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Insm1 cooperates with Neurod1 and Foxa2 to maintain mature pancreatic β-cell function

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 12 January 2015

Thank you very much for submitting your study on Insm1 in cooperating with Neurod1/Foxa2 to maintain maturity and function of pancreatic beta-cells for publication in The EMBO Journal.

I received comments from two scientists that are essentially in favor of publishing the phenotypic analyses, combined with the new insights into the governing transcriptional networks. Ref#1 highlights the thoroughness and suitability for publication, while suggesting a few refinements that may serve to focus and thus strengthen the reported immature phenotype upon Insm1-deletion. Ref#2 takes some issue with the cell line based approach to address cooperativity of Insm1/Neurod1/Foxa2 in determining transcriptional profiles/functional relevant gene expression. S/he therefore recommends to address this by re-ChIP PCR for some of the selected targets in wt versus mutant mouse islet tissue.
I am confident that such amendments are feasible within a very reasonable timeline and are thus delighted to invite formal revisions of your paper for eventual publication in The EMBO Journal.

Please note, that I formally have to remind you that The EMBO Journal allows only one single round of major revisions. Please do not hesitate to get in touch in case of further questions.

I thank you very much for considering The EMBO Journal for presentation of your data and remain

REFEREE REPORTS

Referee #1:

The authors provide a thorough characterization of the function of the beta cell transcription factor Insm1 in adult beta cells. They find that mice have elevated blood glucose levels and glucose stimulated insulin secretion is specifically affected. The authors perform gene expression and chip analysis. Much of the manuscript is solid (particularly Figure 1,2 and 3e-h). Overall, it is an interesting paper and is suitable for publication with appropriate revisions.

Comments (without significance to their sequence)
The writing of the paper is very poor. E.g. in the introduction, sentences do not connect well from one topic to the next.

Figure 1 D and E seems to be a discrepancy. How come randomly fed mice show elevation of insulin secretion, but not after glucose injection?

Fig. 3e-h seems to fit better in topic to Figure 1 and 2. What about granule number per cell?

authors show a 'mild increase in beta cell proliferation', and a mild increase in apoptosis. The paper would benefit from a test to determine regenerative capacity by beta cell ablation and regeneration.

authors say that genes involved in insulin secretion is affected. How then is potassium and arginine-stimulated insulin secretion normal? Do gene expression and chip binding data support the statement that the secretory machinery is not impaired?

the number of 1232 deregulated genes is high. Need to verify false positive and negative rate by other methods and additional samples. The overlap with the chip data is not impressive. "For instance, 16% of all expressed genes and 32% of deregulated genes (FC >1.4) contained an Insm1/Neurod1/Foxa2 binding module within 10 kb." Is this binding module identified computationally or occupied according to CHIP? the difference between all expressed genes and deregulated genes reaches statistical significance, but the reviewer believes this analysis and the conclusions can be strengthened.

the claim that beta cells return to an immature phenotype is not convincing. the overlap is not that extensive, and the authors are starting with a large pool of 1232 genes. The way it is written, it is misleading, just some markers of the mature state are lost. a recent paper from the Melton group (Blum et al. eLife) found that UCN3 is downregulated in islets of T2D mice.

how do authors explain that "Similar numbers of genes associated with Insm1/Neurod1/Foxa2 binding sites were up- and down regulated"? Does Insm1 act as a transcriptional repressor?

This whole transcriptional analysis and chip analysis is a bit shaky, and authors often refer to supplementary data. For instance, why is the statement shown in the supplementary? "Additional computational analysis of different binding site categories confirmed that sites co-occupied by Insm1/Neurod1/Foxa2 correlate best with changes in Insm1-dependent gene expression (Table S5)." Authors appear uncertain and should do sufficient quality controls of their data and analysis to come up with clearer conclusions in the main Figures and Text.
It follows that the further analysis of human sequence variants is not convincing.

what is a 'poised enhancer'?

Referee #2:

In that manuscript Jia et al identify that the combinatorial binding of transcription factor Insm1 with Neurod1 and Foxa2 is crucial to maintain adult b-cell identity. The authors utilize a conditional deletion approach to deplete insulin + cells from insm1. The resultant mutant mouse presents a glucose intolerance phenotype with elevated glucose levels and decreased insulin secretion. The authors establish that the phenotype is due to a reduced b-cell mass with preservation of b-cell numbers. Then, by performing a series of transcriptomic experiments they observe that the

expression profile of the mutant pancreas resembles to a neonate/immature pancreas, and conclude then that phenotype observed in the mutant mouse is due to an improper maturation of b-cells. Then, by using chromatin binding aproach the authors describe that this effect is due to the combinatorial binding of Inms1 with FoxA2 and Neurod1, and not Insm1 alone thus concluding that this triple interaction of the Insm1 with the other master regulators is crucial for maintaining adult b-cell identity.

Overall, the manuscript is of great interest to the pancreas field and highlights the importance of Insm1 and its regulatory network on maintaining cell identity and regulation of insulin secretion. The manuscript is clear and well written and the authors perform state-of-the art techniques to answer their questions.

My main comment relies on the cell line used to perform the Chromatin binding and transcription binding analysis. Why the authors choose to perform these experiments on SJ b-cell line, immortalized by themselves, instead of using native b-cells? They could have used b-cells (isolated by FACS using specific insulin reporter or cell surface antibodies) from a control mouse (not tam induced / wt) and use their mutant mouse as negative control for the binding strategies. That would definitively proof that the binding is responsible for the phenotype observed and essential to keep the mature identity.
The main reason for my concern regarding that experiment, which is the key for the conclusion that Insm1-FoxA2 -Neurod1 are crucial to b-cell mature identity, is that the cell line used has a 10x less secretion when compared to mature islets (compare (Supplementary Fig. S4A, 16.7 sample with figure 3E). The authors argue that "SJ β-cells displays glucose-induced insulin secretion that is very similar to the one of isolated pancreatic islets from adult", however, a 10-fold reduction is definitively significant and should be considered as a potential "immature cell type/". Even more, when we take into account that the comparison is a b-cell like cell (SJ cell) vs a mature islet (which is a mix of several cells), then this 10x difference is even bigger. This point should be addressed or, at least, a re-Chip PCR experiment on these genes should be performed on both wt and mutant islets and the potential drawback of the experiment discussed clearly during the manuscript.

Also, this experiment would perfectly link the 2 sections of the manuscript (mutant mouse phenotype and molecular analsysis) that otherwise seem disconnected.

Other comments:

1) In fig 1A both panels have poor resolution. More important, Fig 1 A has some flaws that need correction:

In the top panel, It seems that all cells in the presented islet are b-cells? . Nuclear co-staining with nuclear marker (e.g. dapi) is missing and would facilitate the evaluation of that.

Bottom panel: The authors claim in the text that Insm1 is expressed in the other cell types (a,d, pp) but this conclusion is impossible to draw from the staining presented. The figure reads as "glc/Stt and PP" in green. I understand from the Suplementary table 9 the authors have used antibodies against glucagon/ Stt and pp all combined together?
the Info regarding the staining protocol followed is missing on the figure legend and is not possible to evaluate how the experiment is being conducted. Without using specific antibodies separately, it is not possible to conclude if Isnm1 is expressed in the other non-b-cell types and in which of them. Again, counterstaining with dapi would be advisable.

Also, What area is represented in the magnifications?

2) by using RIPCRE the authors mention that they selectively delete Insm1 in b-cells" pronounced reduction of Insm1 protein in coInsm1 mice in Pdx1high β-cells by immunohistology or in isolated islets..." (Fig1B). However, Co-staining with a specific b-cell antibody (e.g. insulin) is missing. This is an essential staining control to be able to draw that conclusion. Also taking into account that the entire paper is based on using RIP-CreER to study the role of Insm1 specifically in b-cells this staining becomes essential.

3) in Figure 2 a-b, the protocol used is missing. Also, according to figure legend, n=3 for the

quantification of b-cells, what does it refer to? 3 slides, 3 sections, 3 mice?

3) in table S6 the total number of deregulated genes in the within 50Kb binding sites should be corrected. I guess is 536.

4) Concentrations of secretagoges, e.g. Exendin 4, ...are all missing

5) Check for consistency between "methods" and "experimental procedures"

1st Revision - authors' response 26 February 2015

Referee #1, general comments.

The authors provide a thorough characterization of the function of the beta cell transcription factor Insm1 in adult beta cells. …. Overall, it is an interesting paper and is suitable for publication with appropriate revisions.

The reviewer acknowledges the quality and importance of our findings, but also raises concerns that are not in all cases justified as I explain in more detail below. These concerns might be grounded on some misunderstanding of data and quality controls that were performed. I introduced various changes into the text and figures to better explain these issues.

The writing of the paper is very poor. E.g. in the introduction, sentences do not connect well from one topic to the next.

I introduced various changes into the text to improve the text and its flow.

Figure 1 D and E seems to be a discrepancy. How come randomly fed mice show elevation of insulin secretion, but not after glucose injection?

Fig. 1D and E in the original manuscript analyze blood insulin in two distinct experimental settings. In Fig. 1D (revised Fig. 2C) animals were fed *ad libitum* with a chow containing protein, fat and carbohydrates (around starch 35%), and glucose (around 0.25%). Under these conditions, insulin levels in the blood are controlled by glucose and other nutrients like amino acids as well as hormones (e.g. incretins). Thus, a steady state is monitored in which insulin secretion is controlled in a complex manner (see also introduction of the revised manuscript, second paragraph). In Fig 1E (revised Fig. 2B), we had shown insulin secretion in animals starved for 16 hours that were exposed to a single glucose injection. Thus, we observe a short-term response to a single nutrient, glucose. In Fig. 3H (revised Fig. 4H) we showed that the response to another nutrient, arginine, is intact. The apparent contradiction is thus resolved when the distinct physiological parameters are taken into account. I have rewritten this part on the results (pg. 6 of the revised manuscript), rearranged the figure and introduced changes into the discussion to explain this more clearly in the revised manuscript.

Fig. 3e-h seems to fit better in topic to Figure 1 and 2. What about granule number per cell?

The volume of Insm1 mutant b-cells can be estimated using numbers of nuclei/area (revised Fig. 3E). According to such an estimate, cell volume is decrease around 22%, and this in accordance with the observed reduction in cells mass of 25% (revised Fig. 3B). We did not detect a change in numbers of granules/area (revised Fig. 4G), but taking changed cell size/volume into account, the absolute number of granules is expected to decrease by 25%. When insulin is secreted upon glucose challenge, a small subset of vesicles fuses with the surface membrane during the first phase of insulin secretion (2-15 minutes after glucose injection); insulin secretion in the second phase (15 min- 2 h) depends also on newly generated insulin/secretory vesicles. The estimated decrease in cell size is expected to impinge moderately on overall vesicle numbers, but cannot account for the pronounced physiological effects we observe. This is supported by the fact that mutant b-cells remain fully capable of insulin secretion after challenge with arginine or by global membrane depolarization with exogenous KCl (cf. revised Figs. 4E and H). I have introduced changes into the introduction, results and discussion of the revised manuscript to make this clearer.

authors show a 'mild increase in beta cell proliferation', and a mild increase in apoptosis. The paper would benefit from a test to determine regenerative capacity by beta cell ablation and regeneration.

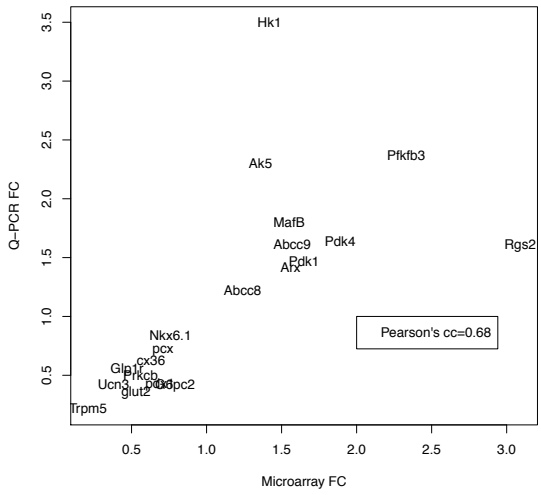
Our work concentrates on the analysis of physiological deficits after Insm1 depletion in the adult, and on the molecular mechanisms of Insm1 function. Proliferation of b-cells is observed in young animals, but the (very) mild increase in turnover can neither account for the deficits in glucose-stimulated insulin secretion nor for the failure to maintain mature gene expression. We therefore did not investigate this further. Analysis of regenerative capacities would diverge the focus of the paper.

authors say that genes involved in insulin secretion is affected. How then is potassium and arginine-stimulated insulin secretion normal? Do gene expression and chip binding data support the statement that the secretory machinery is not impaired?

Among >1.200 differentially expressed genes, we found genes that encode components of the secretory machinery, glucose sensing, glucose-metabolism and mitochondrial function, response to secretagogues and intracellular signaling components. All of these participate in controlling insulin secretion. To determine the responsible mechanism in coInsm1 mutants, we analyzed secretion to various stimuli. We found that glucose-induced insulin secretion (*in vivo* and *in vitro*) and secretagogues response in the presence of glucose (*in vitro*) was changed. Nevertheless, mutant b-cells remain fully capable of insulin secretion after challenge with arginine (*in vitro*) or global membrane depolarization with KCl (*in vitro*). Thus, the primary deficit is restricted to glucose-induced insulin secretion and its modulation by secretagogues, excluding a general deficit in the basic secretory machinery. I have introduced various changes in introduction, results and discussion to make this clearer to the reader, for instance in the result section that describes these data (pg. 9).

the number of 1232 deregulated genes is high. Need to verify false positive and negative rate (?) by other methods and additional samples.

Changes in gene expression were determined by microarray using isolated islets and eight independent microarray experiments for each genotype. The cutoffs chosen were: 1) adjusted (Benjamini-Hochberg) $p < 0.05$ which is more stringent than the normal p value used by many researchers; 2) cutoff for expression level (log2 intensity > 7); 3) fold changes > 1.2 or > 1.4 (most analyses were done twice, i.e. using the two different cutoffs). In addition, the quantile normalization was used to define reliable changes. We re-tested 20 differentially expressed genes (microarray cutoff FC>1.2) also by quantitative PCR using independent mRNA preparations and different animals, and all expression changes were verified (Fig. 4B revised manuscript, see also correlation plot below).



We conclude from this that the microarray experiments and their bioinformatics analyses were

carefully performed, and that the results obtained by microarrays are reproducible using another technique. In addition, the cutoffs used allowed us to retain a very good statistic power when comparing deregulated genes, and binding/not binding of the many possible different transcription factor/transcription factor combinations tested (i.e. Insm1 only, Neurod1 only, Foxa2 only, Insm1/Neurod1, Insm1/Foxa2, Neurod1/Foxa2, Insm1/Neurod1/Foxa2, cf. supplemental Table S5). The statistical power would decrease with smaller gene numbers.

The overlap with the chip data is not impressive. "For instance, 16% of all expressed genes and 32% of deregulated genes (FC >1.4) contained an Insm1/Neurod1/Foxa2 binding module within 10 kb." Is this binding module identified computationally or occupied according to CHIP?

The presence of Insm1/Neurod1/Foxa2 binding modules was determined experimentally using ChIPseq data. The term 'module' was change to 'binding sites' to make this clear.

the difference between all expressed genes and deregulated genes reaches statistical significance, but the reviewer believes this analysis and the conclusions can be strengthened.

Our data provide compelling evidence that the difference between all expressed and deregulated genes is highly significant. In particular, the example cited by the reviewer (16% of all expressed genes and 32% of deregulated genes contained an Insm1/Neurod1/Foxa2 binding module within 10 kb) has a p value of 4.3x10-13, i.e. the chance that this enrichment occurs by chance is 1 in 4x 10.000.000.000.000 (in the previous version of the manuscript the value was given as $4.3e^{-13}$ which is a format used by many programs and corresponds to $4.3x10^{-13}$). Many different computational analyses and comparisons were tested (some of them summarized in Supplementary Table 5), which demonstrated that neither Insm1 binding nor other combinatorial binding sites correlated to such an extent with gene expression changes. In conclusion, we are confident that Insm1/Neurod1/Foxa2 triple binding sites identify a functionally important subset of Insm1 binding sites.

I also want to point out that the correlation between gene expression changes (transcription factor mutants or siRNA-treated cells vs control) and transcription factor binding sites in mammalian cells has remained a difficult research area. Typically, number of binding sites by far exceeds the number of deregulated genes, and therefore factor binding and expression remain difficult to correlate. For instance, the Sander lab recently reported that only 9% of all deregulated genes in Nkx6.1 conditional mutant β-cells had an Nkx6.1 binding site within 10kb, which reached a significance of $p < 0.05$.

the claim that beta cells return to an immature phenotype is not convincing. the overlap is not that extensive, and the authors are starting with a large pool of 1232 genes. The way it is written, it is misleading, just some markers of the mature state are lost. a recent paper from the Melton group (Blum et al. eLife) found that UCN3 is downregulated in islets of T2D mice.

This concern is in our opinion unjustified. We re-tested the overlap of deregulated genes between coInsm1 mutant/wt and P1/adult using a hypergeometric probability test and the following numbers: 11613 genes expressed in wildtype islets (cut-off expression level log2 intensity > 7), 2605 and 1232 genes deregulated in P1 versus adult and coInsm1 mutant vs control, 358 overlapping genes. The p-value for this is $5.64x10^{-9}$ and thus highly significant (i.e. the chance that such an overlap would occur by chance is 1 in 5x 1.000.000.000). We also compared changes in gene expression in coInsm vs control with those recently determined in a diabetes mouse model (diabetic vs non-diabetic; Kluth et al., Diabetes 63, 4230). Among the 2517 differentially expressed genes in diabetic vs. non-diabetic mice, 115 overlapped with our data set (p value = 1).

In conclusion, an overlapping set of genes is deregulated in coInsm1/immature islets or coInsm1/diabetic Islets, but only the overlap between coInsm1 mutant and immature islets is highly significant. Therefore, when we summarize changes we state that mutant mature β-cells resemble immature β-cells in gene expression. We extend the description of this analysis in the text of the revised manuscript to include the comparison with the diabetic model (pg. 8 of the revised manuscript).

how do authors explain that "Similar numbers of genes associated with Insm1/Neurod1/Foxa2 binding sites were up- and down regulated"? Does Insm1 act as a transcriptional repressor?

This indicates that Insm1 acts as a transcriptional regulator and in a context dependent manner as activator or repressor. This is similar to many other transcription factors, and is the most commonly observed situation when ChIPseq and gene expression data are compared to predict direct target genes. We mention this point now in the discussion of the revised manuscript (pg. 17).

This whole transcriptional analysis and chip analysis is a bit shaky, and authors often refer to supplementary data. For instance, why is the statement shown in the supplementary? "Additional computational analysis of different binding site categories confirmed that sites co-occupied by Insm1/Neurod1/Foxa2 correlate best with changes in Insm1-dependent gene expression (Table S5)." Authors appear uncertain and should do sufficient quality controls of their data and analysis to come up with clearer conclusions in the main Figures and Text.
It follows that the further analysis of human sequence variants is not convincing.

This assessment is in our opinion unjustified. As outlined above, the transcriptional data are of very good quality and reproducible. Similarly, the ChIP analysis is of high quality and highly reproducible. For instance, we used very stringent cutoffs to define binding sites compared to other publications in the field. We showed most quality controls for the ChIPseq in Supplementary figures in the original version of the manuscript (supplementary Figs. S4, Supplemental table S5). This was not an attempt to hide quality controls, but a strategy to allow straightforward reading. As the reviewer wishes, we transfer a subset of the quality controls formerly shown in supplemental data into the main figures/tables (revised Figures 5,6,8).

what is a 'poised enhancer'?

Poised enhancers are functionally inactive but are in a poised or predetermined state. This was originally defined in embryonic stem cells where inactive genes encoding early developmental regulators possess bivalent histone modifications and are therefore considered poised for activation. Poised enhancers have also been described in adult tissue and may represent enhancers that respond to external stimuli (Creyghton et al., PNAS 2010 107(50):21931-6). In the revised manuscript, we explain this and include a reference (page 17).

Referee #2, general comments

Overall, the manuscript is of great interest to the pancreas field and highlights the importance of Insm1 and its regulatory network on maintaining cell identity and regulation of insulin secretion. The manuscript is clear and well written and the authors perform state-of-the art techniques to answer their questions.

The reviewer appreciates the quality and the importance of our findings.

Referee #2, specific comments

My main comment relies on the cell line used to perform the Chromatin binding and transcription binding analysis. Why the authors choose to perform these experiments on SJ b-cell line, immortalized by themselves, instead of using native b-cells? They could have used b-cells (isolated by FACS using specific insulin reporter or cell surface antibodies) from a control mouse (not tam induced / wt) and use their mutant mouse as negative control for the binding strategies. That would definitively proof that the binding is responsible for the phenotype observed and essential to keep the mature identity.
The main reason for my concern regarding that experiment, which is the key for the conclusion that Insm1-FoxA2 -Neurod1 are crucial to b-cell mature identity, is that the cell line used has a 10x less secretion when compared to mature islets (compare (Supplementary Fig. S4A, 16.7 sample with figure 3E). The authors argue that "SJ β-cells displays glucose-induced insulin secretion that is very similar to the one of isolated pancreatic islets from adult", however, a 10-fold reduction is definitively significant and should be considered as a potential "immature cell type/".

The reviewer points out that it would have been desirable to use primary cells instead of a model cell line for the ChIPseq analysis. We agree, but the limited availability of primary β-cells makes it

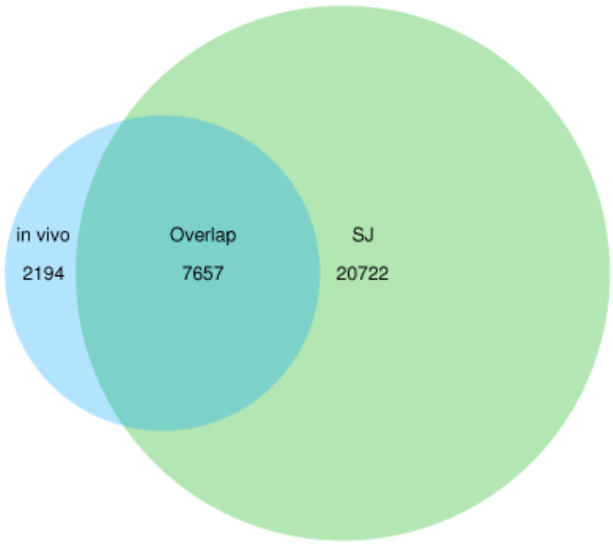
problematic. Thus, we performed all ChIPseq analyses at least twice, ChIPseq was done on three distinct transcription factors, and we had done additional ChIPseq experiments to establish condition and test antibodies. We use about 10 x 10^6 cells/experiment for the final experiments, and would have needed more than 60.000 islets/genotype to isolate the primary β-cells. We usually isolate around 100 islets/mouse, so we would have needed about 600 mice/genotype (total of 1,200 mice!) to perform such experiments on primary cells not taking into account the material required to set up conditions. Thus, the experiments would have required a very large mouse colony not available to us. We verified binding sites in primary pancreatic islets, and could confirm all tested sites.

Other researchers have achieved to perform ChIPseq on smaller amounts of starting material. This depends on the protein analyzed, and often experiments using low amounts of cells were achieved for histone modification and not transcription factors. Histone modifications are present much more frequently in the genome than transcription factor binding sites. Therefore, one order of magnitude more chromatin is precipitated with antibodies directed against histone modifications than with antibodies against transcription factors. ChIPseq for transcription factors has also been done on smaller amounts of cells, but this requires sophisticated techniques established in a few advanced epigenetic laboratories. This is currently not available in our institute.

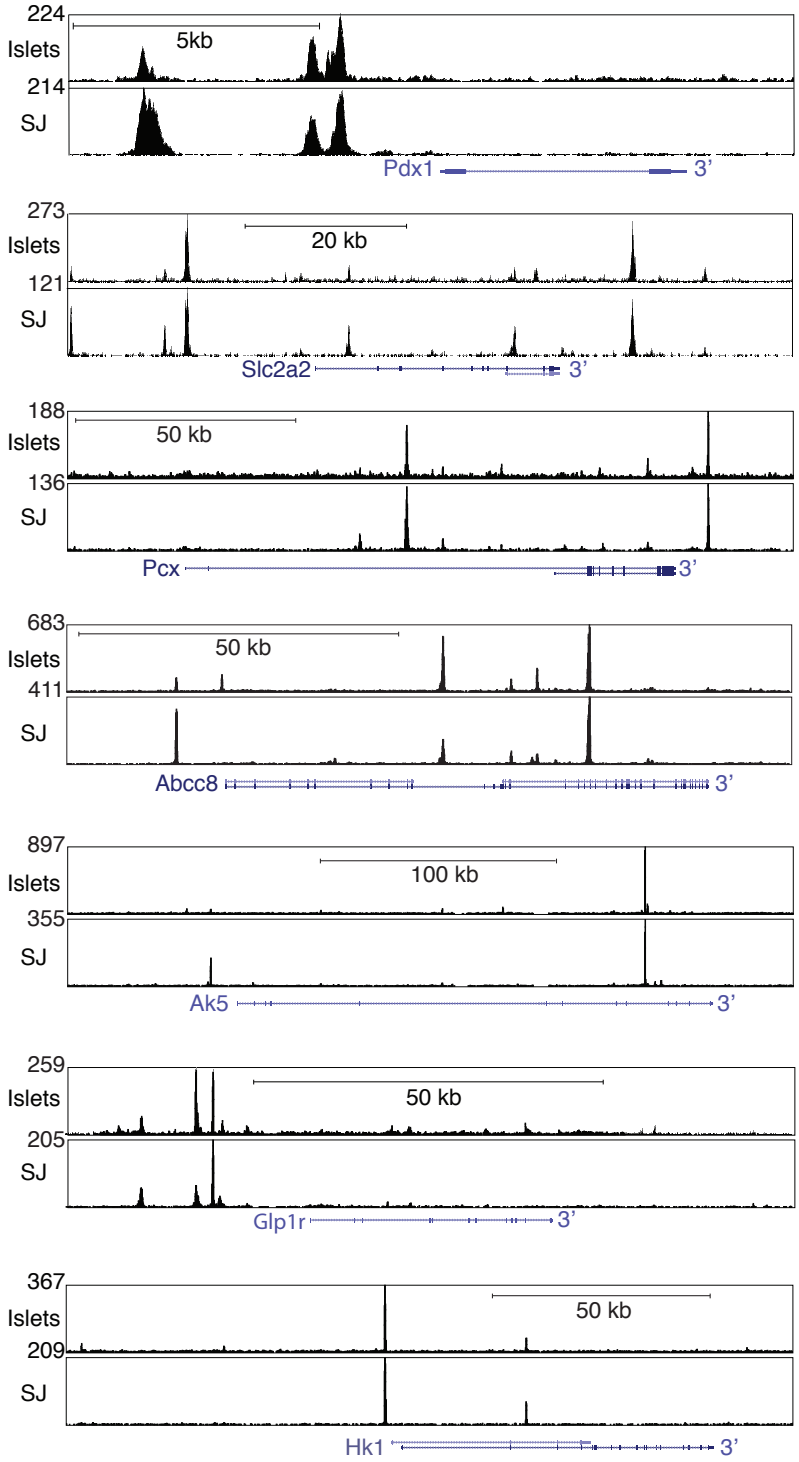
Initial experiments were also performed with Min6 cells that are widely used as β-cell model. Min6 cells available to us showed around 3-4 fold increase in glucose-induced insulin secretion, and we were unsuccessful when we tried to obtain earlier passage numbers that were reported to display better responses. We therefore established a new cells line (SJ β-cells) and used these cells at low passage number. These cells show 8-10 fold increase in glucose-induced insulin secretion, comparable to induction ratios observed using dissociated mature primary b-cells (Halban PA, Endocrinology, 1982). SJ β-cells also respond to incretins like exendin-4 and GIP, which was only observed with particular Min6 subclones.

Even more, when we take into account that the comparison is a b-cell like cell (SJ cell) vs a mature islet (which is a mix of several cells), then this 10x difference is even bigger. This point should be addressed or, at least, a re-Chip PCR experiment on these genes should be performed on both wt and mutant islets and the potential drawback of the experiment discussed clearly during the manuscript.

We have performed one ChIPseq experiment on primary islets using Foxa2 antibodies, which we do not include in the manuscript since no duplicate was done. The background observed in this dataset is higher than background obtained with SJ β-cells; if we normalized the cutoff to the total number of reads mapped as we did before (peak height 80 and FDR of 5%), 77% of the sites defined *in vivo* overlap with sites identified in SJ β-cells. With more stringent cutoffs, the number of binding sites is smaller, but the overlap increases to up to 95%. Thus, Foxa2 binding sites *in vivo* and *in vitro* overlap to a large extent.



Below are included sample traces from *in vivo* and *in vitro* ChIP-seq experiments of Insm1-dependent genes implicated in glucose-dependent insulin secretion.



In addition, we now performed re-chip experiments on primary wild-type islets, which are included in the revised manuscript (Fig.7 B,D). In order to perform these in duplicate, we used about 6.000 islets or material from around 60 mice. In such experiments, we estimated that around 0.5 ng of DNA was precipitated with the first antibody, and 0.05 ng in the re-ChIP. This can no longer be measured, but we know that the IPs worked since the enrichment for sites predicted from the ChIP-Seq experiments but not for control sites were again observed. We did not include a ChIP-reChIP

experiment on mutant material for two reasons. First, we envisaged problems to design appropriate controls. We expect the same outcome regardless whether the experiment would fail (i.e. chromatin would be lost during precipitations), or that regulatory sequences were no longer precipitated by Insm1 antibodies in the coInsm1 mutant. Considerably less efficient precipitations would be expected given the fact that Insm1 protein is very much downregulated in coInsm1 islets. Secondly, we would have needed to generate islets from 60 coInsm1 mutants, which would require an amendment of our current animal experiment permit that covers work on the coInsm1 mutants (our permit to keep mutant mice/introduce a mutation allows the use of a defined mouse number).

Other comments of referee #2

1) In fig 1A both panels have poor resolution. More important, Fig 1 A has some flaws that need correction:

In the top panel, It seems that all cells in the presented islet are b-cells? . Nuclear co-staining with nuclear marker (e.g. dapi) is missing and would facilitate the evaluation of that.

Bottom panel: The authors claim in the text that Insm1 is expressed in the other cell types (a,d, pp) but this conclusion is impossible to draw from the staining presented. The figure reads as "glc/Stt and PP" in green. I understand from the Suplementary table 9 the authors have used antibodies against glucagon/ Stt and pp all combined together?
the Info regarding the staining protocol followed is missing on the figure legend and is not possible to evaluate how the experiment is being conducted. Without using specific antibodies separately, it is not possible to conclude if Isnm1 is expressed in the other non-b-cell types and in which of them.
Again, counterstaining with dapi would be advisable.

The resolution was not optimal and might have been particularly problematic in the PDF provided for the review. The panels labeled *Glc/Stt/Pp* indeed relied on the use of a mixture of antibodies, which was not mentioned. In the revised Fig. 1, we no longer use this mixture and instead show data using the antibodies against each hormone separately. As requested, we include DAPI counter stains. Cells in the islet are positive for Insm1 and glucagon, somatostatin or pancreatic polypeptide and localize to the periphery - their typical distribution in murine islets.

Also, What area is represented in the magnifications?

We indicate the magnified area in the revised Fig. 1.

2) by using RIPCRE the authors mention that they selectively delete Insm1 in b-cells" pronounced reduction of Insm1 protein in coInsm1 mice in Pdx1high β-cells by immunohistology or in isolated islets..." (Fig1B). However, Co-staining with a specific b-cell antibody (e.g. insulin) is missing. This is an essential staining control to be able to draw that conclusion. Also taking into account that the entire paper is based on using RIP-CreER to study the role of Insm1 specifically in b-cells this staining becomes essential.

We now include additional data in the revised Fig. 1 to show that RIP-CreER-dependent recombination is efficient and β-cell specific using an indicator that expresses tomato before and GFP after recombination, respectively. In addition, we show Insm1 and Insulin co-staining without or with recombination (control and coInsm1 islets in revised Fig.1M,O, respectively).

3) in Figure 2 a-b, the protocol used is missing. Also, according to figure legend, n=3 for the quantification of b-cells, what does it refer to? 3 slides, 3 sections, 3 mice?

We used 3 animals, and 6-8 slides from each animal. In the revised manuscript we mention the number of slides/animals in figure legends. The protocol was shortly mentioned in Material and methods of the original manuscript, and extended in the revised manuscript.

3) in table S6 the total number of deregulated genes in the within 50Kb binding sites should be corrected. I guess is 536.

We corrected this mistake.

4) Concentrations of secretagoges, e.g. Exendin 4, ...are all missing.

The used concentrations are included in the revised manuscript (legend to Fig. 4).

5) Check for consistency between "methods" and "experimental procedures"

In the revised manuscript, we use consistently the term ‘Materials and methods’.

Accepted 10 March 2015

I am pleased to inform you that your manuscript has been accepted for publication in The EMBO Journal.

Please allow me to congratulate you to a very nice, insightful and relevant study.

REFEREE REPORT

Referee #2:

I have read the revised version of the manuscript from Birchmeier's lab entitled "Insm1 cooperates with Neurod1 and Foxa2 to maintain mature pancreatic β-cell function" with great interest. After reading the responses of the authors I am convinced that my major concern (why not using islets instead of a cell-line) cannot be address due to technical and cost-effective issues. The new data provided in figure 7 (re-Chip in islets) and the comparisons presented on the letter to the reviewer (Chip-seq Pics) are convincing and reassuring, and partially address this concern.
My other concerns regarding the staining figures and the co-localization with Insm1 are all addressed.

As stated on my previous letter, I believe this manuscript can be of great interest to the pancreas community and highlights the importance of regulatory networks in the maintainance of cell identity in adult tissues, in that case, the pancreas islet. Therefore, I recommend the manuscript for being published at EMBO J.

