Dynamics of Genomic H3K27me3 Domains and Role of EZH2 during Pancreatic Endocrine Specification

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Editor: Thomas Schwarz-Romond

1st Editorial Decision 02 May 2014

Thank you very much for the opportunity to consider your paper on the dynamics of genomic H327me3 also addressing the role of EZH2 in endocrine specification for publication in The EMBO Journal.

The attached comments from three scientists reveal overall merits and definitive interest in these findings. Particularly refs #1 and #3 offer constructive critiques as to more clearly link the presented H3K27me3-dynamics to the functional role of EZH2. It is thus encouraged to integrate your better temporal resolution into conceptually similar, related observations.

Unique and most relevant point of your study is the characterization of EZH2 as executing histone-methyltransferase that would benefit from stronger functional corroboration. While at this stage NOT insisting on employing the inducible PdxCreERT, I kindly ask to attend to ref#3 comments as we would involve this scientist in the assessment of your revised study.

Overall, I am happy to invite a revised manuscript for further consideration at The EMBO Journal.
Please note that we only allow one round of major revisions and the final decision will be reached based on further evaluation from some of the original referees.

Please do not hesitate to get in touch in case I can be of any assistance/you would like to discuss feasibility, amount and timeline of some of the requested experiments.

I am very much looking forward to engage as to facilitate eventual publication of your study at The EMBO Journal and remain with best regards.

REFEREE REPORTS:

Referee #1:

This study examines the dynamics of H3K27me3 during cell fate transitions from endoderm to Pdx1+ pancreatic endoderm to endocrine progenitors. They report that endocrine progenitors undergo a global reduction in H3K27me3 during differentiation, whereas there is a progressive increase in H3K27me3 domains throughout this process (opposite to studies carried out with ESC differentiation protocols). They also show that there is specifically loss of H3K27me3 in endocrine progenitor transcription factors. They then go on to show that the pancreatic Ezh2 KO embryos show increased formation of Ngn3 cells from trunk cells, although there is subsequent glucose intolerance, presumably because of previously described derepression of Cdkn2a. Enhanced endocrine differentiation is recapped by chemical inhibitors. They then show that transient exposure to these chemical inhibitors can also promote endocrine differentiation in human in vitro pancreatic differentiation models. This is an important study that demonstrates a role for EZH2, potentially through one of its repressive function as a H3K27me3 methyltransferase. The findings are significant because of its potential implications for efforts to generate therapeutic beta cells. There are several issues, however, that clearly need to be addressed:

1. The chromatin profiling section is arguably the least exciting. Some parts are quite descriptive and incremental or not entirely novel. Furthermore, at least as written, it does not lead to an easy explanation of the EZH inhibition phenotype. In general I suggest to digest this portion so as to avoid distracting too much attention from the EZH experiments. The title should be modified accordingly. More specific points on this section:

1a. The authors represent the data as Input subtracted from ChIP. This is of course valid, but for some reason the examples shown in Figure 3 appear to depict very scattered reads separated by empty spaces. This is not a typical H3K27me3 profile. Given that the authors have sequenced quite deep, what is the explanation for this?

1b. The authors show that there is selective loss of H3K27me3 in endocrine transcription factor genes such as Ngn3, Neurod1, etc between Pdx1-expressing progenitors and endocrine cells. This is in line with the main conclusion of van Arensbergen et al, 2010. Hypothetically this explains why EZH2 promotes differentiation, given that it prevents repression of endocrine regulators in trunk progenitors. The GO analysis of genes that gained H3K27me3 is also similar to that reported. Although the current study provides greater temporal resolution, it would make sense to discuss this data in light of earlier similar findings.

On the other hand, the finding that Ezh2 inhibits endocrine differentiation is very interesting, but it does not necessarily follow from the chromatin maps as written. As mentioned, it seems that Ezh2 controls the release of repression in endocrine transcription factor genes in trunk cells. However, during endocrine differentiation there is also increased H3K27me3 at progenitor transcription factors like Hnf6. Furthermore, there is an overall increase in H3K27me3-enriched regions during differentiation. It seems necessary to rationalize all of this more clearly.
1. The increased number of H3K27me3 peaks in EPs is likely to represent a true increase, but it could also reflect a better yield of the ChIP, given that the authors have understandably used a suboptimal normal of cells (as few as 50 thousand) and no replicates. What indications do the authors have that this is not technical?

2. Page 11. "In both heterozygous and homozygous floxed Ezh2 embryos, the Ngn3 gene is transcriptionally up-regulated in Ngn3-GFP+ cells, compared to Ezh2 wild type embryos" What is the interpretation of this finding? One might expect a difference in timing or spatial activation of the Ngn3 gene, or simply increased number of cells, but not an increase in expression in purified cells, because presumably H3K27me3 does not repress the Ngn3 gene in these cells.

3. There is a general lack of statistical analysis in several figures.

Fig. 6B needs statistical analysis Furthermore, the analysis shows % of GFP cells, but it should clarify whether the % of NGN3 GFP cells has increased, or if there has been a reduction in non-NGN3 GFP cells.

Glucose tolerance analysis also needs statistical analysis and described in detail.

4. Explant experiments need to be described in detail in the methods section.

5. Figure E1. The validation experiment for the antibody is not great, for all we know this could be recognizing unmethylated H3K27.

6. ChiP-seq protocol. Given that such small amounts of cells were used, it would be useful to provide further details on amounts of DNA used for library construction, stating any modifications of library construction protocol, and providing information on reads/non-clonal reads.

Minor points

1. Intro: "The early differentiated PDX1+ cells are multipotent progenitors that can give rise to ductal cells, acinar cells, and endocrine progenitors (NGN3+) starting from E9.5" It sounds like the authors wish to say that duct and acinar cells become visible as soon as e9.5, I am not sure that this is what the authors wish to convey.

2. Methods. "H3K27me3 peaks were called using software for wide patches of chromatin from the Broad Institute (Guttman et al, 2009)" Does this software have a name, or have the authors repurposed scattered scripts?

3. Figure S5 should correct the allele nomenclature, presumably the Pdx1-Cre mice are also Pdx1+/+ since the are probably not knock-ins. Incidentally it seems that the methods section does not describe the transgenic Pdx1-Cre line.

Referee #2:

Xu et al "Dynamics of Genomic H3K27me3 Domains and Role of EZH2 during Pancreatic Endocrine Specification" assesses changes in the enrichment of H3K27me3 during development and
use the loss of Ezh2 to analyze the dynamics of H3K27me3 enrichment during development and differentiation.

The analysis of enrichment of H3K27me3 during development is important in understanding how to direct ES cells towards beta cells. This study shows that directed ES cells differentiation is quite different from the differentiation in vivo and perhaps points us towards why directed differentiation of ES cells in vitro has been so unsuccessful in generating functional beta cells.

Here are a few points to address

1. It is not clear why Nkx6.1 H3K27me3 enrichment is maintained in pancreatic progenitors and lost only at the endocrine differentiation. This does not match the expression pattern observed during development as Nkx6.1 is expressed throughout the pancreatic progenitors cells similar to Pdx1 and then enriched in endocrine cells fated to become beta cells. Are there different elements directing this expression pattern and can H3k27me3 patterns distinguish these elements?

2. It is not clear to me where the enhanced Ngn3 cells are originating in the Ezh2 f/f Pdx1 cre mice? This needs to investigated and explained better.

3. Increased beta cell area is somewhat subjective and beta cell mass is a better assessment

4. better glucose tolerance in Pdx1-cre Ezh2 f/+ can be due to loss of glucose responsiveness. GSIS of isolated islets needs to be carried out.

5. Further data analysis that correlate repression or derepression with H3k27me3 binding and how this differs from ES cells would helpful.

Referee #3:

The authors characterize histone methylation during development of the mouse pancreas using a low amount of material. Although genome-wide data are inherently difficult to present in a reader-friendly way, they show that pancreatic transcription factors have lower methylation levels. This is not surprising, as the authors state themselves. Using Ezh2 conditional knockout and chemical inhibition, the authors show that the number of ngn3 positive cells increase, and also islet-cell mass. They also show some data supporting the notion that careful timing of ezh2 inhibition might be applicable to improve generation of human beta cells from ES cells. Overall, the work is well conducted, but in the criticism described below, the conclusions are often not solid enough to warrant publication.

Major points:

ChIP data showing a relief of H3K27me3 marks at the Ngn3 locus in the EZH2 deletion background are not presented. I realize that the authors showed that Ngn3 is demethylated by e14.5, but I imagine the methylation marks would be significantly decreased on Ngn3 and other transcription factors at an earlier age compared to WT. what is the effect of ezh2 het or homozygous ko at the pdx1 stage on the epigenetics of the H3K9me3 marks? there is also no methylation analysis upon use of the inhibitors.

Loss of function induces gain of ngn3-gfp. does ezh2 gain of function reduce ngn3-gfp? I think the authors should try to overexpress EZH2 in their explant or iPSC model. It would be interesting to see if the genes promoting beta cell differentiation were silenced and therefore gave rise to fewer beta cells. It appears the homozygous ko does not enhance ngn3 compared to the het knockout. what
Detailed comments.

1. EZH2 suppresses the normal extent of endocrine cell induction
   a. I found it surprising that the authors used the PdxCre instead of an inducible PdxCreER. The Pdx1Cre is on so early (e8.5) that it is likely to have secondary effects
   b. Specifically, the authors wanted to address what controls the methylation changes seen during the transition from pancreatic progenitors to the endocrine progenitor stage. It would therefore have been better to use an inducible Cre and delete EZH2 right before the secondary transition to truly understand the role EZH2 and thus methylation plays in endocrine development.
   c. The authors also found increased Ngn3 RNA in the EZH2 deleted pancreases which they showed was due to an increased number of Ngn3+ low-expressing cells
      i. I think the authors need to show some sort of Ngn3 protein staining here. RNA is good, as is sorting for number of cells, however they never do any antibody staining. They only use the Ngn3-EGFP, which is a transgene. There is a good Ngn3 antibody that I feel would strengthen their data.
   d. The authors need more data to support their claim that extra Ngn3 cells come from the trunks.
      Extended figure 5 is a terrible picture. They should show zoomed in, fluorescent staining of Ngn3, Amylase and Sox9. (They state in the text that they stained for Sox9- where is it?). Also- what age is this picture from?
   e. Figure 5A is also a bad picture. Why not fluorescence and why is the picture so zoomed out? I would like to see a picture of what these islets look like up close and what the insulin staining looks like in the individual endocrine cells. When do the authors start to see more beta cells? During development or only postnatally? Why do they do physiology assays at P9?
   f. There are also no statistics done for Figure 5D. They need AUC or something to claim there is a significant difference in blood glucose when EZH2 is lost. What about serum insulin levels? Was the investigator blinded to the genotype of the mice? This is perhaps the key Figure of the entire manuscript and the highest possible standards of experimentation should be applied.
   g. The authors claim that Ink4A/Arf causes "diabetes" when upregulated in the islets. The authors need further support for this claim. Such as insulin staining corresponding to the age they see up regulation of Ink4/Arf.
      i. Otherwise one could argue that the increase in beta cells seen with the loss of EZH2 were actually dysfunctional to begin with.

2. Histone methyltransferase inhibitors modulate two stages of pancreas differentiation
   a. Figure 6 and Extended figure 6:
      i. One question I had here was that if the loss of EZH2 causes an increase in Pdx1+ cells, then why aren't there more cells in every cell lineage. Pdx1 cells give rise to all pancreatic cell types. Why aren't there more exocrine cells as well? Why isn't the entire pancreas larger?
      ii. What transcription factors are misexpressed or misregulated because of the lack of methylation marks? Do these extra beta cells express markers from other lineages?
   b. Figure 7 B and C need asterisks showing significance
      The authors also never mention Figure 7D in the text. The x-axis shows glucagon but they never mention the lack of double hormone positive cells in the text?
      The increase in c-peptide in human es cells is not that impressive. given the variabilities in this assay, i am not convinced. How many different cell lines were used for this analysis?

The authors say that insight gained in mice can be directly translated to human differentiation. But they also point to differences in the dynamics of the methylation. Is this due to the in vitro differentiation or due to species-specific differences? Did the authors look at mouse ES cells differentiated in vitro? On page 17 top the authors suggest that this is due to in vivo versus in vitro. The discussion is not clear why the authors favor that interpretation.

writing:
"not due to increased cell proliferation or decreased programmed cell."

The authors should make more clear at the start of each chapter/paragraph whether experiments were done on mouse or human cells.
Thank you for your Editorial decision and your specific remarks about how we should change the paper.

The reviewers’ comments are very helpful and I believe we have data to address many of them, and can get more as needed without too much time.

As for the PdxCre we used, those in the field know that it activates significantly later than the endogenous Pdx gene and in fact E10.5, its likely time of first action based on many papers (which we will now cite), is exactly the time we would need to elicit EZH2 deletion and protein decay by a few days later, when endocrine cells normally begin to be specified. In other words, if we used the creER version, we would be pulsing around that time anyway.

We can make these points more clearly in the manuscript, it will certainly help.

With regard to genomics and tag density, our browser views showed only tag mapping points, not the extent to which each full length segment sequenced would, on average, map to the genome (as well as show directionality). We will generate those views which I believe would address this concern.

We also have work in progress to assess beta cell mass at the times and conditions requested; among other additional data to add or reanalyze for the paper.

Thanks again and I hope to be back in touch soon.

Thanks a lot for these reassuring comments. I would be pleased to facilitate rapid publication of an appropriately revised version of your paper.

I look forward to such amendments.
This study examines the dynamics of H3K27me3 during cell fate transitions from endoderm to Pdx1+ pancreatic endoderm to endocrine progenitors. They report that endocrine progenitors undergo a global reduction in H3K27me3 during differentiation, whereas there is a progressive increase in H3K27me3 domains throughout this process (opposite to studies carried out with ESC differentiation protocols). They also show that there is specifically loss of H3K27me3 in endocrine progenitor transcription factors. They then go on to show that the pancreatic Ezh2 KO embryos show increased formation of Ngn3 cells from trunk cells, although there is subsequent glucose intolerance, presumably because of previously described derepression of Cdkn2a. Enhanced endocrine differentiation is recapped by chemical inhibitors. They then show that transient exposure to these chemical inhibitors can also promote endocrine differentiation in human in vitro pancreatic differentiation models. This is an important study that demonstrates a role for EZH2, potentially through one of its repressive function as a H3K27me3 methyltransferase. The findings are significant because of its potential implications for efforts to generate therapeutic beta cells.

There are several issues, however, that clearly need to be addressed:

1. The chromatin profiling section is arguably the least exciting. Some parts are quite descriptive and incremental or not entirely novel. Furthermore, at least as written, it does not lead to an easy explanation of the EZH inhibition phenotype. In general I suggest to digest this portion so as to avoid distracting too much attention from the EZH experiments. The title should be modified accordingly. More specific points on this section:

Response: As described in more detail in 1b, below, our chromatin profiling is distinct from that which has been published. But more importantly, we feel it is very significant for the field to appreciate that the dynamics in H3K27me3, a crucial chromatin mark, differ markedly in our carefully staged cells isolated from embryos compared to that published on embryonic stem cells differentiated in culture. The NIH has invested huge resources in mapping chromatin features in cells differentiated in vitro from ES cells and it is important to know how well, or not well, such may truly model native embryonic development.

1a. The authors represent the data as Input subtracted from ChIP. This is of course valid, but for some reason the examples shown in Figure 3 appear to depict very scattered reads separated by empty spaces. This is not a typical H3K27me3 profile. Given that the authors have sequenced quite deep, what is the explanation for this?

Response: There are two reasons for this. In our original submission, we only showed H3K27me3 tag locations. To address the concern, in the updated manuscript, we remade the figures to show the coverage of each tag extended to 300 bp, the average length of the sequenced fragments (Extended Figure 2); the way many other papers show their browser shots. This gives a better sense of the genomic domains being covered. In addition, our scale in Fig. 3 is spread out compared to that seen in other H3K27me3 published browser views of these lineages. Our image shows 2 or 5 kb in 2 cm, whereas Fig. 3’s of van Arensbergen et al. 2010 or 2013 shows 5 kb in ~1 cm.
1b. The authors show that there is selective loss of H3K27me3 in endocrine transcription factor genes such as Ngn3, Neurod1, etc between Pdx1-expressing progenitors and endocrine cells. This is in line with the main conclusion of van Arensbergen et al, 2010. Hypothetically this explains why EZH2 promotes differentiation, given that it prevents repression of endocrine regulators in trunk progenitors. The GO analysis of genes that gained H3K27me3 is also similar to that reported. Although the current study provides greater temporal resolution, it would make sense to discuss this data in light of earlier similar findings.

Response: We cite van Arensbergen et al. 2010 numerous times, but their study was more focused on how adult beta cells differ from other adult cell types, and their striking result was that beta cells most resemble neural cells even when compared to related endodermal derivatives. This was described in detail in our original manuscript and is retained in the revised version (p. 4, bottom). The 2010 paper presents data for Pdx1+ progenitors, as we do, but they compare the patterns with ES cells and with fully differentiated beta cells; whereas our study is designed to assess the step-by-step transitions from endoderm progenitors to pancreatic progenitors to endocrine progenitors, in vivo. Thus the genome dynamics that we cover have not been assessed previously. The transitions that we mapped do not reveal marked GO profiles of neural differentiation, as van Arensbergen reported. This almost certainly is the simple consequence of us not having assessed the later stage of mature beta cells, which was the focus of the van Arensbergen study. To emphasize this difference and address the reviewer’s concern, we added the following sentences to the Introduction (p. 5, second para.) “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.”

On the other hand, rhe finding that Ezh2 inhibits endocrine differentiation is very interesting, but it does not necessarily follow from the chromatin maps as written. As mentioned, it seems that Ezh2 controls the release of repression in endocrine transcription factor genes in trunk cells. However, during endocrine differentiation there is also increased H3K27me3 at progenitor transcription factors like Hnf6. Furthermore, there is an overall increase in H3K27me3-enriched regions during differentiation. It seems necessary to rationalize all of this more clearly.

Response: As originally noted in the text (p. 7, top), we find that the absence of H3K27me3 tags on HNF6 is transient and confined to the intermediate pancreas progenitor stage (Fig. 1C). Perhaps the reviewer was looking at the entire lane instead of the HNF6 gene, which is to the right of the image. To address the concern, we now clarify our reference to this region with a green dotted line specifically over the HNF6 coding region, to highlight the area of interest. As we note, this pattern of transient HNF6 coverage by H3K27me3 nicely agrees with the Clotman et al. (2002) study of HNF6 expression at these successive developmental stages.

1d. The increased number of H3K27me3 peaks in EPs is likely to represent a true increase, but it could also reflect a better yield of the ChIP, given that the authors have understandably used a
suboptimal normal of cells (as few as 50 thousand) and no replicates. What indications do the authors have that this is not technical?

Response: To address the concern, we added the following sentence to the end of the main paragraph on p. 6: “Notably, among all chromatin samples, the number of patches was not a simple function of the number of aligned reads (Table E1).”

"In both heterozygous and homozygous floxed Ezh2 embryos, the Ngn3 gene is transcriptionally up-regulated in NGN3-GFP+ cells, compared to Ezh2 wild type embryos"

What is the interpretation of this finding? One might expect a difference in timing or spatial activation of the Ngn3 gene, or simply increased number of cells, but not an increase in expression in purified cells, because presumably H3K27me3 does not repress the Ngn3 gene in these cells.

Response: To address the concern, we modified the relevant sentence on p. 11 as follows: “In both 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.”

3. There is a general lack of statistical analysis in several figures.

Fig. 6B needs statistical analysis. Furthermore, the analysis shows % of GFP cells, but it should clarify whether the % of NGN3 GFP cells has increased, or if there has been a reduction in non-NGN3 GFP cells.

Response: On Fig. 6B, apologies, the asterisk layer was hidden in the original image; this has been fixed and the legend indicates that the difference is significant to p < 0.05. To respond to the second concern, we also changed the relevant sentence in the text (p. 14, bottom sentence) to: “...in the percentage of GFP+ cells in DZNep treated explants, without a decrease in GFP- cells...”

Glucose tolerance analysis also needs statistical analysis and described in detail.

Response: We now show a more extensive statistical analysis of the glucose tolerance test, as denoted by the asterisks in Fig. 5D and referred to in Fig. 5 legend. The test itself is described in detail in the methods section. We also added to the main text, p. 13 middle, “...where overnight fasted mice were injected intraperitoneally with glucose and blood glucose levels were measured at various time points thereafter.” Also, we note that the experimenter performing the blood glucose measurements was blinded to the identity of the samples.

4. Explant experiments need to be described in detail in the methods section.

Although we had referred to our previous publications for the methods, we added the following to the updated methods section, p. 20: “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 hours in Dulbecco's modified Eagles medium (DMEM) containing 10% calf
serum (HyClone) (Wandzioch and 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).

5. **Figure E1. The validation experiment for the antibody is not great, for all we know this could be recognizing unmethylated H3K27.**

**Response:** This lot of antibody has been verified by Millipore: (http://www.emdmillipore.com/US/en/product/Anti-trimethyl-Histone-H3-%28Lys27%29-Antibody,MM_NF-07-449#documentation, Lot# JBC1873477). This antibody is H3K27me3 specific and does not recognized unmethylated H3K27 or many other histone modifications tested by the manufacturer. To affirm the manufacturer's claim, our original and revised modifications paper shows a Western blot to verify that this antibody recognizes a single protein in cell extracts (Fig. E1).

6. **ChiP-seq protocol. Given that such small amounts of cells were used, it would be useful to provide further details on amounts of DNA used for library construction, stating any modifications of library construction protocol, and providing information on reads/non-clonal reads.**

**Response:** With the revised manuscript, we now include an 8 page, detailed "Expanded Methods" protocol on our low cell number ChIP-Seq methodology.

**Minor points**

1. **Intro:** "The early differentiated PDX1+ cells are multipotent progenitors that can give rise to ductal cells, acinar cells, and endocrine progenitors (NGN3+) starting from E9.5" It sounds like the authors wish to say that duct and acinar cells become visible as soon as e9.5, I am not sure that this is what the authors wish to convey.

**Response:** Thank you, good point. We deleted "starting from E9.5."

2. **Methods.** "H3K27me3 peaks were called using software for wide patches of chromatin from the Broad Institute (Guttman et al, 2009)" Does this software have a name, or have the authors repurposed scattered scripts?

**Response:** Neither. As stated, we contacted M. Guttman and he kindly provided his program. It has no name and we did not modify it. To address the reviewer's concern, we changed the sentence in the methods to read as follows (p. 23): "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."

3. **Figure S5 should correct the allele nomenclature, presumably the Pdx1-Cre mice are also Pdx1+/+ since the are probably not knock-ins. Incidentally it seems that the methods section does not describe the transgenic Pdx1-Cre line.**
Response: Thank you for pointing this out. We corrected the allele nomenclature in Fig. ES5 and we added the appropriate reference on p. 20 of the methods.

Referee #2:

Xu et al "Dynamics of Genomic H3K27me3 Domains and Role of EZH2 during Pancreatic Endocrine Specification" assesses changes in the enrichment of H3K27me3 during development and use the loss of Ezh2 to analyze the dynamics of H3K27me3 enrichment during development and differentiation.

The analysis of enrichment of H3K27me3 during development is important in understanding how to direct ES cells towards beta cells. This study shows that directed ES cells differentiation is quite different from the differentiation in vivo and perhaps points us towards why directed differentiation of ES cells in vitro has been so unsuccessful in generating functional beta cells.

Here are a few points to address:

1. It is not clear why Nkx6.1 H3K27me3 enrichment is maintained in pancreatic progenitors and lost only at the endocrine differentiation. This does not match the expression pattern observed during development as Nkx6.1 is expressed throughout the pancreatic progenitors cells similar to Pdx1 and then enriched in endocrine cells fated to become beta cells. Are there different elements directing this expression pattern and can H3k27me3 patterns distinguish these elements?

Response: Point well taken, but we see what we see. As a possible explanation, to respond to the reviewer’s valid observation, on p. 10, second para, we now note: “While Nkx6.1 is first activated in pancreatic progenitors and is needed for an endocrine vs. 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).”

2. It is not clear to me where the enhanced Ngn3 cells are originating in the Ezh2f/f Pdx1 cre mice? This needs to investigated and explained better.

Response: To respond to the concern, we added new data panels to Fig. E5, showing that the Ngn3+ cells are originating in the Sox9+ duct compartment at E14.5 in the Pdx1-cre; Ezh2fl/fl pancreata.

3. Increased beta cell area is somewhat subjective and beta cell mass is a better assessment

Response: The method we used to measure beta cell mass is typical for the field. To avoid subjectivity, the investigator was blinded to the genetic identity of the slides. To quantitate beta cell mass in the new Fig. 5A, we analyzed 2-3 intermittent middle sections per pancreas, performed insulin immunohistochemistry, and measured the area of all islets in the sections. We then compared the area of the islets to the whole pancreas areas. We now show much larger sections in 5A.
4. better glucose tolerance in Pdx1-cre Ezh2 f/+ can be due to loss of glucose responsiveness. GSIS of isolated islets needs to be carried out.

Response: To respond to the reviewer's concern and to obtain a better sense of how there is improved glucose tolerance in Pdx1-Cre; Ezh2f/+ and poorer glucose tolerance in Pdx1-Cre; Ezh2fl/fl, we measured the islet mass in adult mice (Fig. 5A, B) and insulin mRNA expression levels in isolated islets. We can see larger islet mass in Pdx1-Cre; Ezh2f/+, while smaller islet mass in Pdx1-Cre; Ezh2fl/fl, compared to WT controls. Both insulin1 and insulin2 expression, per islet, are not affected in Ezh2 homozygous KO and heterozygous KO islets (Fig. 5C). From these results, we conclude that the greater islet mass in Pdx1-Cre; Ezh2f/+ pancreases produces more insulin while the lesser islet mass in Pdx1-Cre; Ezh2fl/fl pancreases produces less insulin. These conclusions fit well with the glucose tolerance phenotypes observed in Fig. 5D.

5. Further data analysis that correlate repression or derepression with H3k27me3 binding and how this differs from ES cells would helpful.

Response: We have performed additional experiments to address this concern. First, we now show in Fig. 4B that "H3K27me3 is greatly diminished in the Ezh2 knock out pancreas and undetectable in nascent endocrine progenitors expressing endogenous NGN3." (p. 11, text). Second, endodermal progenitor cells were incubated with DZNep or GSK-126 for 48 hours and the H3K27me3 mark was examined on the NGN3 promoter by ChIP-qPCR (Fig. E8). We find that treatment of cells with the same dose and time used in the differentiation experiments does have an impact on H3K27me3 levels at this promoter. We feel that these additional data show that the genetic and pharmacological effects are indeed correlated with diminution of H3K27me3.

Referee #3:

The authors characterize histone methylation during development of the mouse pancreas using a low amount of material. Although genome-wide data are inherently difficult to present in a reader-friendly way, they show that pancreatic transcription factors have lower methylation levels. This is not surprising, as the authors state themselves. Using Ezh2 conditional knockout and chemical inhibition, the authors show that the number of ngn3 positive cells increase, and also islet-cell mass. They also show some data supporting the notion that careful timing of ezh2 inhibition might be applicable to improve generation of human beta cells from ES cells. Overall, the work is well conducted, but in the criticism described below, the conclusions are often not solid enough to warrant publication.

Response: We thank the reviewer for his/her comments. As noted in our response to reviewer 1, we would add that our comparison of in vivo embryonic development with in vitro ES cell development, previously published, is an important novel dimension to our study. We show that the in vitro studies do not appropriately model the transitions seen in vivo.

Major points:
ChIP data showing a relief of H3K27me3 marks at the Ngn3 locus in the EZH2 deletion background are not presented. I realize that the authors showed that Ngn3 is demethylated by
e14.5, but I imagine the methylation marks would be significantly decreased on Ngn3 and other transcription factors at an earlier age compared to WT. What is the effect of ezh2 het or homozygous ko at the pdx1 stage on the epigenetics of the H3K9me3 marks? There is also no methylation analysis upon use of the inhibitors.

Response: Given how many embryos need to be harvested for such a study, and that each litter has a mix of different genotypes that would have to be distinguished prior to FACS, it is unfeasible to directly test this on the embryonic samples. On the other hand, we made two changes to the paper to address the concern. First, in the new Fig. 4B, we show that homozygous knockout of Ezh2 results in a marked diminution of H3K27me3, as expected. Second, in a new Fig. E8, we show the effect of EZH2 inhibitors on H3K27me3 at the target NGN3 promoter in human ES cells. As expected, we observe a diminution of H3K27me3 in the drug-treated samples. We feel that these additional data go a long way towards addressing the reviewer’s questions.

Loss of function induces gain of ngn3-gfp. Does ezh2 gain of function reduce ngn3-gfp? I think the authors should try to overexpress EZH2 in their explant or iPSC model. It would be interesting to see if the genes promoting beta cell differentiation were silenced and therefore gave rise to fewer beta cells. It appears the homozygous ko does not enhance ngn3 compared to the het knockout. What is the explanation for this?

Response: Given the extensive additional material added to the paper that enhance our initial claims, we felt that overexpression studies would not add further to the conclusions. With regard to the homozygous knockout not enhancing Ngn3 more than the heterozygous, we now say in the Discussion: “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 Ezh2fl/fl and Ezh2fl/+ pancreas. This suggests that the precise expression level of Ezh2 is critical to restrain endocrine pancreas development.”

Detailed comments.
1. EZH2 suppresses the normal extent of endocrine cell induction
   a. I found it surprising that the authors used the PdxCre instead of an inducible PdxCreER. The Pdx1Cre is on so early (e8.5) that it is likely to have secondary effects
   b. Specifically, the authors wanted to address what controls the methylation changes seen during the transition from pancreatic progenitors to the endocrine progenitor stage. It would therefore have been better to use an inducible Cre and delete EZH2 right before the secondary transition to truly understand the role EZH2 and thus methylation plays in endocrine development.

Response: The endogenous Pdx1 gene is activated at E8.5, but Pdx1-Cre doesn’t function well until about the E9.5-10 stage. This period precedes the normal initiation of induction of endocrine progenitors (E12.5) by a few days, which is why we chose the Pdx1-Cre system. To address this concern, we now clarify the point in the manuscript by adding on p. 11, top, "This would cause precocious loss of Ezh2 prior to E12.5, when endocrine cell production begins."

c. The authors also found increased Ngn3 RNA in the EZH2 deleted pancreases which they
showed was due to an increased number of Ngn3+ low-expressing cells

i. I think the authors need to show some sort of Ngn3 protein staining here. RNA is good, as is sorting for number of cells, however they never do any antibody staining. They only use the Ngn3-EGFP, which is a transgene. There is a good Ngn3 antibody that I feel would strengthen their data.

Response: Since EZH2 works at the level of transcriptional regulation, not protein abundance control, we have focused our quantitative assessments on Ngn3 RNA levels. However, as requested by the reviewer, we now include NGN3 antibody staining in the updated Fig. 4B. In addition, we show co-staining for H3K27me3. While these data nicely show a clean absence of H3K27me3 in the NGN3+ cells, we are reluctant to draw quantitative conclusions about expression levels from the NGN3 immunofluorescence data in WT versus Ezh2 mutant mice.

d. The authors need more data to support their claim that extra Ngn3 cells come from the trunks. 
Extended figure 5 is a terrible picture. They should show zoomed in, fluorescent staining of Ngn3, Amylase and Sox9. (They state in the text that they stained for Sox9- where is it?). Also- what age is this picture from?

Response: We have completely re-done Fig. E5. The top panels show a more zoomed in view of NGN3 and amylase, and the bottom panels show a more zoomed in view of Sox9 and NGN3; all at E14.5. From these data we can clearly conclude that the extra NGN3+ cells come from the trunks.

e. Figure 5A is also a bad picture. Why not fluorescence and why is the picture so zoomed out? I would like to see a picture of what these islets look like up close and what the insulin staining looks like in the individual endocrine cells. When do the authors start to see more beta cells? During development or only postnatally? Why do they do physiology assays at P9?

Response: We used immunohistochemistry instead of fluorescence in Fig. 5A because the former is much better for determining islet mass. On p. 13, we now state: “Islet structure and beta cell morphologies appeared normal in the knockouts (data not shown).” We measured islet mass in adult (2 months old) Ezh2KO pancreas, the same age as we conducted glucose tolerance tests, and the results are in the new Fig. 5A-D. The data for the P9 islet mass measurements have been moved to Fig. E6. Since we observed more endocrine progenitors as early as E14.5 in the Ezh2KO pancreas (Fig. 4), it appears that the increased progenitors give rise to more nascent beta cells during embryonic development.

f. There are also no statistics done for Figure 5D. They need AUC or something to claim there is a significant difference in blood glucose when EZH2 is lost. What about serum insulin levels? Was the investigator blinded to the genotype of the mice? This is perhaps the key Figure of the entire manuscript and the highest possible standards of experimentation should be applied.

Response: We added the statistics in Fig. 5D. Importantly, as now stated in the text on p. 26: “The investigator was blinded as to the identity of the samples during the glucose assays.” We did not test the insulin levels; instead, we now show insulin gene expression levels in islets purified from Ezh2 WT and KO adults; please see our response above to comment 4 of Reviewer 2 for more details of this experiment. We were also blinded with regard to our NGN3-GFP+ flow
cytometry in E14.5 pancreas (Fig. 4D), since we did not know the genotypes of each tissue or animal until we finished the FACS analysis.

g. The authors claim that Ink4A/Arf causes "diabetes" when upregulated in the islets. The authors need further support for this claim. Such as insulin staining corresponding to the age they see up regulation of Ink4/Arf.
i. Otherwise one could argue that the increase in beta cells seen with the loss of EZH2 were actually dysfunctional to begin with.

Responses: The claim that aberrant Ink4A/Arf induction causes mild diabetes is from Seung Kim (Chen et al, Genes and development, 2009, Fig 1) and our data agree. From their images of insulin staining in 23 day old vs. 1 yr pancreas, we cannot see marked differences in insulin staining density. So we think that Ink4A/Arf inhibits beta cell proliferation, as they suggest, rather than insulin expression.

2. Histone methyltransferase inhibitors modulate two stages of pancreas differentiation
a. Figure 6 and Extended figure 6:
i. One question I had here was that if the loss of EZH2 causes an increase in Pdx1+ cells, then why aren't there more cells in every cell lineage. Pdx1 cells give rise to all pancreatic cell types. Why aren't there more exocrine cells as well? Why isn't the entire pancreas larger?
ii. What transcription factors are misexpressed or misregulated because of the lack of methylation marks? Do these extra beta cells express markers from other lineages?

Responses: Figure 6 and Extended Figure 6 focus on two discretely different stages of pancreas development. The original extended Figure 6 (now renumbered to Ext. Fig. 7) focuses on the pancreas vs. liver choice stage in the endoderm at E8.25. In the text, p. 14 middle, we stated that this step was performed to "test small molecule inhibitors of EZH2 for their ability to mimic the Ezh2 conditional knockout," the latter result being published by us previously at E8.25 with an endoderm-cre (Xu et al., Science 2011). We consider it as a positive control for whether the drugs can mimic a previously established genetic response. Having shown that indeed the drugs mimic the effect of the Ezh2 conditional knockout at the pancreas-vs.-liver choice stage, in Fig. 6 we assess the drugs on E12.5 pancreas tissue, at the endocrine induction step. Since as we note on p. 11-12, Extended Fig. 4 shows no significant difference in BrdU incorporation in the Ezh2fl/fl; Pdx-Cre pancreases, it appears that Ezh2 is modulating the endocrine fate choice rather than increasing the total number of all pancreas cell types. In response to these queries, we added the following to p.19 of the Discussion: "Also, the lack of change in proliferation in the Ezh2fl/fl pancreases is consistent with a cell fate control change instead of a putative nonspecific increase in all cells of the organ."

b. Figure 7 B and C need asterisks showing significance

Response: Asterisks with associated p values have been added to Figure 7.

The authors also never mention Figure 7D in the text. The x-axis shows glucagon but they never mention the lack of double hormone positive cells in the text?
Response: Thank you! We now mention the data in Figure 7D in the text of the manuscript (page 16). Prior work published by the Gadue laboratory has demonstrated that endodermal progenitor cells generate mono-hormonal beta-like cells (Cell Stem Cell (2012) 10, 371–384.). We include glucagon expression in Figure 7D to demonstrate that the enhancement of insulin+ cells is not at the expense of generating poly-hormonal cells.

The increase in c-peptide in human ES cells is not that impressive, given the variabilities in this assay, i am not convinced. How many different cell lines were used for this analysis?

Response:
We agree that there is considerable variability during in vitro differentiation experiments. Because of this, we present c-peptide induction as fold-change over control cultures averaged from 10 independent experiments (Figure 7B) in addition to flow cytometric data from a sample experiment in Figure 7D. This enhancement was very significant with a p value of 0.0003. We have added statistical analysis to Figure 7. These results were consistent among 1 human ES cell line and 1 iPS cell line tested. This information has been added to the manuscript.

The authors say that insight gained in mice can be directly translated to human differentiation. But they also point to differences in the dynamics of the methylation. Is this due to the in vitro differentiation or due to species-specific differences? Did the authors look at mouse ES cells differentiated in vitro? On page 17 top the authors suggest that this is due to in vivo versus in vitro. The discussion is not clear why the authors favor that interpretation.

Response: We did not analyze mouse ES cell differentiation. While this is an interesting experiment, we feel it is outside the scope of this manuscript. In addition, mouse ES cell in vitro differentiation into beta like cells is typically of very low efficiency, making interpretation difficult. We agree that we do not have any data arguing whether the differences we see are due to species differences or in vitro vs in vivo effects. Therefore, we have revised the Discussion emphasize this point less.

writing: 
"not due to increased cell proliferation or decreased programmed cell.

The authors should make more clear at the start of each chapter/paragraph whether experiments were done on mouse or human cells.

Response: Done, thank you.