Maternal Ezh1/2 deficiency in oocyte delays H3K27me2/3 restoration and impairs epiblast development responsible for embryonic sub-lethality in mouse

Yinan Zhao, Dandan Bai, You Wu, Dan Zhang, Mengying Liu, Yingpu Tian, Jinhua Lu, Haibin Wang, Shaorong Gao and Zhongxian Lu
DOI: 10.1242/dev.200316

Editor: Haruhiko Koseki

Review timeline

Original submission:	28 October 2021
Editorial decision:	9 December 2021
First revision received:	10 May 2022
Editorial decision:	24 May 2022
Second revision received:	18 June 2022
Accepted:	23 June 2022

Original submission

First decision letter

MS ID#: DEVELOP/2021/200316

MS TITLE: Maternal Ezh1/2 deficiency in oocyte delays H3K27me2/3 restoration and impairs epiblast development responsible for embryonic sub-lethality in mouse

AUTHORS: Yinan Zhao, Dan Zhang, Mengying Liu, Yingpu Tian, Jinhua Lu, Shaorong Gao, Hai-Bin Wang, and Zhongxian Lu

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. Particularly, addition of molecular data showing gene expression status in mutant embryos will be critically important as suggested by reviewers. 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

In this study, the roles of histone H3K27 methylation in the development and lineage determination are investigated by analyzing Ezh1 and Ezh2 KO mice. The maternal deletion of Ezh2 caused the disappearance of H3K27me3 in oocytes and embryos at the early preimplantation stage, whereas H3K27me2 was lost by double KO of Ezh1 and 2. Although the maternal single Ezh2 KO did not affect the development, the litter size was decrease by double KO, which is caused by the deficient differentiation into epiblast. These results would contribute to the understanding the role of Ezh1/2 in H3K27 methylation and the development in mice.

Comments for the author

Although the data provided by this report are potentially interesting, there is a serious discrepancy regarding H3K27me3 imprinting between this report and a previous one. Furthermore, the authors lead some conclusions without providing the clear evidence. These are pointed out below as "major points".

(major points)
A previous report demonstrated that the loss of maternal H3K27me3 caused the growth arrest of embryos after implantation (Inoue et al., 2018), which suggests that the persistence of maternal H3K27me3 imprinting is essential for the development. However, the authors shows that disappearance of H3K27me3 by the deletion of EZh2 did not affect the development. I suppose that one plausible explanation for this discrepancy is that H3K27me3 would remain in some regions involving imprinting in EZh2 KO oocytes, although most of H3K27me3 was lost: the loss of H3K27me3 was checked only by immunohistochemistry. To address this possibility, the transcriptome analysis (RNAseq) for Ezh2 KO embryos should be conducted.
Page 6, line 9-12: It is too immature to suggest that H3K27me2 may be contributed to X chromosome inactivation because some cells had two dots of H3K27me2 staining. Additional evidence should be required to claim it.
Page 6, line 16-17: No evidence is shown that Ezh1 plays an assistant role in H3K27me3 modification in oocytes and early embryos. The analysis for single Ezh1 KO is required to claim it.
Page 10, line 18-24: Although the authors conclude that EPI development is associated with H3K27me3 modification, it is come from the analyses of dKO embryos. Therefore, the possibility cannot be excluded that H3K27me2 but not H3K27me3 would be involved in EPI development. sKO embryos in which only H3K27me3 is lost should be analyzed to investigate the involvement of H3K27me3.

(minor points)
Page 2, line 15: "but not single Ezh1 or Ezh2": The single Ezh2 KO has not been analyzed in this report.
I do not understand what Fig. 3A, C, D, F represent. Are they shown to represent the rates of successful mating? I suppose that Fig. 3B and E are enough to represent the effect of KO on the development.
Page 7, line 5-8: "sFig. 3" should be "sFig. 4".

Reviewer 2

Advance summary and potential significance to field

Remarks to the Author:

In the present study, authors study the function of Ezh1/2 in mouse embryo. The manuscript starts with the investigation of the depletion of Ezh1/2 effect on H3K27me2/3 level during early embryonic development using gene knockout mouse models. Authors find that restoration of H3K27me3 was delayed until late blastocyte by loss of Ezh2 alone and H3K27me2 was reestablished until morulae by deletion of Ezh1 and Ezh2. Then authors investigate the developmental potential of KO embryos. They find that Ezh1/2 KO embryo has a critical effect on fetus and placenta development.

The findings presented in this manuscript are in part important for scientific community and the authors argumentation is partially conclusive. So, I would recommend a publication of this manuscript after some revision:

Comments for the author

Authors concluded that maternal Ezh1/2 are required for the establishment of H3K27me2/3 in in vivo preimplantation embryos and play critical roles in embryonic development in mouse. However, the data do not indicate whether pre-fertilization maternal DNA H3K27me3 levels are important or post-fertilization H3K27me3 reconstruction is important for embryogenesis. Since H3K27me3 in early embryos does not recover in KO mice using WT sperm, Ezh1/2 may not be expressed in the early stages of development. In that case, there are multiple possibilities for the reconstruction mechanism of H3K27me3. For example, it may be supplemented with mRNA or protein stored in the oocyte. The reconstruction of H3K27me3 in the early stage may be led by H3K27me3 of Maternal DNA. In order to clarify these, I think it is better to perform a rescue experiment by injection of Ezh1/2 mRNA.
If Ezh1/2 is needed to reconstruct H3K27me3 in early development, it should be possible to rescue it by injection of Ezh1/2 mRNA into a dKO Zygote. In addition, clarifying the expression level and localization of Ezh1/ in the early development of control embryos will be more convincing. It can be examined by analysis of previously reported RNA-seq data, immunostaining, Western blotting etc.
Authors reported enlarged placentas existed in late development of dKO/+ embryos. Recent report showed that loss of noncanonical H3K27me3 imprinting could be the cause of placental enlargement (Mei et al., Nat Genet. 2021 Apr;53(4):539-550. doi: 10.1038/s41588-021-00820-3). Recently H3K27me3 and Polycomb study was updated (Chen et al., Nat Genet. 2021 Apr;53(4):551-563. doi: 10.1038/s41588-021-00821-2.). Please revise discussion section, including the latest findings.
Please show quantitative data. Please do not just post the photo, but quantify it.
Figure1 and 2; Please measure the fluorescence intensity (H3K27me2/3) and make a graph.
Figure 6B and C; Please count cell number and categorize cell type like Figure S5.
Figure 7E; Please count cell number and categorize cell type.

Reviewer 3

Advance summary and potential significance to field

In this study, Zhao et al. demonstrated that maternal depletion of Ezh1 and Ezh2, which are mutually exclusive core components of PRC2, causes developmental defects of descendent embryos in both embryonic and extraembryonic sides that eventually result sub-lethality at term. Although the redundant functions of Ezh1 and Ezh2 as a maternal protein has been documented by Meng et al. (Nat Commun, 2021) in a partially in vivo system (combined approach of Ezh2 maternal KO and Ezh1 KD by siRNA injection), the biological consequences of such maternal double depletion of Ezh1/2 has not been analyzed yet. Thus, this study by Zhao et al. provides not only a genetic in vivo evidence that Ezh1 and Ezh2 has a redundant function in H3K27 methylation in oocytes and descendent early embryos but also a link between such defective H3K27 methylation and developmental abnormalities in the post-implantation developmental processes.
One unfortunate point, however, is that the molecular relationships between defective H3K27 methylation and developmental abnormalities was not provided; in other word, how the loss of H3K27me3 in Ezh1/2 double maternal KO oocytes or delayed catch-up in the early embryos causes the abnormal gene expression in the embryos? Which gene was more affected? Are such defective

gene regulations direct effect or indirect effect? Since there was no RNA-seq of maternal Ezh1/2 KO embryos, or H3K27me2/3 ChIP-seq of oocytes, such relationships remained unclear.

Comments for the author

[Major comments]
H3K27me3 has been shown to be involved in the regulation of atypical genomic imprinting genes as a maternal factor (Inoue et al., Nature, 2017; Inoue et al., Gene Dev, 2018; Mei et al., Nat Genet, 2021). However, in this paper, the effects of Ezh1/2 KO on such imprinting genes were not analyzed at all, and were not even discussed. In particular, the large placenta phenotype observed in maternal Ezh1/2 KO embryos is assumed to be caused by abnormalities in these imprinting genes.
H3K27me3 appeared to be lost in Ezh2 single maternal KO (FigS1). Despite such situation, the embryos derived from Ezh2 KO oocytes did not show any defects.
Such loss of H3K27me3 does not affect H3K27me3-metiated genomic imprinting?
P5, “Enhanced H3K27me3 staining in inner cell mass (ICM) appeared at sKO/+ late blastocyst stage, along with dot staining in trophectoderm (TE) in some embryos which was comparable to sF/+ embryos (Fig. 1A)”
P6, “Intriguingly, the dot staining of H3K27me2 was strongly increased in dKO/+ embryos and some cells had two dot staining at morula stage (sFig. 3A and 3B),…”
Do these dot stainings represent X chromosome inactivation? If so, please demonstrate that these dots are colocalized with X chromosomes and determine the sex of each embryos as the number of X chr differ between males and females.

[Minor comments]
The authors described that the developmental phenotypes of maternal Ezh1/2 double KO embryos could be classified into three categories, but how such phenotypic variation could happen in the genetically identical embryos? Please provide a possible explanation for this.
Fig S1E, S1F, S2E, and S2F; Please quantify the signal intensities of H3K27 methylation in maternal genome.
P5, "sF/+, sKO/+, dF/+ and dKO/+" What are these words abbreviated to? In particular, sF and dF need to be mentioned. Also, dF is actually a single KO for Ezh1, so it would be better to change the description to make it clearer.

First revision

Author response to reviewers' comments

Response to reviewer #1:

Comment 1： A previous report demonstrated that the loss of maternal H3K27me3 caused the growth arrest of embryos after implantation (Inoue et al., 2018), which suggests that the persistence of maternal H3K27me3 imprinting is essential for the development. However, the authors shows that disappearance of H3K27me3 by the deletion of EZh2 did not affect the development. I suppose that one plausible explanation for this discrepancy is that H3K27me3 would remain in some regions involving imprinting in EZh2 KO oocytes, although most of H3K27me3 was lost: the loss of H3K27me3 was checked only by immunohistochemistry. To address this possibility, the transcriptome analysis (RNAseq) for Ezh2 KO embryos should be conducted.
Answer 1: Thank you for your suggestion. Unfortunately, we could not complete the transcriptome analysis (RNAseq) for Ezh2 KO embryos test because we didn't get enough Ezh2 KO embryos. However, we have performed the RNA-seq on Ezh1/2 KO embryos and found that Ezh1/2 KO affects H3K27me3 imprinting genes and development (Fig. 8). The reason that single Ezh2 KO does not contribute to defective development maybe as follows:
(1) H3K27me3 still enriched in locus of imprinting genes despite loss of H3K27me3 staining; for this case, Ezh1 may safeguard this modification. (2) some genes that are not critical to development are loss of imprinting. (3) extra epigenetic regulator probably

guards imprinting genes by Ezh2 KO. These hypotheses are all need to be investigated.

Comment 2 : Page 6, line 9-12: It is too immature to suggest that H3K27me2 may be contributed to X chromosome inactivation because some cells had two dots of H3K27me2 staining. Additional evidence should be required to claim it.
Answer 2: We agree that more evidences are need to suggest that H3K27me2 may be contributed to X chromosome inactivation. We tried DNA-FISH with IF to determine this, but this experiment could not be performed successfully. We deleted this claim in the revised manuscript. Whether H3K27me2 is contributed to X chromosome inactivation will be part of our ongoing work.

Comment 3: Page 6, line 16-17: No evidence is shown that Ezh1 plays an assistant role in H3K27me3 modification in oocytes and early embryos. The analysis for single Ezh1 KO is required to claim it
Answer 3: You are right. Our current results are enough to support the conclusion "Ezh1 plays an assistant role in H3K27me3 modification in: oocytes and early embryos". We have modified this conclusion in the revised manuscript: "maternal Ezh1 plays assistant roles in H3K27me2 modification".

Comment 4: Page 10, line 18-24: Although the authors conclude that EPI development is associated with H3K27me3 modification, it is come from the analyses of dKO embryos. Therefore, the possibility cannot be excluded that H3K27me2 but not H3K27me3 would be involved in EPI development. sKO embryos in which only H3K27me3 is lost should be analyzed to investigate the involvement of H3K27me3.
Answer 4: Thank you for your valuable advice. We have investigated the EPI development in sKO embryos and found "strong correlations for Nanog+ cells and H3K27me3 modification from both dF/+ and dKO/+ embryos (sFig. 7D). However, Nanog and H3K27me3 cell numbers showed no notable difference between sF/+ and sKO/+ late blastocysts, although they exhibited obvious correlation in sF/+ and sKO/+ embryos (sFig.8)."

Comment 5: Page 2, line 15: "but not single Ezh1 or Ezh2": The single Ezh2 KO has not been analyzed in this report.
Answer 5: We are sorry for our unclear descriptions. We have analyzed the reproductive ability of single Ezh2 KO and found the single Ezh2KO has normal litter size (**sKO** in Fig. 3A-C). Thank you for point this out.

Comment 6: I do not understand what Fig. 3A, C, D, F represent. Are they shown to represent the rates of successful mating? I suppose that Fig. 3B and E are enough to represent the effect of KO on the development.
Answer 6: We are sorry for our unclear descriptions again. The accumulated pups over time were described in Fig. 3A and D. The average time of pregnancy or parturition for female mice was described in Fig. 3C. We referred to the classic papers about reproductive to describe our results, such as：Claudia Andreu-Vieyra et al., Molecular Endocrinology 22 (9):2141-2161, doi: 10.1210/me.2008-0033.

Comment 7: Page 7, line 5-8: "sFig. 3" should be "sFig. 4".
Answer 7: Thank you for point this out. We have corrected it in the revised manuscript.

Response to reviewer #2:

Comment 8：authors concluded that maternal Ezh1/2 are required for the establishment of H3K27me2/3 in in vivo preimplantation embryos and play critical roles in embryonic development in mouse. However, the data do not indicate whether pre-fertilization maternal DNA H3K27me3 levels are important or post-fertilization H3K27me3 reconstruction is important for embryogenesis. Since H3K27me3 in early embryos does not recover in KO mice using WT sperm, Ezh1/2 may not be expressed in the early stages of development. In that case, there are multiple possibilities for the reconstruction mechanism of H3K27me3. For example, it may be supplemented with mRNA or protein

stored in the oocyte. The reconstruction of H3K27me3 in the early stage may be led by H3K27me3 of Maternal DNA. In order to clarify these, I think it is better to perform a rescue experiment by injection of Ezh1/2 mRNA. If Ezh1/2 is needed to reconstruct H3K27me3 in early development, it should be possible to rescue it by injection of Ezh1/2 mRNA into a dKO Zygote.
Answer 8: Thank you for this suggestion. How H3K27me3 is reconstructed in KO embryos is unknown and need to be investigate. But, we are sorry we are not successful to perform that experiments due to technical and time limitation in terms of our current conditions.

Comment 9 : In addition, clarifying the expression level and localization of Ezh1/2 in the early development of control embryos will be more convincing. It can be examined by analysis of previously reported RNA-seq data, immunostaining, Western blotting, etc.
Answer 9: Thanks for your helpful advice. we could refer a recent report (Tie-Gang Meng et al., Nat Commun. 2020 Dec 11;11(1):6354.doi: 10.1038/s41467-020-20242-9.). In this paper, Ezh2 protein is in oocyte and early embryos, whereas Ezh1 protein level is lower and are reduced rapidly after fertilization.

Comment 10: Authors reported enlarged placentas existed in late development of dKO/+ embryos. Recent report showed that loss of noncanonical H3K27me3 imprinting could be the cause of placental enlargement (Mei et al., Nat Genet. 2021 Apr;53(4):539-550. doi: 10.1038/s41588-021-00820-3). Recently H3K27me3 andPolycomb study was updated (Chen et al., Nat Genet. 2021 Apr;53(4):551-563. doi 10.1038/s41588-021-00821-2.). Please revise discussion section, including the latest findings.
Answer 10: Thank you for your advice. We have rediscussed this in this part with these latest findings.

Comment 11. Please show quantitative data. Please do not just post the photo, but quantify it. Figure1 and 2; Please measure the fluorescence intensity (H3K27me2/3) and make a graph.Figure 6B and C; Please count cell number and categorize cell type like Figure S5.Figure 7E; Please count cell number and categorize cell type.
Answer 11: Thank you for this suggestion. We quantified these results (sFig.7).

Response to reviewer #3:

Comment 12： One unfortunate point, however, is that the molecular relationships between defective H3K27 methylation and developmental abnormalities was not provided; in other word, how the loss of H3K27me3 in Ezh1/2 double maternal KO oocytes or delayed catch-up in the early embryos causes the abnormal gene expression in the embryos? Which gene was more affected? Are such defective gene regulations direct effect or indirect effect? Since there was no RNA-seq of maternal Ezh1/2 KO embryos, or H3K27me2/3 ChIP-seq of oocytes, such relationships remained unclear.
Answer 12: This is a grate suggestion. We have done the RNA-seq for determine the effect of Ezh1/2 on imprinting genes and identified many putative H3K27me3-dependent imprinting genes (Fig 8 in P14).

Comment 13 : H3K27me3 has been shown to be involved in the regulation of atypical genomic imprinting genes as a maternal factor (Inoue et al., Nature, 2017; Inoue et al.,Gene Dev, 2018; Mei et al., Nat Genet, 2021). However, in this paper, theeffects of Ezh1/2 KO on such imprinting genes were not analyzed at all, and werenot even discussed. In particular, the large placenta phenotype observed inmaternal Ezh1/2 KO embryos is assumed to be caused by abnormalities in theseimprinting genes.
Answer 13: Thank you for pointing this out. We have the effects of Ezh1/2 KO on imprinting genes by the RNA-seq and identified many putative H3K27me3-dependent imprinted genes (Fig 8 in P14). We have also rediscussed "large placenta phenotype" in P18 (line6-14) in the revised manuscript.

Comment 14 : H3K27me3 appeared to be lost in Ezh2 single maternal KO (FigS1). Despite such situation, the embryos derived from Ezh2 KO oocytes did not show any defects. Such loss of H3K27me3 does not affect H3K27me3-metiated genomic imprinting?
Answer 14: Thank you for your good question. Our revised results (Fig. 8) showed that Ezh1/2 KO affects H3K27me3 imprinting genes and development. The reason that single Ezh2 KO does not contribute to defective development maybe as follows: (1) H3K27me3 still enriched in locus of imprinting genes despite loss of H3K27me3 staining; for this case, Ezh1 may safeguard this modification. (2) some genes that are not critical to development are loss of imprinting. (3) extra epigenetic regulator probably guards imprinting genes by Ezh2 KO. These hypotheses are all need to be investigated.

Comment 15: P5, "Enhanced H3K27me3 staining in inner cell mass (ICM) appeared at sKO/+ late blastocyst stage, along with dot staining in trophectoderm (TE) in some embryos, which was comparable to sF/+ embryos (Fig. 1A)" P6, "Intriguingly, the dot staining of H3K27me2 was strongly increased in dKO/+ embryos and some cells had two dot staining at morula stage (sFig. 3A and 3B)" Do these dot stainings represent X chromosome inactivation? If so, please demonstrate that these dots are colocalized with X chromosomes and determine the sex of each embryos as the number of X chr differ between males and females.
Answer 15: Thank you for your important point and good suggestion. We tried DNA-FISH with IF to determine this, but this experiment could not be performed successfully. We have deleted our claim in the revised manuscript:

Comment 16: The authors described that the developmental phenotypes of maternal Ezh1/2 double KO embryos could be classified into three categories, but how such phenotypic variation could happen in the genetically identical embryos? Please provide a possible explanation for this.
Answer 16: Thank you for the suggestion. We have added explanations in P17-18 (discussion in "Maternal Ezh1/2 in placental development and imprinted genes").

Comment 17: Fig S1E, S1F, S2E, and S2F; Please quantify the signal intensities of H3K27 methylation in maternal genome.
Answer 17: Thank you for your advice, we quantified the signal intensities and showed these data in sFig 3.

Comment 18: P5, "sF/+, sKO/+, dF/+ and dKO/+" What are these words abbreviated to? In particular, sF and dF need to be mentioned. Also, dF is actually a single KO for Ezh1, so it would be better to change the description to make it clearer.
Answer 18: We are sorry for our unclear descriptions. Following your suggestion, we have improved our description in the revised manuscript: "Embryos from sCtrl, sKO, dCtrl and dKO females mated with wild type males were referred to as sF (no Ezh1 and Ezh2 KO) /+, sKO(single EZH2 KO)/+, dF(single EZH1 KO)/+ and dKO (Ezh1 and Ezh2 KO)/+, F represents flox)"

Second decision letter

MS ID#: DEVELOP/2021/200316

MS TITLE: Maternal Ezh1/2 deficiency in oocyte delays H3K27me2/3 restoration and impairs epiblast development responsible for embryonic sub-lethality in mouse

AUTHORS: Yinan Zhao, Dandan Bai, You Wu, Dan Zhang, Mengying Liu, Yingpu Tian, Jinhua Lu, Shaorong Gao, Hai-Bin Wang, and Zhongxian Lu

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 still 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. If it would be helpful, you are welcome to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating your plans for addressing the referee's comments, and we will look over this and provide further guidance.

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

In this study, the roles of histone H3K27 methylation in the development and lineage determination are investigated by analyzing Ezh1 and Ezh2 KO mice. The maternal deletion of Ezh2 caused the disappearance of H3K27me3 in oocytes and embryos at the early preimplantation stage, whereas H3K27me2 was lost by double KO of Ezh1 and 2. Although the maternal single Ezh2 KO did not affect the development, the litter size was decrease by double KO, which is caused by the deficient differentiation into epiblast. These results would contribute to the understanding the role of Ezh1/2 in H3K27 methylation and the development in mice.

Comments for the author

The authors have addressed satisfactorily most of issues raised in the initial review. However, some minor issues still remain to be addressed.

Fig. 3 : I do not still understand what "Litters per mouse" stands? More detailed explanation for Fig. 3C and 3F would be required.

Page 2, line 10-11: I made a wrong description on the previous comment as shown below. The correct one is: The single "Ezh1" KO has not been analyzed in this report. Therefore, the authors cannot describe "but not single Ezh1 or Ezh2". They can mention only Ezh2 but not Ezh1.
>Comment 5: Page 2, line 15: "but not single Ezh1 or Ezh2": The single Ezh2 KO has not been
>analyzed in this report.
>Answer 5: We are sorry for our unclear descriptions. We have analyzed the reproductive
>ability of single Ezh2 KO and found the single Ezh2 KO has normal litter size
>(**sKO**inFig.3A-C). Thank you for point this out.

Reviewer 2

Advance summary and potential significance to field

In the revised version of this study, authors added quantification data and RNA-seq data.
Quantification data made the figure easier to understand and more convincing.
From RNA-seq data, authors showed maternal Ezh1/2 KO embryos has critical effect on expression of essential genes associated with stem cell and embryonic development and H3K27me3-dependent imprinting.

Comments for the author

The authors have addressed my concerns and the manuscript is now suitable for publication.

Reviewer 3

Advance summary and potential significance to field

In this study, Zhao et al. demosntrated that maternal depletion of Ezh1 and Ezh2, which are mutually exclusive core components of PRC2, causes developmental defects of descendent embryos in both embryonic and extraembryonic sides that eventually result sublithality at term. Although the redundunt functions of Ezh1 and Ezh2 as a maternal protein has been documented by Meng et al. (Nat Commun 2021) in a partially in vivo system (combined approach of Ezh2 maternal KO and Ezh1 KD by siRNA injection), the biological consequences of such maternal double depletion of Ezh1/2 has not been analyzed yet. Thus, this study by Zhao et al. provides not only a genetic in vivo evidence that Ezh1 and Ezh2 has a redundunt function in H3K27 methylation in oocytes and descendent early embryos, but also a link between such defective H3K27 methylation and developmental anormalities in the postimplantation developmental processes.
One unfortunate point, however, is that the molcular relashionships between defective H3K27 methylation and developmental abnormalities was not provided; in other word, how the loss of H3K27me3 in Ezh1/2 double maternal KO oocytes or delayed catch-up in the early embryos causes the abnormal gene expression in the embryos? Which gene was more affected? Are such defective gene regulations direct effect or indirect effect? Since there was no RNA-seq of maternal Ezh1/2 KO embryos, or H3K27me2/3 ChIP-seq of oocytes, such relationships remained unclear.

In the revised manuscript, the authors demonstrated by RNA-seq that Ezh1/2 maternal KO embryos showed abnormal expression of stem cell factors, such as Fgf4 and Sox2, after implantation. However, some points the reviewer(s) raised were not properly answered, and the results are unclear due to inappropriate analysis. In addition, there are a number of inaccurate sentences in the text so I believe that a professional proof reading should be conducted.

Comments for the author

1. Regarding the analysis of H3K27me3 imprinting genes, the experimental methods and analyses performed are unclear. It should be described more clearly how many of the H3K27me3 imprinting genes reported so far show loss-of-imprint in Ezh1/2 maternal KO embryos (and Ezh2 maternal KO embryos).
2. It is unfortunate that no analysis was successfully performed on the lack of phenotype in Ezh2 single maternal KO despite the loss of H3K27me3. It would be better to at least include potential explanations in the Discussion; " (1) H3K27me3 still enriched in locus of imprinting genes despite loss of H3K27me3 staining; for this case, Ezh1 may safeguard this modification. (2) some genes that are not critical to development are loss of imprinting. (3) extra epigenetic regulator probably guards imprinting genes by Ezh2 KO. These hypotheses are all need to be investigated."
3. The request for a description of phenotypic variation in Ezh1/2 double MKO was not answered, and the molecular background for the placental phenotype described in "Maternal Ezh1/2 in placental development and imprinted genes" appeared unclear to me.

Second revision

Author response to reviewers' comments

Response to reviewer #1:

Comment 1 : Fig. 3： I do not still understand what "Litters per mouse" stands? More detailed explanation for Fig. 3C and 3F would be required.
Answer 1: We are sorry for our unclear descriptions. "Litters per mouse" means the number of parturitions per mouse during fertility study. We have added more detail in P20 (line 1-5) in the revised manuscript.

Comment 2: Page 2, line 10-11: I made a wrong description on the previous comment as shown below. The correct one is: The single “Ezh1” KO has not been analyzed in this report. Therefore, the authors cannot describe “but not single Ezh1 or Ezh2”. They can mention only Ezh2 but not Ezh1.
Answer 2: We are sorry for our unclear descriptions again. Actually, the single “Ezh1” KO is the dCtrl or dF/+ group. We have analyzed the fertility ability of dCtrl (Ezh1 KO) females in Fig 3. The litter size of dCtrl females is similar to that of sCtrl (wildtype) females.

Response to reviewer #3:

Comment 3 : Regarding the analysis of H3K27me3 imprinting genes, the experimental methods and analyses performed are unclear. It should be described more clearly how many of the H3K27me3 imprinting genes reported so far show loss-of-imprint in Ezh1/2 maternal KO embryos (and Ezh2 maternal KO embryos).
Answer 3: Thank you for your helpful suggestion. We have added more clear description of the analysis of H3K27me3 imprinting genes in section “Materials and Methods” in P23 (line27-30) and P24 (line1-5). So far, there are no other report on the H3K27me3 imprinting genes in Ezh1/2 maternal KO embryos (and Ezh2 maternal KO embryos). We have discussed that information in P18 (line 23-30).

Comment 4 : It is unfortunate that no analysis was successfully performed on the lack of phenotype in Ezh2 single maternal KO despite the loss of H3K27me3. It would be better to at least include potential explanations in the Discussion; “(1) H3K27me3 still enriched in locus of imprinting genes despite loss of H3K27me3 staining; for this case, Ezh1 may safeguard this modification. (2) some genes that are not critical to development are loss of imprinting. (3) extra epigenetic regulator probably guards imprinting genes by Ezh2 KO. These hypotheses are all need to be investigated.”
Answer 4: Thank you for your good suggestion. We have added the potential explanations in the Discussion in Page 15 (line 11-19) in the revised manuscript.

Comment 5 : The request for a description of phenotypic variation in Ezh1/2 double MKO was not answered, and the molecular background for the placental phenotype described in "Maternal Ezh1/2 in placental development and imprinted genes" appeared unclear to me.
Answer 5: We are sorry for our unclear answer for how the phenotypic variation could happen in the genetically identical embryos in our study. The reason may come from the epigenetic modifications of maternal PRC2 on fetal and placental development. Multiple factors, including the imprinting of many genes and the development of multiple cell types, were involved in this process. Therefore, the long-standing effects of maternal Ezh1/2 KO may vary based on the context in each embryo. We have explained the phenotypic variation (embryo and placenta) in the section “Discussion” in page 18 (line 2-22).
Based on the important role of H3K27me3-modified imprinted genes in placental development, we have discussed the placental phenotypic variation and imprinted genes together in "Maternal Ezh1/2 and imprinted genes in placental development " in page 18 and 19 in the revised manuscript.

Comment 6 : In addition, there are a number of inaccurate sentences in the text, so I believe that a professional proof reading should be conducted.
Answer 6: Following your advice, our manuscript has been polished by Nature Publishing Group Language Editing. The certification No: 927F-583A-D652-8C26- 15DF. (secure.authorservices.springernature.com/certificate/verify).

Third decision letter

MS ID#: DEVELOP/2021/200316

MS TITLE: Maternal Ezh1/2 deficiency in oocyte delays H3K27me2/3 restoration and impairs epiblast development responsible for embryonic sub-lethality in mouse

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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.