EMBO reports

Manuscript EMBO-2015-40538

DAZL regulates Tet1 translation in murine embryonic stem cells

Maaike Welling, Hsu-Hsin Chen, Javier Munoz, Michael U. Musheev, Lennart Kester , Jan Philipp Junker, Nikolai Mischerikow, Mandana Arbab, Ewart Kuijk, Lev Silberstein, Peter V. Kharchenko, Mieke Geens, Christof Niehrs, Hilde van de Velde, Alexander van Oudenaarden, Albert J.R. Heck and Niels Geijsen

Corresponding author: Niels Geijsen, Hubrecht Institute

Review timeline:

Transfer date:	05 May 2015
Editorial Decision:	07 May 2015
Revision received:	08 May 2015
Accepted:	08 May 2015

Editor: Barbara Pauly

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.)

Transfer Note:

Please note that this manuscript was originally submitted to the EMBO Journal where it was peer-reviewed and revised. It was then transferred to EMBO reports with the original referees comments attached. (Please see below)

Authors' response to original referees' comments - EMBO Journal

Response to Review comments:

We would like to thank the reviewers for the time taken to thoroughly read our manuscript and provide useful feedback. All three reviewers thought our data were interesting but too preliminary. In hindsight, we can only agree. We have now revised our manuscript and included mass-spec analysis of cytosine methylation and hydroxymethylation, which independently support our finding

that Dazl is an important regulator of Tet1-mediated hydroxymethylation. In our previous submission we had already included data indicating that loss of Dazl expression stunts cytosinehydroxymethylation. In this revised manuscript we have included mass-spec analysis to independently back-up this observation and in addition, we have added data demonstrating that, as expected, ectopic Dazl expression induces elevated Tet1 protein levels and cytosine hydroxymethylation.

We are grateful for the supportive feedback provided, as the reviewers' comments have been helpful in further strengthening the conclusions in our manuscript.

Below, we address prior concerns in a point by point fashion.

Referee #1:

This manuscript by Welling et al. studies Dazl, a RNA-binding protein, in the process of a naïve transition of embryonic stem cell cultures (ESCs) upon treatment with GSK3β and MEK inhibitors (2i conditions). The authors identified Dazl as a marker of a subpopulation of ESCs that is actively transitioning towards naïve pluripotency. They claim that Dazl plays an essential role in the active reprogramming of cytosine methylation via the induction of Tet gene expression, which is required for hydroxylation of 5-methylcytosine. They further propose that cells in 2i conditions lacking Dazl show impaired 5-hydroxymethylation, and that demethylation occurs in a delayed fashion and passively in these cells through downregulation of DNMTs. Overall, the paper is well written and addresses a relevant and interesting scientific topic. However, some of the experiments are in a too preliminary stage and some do not fully support the proposed model. In addition, the authors tend to overstate some of their findings. The following points need to be addressed:

Major points

1. Figure 1A, 1B and Supplementary Figure 1B: The authors show upregulation of Dazl in cultured ESC cells under 2i conditions. It would be interesting to see whether Dazl is upregulated when pluripotency is induced by other means, such as in the production of iPSCs. *Dazl is expressed in ESCs as well as in primordial germ cells, but absent in somatic cells. During reprogramming of somatic cells to iPS cells, Dazl can only be detected at the final stage of the process, when cells become stably reprogrammed. Ideally, one would like to perform single-cell analysis of the link between Dazl expression and hydroxymethylation levels, but this is technically not feasible due to the scarcity of the material, the heterogeneous and infrequent nature of the reprogramming process and the inability to prospectively identify successfully reprogramming cells.*

2. Figure 3B/upper panel: The authors show decreased 5meC levels upon 2i conditions by dot blot. The authors should confirm the observed differences in 5meC using an additional method, such as mass spectrometry.

We agree. We have now independently quantified 5mC levels in Dazl positive and Dazl negative cells in serum as well as in 2i conditions using mass spec, as shown in figure 3C. We found that global 5meC levels are indeed deceased in 2i culture conditions.

3. Figure 3B/lower panel: The authors demonstrate that Dazl positive ESCs in serum are more highly hydroxymethylated compared to Dazl negative ESCs in serum. Again, the authors should confirm the observed differences in 5hmeC levels by mass spectrometry. It is further suggested that the authors characterize the difference in 5hmeC between Dazl positive and Dazl negative ESCs on a genome-wide level using 5hmeC sequencing. This approach could provide informative target regions that are differentially hydroxymethylated in the presence or absence of Dazl and these regions could be further explored, e.g. gene ontology analyses, to better understand the role of Dazl in the process of TET-mediated reprogramming to a naïve pluripotent state.

We have quantified 5hmC levels in Dazl positive and Dazl negative cells in serum as well as in 2i conditions as shown in figure 3C. We found that global 5hmC levels are significantly higher in Dazl positive cells in serum culture.

The reviewer addresses an interesting question with the suggestion of sequencing for 5hmC regions in Dazl positive and Dazl negative cells in serum. Hackett et al. recently demonstrated that pluripotency and germline genes are highly hydroxymethylated in serum cultured ESCs. Our gene expression data in Dazl positive ESCs (Figure 3A) corroborates these findings at the gene expression level, demonstrating essential differences between Dazl positive and Dazl negative cells.

4. Figure 3D: The authors show an increase in 5hmeC in Dazl negative cells under 2i conditions indicating TET-mediated 5-hydroxymethylation and active demethylation. They mention but do not discuss such increase. Specifically, the authors should discuss this observation in the context of passive DNA demethylation, which they discuss as the solely mechanism of demethylation in Dazl negative cells (Page 2: "As a result, when Dazl-knockout ESCs are cultured in 2i conditions, active 5-hydroxymethylation is impaired and cytosine demethylation occurs solely through passive mechanisms").

We agree, and have further clarified this in the text. Dazl negative cells are sorted from a cell population in which Dazl is heterogeneously expressed. We included an overview figure of the experimental setup (Figure 3D). We find that Dazl is upregulated upon conversion to 2i conditions. Supplementary figure 4 describes our analysis of DNA demethylation in Dazl knockout ESCs, and demonstrates that hydroxymethylation is impaired in these cells.

5. Figure 4C: The differences in DNMTs expression between Dazl +/- and Dazl-/- cells are not convincing. The authors overstate their findings by concluding that this implies an increased passive

demethylation in Dazl -/- cells. The authors should increase the number of samples or provide an explanation. They should also discuss the fact that Dazl +/- cells show downregulation of DNMTs, especially DNMT3a and DNMT3b, upon 2i conditions indicating passive demethylation.

The transition of serum cultured ESCs to the 2i condition coincides with DNA demethylation. This occurs via active and passive demethylation pathways and indeed also with the downregulation of de novo methyltransferases (DNMT3s). We have clarified this downregulation of DNMTs in the text and have increased the number of repeat experiments (N= at least 4 biological replicates) for more biological replicates with other Dazl -/-, Dazl +/- and Dazl +/+ ESCs lines from the same background.

6. Figure 4D: The enrichment for Tet mRNAs in RIP experiments is somewhat weak, especially for Tet2. Again, the results of this experiment are overstated and appropriate controls are lacking. First, a t-test is suggested to evaluate the significance of the differences. The authors should also include positive and negative (mRNAs that are not enriched) controls into RIP experiments. Further, it would be interesting if the authors could perform the reciprocal experiment (biotinylated Tet1 and Tet2 mRNA-pull down followed by Dazl western blot) to prove the binding of Dazl to Tet1 and Tet2 mRNAs. Lastly, the authors should perform experiments to show that Dazl regulates Tet1 and Tet2 mRNAs as claimed in the discussion (Page 11: "Dazl plays an essential role in the process by binding and regulating Tet1 and Tet2 mRNAs..."). This could be achieved through ribosome/polysome fractionation followed by qPCR or sequencing.

We have further optimized our RNA-IP protocol by including a blocking step with yeast tRNA and also performed IP for GFP as an additional control. We have performed 3 biological replicates for both the UV-crosslinking RNA-IP as well as for the native RNA-IP. Our results demonstrate a significant enrichment of Tet1 mRNA in the Dazl RNA-IP indicating that Dazl binds Tet1 (figure 4C). In contrast, Tet2 mRNA was not enriched (Supplementary figure S4B). To test whether Dazl also induces Tet1 protein translation, we made an ES-cell line in which the expression of Dazl is controlled by a doxycycline inducible promoter. Using this line, we find that Dazl overexpressed results in upregulation of Tet1 protein levels, while Tet1 mRNA levels remain stable, further corroborating our conclusion that Dazl regulates Tet1 protein levels (Figure 4D).

7. Figure 5: This model is not fully supported by the presented data. The authors make a too simplistic division of DNA demethylation pathways, especially as they suggest only the active demethylation pathway for Dazl positive cells. This is in contrast to Figure 4C, where Dazl positive cells show downregulation of DNMTs, especially DNMT3a and DNMT3b, upon 2i conditions indicating passive demethylation in these cells.

We did not mean to claim that Dazl positive cells only undergo active demethylation, but realize this was not very clear. We made textual clarifications that 5hmC can be followed by active as well as passive demethylation mechanisms, since 5hmC is not recognized by DNMT1 and is replicatively lost. We also changed our model figure (figure 5) to emphasize that the role of Dazl mainly impacts Tet1 mRNA translation which results in increased hydroxymethylation, but also shows that DNMTs

remain important and that global DNA demethylation does not take place if DNMT3s remain upregulated.

8. Supplementary Figure 3B: This figure shows transcriptional upregulation of naïve genes in Dazl positive as compared to Dazl negative cells. It would be of interest to show methylation and hydroxymethylation states of these genes with or without 2i condition and with or without Dazl.
The observation that naive genes are upregulated in Dazl positive cells in serum conditions, does not necessarily mean that these genes are also directly regulated by Dazl or hydroxymethylaion. There might be additional upstream or downstream factors involved supporting the demethylation and expression of these genes in serum. While we agree that whole genome bisulfite sequencing of the promotors of genes in DazlGFP+ and DazlGFP- cells could provide interesting insights in the regulation of demethylation in different populations of ESCs in serum, we feel that this is a separate study that falls outside the scope of this paper.

Minor points

• Page 5 and Supplementary Figure S1A: " Upon FACS separation of DAZL-GFP-positive from the DAZL-GFP-negative cells, the sorted cells re-establish the original equilibrium within a few days...". D0 or D1 should be shown as a time point in that figure. That would make the re-establishment of the original equilibrium much clearer.
We modified this figure and included D0, D1, D2 and D3 time points to clarify the re-establishment of the original equilibrium.

• Page 6: "In contrast, we find that a series of germ-cell related genes, including Stella, Lefty1 and Tcfap2c...". If it is supposed to be in Figure 1D, this was not seen because the resolution was not good enough.
These genes are among the genes represented by the heatmaps in figure 1C. The genes belonging to each cluster can be found in supplementary table S3.

• Page 8: "5-methylcytosine can be oxidized to 5-hydroxymethylcytosine (5hmC) by the Tet enzymes (Tet1-3) preceding loss of DNA methylation". Authors should mention that the conversion of 5mC to 5hmC isn't always a step towards demethylation. 5hmC has its own reader proteins and is increasingly regarded as a independent epigenetic mark as well.
We now added a more detailed description of the regulation of DNA demethylation in ESCs.

• Page 12: "Loss of global cytosine demethylation, which takes place when ESCs transition to a naïve pluripotent state...". It is suggested to change "cytosine demethylation" to "cytosine methylation" since cytosine demethylation does not make sense in that context.
We thank the reviewer for the suggestion and have modified the sentence.

• Figure 3B: It needs to be more clearly indicated what are Dazl positive and Dazl negative ESCs in this figure.

We added DazlGFP+ and DazlGFP- to the figure for clarification.

• Figure 3C: Has the p-value been computed for DNMT3b expression between DazlGFP+ and DazlGFP- as well as between 2iDazlGFP+ and 2iDazlGFP- cells? The values need to be displayed.

We have moved the figure to supplementary figure S3C. The corresponding p-values are now displayed and are not significant.

• Figure 4A: For the hmC dot blot, the background signal is higher than in Figure 3D. This makes it difficult to evaluate if the hmC is truly not there in Dazl -/- cells at d5 and d6.

We performed new dotblot analysis (supplementary figure S4D) and also quantified 5mC and 5hmC by mass spectrometry for some of the time points (Figure S4C), which more clear and reliable insight in the differences between Dazl -/- and Dazl +/+ ESCs in 2i conditions.

Referee #2:

In this manuscript the authors report on the expression a BAC-derived DAZL-GFP reporter in mouse embryonic stem (ES) cells in serum/LIF and under 2i culturing conditions. They suggest that DAZL expression marks a subpopulation of ESCs that is actively transitioning toward naïve pluripotency. Less convincingly they suggest that Dazl plays an essential role in the active reprogramming of cytosine methylation, when ES cells transition to the 2i state by regulating TET expression; which are required for hydroxylation of 5-methylcytosine. The data demonstrating that that Tet1 and Tet2 are mRNA targets of Dazl is poor and not backed up by targeted mutation experiments. The experiments presented do not robustly support statements in the abstract and throughout the manuscript.

This issue was also raised by Reviewer #1 and we agree that our data on the Dazl-regulation of Tet1 could be further strengthened. We have performed additional RNA-IP experiments with more biological replicates and now demonstrate robust p-values in our Dazl-mRNA co-IP experiments. We performed 3 biological replicates for both the UV-crosslinking RNA-IP (allows more stringent washing steps, but crosslinking could results in false positives) as well as for the native RNA-IP (more sensitive to washing steps so may results in false negatives). We demonstrate that the interaction between Dazl and Tet1 mRNA is robust in both methods, while Tet2 mRNA is not enriched.

The Dazl binding motif is abundantly present in the 3'UTR of Tet1, but since this 3'UTR sequence is very long (>6kb) and includes >100 predicted Dazl binding sites, targeted mutation of Dazl binding sites is unfeasible. Instead, we have made an ES-cell line in which Dazl expression can be induced by doxycycline. Using this cell line, we find that Dazl overexpressed results in increased Tet1 protein levels and elevated DNA hydroxymethylation (figure 4 and 4D), while Tet1 mRNA levels

remain constant. We believe that these results strongly support the role of Dazl Tet1-mediated hydroxymethilation in mESCs.

ES cells are derived from mammalian embryos during the transition from totipotency (when individual blastomeres can make all lineages) to pluripotency, when they are competent to make only embryonic lineages. When maintained with inhibitors of MEK and GSK3 (2i) ES cells are thought to represent an embryonically restricted ground state. However recent work has shown that the extraembryonic endoderm marker Hex exhibits heterogenous expression in 2i ES cultures and this fraction is 'primed' to differentiate into trophoblast and extraembryonic endoderm. Thus like cells grown in Lif/serum, heterogeneity also exists in the 2i state. This challenges some of the statements in the introduction of this manuscript.

We agree with the reviewer that heterogeneity in gene expression is also observed in 2i cultured ESCs. However, compared to ESCs cultured in conventional serum conditions, this heterogeneity very much decreased. We have further clarified this further in the introduction.

A major problem with this study is that it is based on a transgenic DAZL promoter driven GFP construct. Since the 'crux' of this paper depends on endogenous protein function, the authors need to show that DAZL protein expression matches their reporter expression in ES cells and in embryos. There is no direct evidence that their reporter faithfully expresses endogenous DAZL patterns. The data in figure 1B is not convincing. In addition their Nanog patterns do not match previously reported staining profiles in ES cells grown in serum/Lif versus 2i cultured ES cells. Although the use of Nanog-GFP and Stella-GFP reporter ESCs is reported in the text, they are not mentioned in the materials and methods.

While revising this manuscript, our derivation of the Dazl-GFP cell line was published (Chen et al. 2014, Stem Cell Reports). This paper demonstrates that Dazl expression is observed exclusively in the developing germline and in ESCs. In this manuscript we performed single molecule FISH on wildtype blastocysts and find mRNA expression in a similar fashion as observed for the Dazl-GFP blastocyst embryos. We now included an additional figure showing the correlation between Dazl mRNA expression and Dazl-GFP expression in the Dazl-GFP ESCs (supplementary figure S1A).

The Nanog-GFP and Stella-GFP ESC-lines we used were previously published (Nanog-GFP, Hatano et al 2005) (Stella-GFP, Payer et al 2006). We observe an increase in Nanog-GFP expression upon switching to 2i culture conditions which is in accordance with previous reports (Miyanari et al 2012).

It is not clear why they are 'surprised' to find that 2i culture conditions increased the expression of Dazl-GFP, since the same conditions have been reported multiple times to increase endogenous Dazl transcripts in hypomethylated 2i treated ES cells.

While we knew that Dazl was previously reported to be upregulated in 2i conditions, we did not expect such a large increase from 5% of the cells in serum to 80% of the cells in 2i. This could

indicate that Dazl plays an important role in naïve pluripotency while the role of Dazl in pluripotency has never been reported before.

The conclusion that 'Dazl-expression in cultured ESCs is not the result of aberrant gene expression, but reflects the expression of this gene in the late pre-implantation blastocyst' makes no logical sense, it is an extrapolation without obvious foundation.
We agree with the reviewer that this statement is too speculative and have removed this sentence from the manuscript.

The section on DNA modification is also weak. Loss of DNA methylation is a hallmark of 2i exposure but requires a long period of 2i exposure (up to 21 days) to reduce global 5mC levels by over 60%. It is not clear if this is a driver or a consequence of this in vitro process. The methods to investigate 5mC and 5hmC levels are qualitative, not done in multiple biological replicates and no loading controls are presented. The data is weak at best. This has a subsequent negative impact on the data presented in figure 3D, which are again presented without proper controls. This criticism precludes from saying that 'Dazl marks a subpopulation of ESCs that is more akin to 2i-induced naïve pluripotent stem cells' as much of the supporting data is qualitative at best and in reality has not been performed to a high standard.
While it was reported that culture up to 12 days of ESCs in 2i conditions is necessary to achieve a naïve demethylated ground state, many DNA methylation marks are already removed by day 3 and most by day 6-8 in 2i conditions (Ficz et al 2013). DNA methylation was analyzed by dotblot and respresentative images of at least 3 independent experiments were shown. Each slot in the dotblot was loaded with equal amounts of genomic DNA of which the concentration was accurately measured using Qubit. For clarification we added the amount of DNA loaded per slot to the figures. Also we now included LS-MS quantification of at least 3 biological replicates per sample to verify dotblot results (Figure 3C, supplementary figure S3B). Now that we added these quantification values, our claim that Dazl positive cells have high hydroxymethylation and are actively reprogramming to a naïve pluripotent state is much stronger.

There are similar problems with the idea that DAZL binding stabilizes Tet genes. To test this in reality the authors have to identify the DAZL binding sites in the TET transcripts and demonstrate that mutation of these sites abolishes their dependence on Dazl for 'stabilisation'. The RNA-IP is not believable, being barely above background and not significantly stabilised by UV x-linking. The error-bars for this data like many other data analysis in this manuscript are so large in scale that the veracity of the data is brought into question.
We have now optimized our RNA-IP protocols by the adding an additional blocking step using yeast tRNA reducing the chances of false positive results. Also, repeated the RNA-IP experiments with 3 biological replicates for both the UV-IP as well as the native IP and can now present convincing data for the enrichment of Tet1, but not Tet2 genes in Dazl protein complexes with reliable significant p-values (Figure 4C, supplementary figure S4B). We agree with the reviewer that

showing an enrichment of Dazl binding to mRNA molecules of a particular gene is not sufficient to claim a role for Dazl in stabilization of this gene. Additional data in Dazl overexpression experiments and Dazl knockout cells result in a clear upregulation of Tet1 protein, but not Tet1 transcription, when Dazl is overexpressed. Together we can conclude that Dazl binding to Tet1 mRNAs supports Tet1 translation.
The large size of error bars in some qRT-PCR results is due to heterogeneous starting cultures which are vary in pluripotency state when an experiment is started. Therefore, the use of independent biological replicates can result in large error bars but similar trends between different ESC-lines and p-values are calculated by t-test based on at least 3 biological replicates per sample.

The data and ideas presented in this manuscript are preliminary at best. It requires a more thorough investigation to support the exaggerated conclusions presented here.
The generation of an inducible Dazl ESC-line, the addition of more biological replicates per sample and the quantification of 5mC and 5hmC levels using mass spectrometry provide more qualitative and quantitative data to support our claim and gives more detailed insight in the role of Dazl in hydroxymethylation in serum cultured ESCs.

Referee #3:

In this manuscript, the authors describe that ES cultured in serum can be sorted into two populations depending on Dazl expression. Furthermore, Dazl positive cells correspond to a more naïve state according to their transcriptomic and proteomic profile as well as to their low methylation level. Finally, the authors suggest that this low methylation level is due to enhanced Tet1 and Tet2 translation by Dazl.

The hypothesis of this manuscript is very interesting and very provocative. However, I think that some points need to be clarified and that some conclusions need to be better supported:

-In the abstract, the authors state "Dazl marks a subpopulation of ES that is actively transitioning toward naïve pluripotency". As Dazl is expressed in the final naïve state, a Dazl positive cell can be both a transitioning-cell or a fully reprogrammed-cell. In this scenario, is it accurate to consider Dazl a marker for cells that are actively transitioning?
While gene expression of Dazl positive cells in serum already present a pattern more similar to naïve ESCs in 2i conditions, these cells still exhibit high 5mC levels similar to Dazl negative ESCs in serum. While we do observe significant higher levels of 5hmC in Dazl positive cells, they have not reached a demethylated ground state yet in serum culture. However, we show that these high levels of 5hmC in Dazl-positive ESCs facilitate their rapid transition to a naïve ground state.

-Figure 2. To confirm that Dazl-GFP reporter follows endogenous Dazl expression, the authors

performed a single molecule RNA FISH. However, the presence of mRNA does not always correlate with the protein. As there are many DAZL available antibodies, the expression of DAZL protein should be confirmed by ICC in embryos and ES.

While we were doing revision experiments for this paper, the paper describing the derivation of the Dazl-GFP cell line was published (Chen et al. 2014, Stem Cell Reports). In that paper we show that Dazl expression is observed in the developing germline and in ESCs faithfully recapitulating endogenous gene expression, and that it is absent in other tissues. In this manuscript we performed single molecule FISH on wildtype blastocysts and find mRNA expression in a similar fashion as observed for the Dazl-GFP blastocyst embryos. To further confirm the linear relationship between Dazl mRNA and protein expression, we have included an additional figure showing the correlation between Dazl mRNA expression and Dazl-GFP expression in the Dazl-GFP ESCs (supplementary figure S1A). Furthermore, we have performed mass spectrometry on ESCs transitioning in 2i conditions at different time point, and demonstrate the upregulation of endogenous Dazl protein in a similar fashion as we have seen for our GFP reporter using FACS and immunofluorescent stainings together indicating our reporter expression carefully recapitulates endogenous Dazl expression (Figure 1C, supplementary table S3).

-The authors state "Dazl marks unique naïve cells within the heterogeneous ES population akin to the cells in the naïve ICM". However, 2i naïve ES also exhibit a Dazl negative population, thus, do 2i-Dazl negative ES correspond to primed pluripotent ES? If yes, would it mean that 2i naïve state still contains a primed subpopulation? In Figure 2A and 2B, Dazl is only expressed in some ICM cells, are the Dazl-negative ICM cells not naïve pluripotent cells?

Dazl positive and Dazl negative cells in 2i conditions both show global DNA demethylation. We now included additional qRT-PCR analysis for primed pluripotency genes to test if Dazl negative cells in 2i cultured ESCs are in a primed pluripotent state. We observe no difference in the expression of primed pluripotent marker genes compared to Dazl positive cells in 2i conditions. Indeed, in supplementary figure S3A we show that there is no difference in the expression of naïve pluripotency genes between Dazl-GFP positive and Dazl-GFP negative cells in 2i conditions. Therefore, in stable 2i conditions both Dazl-negative and positive ESCs are in a naïve state.

-Figure 3A. The authors compare serum-Dazl positive, serum-Dazl negative and 2i-Dazl positive cells at the proteomic level. However, 2i-Dazl negative cells are missing in this comparison and should be included together with EpiSCs, as a control for primed pluripotency. Furthermore, presenting the proteomic profile of all 5 populations in an unsupervised hierarchical clustering and in a principal component analysis will help the reader to evaluate the similarities between them. In addition, the analysis of several naïve and primed pluripotent markers by qRT-PCR in all 5 populations is also required to ensure that Dazl is a marker of naïve cells.

While ESCs cultured in 2i conditions are more comparable to each other and have homogenous gene expression levels, some heterogeneity is still observed, but at much lower levels than in serum cultured ESCs. The observation that Dazl is expressed in 80% of 2i cultured ESCs reflect this low

heterogeneity in 2i conditions, but transcript levels of other genes are generally similar in both populations. Therefore, we expect that the additional analysis of DazlGFP negative cells in 2i would results in a similar protein expression pattern as observed for DazlGFP positive cells in 2i and would provide limited value.

EpiSCs are pluripotent, but represent a very different pluripotent cell type in which Dazl is not expressed (Tesar et al 2007). We therefore feel that analysis of EpiSCs is outside the scope of this manuscript, which focuses on the role of Dazl in regulating Tet1-mediated DNA hydroxymethylation.

-Figure 3B. As 2i-Dazl positive and negative cells express the same 5hmC levels, how can the authors exclude that the 5hmC differences between serum-Dazl positive and negative cells are not due to a gene other than Dazl? Are the levels of Tet1 and Tet2 statistically different between serum-Dazl negative and 2i-Dazl positive and negative cells? The error bars in Tet1 chart are too high to draw conclusions.

We did not mean to claim that Dazl is the only gene in ESCs involved in the observed differences in 5hmC and Tet gene expression. However, our additional RNA-IP experiments as well as Dazl overexpression in ESCs now provide further support for the role of Dazl in regulating Tet1 expression and hydroxymethylation in serum cultured ESCs. We have modified the text to clarify this point.

-Sup Fig 3B. Dazl negative cells at day 10 express 8-times more Dazl than Dazl negative cells at day 0. In fact, Dazl negative cells express equivalent Dazl levels than Dazl positive cells at d10. The authors should comment on this observation since these results suggest that the Dazl-GFP reporter does not faithfully correlate with Dazl expression. Finally, the authors state that 2i-Dazl positive cells are more similar to naïve ES than 2i-Dazl negative cells after 3 days of culture in 2i medium. However, Prdm14, Tfcp2l1 and Dazl differences between Dazl positive and negative cells are not significant at day 3. In addition, the level of these 3 genes at day 3 is lower than at day 0, and in consequence, more distant to day 10. The authors should comment on this.

We would like to thank the reviewer for this comment since this made us realize that our figure is probably not sufficiently clear. The qPCR data shown in supplementary figure S3C shows expression levels in Dazl-positive and Dazl-negative cells that were sorted at D0 and are subsequently culture in 2i conditions. Therefore, by d10 also the cells that were originally grown from a Dazl-GFP-negative cell population, have re-established their Dazl expression at this time point to similar levels as cells that are derived from Dazl-GFP-positive cells. We adjusted the legend in this figure to clarify the point that Dazl-GFP+ or Dazl-GFP-negative relates to the status of the cells at day 0.

While global transcript levels go down in ESCs cultured in 2i conditions (published by Grun et al 2013), individual transcripts are more homogeneously distributed in 2i conditions. In serum conditions, expression levels of pluripotency genes are much more heterogeneous, but global levels of expression will not show this heterogeneity in qPCR results. Therefore, we performed smFISH of

Dazl-GFP+ and Dazl-GFP- cells sorted at d0 and cultured for 3 days in 2i to show transcript variation between these populations with different origin. Figure 3F shows more homogeneity in transcript expression between different cells in cells that had Dazl-GFP+ cells as a starting population, but qPCR results as shown in supplementary figure S3C is based on total RNA levels, making it more difficult to draw conclusions about heterogeneity in gene expression. As ESCs are still transitioning towards a naïve pluripotent state at day 3 in 2i conditions, the global gene expression levels have not reached their final levels (as found by day 10) yet, but cells do already show the establishment of more homogeneity.

-Dazl cannot affect transcription since it is not a transcription factor. In contrast, Dazl is located in the cytoplasm where it plays a role in mRNA stabilization and translation. Indeed, the authors suggest that Dazl promotes translation. In this scenario, the mRNA levels of Dazl target genes should not be affected by the presence or absence of Dazl and only the protein levels of Dazl target genes should be affected by the presence or absence of Dazl. However, most of the results of this manuscript are based on mRNA changes and not on protein changes. The authors should comment on this.

The reviewer addresses an excellent point. Dazl in a mRNA binding protein and therefore observed transcriptional changes are indirect, potentially via hydroxymethylation followed by demethylation of the promoters of the genes upregulated in Dazl positive cells. Dazl has been reported to function as a translational enhancer in germ cells and therefore we investigated the effects on Dazl overexpression on actual protein levels. Since we observed high hydroxymethylation values in Dazl positive cells, we hypothesized that Tet genes were involved in the upregulation of 5hmC by Dazl. We have now included data demonstrating that while Tet1 mRNA levels remain relatively constant upon induction of Dazl, Tet1 protein is induced with concomitant elevation of DNA-hydroxymethylation when Dazl is overexpressed. Together, these results strongly support the role of Dazl in stabilizing Tet1 mRNA protein translation.

-Figure 4B. If Tet1 and Tet2 transcript levels directly depend on DAZL, shouldn't be the 'Tet1 mRNA in Dazl KO'/ 'Tet1 mRNA in Dazl WT' ratio constant? As it is not, do these results suggest that the Dazl effect on Tet1 is an indirect effect? The same for Tet2.

We performed additional qPCR experiments on several Dazl knockout, Dazl heterozygous and also Dazl WT lines from the same background with at least 3 biological replicates per cell line to address this point. We observed no significant differences in Tet1 and Tet2 transcript levels between all of these lines in serum conditions (supplementary figure S4E). However, when Dazl KO cells are cultured in 2i conditions they display a delayed and lower activation of Tet1 protein levels compared to Dazl WT cells. This indicated that Dazl is essential for the timely Tet1 mRNA translation in 2i conditions. In Figure 4E we further analyse the role of Dazl in regulating Tet1 protein levels and demonstrate that induction of Dazl indeed results in an upregulation of Tet1 protein while leaving Tet1 mRNA expression unchanged.

-Figure 4D. Are these results statistically significant? A gene known of being bound by DAZL should be included as a positive control to help the reader to interpret the data.

We optimized our RNA-IP protocol by including an additional blocking step with yeast tRNA and performed both the UV-IP as well as the native IP again with 3 biological replicates. Our results now show robust and significant enrichment of Tet1 mRNA in Dazl complexes, while this is not observed for tet2 (Figure 4C, supplementary figure S4B).

Other comments:

- Sup Fig 1A, the resolution of histogram images is low and the quantifications can barely be read. It will also be informative to include D1 to better understand how Dazl-GFP positive cells transit from 100% at D0 to 10% at D3.

We would like to thank the reviewer for this suggestion and we now modified this figure and included D0, D1, D2 and D3 time points which more accurately shows the re-establishment of the original equilibrium.

-Legends. Many experiments have been performed with 2 or less samples, how can statistics be calculated with one sample? How have the error bars been calculated? In general, most qRT-PCRs show very high error bars, hindering data interpretation.

We now included additional biological replicates for all the qRT-PCRs performed resulting in at least 3 biological replicates per sample. For the Dazl KO ESCs, we also included biological replicates from additional different ESC-lines. P-values were calculated based on the averages of the technical repliactes from each biological replicate and calculated by T-test.

Editorial Decision – EMBO Journal

Thank you for returning a strongly revised paper that has now been assessed from two of the original referees.

As you will recognize, both scientists find the study overall interesting and of relevance. However, both insist on assessing protein level in the blastocyst and ref#1 critically demands genome-wide 5hmC-Seq data plus further experimental substantiations for the surrogate mass-spec results.
As these appear experimentally challenging and to not delay presentation of your intriguing proposal of Dazl being required for Tet1-mediated reprogramming to the very fast moving stem cell field too much, I took the liberty to inquire with our sister title EMBO Reports that indeed thrive on short, concise and conceptually novel observations without requesting significant molecular-mechanistic corroboration. I am happy to inform you that their scientific editor Barbara Pauly would be delighted to rapidly engage to facilitate efficient presentation of your study, though conditioned on analyzing Dazl-protein expression in the blastocyst stage.

I sincerely hope that this presents a suitable and viable alternative to enable presentation of your data in a similarly general and visible journal.

REFEREE REPORTS:

Referee #1:

Overall, the revised manuscript from Welling et al. shows improvements. However, some of our key comments have unfortunately not been addressed by the authors or do not provide conclusive evidence. Specifically:

1) We are delighted to see that the authors followed our suggestion to confirm the differences in 5mC and 5hmC levels with mass spectrometry analysis. However, the differences observed are not convincing.

2) In response to our proposal to characterize the difference in 5hmC between Dazl+ and Dazl- cells on a genome-wide level using 5hmC sequencing, the authors discussed resent results by Hackett et al. showing that pluripotency and germline genes are highly hydroxymethylated in serum cultured ESCs. While of interest, this does not directly address our point. We still think that 5hmC-Seq would be crucial for the paper.

3) In response to our suggestion to show methylation and hydroxymethylation states of genes that are differential expressed between Dazl+ and Dazl- cells the authors responded that this falls outside the scope of this study. We would understand that 5mC would not be investigated in the present study. However, as mentioned in point 2, we still feel that it would be essential to perform 5hmC-Seq to support the suggested model (cf. Figure 5).

Minor points:

1) We cannot find table S3 in the provided document.

2) The authors stated that they performed new dot blot analysis and make the results now available in supplementary figure S4D. But in supplementary figure S4D we see exactly the same dotblot experiment (not a new one) that was shown in the original submitted manuscript in figure 4A. We wonder whether the .png files got switched as figures were rearranged.

3) Page 9, line 1: "day 3 followed by DNA demethylation in the next days (Figure 3D, Supplementary Figure S3B) (Ficz et al. 2013; Leitch et al. 2013)" should be changed to "day 3 followed by DNA demethylation in the next days (Figure 3E, Supplementary Figure S3B) (Ficz et al. 2013; Leitch et al. 2013)".

4) Page 11, line 13: "Indeed, while the expression of Tet1 and Tet2 genes does not change upon Dazl overexpression (Supplementary figure S4A)". Tet1 and Tet2 expression data are not shown in the named figure.

Referee #3:

While we were doing revision experiments for this paper, the paper describing the derivation of the Dazl-GFP cell line was published (Chen et al. 2014, Stem Cell Reports). In that paper we show that Dazl expression is observed in the developing germline and in ESCs faithfully recapitulating endogenous gene expression, and that it is absent in other tissues. In this manuscript we performed single molecule FISH on wildtype blastocysts and find mRNA expression in a similar fashion as observed for the Dazl-GFP blastocyst embryos. To further confirm the linear relationship between Dazl mRNA and protein expression, we have included an additional figure showing the correlation between Dazl mRNA expression and Dazl-GFP expression in the Dazl-GFP ESCs (supplementary figure S1A). Furthermore, we have performed mass spectrometry on ESCs transitioning in 2i conditions at different time point, and demonstrate the upregulation of endogenous Dazl protein in a similar fashion as we have seen for our GFP reporter using FACS and immunofluorescent stainings

together indicating our reporter expression carefully recapitulates endogenous Dazl expression (Figure 1C, supplementary table S3).

The expression of Dazl in the blastocyst-stage embryo has not been checked in the report recently published by the authors. I think that showing Dazl protein in the blastocyst is essential to demonstrate that Dazl is expressed in the late blastocyst embryo, which is an entire section of Results.

While global transcript levels go down in ESCs cultured in 2i conditions (published by Grun et al 2013), individual transcripts are more homogeneously distributed in 2i conditions. In serum conditions, expression levels of pluripotency genes are much more heterogeneous, but global levels of expression will not show this heterogeneity in qPCR results. Therefore, we performed smFISH of Dazl-GFP+ and Dazl-GFP- cells sorted at d0 and cultured for 3 days in 2i to show transcript variation between these populations with different origin. Figure 3F shows more homogeneity in transcript expression between different cells in cells that had Dazl-GFP+ cells as a starting population, but qPCR results as shown in supplementary figure S3C is based on total RNA levels, making it more difficult to draw conclusions about heterogeneity in gene expression. As ESCs are still transitioning towards a naïve pluripotent state at day 3 in 2i conditions, the global gene expression levels have not reached their final levels (as found by day 10) yet, but cells do already show the establishment of more homogeneity.

The statement "Dazl-GFP positive cells cultured in 2i conditions for 3 days are also more similar to naïve ESCs than Dazl-GFP negative cells by day 3 of 2i culture (Supplementary figure S3C).", page 10, is based on qRT-PCR data. As the authors indicate in the response to reviewers that: "qPCR results as shown in supplementary figure S3C is based on total RNA levels, making it more difficult to draw conclusions about heterogeneity in gene expression", then the statement on page 10 has to be removed since that conclusion cannot be obtained from qRT-PCR data.

New comments:
- Page 8: "Dazl positive cells in serum cluster together with Dazl positive cells in 2i while Dazl positive and Dazl negative cells in 2i conditions are transcriptionally similar".

I am not sure whether this statement can be concluded from the expression analysis of 8 genes. Specially considering that: Dnmt3a is lower in 2iDazlGFP+ than in DazlGFP+; Tet2 and Dnmt3b are quite similar between DazlGFP- and their 2i counterparts; and that the error bars are very high in Tet1, Prdm14 and Tfcp2l1, so no conclusions can be extracted.

- Page 11: "elevated Tet1 protein levels are observed in ESCs from 2 days onwards after the induction of Dazl with doxycycline indicating that Dazl supports more efficient translation (Figure 4D).

Considering the error bars, is there a significant increase of Tet1 protein after Dazl overexpression? Is 4D performed in 2i medium?

Correspondence - authors

Thank you for your careful consideration of our manuscript, and your interest in publishing our results in EMBO reports. I've consulted with the first author and we think EMBO reports is a good platform for our manuscript.

Transfer to EMBO reports – revised manuscript and authors' response to original comments 05 May 2015

We thank the reviewers for their time reading our manuscript as well as for their constructive comments.

Referee #1:

Overall, the revised manuscript from Welling et al. shows improvements. However, some of our key comments have unfortunately not been addressed by the authors or do not provide conclusive evidence. Specifically:

1) We are delighted to see that the authors followed our suggestion to confirm the differences in 5mC and 5hmC levels with mass spectrometry analysis. However, the differences observed are not convincing.

While the differences in Cytosine methylation and hydroxymethylation observed in our initial dot-blot assays indeed seemed larger than during our further analysis by mass spectrometry, we disagree that the latter are not convincing. Our mass spec results are consistent over multiple technical as well as biological replicates (6 biological replicates for DazlGFP+ and DazlGFP- sorted serum cultured ES cells) as indicated in the figures. Since serum cultures ES cells are heterogeneous, their global 5hmC and 5mC levels vary between biological replicates from different time points. However, 5hmC values were consistently higher in DazlGFP+ cells.

2) In response to our proposal to characterize the difference in 5hmC between Dazl+ and Dazl- cells on a genome-wide level using 5hmC sequencing, the authors discussed resent results by Hackett et al. showing that pluripotency and germline genes are highly hydroxymethylated in serum cultured ESCs. While of interest, this does not directly address our point. We still think that 5hmC-Seq would be crucial for the paper.

The main focus of our manuscript is the demonstration that Dazl is a regulator of Tet1, and as such an indirect regulator of DNA hydroxymethylation, an observation that is robustly supported by knock-out, overexpression and biochemical analyses. While whole-genome 5hmC analysis would be interesting indeed, we feel it is outside the scope of this paper.

3) In response to our suggestion to show methylation and hydroxymethylation states of genes that are differential expressed between Dazl+ and Dazl- cells the authors responded that this falls outside the scope of this study. We would understand that 5mC would not be investigated in the present study. However, as mentioned in point 2, we still feel that it would be essential to perform 5hmC-Seq to support the suggested model (cf. Figure 5).

We feel that our data provide strong and convincing support for the regulatory role of Dazl in modulating Tet1 expression. As explained above, the paper is about Dazl interaction with Tet1 mRNA, not about the nature of the Tet1 target genes. Exploring this will be part of a future study. Our proteomics data for DazlGFP+ and DazlGFP- cells as well as our Dazl overexpression studies followed by qRT-PCR show that naïve pluripotency genes are upregulated in cells that express higher levels of Dazl thus supporting our model in figure 5.

Minor points:

1) We cannot find table S3 in the provided document.

We have corrected this error

2) The authors stated that they performed new dot blot analysis and make the results now available in supplementary figure S4D. But in supplementary figure S4D we see exactly the same dotblot experiment (not a new one) that was shown in the original submitted manuscript in figure 4A. We wonder whether the .png files got switched as figures were rearranged.

We didn't change the figure, since the repeat experiments showed the exact same results.

We've appended the results of the additional experiment to demonstrate this

3) Page 9, line 1: "day 3 followed by DNA demethylation in the next days (Figure 3D, Supplementary Figure S3B) (Ficz et al. 2013; Leitch et al. 2013)" should be changed to "day 3 followed by DNA demethylation in the next days (Figure 3E, Supplementary Figure S3B) (Ficz et al. 2013; Leitch et al. 2013)".

We have corrected the text as per the reviewer's suggestion, thank you

4) Page 11, line 13: "Indeed, while the expression of Tet1 and Tet2 genes does not change upon Dazl overexpression (Supplementary figure S4A)". Tet1 and Tet2 expression data are not shown in the named figure.

We apologize for referring to the wrong figure. Tet1 and Tet2 expression data can be found in supplementary figure S4E. We modified the text accordingly.

Referee #3:
The expression of Dazl in the blastocyst-stage embryo has not been checked in the report recently published by the authors. I think that showing Dazl protein in the blastocyst is essential to demonstrate that Dazl is expressed in the late blastocyst embryo, which is an entire section of Results.

While we were doing revision experiments for this paper, the paper describing the derivation of the Dazl-GFP cell line was published (Chen et al. 2014, Stem Cell Reports). In that paper we show that Dazl expression is observed in the developing germline and in ESCs faithfully recapitulating endogenous gene expression, and that it is absent in other tissues. In this manuscript we performed single molecule FISH on wildtype blastocysts and find mRNA expression in a similar fashion as observed for the Dazl-GFP blastocyst embryos. To further confirm the linear relationship between Dazl mRNA and protein expression, we have included an additional figure showing the correlation between Dazl mRNA expression and Dazl-GFP expression in the Dazl-GFP ESCs (supplementary figure S1A). Furthermore, we have performed mass spectrometry on ESCs transitioning in 2i conditions at different time point, and demonstrate the upregulation of endogenous Dazl protein in a similar fashion as we have seen for
our GFP reporter using FACS and immunofluorescent stainings together indicating our reporter expression carefully recapitulates endogenous Dazl expression (Figure 1C, supplementary table S3).

In the past, we have tested several anti-Dazl antibodies which are commercially available on testis sections and have found that (perhaps in addition to Dazl itself) these antibodies recognize another protein which is not Dazl, since sections of Dazl-knockout testis also yield a positive signal. This was the reason we generated and used the Dazl-GFP reporter mouse. As demonstrated above, we have demonstrated a linear relationship between mRNA expression of endogenous Dazl and Dazl-GFP signal. Furthermore, in addition to demonstrating Dazl-GFP expression in the blastocyst, we have performed single mRNA fish to demonstrate the presence of endogenous Dazl transcript in the ICM of late blastocysts as well.

While global transcript levels go down in ESCs cultured in 2i conditions (published by Grun et al 2013), individual transcripts are more homogeneously distributed in 2i conditions. In serum conditions, expression levels of pluripotency genes are much more heterogeneous, but global levels of expression will not show this heterogeneity in qPCR results. Therefore, we performed smFISH of Dazl-GFP+ and Dazl-GFP- cells sorted at d0 and cultured for 3 days in 2i to show transcript variation between these populations with different origin. Figure 3F shows more homogeneity in transcript expression between different cells in cells that had Dazl-GFP+ cells as a starting population, but qPCR results as shown in supplementary figure S3C is based on total RNA levels, making it more difficult to draw

conclusions about heterogeneity in gene expression. As ESCs are still transitioning towards a naïve pluripotent state at day 3 in 2i conditions, the global gene expression levels have not reached their final levels (as found by day 10) yet, but cells do already show the establishment of more homogeneity.

The statement "Dazl-GFP positive cells cultured in 2i conditions for 3 days are also more similar to naïve ESCs than Dazl-GFP negative cells by day 3 of 2i culture (Supplementary figure S3C).", page 10, is based on qRT-PCR data. As the authors indicate in the response to reviewers that: "qPCR results as shown in supplementary figure S3C is based on total RNA levels, making it more difficult to draw conclusions about heterogeneity in gene expression", then the statement on page 10 has to be removed since that conclusion cannot be obtained from qRT-PCR data.

We have corrected this statement according to the reviewer's suggestion.

New comments:
- Page 8: "Dazl positive cells in serum cluster together with Dazl positive cells in 2i while Dazl positive and Dazl negative cells in 2i conditions are transcriptionally similar".

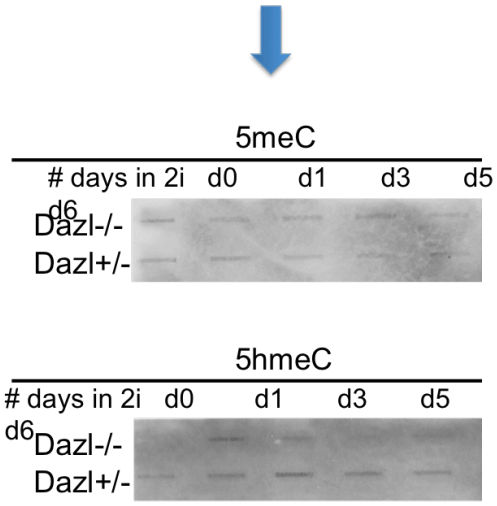
I am not sure whether this statement can be concluded from the expression analysis of 8 genes. Specially considering that: Dnmt3a is lower in 2iDazlGFP+ than in DazlGFP+; Tet2 and Dnmt3b are quite similar between DazlGFP- and their 2i counterparts; and that the error bars are very high in Tet1, Prdm14 and Tfcp2l1, so no conclusions can be extracted.

This sentence is unclear and can be interpreted in multiple ways. We meant to clarify that Dazl-GFP+ and DazlGFP- cells in 2i represent similar populations in gene expression instead of comparing serum cultured Dazl-GFP+ cells to 2i cultured cells. Furthermore, 2i cultured ESCs have been very well investigated and are known to consist of homogeneous cell populations. We modified this sentence to avoid further confusion.

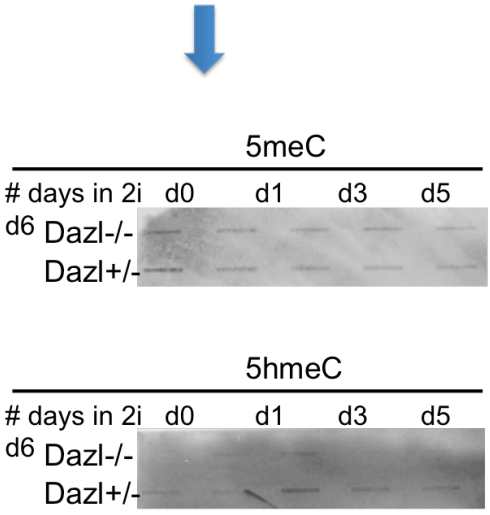
- Page 11: "elevated Tet1 protein levels are observed in ESCs from 2 days onwards after the induction of Dazl with doxycycline indicating that Dazl supports more efficient translation (Figure 4D).
Considering the error bars, is there a significant increase of Tet1 protein after Dazl overexpression? Is 4D performed in 2i medium?

We performed this experiment in serum + LIF and not in 2i conditions since 5hmC in elevated in Dazl+ cells in serum compared to Dazl- cells in serum while this difference is not observed in 2i conditions. This is probably due to the fact that 2i cultured ESCs already have low DNA methylation levels and high expression of naïve pluripotency genes.
We performed the Dazl overexpression experiment followed by western for Tet1 for 3 biological replicates and there is already significant increase (p=0.02) in Tet1 protein by day 2 of Dazl overexpression which remains elevated upon continuous dox induction.

Figure S4D



Additional experiment



1st Editorial Decision – EMBO reports 07 May 2015

Many thanks for submitting your revised study and the detailed point by-point response to our editorial office. I had sent the documents back to one of the original referees and s/he has now confirmed that his/her concerns have been adequately addressed.

The only point s/he raises is that apparently, you didn't change the wording in the main text in two instances, even though you discuss this issue in the point-by-point response.

Could you please modify the text accordingly and send us the final version by email?

For your convenience and to make the textual changes easier to identify for you, I am pasting this referee's comment here:

"In the rebuttal letter, the authors indicate that the sentences: "Dazl-GFP positive cells cultured in 2i conditions for 3 days are also more similar to naïve ESCs than Dazl-GFP negative cells by day 3 of 2i culture (Supplementary figure S3C)." on page 10 and "Dazl positive cells in serum cluster together with Dazl positive cells in 2i while Dazl positive and Dazl negative cells in 2i conditions are transcriptionally similar" on page 8 have been corrected. However, they have not been modified in the revised version of the manuscript."

Once we have received the final version, I can proceed with the production. It would be great if you could send it by tomorrow, so that we can still meet the dealing for the next issue. I don't think it will be too difficult to change the two sentences in the text. I could do it myself, but I also do not want to mis-interpret your data, so it would be best if you could do it.

1st Revision – authors' response 08 May 2015

Glad to hear the reviewer was happy with our changes and apologies for the error.
Please find attached the manuscript with the last two textual changes.

2nd Editorial Decision 08 May 2015

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

