

Mechanism of EHMT2-mediated genomic imprinting associated with Prader-Willi syndrome

Corresponding Author: Professor Yong-hui Jiang

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)

Wang et al. reported comprehensive study on EHMT2 or G9a to the maintenance of the imprinting status of maternally derived 15q11.2-q13 imprinting domain. They demonstrated that EHMT2 mediated H3K9 methylation and chromosomal regulation maintains silencing of imprinted genes including SNRPN distal to PWS-IC, but not for imprinted genes proximal to PWS-IC, independent from DNA methylation of PWS-IC. They further demonstrated that maternal specific expression of non-coding RNA upstream of PWS-IC recruits EHMT2 complex to PWS-IC as a new mechanism of silencing imprinted genes on the maternal 15q11.2-q13 imprinting domain.

This is an interesting manuscript demonstrating important mechanism of the silencing of imprinted genes independent from DNA methylation, which has been believed to be the major factor of silencing. Furthermore, allele specific expression of non-coding RNA in cis was shown to be the critical for recruitment of EHMT2 to PWS-IC, which would provide a new paradigm of mechanism of genomic imprinting.

I have following comments.

Major

- 1) EHMT2 may play a role other than PWS critical region. Therefore, it is good to demonstrate detailed phenotypes of Nestin-Cre/Ehmt2^{flox/flox} mice. Also, genomewide changes of H3K9me2 levels are good to be demonstrated. I am curious on the changes of H3K9me2 levels at other imprinting domains.
- 2) Unsilencing of IC transcript may repress the expression of maternal Ube3a. How about the expression change of Ube3a?
- 3) Drug induced DNA demethylation of PWS-IC is shown to induce expression of maternal SNURF-SNRPN. If unsilencing of maternal imprinted genes are mediated by EHMT2 complex independent of DNA methylation, how such DNA demethylation inducing unsilencing is explained?
- 4) The authors showed interaction of non-coding RNA 280118 and EHMT2, and reduction of the interaction by inhibition of EHMT2. However, causal relationship is not clearly shown yet. Genome editing study interrupting the TSS4 would be of great value.

Minor

- 1) Fig 1C; f/f;p+/mS-E appears decreased H3K9me2 level, which is not expected.

Reviewer #2

(Remarks to the Author)

This study aims to investigate the mechanisms underlying genomic imprinting at the 15q11.2-q13 region using mouse models and patient-derived cell lines. Through epigenomic profiling and molecular experiments, the authors propose a model in which a long non-coding RNA that is preferentially expressed from the maternal chromosome recruits the histone methyltransferase EHMT2/G9a to the imprinted region, followed by heterochromatin formation and gene silencing. If true, this would be interesting as EHMT2 recruitment is independent of DNA methylation, but functional validation of this model is lacking. The work also examines allele-specific 3D genome folding, but the relevance of the observed structural differences to imprinted expression remains unclear. Additionally, more focused exploration is needed to clarify the distinctions in proximal versus distal gene regulation. Some additional considerations are below:

Other Points:

Figure 1d is the only panel that directly shows allele-specific SNRPN expression as a result of EHMT2 deletion in the mouse model. This panel should be quantified and plotted like the other blots in this manuscript. In order to conclude that the signal differs between the Nestin-Cre⁺;f/f;p+/mS-E and the f/f;p+/mS-E conditions, the authors need to show the values for more than one replicate of the latter condition.

In order to more concretely show that DNA methylation is unaffected in Figure 1g, could the methylation analysis be performed on GFP⁺ sorted cells. Methylation changes in the subpopulation of Ehmt2-deleted neurons may be masked by other cell types lacking Nestin expression within the tissue.

It is difficult to observe any structural differences between the parental alleles in Figure 4b. Can a subtraction plot be provided to highlight any differences?

Regarding Figure 6, can you explain the discrepancy in TSS4-associated RNA levels in PWS and AS cells using bulk versus chromatin-associated RNA-seq? In other words, the TSS4-associated RNA-seq signal looks bi-allelic in the bulk RNA, but maternally-biased in chrRNA. Can you provide additional evidence that you've detected a new non-coding RNA? How long is this ncRNA? Is it nuclear retained? Can you provide evidence that this is a separate transcript and not just another upstream TSS for Snrpn? In Figure 6C, why does the control sample not show association between the ncRNA and EHMT2 by RIP?

The conclusion "maternal non-coding RNAs recruit EHMT2 to PWS-IC to silence the expression of SNRPN/SNHG14 in the maternal chromosome" is not fully supported by the data. The statement should either be updated to reflect the data shown, or additional experiments should be performed to support the conclusion as stated. For example, one could observe the effect of ncRNA-knockdown on maternal expression of SNRPN.

Reviewer #3

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4

(Remarks to the Author)

In this manuscript, Prof. Jiang and colleagues revealed a new mechanism model for imprinting maintenance regulation of the PWS imprinted domain on 15q11.2-q13.1 where allele-specific recruitment of EHMT2 could play a key role. They used Ehmt2-deficient mouse, PWS/AS patient-derived fibroblasts for delineating the comprehensive molecular and high-resolution chromatin analyses and found a different 3D chromatin folding confirmation in the maternal PWS imprinting domain. Moreover, they showed EHMT2 might be recruited and interacted with a maternal ncRNA transcribed upstream of the PWS-IC and formed a heterochromatin complex to silence SNRPN expression in cis on maternal chromosome. These findings are unique and very interesting.

Here I have several questions and suggestions:

1. The first result part suggested EHMT2 could act as both repressor and activator in regulating different paternally expressed genes from other genomic imprinted domain. I wonder the expression pattern of maternally expressed genes in Ehmt2 deficiency model. The discussion on the possible mechanism and also underlying influence analysis are needed.
2. In addition to RIP, supplemented CLIP experiments would also be useful in detecting the binding between the maternal ncRNAs and EMHT2. Deploying the binding sites is better.
3. Actually, colocalization assay is essential to identify and characterize direct the interactions between ncRNA (280118) or other chromatin regulators between the EMHT2 protein in cells.
4. The research limitations should be analyzed and presented in the discussion content.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

I appreciate the authors' efforts to address the reviewers' comments. the EHMT2 and H3K9me2 ChIP assay results are intriguing, as they demonstrate that the role of EHMT2 in imprinting is not universal. Additional experiments involving TSS4 knockout (KO) provide compelling evidence for a causal relationship between noncoding RNA and the maintenance of imprinting of SNRPN and SNORD116.

I have one question: In the TSS4 KO experiments, did you examine the DNA methylation of the PWS-IC? It would be important to determine whether EHMT2-mediated imprinting is independent of DNA methylation.

Reviewer #5

(Remarks to the Author)

The authors reported that 1) the DNA methylation of PWS-IC serves in the establishment of imprinting but is not essential for imprinting maintenance; 2) a different mechanism operates to silence expression of paternally expressed genes distal or proximal to the PWS-IC on the maternal chromosome; 3) maternal non-coding RNA of TSS4-386 280118 upstream of PWS-IC interact with and recruit EHMT2 to PWS-IC, form a heterochromatin repressor complex, and silence the expression of SNRPN/SNHG14 on the maternal chromosome.

The study for the 15q11-13 imprinted region has been conducted since the 1990s, but the detailed regulatory mechanisms are unknown. It is very interesting that the binding of non-coding RNA to catalyze related to histone modification plays a major role in the imprinting of the maternal allele PWS-IC.

In particular, the authors revealed that the non-coding RNAs TSS4-280118 and EHNT2 are co-localized in the nucleus, and that knock out of ENSG00000280118 by the CRISPR/Cas9 system in the skin fibroblasts of AS deletion cases and PWS deletion cases resulted in unsilencing of gene expression of SNRPN and SNORD116. These findings show that TSS4-280118 has an important role in silencing paternally expressed genes in the 15p11-13 imprinted region on the maternal allele.

Comment 1

The imprinted regions contain many non-coding RNAs with unknown functions. I want to know how the authors target ENSG00000280118 from among the many non-coding RNAs. Please provide more description about them. Did you also examine microRNAs and snoRNAs?

Comment 2

Could you describe the reason for the discrepancy in TSS4-associated RNA levels in PWS and AS cells using bulk versus chromatin-associated RNA-seq in the main text? The TSS4-associated RNA-seq signal looks bi-allelic in the bulkRNA, but maternally-biased in chrRNA.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

I have no further comments.

Reviewer #5

(Remarks to the Author)

The authors modified the manuscript in response to the reviewers' comments. The reviewer has no further comments.

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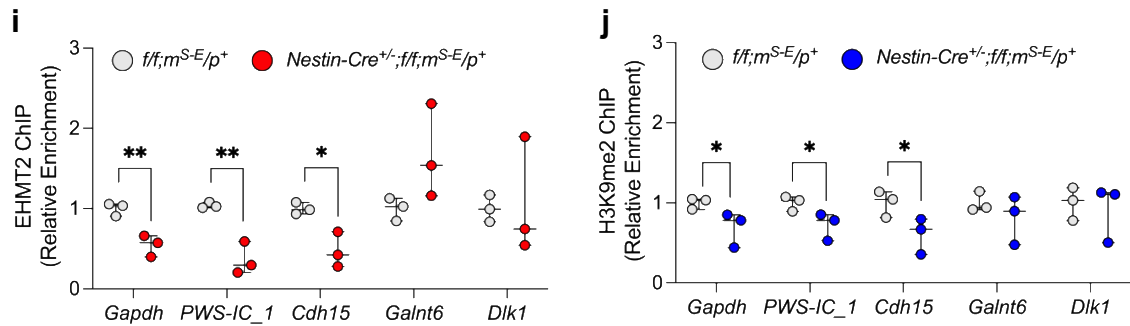
Point-by-Point Responses to Reviewer's Comments

Reviewer #1 (Remarks to the Author):

Reviewer's comment: EHMT2 may play a role other than PWS critical region. Therefore, it is good to demonstrate detailed phenotypes of Nestin-Cre/Ehmt2^{flox/flox} mice. Also, genomewide changes of H3K9me2 levels are good to be demonstrated. I am curious on the changes of H3K9me2 levels at other imprinting domains.

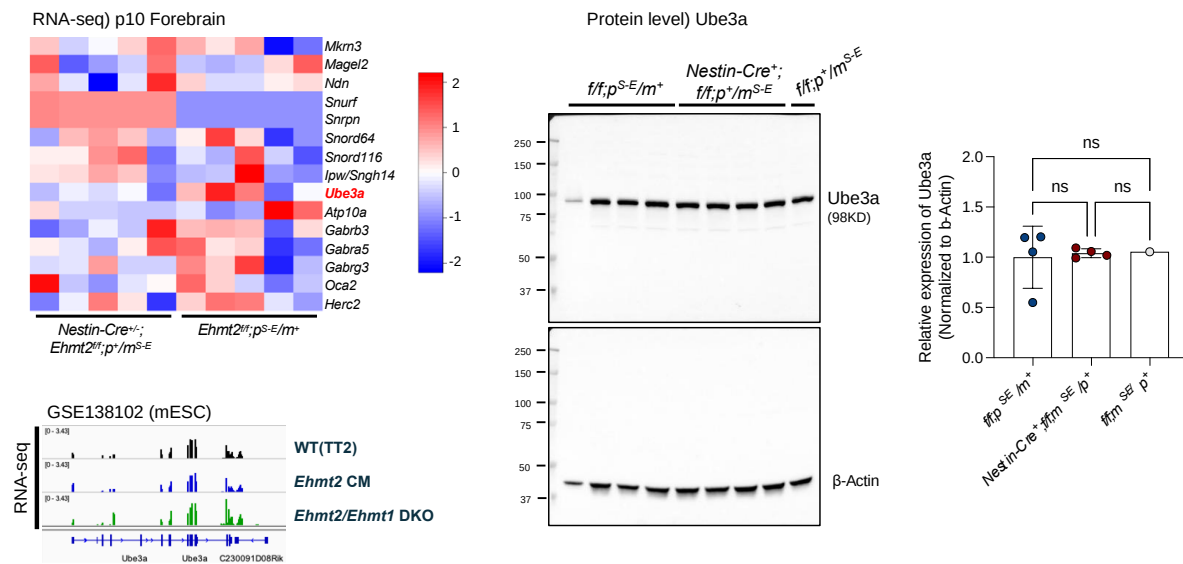
Response: Previous studies have shown the phenotype of Ehmt2(G9a) conditional KO (cKO) in the brain driven by *CamKii α -Cre*, *Drd1-Cre*, or *Drd2-Cre* (PMID: 20005824)¹. They reported that postnatal, neuron-specific deficiency of Ehmt2/G9a leads to transcriptional alteration and complex behavioral phenotypes including impairments in learning and memory, reduced exploration motivation, and environmental adaptation. In addition, the transcriptional dysregulation and behavioral phenotypes of nestin-Cre mediated Ehmt1 cKO were also described (PMID: 34258564)². The focus of this study was to investigate mechanism of imprinting maintenance during early development, we believe that the detailed phenotypic analysis of Nestin-Cre mediated cKO of Ehmt2 may deserve an independent study in future. We will discuss the reports in literature briefly in discussion. We hope that the reviewer agrees.

To examine the H3K9me2 levels at other imprinting domains, chromatin immunoprecipitation assay of EHMT2 and H3K9me2 was performed using the forebrain tissues of p10 *Nestin-Cre^{+/-};Ehmt2^{ff};m^{S-E}/p⁺* and *Ehmt2^{ff};m^{S-E}/p⁺* as a control for domains associated with imprinting genes of and *Dlk1* in chromosome 12, *Cdh15* in chromosome 8, *Galnt6* in chromosome 15 (**Fig. 1i & 1j**). These loci were chosen because the expression changes of *Cdh15*, *Galnt6*, and *Dlk1* was shown in transcriptome analysis (**Extended Data Fig. 1b**). The *Dlk1* locus is at a well characterized imprinting domain that mirror the imprinting domain of homologous imprinting domain of PWS in mice. EHMT2 binding affinity was reduced with H3K9me2 in PWS-IC and regulatory region of *Cdh15*, accompanying with increase of each gene expression (**Fig. 1i**). Meanwhile, *Galnt6* and *Dlk1*, decreased transcripts, were not directly regulated by EHMT2 (**Fig. 1j**).



Reviewer's comment: Unsilencing of IC transcript may repress the expression of maternal Ube3a. How about the expression change of Ube3a?

Response: We appreciate the reviewer's comments. RNA-seq data did not reveal any significant changes of *Ube3a* expression in the forebrain of *Ehmt2* cKO mice (**Fig.1h**). Similarly, *Ube3a* expression is not altered in embryonic stem cells (ESC) derived from *Ehmt2* catalytic mutant (CM) or *Ehmt2/Ehmt1* double KO (DKO) mice (**Extended Data Fig.1e**) (PMID: 33113380)³. We also performed analysis of UBE3A protein. The UBE3A protein in the forebrain is comparable between *Ehmt2* cKO and control (**Extended Data Fig. 1d**).



Extended Data Fig. 1e

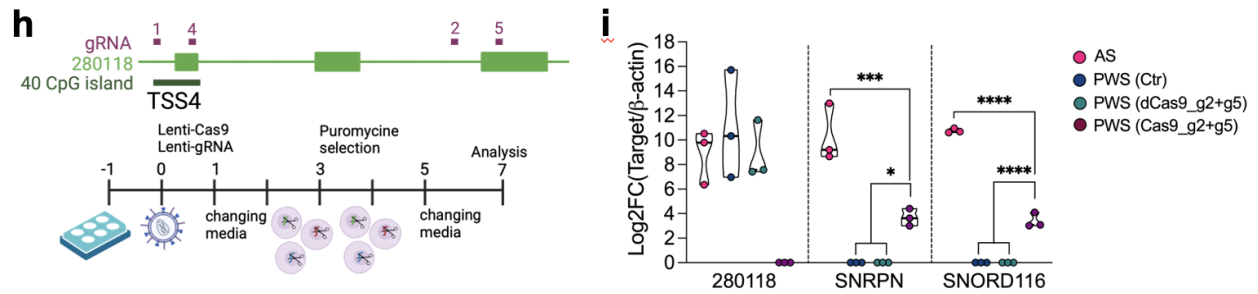
Reviewer's comment: Drug induced DNA demethylation of PWS-IC is shown to induce expression of maternal SNURF-SNRPN. If unsilencing of maternal imprinted genes are mediated by EHMT2 complex independent of DNA methylation, how such DNA demethylation inducing unsilencing is explained?

Response: We appreciated the reviewer's comments. Indeed, this was question that we have presented and discussed in detail Discussion section as following. *Loss of methylation of PWS-IC is observed in both mouse Dnmt1^{-/-} ESC and embryos. However, expression of Snrpn is mono-allelic in ESC but biallelic in embryos of Dnmt1^{-/-}. In contrast, expression of Snrpn is biallelic in Ehmt2^{-/-} ESC and embryos. Interestingly, methylation of PWS-IC in the maternal chromosome is lost in ESC but intact in embryos of Ehmt2^{-/-}. These results indicate both DNMT1 and EHMT2 contribute to the methylation of PWS-IC in ESC but only DNMT1 is involved in the embryo. These are consistent with other reports that DNMT1 and EHMT2 interact directly. This is also in line with the finding that different epigenetic machinery proteins are implicated in the epigenomic reprogramming of demethylation during early development. Consistent with these findings, we reported that EHMT2 inhibitors unsilence the paternally expressed SNRPN/SNHG14 from the imprinted genes on maternal chromosome without changing the DNA methylation of PWS-IC.*

To our knowledge, the unsilencing of SNRPN gene by DNA methylation inhibitors from the maternal chromosome has been described in vivo. As our data indicated, the methylated CpG island may serve an allelic specific mark for upstream maternal non-coding transcript to form a repressor complex. DNA methylation inhibitors reduce the DNA methylation but will affect the formation of repressor complex that may be responsible for the unsilence the expression of SNRPN from the maternal chromosome (PMID: 22781841)⁴. We have revised the text and included additional discussion

Reviewer's comment: The authors showed interaction of non-coding RNA 280118 and EHMT2, and reduction of the interaction by inhibition of EHMT2. However, causal relationship is not clearly shown yet. Genome editing study interrupting the TSS4 would be of great value.

Response: We appreciate the reviewer's constructive comments. To establish the causality, we conducted a critical experiment of using CRISPR/Cas9 to inactivate the non-coding RNA 280118 and then examined the unsilencing of SNRPN. We did co-transduction of Lenti-Cas9 and two sgRNA targeting non-coding transcript of RNA 280118 in PWS (maternal chr15 q11-q13) fibroblasts, used AS fibroblasts (paternal chr15, normal gene expression) as a positive control, and dCas9 with sgRNAs were used as a negative control. As a result, inactivation of RNA 280118 drove unsilencing of maternal SNRPN and SNORD116 genes. These new data are incorporated in Fig 6h-i.



Minor

Reviewer's comment: Fig 1C; *f/f;p+/mS-E* appears decreased H3K9me2 level, which is not expected.

Response: We agree with the reviewer's comment. This lane was considered as negative control. However, unintentionally, the loading amount of *f/f;p+/mS-E* was significantly less than other lanes based on H3 loading control. The reduction of H3K9me2 is most likely due to the loading. We believe that this negative control is not essential for the interpretation for data. We removed the lane in revised Fig 1C. Hope reviewer agree.

Reviewer #2 (Remarks to the Author):

Reviewer's comment: This study aims to investigate the mechanisms underlying genomic imprinting at the 15q11.2-q13 region using mouse models and patient-derived cell lines. Through epigenomic profiling and molecular experiments, the authors propose a model in which a long non-coding RNA that is preferentially expressed from the maternal chromosome recruits the histone methyltransferase EHMT2/G9a to the imprinted region, followed by heterochromatin formation and gene silencing. If true, this would be interesting as EHMT2 recruitment is independent of DNA methylation, but functional validation of this model is lacking. The work also examines allele-specific 3D genome folding, but the relevance of the observed structural differences to imprinted expression remains unclear. Additionally, more focused exploration is needed to clarify the distinctions in proximal versus distal gene regulation. Some additional considerations are below:

Response: We thank the reviewer for the constructive criticism and suggestion. We have addressed these comments and suggestions by conducting additional experiments described below or revise the discussion for clarity.

Other Points:

Reviewer's comment: Figure 1d is the only panel that directly shows allele-specific SNRPN expression as a result of EHMT2 deletion in the mouse model. This panel should be quantified and plotted like the other blots in this manuscript.

Response: We apologize for not labeling the data clearly. We would like to point out that we already included quantitative PCR data in **Fig. 1e** while **Fig. 1d** showed a result from RT-PCR. We re-labeled the Figure to make clearer.

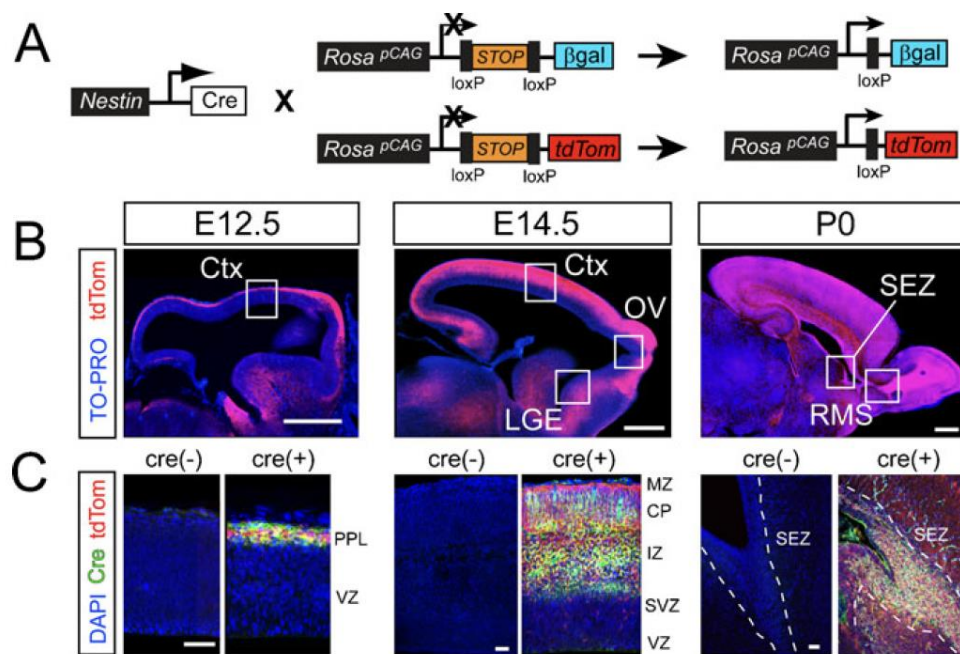
Reviewer's comment: In order to conclude that the signal differs between the Nestin-Cre⁺;f/f;p⁺/mS-E and the f/f;p⁺/mS-E conditions, the authors need to show the values for more than one replicate of the latter condition.

Response: We appreciate the reviewer's concern. We wish that we have described the data better. Our study indeed examine the molecular changes of forebrain tissues from two different time points of E18 (**Fig. 1f, 1g, Extended Data Fig. 1a**) and p10. (**Fig. 1b-1e, 1h-1j**) mice. We described the age of brain tissue used in detail in Figure legend. The difference of H3K9me2 level

on PWS-IC between cKO mice and control is less prominent at p10 (**Fig. 1j**) than that of E18 (**Fig. 1f**). This comment is incorporated in revised manuscript.

Reviewer's comment: In order to more concretely show that DNA methylation is unaffected in Figure 1g, could the methylation analysis be performed on GFP+ sorted cells. Methylation changes in the subpopulation of Ehmt2-deleted neurons may be masked by other cell types lacking Nestin expression within the tissue.

Response: We appreciate the reviewer's comment. Nestin-Cre drives the Cre expression in both neuronal and glial cell precursors. We used the forebrain tissues from E18 forebrain for DNA methylation. The expression of Nestin-Cre is reported peaked at P0 (see Figure below from PMID: 23259054)⁵. The tissues from E18 for DNA methylation assay are appropriate for DNA methylation analysis. Furthermore, no methylation change is reported in embryos of Ehmt2 conventional KO and G9a inhibitor data in fibroblasts in vitro. Thus, the concern for DNA methylation changes that may be masked by a small cell population is minimal if any and the experiment of using GFP+ sorted cells may not add little value.



(A) Breeding schemes to generate *Nestin-cre* transgenic mice (Jax: B6.Cg-Tg(Nes-cre)1 Kln/J) on either a *LacZ* or *tdTomato* (*tdTom*) reporter background. *LacZ* or *tdTom* are conditionally expressed under the chicken β -actin promoter, which was knocked into the *Rosa* locus to track cre-mediated recombination. Two loxP sites flank a STOP codon upstream of the reporter

cassettes, preventing reporter expression in the absence of cre-mediated recombination. *Nestin-cre* mediated recombination deletes the stop codon allowing the expression of the reporters. (B) Sagittal views of the developing CNS illustrating tdTom reporter expression mediated by *Nestin-cre* at embryonic stages E12.5, E14.5, and at birth (P0). (C) Analysis of cre expression at ages corresponding to sections in B, using a cre-specific antibody (green), in combination with tdTom and DAPI (blue). Cre(-) controls were obtained from brains of mice negative for Nestin-cre, but genetically positive for tdTom reporter.

Reviewer's comments: It is difficult to observe any structural differences between the parental alleles in Figure 4b. Can a subtraction plot be provided to highlight any differences?

Response: We agree that there was no significant structural difference for TAD between the paternal and maternal chromosome in **Fig. 4b** and also **Extended Data Fig. 4a**). These results are consistent with the scarcity of CTCF binding in this region that is in contrast with other imprinting domains such as H19-IGF2 in human chromosome 11p15 region⁴⁵ and Peg13-Kcnk9 in mouse chromosome 15 (**Fig. 4a**). As discussed in Discussion section, the negative results could also be due to the resolution of 5kb distance is still not sufficient to detect the more subtle difference at 3D chromatin level. However, we observed significant difference for the maternal and paternal specific chromatin loops in the 15q11-q13 domain between paternal and maternal (**Fig. 4c**).

Reviewer's comments: It Regarding Figure 6, can you explain the discrepancy in TSS4-associated RNA levels in PWS and AS cells using bulk versus chromatin-associated RNA-seq? In other words, the TSS4-associated RNA-seq signal looks bi-allelic in the bulkRNA, but maternally-biased in chrRNA. Can you provide additional evidence that you've detected a new non-coding RNA? How long is this ncRNA? Is it nuclear retained? Can you provide evidence that this is a separate transcript and not just another upstream TSS for Snrpn?

Response: We appreciate the reviewer's comments. The difference between Bulk RNA-seq and chromatin specific RNA-Seq support that the TSS4 is more associated with maternal chromatin. The key point for the hypothesis and experiments. TSS4 represents a new species of non-coding RNA since it is transcribed preferentially from the maternal chromosome in somatic cells that is contrast to other paternally transcripts from upstream of PWS-IC. TSS4-associated ncRNA 280118 is approximately 3.8 Kb in length deposited in genome browser. But like other non-coding transcripts in the region, we could not determine the exact size of transcript. It could be much

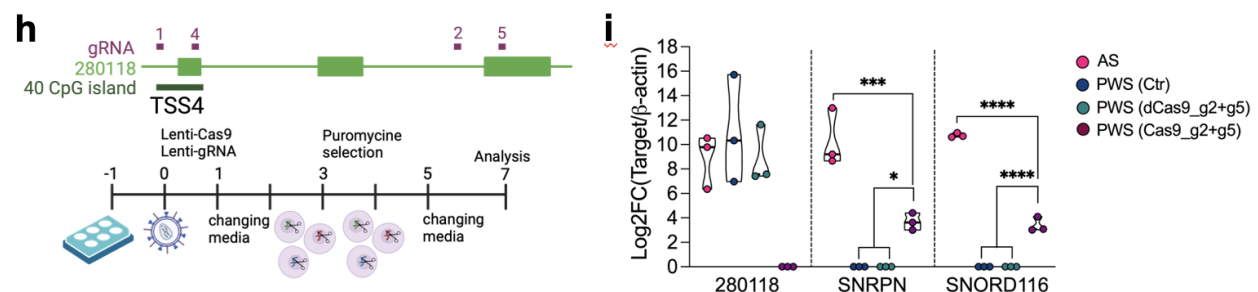
longer. We used a complementary RNA fluorescence in situ hybridization (FISH) assay to confirm the expression of TSS4-280118. The new data is incorporated in **Fig. 6e**.

Reviewer's comment: In Figure 6C, why does the control sample not show association between the ncRNA and EHMT2 by RIP?

Response: Higher value of Log₂FC means that 280118 RNA is enriched in IP over input. While, lower value of Log₂FC does not mean that there is no association of ncRNA and EHMT2 or no expression at transcript level. In **Fig 6c**, control samples (AS, WT) had values around 0.7, suggesting less association compared to PWS sample.

Reviewer's comment: The conclusion “maternal non-coding RNAs recruit EHMT2 to PWS-IC to silence the expression of SNRPN/SNHG14 in the maternal chromosome” is not fully supported by the data. The statement should either be updated to reflect the data shown, or additional experiments should be performed to support the conclusion as stated. For example, one could observe the effect of ncRNA-knockdown on maternal expression of SNRPN.

Response: Appreciate the reviewer's constructive comments. See the response to the reviewer #1 number 4 comment. We have conducted a critical experiment of using CRISPR/Cas9 to inactivate the non-coding RNA 280118 and then examined the unsilencing of SNRPN. The result showed that inactivation of RNA 280118 drove unsilencing of maternal *SNRPN* and *SNORD116* genes. These new data are incorporated in **Fig. 6h-i**.



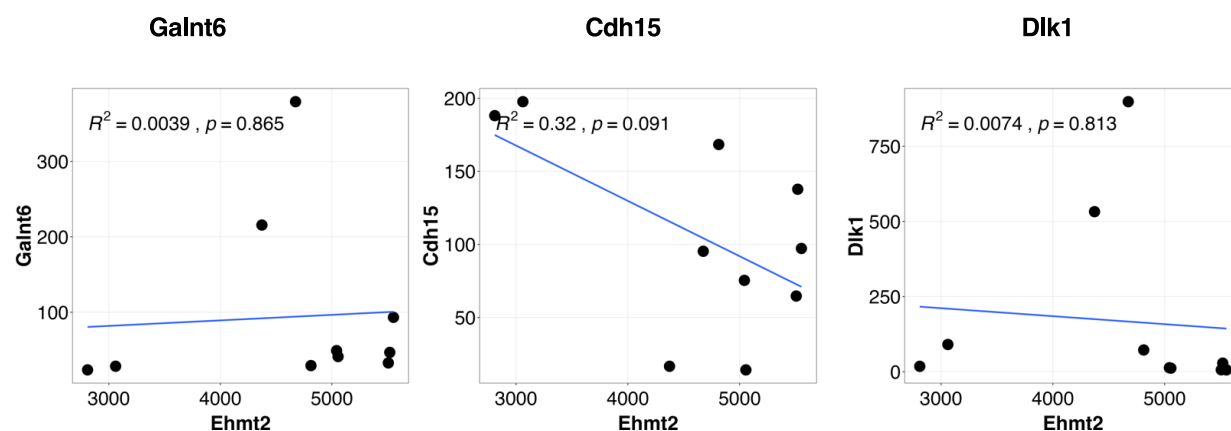
Reviewer #4 (Remarks to the Author):

In this manuscript, Prof. Jiang and colleagues revealed a new mechanism model for imprinting maintenance regulation of the PWS imprinted domain on 15q11.2-q13.1 where allele-specific recruitment of EHMT2 could play a key role. They used Ehmt2-deficient mouse, PWS/AS patient-derived fibroblasts for delineating the comprehensive molecular and high-resolution chromatin analyses and found a different 3D chromatin folding confirmation in the maternal PWS imprinting domain. Moreover, they showed EHMT2 might be recruited and interacted with a maternal ncRNA transcribed upstream of the PWS-IC and formed a heterochromatin complex to silence SNRPN expression in cis on maternal chromosome. These findings are unique and very interesting.

Response: We thank the reviewer for the kind assessment and the suggestions. We agree that additional analysis is necessary to suggest clarified function of EHMT2 on imprinting maintenance.

Reviewer's comment: The first result part suggested EHMT2 could act as both repressor and activator in regulating different paternally expressed genes from other genomic imprinted domain. I wonder the expression pattern of maternally expressed genes in Ehmt2 deficiency model. The discussion on the possible mechanism and also underlying influence analysis are needed.

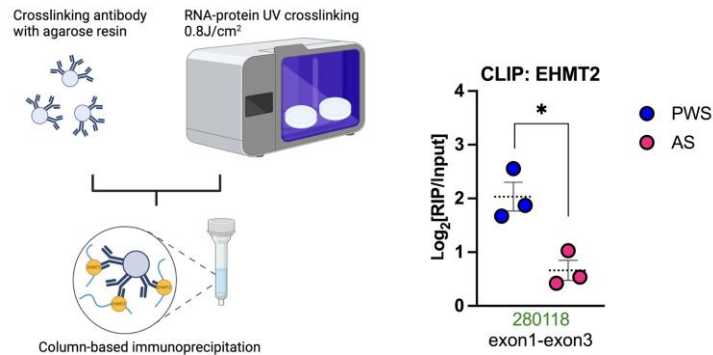
Response: We thank for the reviewer's suggestion. Using RNA-seq data, we performed influence analysis depending on Ehmt2 expression. We investigated the paternal imprinted genes/maternal expressed gene set including *Galnt6*, *Cdh15*, and *Dlk1*. *Ehmt2* deficiency altered transcription level of these genes in forebrain at p10. However, only *Cdh15* gene showed negative correlation with Ehmt2 at mRNA level, suggesting that EHMT2 may contribute to the imprinted expression of *Cdh15* gene. This data is incorporated in **Extended data Fig. 1c**.



Reviewer's comment In addition to RIP, supplemented CLIP experiments would also be useful in detecting the binding between the maternal ncRNAs and EMHT2. Deploying the binding sites

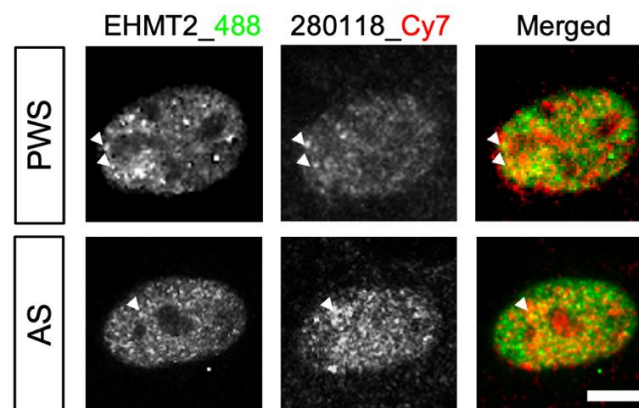
is better.

Response: Following the reviewer's suggestion, we performed the crosslinking and immunoprecipitation (CLIP) experiments to reproduce the RIP data (**Extended Data Fig. 5b**). Consistent with the data in **Fig. 6c**, we were able to detect binding between ncRNA 280118 and EHMT2 shown in Response Figure 2. This data is incorporated in **Fig. 6d**.



Reviewer's comment: Actually, colocalization assay is essential to identify and characterize direct the interactions between ncRNA (280118) or other chromatin regulators between the EHMT2 protein in cells.

Response: We thank for the reviewer's comments. Actually, the RIP experiment is better to detect the direct interaction between ncRNA and EHMT2 protein. However, we agree that colocalization may complement to the RIP experiment or a replication. For colocalization assay, we used RNA FISH assay with immunofluorescence. We newly designed the probes binding to ncRNA 280118 (**Supplementary Table 4**) and imaged 280118 with EHMT2 at the same time. We indicated clear colocalized foci as white arrowhead in images below. These data are incorporated in **Fig. 6e**.



4. The research limitations should be analyzed and presented in the discussion content.

We appreciate the suggestions and we have discussed the following limitations in revised

manuscript

Response: We deciphered chromatin training in fibroblasts in vitro only. It would be desirable to conduct the same study using mouse tissues which is technically challenging currently. Our study suggested that the mechanism of imprinting maintenance of *MKRN3*, *MAGEL2*, *NDN* genes proximal to PWS_IC is different from SNRPN. Future study is warranted. These comments are incorporated into the discussion of revised manuscript (page 15).

Reference

- 1 Schaefer, A. *et al.* Control of cognition and adaptive behavior by the GLP/G9a epigenetic suppressor complex. *Neuron* **64**, 678-691 (2009). <https://doi.org:10.1016/j.neuron.2009.11.019>
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- 3 Jiang, Q. *et al.* G9a Plays Distinct Roles in Maintaining DNA Methylation, Retrotransposon Silencing, and Chromatin Looping. *Cell Rep* **33**, 108315 (2020). <https://doi.org:10.1016/j.celrep.2020.108315>
- 4 Moore, L. D., Le, T. & Fan, G. DNA methylation and its basic function. *Neuropsychopharmacology* **38**, 23-38 (2013). <https://doi.org:10.1038/npp.2012.112>
- 5 Liang, H., Hippenmeyer, S. & Ghashghaei, H. T. A Nestin-cre transgenic mouse is insufficient for recombination in early embryonic neural progenitors. *Biol Open* **1**, 1200-1203 (2012). <https://doi.org:10.1242/bio.20122287>

Point-by-Point Responses to Reviewer's Comments

Reviewer #1 (Remarks to the Author):

I appreciate the authors' efforts to address the reviewers' comments. the EHMT2 and H3K9me2 ChIP assay results are intriguing, as they demonstrate that the role of EHMT2 in imprinting is not universal. Additional experiments involving TSS4 knockout (KO) provide compelling evidence for a causal relationship between noncoding RNA and the maintenance of imprinting of SNRPN and SNORD116.

I have one question: In the TSS4 KO experiments, did you examine the DNA methylation of the PWS-IC? It would be important to determine whether EHMT2-mediated imprinting is independent of DNA methylation.

Response: We appreciate the reviewer's comment. Several lines of evidence from our current and prior studies supporting that EMHT2-mediated imprinting is independent of DNA methylation at PWS-IC. *First*, DNA methylation of PWS-IC is maintained in *Ehmt2* conditional knockout (cKO) and paternal *Snrpn-Ube3a* deletion mice (**Fig. 1g**). *Second*, DNA methylation of PWS-IC is intact in 9.5 day embryos of *Ehmt2* conventional knockout (PMID 12586828). *Third*, in our previous study published in Nature Medicine (PMID: 28024084), EHMT2 inhibitor treatments in PWS mouse model and PWS patient derived fibroblasts do not have any impact on DNA methylation of PWS-IC. *Lastly*, our data support a hypothesis that the transcript of TSS4 mediate the recruitment of EHMT2 to PWS-IC that is independent of the status of PWS-IC. We have showed that DNA methylation inhibitor treatments does not affect the level of H3K9me2 at PWS-IC by ChIP-qPCR. For these reasons, we did not check DNA methylation of PWS-IC in TSS4 knockdown and feel that it is not essential or justified.

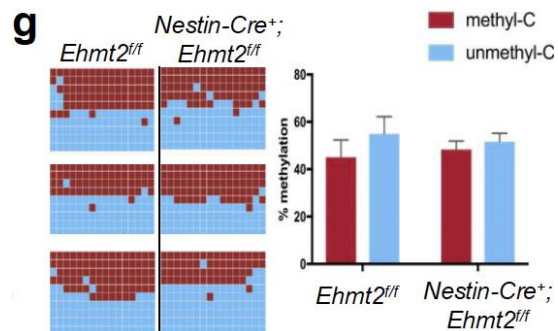
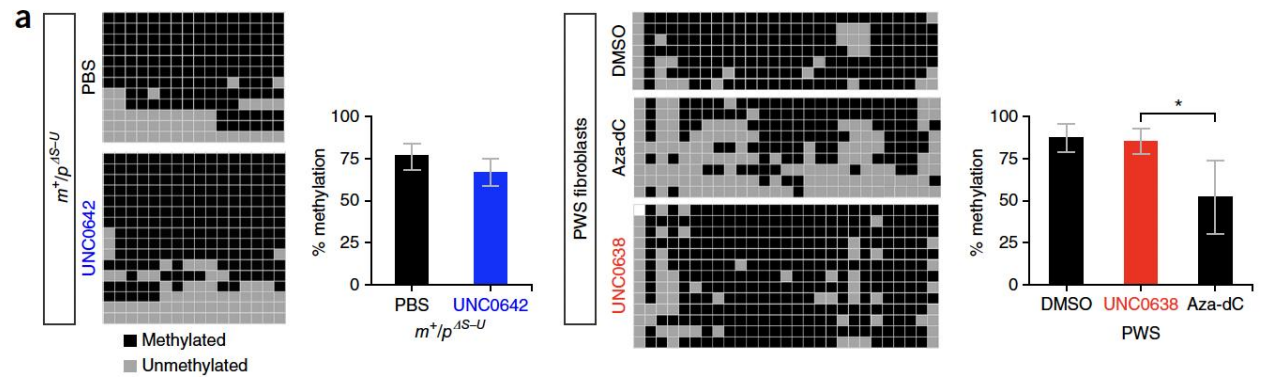


Fig. 1g) Comparison of the DNA methylation in PWS-IC between control and *Ehmt2* cKO mice at E18.



(a) Comparison of the DNA methylation in PWS-IC between vehicle- and UNC0642- or UNC0638- treated in liver of $m^+/p^{\Delta snrpn-ube3a}$ mice and in a human PWS fibroblast cell lines (PMID: 28024084).

Reviewer #5 (Remarks to the Author):

The authors reported that 1) the DNA methylation of PWS-IC serves in the establishment of imprinting but is not essential for imprinting maintenance; 2) a different mechanism operates to silence expression of paternally expressed genes distal or proximal to the PWS-IC on the maternal chromosome; 3) maternal non-coding RNA of TSS4-386 280118 upstream of PWS-IC interact with and recruit EHMT2 to PWS-IC, form a heterochromatin repressor complex, and silence the expression of SNRPN/SNHG14 on the maternal chromosome. The study for the 15q11-13 imprinted region has been conducted since the 1990s, but the detailed regulatory mechanisms are unknown. It is very interesting that the binding of non-coding RNA to catalyze related to histone modification plays a major role in the imprinting of the maternal allele PWS-IC. In particular, the authors revealed that the non-coding RNAs TSS4-280118 and EHMT2 are co-localized in the nucleus, and that knock out of ENSG00000280118 by the CRISPR/Cas9 system in the skin fibroblasts of AS deletion cases and PWS deletion cases resulted in unsilencing of gene expression of SNRPN and SNORD116. These findings show that TSS4-280118 has an important role in silencing paternally expressed genes in the 15p11-13 imprinted region on the maternal allele.

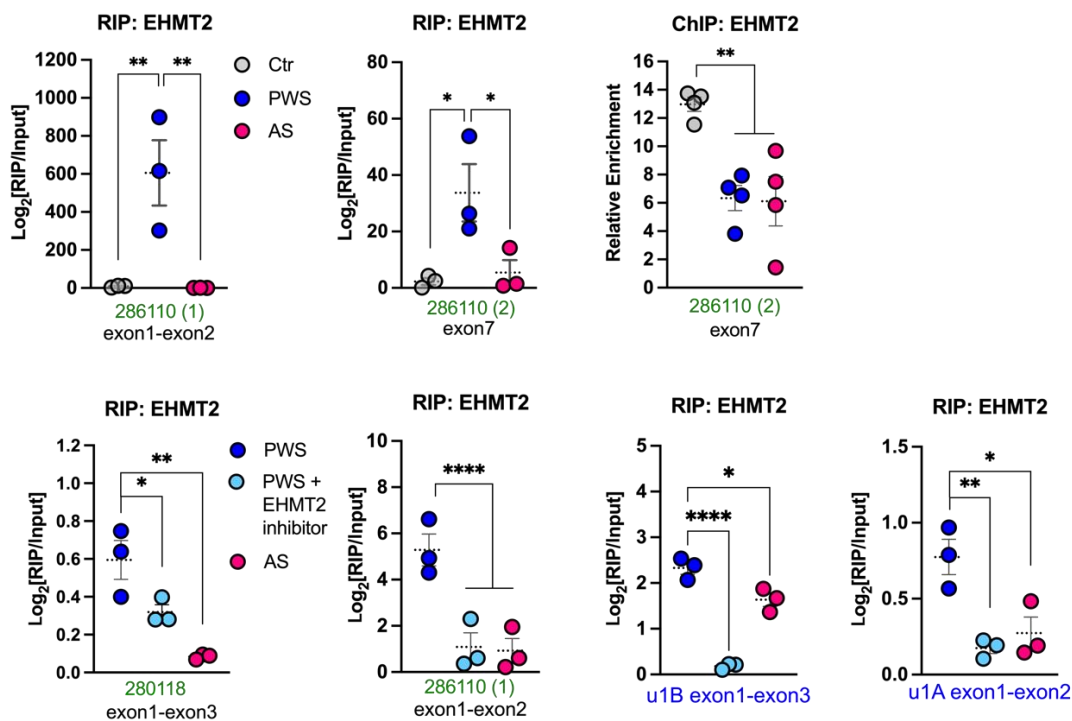
Comment 1

The imprinted regions contain many non-coding RNAs with unknown functions. I want to know how the authors target ENSG00000280118 from among the many non-coding RNAs. Please provide more description about them. Did you also examine microRNAs and snoRNAs?

Response: We agree with the reviewer's comments that there are many non-coding RNAs including microRNAs and SnoRNAs within the PWS imprinting domain. The characterization of 5' UTR of the *SNRPN* gene has been described previously. However, the role of non-coding RNAs transcribed from the upstream of PWS-IC is largely unknown. Previous studies described the oocyte-specific expression for one transcript in mouse (PMID: 16429232). Our initial focus is on non-coding RNAs or transcripts upstream of PWS-IC and part of upstream of SNRPN/SNHG14 transcripts. Our *in silico* analysis identified ENSG00000280118 and many other transcripts in an extended region between PWS-IC and NDN. For these reasons, we targeted ENSG00000280118 as a novel transcript within the 5' UTR of the *SNRPN* gene and also associated with a CpG island (CGI-40), suggesting a new transcriptional starting site (TSS). Additionally, RNA-IP experiments using EHMT2 antibody confirmed the interaction of EHMT2 and ENSG00000280118 or other

non-coding RNAs (ENSG00000286110, SNRPN transcript including u1B or u1A) in the region. More importantly, the expression of ENSG00000280118 via chromatin associated RNA-seq was shown a preferential expression from the maternal chromosome.

These together led the hypothesis of transcript of ENSG00000280118 from TSS4 mediated the recruitment of EHMT2 to PWS-IC. The role of the miRNAs or snoRNAs remains to be studied in future investigations. We addressed in result and discussion section to provide more context for this statement.



Comment 2

Could you describe the reason for the discrepancy in TSS4-associated RNA levels in PWS and AS cells using bulk versus chromatin-associated RNA-seq in the main text? The TSS4-associated RNA-seq signal looks bi-allelic in the bulkRNA, but maternally-biased in chrRNA.

Response: Thanks for the reviewer's careful review. The difference between bulk-RNA and chrRNA (chromatin-associated RNA) is because of the different nature and sensitivity of two methods (PMID: 29648534, 36084040). chrRNA is usually not detectable in total RNA-seq that are used for bulk RNA-seq. chrRNA-seq is capable of detecting the active nascent transcription across the genome. Thus, the total RNA-seq may not be able to detect the maternally biased expression largely since the captured read number on that region was too small in total RNA-seq.

including the RNA in cytosol.