIP-DTR* transgene. In these animals, the human diphtheria toxin receptor (DTR) is expressed on β -cell surface, so that DT administration triggers a near-total (> 99%) β -cell ablation, resulting in severe diabetes (Thorel et al., 2010).

We injected DT in young adult *α DNMT1^{fl/fl}* and *δ Dnmt1^{fl/fl}* mice and analyzed the pancreata 7 weeks and 10 months later to measure the rate of α -to- β and δ -to- β cell conversion, that is the percentage of YFP-labeled α -, respectively δ -, cells that started to express insulin during this period (**Figure 4A**). The rate of α -to- β conversion was similar in *α Dnmt1^{fl/fl}* and *α Dnmt1^{+/+}* animals (**Figure 4B, D**). Similarly, δ -to- β cell conversion occurred at a comparable frequency in *δ Dnmt1^{fl/fl}* and *δ Dnmt1^{+/+}* mice (**Figure 4C, E**). Finally, the lack of *Dnmt1* did not cause an upregulation of β -cell genes in YFP⁺ cells sorted from *α Dnmt1* and *δ Dnmt1* animals (**Figure 4G-H**). Together, these results indicate that the lack of *Dnmt1* does not enhance α - or δ -cell plasticity toward a β -like fate.

3.5. Normal α -cell number and unaltered α -cell fate after *Ezh2* inactivation

Ezh2, the catalytic enzyme of the PRC2 complex, catalyzes H3K27 trimethylation, a histone mark that was shown to contribute to the repression of β -cell genes in human α -cells (Bramswig et al., 2013). To investigate the role of *Ezh2* in α -cell development and plasticity, we generated *RIP-DTR;Glucagon-rtTA;TetO-Cre;Rosa26-YFP,Ezh2^{fl/fl}* mice. Using a similar strategy as for *α Dnmt1* animals, we administered Dox through drinking water of breeding cages to induce irreversible YFP-labeling and *Ezh2* deletion in developing α -cells (*α Ezh2^{fl/fl}*) (**Figure 5A**).

α -Cell labeling was efficient in mice born from Dox-treated females with over 87% of Gcg+ cells co-expressing YFP (**Figure 5B**). We observed a nine-fold downregulation of *Ezh2* in YFP⁺ cells sorted from *α Ezh2^{fl/fl}* animals as compared with YFP⁺ cells from *α Ezh2^{+/+}* controls; expression levels of *Ezh1*, which can replace *Ezh2* in the PRC2 complex, were unchanged (**Figure 5C**). Despite efficient *Ezh2* inactivation, the number of α -cells per islet section was similar in adult *α Ezh2^{+/+}* and *α Ezh2^{fl/fl}* mice (**Figure 5D**). This suggests that, contrary to what is observed for β -cells (Chen et al., 2009), *Ezh2* is not needed for the generation of an adequate α -cell number in adult mice.

We then sought to determine whether loss of *Ezh2* enhances α -cell reprogramming towards insulin production. After *Ezh2* inactivation, YFP-labeled α -cells maintained glucagon expression (**Figure 5E**), and mRNA levels of glucagon and the main determinants of the α -cell programme, namely *Arx* and *MafB*, were not affected (**Figure 5F**). In addition, *Ezh2*

loss did not trigger an upregulation of insulin or of transcription factors important for β -cell differentiation and function, such as Pdx1 or Nkx6.1. Finally, to further explore the plasticity of α -cells lacking *Ezh2*, we treated young adult mice with DT in order to kill β -cells. After six weeks of regeneration, the percentage of α -cells that had started to express insulin (YFP⁺Ins⁺) was similar in *α Ezh2*^{+/+} and *α Ezh2*^{-/-} diabetic animals (Figure 5G-H).

Together, these results indicate that *Ezh2* inactivation does not promote the conversion of α -cells toward a β -like phenotype.

4. Discussion

Although inactivation of *Dnmt1* or *Dnmt3a* in β -cells triggers a β -to- α cell reprogramming (Dhawan et al., 2011; Zhang et al., 2014), we did not observe the opposite cell fate change after loss of this enzyme in α - or δ -cells, even after massive β -cell ablation, a context known to enhance α - and δ -cell plasticity (Chera et al., 2014; Thorel et al., 2010). Although the number of animals was limited in these experiments, due to their complex genotype and to the difficulty of keeping them alive with severe diabetes for long periods of time, the rate of α -to- β and δ -to- β conversion remained below 3% in all mice. Other mechanisms must therefore prevent α - and δ -cells from losing their fate. For instance, it is possible that *Arx*, the re-expression of which is central in driving β -to- α cell conversion, is sufficient to maintain the α -cell phenotype despite the lack of *Dnmt1*. *Hhex* might play an analogous role in δ -cells. Similarly, *Ezh2* inactivation did not enhance α -to- β cell conversion after near-total β -cell destruction. Previously, treatment with the histone methyltransferase inhibitor Adox was shown to induce the appearance of glucagon-insulin bihormonal cells, presumably through removal of the H3K27me3 repressive histone marks on β -cell genes (Bramswig et al., 2013). As Adox is not specific for *Ezh2*, inhibition of other histone methyltransferases may have played a role in this process. Alternatively, as the Adox experiments were performed *in vitro*, it is possible that α -cell differentiation can be reverted more easily in this context than *in vivo*.

We observed a gradual loss of α -, but not δ -cells in adult mice after *Dnmt1* inactivation. As *Dnmt1* is a maintenance methyltransferase, several rounds of replication are needed to dilute methylated cytosines in cells lacking this enzyme. Loss of *Dnmt1* in fast cycling cells was shown to cause global DNA demethylation, cell cycle arrest and death (Chen et al., 2007; Liao et al., 2015). This was also observed in pancreatic progenitors where inactivation of *Dnmt1* caused a rapid apoptosis, resulting in pancreatic aplasia (Georgia et al., 2013). By contrast, islet cells divide very slowly, even during development (Desgraz and Herrera, 2009), therefore derepression of silenced genes is expected to take longer in these cells. In support of this hypothesis, *Arx* derepression and β -to- α conversion was not observed before 8 months of age after inactivation of *Dnmt1* in fetal β -cells (Dhawan et al., 2011). It is also possible that *Dnmt3a* and *Dnmt3b* can partially compensate for the absence of *Dnmt1* in young α -cells, which would explain why we started to observe a reduction in α -cell number only after 1 month of age.

Why then did we not observe any phenotype after *Dnmt1* deletion in δ -cells? While the number of α -cells per islet section increased by 20% between 4 and 12 months of age, we did not detect any increase in the number of δ -cells during this period. A lower rate of

replication in adult δ -cells may thus lead to a slower removal of methylated cytosines than in α -cells. It is also possible that Bcl-2 downregulation in *α Dnmt1*^{-/-} cells contributes to α -cell apoptosis.

Interestingly, inactivation of *Dnmt1* in fetal α -cells or *Ezh2* in fetal β -cells do not cause a developmental phenotype but result in a postnatal reduction in α - and β -cell numbers, respectively (Chen et al., 2009). *Ezh2* silences the cell cycle inhibitors p16^{Ink4a} and p19^{Arf} in young β -cells (Chen et al., 2011; Chen et al., 2009; Dhawan et al., 2009). As a consequence, *Ezh2* inactivation in embryonic β -cells leads to derepression of p16 and p19, resulting in a reduced β -cell mass (Chen et al., 2009). By contrast, we did not observe any reduction in α -cell number after loss of *Ezh2* in α -cells. This suggests that different mechanisms regulate the proliferation of α - and β -cells. In support of this idea, it was shown that preventing p16-mediated Cdk4 inhibition results in β -cell hyperplasia but does not affect α - or δ -cells (Rane et al., 1999). Similarly, constitutive Cdk4 deletion leads to a severe reduction in β -cell mass, but not in α - or δ -cell mass. Therefore, the *Ezh2*-p16-Cdk4 axis is important for β -cell mass regulation but does not seem as crucial for controlling the α -cell number. Because most studies have focused on β -cell proliferation, the molecular mechanisms that govern α - and δ -cell proliferation are largely unknown. Our results support the idea that major differences exist in this area between islet cell types.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Cre	Cre recombinase
Dnmt1	DNA methyltransferase 1
Dox	Doxycycline
DT	Diphtheria toxin
DTR	Diphtheria toxin receptor
Ezh2	Enhancer of zeste homolog 2
Gcg	Glucagon
IAP	Intracisternal A-particle

Ins	Insulin
m-o	month-old
PRC	Polycomb repressive complex
RIP	Rat insulin promoter
rtTA	Reverse tetracyclin transactivator
Sst	Somatostatin
TetO	Tet operator sequence
YFP	Yellow fluorescent protein

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Highlights

- *Dnmt1* is dispensable for pancreatic α - and δ -cell development and maturation
- Inactivation of *Dnmt1* in fetal α -cells leads to a gradual α -cell loss later in life
- Loss of *Dnmt1* does not enhance α - or δ -cell plasticity after massive β -cell death
- *Ezh2* is dispensable for α -cell development, fate maintenance and survival

FIGURE 1. *Dnmt1* inactivation in fetal α -cells leads to a gradual α -cell loss in adult mice

A. List of transgenes for Dox-inducible and irreversible α -cell-specific labeling and *Dnmt1* inactivation. Red triangles represent loxP sites. **B.** Experimental timeline. **C.** Left: islet sections from 12-m-o mice showing a reduction in YFP-labeled α -cells (yellow arrowheads) in *αDnmt1*^{-/-} mice as compared to *αDnmt1*^{+/+} controls. Right: higher magnification of the areas in white boxes show loss of glucagon-expression in a fraction of YFP⁺ cells from *αDnmt1*^{-/-} mice. Scale bars: 25 μ m. **D-E.** Number of YFP⁺Ins⁻ (D) and Gcg⁺ (E) cells per islet section in *αDnmt1* mice. **F.** Expression levels of α -cell markers in YFP⁺ cells FACS-sorted from 3-m-o *αDnmt1*^{+/+} and *αDnmt1*^{-/-} animals (N=4-5 mice per group). **G.** Percentage of YFP-labeled α -cells that co-express glucagon in 1-, 4- and 12-m-o *αDnmt1*^{+/+} and *αDnmt1*^{-/-} mice. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$; t -test with Welch's correction.

FIGURE 2. Loss of *Dnmt1* in embryonic δ -cells does not affect δ -cell differentiation or survival
A. List of transgenes for irreversible δ -cell-specific labeling and *Dnmt1* inactivation. Red triangles represent loxP sites. **B.** Experimental timeline. **C.** Islet sections from 12-m-o. δ Dnmt1 mice. Areas indicated by white boxes are shown at higher magnification on the right. Scale bars: 25 μ m. **D-E.** Number of YFP+Ins⁻ (D) and Sst⁺ (E) cells per islet section in δ Dnmt1 animals. **F.** Percentage of YFP-labeled cells that co-express somatostatin. **G.** Expression levels of the δ -cell markers somatostatin and Hhex in YFP⁺ cells sorted from 3-m-o δ Dnmt1 mice (N=4-5 mice per group). n.s., not significant ($P>0.05$); t -test with Welch's correction.

Figure 3: Expression of genes related to apoptosis, cell cycle and p53 pathway

FIGURE 3. Lack of Dnmt1 causes p21 derepression in both α - and δ -cells, but Bcl-2 downregulation only in α -cells

A-B. Gene expression levels in YFP⁺ cells FACS-sorted from 3-m-o *aDnmt1* (A) and *δDnmt1* (B) mice (N=4-5 mice per group). *, $P < 0.05$; multiple *t*-tests with Holm-Sidak post-hoc correction.

FIGURE 4. Loss of *Dnmt1* does not enhance α - or δ -cell reprogramming after massive β -cell ablation

A. Experimental timeline. **B-C.** Islet from α *Dnmt1* Δ/Δ (B) and δ *Dnmt1* Δ/Δ (C) mice after β -cell ablation. Split channels are shown on the right to highlight co-expression of YFP and insulin in cells indicated by yellow arrowheads. Scale bars: 15 μ m. **D-E.** Percentage of YFP-labeled cells that co-express insulin in DT-treated α *Dnmt1* (D) and δ *Dnmt1* (E) animals. **F-G.** Expression levels of β -cell markers in YFP⁺ cells sorted from 4-mo α *Dnmt1* (F) and δ *Dnmt1* (G) mice (N=4-5 mice per group).

FIGURE 5. Normal α -cell number and unaltered α -cell fate after *Ezh2* inactivation
A. Experimental timeline. **B.** Efficiency of labeling in *αEzh2* animals. **C.** *Ezh2* and *Ezh1* gene expression in YFP+ cells sorted from 4-m-o *αEzh2* mice (N=4-5 mice per group). **D-E.** Number of YFP-labeled α -cells per islet section (D) and percentage of YFP+ cells that co-express glucagon (E) in non-ablated 4-m-o *αEzh2* mice. **F.** Expression levels of α - and β -cell markers in YFP+ cells FACS-sorted from non-ablated *αEzh2* animals (N=4-5 mice per group). **G.** Islet sections from 4-m-o DT-treated *αEzh2* mice. Areas in white boxes are shown at higher magnification on the right. Yellow arrowheads indicate YFP+Ins+ cells. Scale bars: 15 μ m. **H.** Percentage of reprogrammed (Ins+YFP+) α -cells in DT-treated *αEzh2* mice. n.s., not significant ($P>0.05$); t -test with Welch's correction.