

Germline epigenome editing identifies H3K9me3 as a mediator of intergenerational DNA methylation recovery in mice

Corresponding Author: Professor Takuro Horii

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)

As the authors correctly state transgenerational epigenetic inheritance is a controversial topic. In order to avoid just adding another "controversial paper on this topic to the literature, it would be needed that the results presented in this study are supported by strong experimental data and conceptual power. Moreover, writing should be as clear as possible to avoid misunderstanding and inaccuracies. Unfortunately, both these conditions are not full-filled.

1) One of the critical claims made by the authors is that „DNA methylation was fully lost in the sperm“... but „it was partially restored during pre-implantation development, suggesting the existence of epigenetic memory that instructs de novo DNA methylation.“ This finding must be seen in the well-established context of massive global de novo DNA methylation ongoing in the early development. While it is shown that DNA methylation is restored in the early embryo (lines 172-190), the author do not provide convincing evidence that this remethylation is instructed by methylation information present in the sperm and later the paternal pronucleus and paternal genome.

2) Of note, intergenerational inheritance in this paper is studied in the context of an imprinted locus. It will be established that several methylation marks at DMRs act together to mediate biological responses. Hence it is well-conceivable that other differentially methylated genomic regions that were not affected by epigenome editing were responsible for the recovery of methylation at the H19 locus.

3) „Tri-methylation at lysine 9 of histone H3 (H3K9me3) was required for de novo DNA methylation.“ This is correct and convincingly shown in lines 231-242, but this is expected in the context of „normal“ de novo DNA methylation. There are no data providing evidence for a role of H3K9me3 in transgenerational inheritance.

4) „...our study ... identifies H3K9me3 as a mediator of intergenerational DNA methylation recovery.“ This claim is not supported by data. There is no experimental evidence showing that H3K9me3 transmits epigenetic information through generations.

5) Investigation of transgenerational inheritance would require the presence of the epimutation in the germline of the F1 generation and in F2. The authors demonstrate that this is not happening (Line 165-170). So claims on transgenerational inheritance are unfounded.

6) Line 130: Fig. 2C also shows some expression of the epigenome editor in oocytes, different than stated by the authors. Oocyte DNA methylation data should be provided as well. Moreover, expression should be tested in the zygote and early embryo.

My conclusion is that the authors have presented an epigenome editing system in the germline, though some doubts remain on the germline-only expression of the factors. The application of the system to study intergenerational epigenetic inheritance is not convincing and the conclusions are not justified by the provided data.

Minor comments:

There are language inaccuracies suggesting that concepts are not clear to the authors. For example:

Line 55: Mendel did not know about DNA based inheritance.

Line 58: This is only true if the absence of genetic changes is convincingly documented, which is not the case in many of these reports.

Reviewer #2

(Remarks to the Author)

The manuscript "Germline epigenome editing identifies H3K9me3 as a mediator of intergenerational DNA methylation recovery" by Horii et al. addresses several longstanding and critical questions regarding epigenetic inheritance, the regulation of imprints, and DNA methylation stability. Importantly, the authors tackle these issues using state-of-the-art methodologies, including in vivo epigenetic engineering, which represents a significant technical and conceptual advance in the field. The combination of cutting-edge approaches and well-designed, convincing strategies makes this study a highly valuable contribution to the field of epigenetics.

This is an exciting and timely manuscript that deserves publication. The results presented are not only robust but also, in some cases, unexpected, particularly regarding the recovery of DNA methylation. While the authors do not yet present a fully conclusive mechanism underlying these observations, this should not detract from the significance of their findings. In fact, the partially surprising behaviors of DNA methylation, particularly on differentially methylated regions (DMRs) following perturbations, are somewhat consistent with previous unresolved phenomena observed in the imprinting field. These findings further underscore the complexity of DNA methylation dynamics and open new avenues for investigation.

The authors' use of epigenome editing techniques, specifically for targeted DNA de-methylation and H3K9 modification, is noteworthy. These approaches are not only difficult to establish but also significantly more specific and precise compared to previous methodologies. The surprising nature of some of the findings only adds to the intrigue and importance of the work. Overall, this manuscript provides an impressive array of insights into the mechanisms governing DNA methylation stability and epigenetic regulation. I do not have any major concerns, as the study is well-conceived and executed. However, there are a few minor points that the authors could address to further enhance the clarity and comprehensiveness of their presentation:

- "DNA methylation recovery occurred specifically at the paternal, but not the maternal, DMR after fertilisation"

It stays unclear on which basis this statement has been made. The data I found concerning DNA methylation, Fig.2, Fig.3, Fig.4, EXF1, EXF2, EXF4 do not distinguish the parental alleles. If there is information about the allelic dynamics of recovery this should be clearly and prominently analysed and depicted.

- There are some typos in the figure legends

Reviewer #3

(Remarks to the Author)

The stable epigenetic inheritance across cell division of 5-cytosine DNA methylation (5meC) makes it a compelling vector for the transmission of non-genetic information across generations. However, DNA methylation reprogramming in the mammalian embryo and germline makes transmission of the mark inter/transgenerationally difficult in theory. Recently, a study from the Belmonte lab showed that artificially induced 5meC indeed is lost during germline reprogramming but, in some contexts, returns during the DNA methylation establishment phase (PMID: 36754048). This is a very compelling result, as it implies there is another epigenetic signature in addition to 5meC that allows for a memory of gene silencing. Horii, Hatada and colleagues have been leaders in the epigenome editing field since their breakthrough paper describing the dCas9-SunTag/TET in 2016 (PMID: 27571369). Here, they adapt their system to make its expression male germline-specific (Stra8 promoter), and target demethylation to the H19-Igf2 paternal imprint. In this way, they can demonstrate if sperm-specific demethylation is sufficient to eliminate transmission of the imprint, which results in a Silver-Russell Syndrome-like phenotype. As they previously showed (PMID: 32234052), their system is very efficient at in vivo removal of the methyl-mark. Now, they show that this signature can be maintained intergenerationally, and lead to a phenotype. While this is a satisfying result, it was also somewhat expected. More surprisingly, they went on to show that regions of the H19-Igf2 DMR reacquire DNA methylation in the preimplantation embryo. Furthermore, by targeting an H3K9me3-demethylase (KDM4D) using dCas9-SunTag, they eliminate his DNA methylation gain. Hence, H3K9me3 is a second epigenetic signature that contributes to imprint regulation.

I really appreciated this paper. The experiments are very straightforward, the data are clear, and the authors do a good job of explicating their study. Also, they make a breakthrough by establishing a link with H3K9me3 methylation and imprint transmission. This finding demonstrates the power of the dCas9-SunTag tool, and also is a strong hint at the mechanism underlying the observations made in the Belmonte paper mentioned above. This paper should be added to the list of important advances made in epigenome editing by Horii and Hatada. A major question, and one for future studies, is what is the signal for de novo deposition of H3K9me3 in the early embryo? I do have a list of ideas/concerns that I would like to see addressed:

- The authors mentioned that CTCF protection could be an explanation for why the maternal imprint was not H3K9me3-methylated. However, they failed to mention the study from Burton et al. (PMID: 32601371) that showed SUV39H2 is required for H3K9me3 deposition on the paternal genome. This may be akin to the paternal-specific DNMT3A activity reported by Richard Albert et al, and already cited. In other words, protection of the maternal allele is one potential mechanism, but active targeting of the paternal allele is a non-exclusive second mechanism.

- On this note, do the authors propose the signal for this deposition? Genetic? Epigenetic? If it's a genetic signal, the 5' of the DMR could be injected into paternal pronuclei, and observed if H3K9me3 accumulates. If it's epigenetic, what could that signal be? Another histone mark or chromatin complex?

- One experiment I felt was lacking was phenotypic characterization of the TG+KDM4D pups. Was the Silver-Russell phenotype exacerbated? This would be important to show the physiological relevance of the embryonic H3K9me3

deposition.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

In general, the authors made a good job to clarify the paper and add additional data and they resolved most of my concerns. Overall, this is a very nice and convincing paper. I only see little room for improvement at few places in the manuscript. I recommend acceptance of an appropriately adjusted manuscript.

Comments

- 1) The authors showed at their model locus full establishment of a DNA methylation epimutation. Still there was "incomplete" intergenerational inheritance and no transgenerational inheritance. These important conclusions should be spelled out clearly in the abstract to become visible to the community.
- 2) It would be of interest, if editing of DNA methylation and H3K9me3 leads to transgenerational inheritance of the combined epimutation.
- 3) H3K9me3 has been shown to support incorporation of DNA methylation in general. Please clarify if there is a specific effect at imprinted loci that goes beyond that and adjust the writing accordingly.
- 4) Line 39: "validate" implies the authors aim to show the concept is correct. I recommend more neutral wording like "investigate", "examine" or even "challenge", which comes more from the friendly critical side.
- 5) Line 50: Maybe it is better to say: "H3K9me3 as a mediator for DNA methylation recovery also acting at imprinted loci"?
- 6) Line 58 and based on the rebuttal letter. One may state: "germline. While these data are still under discussion because of difficulties in showing the absence of mutations, DNA methylation..."
- 7) Line 68: It will be good here to provide the definition of inter and transgenerational inheritance used in this paper.
- 8) Line 298: "Candidates include those *factors* known to be ..."

Reviewer #2

(Remarks to the Author)

The authors clarified all my concerns.

Reviewer #3

(Remarks to the Author)

The authors have satisfactorily responded to my comments. I look forward to publication of these exciting results.

Maxim Greenberg

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

All my comments were addressed. I recommend acceptance of this paper.

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Ref.: NCOMMS-24-84758

Title: Germline epigenome editing identifies H3K9me3 as a mediator of intergenerational DNA methylation recovery

Responses to Reviewers' Comments

Reviewer #1 (Remarks to the Author):

We are grateful to reviewer #1 for the critical comments and useful suggestions that have helped us to improve our paper. As indicated in the responses that follow, we have taken all these comments and suggestions into account in the revised version of our paper.

As the authors correctly state transgenerational epigenetic inheritance is a controversial topic. In order to avoid just adding another “controversial paper on this topic to the literature, it would be needed that the results presented in this study are supported by strong experimental data and conceptual power. Moreover, writing should be as clear as possible to avoid misunderstanding and inaccuracies. Unfortunately, both these conditions are not full-filled.

1) One of the critical claims made by the authors is that „DNA methylation was fully lost in the sperm“... but „it was partially restored during pre-implantation development, suggesting the existence of epigenetic memory that instructs de novo DNA methylation.“ This finding must be seen in the well-established context of massive global de novo DNA methylation ongoing in the early development. While it is shown that DNA methylation is restored in the early embryo (lines 172-190), the author do not provide convincing evidence that this remethylation is instructed by methylation information present in the sperm and later the paternal pronucleus and paternal genome.

Response: We sincerely apologize for any lack of clarity in our previous response. The central objective of this study is to validate the applicability of our gamete-specific epigenome editing system in assessing whether inter/transgenerational epigenetic inheritance occurs at specific genomic loci. Using this system, we directly revealed that epigenetic information of H19-DMR is inherited intergenerationally but not transgenerationally. Through targeted and global H3K9me3 removal experiments, we observed that H3K9me3 plays a pivotal role in mediating the restoration of DNA methylation at the H19-DMR. We believe this is a major discovery that shows how two key epigenetic marks—DNA methylation and H3K9me3—work together to control genomic imprinting in early embryos.

As the reviewer rightly pointed out, our data suggest that H3K9me3 itself may not serve

as the intergenerational epigenetic mark. Instead, it is plausible that an upstream regulatory factor—yet to be identified—initiates this process. We acknowledge this as an important avenue for future investigation. Accordingly, we have incorporated the following clarification into the Discussion section to better reflect our interpretation and the future direction of our research:

“Target-specific and global H3K9me3 removal experiments in zygotes revealed that H3K9me3 mediates DNA methylation recovery in the H19-DMR. By contrast, H3K9me3 is nearly absent at the H19-DMR in sperm, suggesting that H3K9me3 is not the primary intergenerational memory directly transmitted across generations, but rather that some other factor(s) upstream of H3K9me3 is involved. Identification of the upstream factors, which could be genetic or epigenetic, awaits further investigations. Future studies will be directed toward uncovering the mechanism of these upstream signals to further advance our understanding of epigenetic inheritance (L291-L298).”

2) Of note, intergenerational inheritance in this paper is studied in the context of an imprinted locus. It will be established that several methylation marks at DMRs act together to mediate biological responses. Hence it is well-conceivable that other differentially methylated genomic regions that were not affected by epigenome editing were responsible for the recovery of methylation at the H19 locus.

Response: We are grateful to the reviewer for highlighting this possibility. In this study, we found that removal of H3K9me3 from the H19-DMR prevented DNA methylation recovery after fertilization. Therefore, we believe that it is not necessary to consider the involvement of other potential DMRs in methylation recovery. We apologize if our response does not appropriately address or misses the point of the reviewer's comment.

3) „Tri-methylation at lysine 9 of histone H3 (H3K9me3) was required for de novo DNA methylation.“ This is correct and convincingly shown in lines 231-242, but this is expected in the context of „normal“ de novo DNA methylation. There are no data providing evidence for a role of H3K9me3 in transgenerational inheritance.

Response: We sincerely apologize for any lack of clarity in our previous response. As the reviewers commented, it is assumed that H3K9me3 itself may not be the primary epigenetic mark transmitted across generations, but rather that it is an early-deposited mediator required for subsequent DNA methylation recovery. There is likely to be another factor(s) further upstream of H3K9me3 that intergenerationally transmits the information across generations. It could be epigenetic marks or genetic sequence, and determining which one it is will be an important avenue for future investigations. The text in the

Abstract pointed out by the reviewer has been corrected as follows: “Tri-methylation at lysine 9 of histone H3 (H3K9me3), which is deposited shortly after fertilization, is required for subsequent de novo DNA methylation at the H19-DMR (L47-L49).” In addition, due to technical challenges and low throughput of germline targeted DNA demethylation, we admit that it currently remains unknown how much H3K9me3-mediated recovery operates generally at other loci. We have mentioned this limitation in the Discussion as follows: “Our study suggests that de novo H3K9me3 in zygotes is a prerequisite for subsequent de novo DNA methylation, although the degree to which this mechanism is shared at loci other than H19-DMR is currently unknown (L321-L324).”

4) “..our study ... identifies H3K9me3 as a mediator of intergenerational DNA methylation recovery.” This claim is not supported by data. There is no experimental evidence showing that H3K9me3 transmits epigenetic information through generations.

Response: We apologize for any confusion caused by the lack of clarity in our previous response. In this context, the term ‘mediator’ refers to a factor that receives epigenetic memory from upstream signals and transmits it downstream to facilitate functional outcomes. H3K9me3 is a “mediator,” but is not necessarily the primary memory in sperm. There may be other factor(s) operating upstream of H3K9me3. At present, it remains unclear whether this upstream signal is genetic or epigenetic in nature. We acknowledge that this as an important avenue for future investigations.

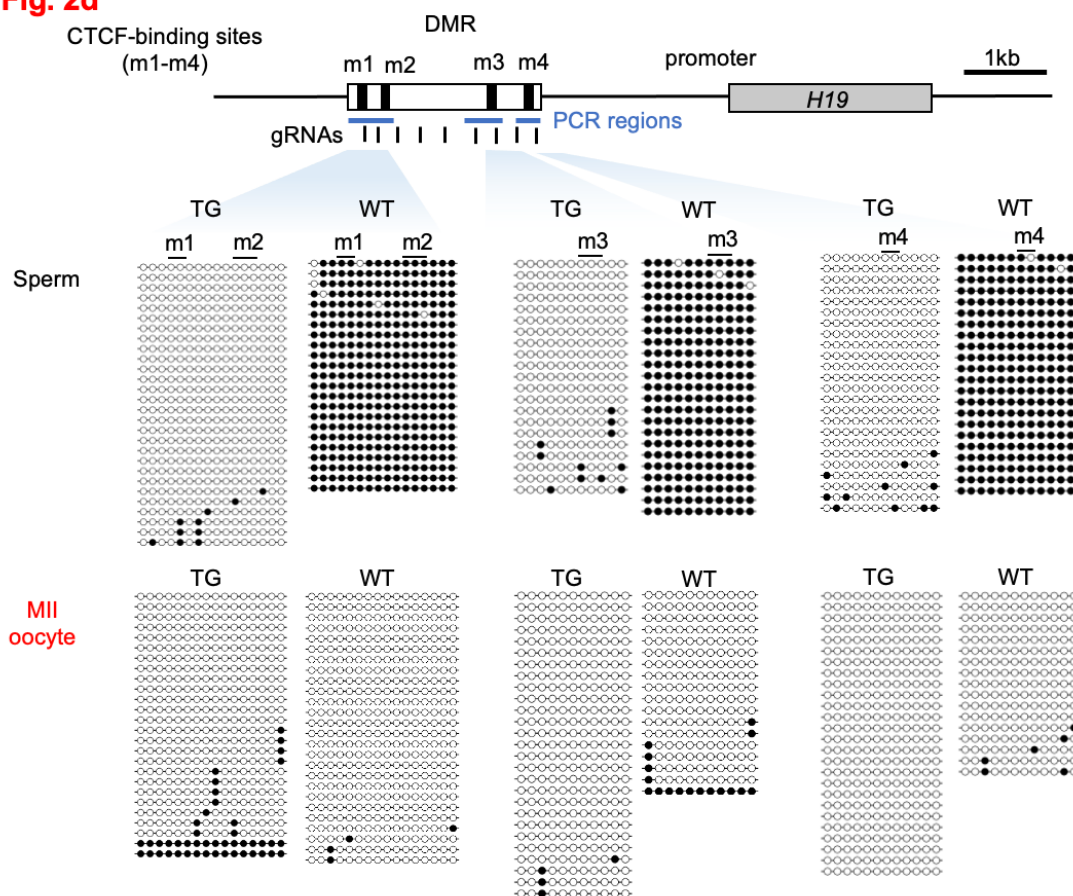
5) Investigation of transgenerational inheritance would require the presence of the epimutation in the germline of the F1 generation and in F2. The authors demonstrate that this is not happening (Line 165-170). So claims on transgenerational inheritance are unfounded.

Response: We apologize for not being able to explain clearly what we meant previously. We would like to argue that our gamete-specific epigenome editing system can be applied to verify whether inter/transgenerational inheritance occurs at a target region. In this study, the germline-specific epigenome editing system revealed that the epigenetic information of H19-DMR is inherited intergenerationally but not transgenerationally. To avoid any confusion, we have modified the manuscript as follows: In this study, we developed a germline-specific DNA methylation editing system and applied it to a genomic imprinting disorder disease model, to investigate inter/transgenerational inheritance of epimutations in sperm (L104-L106). We hope that revised text in the manuscript is now clearer.

6) Line 130: Fig. 2C also shows some expression of the epigenome editor in oocytes, different than stated by the authors. Oocyte DNA methylation data should be provided as well.

Response: We agree that DNA methylation data of oocytes was missing from the manuscript and should be added to bolster the findings of the study. Accordingly, we performed bisulfite sequencing analysis of both TG and WT mother-derived MII oocytes, and confirmed that H19-DMR is originally hypomethylated in WT oocytes and that this hypomethylation is not altered in TG-derived oocytes (**Fig. 2d**).

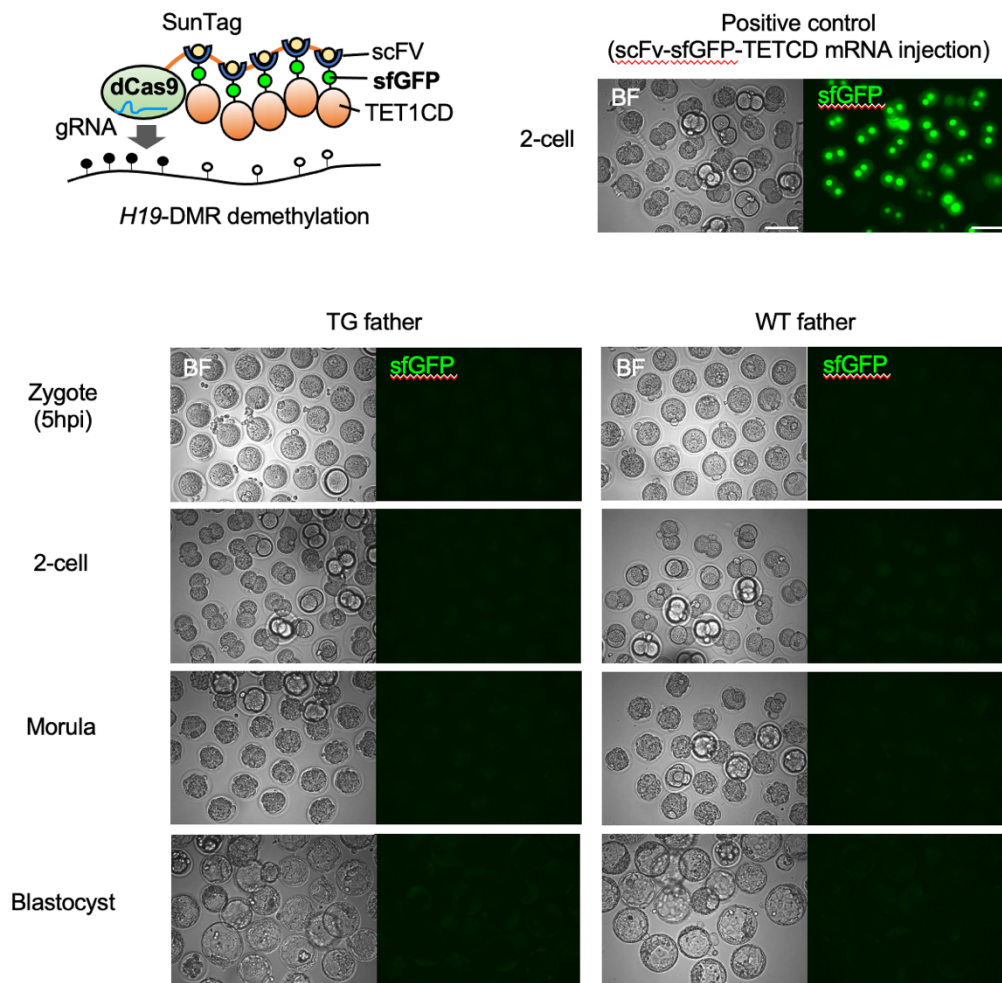
Fig. 2d



Moreover, expression should be tested in the zygote and early embryo.

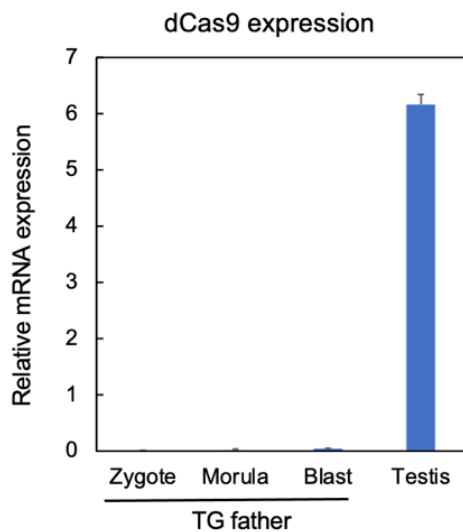
Response: We also performed fluorescent microscopy analysis and RT-qPCR to detect expression of the epigenome editor in zygotes and early embryos. Fluorescence microscopy analysis to detect sfGFP revealed no significant differences in fluorescence intensity between TG and WT samples (**Supplementary Fig. 1a**).

Supplementary Fig. 1 a



Furthermore, RT-qPCR analysis did not detect the expression of the epigenome editor dCas9 at any developmental stage. Therefore, we concluded that the epigenome editors were hardly expressed in zygotes or early embryos.

Supplementary Fig. 1 b



Minor comments:

There are language inaccuracies suggesting that concepts are not clear to the authors.

For example:

Line 55: Mendel did not know about DNA based inheritance.

Response: We agree with the reviewer's suggestion. This sentence has been changed to "Phenotypic inheritance is believed to be achieved via the transmission of genomic DNA (L53)."

Line 58: This is only true if the absence of genetic changes is convincingly documented, which is not the case in many of these reports.

Response: We thank the reviewer and fully agree with the comments. Indeed, genetic mutations might have occurred, but this has not been documented in many of these reports. Therefore, we are considering adding the following sentence to the Introduction "This is only true if the absence of genetic changes is convincingly documented, which is not the case in many of these reports". However, we think that adding this sentence could disrupt the flow of the surrounding text, so we decided not to add it to the revised manuscript. However, if the reviewer could provide us with advice about how to incorporate it, we'd be happy to add it to the manuscript. .

Reviewer #2 (Remarks to the Author):

We are grateful to reviewer #2 for the critical comments and useful suggestions that have helped us to improve our paper. As indicated in the responses that follow, we have taken

all these comments and suggestions into account in the revised version of our paper.

The manuscript "Germline epigenome editing identifies H3K9me3 as a mediator of intergenerational DNA methylation recovery" by Horii et al. addresses several longstanding and critical questions regarding epigenetic inheritance, the regulation of imprints, and DNA methylation stability. Importantly, the authors tackle these issues using state-of-the-art methodologies, including in vivo epigenetic engineering, which represents a significant technical and conceptual advance in the field. The combination of cutting-edge approaches and well-designed, convincing strategies makes this study a highly valuable contribution to the field of epigenetics.

This is an exciting and timely manuscript that deserves publication. The results presented are not only robust but also, in some cases, unexpected, particularly regarding the recovery of DNA methylation. While the authors do not yet present a fully conclusive mechanism underlying these observations, this should not detract from the significance of their findings. In fact, the partially surprising behaviors of DNA methylation, particularly on differentially methylated regions (DMRs) following perturbations, are somewhat consistent with previous unresolved phenomena observed in the imprinting field. These findings further underscore the complexity of DNA methylation dynamics and open new avenues for investigation.

The authors' use of epigenome editing techniques, specifically for targeted DNA demethylation and H3K9 modification, is noteworthy. These approaches are not only difficult to establish but also significantly more specific and precise compared to previous methodologies. The surprising nature of some of the findings only adds to the intrigue and importance of the work.

Overall, this manuscript provides an impressive array of insights into the mechanisms governing DNA methylation stability and epigenetic regulation. I do not have any major concerns, as the study is well-conceived and executed. However, there are a few minor points that the authors could address to further enhance the clarity and comprehensiveness of their presentation:

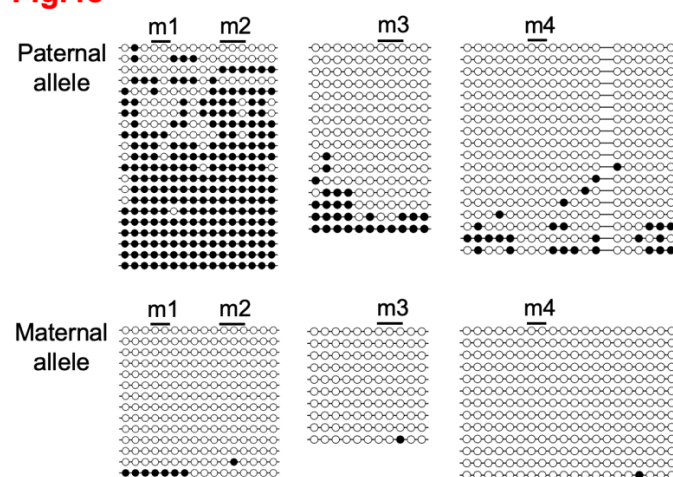
- "DNA methylation recovery occurred specifically at the paternal, but not the maternal, DMR after fertilisation"

It stays unclear on which basis this statement has been made. The data I found concerning DNA methylation, Fig.2, Fig.3, Fig.4, EXF1, EXF2, EXF4 do not distinguish the parental alleles. If there is information about the allelic dynamics of recovery this should be clearly and prominently analysed and depicted.

Response: We thank the reviewer for this helpful suggestion. As the reviewers pointed

out, it is important to investigate allele-specific methylation in this study. In order to distinguish parental alleles, it is necessary to create TG mice with sub-species background (e.g., PWK strain). To this end, we attempted to backcross TG mice with sub-strain PWK mice, but the reproductive ability of hybrid TG mice was extremely low, and thus, we were unable to prepare them in time for the initial submission. Subsequently, we obtained a male TG mouse that had been backcrossed twice with the PWK strain, which allowed us to perform bisulfite sequencing analysis using this mouse. Finally, we confirmed that DNA methylation recovery occurs in the paternal allele but not in the maternal allele (**Fig. 4c**).

Fig.4c



- There are some typos in the figure legends

Response: We thank the reviewer for this suggestion. We have proofread the manuscript again to remove the typos.

Reviewer #3 (Remarks to the Author):

We are grateful to reviewer #3 for the critical comments and useful suggestions that have helped us to improve our paper. As indicated in the responses that follow, we have taken all these comments and suggestions into account in the revised version of our paper.

The stable epigenetic inheritance across cell division of 5-cytosine DNA methylation (5meC) makes it a compelling vector for the transmission of non-genetic information across generations. However, DNA methylation reprogramming in the mammalian embryo and germline makes transmission of the mark inter/transgenerationally difficult

in theory. Recently, a study from the Belmonte lab showed that artificially induced 5meC indeed is lost during germline reprogramming but, in some contexts, returns during the DNA methylation establishment phase (PMID: 36754048). This is a very compelling result, as it implies there is another epigenetic signature in addition to 5meC that allows for a memory of gene silencing. Horii, Hatada and colleagues have been leaders in the epigenome editing field since their breakthrough paper describing the dCas9-SunTag/TET in 2016 (PMID: 27571369). Here, they adapt their system to make its expression male germline-specific (Stra8 promoter), and target demethylation to the H19-Igf2 paternal imprint. In this way, they can demonstrate if sperm-specific demethylation is sufficient to eliminate transmission of the imprint, which results in a Silver-Russell Syndrome-like phenotype. As they previously showed (PMID: 32234052), their system is very efficient at in vivo removal of the methyl-mark. Now, they show that this signature can be maintained intergenerationally, and lead to a phenotype. While this is a satisfying result, it was also somewhat expected. More surprisingly, they went on to show that regions of the H19-Igf2 DMR reacquire DNA methylation in the preimplantation embryo. Furthermore, by targeting an H3K9me3-demethylase (KDM4D) using dCas9-SunTag, they eliminate his DNA methylation gain. Hence, H3K9me3 is a second epigenetic signature that contributes to imprint regulation.

I really appreciated this paper. The experiments are very straightforward, the data are clear, and the authors do a good job of explicating their study. Also, they make a breakthrough by establishing a link with H3K9me3 methylation and imprint transmission. This finding demonstrates the power of the dCas9-SunTag tool, and also is a strong hint at the mechanism underlying the observations made in the Belmonte paper mentioned above. This paper should be added to the list of important advances made in epigenome editing by Horii and Hatada. A major question, and one for future studies, is what is the signal for de novo deposition of H3K9me3 in the early embryo? I do have a list of ideas/concerns that I would like to see addressed:

- The authors mentioned that CTCF protection could be an explanation for why the maternal imprint was not H3K9me3-methylated. However, they failed to mention the study from Burton et al. (PMID: 32601371) that showed SUV39H2 is required for H3K9me3 deposition on the paternal genome. This may be akin to the paternal-specific DNMT3A activity reported by Richard Albert et al, and already cited. In other words, protection of the maternal allele is one potential mechanism, but active targeting of the paternal allele is a non-exclusive second mechanism.

Response: We are grateful for the reviewer's request to include the study by Burton et al., and we apologize for our oversight in not referencing this important work earlier. We agree that the DNA methylation recovery of H19-DMR via H3K9me3 may occur through the mechanism outlined by the reviewer. Therefore, we have now cited this paper, and added the following sentence to the Discussion:

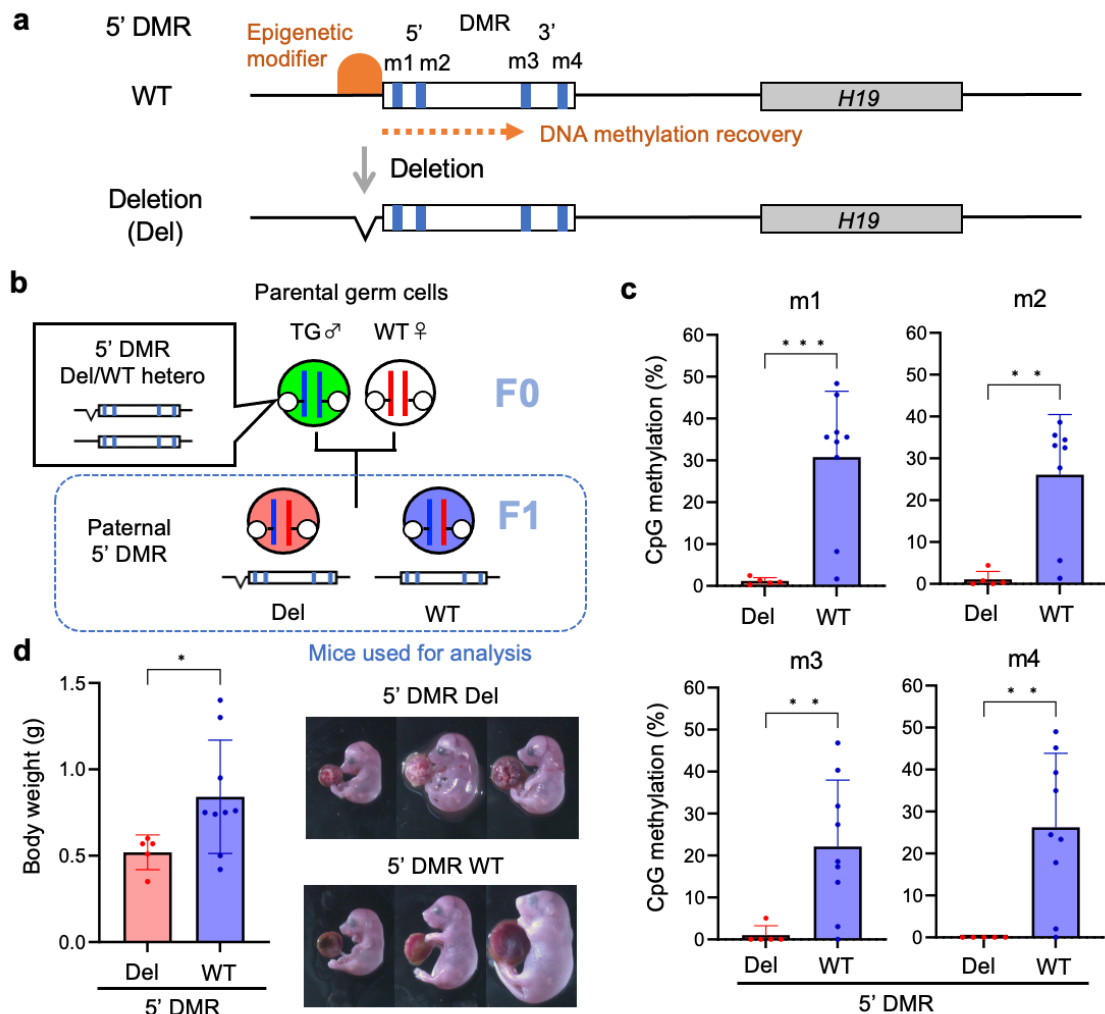
"Burton et al. reported that de novo H3K9me3 catalyzed by SUV39H2 (H3K9 methyltransferase) occurs in the paternal genome immediately after fertilization, which is consistent with our observation that H3K9me3 emerges on the paternal allele of H19-DMR in early zygotes (L315-L318)."

- On this note, do the authors propose the signal for this deposition? Genetic? Epigenetic? If it's a genetic signal, the 5' of the DMR could be injected into paternal pronuclei, and observed if H3K9me3 accumulates. If it's epigenetic, what could that signal be? Another histone mark or chromatin complex?

Response: We thank the reviewer for the insightful suggestion. At this point, it is unclear whether this is a genetic or an epigenetic signal, but we have obtained preliminary data that we think potentially addresses this point. We generated mice lacking the 5' region of the H19-DMR, and examined phenotypes of the offspring of the TG father lacking the 5' region of the DMR (Figures **a**, **b**). The results showed no DNA methylation recovery in these offspring (Figure **c**) and the offspring had a more severe Silver-Russell syndrome-like phenotype, including intrauterine growth retardation at E18.5 (Figure **d**). This indicates that the genetic sequence of the 5' DMR is necessary for DNA methylation recovery. On the other hand, it cannot be ruled out that histone marks other than H3K9me3 and chromatin complexes are transmitted from sperm. We hope to clarify this in a future study.

In addition, we added the following description to the Discussion:

"Target-specific and global H3K9me3 removal experiments in zygotes revealed that H3K9me3 mediates DNA methylation recovery at the H19-DMR. By contrast, H3K9me3 is nearly absent at the H19-DMR in sperm, suggesting that H3K9me3 is not the primary intergenerational memory directly transmitted across generations, but rather that some other factor(s) upstream of H3K9me3 is involved. Identification of the upstream factors, which could be genetic or epigenetic, awaits further investigations. Future studies will be directed toward uncovering the mechanism of these upstream signals to further advance our understanding of epigenetic inheritance (L291-L298)."



Extended Data Figure: The 5' region of *H19*-DMR is crucial for DNA methylation recovery.

a. The 5' region of *H19*-DMR deletion (5' DMR Del) mice were generated by CRISPR-Cas9 genome editing. **b.** Male TG mice carrying 5' DMR Del/WT heterozygous were crossed with female WT mice. The F1 offspring carrying the 5' DMR Del and WT alleles were then compared at E18.5. **c.** DNA methylation levels in *H19*-DMR were not recovered in upstream of DMR Del offspring derived from TG father. **d.** Body weight and appearance of 5' DMR Del mice (E18.5) derived from TG father. **c, d.** *P* values were obtained using a Welch's *t* test (two-tailed test). **p* < 0.05, ***p* < 0.01, ****p* < 0.001

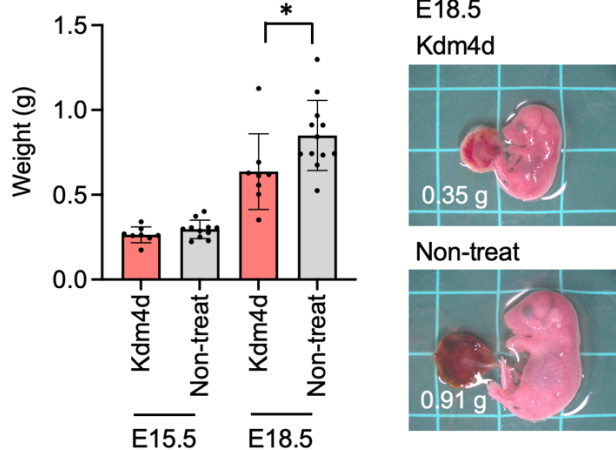
Regarding the reviewer's proposal that we perform DNA fragment injection followed by H3K9me3 ChIP-seq, we think that this would be pose many problems, not least because such an experiment would be completely new and to the best of our knowledge, has not been previously performed in this field of research. There are too many concerns to overcome, including optimization of DNA concentration, fragment length, protection chemistry for the fragment (otherwise it could be degraded too soon), and ChIP-seq optimization for the injected fragments (we don't know if our protocol optimized for chromatin will work for a DNA fragment). So, we'd like to avoid performing this

experiment, at least until after the publication of this work. We think the question about whether the signal is genetic or epigenetic can be left open for investigation in a future study.

- One experiment I felt was lacking was phenotypic characterization of the TG+KDM4D pups. Was the Silver-Russell phenotype exacerbated? This would be important to show the physiological relevance of the embryonic H3K9me3 deposition.

Response: We thank the reviewer for this suggestion. The weights of TG+KDM4D embryos were examined at E15.5 and E18.5. We found that removal of H3K9me3 by target-specific histone editing resulted in more severe intrauterine growth retardation at E18.5 (**Fig. 6c**), demonstrating the physiological relevance of H3K9me3 deposition.

Fig. 6c



Ref.: NCOMMS-24-84758A

Title: Germline epigenome editing identifies H3K9me3 as a mediator of intergenerational DNA methylation recovery

Responses to Reviewers' Comments

Reviewer #1 (Remarks to the Author):

We are grateful to reviewer #1 for the critical comments and useful suggestions that have helped us to improve our paper. As indicated in the responses that follow, we have taken all these comments and suggestions into account in the revised version of our paper.

In general, the authors made a good job to clarify the paper and add additional data and they resolved most of my concerns. Overall, this is a very nice and convincing paper. I only see little room for improvement at few places in the manuscript. I recommend acceptance of an appropriately adjusted manuscript.

Comments

1) The authors showed at their model locus full establishment of a DNA methylation epimutation. Still there was "incomplete" intergenerational inheritance and no transgenerational inheritance. These important conclusions should be spelled out clearly in the abstract to become visible to the community.

Response: Thank you for the helpful suggestion. We consider it essential to highlight the findings from the inter/transgenerational inheritance experiments at the H19-DMR locus in the abstract section. While we mostly agree, we felt that "incomplete" could sound slightly negative, so we replaced it with the more balanced term "partial" as shown below:

"Our study provides a robust germline editing tool, which reveals partial intergenerational inheritance and no transgenerational inheritance at the model locus." (L50-L51)

2) It would be of interest, if editing of DNA methylation and H3K9me3 leads to transgenerational inheritance of the combined epimutation.

Response: We appreciate the reviewer's insightful comment. While this intriguing possibility has not yet been investigated in our current study, we consider it a compelling direction for future research and intend to explore it further.

3) H3K9me3 has been shown to support incorporation of DNA methylation in general.

Please clarify if there is a specific effect at imprinted loci that goes beyond that and adjust the writing accordingly.

Response: We appreciate the reviewer's thoughtful comment. While H3K9me3 is indeed known to generally support DNA methylation, our observations suggest that its role at imprinted loci may involve additional specificity. However, further investigation is required to determine whether this effect goes beyond the general mechanism. Therefore, we revised manuscript as follows:

"Although H3K9me3 is generally recognized for its role in supporting DNA methylation, further research is necessary to determine whether this H3K9me3-mediated DNA methylation recovery is a general phenomenon or specific to imprinted loci." (L335-L338)

4) Line 39: "validate" implies the authors aim to show the concept is correct. I recommend more neutral wording like "investigate", "examine" or even "challenge", which comes more from the friendly critical side.

Response: Thank you for the helpful suggestion. In accordance with the reviewer's recommendation, we have revised "validate" to "investigate". (L39)

5) Line 50: Maybe it is better to say: "H3K9me3 as a mediator for DNA methylation recovery also acting at imprinted loci"?

Response: Following the reviewer's comment, we revised the sentence as follows: "Furthermore, we identify H3K9me3 as a mediator for DNA methylation recovery also acting at imprinted loci." (L51-L52)

6) Line 58 and based on the rebuttal letter. One may state: "germline. While these data are still under discussion because of difficulties in showing the absence of mutations, DNA methylation..."

Response: We truly appreciate the excellent phrasing the reviewer provided. We have rephrased it as follows. "...germline. While these data are still under discussion because of difficulties in showing the absence of genetic mutations, DNA methylation..." (L59-L60)

7) Line 68: It will be good here to provide the definition of inter and transgenerational inheritance used in this paper.

Response: We thank the reviewer for this valuable suggestion. To improve clarity, we have now included definitions of intergenerational and transgenerational inheritance in the revised manuscript as follows:

“In this study, we define intergenerational inheritance as the transmission of epigenetic alterations originating in the gametes to the immediate offspring (F1 generation). By contrast, transgenerational inheritance refers to the sustained presence of these modifications in subsequent generations (e.g., F2 and beyond) without continued exposure to the initial trigger.” (L73-L77)

8) Line 298: “Candidates include those *factors* known to be ...”

Response: Following the reviewer’s suggestion, we included the term “factors”. (L309)