nature portfolio

Peer Review File

H3K27me3-dependent imprinting and transcriptional regulation in early mouse embryos requires EZHIP-mediated restriction of PRC2 activity

Corresponding Author: Dr Raphael Margueron

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This study is a follow-up to previous work by the Margueron lab, where they characterized the role of EZHIP, an EZH2-interacting protein that inhibits its activity, in germline development. In this study, they demonstrate that nuclear EZHIP, translated from the mRNA pool in oocytes, is essential for preventing the aberrant accumulation of H3K27me3 in early embryos. Overall, the work is well-designed and well-executed, with high-quality data. The paper is well-written and easy to follow. However, I have some comments that could be considered for the revised version of the manuscript.

Major points
1. Although the impact of Ezhip deletion on the oocyte transcriptome is modest, with almost no correlation between the aberrant gain of H3K27me3 and transcriptome changes, a substantial number of genes are misregulated in Ezhip-mat KO 2-cell embryos and blastocysts. Unlike in oocytes, is there a correlation between the aberrant gain of H3K27me3 and gene downregulation in these cells?
2. It is intriguing that the pattern of H3K27me3 between the maternal and paternal alleles becomes similar in Ezhip-mat KO blastocysts. Does the aberrant gain of H3K27me3 on the paternal allele occur randomly, or does it preferentially target regions that exhibit H3K27me3 on the maternal allele?
3. The authors note that some embryos die around E6.5, with a reduction in litter size observed at E13.5. Including images of Ezhip-mat KO E6.5 and E13.5 embryos alongside their control littermates would help determine if there are any obvious developmental defects in the embryos. Related to this, can live pups be obtained from Ezhip KO mothers? If so, do the offspring develop to term and successfully produce the next generation? I am curious whether the aberrant accumulation of H3K27me3 in the Ezhip-mat KO offspring could affect subsequent generations. To clarify, I am not suggesting additional breeding experiments, as they would require considerable time to address this question.
4. The authors find that many H3K27me3-dependent imprinted genes display biallelic expression in the Ezhip-mat KO blastocysts and E6.5 ExEs. Although Figure 4b shows the allelic ratio as a heatmap, it is unclear whether an increase in the allelic ratio reflects an increase in maternal reads or a decrease in paternal reads. If the latter is true, a plausible explanation for EZHIP's role might be that this protein prevents the ectopic accumulation of H3K27me3 on the paternal allele in pre- and post-implantation embryos, rather than directly contributing to H3K27me3-dependent imprinting established in oocytes.
5. The authors observe aberrant expression of Xist on the maternal allele in the Ezhip-mat KO female embryos. Would it be possible that a decrease of H3K27me3 at the Xist locus in Ezhip KO oocytes cause for the loss of allele-specific Xist expression?

Minor points
What do the white columns in Figure 5c and 5d indicate? If they represent genes that do not meet the criteria for SNP analysis, presenting this data as a heatmap in Figure 4 would make it easier to interpret.

Reviewer #2

(Remarks to the Author)
This manuscript by Diop et all studies the role of PRC2 activity inhibitor EZHIP on genomic imprinting and gene expression in general during early pre and post implantation embryonic development. In their studies the authors show that maternal

depletion of EZHIP results in global changes in H3K27me3 deposition, with an marked increase of H3K27me3 on the paternally inherited chromosomes. This mis-regulation is associated with loss of DNA methylation independent imprinted gene expression. Finally the authors show that imprinted X chromosome inactivation is affected with a reversal of imprinted X chromosome inactivation to a form of X chromosome inactivation where maternal Xist is expressed and X linked genes appear to be randomly inactivated. Overall this is an interesting manuscript that needs a lot of work to understand the mechanism leading to the reported phenotype. I have listed my questions and concerns below that may help the authors to strengthen their conclusions.
The immune shown in 2a is not quantitative, and it is hard to conclude anything except distribution changes but these have not been studied.
In 2d-e expression data is shown, it would be helpful and interesting to relate these changes to the H3K27me3 status of the genes analyzed.
The conclusion that the effects of Kdm6a and EZHIP KOs are very different are expected as Kdm6a is part of COMPASS and involved in local removal of H3K27me3 at enhancers, whereas EZHIP plays a role in broader H3K27me3 domain formation.
In 3b besides native H3 an H3K27me3 template is provided. Upon addition of EZHIP H3K27me3 is lost, what does this enzymatically mean, is there demethylase activity present that cannot be overcome by the inhibiting activity of EZHIP, what is the working model here? What is the difference between the PRC2 complexes?
The study highlighted in 4a shows an increase of H3K27me3 on the paternal imprinted loci. Why would this lead to more maternal expression of imprinted genes? Is this because the paternal expression is shut down, and you are now able to pickup low levels of maternal expression. Again what is the model here?
In 4d it is concluded that the placental size is similar between WT and KO but that the EZHIP KO embryos are smaller. It is concluded that there may be a placental disfunction here. One could as well conclude that there is a problem with embryonic development independent of the placenta.
In 5a it is concluded that Xist is robustly expressed in male KO embryos, what is the expression relative to female embryos here (to be able to conclude that expression is robust).
This figure misses RNA-FISH or scRNA-seq data to fully understand the phenotype (random Xist expression or bi-allelic, same for silencing).
A switch from imprinted to random X inactivation has been described before, for instance for the Tsix KO.
Finally, what I miss in this chapter is a clear rationale of what is going wrong. The Xist H3K27me3 imprint is inherited through the maternal germline, and in the EZHIP KO more H3K27me3 is reported suggesting increased H3K27me3 on the paternal Xist locus (this is not but should be shown). Then why would this lead to bi-allelic or female specific Xist expression?

Reviewer #3

(Remarks to the Author)
Diop and colleagues study the role of Ezhip, an inhibitor of PRC2, finding that the oocyte derived maternal pool of Ezhip is required for correct polycomb deposition in mice. Without maternal Ezhip, H3K27me3 becomes enriched in the oocyte. H3K27me3 also becomes enriched in the embryo, particularly on the paternally derived chromosomes of the blastocyst, suggesting the maternal pool of Ezhip is required to limit PRC2 activity in the embryo. IF confirms that Ezhip is present in the zygote and early embryo, however it is predominantly cytoplasmic. Counterintuitively, the increased H3K27me3 results in loss of H3K27me3-mediated imprinting, leading to biallelic expression of the imprinted genes. One such imprinted gene is the X inactivation regulator Xist, which becomes biallelic, inducing X inactivation of both X chromosomes in a subset of female blastocysts. These findings are of high interest and are important for understanding development, unfortunately a lack of appropriate controls and replication mean the findings are largely unsupported by the data. My concerns are detailed below.

Major Concerns

1. The genetics of the Ezhip-matKO are not accurately described, however it seems that it is deleted in the whole mother rather than specifically in the oocyte. If it is deletion in the whole mother, then embryos are heterozygous for Ezhip, while control embryos are WT. Therefore, the observations may be due to maternal Ezhip, as suggested, or may be a heterozygous effect. To rule out a heterozygous effect the reciprocal cross must be performed, to make control embryos that are also heterozygous receiving the deleted allele from the father. All experiments would need to be repeated in these control mice to determine which effects are due to loss of maternal Ezhip and which are a heterozygous effect.

2. There is no increase in total expression of Xist, that accompanies activation of the maternal allele, which would be expected. The authors suggest biallelic Xist leads to inactivation of both X's, however confusingly biallelic expression is observed. These data are obtained in blastocyst, where X reactivation occurs, such that only developmentally early blasts show imprinted XCI in the whole embyo, but more developed blastocysts show imprinted XCI only in extraembryonic tissue and biallelic expression in the ICM. Therefore, a more logical explanation for biallelic Xist and X-linked expression in matKO blastocysts is that there's a change in developmental timing of the embryos, where development is accelerated in the matKO samples. The authors should address a potential developmental timing change, or look in morula, where XCI is strictly imprinted, or in extraembryonic tissue. IF for H3K27me3, or FISH for XIST, would be expected to show 2 foci (and 1 foci in male) if they're observing precious XCI of the maternal X, this should be performed.

3. The biochemical assays shown in Figure 3F, suggest that Ezhip does not bind chromatin, however this assay lacks a positive control. Does PRC2 bind in this assay? What is the DNA and chromatin used in this assay, is it a region that

polycomb should bind? Does Ezhip require PRC2 in the assay to bind chromatin?

4. Experiments are frequently only superficially described, making it hard to understand the importance of each. More specific details should be included in the text, the methods and on the figures themselves.

5. Data is frequently presented as only one replicate, even when multiple replicates have been performed, this includes genomics, IF and biochemical assays. All experiments should be quantified and a statistical analysis of the replicate measurements performed, even where representative images must be shown.

6. Of particularly concern is that the cut'n'run data appears to be only one replicate, when it is these results that form the basis of the study. Showing a global change is difficult and the authors should be commended for using a spike-in, however this gives a lot of weight to the single spike in measurement. How much of the global difference is due to the spike in? Replicates need to be performed to ensure these results aren't an anomaly.

Minor concerns

1. Fig 1g. What's a HiC cluster? What's the difference between a polycomb associated domain and a polycomb enriched domain.

2. Fig 3d. What is PRC2/J2/A? This is not defined or described in the text.

3. Authors state that Ezhip-matKO does not alter the asymmetric distribution of H3K27me3 between the gametes since it is increased in the oocyte, however no formal analysis of this was presented.

4. Fig 4a, how far does the scale extend beyond the TSS and TES? Making this longer, so you can tell if you're seeing a peak or background would help these plots. How many genes are these based on and how were the imprinted genes decided on?

5. Fig 4c, these genes seem cherry picked. This should be a more formal statistical analysis, probably using gene set testing.

6. Show the H3K27me3 data for the imprint at the Xist locus.

7. The rescue of the sex ratio in Fig S5b is compelling data. Expression of maternal Xist should be confirmed in these embryos. Also, XCI should be assessed by XIST or H3K27me3 staining.

Reviewer #4

(Remarks to the Author)
The manuscript by Diop et al investigates the role of EZHIP in regulating PRC2 activity during early embryogenesis and provides insights into the mechanisms of H3K27me3-dependent imprinting and transcriptional regulation. The authors convincingly demonstrate that EZHIP restricts PRC2 activity, and that the loss of EZHIP affects H3K27me3 landscapes, parental genome asymmetry and thereby proper embryonic development. This is demonstrated through a combination of genetic deletion models, transcriptomic analyses, and chromatin profiling, all of which comprehensively support the authors' conclusions. Overall, the work addresses a critical aspect of epigenetic regulation during mammalian development and its consequences on genomic imprinting and X-chromosome inactivation. Below, I outline my comments and suggestions for improving the clarity and the impact of the manuscript.

1) Figure 1A (and other panels displaying chromatin enrichment tracks): The y-axis representation is not clearly defined. Is it FPKM, log2 fold change, or C&R normalized to IgG? Providing this information would help to clarify the presented data and aid in understanding the magnitude of H3K27me3 enrichment changes. Also, it is not clear from the manuscript in how many biological replicates were the assays performed.

2) Figure 2D: It would be useful if the authors could comment on the GO terms of the differentially expressed genes. Also, could the authors clarify whether there is an overlap of affected genes between the different developmental stages (e.g., 2C and blastocyst)? Specifically, do the 250 downregulated and 362 upregulated genes in the 2C stage remain affected in the blastocyst or at later stages?

3) Figure 4: The authors provide follow-up analyses on a subset of 16 genes that remain aberrantly enriched in H3K27me3 post-implantation (E6.5). Nevertheless, it is not clear what happens with the global H3K27me3 landscape in E6.5 matKO embryos. Is the global landscape restored to the wt state (except for the small number of genes that are discussed)? Global assessment by C&R at later stages would have been useful to better understand the matKO phenotype. Also, at E6.5 global DNA methylation should already be largely restored after the first reprogramming event. Could the authors check, using available WGBS data, whether the 16 targets reside in hypomethylated domains? Or is there any other specific sequence feature that characterises this gene group?

4) Discussion: The authors could consider including a brief section on potential implications of EZHIP regulation beyond

preimplantation development, particularly in later developmental stages or potentially in pathological contexts such as imprinting disorders? A sentence or two on the evolutionary conservation of EZHIP would also be useful.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have fully addressed all my concerns, and I have no further comments.

Reviewer #2

(Remarks to the Author)
The authors addressed most of my questions. I have a few more concerns/suggestions:
Regarding the relationship between changes in H3K27me3 and gene expression the authors show in their rebuttal figure 1 that there is no such correlation. I think this is an important result that should be presented in the manuscript and not only to the reviewers.

Supplementary Figure 5 refers to chr6 but is showing the X-linked X inactivation center.
Second this figure show a decrease in H3Kme3 at the Xist regulatory region, which may explain maternal Xist activation, has this been done in an allelic setting?

Finally, the reviewer figure 5 showing Xist expression should be included as it shows the expression is lower than observed in female embryo's and might be the consequence of an impact of the mutation on other mechanisms that regulate Xist expression, for instance increase levels of RLIM would give a similar phenotype.

Reviewer #3

(Remarks to the Author)
The authors have performed a commendable number of additional experiments, which have made the manuscript more compelling. My primary concern remains however. I'm happy to accept the authors explanation that comparing heterozygous embryos to WTs is valid if there is no embryonically derived Ezhip, however currently provided data falls short of justifying this. The addition of Ezhip qPCR is useful, however RNA-seq would do a better job of detecting low level embryonic expression - can you see paternally derived Ezhip reads in the blastocyst RNA-seq from Fig2? The Ezhip IF should be done in blastocysts as well. In the rebuttal, authors state that there is no obvious heterozygous effect when the Ezhip deletion is paternally inherited. This data should be shown. A measurement of H3K27me3 in a blastocyst with a paternally inherited Ezhip deletion would be the most compelling addition. Finally, it should be clearly stated in the text that controls are WT and tests heterozygous, with the evidence for why this is a fair comparison. Presently this is not made clear.

Reviewer #4

(Remarks to the Author)
The authors have addressed the main points raised in the earlier review. The manuscript now reads well and in my opinion is suitable for publication.

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
The authors addressed all my concerns/questions.

Reviewer #3

(Remarks to the Author)
The authors have sufficiently addressed my concerns, and I would support publication.

We would like to thank the reviewers for their time and valuable comments. We have strived to address their comments and believe that this has significantly improved the quality of the study. We hope that they will share our opinion that the manuscript is now ready for publication.

REVIEWER COMMENTS

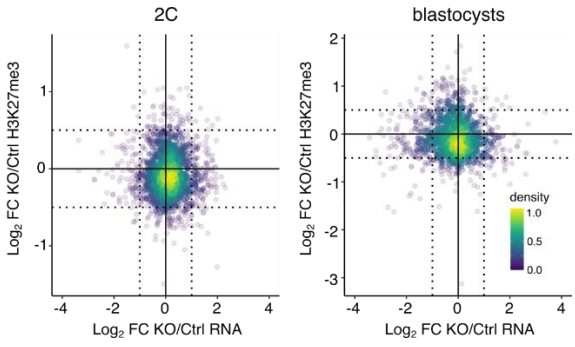
Reviewer #1 (Remarks to the Author):

This study is a follow-up to previous work by the Margueron lab, where they characterized the role of EZHIP, an EZH2-interacting protein that inhibits its activity, in germline development. In this study, they demonstrate that nuclear EZHIP, translated from the mRNA pool in oocytes, is essential for preventing the aberrant accumulation of H3K27me3 in early embryos. Overall, the work is well-designed and well-executed, with high-quality data. The paper is well-written and easy to follow. However, I have some comments that could be considered for the revised version of the manuscript.

Major points

1. Although the impact of Ezhip deletion on the oocyte transcriptome is modest, with almost no correlation between the aberrant gain of H3K27me3 and transcriptome changes, a substantial number of genes are misregulated in Ezhip-mat KO 2-cell embryos and blastocysts. Unlike in oocytes, is there a correlation between the aberrant gain of H3K27me3 and gene downregulation in these cells?

This is an important question that we did not include considering that H3K27me3 enrichment at the 2-cell stage is very low (see H3K27me3 density at imprinted genes in Figure 4a and supplementary Figure 4a which are representative of what happens genome wide, same result can be seen in other studies such as Zheng et al., Mol Cell, 2016). We were therefore afraid that the correlation could be biased by noisy signal. To limit this problem, we considered only genes within regions enriched for H3K27me3 (Fold change >1.5 as determined by csaw and cross-validation based on an independent data set (GSE76687)) and computed the correlation between transcription and H3K27me3 enrichment. The results are shown below (Reviewer Figure 1):



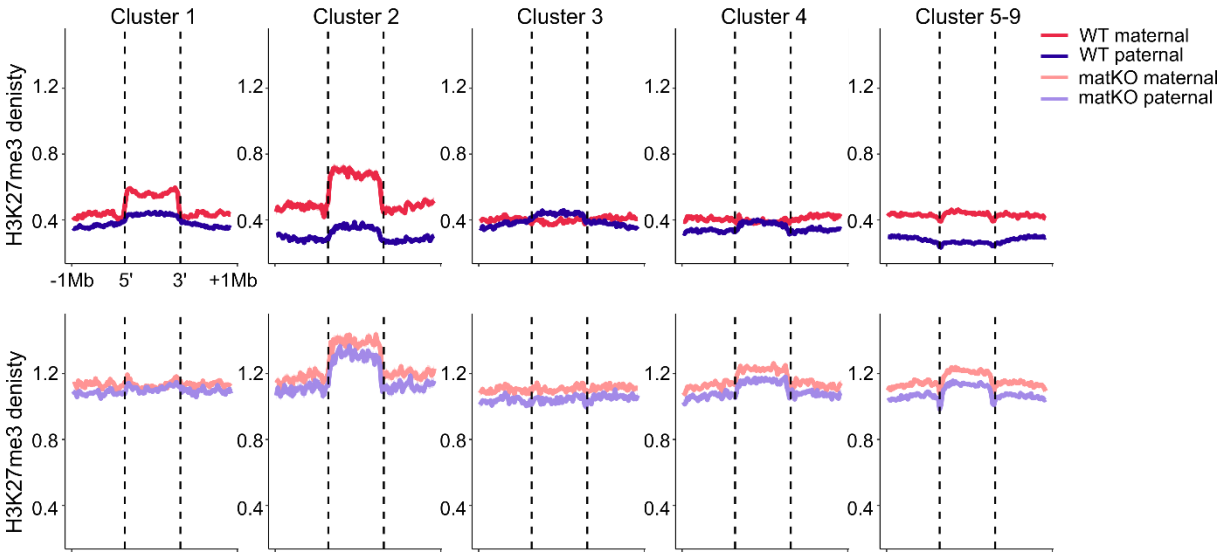
Reviewer Figure 1: Density scatter plot showing the interplay between Log₂(FC) H3K27me3 CUT&RUN signal (y axis) and Log₂(FC) RNAseq data expression (x axis) when comparing Ctr and Ezhip KO at the 2C and blastocyst stages, each dot represents one single genomic region.

This did not reveal any clear link between transcriptional change and altered H3K27me3 deposition. This is consistent with the observation that H3K27me3 increased in *Ezhip-mat*KO

embryos occurs genome wide (See Figure 2A) and is associated with a global reduction of H3K27me3 peaks (due to the flattening of the signal). It appears therefore almost uncoupled from the transcriptional changes.

2. It is intriguing that the pattern of H3K27me3 between the maternal and paternal alleles becomes similar in Ezhip-mat KO blastocysts. Does the aberrant gain of H3K27me3 on the paternal allele occur randomly, or does it preferentially target regions that exhibit H3K27me3 on the maternal allele?

We assume that the reviewer implied *"(...) or does it preferentially target regions that exhibit H3K27me3 on the maternal allele* ***in Ctr embryos****?"*
As shown in Figure 2b, and alluded to in previous response, H3K27me3 pattern in the *Ezhip-matKO* embryos changes on both alleles. The pattern becomes rather flat, covering broadly the genome and similar on both alleles. Therefore, the gains on the paternal alleles are not specific to regions that are normally enriched for H3K27me3 in Ctr embryos.
Another way to address this question is to use the clusters defined in Collombet et al. based on the dynamics of topological-associated domains regulation from oocytes to blastocysts (see also response to reviewers #3, minor point 1). Importantly, the first two clusters are maternally enriched for H3K27me3 and cluster 3 slightly paternally enriched. Clusters 4 to 9 are not enriched for H3K27me3 at the considered developmental stages. In the *Ezhip-matKO* blastocyst, all the clusters have gained H3K27me3, and maternal and paternal alleles are not distinguishable anymore (Reviewer Figure 2).

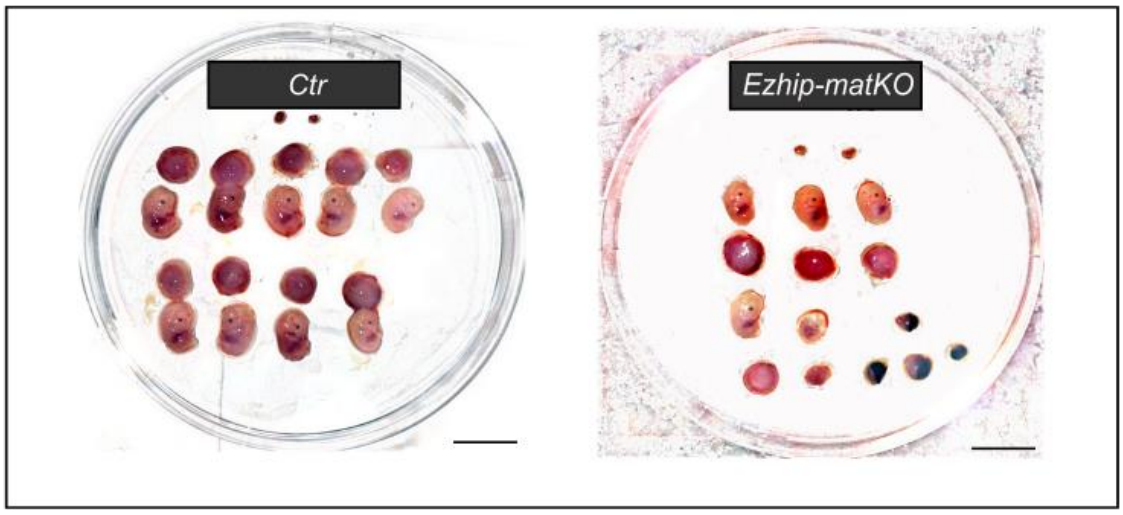


Reviewer Figure 2: H3K27me3 enrichment over clusters of TAD defined in Collombet et al. 2020 in Ctr versus Ezhip-matKO blastocysts.

3. The authors note that some embryos die around E6.5, with a reduction in litter size observed at E13.5. Including images of Ezhip-mat KO E6.5 and E13.5 embryos alongside their control littermates would help determine if there are any obvious developmental defects in the embryos.

For practical reasons (harvesting the extraembryonic tissues as fast as possible), we did not take pictures of embryos at E6.5. Below is a picture of a litter Ctr or *Ezhip-MatKO* at E13.5 dpc which

shows that almost half of the *Ezhip-MatKO* embryos appear wildtype while the other half tends to be fully resorbed (Reviewer Figure 3).



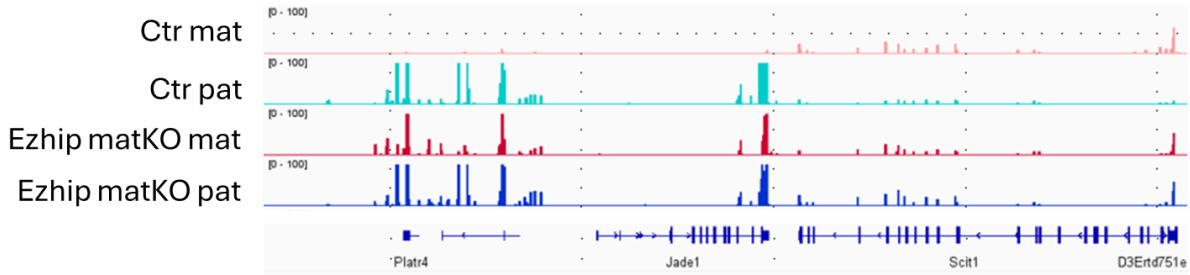
Reviewer Figure 3: Representative pictures of embryos at E13.5 dpc either Ctr or Ezhip-MatKO.

Related to this, can live pups be obtained from Ezhip KO mothers? If so, do the offspring develop to term and successfully produce the next generation? I am curious whether the aberrant accumulation of H3K27me3 in the Ezhip-mat KO offspring could affect subsequent generations. To clarify, I am not suggesting additional breeding experiments, as they would require considerable time to address this question.

Indeed, about half the embryos give rise to live pups that develop mostly normally. We know that the second generation can be fertile, but we have not done a comprehensive analysis to determine whether there are any more subtle phenotypes.

4. The authors find that many H3K27me3-dependent imprinted genes display biallelic expression in the Ezhip-mat KO blastocysts and E6.5 ExEs. Although Figure 4b shows the allelic ratio as a heatmap, it is unclear whether an increase in the allelic ratio reflects an increase in maternal reads or a decrease in paternal reads. If the latter is true, a plausible explanation for EZHIP's role might be that this protein prevents the ectopic accumulation of H3K27me3 on the paternal allele in pre- and post-implantation embryos, rather than directly contributing to H3K27me3-dependent imprinting established in oocytes.

We have now included in Figure 4c a scheme showing how the mRNA of H3K27me3-dependent imprinted genes varies upon maternal deletion of *Ezhip*. It enables to indirectly infer how each allele is regulated. Indeed, since the maternal deletion of *Ezhip* is not necessarily associated to a global increase of gene expression, this indicates that the biallelic expression reflects mostly an increase of maternal expression (and regulatory loops that, in turn, limit paternal expression).
In addition to the example of *Xist* that is shown in Figure 5a, the reviewer will find below the example of Platr4/Jade1 (Reviewer Figure 4).



Reviewer Figure 4: Genome browser view of RNA-seq showing gene expression around the Platr4/Jade1 locus in an allele specific manner in blastocyst either Ctr or Ezhip-MatKO.

5. The authors observe aberrant expression of Xist on the maternal allele in the Ezhip-mat KO female embryos. Would it be possible that a decrease of H3K27me3 at the Xist locus in Ezhip KO oocytes cause for the loss of allele-specific Xist expression?

We are now providing a genome browser view of H3K27me3 enrichment at the Xist locus in oocytes (Supplementary Data Figure 5). This does not support the hypothesis of a loss of H3K27me3 in the mutant condition that would explain its post-fertilization bi-allelic expression.

Minor points
What do the white columns in Figure 5c and 5d indicate? If they represent genes that do not meet the criteria for SNP analysis, presenting this data as a heatmap in Figure 4 would make it easier to interpret.

We apologize for the lack of clarity in the legends that we have now corrected. White square in Figure 5c means absence of allelic information (biallelic is yellow). In Figure 5d, white means no relative change of expression in the mutant as compared to the ctr.

Reviewer #2 (Remarks to the Author):

This manuscript by Diop et all studies the role of PRC2 activity inhibitor EZHIP on genomic imprinting and gene expression in general during early pre and post implantation embryonic development. In their studies the authors show that maternal depletion of EZHIP results in global changes in H3K27me3 deposition, with a marked increase of H3K27me3 on the paternally inherited chromosomes. This mis-regulation is associated with loss of DNA methylation independent imprinted gene expression. Finally, the authors show that imprinted X chromosome inactivation is affected with a reversal of imprinted X chromosome inactivation to a form of X chromosome inactivation where maternal Xist is expressed and X linked genes appear to be randomly inactivated. Overall, this is an interesting manuscript that needs a lot of work to understand the mechanism leading to the reported phenotype. I have listed my questions and concerns below that may help the authors to strengthen their conclusions.

The immune shown in 2a is not quantitative, and it is hard to conclude anything except distribution changes, but these have not been studied.

To address the reviewer comments, we have repeated part of the Figure 2a (IF on 2C embryos) to increase the *n* and be able to quantify the IF. This confirms a marked increase of H3K27me3 signal in the *Ezhip-matKO* embryos as compared to the Ctr, which is still observable at the early blastocyst stage.

In 2d-e expression data is shown, it would be helpful and interesting to relate these changes to the H3K27me3 status of the genes analyzed.

Please see the response to reviewer #1 Major Point 1 (Reviewer Figure 1).

The conclusion that the effects of Kdm6a and EZHIP KOs are very different are expected as Kdm6a is part of COMPASS and involved in local removal of H3K27me3 at enhancers, whereas EZHIP plays a role in broader H3K27me3 domain formation.

We would like to stress that we are not referring to *kdm6A* KO but to kdm6b overexpression in zygote after microinjection, a tool that was used in *Inoue et al. 2017* and *Chen et al. 2021* to globally erase the maternal contribution of H3K27me3. The rationale to compare both transcriptomes is that in both cases (*Ezhip* maternal deletion and *kdm6b* overexpression) H3K27me3-dependent imprinting is lost. Although the underlying mechanisms are different, impairing H3K27me3-dependent imprinting could have had similar transcriptional outcome.

In 3b, besides native H3 an H3K27me3 template is provided. Upon addition of EZHIP H3K27me3 is lost, what does this enzymatically mean, is there demethylase activity present that cannot be overcome by the inhibiting activity of EZHIP, what is the working model here? What is the difference between the PRC2 complexes?

The reviewer is right that we did not provide enough details to follow the assay. Importantly, the assay is performed with purified recombinant proteins and include only what is indicated in the legend. Therefore, we can rule out the presence of demethylase.
"PRC2" refers to the minimal core complex required for the enzymatic activity (EZH2/SUZ12/EED/RBAP48). The core complex has a weak enzymatic activity by itself that can be enhanced either by H3K27me3 peptide or through interaction with cofactors. "PRC2/J2/A" refers to PRC2 in presence of equimolar amount of JARID2 and AEBP2.
The substrate for the enzymatic activity is recombinant nucleosome. The nucleosome is reconstituted with histones expressed in bacteria which therefore do not carry any methyl mark.
The figure shows the inhibitory effect that EZHIP has on PRC2 basal enzymatic activity and on its stimulated activity. The main message is that EZHIP inhibits PRC2 in all tested conditions. Yet the inhibition is partial, therefore when PRC2 is in presence of factors that promote its activity, the residual activity in presence of EZHIP remains higher (See Supplementary Data Figure 3C for quantification).

The study highlighted in 4a shows an increase of H3K27me3 on the paternal imprinted loci. Why would this lead to more maternal expression of imprinted genes? Is this because the paternal expression is shut down, and you are now able to pickup low levels of maternal expression. Again what is the model here?

We agree with the reviewer that the expected result was that both alleles would become more repressed in the absence of EZHIP since H3K27me3 enrichment increases at the H3K27me3-

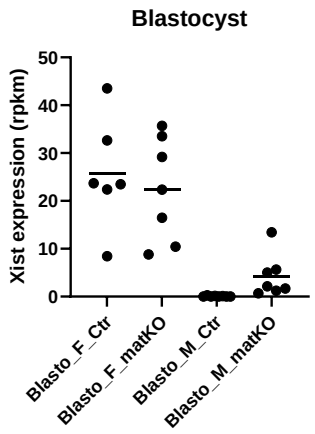
dependent imprinted genes. However, we observed the opposite, and this is an important result of the study. To make this clearer, we have now included the Log_2 RNA-seq FC for the imprinted genes (Figure 4C) which shows that total RNA levels tend to be either increased or to not change for the genes that become biallelically expressed (e.g. *Smoc1, Rbms1*…). This implies that biallelic expression cannot be only explained by paternal expression being reduced and, that maternal expression is necessary increased (See also Reviewer #1, major point 4, Reviewer Figure 4). This result is further supported at the single cell levels by the smRNA-FISH experiment to detect *Xist* which shows that in a number of cells from female *Ezhip-mat*KO blastocysts, *Xist* is expressed from both alleles (New Figure 5b).
In the result section describing Figure 2, we stressed that, H3K27me3 increase in the mutant blastocysts is associated with a flattening of the pattern and with loss of well-defined H3K27me3-enriched peaks. We speculate in the discussion that, the fact that increased deposition of H3K27me3 mitigates transcriptional repression, could be due to an indirect effect on H3K27me3 readers such as CBXs that become diluted throughout the genome due to the lack of clear peaks.

In 4d it is concluded that the placental size is similar between WT and KO but that the EZHIP KO embryos are smaller. It is concluded that there may be a placental disfunction here. One could as well conclude that there is a problem with embryonic development independent of the placenta.

We agree that the impaired ratio between placenta and embryo can be interpreted both ways. We reformulate the conclusion accordingly.

In 5a it is concluded that Xist is robustly expressed in male KO embryos, what is the expression relative to female embryos here (to be able to conclude that expression is robust).

The expression in males is on average one fifth the corresponding expression in females (See Reviewer Figure 5 representing individual values).



Reviewer Figure 5: Xist expression (rpkm) in female and male blastocysts, either Ctr or Ezhip matKO (data extracted from RNAseq presented in Figures 2 and 4).

This figure misses RNA-FISH or scRNA-seq data to fully understand the phenotype (random Xist expression or bi-allelic, same for silencing).

We thank the reviewer for this incentive to perform this missing experiment, probing *Xist* expression in blastocysts, female or male, control or upon maternal deletion of *Ezhip (Figure 5d*

and 5e). This new experiment reveals that about 25% of the cells of female *Ezhip-Mat*KO blastocyst displays two clear clouds and about 50% of the cells of male *Ezhip-Mat*KO blastocyst displays one cloud. This confirms the aberrant expression of *Xist* from the maternal X upon maternal deletion of *Ezhip*.

A switch from imprinted to random X inactivation has been described before, for instance for the Tsix KO.

The RNA-FISH described above argues against the hypothesis of a switch from imprinted to random X inactivation, besides it would not explain the phenotype in males.

Finally, what I miss in this chapter is a clear rationale of what is going wrong. The Xist H3K27me3 imprint is inherited through the maternal germline, and in the EZHIP KO more H3K27me3 is reported suggesting increased H3K27me3 on the paternal Xist locus (this is not but should be shown).

See new Supplementary Data Figure 5 for H3K27me3 enrichment in oocytes at *Xist* locus.

Then why would this lead to bi-allelic or female specific Xist expression?

Our hypothesis is that the absence of EZHIP has two distinct consequences. On the one hand, it results in the loss of the asymmetric distribution of H3K27me3 post-fertilization which could explain the alteration of H3K27me3-dependent imprinting. On the other hand, H3K27me3 is globally altered in the blastocyst, with a flattened pattern and a broader distribution (*i.e.* classical peaks are replaced by a rather blanket-type distribution) which we speculate to dilute any potential H3K27me3 readers and, therefore, *in fine* impairs H3K27me3 mediated transcriptional repression.

Reviewer #3 (Remarks to the Author):

Diop and colleagues study the role of Ezhip, an inhibitor of PRC2, finding that the oocyte derived maternal pool of Ezhip is required for correct polycomb deposition in mice. Without maternal Ezhip, H3K27me3 becomes enriched in the oocyte. H3K27me3 also becomes enriched in the embryo, particularly on the paternally derived chromosomes of the blastocyst, suggesting the maternal pool of Ezhip is required to limit PRC2 activity in the embryo. IF confirms that Ezhip is present in the zygote and early embryo, however it is predominantly cytoplasmic. Counterintuitively, the increased H3K27me3 results in loss of H3K27me3-mediated imprinting, leading to biallelic expression of the imprinted genes. One such imprinted gene is the X inactivation regulator Xist, which becomes biallelic, inducing X inactivation of both X chromosomes in a subset of female blastocysts. These findings are of high interest and are important for understanding development, unfortunately a lack of appropriate controls and replication mean the findings are largely unsupported by the data. My concerns are detailed below.

Major Concerns

1. The genetics of the Ezhip-matKO are not accurately described, however it seems that it is deleted in the whole mother rather than specifically in the oocyte. If it is deletion in the whole mother, then embryos are heterozygous for Ezhip, while control embryos are WT. Therefore, the observations may be due to maternal Ezhip, as suggested, or may be a heterozygous effect. To rule out a heterozygous effect the reciprocal cross must be performed, to make control embryos that are also heterozygous receiving the deleted allele from the father. All experiments would need to be repeated in these control mice to determine which effects are due to loss of maternal Ezhip and which are a heterozygous effect.

The reviewer's comment led us to realize that we did not sufficiently introduce the model which ultimately resulted in some confusion. We have modified the text to clarify this point.
The mouse model that we used was described in a previous publication to which we referred, it is indeed based on a constitutive deletion of *Ezhip* in the mother. Importantly, *Ezhip* is mostly expressed in the germline (See Ragazzini et al. 2019), *Ezhip* mRNA is very abundant in oocytes and transmitted to the zygote within the maternal pool. *Ezhip* expression from the pre-implantation embryos is negligeable. Therefore, the deletion of *Ezhip* in the oocytes translate into a quasi-deletion of *Ezhip* mRNA in the pre-implantation embryos.
We added a new panel (Supplementary Data Figure 2a) to show *Ezhip* mRNA levels at the 2C and blastocyst stages, and it was already shown by IF that EZHIP protein could not be detected at the zygote, 2C and morula stages in the *Ezhip*-matKO embryos.
Finally, although we are not showing it here, it is noteworthy that the paternal deletion of *Ezhip* has no obvious phenotype on embryonic development further ruling out the hypothesis of a phenotype due to the heterozygous deletion of *Ezhip* in the embryos.

2. There is no increase in total expression of Xist, that accompanies activation of the maternal allele, which would be expected. The authors suggest biallelic Xist leads to inactivation of both X's, however confusingly biallelic expression is observed. These data are obtained in blastocyst, where X reactivation occurs, such that only developmentally early blasts show imprinted XCI in the whole embyo, but more developed blastocysts show imprinted XCI only in extraembryonic tissue and biallelic expression in the ICM. Therefore, a more logical explanation for biallelic Xist and X-linked expression in matKO blastocysts is that there's a change in developmental timing of the embryos, where development is accelerated in the matKO samples. The authors should address a potential developmental timing change, or look in morula, where XCI is strictly imprinted, or in extraembryonic tissue. IF for H3K27me3, or FISH for XIST, would be expected to show 2 foci (and 1 foci in male) if they're observing precious XCI of the maternal X, this should be performed.

We thank the reviewer for these comments which prompted us to perform additional experiments, it should unambiguously address this point.
Of note, while developmental timing, or a switch from imprinted to random X inactivation as also suggested by reviewer #2, could indeed contribute to the bi-allelic expression of *Xist* in female embryos, this would not explain the aberrant *Xist* expression in male embryos.
Regarding the developmental timing, we collected the embryos at 3.5 dpc, therefore before the reduction of *Xist* expression that occurs at 4 dpc (See *Borensztein et al. Nat Comm, 2017* for a precise developmental characterization). In the *Ezhip-mat*KO embryos, we did not observe any change in the aspect, at 3.5 dpc, that would suggest a developmental alteration. We are now

providing a more comprehensive analysis of expression of genes involved in development (Supplementary Data Figure 4c), which shows that, while some genes are dysregulated in the mutant embryos, this is not a homogenous trend. Moreover, a change in the developmental timing, would not explain the global reduction in expression of genes from the X observed both in males and females (Figure 6a) but rather the opposite (reactivation).
Finally, we set up smRNA-FISH to detect *Xist* cloud in blastocysts. The experiment, now included in Figures 5b and 5c, shows the presence of 2 clouds in a fraction of cells in female *Ezhip*-matKO embryos and 1 cloud in the mutant male embryos.

3. The biochemical assays shown in Figure 3F, suggest that Ezhip does not bind chromatin, however this assay lacks a positive control. Does PRC2 bind in this assay? What is the DNA and chromatin used in this assay, is it a region that polycomb should bind? Does Ezhip require PRC2 in the assay to bind chromatin?

PRC2 has little sequence specificity in terms of DNA binding except for a bias toward GC-rich regions. Therefore, biochemical characterization of PRC2 binding to chromatin are generally made with repeated DNA sequences carrying a nucleosome positioning sequence enabling homogenous nucleosome spacing (See for example structural characterization of PRC2 from E. Nogales, Poepsel *et al.* NSMB 2018 or the biochemical approach from T. Ceck, Wang *et al.* NSMB 2018).
Here, we have used 3 repeats of the 601 Widom positioning sequence DNA. Using this substrate, we could indeed see a one fraction shift of PRC2 in presence of chromatin as we had reported previously (Margueron et al., Mol Cell, 2008).
We also investigated whether PRC2 could favor the binding of EZHIP, but even in the presence of PRC2, EZHIP has little affinity for chromatin. This result is now included in Supplementary Data Figure 3c.

4. Experiments are frequently only superficially described, making it hard to understand the importance of each. More specific details should be included in the text, the methods and on the figures themselves.

We have updated the legends and the Material and Methods to make the experiment clearer to understand (See for instance Figure 3 and, HMT assays or sucrose gradient in Material and Methods).

5. Data is frequently presented as only one replicate, even when multiple replicates have been performed, this includes genomics, IF and biochemical assays. All experiments should be quantified and a statistical analysis of the replicate measurements performed, even where representative images must be shown.

For the CUT&RUN experiments, each experiment is the aggregation of individual experiments/embryos, which by themselves have a very low coverage but together they can be exploited (*i.e.* pseudo bulk). We have added a table (Supplementary Table 1) summarizing the *n* for each experiment.
We added statistics/quantification to Figure 2a, Figure 3d and 3e, Figure 4c, Figure 6d.

6. Of particular concern is that the cut'n'run data appears to be only one replicate, when it is these results that form the basis of the study. Showing a global change is difficult and the authors should be commended for using a spike-in, however this gives a lot of weight to the single spike in measurement. How much of the global difference is due to the spike in? Replicates need to be performed to ensure these results aren't an anomaly.

As explained above, CUT&RUN experiment results represent the aggregation of many different experiments together and are not a unique replicate. The precise number of embryos/oocytes is now provided in Supplementary Table 1. This is a standard practice for early embryo studies considering the limiting amount of starting materials.
Regarding the role of Spike-in, our main conclusions are actually independent of Spike-in correction. Clustering presented in Figure 2c is by nature independent of factor correction. Comparison of symmetry between maternal and paternal genome is intrinsically normalized since both alleles originate from the same embryo. The shape of the peaks is also independent of Spike-in normalization.

Minor concerns

1. Fig 1g. What's a HiC cluster? What's the difference between a polycomb associated domain and a polycomb enriched domain.

HiC clusters were defined by Collombet et al., they regroup TAD (topological-associated domains) with similar properties based on dynamic regulation and allele specific regulation in embryos from zygote to 8C stage. Two clusters were shown to have a specific enrichment for H3K27me3 and were labelled for simplicity "Polycomb Enriched Domains".
The Polycomb associated domains were defined by Du et al. based on H3K27me3 enrichment in oocytes.
We kept both types of domains since the PAD presents the advantage of being defined in oocytes which is more relevant at this stage. The HiC clusters are interesting because we can compare the clusters enriched for H3K27me3 to the ones that tend to be depleted.

2. Fig 3d. What is PRC2/J2/A? This is not defined or described in the text.

We have updated the HMT assays description that was indeed incomplete.

3. Authors state that Ezhip-matKO does not alter the asymmetric distribution of H3K27me3 between the gametes since it is increased in the oocyte, however no formal analysis of this was presented.

Our sentence was probably unclear, we modified it. We meant to express that since the maternal deletion of EZHIP overall increases H3K27me3 in the allele inherited from the oocytes and has no impact on the allele inherited from sperm, the asymmetry is necessarily maintained, if not enhanced. This is illustrated by Figure 4a and by Supplementary Data Figure 4 looking at the different types of imprinted genes.

4. Fig 4a, how far does the scale extend beyond the TSS and TES? Making this longer, so you can tell if you're seeing a peak or background would help these plots. How many genes are these based on and how were the imprinted genes decided on?

We apologize for inadvertently removing part of the legend while making the figure. As for the Supplementary data Figure 4a, the scale extends 5kb upstream and downstream of TSS/TES. Increasing the scale does not change the shape of the plot.
22 genes are considered for this figure. They were selected based on the list of 28 genes described in *Inoue et al., Nature, 2017*, the original publication reporting the H3K27me3-depepndent imprinting and filtered for genes that had sufficiently SNP to infer allelic information.

5. Fig 4c, these genes seem cherry picked. This should be a more formal statistical analysis, probably using gene set testing.

The genes were indeed selected to support the idea that genes involved in development are affected. We have now added a more comprehensive analysis of genes involved in the control of three different cell populations that constitute the blastocyst (Supplementary Data Figure 4c). This shows that the expression of some developmental gene changes in the *Ezhip-Mat*KO mutant whereas others don't, it also illustrates the important degree of heterogeneity when comparing developmental gene expressions among embryos.

6. Show the H3K27me3 data for the imprint at the Xist locus.

See new Supplementary Data Figure 5 for H3K27me3 enrichment in oocytes at *Xist* locus.

7. The rescue of the sex ratio in Fig S5b is compelling data. Expression of maternal Xist should be confirmed in these embryos. Also, XCI should be assessed by XIST or H3K27me3 staining.

We agree that this would have constituted interesting additional controls. Yet, our smRNA-FISH now shows that *Xist* is expressed from both alleles in a fraction of cells in blastocyst *Ezhip-mat*KO therefore there is little doubt that when *Xist* is deleted from the paternal side, we will observe some expression from the maternal side. In reason of limitation in the size of our animal colony, we did not keep this mouse line alive, we therefore hope that the reviewer will agree that this control is not worth one year of mouse work.

Reviewer #4 (Remarks to the Author):

The manuscript by Diop et al investigates the role of EZHIP in regulating PRC2 activity during early embryogenesis and provides insights into the mechanisms of H3K27me3-dependent imprinting and transcriptional regulation. The authors convincingly demonstrate that EZHIP restricts PRC2 activity, and that the loss of EZHIP affects H3K27me3 landscapes, parental genome asymmetry and thereby proper embryonic development. This is demonstrated through a combination of genetic deletion models, transcriptomic analyses, and chromatin profiling, all of which

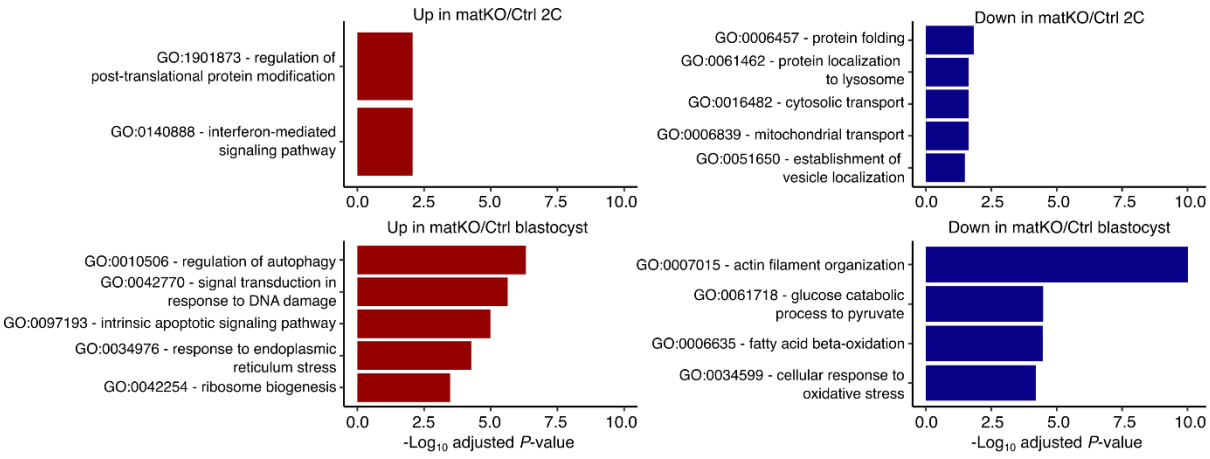
comprehensively support the authors' conclusions. Overall, the work addresses a critical aspect of epigenetic regulation during mammalian development and its consequences on genomic imprinting and X-chromosome inactivation. Below, I outline my comments and suggestions for improving the clarity and the impact of the manuscript.

1) Figure 1A (and other panels displaying chromatin enrichment tracks): The y-axis representation is not clearly defined. Is it FPKM, log2 fold change, or C&R normalized to IgG? Providing this information would help to clarify the presented data and aid in understanding the magnitude of H3K27me3 enrichment changes. Also, it is not clear from the manuscript in how many biological replicates were the assays performed.

The scale for the genome browser view is based on RPKM. We have added the Supplementary Table 1 which provides the precise number of oocytes/embryos/litter that were analyzed for each CUT&RUN and RNA-seq experiments.

2) Figure 2D: It would be useful if the authors could comment on the GO terms of the differentially expressed genes. Also, could the authors clarify whether there is an overlap of affected genes between the different developmental stages (e.g., 2C and blastocyst)? Specifically, do the 250 downregulated and 362 upregulated genes in the 2C stage remain affected in the blastocyst or at later stages?

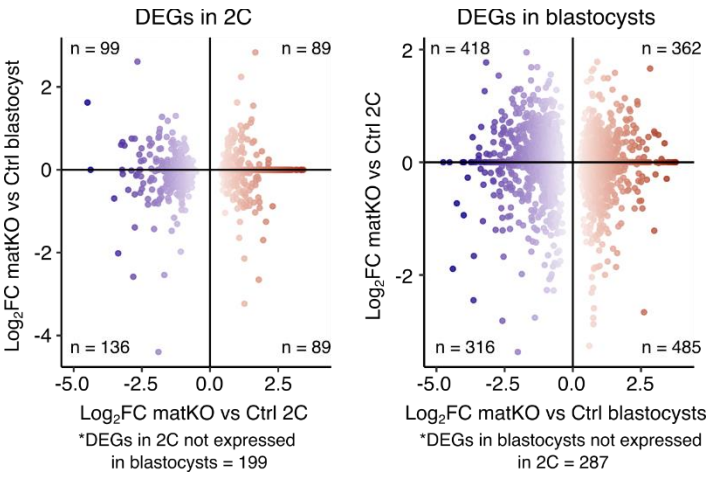
Following the reviewer suggestion, we have analyzed gene ontology in the different set of DEGs at the 2C and blastocyst stages.
The results, shown below (Reviewer Figure 6), did not reveal very specific GO terms at the 2C stage (adjusted P-value is quite weak for the top GO terms). At the blastocyst stage, several GO terms are related to stress (response to DNA damage, apoptotic signaling, ER stress, oxidative stress) which we speculate reflect transcriptomic stress. However, no specific developmental pathway seems to be affected in the mutant embryos.



*Reviewer Figure 6: Top GO terms, manually curated to avoid redundancy, gene differentially expressed at the 2C and blastocyst stage when comparing Ctr and Ezhip-mat*KO *embryos.*

We then analyzed how genes that were found differentially expressed at the 2C stage when comparing Ctr to *Ezhip-mat*KO, are regulated at the blastocyst stage (Reviewer Figure 7).
Overall, differential expression at one stage is not predictive of what is happening at the other stage. Of note, a number of genes that are expressed at one stage are not expressed at the other.

We interpret this result as reflecting some global alteration in the transcriptomic program rather than a default in a specific pathway.



Reviewer Figure 7: Dot blot representing gene expression changes of differentially expressed genes in 2C versus blastocyst. Note that 199 DEGs in 2C are not expressed in blastocysts whereas 287 DEG in blastocysts are not expressed in 2C. Data from RNAseq presented in Figures 2d and 2e.

3) Figure 4: The authors provide follow-up analyses on a subset of 16 genes that remain aberrantly enriched in H3K27me3 post-implantation (E6.5). Nevertheless, it is not clear what happens with the global H3K27me3 landscape in E6.5 matKO embryos. Is the global landscape restored to the wt state (except for the small number of genes that are discussed)? Global assessment by C&R at later stages would have been useful to better understand the matKO phenotype. Also, at E6.5 global DNA methylation should already be largely restored after the first reprogramming event. Could the authors check, using available WGBS data, whether the 16 targets reside in hypomethylated domains? Or is there any other specific sequence feature that characterises this gene group?

Studies from the Kelsey and Zhang labs have shown that the subset of genes whose H3K27me3-dependent imprinting is maintained post-implantation in the extraembryonic tissue are not enriched for H3K27me3 anymore at that stage. Instead, the maternal allele is DNA methylated suggesting that DNA methylation maintains this imprinting post-implantation (Hanna et al., 2019; Chen et al., 2019). Besides, it was suggested that H3K27me3 alteration due to the maternal deletion of *Eed* are also resolved at that stage (Inoue et al., 2018). This led us to speculate that the global landscape of H3K27me3 at E6.5 is likely to be restored but this needs, indeed, to be formally demonstrated.

4) Discussion: The authors could consider including a brief section on potential implications of EZHIP regulation beyond preimplantation development, particularly in later developmental stages or potentially in pathological contexts such as imprinting disorders? A sentence or two on the evolutionary conservation of EZHIP would also be useful.

We have updated the discussion following the reviewer's suggestion.

We thank the reviewers for taking again the time to go through our manuscript. We have now updated our manuscript according to their last comments and hope that the study is now ready for publication.

Reviewer #1 (Remarks to the Author):

The authors have fully addressed all my concerns, and I have no further comments.

Reviewer #2 (Remarks to the Author):

The authors addressed most of my questions. I have a few more concerns/suggestions:

Regarding the relationship between changes in H3K27me3 and gene expression, the authors show in their rebuttal figure 1 that there is no such correlation. I think this is an important result that should be presented in the manuscript and not only to the reviewers.

We moved the reviewer figure #1 to supplementary Figure 2e as requested.

Supplementary Figure 5 refers to chr6 but is showing the X-linked X inactivation center.

We corrected this mislabeling.

Second, this figure shows a decrease in H3K27me3 at the Xist regulatory region, which may explain maternal Xist activation, has this been done in an allelic setting?

The supplementary Figure 5 showed the XIC in GV oocytes as we thought that the reviewer wished to see the “transmitted” H3K27me3 enrichment. We have now added a second panel with H3K27me3 in blastocyst in a sex and allele specific manner (supplementary Figure 5b). We recommend interpreting this subpanel with caution considering the scarcity of the signal in this condition.

Finally, the reviewer figure 5 showing Xist expression should be included as it shows the expression is lower than observed in female embryo's and might be the consequence of an impact of the mutation on other mechanisms that regulate Xist expression, for instance increase levels of RLIM would give a similar phenotype.

We moved the reviewer figure #5 to supplementary Figure 5c as requested.

Reviewer #3 (Remarks to the Author):

The authors have performed a commendable number of additional experiments, which have made the manuscript more compelling. My primary concern remains however. I’m happy to accept the

authors explanation that comparing heterozygous embryos to WTs is valid if there is no embryonically derived Ezhip, however currently provided data falls short of justifying this. The addition of Ezhip qPCR is useful, however RNA-seq would do a better job of detecting low level embryonic expression - can you see paternally derived Ezhip reads in the blastocyst RNA-seq from Fig2?

First, we would like to emphasize that *Ezhip* is on the X chromosome therefore male embryos are not heterozygous but KO (KO/Y).

Second, we provided the RNAseq count in the previous revision (Supplementary Figure 2a) as described in the figure legends:

"**Supplementary Data Figure 2: a** *Ezhip* expression (rpkm) in embryos Ctr or *Ezhip*-matKO, at the 2C and blastocyst stages. Values extracted from RNAseq presented in Figures 2d and 2e."

Finally, we provided a source data file containing the raw values that were used to generate the graph. In case, this was not transmitted to the reviewer, we copy below the raw data:

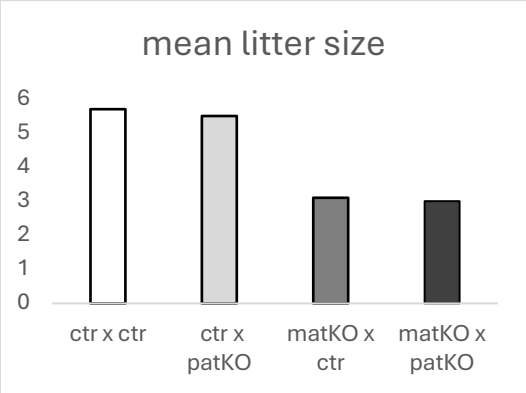
Ctr-2C	matKO-2C	Ctr-Blasto	matKO-Blasto
66,1847858	0,4337701	0	1,65318318
14,2242243	3,56263297	0,06016853	0
32,7696881	0,99333773	0,05849899	0
61,2927183	1,88637598	2,4110669	0,13284988
157,550276	1,6711694	0	0,29711155
117,942055	0	0,28821858	0,74639646
110,047871	0,49446717	0,42316532	0,16641127
42,2085155	0	0	0
85,0155152	0,23870551	0,58237732	0,20650078
58,7890419	0	0,57101835	0,26750364
53,2619243	0	0,09087058	0
	8,66909609	18,8484398	0,64711571
		7,41548738	0,29572909
		2,44907858	

The Ezhip IF should be done in blastocysts as well.

We hope that the reviewer will now agree that *Ezhip* expression from the paternal genome at early stages post-fertilization is either null (male have a Y contribution from father, therefore without *Ezhip*) or negligeable and that, since the protein is not detected by IF at the zygote, 2C and morula stages, it is reasonable to assume that it will not be any different at the blastocyst stage.

In the rebuttal, authors state that there is no obvious heterozygous effect when the Ezhip deletion is paternally inherited. This data should be shown. A measurement of H3K27me3 in a blastocyst with a paternally inherited Ezhip deletion would be the most compelling addition.

Below is a figure showing that while *Ezhip*-KO females have a reduced fertility, *Ezhip*-deletion in males has no obvious impact on the size of the litter.



Reviewer Figure 1: Bar plot representing the average litter size at 10 days post birth. PatKO means that father was *Ezhip* KO/Y, matKO means that mother was *Ezhip* KO/KO.

Finally, it should be clearly stated in the text that controls are WT and tests heterozygous, with the evidence for why this is a fair comparison. Presently this is not made clear.

We further modified the text to clear up any possible remaining ambiguity.
In the result section, we wrote:
"Importantly, *Ezhip* RNA is very abundant in the maternal pool of RNAs transmitted by the oocyte but barely expressed post-fertilization in the embryos ([14] and Supplementary Data Figure 2a). *Ezhip*-matKO embryos are therefore *Ezhip* wt/- for females and -/Y for males, yet *Ezhip* mRNA is mostly absent in both cases."

Reviewer #4 (Remarks to the Author):

The authors have addressed the main points raised in the earlier review. The manuscript now reads well and in my opinion is suitable for publication.