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Peer Review File

Allele-specific chromatin architecture shapes imprinted domains and coordinates a distal enhancer and antisense transcription at the mouse *Mest-Copg2* domain

Corresponding Author: Dr Amanda Whipple

This manuscript has been previously reviewed at another journal. This document only contains information relating to versions considered at Nature Communications.

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)
The authors addressed all the points raised by this reviewer. The textual changes and the additional data look convincing and reinforce the study. Overall, the manuscript has become much improved.

Reviewer #2

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The authors have performed an extensive revision of their manuscript in response to my comments. They have clarified technical details, toned down overstatements, added control experiments (e.g., H3K9me3 CUT&Tag for CRISPRi efficiency, reciprocal cross validations, nanopore sequencing for deletions), and provided more nuanced interpretations, particularly regarding the DNMTi experiments and the unresolved mechanism of MestXL action.
However, several points still require attention before final acceptance:

1. E105 is probably the most important new element in the manuscript. The current explanation is reasonable: E105 inhibition may reduce maternal Copg2 activation while also relieving repression of paternal Copg2, so total Copg2 expression stays roughly unchanged. The allele-specific ddPCR and RNA-seq data in Figure 6 help support this idea. As an example, that will be repeatedly referred to, “reasonable” is not quite enough. The authors should make the size of the effect much clearer. Specifically, they should state how large the E105 effect on Copg2 allelic bias is, how much of the normal Copg2 imprinting bias this accounts for, and whether a roughly 10% change in maternal fraction has any known developmental or neuronal relevance.
2. In Figure 6F, why are there no significant increases in paternal Copg2expression following enhancer inhibition? This result appears inconsistent with the observations in Figure S14E. Furthermore, what is the mechanistic basis for the observed decrease in paternal Copg2 expression under the combined inhibition of E105 and XL. More conclusive evidence is required to establish the regulatory mechanism of E105 on paternal Copg2expression. Additionally, what is the sample size (n value) for the analysis presented in Figure 6F?
3. The assertion in Figure 6 that Mest-XL independently regulates Copg2 is fundamentally compromised by the lack of direct quantification for the canonical Mest transcript following ASO treatment. The authors’ indirect quantitative inference for ASO specificity relies on an overly simplified two-isoform model, which starkly contradicts the GRCm39.115 annotation cataloging up to 11 distinct Mest isoforms. Given the high transcriptional complexity of this locus, a simple deduction based on transcript proportions is insufficient to rule out non-specific degradation or off-target interference on other overlapping variants. To definitively prove the absolute specificity of the ASO targeting and resolve the true transcriptomic landscape, the authors could need to employ full-length transcriptome sequencing (e.g., PacBio Iso-Seq or Oxford Nanopore) on the neuronal samples. Without this long-read validation to unequivocally confirm the unchanged absolute abundance of all other Mest isoforms, the current mechanistic conclusions remain highly speculative.
4. About Response to the Reply to Issue 21 of reviewer2: Using undifferentiated NPCs as negative controls for tissue-specific imprinting can partially illustrate certain patterns. Nevertheless, I still recommend supplementing the results from

non-neural tissues (e.g., mouse liver and myocardium) as additional negative controls of tissue-specific imprinting.
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Reviewer #3

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The authors addressed my questions, as well as many of the comments raised by the other reviewers. In the revision, I do take issue with the response to reviewer 1 (comment 6) regarding observations at the Meg3-Rian-Mirg domain (lack of H3K27 deposition by a non-coding RNA etc). The authors have oversimplified this complex locus. More importantly, the use of the DNA methylation data of Xie et al is not really relevant, given the use of bisulfite methylation analyses, which do not distinguish mC from hmC. These two modifications are associated with very different properties and depicting all modified C as mC inaccurate.

Reviewer #4

(Remarks to the Author)
I am happy with the way that the authors have addressed my comments

Version 1:

Reviewer comments:

Reviewer #2

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The authors have adequately addressed all of my comments and concerns. I have no further issues to raise.

(Remarks on code availability)
The code is well documented and usable, and appears sufficient to reproduce the main results reported in the manuscript, with no critical issues affecting reproducibility.

REVIEWER COMMENTS

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The authors addressed all the points raised by this reviewer. The textual changes and the additional data look convincing and reinforce the study. Overall, the manuscript has become much improved.

Thank you.

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To more clearly convey the magnitude of the E105 effect on *Copg2* allelic bias, we have added additional quantification to the Results section (see page 10 lines 19-21 and page 12 lines 18-22).

E105 inhibition significantly reduced the maternal-to-paternal *Copg2* expression ratio from 2.0 to 1.3 (Figure 5D), corresponding to a decrease in maternal bias from 67% to 57% (Supplementary Figure 13D). Thus, relative to biallelic baseline (50% maternal expression), E105 inhibition reduced approximately 60% of the neuron-specific maternal bias of *Copg2*.

Similar effect sizes were observed in two additional independent experiments and by two orthogonal assays (RT-ddPCR and RNA-seq).

Table: Quantification of the E105 contribution to neuron-specific *Copg2* maternal bias

Assay (Figure)	% maternal, control	% maternal, E105i	% change	% maternal bias accounted for by E105
ddPCR (Fig 5D,S13D)	67%	57%	-10%	59%
ddPCR (Fig S14E)	73%	60%	-13%	57%
RNA-seq (Fig S14F)	67%	57%	-10%	59%

The functional significance of this shift in allelic bias remains unknown, and we have therefore avoided making claims regarding developmental or neuronal significance. Instead, our conclusions focus on the role of E105 in establishing a substantial fraction of the neuron-specific maternal bias of *Copg2* expression.

2. In Figure 6F, why are there no significant increases in paternal Copg2 expression following enhancer inhibition? This result appears inconsistent with the observations in Figure S14E. Furthermore, what is the mechanistic basis for the observed decrease in paternal Copg2 expression under the combined inhibition of E105 and XL. More conclusive evidence is required to establish the regulatory mechanism of E105 on paternal Copg2 expression. Additionally, what is the sample size (n value) for the analysis presented in Figure 6F?

We agree that the increase in paternal *Copg2* expression following E105 inhibition is less pronounced in Figure 6F than in the independent allele-specific RT-ddCPR and RNA-seq analyses presented in Figures S14E,F.

To address this concern, we have revised the text to more accurately describe the data. Across all experiments, E105 inhibition consistently reduced maternal *Copg2* expression and shifted overall allelic bias toward a more balanced state. In independent RT-ddPCR and RNA-seq experiments (Figures S14E,F), this shift was accompanied by an increase in paternal *Copg2* expression. In Figure 6F, the direction of the effect is similar, but the magnitude of the paternal increase is smaller and does not reach significance under E105 inhibition alone. We therefore focus our conclusions on the reproducible reduction in maternal *Copg2* expression and the resulting decrease in allelic bias.

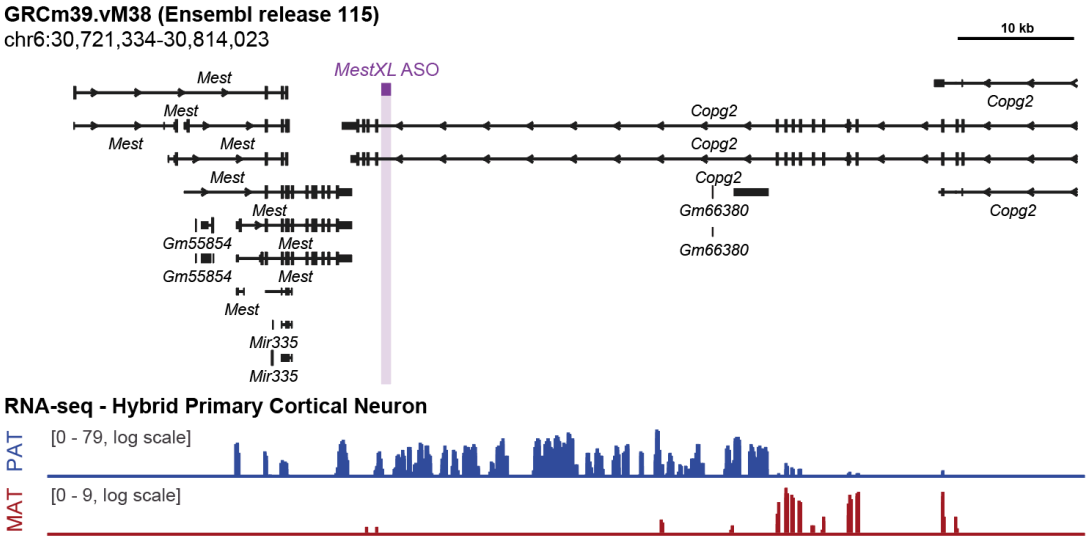
Regarding the combined inhibition of E105 and *MestXL*, we note that paternal *Copg2* expression remains elevated relative to the Scrb + Ctrl ASO condition and is not significantly different than *MestXL* inhibition alone. We have revised the text to clarify this point and to avoid implying a direct mechanistic role for E105 in promoting paternal *Copg2* expression.

Accordingly, we now state that the data support a model in which *Mest*/*MestXL* contributes to repression of paternal *Copg2*, whereas E105 promotes maternal *Copg2* expression. The sample size for Figure 6F (n = 4 biological replicates per condition) has been added to the figure legend.

3. The assertion in Figure 6 that Mest-XL independently regulates Copg2 is fundamentally compromised by the lack of direct quantification for the canonical Mest transcript following ASO treatment. The authors' indirect quantitative inference for ASO specificity relies on an overly simplified two-isoform model, which starkly contradicts the GRCm39.115 annotation cataloging up to 11 distinct Mest isoforms. Given the high transcriptional complexity of this locus, a simple deduction based on transcript proportions is insufficient to rule out non-specific degradation or off-target interference on other overlapping variants. To definitively prove the absolute specificity of the ASO targeting and resolve the true transcriptomic landscape, the authors could need to employ full-length transcriptome sequencing (e.g., PacBio Iso-Seq or Oxford Nanopore) on the neuronal samples. Without this long-read validation to unequivocally confirm the unchanged absolute abundance of all other Mest isoforms, the current mechanistic conclusions remain highly speculative.

We respectfully disagree that full-length transcriptome sequencing is required to support the conclusions of this study.

The ASO was designed against the unique 3′ extension of the *MestXL* transcript and does not overlap regions common to any other annotated *Mest* isoforms. The position of the ASO aligned to the current GRCm39.115 annotations is shown below.



We agree that the *Mest* locus is transcriptionally complex and that additional unannotated transcripts may exist. For this reason, we have further moderated our interpretation in the manuscript and now refer to this ASO perturbation as *Mest*/*MestXL* knockdown rather than attributing the effect exclusively to *MestXL*.

Importantly, our central conclusion does not depend on demonstrating that the ASO exclusively affects a single annotated transcript. Rather, the data show that perturbation of transcription from the *MestXL* extended region increases paternal *Copg2* expression and alters allelic bias. We therefore interpret the results conservatively as evidence that *Mest*/*MestXL* transcription contributes to repression of paternal *Copg2*.

While long-read transcriptome sequencing would provide a more complete description of transcript diversity at this locus, we believe such experiments are beyond the scope of the present study and are not required to support the conclusions drawn from the current data.

4. About Response to the Reply to Issue 21 of reviewer2: Using undifferentiated NPCs as negative controls for tissue-specific imprinting can partially illustrate certain patterns. Nevertheless, I still recommend supplementing the results from non-neural tissues (e.g., mouse liver and myocardium) as additional negative controls of tissue-specific imprinting.

We agree that analysis of additional non-neural tissues would provide useful context regarding the tissue specificity of the observed effects. However, the goal of this study is to define the mechanisms underlying imprinted expression at the *Mest–Copg2* locus in neurons. Accordingly, we focused our experimental analyses on neuronal systems, including cortex, primary cortical neurons, and NPC-derived neurons.

As noted during the transfer of this manuscript from *Nature Structural & Molecular Biology*, expansion of the study to additional tissues was not considered necessary to support the central

conclusions. Consistent with this guidance, we prioritized strengthening the mechanistic analyses within the neuronal context rather than broadening the scope of the study.

We have nevertheless included undifferentiated NPCs as a negative control and show that perturbation of E105 has minimal effects prior to neuronal differentiation (**Supplementary Figure 14H**), supporting the neuron-specific nature of the regulatory mechanism. We therefore believe that additional analysis of liver or myocardium would be unlikely to alter the central conclusions of the study.

5. Code availability: The manuscript does not state whether custom analysis scripts (e.g., for allele-specific Hi-C matrix generation, virtual 4C, insulation score calculation, ChromHMM integration) have been deposited in a public repository. To ensure reproducibility, I strongly recommend uploading all relevant code to GitHub or Zenodo and citing the DOI in the Data Availability or Methods section.

Analysis scripts have now been deposited to GitHub (github.com/Whipple-Lab/capture-hic-imprinting).

6. The format of the references should be consistent with that of Nature Communications.

We believe references are now correctly formatted but are happy to work with the editorial staff to ensure compliance.

Reviewer #3 (Remarks to the Author):

The authors addressed my questions, as well as many of the comments raised by the other reviewers. In the revision, I do take issue with the response to reviewer 1 (comment 6) regarding observations at the Meg3-Rian-Mirg domain (lack of H3K27 deposition by a non-coding RNA etc). The authors have oversimplified this complex locus. More importantly, the use of the DNA methylation data of Xie et al is not really relevant, given the use of bisulfite methylation analyses, which do not distinguish mC from hmC. These two modifications are associated with very different properties and depicting all modified C as mC inaccurate.

We thank Reviewer #3 for highlighting this limitation. We have revised the text to acknowledge that the bisulfite-based MethylC data from Xie et al. do not distinguish 5mC and 5hmC. However, we believe the data remain informative for the purposes used in this study, namely identifying allele-specific patterns of cytosine modification and their relationship to chromatin structure. To avoid overinterpretation, we now explicitly acknowledge that the observed signal may reflect a combination of 5mC and 5hmC and have revised the corresponding text and figure legends (see page 8 lines 2-4).

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I am happy with the way that the authors have addressed my comments

Thank you.

REVIEWERS' COMMENTS

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The authors have adequately addressed all of my comments and concerns. I have no further issues to raise.

Thank you.

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The code is well documented and usable, and appears sufficient to reproduce the main results reported in the manuscript, with no critical issues affecting reproducibility.

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