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The Sm protein methyltransferase PRMT5, is not required for primordial germ cell specification in mice

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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.)

Editor: Thomas Schwarz-Romond

1st Editorial Decision

14 July 2014

Thank you very much for submitting your study that assesses the developmental role of Prmt5 in the mammalian germline for consideration to The EMBO Journal editorial office.

Two scientists that are familiar with the topic commented on potential suitability of your submission for The EMBO Journal. While we notice general interest for eventual publication of your results, particularly ref#1 takes issue with the mostly descriptive outcome of Prmt5's functional abrogation. S/he would like to see some insight into the exact nature of the reported defect before being able to support eventual publication in The EMBO Journal.

While certainly aware of the necessity for rather timely pursuit, I would like to give you the opportunity to incorporate possibly already existing results, respective prioritize experiments that may surface such further reaching insights for timely incorporation.

Conditioned on a satisfactorily response to this issue, I am happy to invite revisions/relevant amendments to your paper for a final assessment.

Please do not hesitate to get in touch with regard to feasibility and anticipated timeline for the necessary revisions (due to time constrains preferably via e-mail).

I also have to formally remind you that The EMBO Journal only considers one major round of revisions and look forward to hear from you/receive a suitably revised version of your manuscript!

REFEREE REPORTS:

Referee #1:

The submitted manuscript by Li et al addresses a potential role of Prmt5 during PGC specification and early germ line development in mice. Prmt5 has been previously shown to interact with Prdm1/Blimp1 - a key germline determinant (Ancelin et al, 2006). As a mutation in *dart5* (a *Drosophila* Prmt5 homologue) causes fly sterility (Gonsalvez et al, 2006); Prmt5 has been suggested to play an important role also during the mouse germline specification and development. The authors examine this hypothesis by generating mouse germline specific Prmt5 conditional knockout. They show that PGCs lacking Prmt5 are lost between E9.5 and E11.5 and suggest that this is due to the failure to exit from the G2/M arrest. In order to further address the role of Prmt5 during PGC specification, the authors use inducible Prmt5 deletion in combination with the PGCIC system in vitro (Hayashi et al, 2011). They show that PGCIC can be induced in the absence of Prmt5, albeit with a slightly low efficiency ; however the resulting cells display abnormalities in gene expression. The authors conclude, that Prmt5 is not important for PGC specification, but acts "at the interphase between reprogramming I and II" during PGC development.

Although the presented data are undoubtedly interesting, my main criticism is the lack of understanding of the exact nature of the observed defect caused by the Prmt5 deletion. The authors claim that the loss of PGCs is caused by a failure to exit from the G2/M arrest, however this is not very convincing. The difference shown in Fig4F is only slight and not statistically significant. As mutant PGCs fail to upregulate *mvh/ddx4* the findings are consistent with Prmt5 deficient PGCs failing to execute a correct developmental programme. In this context it is difficult to argue, whether the PGC specification is not affected at all, since the resulting PGCs are aberrant.

The same is true for the PGCIC in vitro system: are the observed differences shown in Fig5G statistically significant? Fig6 clearly shows that the expression of at least some of the PGC markers is affected by the loss of Prmt5. The exact nature of the defect would thus deserve much more in depth characterisation.

In view of this, I am also not sure whether the title of the manuscript is justified.

Additional comments:

Can the observed effect on embryo size (Fig 3H) be caused by Prmt5 deletion outside the germ line? Prdm1Cre is known to be expressed in visceral endoderm.

The authors claim that Prmt5 staining shows presence of this protein in the P bodies in the male germline after E16.5 . This would need to be shown using specific markers....

Relative expression of Prmt5 in male vs female gonads - to make any claims, it would be important to analyse the expression level by qPCR. It is possible that the somatic expression of Prmt5 differs between male and female gonads.

Referee #2:

PRMT5 is an arginine methyltransferase that in flies is required for specification of primordial germ cells (PGCs). PGC specification in flies and mice is drastically different, with fly PGCs being specified early in development by the localization of molecular determinants, while in mice PGCs are specified through inductive signals. Although the mechanisms that establish PGCs in flies and mice are different, whether the molecular players involved in fly PGCs have conserved roles in mouse PGCs is an interesting area of investigation that can lead to a better understanding of how

somatic and germ lineages are established and maintained.

In this manuscript, Li and colleagues investigate the requirement of PRMT5 in mouse PGC development using both embryos and stem cell models. The authors demonstrate that PRMT5 is expressed in a dynamic pattern during PGC development and is required for the presence of germ cells at birth. Further investigation of Prmt5 null PGCs demonstrates that unlike in flies, PRMT5 is not required for the specification of PGCs in the mouse, but instead is required to maintain cell cycle progression, survival, and normal gene expression of mouse PGCs.

This manuscript contains definitive findings and shows that the role of mammalian PRMT5 differs from that of the fly orthologue, and is the first molecule that acts to promote exit from the G2/M pause in the germline. The manuscript should therefore be considered for publication in EMBO journal if the following major and minor issues can be addressed.

1. The title could be stronger and clearer. Later in the paper, the authors use the term "checkpoint" rather than reprogramming interface, which is easier to understand.
2. The Intro should include a description of the timing of PGC specification, migration, and gonad colonization.
3. In the abstract, the phrasing, "Using an inducible deletion" is awkward.
4. On p. 3, "In pluripotency" is vague. Change "precedence" to "precedent." The description of the roles of Prmt5 in male and female flies is confusing.
5. Throughout the manuscript, change "drosophila" to "Drosophila" (ital).
6. On p. 4, "...PRMT5 is an ancient protein that unifies the two modes of PGC..." might be more clear as "...PRMT5 function could be conserved."
7. The following subheading is incorrect "Male and Female mice are born without germ cells."
8. On p. 6, "exhibit" is not best word choice.
9. On p. 7 "cell-autonomously dying" is not best word choice.
10. On p. 8, "In addition to exiting the cell cycle..." The paragraph is not as well written as some of the others - please revise. Similarly awkward paragraph on p. 10 "Given that deleting PRMT5 in ESCs..."
11. On p. 9, "To address this.." Please clarify what is being addressed.
12. Throughout the manuscript, "data reveals" and "data shows." Please remember data are plural.
13. On p. 12, "iPKO#2 PGCLC have no difference in..." compared to what?
14. On p. 12 (and also implied in the abstract) "...expression of HoxB1 and HoxA1 is also repressed in Prmt5 negative iPKO PGCLCs." Statement is not supported by the evidence presented (Fig. 6).
15. On p. 12 "...Stella is not required for..." please cite.
16. On p. 13, italicize "C. elegans." For symmetry, only genus name could be given, since this is the authors' convention when flies are discussed.
17. On p. 13, "Our data indicate that PRMT5 has evolved a new role..." is overstated.
18. On p. 14, "...PRMT5 acts after PGC specification and not together with BLIMP1..." is overstated. PRMT5 could still work with BLIMP1, but the role has not been revealed by removal of PRMT5 alone.

On p. 14, please elaborate on/define 'pull the trigger' in the final paragraph of the discussion.

In all figures, the authors should include the number of embryos examined, and they should define Ctrl (wild type embryos, embryos carrying the BC allele, or PRMT5 fl embryos?).

Figure 1: the authors should include staining for PRMT5 in E9.5 PCKO mice to confirm the specificity of their PRMT5 staining at this stage.

Figure 2, legend: Germ cells are absent in the gonads of newborn pups. Do the authors mean PCKO newborn pups?

Figure 3B: panels are too dark to appreciate.

Figure 3G the authors do not specify how PGCs were identified at each stage.

Figure 4B: it is unclear whether the standard error is across individual embryos or per 10 PGCs.

Figure 4 and legend uses CCNB1 and CyclinB1 - please choose one convention.

Figure 5D, E: Are PRMT5 and DAPI both in green? DAPI channel seems to be missing.

Figure 5G: please provide p value for the comparison. This comparison is critical for showing that the ES cell model is a useful way to evaluate the role of PRMT5 in PGC development, especially given that the deletion of PRMT5 was so inefficient in ES cells. Please also define error bars (replicate differentiation experiments?). If the difference in Fig. 5G is not statistically discernable, then the authors may want to eliminate the ES cell model, focusing instead on the strengths of their in vivo system, but also sorting PGCs from embryos (Vincent et al., 2011), and then performing the single cell RT-PCR to evaluate stem cell and differentiation markers (as in Fig. 6). Alternatively, could the authors restrict the comparison in Fig. 5G to only those cells in which PRMT5 protein is detectable (as a way to exclude those cells in which deletion did not occur)?

Figure 6C-H, legend: do the authors really mean semi-quantitative?

1st Revision - authors' response

31 July 2014

We would like to thank the reviewers for their thoughtfulness and time to review our work. We agree with all of your comments and have both performed new experiments as well as removed experiments that you recommended. As a result of our (including your function as anonymous reviewers) collective work, we think the manuscript has been significantly improved.

Please find below the specific comments.

Referee #1:

The submitted manuscript by Li et al addresses a potential role of Prmt5 during PGC specification and early germ line development in mice. Prmt5 has been previously shown to interact with Prdm1/Blimp1 - a key germline determinant (Ancelin et al, 2006). As a mutation in dart5 (a Drosophila Prmt5 homologue) causes fly sterility (Gonsalvez et al, 2006); Prmt5 has been suggested to play an important role also during the mouse germline specification and development.

The authors examine this hypothesis by generating mouse germline specific Prmt5 conditional knockout. They show that PGCs lacking Prmt5 are lost between E9.5 and E11.5 and suggest that this is due to the failure to exit from the G2/M arrest. In order to further address the role of Prmt5 during PGC specification, the authors use inducible Prmt5 deletion in combination with the PGCLC system in vitro (Hayashi et al, 2011). They show that PGCLC can be induced in the absence of Prmt5, albeit with a slightly low efficiency; however the resulting cells display abnormalities in

gene expression. The authors conclude, that Prmt5 is not important for PGC specification, but acts "at the interphase between reprogramming I and II" during PGC development.

Although the presented data are undoubtedly interesting, my main criticism is the lack of understanding of the exact nature of the observed defect caused by the Prmt5 deletion. The authors claim that the loss of PGCs is caused by a failure to exit from the G2/M arrest, however this is not very convincing. The difference shown in Fig4F is only slight and not statistically significant. As mutant PGCs fail to up-regulate mvh/ddx4 the findings are consistent with Prmt5 deficient PGCs failing to execute a correct developmental program. In this context it is difficult to argue, whether the PGC specification is not affected at all, since the resulting PGCs are aberrant.

We would like to thank the reviewer for their thoughtful comments on our manuscript and agree that more evidence is required to support the conclusion that germline specification is largely unaffected. To do this, we performed new experiments to evaluate the first epigenetic PGC reprogramming event, which is uniquely linked to the specification phase of PGC formation, and involves the removal of 5 methyl cytosine (5mC) and Histone H3 lysine 9 dimethylation (H3K9me2) from the genome and chromatin respectively. Therefore if PGC specification is abnormal, the normal removal of 5mC and/or H3K9me2 in PGCs would not occur (as was the case when Prdm14 was knocked out. Analysis of 5mC and H3K9me2 in individual PGCs of PCKO and control embryos revealed that both 5mC and H3K9me2 are normally repressed in PCKO PGCs similar to controls (Please refer to new Fig 4E and 4F). This new data confirms that PRMT5 has no or limited effects on PGC specification, or the unique global epigenetic PGC reprogramming event that is linked to PGC specification. However, we acknowledge that although PGC specification appears largely unaffected *in vivo*, some aspects of early PGC development beyond epigenetic reprogramming may not be normal, and we have indicated this possibility in the discussion (Please refer to page 12).

With regard to the mechanism by which PRMT5 causes cells exit from the cell cycle, we recognize that this was a weakness of our first manuscript and have therefore deleted any reference to specifically affecting G2 as we have no direct evidence for this. In order to perform molecular studies, we turned to the embryonic stem cell (ESCs) model and induced a deletion in *Prmt5* using tamoxifen. We did this because of the difficult nature of studying PGCs in the embryo at E9.5 and E10.5, particularly when only 1 in 4 animals are mutants, and only 100-200 PGCs are found in each embryo at this stage. In previous work we performed single cell analysis on PGCs during this time period (Vincent et al., 2011). However this experiment involved first pooling embryos to have sufficient cells to sort through the FACS machine to gate on germ cells and sort single PGCs, therefore it is not easily applicable to the current study which would require individually sorting embryos. Ground state ESCs (cultured in presence of 2i inhibitors with LIF) have been used to model immature PGCs in many studies including Grabole et al. 2013 and Ficz et al. 2011), and similar to PGCs at E9.5-E10.5, ground state ESCs are globally hypomethylated and express uniform levels of *nanog*, *oct4* and *sox2*. Furthermore, we found that similar to PGCs, a deletion in *Prmt5* in ESCs also resulted in exit from the cell cycle, increased cPARP and decreased growth (Please refer to new Fig 5B-D). Given that Prmt5 is a Sm protein methyltransferase we hypothesized that Prmt5 dramatically affects splicing in these cells. By performing a western blot using the SYM-10 antibody, we first showed that arginine methylation of Smd1/D3 proteins is depleted following deletion of *Prmt5* (Please refer to Figure 5E). Next we performed paired end RNA-Sequencing to prove that a deletion in *Prmt5* functionally causes a defect in splicing of genes involved in RNA processing, cell cycle and chromatin organization.

The same is true for the PGCLC in vitro system: are the observed differences shown in Fig5G statistically significant? Fig6 clearly shows that the expression of at least some of the PGC markers is affected by the loss of Prmt5. The exact nature of the defect would thus deserve much more in depth characterisation.

We agree with the reviewer's comments and therefore decided to remove the PGCLC differentiation experiments from this paper as it detracts from the main findings during *in vivo* PGC differentiation. This same sentiment was also shared by reviewer 2. In the *in vivo* PGCs with a deletion in *Prmt5* we

saw no effect on STELLA expression (meaning all OCT4 positive cells were STELLA positive at E9.5). In contrast, as the reviewer points out there were some differences, including the finding that some OCT4 positive PGCLCs are negative for STELLA *in vitro*. This suggests that the *in vitro* and *in vivo* systems have slightly different sensitivities to loss of *Prmt5*. The purpose of the PGCLC experiment was to examine whether *Hoxb1* and *Hoxa1* (which are the targets of BLIMP1), and still repressed in the absence of PRMT5, and we found that *in vitro* the HOX genes were repressed in both STELLA positive and STELLA negative PGCLCs which no longer expressed *Prmt5*. Therefore, to avoid confusion we have removed the PGCLC differentiation model from the paper in light of the new data that better supports the lack of phenotype in PGC specification which is to demonstrate that PRMT5 mutant PGCs undergo PGC reprogramming I and loss of 5mC and H3K9me2

In view of this, I am also not sure whether the title of the manuscript is justified.

In light of the new data we have changed the title to: The Sm protein methyltransferase PRMT5, is not required for primordial germ cell specification in mice.

Additional comments:

Can the observed effect on embryo size (Fig 3H) be caused by Prmt5 deletion outside the germ line? Prdm1Cre is known to be expressed in visceral endoderm.

Yes, we have included in the results the following comments and references about embryo size in the *Prmt5* mutants. “The growth retardation phenotype could be due to a number of factors most likely related to the fact that Blimp1 is also expressed in non-germline cells of the early mouse embryo including the prechordal plate, visceral endoderm as well as Ter119 positive erythroid cells.”

The authors claim that Prmt5 staining shows presence of this protein in the P bodies in the male germline after E16.5. This would need to be shown using specific markers....

We have removed the reference to P-bodies as we do not have data to support this statement and instead refer to the PRMT5 foci as “MVH negative PRMT5 positive foci”.

Relative expression of Prmt5 in male vs female gonads - to make any claims, it would be important to analyse the expression level by qPCR. It is possible that the somatic expression of Prmt5 differs between male and female gonads.

We agree with your comment, therefore we have removed any reference to relative expression levels of PRMT5 in male and female gonads, as this is not the main point of the work. Instead we focus only on the intracellular localization of PRMT5 (which is different between the sexes) and do not mention expression levels.

Referee #2:

PRMT5 is an arginine methyltransferase that in flies is required for specification of primordial germ cells (PGCs). PGC specification in flies and mice is drastically different, with fly PGCs being specified early in development by the localization of molecular determinants, while in mice PGCs are specified through inductive signals. Although the mechanisms that establish PGCs in flies and mice are different, whether the molecular players involved in fly PGCs have conserved roles in mouse PGCs is an interesting area of investigation that can lead to a better understanding of how

somatic and germ lineages are established and maintained.

In this manuscript, Li and colleagues investigate the requirement of PRMT5 in mouse PGC development using both embryos and stem cell models. The authors demonstrate that PRMT5 is expressed in a dynamic pattern during PGC development and is required for the presence of germ cells at birth. Further investigation of Prmt5 null PGCs demonstrates that unlike in flies, PRMT5 is not required for the specification of PGCs in the mouse, but instead is required to maintain cell cycle progression, survival, and normal gene expression of mouse PGCs.

This manuscript contains definitive findings and shows that the role of mammalian PRMT5 differs from that of the fly orthologue, and is the first molecule that acts to promote exit from the G2/M pause in the germline. The manuscript should therefore be considered for publication in EMBO journal if the following major and minor issues can be addressed.

Thank you for the time you spent to carefully go through this manuscript. The specific details you have highlighted and asked us to correct, change, clarify and remove have undoubtedly made the manuscript significantly stronger.

1. The title could be stronger and clearer. Later in the paper, the authors use the term "checkpoint" rather than reprogramming interface, which is easier to understand.

The Sm protein methyltransferase PRMT5, is not required for primordial germ cell specification in mice.

2. The Intro should include a description of the timing of PGC specification, migration, and gonad colonization.

We have now included a time line of PGC specification, migration and colonization as recommended.

3. In the abstract, the phrasing, "Using an inducible deletion" is awkward.

We have removed the phrase.

4. On p. 3, "In pluripotency" is vague. Change "precedence" to "precedent." The description of the roles of dart5 in male and female flies is confusing.

We have changed "in pluripotency" to "in mouse embryonic stem cells" and precedence to "precedent". We have also simplified the description of *dart5* in PGC specification as follows. "The precedent for PRMT5 regulating PGC specification comes from work in *Drosophila melanogaster* where a homozygous mutation in the homologue of *Prmt5* in females leads to progeny that completely lack PGCs (Gonsalvez *et al*, 2006, Anne *et al.*, 2007)."

5. Throughout the manuscript, change "drosophila" to "Drosophila" (ital).

We have changed as recommended.

6. On p. 4, "...PRMT5 is an ancient protein that unifies the two modes of PGC..." might be more clear as "...PRMT5 function could be conserved."

We have changed the phrasing of this entire paragraph and deleted the sentence “PRMT5 is an ancient protein that unifies the two modes of PGC”. Instead we have written “Given the fundamental differences between PGC specification in the preformation and inductive models, it is unclear whether PRMT5 will have a conserved role in PGC specification as predicted from a Prmt5 mutation in *Drosophila*.”

7. *The following subheading is incorrect "Male and Female mice are born without germ cells."*

We have changed the sentence to “Male and Female PCKO mice are born without germ cells.”

8. *On p. 6, "exhibit" is not best word choice.*

We changed exhibit to “have”.

9. *On p. 7 "cell-autonomously dying" is not best word choice.*

Changed to “dying in a cell-autonomous manner”.

10. *On p. 8, "In addition to exiting the cell cycle..." The paragraph is not as well written as some of the others - please revise. Similarly awkward paragraph on p. 10 "Given that deleting PRMT5 in ESCs..."*

We have removed this sentence.

11. *On p. 9, "To address this.." Please clarify what is being addressed.*

We have deleted this sentence and instead write “Finally to determine whether the gonadal germline program has been activated we evaluated MVH, which is expressed in murine PGCs from E10.5”.

12. *Throughout the manuscript, "data reveals" and "data shows." Please remember data are plural.*

We have corrected the grammar to indicate data is plural.

13. *On p. 12, "iPKO#2 PGCLC have no difference in..." compared to what?*

Given that both reviewer 1 and reviewer 2 indicated that the PGC analysis is more convincing using the *in vivo* model, we removed the PGCLC differentiation from this manuscript.

14. *On p. 12 (and also implied in the abstract) "...expression of HoxB1 and HoxA1 is also repressed in Prmt5 negative iPKO PGCLCs." Statement is not supported by the evidence presented (Fig. 6).*

We removed the PGCLC figure (Fig 6).

15. *On p. 12 "...Stella is not required for..." please cite.*

Given we have removed the Fig 6, therefore, we have also no need to cite this reference as Stella protein expression is not affected *in vivo* (all OCT4 positive cells are Stella positive).

16. On p. 13, italicize "*C. elegans*." For symmetry, only genus name could be given, since this is the authors' convention when flies are discussed.

We have italicized *Caenorhabditis* as recommended.

17. On p. 13, "Our data indicate that PRMT5 has evolved a new role..." is overstated.

We have deleted the sentence.

18. On p. 14, "...PRMT5 acts after PGC specification and not together with BLIMP1..." is overstated. PRMT5 could still work with BLIMP1, but the role has not been revealed by removal of PRMT5 alone.

You are correct to point this out, and we have added a new sentence to read "Although our results demonstrate that PRMT5 has a different role to what was previously reported for BLIMP1 in PGC specification, it is still conceivable that PRMT5 works together with BLIMP1, which was is revealed by removal of PRMT5 alone."

On p. 14, please elaborate on/define 'pull the trigger' in the final paragraph of the discussion.

We have deleted this sentence.

In all figures, the authors should include the number of embryos examined, and they should define Ctrl (wild type embryos, embryos carrying the BC allele, or PRMT5 fl embryos?).

The information on number of embryos analyzed, and the genotype of the control samples have now been added to the figure legends.

Figure 1: the authors should include staining for PRMT5 in E9.5 PCKO mice to confirm the specificity of their PRMT5 staining at this stage.

The E9.5 PGC expression data is the same as the previously published expression pattern for PRMT5 in pregonadal PGCs (Ancelin et al., 2006).

Figure 2, legend: Germ cells are absent in the gonads of newborn pups. Do the authors mean PCKO newborn pups?

We have changed the title to indicate PCKO newborn pups.

Figure 3B: panels are too dark to appreciate.

We have selected a different Figure.

Figure 3G the authors do not specify how PGCs were identified at each stage.

We have corrected the figure legend to indicate the marker used to identify PGCs at each developmental stage and in each panel.

Figure 4B: it is unclear whether the standard error is across individual embryos or per 10 PGCs.

We clarified that the standard error is across 4 fields containing 59 and 43 total PGCs analyzed for Ctrl (n=2) and PCKO (n=2) respectively.

Figure 4 and legend uses CCNB1 and CyclinB1 - please choose one convention.

This figure has been removed, as reviewer 1 did not feel that we had strong enough evidence to indicate that the PGCs fail to exit the G2/M pause. Instead, we have concluded that loss of PRMT5 causes cells to exit the cell cycle, as this interpretation is a more accurate reflection of the data both in PGCs as well as in the ESC model.

Figure 5D, E: Are PRMT5 and DAPI both in green? DAPI channel seems to be missing.

Figure 5D and E have been removed from the manuscript on account of the PGCLC experiments being removed.

Figure 5G: please provide p value for the comparison. This comparison is critical for showing that the ES cell model is a useful way to evaluate the role of PRMT5 in PGC development, especially given that the deletion of PRMT5 was so inefficient in ES cells. Please also define error bars (replicate differentiation experiments?). If the difference in Fig. 5G is not statistically discernable, then the authors may want to eliminate the ES cell model, focusing instead on the strengths of their in vivo system, but also sorting PGCs from embryos (Vincent et al., 2011), and then performing the single cell RT-PCR to evaluate stem cell and differentiation markers (as in Fig. 6). Alternatively, could the authors restrict the comparison in Fig. 5G to only those cells in which PRMT5 protein is detectable (as a way to exclude those cells in which deletion did not occur)?

We have removed Figure 5G.

Figure 6C-H, legend: do the authors really mean semi-quantitative?

We have removed Figure 6.

2nd Editorial Decision

25 August 2014

I received the comments enclosed below and am rather certain that you will be able to address these constructively within a reasonable time.

REFEREE REPORT:

Referee 1

The revised manuscript has addressed most of my questions. Unfortunately, although the new version became stronger on the PGC side, I am not convinced about the direct mechanistic links the authors are making between 2i grown ES cells and PGCs in vivo.

The new added data on Prmt5 deletion in mouse ESCs and the resulting splicing defects are interesting and the connection to the phenotype observed in PGCs likely. However, I would have expected to see at least some validation in the PGC system of the splicing defects observed in the cultured ESCs. Contrary to the authors' claims, FACS purification of PGCs from individual embryos

at E9.5 is feasible. The authors could have also attempted to validate the splicing defects of some of the targets at the level of altered protein expression.

Although there are clear parallels between ESCs grown in 2i and PGCs in vivo, especially regarding some of the epigenetic features, PGCs are under very tight control of proliferation (as opposed to rapidly proliferating pluripotent ESCs). Additionally, the germline transcriptional programme is not identical to that observed in mouse ESCs.

Additional comments:

Some figures lack scale bars.

Fig 1A: the quality of Prmt5 staining in E9.5 embryos is poor

The text requires carefully proofreading some sentences do not make sense (missing words etc).

"frail PGCs" (p4) is an awkward expression

p8 second sentence "the growth retardation..." presumably the authors mean Prdm1Cre or Prdm1 ?

p8 second paragraph "cPARP positive cells were identified in both control and PCKO mutant PGCs..." unclear, perhaps "amongst mutant PGCs.."?

p8 last paragraph: "PGCs are still in cycle" an awkward expression

Additional author correspondence

10 September 2014

I thought I would touch base with you about where we are on this paper.

We are at the point of now doing the splicing analysis in PGCs! It took us a year to get the PGC reporter allele bred into the model. Our first analysis will be a week from Friday but if we don't get any mutants (which sometimes happens because the litters are small) it could be a few months before we get the next set of mice. The challenge is that we are going to try to do a splicing analysis on around 100 cells isolated by FACS from a germ cell reporter embryo at E10.5.

Splicing on small cell numbers has been done before using cultured cells titrated down, but not from individual embryo's after a sort. So the experiment is not totally not straight forward. None the less we are trying! I will be able to give you a better sense of whether the experiment is feasible in a few weeks.

Is this time frame still OK?

Additional editorial correspondence

10 September 2014

I would like to be very pragmatic about this certainly intriguing, though seemingly not easy to pursue experimental opportunity.

Please keep me posted as soon as you can be certain (i) that you obtained the relevant mutant strain (ii) would be able to collect sufficient material for the splicing analysis (either at transcript or protein level).

Referee #1:

The revised manuscript has addressed most of my questions. Unfortunately, although the new version became stronger on the PGC side, I am not convinced about the direct mechanistic links the authors are making between 2i grown ES cells and PGCs in vivo.

The new added data on Prmt5 deletion in mouse ESCs and the resulting splicing defects are interesting and the connection to the phenotype observed in PGCs likely. However, I would have expected to see at least some validation in the PGC system of the splicing defects observed in the cultured ESCs. Contrary to the authors' claims, FACS purification of PGCs from individual embryos at E9.5 is feasible. The authors could have also attempted to validate the splicing defects of some of the targets at the level of altered protein expression.

Although there are clear parallels between ESCs grown in 2i and PGCs in vivo, especially regarding some of the epigenetic features, PGCs are under very tight control of proliferation (as opposed to rapidly proliferating pluripotent ESCs). Additionally, the germline transcriptional programme is not identical to that observed in mouse ESCs.

To address this major comment we have now proved that RNA splicing is affected in PRMT5 mutant PGCs (Please see Fig 5J-L)

Additional comments:

Some figures lack scale bars.

All figures now have scale bars.

Fig 1A: the quality of Prmt5 staining in E9.5 embryos is poor

Given that the E9.5 expression pattern is already known (Ancelin et al., 2006), we removed this panel from the figure and figure legend and all references to it in the text.

The text requires carefully proofreading - some sentences do not make sense (missing words etc.)

The text has been proof read, missing words were added, and the recommended corrections as detailed below have also been made.

"frail PGCs" (p4) is an awkward expression

Changed to fragile.

p8 second sentence "the growth retardation..." - presumably the authors mean Prdm1Cre or Prdm1?

The Prdm1Cre itself does not have a growth retardation phenotype, however PRMT5 Conditional knockout (PRKO) embryos do have a growth retardation phenotype. Therefore we have changed the sentence to '.....growth retardation phenotype of PCKO embryos' for clarity.

p8 second paragraph "cPARP positive cells were identified in both control and PCKO mutant PGCs..." unclear, perhaps "amongst mutant PGCs..?"

Changed to “amongst” as recommended.

p8 last paragraph: "PGCs are still in cycle" an awkward expression

Changed to ‘... are still in cell cycle...’ for clarity.