Full methylation of H3K27 by PRC2 is dispensable for initial embryoid body formation but required to maintain differentiated cell identity

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MS TITLE: H3K27me3 is dispensable for early differentiation but required to maintain differentiated cell identity

AUTHORS: Sara Miller, Manashree Damle, and Robert E Kingston

I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

We are aware that you may be experiencing disruption to the normal running of your lab that make experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The Polycomb repressive complex 2 (PRC2) is essential for maintaining cellular identity during development. PRC2 is comprised of core components, SUZ12 and EED and its catalytic component (either EZH1 or EZH2) and is responsible for the di- and tri- methylation of Histone H3 (H3K27me2/3). Miller et al wished to explore the role of the PRC2 mediated H3K27me3 modification in the repression of genes during differentiation of mouse embryonic stem cells (ESCs) to embryoid bodies (EBs). They address this by blocking the PRC2 mediated H3K27me3 using the small molecule inhibitor GSK343 or introducing an EZH2 point mutation. They performed RNA-seq, H3K27me3/PRC2 CUT&RUN-seq, as well as biochemistry and differentiation assays to investigate the contribution of H3K27me3 to both gene repression and maintenance of the differentiated state. They conclude that H3K27me3 is not required for the repression of many PRC2 target genes, but is required during early differentiation for the maintenance of the differentiated state.

Comments for the author

Major points:
1. In Figure 1E, the authors present RNA-seq analyses to present the differential genes in DMSO control treated versus GSK343 treated cells. Could the authors indicate on the panel how many genes are direct PRC2 target genes. This would inform on the direct and indirect consequences.
2. In Figure 2C, the authors should perform a ChIP-qPCRs or ChIP-seq of H3K27me2 and H3K27me3 to more comprehensively evaluate the consequences of this mutation (compared to a wild-type EZH2 control) on these two modifications. Shen et al (2008 Mol Cell) reported that Ezh1 may compensate for loss of Ezh2 to a certain extent and PRC2 targets did maintain at least some H3K27me3 in Ezh2 KO ESCs.
3. In Figure 2E-F, there might be that is an issue with the SUZ12 CUT&RUN-seq experiment. For example, the Figure 2E panel shows that the number of SUZ12 target genes in wild-type ESCs is only 519. However, very many studies since 2006, using ChIP-on-Chip, then ChIP-seq, and more recently ChIP-RX, have established that the number of SUZ12 target genes in ESCs (and also differentiated cells) is between 2,000 and 4,000. Similarly, the numbers of Ezh2, Suz12 and Ring1b targets in Figure 3B are relatively small, compared to several prior studies using ChIP-seq and ChIP-RX approaches. The authors should address this apparent discrepancy and also present their Suz12, Ezh2 and Ring1b CUT&RUN tracks in UCSC or IGV CUT&RUN format to confirm the data quality.
4. In Figure 5, it is a challenging to follow the experiment and perhaps the color-coding of Figure 5C could be matched with Figure 5A.

Minor points:

1. The authors should also present images for Day 8 of EB differentiation in Figures 1C and 2D.
2. In Figure 3D, the gene expression patterns of Mtase dependent or independent genes look very similar. The authors should present some examples genes from each cluster (e.g. activated or repressed, Mtase dependent or independent genes) and also present some quantitative average analysis in addition to the blue/red Heatmap visuals. This comment is also relevant for Figure 1E).
3. The correlation score between two different samples should be added to Supplementary Figure 1C (as done for Supplemeentary Figures 4 and 5).
4. The figures have no titles in the figure legends, making it more challenging to appreciate the outline and of the paper and the purpose behind each of the experiments.
5. There are some typographical errors, e.g. Line 407, “K3K27me3” should be H3K27me3; Line 140, “and Fig. 2E.” should be Fig. 2D; “Lavarone et al generated the EZH2-722D mutation ESCs” should instead refer to the EZH2-726D mutation.

Reviewer 2

Advance summary and potential significance to field

This study describes the role of H3K27me3 both in mouse ESC differentiation and in the maintenance of differentiated states. To this end, the authors use a catalytic mutant of Ezh2

unable to deposit the third methyl group to a di-methylated H3K27 as well as an inhibitor of Ezh2 methyltransferase activity. They note that the resulting loss of H3K27me3 does not block differentiation of mESCs into embryoid bodies, unlike what is reported in the literature for deletions of PRC2 core complex members. However, embryoid bodies devoid of H3K27me3 can revert more easily to an ESC like state. The authors define sets of genes that either depend or do not depend on the methyltransferase activity of PRC2 for their regulation. Finally, they identify a time window during differentiation where PRC2 methyltransferase activity is important for the maintenance of differentiated states.

Comments for the author

Some of the conclusions are not supported convincingly enough and the main conclusion in particular is grossly overstated: “H3K27me3 is dispensable for early differentiation but required to maintain differentiated cell identity”. The proposed dispensability of H3K27me3 for early differentiation cannot be generalized based solely on a morphological phenotype in an in vitro system like embryoid bodies; additional (and better defined) differentiation models would need to be explored to make such a broad conclusion. Moreover, the very substantial transcriptomic variations in the absence of H3K27me3 that the authors uncover in their RNA-seq undermine their interpretation even as it applies to EBs.

The quantitative analysis on proteins should at least rely on high quality western blots. Thus, the western blots in figures 1B, 2C and supp 7B should be performed more carefully to avoid signal saturation and problems in migration. Western blots in Lavarone et al. (2019) show a low level of residual H3K27me3 in cells expressing Ezh2-R681C, clearly higher than the Ezh2 knockout or the Y722D null mutant. Assuming that the same is true in the present study, the authors have not convincingly shown that the apparent differentiation of their ESCs (with the caveats mentioned above) occurs in the absence of H3K27me3 enrichment at target genes or a subset of target genes, a possibility which the metaplots in figure 3 do not rule out and may in fact partly obscure by averaging many genes together.

Some results from the RNA-seq experiments are not convincing:
- The activation of “MTase-independent activated genes” in both GSK343-treated and mutant cells actually seems to be much less robust than in wild-type cells. “Partially dependent” would be more accurate. It would be interesting to see gene expression at D4 and D8 as a fold change as compared to D0 expression.
- For Ezh2 mutants, the authors note that “the day of differentiation is a more relevant predictor of similarity rather than the mutant strain.” I would add to this that the replicate number is an even better predictor than day of differentiation, suggesting a problem of reproducibility that casts doubt on the rest of the analysis (supp figure 4B).

The authors propose that for maintenance of differentiated states, PRC2 methyltransferase activity is required early during differentiation between D0 and D4 (figure 5c). Treating differentiating cells with GSK343 only during this time window or in even smaller time windows would be required to support their hypothesis. Indeed I would have a slightly different interpretation of the presented data. Withdrawal of GSK343 from D4 (GSK343-DMSO D4) produces similar results as GSK343-DMSO D0. This suggests that a pulse of methyltransferase activity is needed between D0 and D8 and not only between D0 and D4. It would be also be interesting to present the data from D8 and D14 separately rather than pooling it in “D8 or D14”.

Reviewer 3

Advance summary and potential significance to field

The study by Miller et al investigates the role of H3K27 methylation by PRC2 during differentiation of mouse ES cells into embryoid bodies. Specifically, the authors attempt to reduce rather than abolish H3K27me3 levels by use of a small molecule inhibitor of PRC2 or by introducing a point mutation in Ezh2 that reduces its enzymatic activity. Unlike cells with PRC2 KO mutations that fail to form embryoid bodies, cells with compromised but not abolished PRC2 catalytic activity retain the ability to differentiate and form embryoid bodies.

The authors show that dissociated embryoid body cells, if cultured in ES cell media, can to some extent return to adopt ES cell properties, suggesting that the cells with reduced H3K27me3 levels can differentiate but are unable to stably maintain their differentiated state. The authors note, however, that the ability of embryoid body cells to return to an ES cell-like state is only possible during a certain time window after the initiation of EB formation.

Overall, this study is highly similar to a previous study from the Pasini lab (Lavarone et al, Nat. Comm. 2019) with very similar findings and conclusions. The study by Miller et al, extends these earlier findings by investigating the stability of the Ezh2 mutant cells that make up differentiated embryoid bodies. Independent studies that arrive at similar conclusions as previous studies are of great value and I am therefore, in principle, supportive of publication of the present study in Development. However, I feel that Miller et al should improve the documentation of some of the effects they report (points 1-3 below), and they should be fully transparent and acknowledge that Lavarone et al had also analyzed Ezh2 mutant cells with the same Ezh2 point mutation (Ezh2R685C in the Lavarone study corresponds to Ezh2R681C in this study) and had not just Ezh2 KO cells as implied in lines 85-89.

Comments for the author

Overall, this study is highly similar to a previous study from the Pasini lab (Lavarone et al, Nat. Comm. 2019) with very similar findings and conclusions. The study by Miller et al, extends these earlier findings by investigating the stability of the Ezh2 mutant cells that make up differentiated embryoid bodies. Independent studies that arrive at similar conclusions as previous studies are of great value and I am therefore, in principle, supportive of publication of the present study in Development. However, I feel that Miller et al should improve the documentation of some of the effects they report (points 1-3 below), and they should be fully transparent and acknowledge that Lavarone et al had also analyzed Ezh2 mutant cells with the same Ezh2 point mutation (Ezh2R685C in the Lavarone study corresponds to Ezh2R681C in this study) and had not just Ezh2 KO cells as implied in lines 85-89.

Point 1
Figure 1B. For these types of analyses, it is standard to provide some semi-quantitative data like a Western blot analysis on serial dilutions of cell extracts. This would help the reader to assess to what extent H3K27me3 levels are reduced. I understand that the authors might not have/ be able to generate any better quality WB data for the revision. But at a minimum, they should then tone down their conclusions. In particular, it does not seem wise to use the differences in transcriptome phenotypes (i.e. comparing genetic PRC2 knock-out and inhibitor-treated cells) to draw bold conclusions about dependency on PRC2 protein versus PRC2 catalytic activity (lines 151-157).

Point 2
Figure 2A/ mutated Ezh2 residues. This is completely confusing. In the alignment on the right, the Arg boxed in red corresponds to Arg685 in human EZH2, this residue stabilizes the orientation of the EZH2 loop that binds SAM. The text in line 166 says the boxed residue corresponds to “R635 in human EZH2” (???). The numbering in the alignment shown on the right ends with a Gln that corresponds to EZH2 Gln730 but is numbered as Gln670. So it seems that the numbering in the alignment here is shifted by 50 residues. The ribbon diagram on the left - cropped from Figure 6 of the Antonysamy paper - contains a red circle around Ala692; what is that supposed to show? The authors should use PyMOL to generate their own figure where they illustrate the position of the mutated Arg685 residue in the structure. They should also show an alignment of mouse Ezh2 (currently not shown in the alignment) and human EZH2 with the residue mutated in Ezh2 numbered correctly (it may be useful to use a protein ID number in case there are several isoforms).

Point 3:
What is the loading control in Figure 2C and Suppl Figure 3? Without a control of e.g. a WB against an unrelated protein, it is impossible to judge whether the authors loaded comparable amount of extract in the different lanes and whether the Ezh2R681C protein is present at wild-type levels. Adding a control here is essential. It is known that Ezh2 levels in Eed mutant cells are reduced and, so again, a loading control of an unchanged protein is needed. The lower number of Ezh2 CUT&RUN peaks in Ezh2R681C mutant cells shown in Figure 2 E&F could simply reflect lower levels of

Ezh2R681C protein in the mutant cells and have nothing to do with reduction of H3K27me3 levels that the authors put forward as their interpretation of the reduced Ezh2R681C binding (lines 213-129).

Moreover, Lavarone et al (Nat. Comm. 2019) show in Fig. 7B of their paper that the reduction of H3K27me3 in their Ezh2R685C cells (Ezh2R685C in the Lavarone study corresponds to Ezh2R681C in this study) is not as severe as implied by the data shown in Fig. 2C in this study. So the authors may want to improve the documentation of the actual reduction of H3K27me3 levels seen in their experiment.

Minor point 1:
Lines 115-117: Pasini reported the phenotype of Suz12 KO mice. Please correct or cite O'Carroll et al, 2001 for EZH2 KO phenotype.

Minor point 2:
Lines 125 etc.: "PRC2 has been shown to play regulatory roles independent of H3K27 tri-methylation, including H3K27 di-methylation and proposed non-methyltransferase functions". What is the evidence that "showed" the non-methyltransferase functions? Either describe the experimental evidence that showed this and cite the paper(s) or tone down.

First revision

Author response to reviewers' comments

General comments:

1) The reviewers noted (and we cited in the original submission) that the point mutation we characterize in detail, R681C, is the same point mutation characterized in a paper from the Pasini laboratory (Lavarone et al., 2019). That paper was published when we were well along in characterizing these mutant cell lines. Our data on this mutation are similar to those reported, so we focused the current paper on experiments that explore the phenotype of this mutation in greater detail both at the molecular level and at the cell growth level than what was done in the published work. Most of the data in the paper, especially Figs. 3-5, covers areas not covered in the Lavarone et al. work. We agree with Reviewer 3 that the fact that the two laboratories independently reached the same initial conclusions is a strength. However, the main strength in our work is that we were able to use this hypomorph to distinguish the impact of H3K27 methylation on differentiation versus maintenance and were able to identify genes that differed in their requirement for methylation of H3K27. We believe these findings constitute significant and important extensions of what has been published previously.
2) In response to technical criticisms, we have redone Westerns blots and added substantial new analysis of changes in methylation patterns and expression patterns as detailed below in our point-by-point rebuttal.

Reviewer 1 Comments for the Author:

Major points:

1. In Figure 1E, the authors present RNA-seq analyses to present the differential genes in DMSO control treated versus GSK343 treated cells. Could the authors indicate on the panel how many genes are direct PRC2 target genes. This would inform on the direct and indirect consequences.

All genes presented in Figure 1E were PRC2 targets. We have added this to both the Figure and Figure legend.

2. In Figure 2C, the authors should perform a ChIP-qPCRs or ChIP-seq of H3K27me2 and

H3K27me3 to more comprehensively evaluate the consequences of this mutation (compared to a wild-type EZH2 control) on these two modifications. Shen et al (2008 Mol Cell) reported that Ezh1 may compensate for loss of Ezh2 to a certain extent and PRC2 targets did maintain at least some H3K27me3 in Ezh2 KO ESCs.

We now show CUT&RUN data for H3K27me3 on 7190 genes in the new Fig. 3C. The level of this modification was greatly reduced at D4 though there was some residual H3K27me3 detected at D8 and especially at D0, consistent with Shen et al. We have not done a similar detailed analysis of the location of H3K27me2 by CUT&RUN due to issues raised by COVID restrictions and because our analysis shows incomplete reduction in this modification as analyzed by western (see the new Fig. 2C). We are not focused on any specific genes in this study, but rather on the entirety of the impacted transcriptome; Chip-qPCR on individual genes does not meet that goal while westerns and genome wide analysis does.

3. In Figure 2E-F, there might be that is an issue with the SUZ12 CUT&RUN-seq experiment. For example, the Figure 2E panel shows that the number of SUZ12 target genes in wild-type ESCs is only 519. However, very many studies since 2006, using ChIP-on-Chip, then ChIP-seq, and more recently ChIP-RX, have established that the number of SUZ12 target genes in ESCs (and also differentiated cells) is between 2,000 and 4,000. Similarly, the numbers of Ezh2, Suz12 and Ring1b targets in Figure 3B are relatively small, compared to several prior studies using ChIP-seq and ChIP-RX approaches. The authors should address this apparent discrepancy and also present their Suz12, Ezh2 and Ring1b CUT&RUN tracks in UCSC or IGV CUT&RUN format to confirm the data quality.

We acknowledge that our method for calling peaks in our SUZ12 CUT&RUN data (as well as the other PRC2 subunits) leaves out some of the previous targets identified in other studies. We purposefully chose to analyze high confidence targets in examining the effects of the R681C mutation as our primary goal was to identify genes where PRC2 methylation was differentially required for regulation, which we felt would be most clear for genes that are high confidence targets. Therefore we used a more stringent cut-off than has been used previously. We have added a comparison of our data to the previously published ChIP-seq data from Li et al 2017 (Fig. S3C) to demonstrate that they are consistent especially among the high confidence targets.

4. In Figure 5, it is a challenging to follow the experiment and perhaps the color-coding of Figure 5C could be matched with Figure 5A.

We apologize for the for the confusion with Figure 5 and have matched the color coding as the reviewer suggested.

Minor points:

1. The authors should also present images for Day 8 of EB differentiation in Figures 1C and 2D.
We have added Day 8 embryoid body images of WT and 681C mutant cells to Fig. S2F.

2. In Figure 3D, the gene expression patterns of Mtase dependent or independent genes look very similar. The authors should present some examples genes from each cluster (e.g. activated or repressed, Mtase dependent or independent genes) and also present some quantitative average analysis in addition to the blue/red Heatmap visuals. This comment is also relevant for Figure 1E).
Per the reviewer's suggestion we have added line graphs showing the relative expression patterns of DMSO/GSK343 treated cells as well as WT/681C mutant cells to Fig. 1F, Fig. S1F, and Fig. S4C. These Figures display all of the individual genes in each cluster. These Figures show clear differences in the patterns of expression of genes between the MTase dependent and the independent classes.

3. The correlation score between two different samples should be added to Supplementary Figure 1C (as done for Supplementary Figures 4 and 5).
We have added the correlation score to the former Fig. S1C (now S1D).

4. The figures have no titles in the figure legends, making it more challenging to appreciate the outline and of the paper and the purpose behind each of the experiments.
We have added titles to all of the main Figure legends.

5. There are some typographical errors, e.g. Line 407, "K3K27me3" should be H3K27me3; Line 140, "and Fig. 2E." should be Fig. 2D; "Lavarone et al generated the EZH2-722D mutation ESCs" should instead refer to the EZH2-726D mutation.
We have corrected the typographical errors noted by the reviewer and thank them for their careful reading of our manuscript. The discrepancy between amino acid numbers in our work versus Lavarone et al comes from using different references. We are using UCSC's annotation which corresponds with our own sequencing of cloned Ezh2 from our cells. NCBI's annotation includes additional residues outside of the SET domain.

Reviewer 2 Comments for the Author:
Some of the conclusions are not supported convincingly enough and the main conclusion in particular is grossly overstated: "H3K27me3 is dispensable for early differentiation but required to maintain differentiated cell identity". The proposed dispensability of H3K27me3 for early differentiation cannot be generalized based solely on a morphological phenotype in an in vitro system like embryoid bodies; additional (and better defined) differentiation models would need to be explored to make such a broad conclusion.
We did not mean to imply that the results are generalizable to all situations and have clarified the title to state that our results concern EB differentiation.

Moreover, the very substantial transcriptomic variations in the absence of H3K27me3 that the authors uncover in their RNA-seq undermine their interpretation even as it applies to EBs.
As detailed below, we disagree with the characterization of 'substantial transcriptomic variations' in the absence of K27me3.

The quantitative analysis on proteins should at least rely on high quality western blots. Thus, the western blots in figures 1B, 2C and supp 7B should be performed more carefully to avoid signal saturation and problems in migration. Western blots in Lavarone et al. (2019) show a low level of residual H3K27me3 in cells expressing Ezh2-R681C, clearly higher than the Ezh2 knockout or the Y722D null mutant. Assuming that the same is true in the present study, the authors have not convincingly shown that the apparent differentiation of their ESCs (with the caveats mentioned above) occurs in the absence of H3K27me3 enrichment at target genes or a subset of target genes, a possibility which the metaplots in figure 3 do not rule out and may in fact partly obscure by averaging many genes together.
We have redone the western blots from mutant and drug treated cells in the new Fig. 2C and Fig. S7A and included loading controls separate from the Ezh2 level in order to address the reviewer's concerns. These data show similar levels of H3K27me3 in our two R681C clones as are seen in an *Eed* null. It is also not our contention that we have completely eliminated all of this modification, but that we have substantively decreased it in these mutant lines in agreement with Lavarone et al. Based on methyltransferase assays the mutant reduces activity *~50 fold, which is in rough agreement with the signals that we see in westerns which have some inherent background due to the antisera reacting weakly with unmodified histones. We agree that the metaplots might be misleading concerning changes in individual genes, and now show all individual genes, which indicate uniform impacts in the two mutant lines at D4 and D8 of EB differentiation (new Fig. 3C).*

Some results from the RNA-seq experiments are not convincing:
-The activation of "MTase-independent activated genes" in both GSK343-treated and mutant cells actually seems to be much less robust than in wild-type cells. "Partially dependent" would be more accurate. It would be interesting to see gene expression at D4 and D8 as a fold change as compared to D0 expression.
We have included fold change over Day 0 analyses for all RNA-seq data as the reviewer requested in Fig. S1E and Fig. S3B. We have qualified the characterization of the strength of the impacts in the results, as it does differ to some degree between individual genes, however this does not change our major conclusion that there is a distinct subset of genes whose regulation is substantively unaffected by loss of methylation. Each gene we classified as repressed was at least

1.5-fold reduced D4 over D0 or D8 over D4.

-For Ezh2 mutants, the authors note that "the day of differentiation is a more relevant predictor of similarity rather than the mutant strain." I would add to this that the replicate number is an even better predictor than day of differentiation, suggesting a problem of reproducibility that casts doubt on the rest of the analysis (supp figure 4B).
We do not agree with the reviewer that there is a problem of reproducibility. We examined EB differentiation, which is an inherently variable process between experiments set up on different days. Each of the replicates was set up in parallel, so one expects the two mutant clones with identical genotype to be more similar within a replicate than between replicates. In Fig. 4A we had shown that the two mutant lines showed similar expression profiles at D0, D4 and D8 that have R values of 0.99 when the three replicates are averaged. In Fig. 4B we show that each individual replicate clusters together as they are expected to because they are done in parallel. That demonstrates strong reproducibility between biological replicates. Second, all of the D0 data cluster in one group during unsupervised clustering, while, with one exception (both of the mutant D4 replicate 3 lines) all of the D4 and D8 data cluster in a separate group. We therefore do not believe the reviewers statement that 'replicate number is an even better predictor' is supported by the data.

The authors propose that for maintenance of differentiated states, PRC2 methyltransferase activity is required early during differentiation between D0 and D4 (figure 5c). Treating differentiating cells with GSK343 only during this time window or in even smaller time windows would be required to support their hypothesis. Indeed I would have a slightly different interpretation of the presented data. Withdrawal of GSK343 from D4 (GSK343-DMSO D4) produces similar results as GSK343-DMSO D0. This suggests that a pulse of methyltransferase activity is needed between D0 and D8 and not only between D0 and D4. It would be also be interesting to present the data from D8 and D14 separately rather than pooling it in "D8 or D14".
To avoid confusion with the data presented in Fig. 5C, we have separated Embryoid bodies differentiated for 8 days (new Fig. 5C) from those differentiated for 14 days (new Fig. 5D) and added further replicates to improve the data. These data were never pooled as the reviewer suggested, but we apologize for not making that clear in our original submission. The reviewer is correct in stating that withdrawal of GSK343 at D0 or D4 produces increased levels of AP staining after 14 days of differentiation (new panel 5D), which differs to some degree to the result seen when cells differentiate for 8 days (new panel 5C). We note that adding GSK343 to the media at D4 does not increase AP staining in the D8 data but does result in a small but significant increase in the D14 data. We have qualified the conclusions to reflect that the effective window is through D4 but extends slightly when cells are allowed to differentiate for 14 days. The conclusions that we believe are important do not concern the precise timing (e.g., closing of the window at D4 vs a bit later), but rather that it is well before when cells are *challenged to de-differentiate. The key data to support that point is that when we remove GSK343 well before we de-differentiate we still observe substantive de-differentiation. We have qualified that conclusion in the manuscript.*

Reviewer 3 Comments for the Author:
Overall, this study is highly similar to a previous study from the Pasini lab (Lavarone et al, Nat. Comm. 2019) with very similar findings and conclusions. The study by Miller et al, extends these earlier findings by investigating the stability of the Ezh2 mutant cells that make up differentiated embryoid bodies. Independent studies that arrive at similar conclusions as previous studies are of great value and I am therefore, in principle, supportive of publication of the present study in Development. However, I feel that Miller et al should improve the documentation of some of the effects they report (points 1-3 below), and they should be fully transparent and acknowledge that Lavarone et al had also analyzed Ezh2 mutant cells with the same Ezh2 point mutation (Ezh2R685C in the Lavarone study corresponds to Ezh2R681C in this study) and had not just Ezh2 KO cells as implied in lines 85-89.
We consider the Lavarone et al. paper to be important, and cited it numerous times in our original submission, including explicitly mentioning that we are looking at the same mutation. We have included that statement earlier in the paper as requested.

Point 1
Figure 1B. For these types of analyses, it is standard to provide some semi-quantitative data

like a Western blot analysis on serial dilutions of cell extracts. This would help the reader to assess to what extent H3K27me3 levels are reduced. I understand that the authors might not have/ be able to generate any better quality WB data for the revision. But at a minimum, they should then tone down their conclusions. In particular, it does not seem wise to use the differences in transcriptome phenotypes (i.e. comparing genetic PRC2 knock-out and inhibitor-treated cells) to draw bold conclusions about dependency on PRC2 protein versus PRC2 catalytic activity (lines 151-157).

We have redone the western blots, as outlined in response to Reviewer 2 point 2, in Figs. 2C and S7A. We believe that examining the H3K27me3 signal on individual genes is more instructive than bulk westerns to make the point about decreased H3K27me3 and now show (new Fig. 3C) how H3K27me3 levels are reduced at individual genes at each time point.

Point 2

Figure 2A/ mutated Ezh2 residues. This is completely confusing. In the alignment on the right, the Arg boxed in red corresponds to Arg685 in human EZH2, this residue stabilizes the orientation of the EZH2 loop that binds SAM. The text in line 166 says the boxed residue corresponds to "R635 in human EZH2" (???). The numbering in the alignment shown on the right ends with a Gln that corresponds to EZH2 Gln730 but is numbered as Gln670. So it seems that the numbering in the alignment here is shifted by 50 residues. The ribbon diagram on the left - cropped from Figure 6 of the Antonysamy paper - contains a red circle around Ala692; what is that supposed to show? The authors should use PyMOL to generate their own figure where they illustrate the position of the mutated Arg685 residue in the structure. They should also show an alignment of mouse Ezh2 (currently not shown in the alignment) and human EZH2 with the residue mutated in Ezh2 numbered correctly (it may be useful to use a protein ID number in case there are several isoforms).

We have updated Fig. 2A as well as the text describing that figure as the reviewer suggested and are sorry for the confusion. Some of the discrepancies were due to the issue of UCSC and *NCBI having different annotations as we mentioned in response to Reviewer 1 minor point 5, others were simply an error in putting together the final Figure that we missed due to overfamiliarity when proofreading the paper. We appreciate the comment.*

Point 3:

What is the loading control in Figure 2C and Suppl Figure 3? Without a control of e.g. a WB against an unrelated protein, it is impossible to judge whether the authors loaded comparable amount of extract in the different lanes and whether the Ezh2R681C protein is present at wild-type levels. Adding a control here is essential. It is known that Ezh2 levels in Eed mutant cells are reduced and, so again, a loading control of an unchanged protein is needed. The lower number of Ezh2 CUT&RUN peaks in Ezh2R681C mutant cells shown in Figure 2 E&F could simply reflect lower levels of Ezh2R681C protein in the mutant cells and have nothing to do with reduction of H3K27me3 levels that the authors put forward as their interpretation of the reduced Ezh2R681C binding (lines 213-129).

As mentioned above , we have redone these westerns including TBP as a loading control. When normalized against this unchanging protein, the 681C-99 and 681C-102 mutant cells show 37% and 17% reduction in EZH2 expression, respectively. This is in contrast to the 50% reduction seen in EED null cells. It is possible that the reduction in the number of SUZ12 peaks detected in the mutant cells was due to many factors, including the amount of complex available and H3K27me3 levels. The point we were focused on with that data was that PRC2 localized to largely overlapping sites and so the phenotypes are not due to mislocalization of the complex in mutant cells.

Moreover, Lavarone et al (Nat. Comm. 2019) show in Fig. 7B of their paper that the reduction of H3K27me3 in their Ezh2R685C cells (Ezh2R685C in the Lavarone study corresponds to Ezh2R681C in this study) is not as severe as implied by the data shown in Fig. 2C in this study. So the authors may want to improve the documentation of the actual reduction of H3K27me3 levels seen in their experiment.

We show new western blots on this point of Fig. S7C. These data show similar levels of H3K27me3 in our two R681C clones as are seen in an *Eed* null. It is also not our contention that we have completely eliminated all of this modification, but that we that we have decreased its level in these mutant lines in agreement with Lavarone et al. Based on methyltransferase assays the

mutant reduces activity ~50 fold, which we believe is in rough agreement with the signals that we see in westerns which have some inherent background due to the antisera reacting weakly with unmodified histones. As mentioned above, we believe that examining the H3K27me3 signal on individual genes is more instructive than bulk westerns to make the point about decreased H3K27me3 and now show (new Fig. 3C) how H3K27me3 levels are reduced at individual genes at each time point.

Minor point 1:
Lines 115-117: Pasini reported the phenotype of Suz12 KO mice. Please correct or cite O'Carroll et al, 2001 for EZH2 KO phenotype.
Thank you, we have corrected our citing error and now refer to O'Carroll et al.

Minor point 2:
Lines 125 etc.: "PRC2 has been shown to play regulatory roles independent of H3K27 tri-methylation, including H3K27 di-methylation and proposed non-methyltransferase functions". What is the evidence that "showed" the non-methyltransferase functions? Either describe the experimental evidence that showed this and cite the paper(s) or tone down.
We agree that the word 'shown' is not accurate and have removed that sentence . We were citing previous papers that led us to examine the role for methyltransferase activity more thoroughly in order to give credit to that previous work. We believe this shows a context for the complex having roles that are independent of its catalytic activity.

Second decision letter

MS ID#: DEVELOP/2020/196329

MS TITLE: Full methylation of H3K27 by PRC2 is dispensable for initial embryoid body formation but required to maintain differentiated cell identity

AUTHORS: Sara Miller, Manashree Damle, Jongmin Kim, and Robert E Kingston

I have now received all the referees reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Reviewer 1

Advance summary and potential significance to field

N/A

Comments for the author

The authors satisfactorily addressed all my comments and feedback.

Reviewer 2

Advance summary and potential significance to field

We read carefully the response to reviewers.
The change to the title is a good step.
Nonetheless, we remain unconvinced by the experimental design and consequently believe that the contribution to the field of this study is limited.

Comments for the author

Not applicable. See above.

Reviewer 3

Advance summary and potential significance to field

Miller et al present a revised version of their paper investigating the role of H3K27 methylation by PRC2 during differentiation of mouse ES cells into embryoid bodies.
The authors have addressed the technical shortcomings in the original submission and the new data have improved the quality of the manuscript. I support publication of this version of the paper in Development, pending that the authors address one minor comment.

Comments for the author

I have only one minor comment of editorial nature. Back in 2002, when the Kingston lab first characterized PRC2 (Müller et al, Cell 2002), they had mutated the same residue in Drosophila E(z) (i.e. R699 in E(z) corresponds to R681 in EZH2). Kingston and co-workers had reported that an R699A or R699H mutation drastically diminishes HMTase activity of fly PRC2 on nucleosomes in vitro and they showed that these mutations fail to support Polycomb repression at homeotic genes in Drosophila. It seems that those original results from the author's lab (from 20 years ago!) could at least be mentioned when discussing the reduced HMTase activity of mammalian PRC2 with EZH2 R681C in vitro and the inability of the mutant protein to maintain the differentiated state of EB cells.

Second revision

Author response to reviewers' comments

Thank you for the comment, That mutation was actually made in Juerg Mueller’s group (we did the biochemistry of that paper) and we are happy to comment upon and reference the paper (lines 106-108).

Third decision letter

MS ID#: DEVELOP/2020/196329

MS TITLE: Full methylation of H3K27 by PRC2 is dispensable for initial embryoid body formation but required to maintain differentiated cell identity

AUTHORS: Sara Miller, Manashree Damle, Jongmin Kim, and Robert E Kingston
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.